Association between reproductive factors and Colorectal Cancer; Identification and Characterization of β-catenin *(CTNNB1)* gene and modulatory potential of Taxifolin and Nano-particle with vitamin D (NVD) against colorectal cancer in Pakistani population.

Doctor of Philosophy

By

Suhail Razak

Department of Animal Sciences,

Quaid-i-Azam University, Islamabad

Pakistan.

2020

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A dissertation submitted for the fulfillment of the degree of

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Certificate of Approval

This is to certify that the research work presented in this thesis, entitled "Association Between Reproductive Factors and Colorectal Cancer; Identification and Characterization of β-catenin *(CTNNB1)* Gene and Modulatory potential of Taxifolin and Nano-particle with Vitamin D (NVD) against Colorectal Cancer in Pakistani Population" was conducted by **Mr. Suhail Razak** under the supervision of **Dr. Sarwat Jahan**. No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Department of Zoology of Quaid-i-Azam University, Islamabad in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Field of Human Genetics.

Student Name: **Mr. Suhail Razak** Signature: Suhail

Examination Committee:

a) External Examiner 1:

Dr. Allah Nawaz
(Ex-Principal biochemist)
H. No. 8, (FF) St. No B-17, F-Block,
Naval Anchroge, Islamabad

Signature:

Sultana
18-06-2020

b) External Examiner 2:

Dr. Tayyaba Sultana
Professor, Department of Zoology,
GC University, Faisalabad

Signature:

Supervisor Name: **Prof. Dr. Sarwat Jahan** Signature: Sarwat Jahan

Name of HOD: **Prof. Dr. Sajid Malik** Signature:

Date: 18-06-2020

IN THE NAME OF ALLAH, THE MOST COMPASSIONATE, THE MOST MERCIFUL,

“On the earth are diverse tracts, adjoining one another: vineyards and cornfields and groves of palm, the single and the clustered. Their fruits are nourished by the same water; yet We make the taste of some excel that of others. In this also are signs for people who understand.”

(Sura Al Ra’d, Ayat 4)

Author's Declaration

I **Mr. Suhail Razak** hereby state that my Ph.D. thesis titled "Association Between Reproductive Factors and Colorectal Cancer; Identification and Characterization of β-catenin *(CTNNB1)* Gene and Modulatory potential of Taxifolin and Nano-particle with Vitamin D (NVD) against Colorectal Cancer in Pakistani Population" is my own work and has not been submitted previously by me for taking any degree from Quaid-i-Azam University, Islamabad, Pakistan.

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I solemnly declare that research work presented in the thesis titled "Association Between Reproductive Factors and Colorectal Cancer; Identification and Characterization of β-catenin *(CTNNB1)* Gene and Modulatory potential of Taxifolin and Nano-particle with Vitamin D (NVD) against Colorectal Cancer in Pakistani Population" is solely my research work with no significant contribution from any other person. Small contribution / help wherever taken has been duly acknowledged and that complete thesis has been written by me.

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Dedication

I dedicate my dissertation work to my parents for their support, encouragment and for earning an honest living for us.

To my supporting wife, who assisted me in every aspect of my life, taught me to trust in Allah, believe in hard work and affection and love.

I dedicate this work and give special thanks to my friend Dr. Asad Ullah, who have supported me throughout the process.

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Suhail Razak

- **LIST OF ABBREVIATIONS**

Full name	Abbreviations
Adenomatous polyposis coli	(*APC*)
Alpha	(α)
Beta	(β)
Bovine serum albumin	(BSA)
Cancer stem cells	(CSCs)
Casein kinase 2	(CK2)
Chromosomal instability	(CIN)
Colorectal cancer	(CRC)
Cpg Island Methylator Phenotype	(CIMP)
C-terminal binding protein	(CtBP)
Deoxyribonucleic acid	(DNA)
Diabetic cardiomyopathy	(DCM)
Diamminnobenzidine	(DAB)
Dihydrotestosterone	(DHT)
Dimethyl hydrazine	(DMH)
Dimethyl sulfoxide	(DMSO)
Diphenylamine	(DPA)
Distyrene, plasticizer, and Xylene	(DPX)
EGF receptor	(EGFR)
Enzyme linked immunosorbant assay	(ELISA)
Epidermal growth factor	(EGF)
Epithelial– mesenchymal transition	(EMT)
Ethylenediaminetetraacetic acid	(EDTA)
Extracellular-regulated kinase	(ERK)
Familial adenomatous polyposis	(FAP)

Fifty percent inhibition concentration …….. ...(IC_{50})

Follicle stimulating hormones …….. ...(FSH)

Formalin-fixed paraffin-embedded…….……..…….. (FFPE)

Glycogen synthase kinase 3-B ……..….....(GSK3)

Hazard ratio…….…….…….. (HR)

Hematoxylin and Eosin ……..... ..(H&E)

Heme Oxygenase…….....(HO-1)

Hereditary non-polyposis colorectal cancer…….…….. (HNPCC)

Hormone replacement therapy…….....….. (HRT)

Horse Reddish peroxidease…… …….....….. (HRP)

Human serum albumin……..……..…..... (HSA)

IGF-binding proteins……... ….(IGFBP)

Inflammatory bowel disease……..…..…….. (IBD)

Institutional Review Board …….……...…….....… (IRB)

Insulin growth factor 2……...……..… (IGF2)

Janus Kinase ……..……..…….(JAK)

Kilogram ……..…….…..... …(Kg)

Liquid cover slip……..……..…..…….. (LCS)

Loss of heterozygosity ……..…(LOH)

Luteinizing hormones ……...……..….. ...(LH)

Magnesium chloride.……..... ($MgCl_2$)

Manosonication…….…….....….. (MS)

Mano-thermosonication……..…..…….…..... (MTS)

Micro gram……..……..…….…… (µg)

Micro liter ……..……..…….…..... (µl)

Micromolar ……..……...……..(µM)

Microsatellite Instability …….…….....…..(MSI)

Microsatellite instability……..... (MSI)

Milimeter …….…….....…(mm)

Milli gram …….....…. (mg)

Milli molar……..…..... (mM)

Milliliter ……..…..…...…...(ml)

Mismatch repair ……..... (MMR)

Mitogen-activated protein kinase...……...……. ……(MAPK)

Molar..……..…..…..… (M)

Molecular dynamic……..…...……..... (MD)

Multidrug resistance 1…….……..... (*MDR1)*

Myeloperoxidase……..……..…… ..….(MPO)

Nanoparticle plus vitamin D …….…….....(NVD)

Nuclear accumulation……..……..…..…….….(NA)

Ornithine decarboxylase…..……..……...……..... (ODC)

Peroxisome proliferator-activated receptor ά ……...……..... (PPARά)

P-glycoprotein-1……..…….…….. (PGP-1)

Phosphatase and Tensin homolog…….…….….... (PTEN)

Phosphate buffer saline …….…….…….…..... (PBS)

phosphoinositol kinases……..……..……..... (PI3K)

Poly (ADP-ribose) polymerase……..……..…….....….... (PARP)

Polymerase chain reaction…….…….…….....… (PCR)

Reactive oxygen species…….……..…..…..... (ROS)

Revolution per minute ……..……...…..… (rpm)

Root mean square deviation ……..…..……..……... (RMSD)

Root mean square fluctuation……..……..……..... (RMSF)

Signal Transducer and Activator of Transcription…… .(STAT)

Thiobarbituric acid…….……....….. .(TBA)

Sodium acetate …….…….. ….(NaAc)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis…….. (SDS-PAGE)

Sodium dodecyl sulphate …….…….……...… (SDS)

Streptozotocin……..…….……...….(STZ)

Superoxide dismutase …….……...…. (SOD)

Taxifolin …….…..... ….(TAX)

T-cell factor/lymphoid enhancer-binding factor…….. ….(TCF/LEF)

Testosterone albumin conjugates……...…….....(TACs)

The Chromosomal Instability Pathway……..(CIN)

Thermosonication……..…….…….……(TS)

Transducin containing protein1……....…..(TrCp1)

Transforming growth factor……..(TGF)

Tris buffer saline with tween…….…….……...(TBS-T)

Tumor protein53……..…(TP53)

Ultra violet …….…….....(UV)

Vitamin D receptor……...…….…...…..... (VDR)

WHI Observational Study ……...…….…..... (WHIOS)

Women's Health Initiative…….…… …...(WHI)

World Health Organization…….…… ..…..…. (WHO)

β -catenin gene……...……..... ...…(*CTNNB1*)

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Abstract

Abstract

Introduction: Colorectal cancer (CRC) is categorized by alteration of vital pathways such as β-catenin *(CTNNB1*) mutations, *WNT* signaling activation, tumor protein 53 (*TP53*) inactivation, *BRAF*, Adenomatous polyposis coli (*APC*) inactivation, *KRAS*, dysregulation of epithelial to mesenchymal transition (*EMT*) genes, *MYC* amplification etc. The assorted molecular outlines of colorectal cancer (CRC) and the necessity to categorize patients which could efficiently take clinical benefit from combined chemotherapies ignited the categorization of the mechanisms accountable for sensitivity and confrontation to treatments. More than the precedent two decades, the question of whether vitamin D has a role in cancer frequency, development, and transience has been premeditated fully. Colorectal, breast, and prostate cancers have been a scrupulous spot of center, in concert, these three malignancies report for approximately 35% of cancer cases and 20% of cancer demises in the United States, and as such are a chief public health apprehension. In the present study an attempt was made to screen *CTNNB1* gene in colorectal cancer samples from Pakistani population and investigated the association of *CTNNB1* gene mutations in the development of colorectal cancer. Data on 25-OH VD concentrations and the associated factors in colorectal cancer (CRC) patients are scarce and need to be investigated and to evaluate and equate antitumor activity of taxifolin (TAX) and nanoparticle plus vitamin D (NVD) in colorectal cancer cell lines and HCT116 xenograft model in a comprehensive approach.

Materials and Methods: 200 colorectal tumors approximately of male and female patients with sporadic or familial colorectal tumors and normal tissues were included. DNA was extracted and amplified through Polymerase chain reaction (PCR) and subjected to exome sequence analysis. Immunohistochemistry was done to study protein expression. Molecular dynamic (MD) simulations of $CTNNB1^{WT}$ and mutant S33F and T41A were performed to evaluate the stability, folding, conformational changes and dynamic behaviors of CTNNB1 protein. A total of 200 CRC patients participated in this cross-sectional study conducted in Pakistan. Socio- demographic and other health data were collected in a pretested questionnaire. Serum measurements of Vitamin D (1, 25(OH)2 D3) levels and hormones were performed. Association of age, sex, primary site, and stage of disease and effects of hormone therapy and selected reproductive health indicators on vitamin D status were initially investigated by univariate analysis**.** Two human colorectal cancer cell lines HCT116 and HT29

(gained from College of Pharmacy, King Saud University, KSA were grown. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazoliumbromide protocol were performed to show the impact of TAX, NVD and β- catenin inhibitor (FH535) on the viability of HCT116 and HT29 cell lines. Apoptosis /cell cycle assay was performed. Analysis was done with a FACScan (Becton Dickinson, NJ). About 10,000 cells per sample were harvested and Histograms of DNA were analyzed with ModiFitLT software (verity Software House,ME,USA). Western blotting and RT-PCR were performed for protein and gene expression respectively in *in vitro* and *in vivo.*

Results: Sequence analysis revealed two activating mutations (S33F and T41A) in Exon 3 of *CTNNB1* gene involving transition of C.T and A.G at amino acid position 33 and 41 respectively (p.C33T and p.A41G). Immuno-histochemical staining showed the accumulation of β-catenin protein both in cytoplasm as well as in the nuclei of cancer cells when compared with normal tissue. Further molecular modeling, docking and simulation approaches revealed significant conformational changes in the N-terminus region of normal to mutant *CTNNB1* gene critical for binding with Glycogen synthase kinase 3-B (GSK3) and transducin containing protein1 (TrCp1). The blood samples of 200 colorectal cancer patients, males and females were taken under consideration. The mean age of the population was 55.3 years (±15.6; Range: 20-90 years). Estradiol concentration were significantly higher in young female as compared to young male patients ($p<0.001$). The concentrations of FSH, LH testosterone and estradiol were significantly lower in post-menopausal female CRC patients as compared to their male counterparts of old age (p, for all trends <0.05). Both LH and FSH showed significant gender difference only in older ages. Level of estrogen is markedly decreased in older post-menopausal CRC patients compared to premenopausal CRC cohorts, which might be associated with CRC progression. In the group of women who ever used hormone therapy had differences of statistical significance (p, for all trends <0.05) in their mean serum 25-OH VD concentrations, while in the group of women who never used hormone therapy had non-significant differences in their mean serum 25-OH VD concentrations (p, for all trends>0.05). High 25-OH VD concentrations were observed in women who had their menarche at the age 15 years or more. Nulliparous women had the highest mean 25-OH VD concentrations as compared to unparious or multiparious women. Women having their menopause at 40-44 years of age had the highest 25-OH VD concentrations, although the difference was not significant ($p=0.08$). Women who never used any oral contraceptive had higher 25-OH VD concentrations as compared to those who ever

used oral contraceptives. We found that TAX and NVD induced cytotoxicity in colorectal cells in a dose-dependent manner and time dependent approach. Further, our data validated that TAX and NVD administration of human colorectal cancer HCT116 and HT29 cells resulted in cell growth arrest, alteration in molecules regulating cell cycle operative in the G2 phase of the cell cycle and apoptosis in a dose dependent approach. Further our results concluded that TAX administration decreases expression of β-*catenin* gene, *AKT* gene *and Survivin* gene and protein expression in *in vitro* and *in vivo*

Conclusion: Screening of Pakistani population revealed association of two non-synonymous polymorphisms in CTNNB*1 gene* with colorectal cancer. These genetic variants led to the accumulation of *CTNNB1*, a hallmark of tumor development. Also analysis of structure to function alterations in *CTNNB1* gene is crucial in understanding downstream biological events. The results of this analysis support a role for hormones in 25-OH VD concentrations. Further prospective studies that directly evaluate levels of circulating hormones and hormone therapy in women in relation to 25-OH VD concentrations as well as their possible role in colorectal cancer risk would be highly informative. Also our findings suggest that targeting *β-catenin* gene may encourage the alterations of cell cycle and cell cycle regulators. Wnt/ *β-catenin* signaling pathway possibly takes part in the genesis and progression of colorectal cancer cells through regulating cell cycle and the expression of cell cycle regulators.

Keywords: Wnt/ β-catenin; Taxifolin, cell cycle. CTNNB1, Colorectal cancer, Immunohistochemistry, Vitamin D (1, 25(OH)2 D3), sex hormones.

Chapter 1
Introduction

1. Introduction:

The all-inclusive frequency of colorectal cancer (CRC) is third amid tumor proportion of relapse in males as well as fourth amid females (Jemal et al., 2010, Parkin et al., 2005). Furthermore, colonic adenocarcinoma versions aimed at 37–45% of entirely metastatic ovarian cancers. Although turn down in the demise rates was seen aimed at colorectal cancers from 2000 to the existing and in spite of progresses in program and surgical conduction, no antidote has been revealed for metastatic cancer. The 5-year continued existence frequency is regretfully low (about 8%). Such startling ineptitude of anti-cancer standard therapies has been characterised towards the survival of comparatively exceptional, exceedingly resistance to drug, inert or sluggish reproducing cells with stem-similar possessions: cancer stem cells (CSCs). Contemporary validations considers cancers as complex assorted organ-like systems with a hierarchical cellular organization, more willingly than simply as assortments of homogeneous tumor cells (Dalerba et al., 2007, Mimeault et al., 2007). Colorectal cancer arises during the continuing accrual of amendments in cancer related genes in addition to tumor suppressor genes. The accretion of alterations frequently occur due to cumulative consequences of numerous epigenetic modifications and genetic mutations concerning genes that standardize cell expansion as well as segregation (Chung and Fleshman, 2004).

1.1 Aetiology

Onset of CRC is allied to the mutations in particular genes, as occur in other sort of cancer. Usually the mutations arise in genes allied to DNA repair systems, tumour suppressor genes, and oncogenes (Fearon and Vogelstein, 1990). Colorectal carcinomas have been categorized, on the basis of origin of mutation as familial, sporadic, and inherited. Point mutations, emerging for a period of life, are not linked with innated syndromes. They merely involve particular cells and their inheritors. Sporadic cancers are consequences of point mutations and comprises about 70% of the entire CRC. The cellular pathogenesis of infrequent cancer is assorted as transformations could aim diverse genes (Fearon and Vogelstein, 1990). On the contrary, majority of CRC cases

(70%) pursue a precise series of mutations which are decoded into a detailed morphological sequence, leading to the adenoma and concluding in the carcinoma state.

The second category- inherited cancers share 5% of all colorectal cancer cases. Inherited mutations manipulate mutated gene's one of the alleles. In the additional allele, Point mutation resolve prompt the specter of the tumor cell and consequently, the carcinoma. Inherited cancers are further sub- categorized into two classes, polyposis & non-polyposis types. The polyposis deviations primarily occupy familial adenomatous polyposis (FAP). FAP being distinguished through the expansion of frequent prospectively malicious growths in the colon (Lynch and De la Chapelle, 2003). On the contrary, the mutations in DNA repair systems are allied to HNPCC. Lynch syndrome being the foremost origin related to HNPCC, arises by innate mutations in proteins responsible for DNA repair for example PMS1, PMS2, MLH6 MSH2, & MLH1. Of all CRC cases, Lynch syndrome accounts 3% thus, considered most frequent syndrome in HNPCC class (Lynch and De la Chapelle, 2003, Umar et al., 2004). Approximately 24% CRC diagnosed cases fall under familial colorectal cancer arised by inherited mutations (Stoffel and Kastrinos, 2014).

1.2 Colorectal Cancer and Molecular pathways

Genomic instability is crucial characteristic contributory colorectal cancer. The pathogenic mechanisms that results in CRC have been categorized into diverse pathways, namely CpG island methylator phenotype (CIMP), microsatellite instability (MSI), and chromosomal instability (CIN).

1.3 The Chromosomal Instability Pathway (CIN)

The chromosomal instability corridor remains cutting-edge recognized as classical pathway as it accounts the origin of 80% to 85% of all colorectal cases (Grady and Carethers, 2008), is exemplified by discrepancies in the chromosomal number, resulting to loss of heterozygosity (LOH) and aneuploydic tumours. The engaged systems of CIN involve amendments in DNA damage, chromosomal segregation, and telomere dysfunction, influencing crucial genes engaged in the continuance of approved cell activity for instance TP53, PI3K, KRAS, and APC, among additionals. The transfer of beta-catenin to the nucleus results due to APC mutations and impel the gene transcription

associated in tumourigenesis and incursion, while *KRAS* and *PI3K* mutations results in unvarying commencement of MAP kinase, hence inclining advancement of cell. In the cell cycle, the loss-of-function alterations cutting-edge TP53 encoding p53, the crucial cell-cycle patternsocket, results an uninhibited access (Pino and Chung, 2010).

1.3.1 Microsatellite Instability (MSI)

Besides chromosomal Instability pathway, about 12–16% of sporadic colorectal cancer are owing to the MSI. Microsatellite instability pathway is the state of genetic hypermutability acquiring due to damaged DNA mismatch repair (MMR). In evident definition, Microsatellite instability is the phenotypic confirmation that DNA mismatch repair is performing defectively (Armaghany et al., 2012). The capability to restore short DNA chains or tandem repeats is diminished in malignancies through micro satellite Instability consequently; mutations have a tendency to mount up in those areas. Codifying microsatellites as well as non-coding areas are influenced by these mutations and tumors progresses. These mutations can influence both codifying microsatellites and non-coding regions. Consequently, leads to tumor progression subsequent to alteration in the tomor suppressor genes codified in microsatellite or reading frames of oncogenes. Due to germinal mutations or natural occurrences (promoter hypermethylation) as occur in Lynch syndrome, results in Loss of expression of mismatch repair genes (MMR). This type of tumors is primarily diploid and anchorage fewer Loss of heterozygosity. PMS1, PMS2, MSH2, MSH6, and MLH2 are mutant genes in tumors woth microsatellite instability (Lynch and De la Chapelle, 2003, Boland and Goel, 2010).

1.3.2 Cpg Island Methylator Phenotype (CIMP).

Epigenetic instability being featured accountable for the CIMP, which is said to be frequent trait in CRC. Hypermethyaltion of oncogene promoters is considered as major feature of CIMP tumors that results to loss of protein expression and genetic silencing. Both genetics and epigentics collaborate in the progression of CRC, with additional methylation proceedings than point mutations frequently being found (Lao and Grady, 2011). In progression of CRC, joint operation of genetics and epigenetics can be

observed in *BRAF* mutations and MSI in various CIMP tumors (Weisenberger et al., 2006).

1.4 β-Catenin (*CTNNB1*) Gene

The gene encompasses 23.2kb of DNA, consists of 16 exons with mRNA transcript around 2343 bp. In humans, the *CTNNB1* gene encodes 781 amino acid residue CTNNB1 protein (Kraus et al., 1994, MacDonald et al., 2009), whereas, the homologous protein in Drosophila being recognized equally armadillo (Peifer et al., 1991). Beta-catenin (β-catenin) being multitask, 90 kD protein, entailed in check and control of cell expansion in usual physiological state (Lien and Fuchs, 2014). β-Catenin being vital transcriptional feature in Wingless–Int (Wnt) signaling. Also has crucial function in stem cell regeneration as well as tissue revival (Trompouki et al., 2011). Also β-catenin is crucial an oncogenic transcription factor acts only after restricted in the nucleus (REF). The CTNNB1 protein is a membrane of multifarious proteins, frame up adherens junctions (AJs). These AJs are vital for the epithelial cell layer formation and regulation by controlling cell expansion and cell to cell linkage. The CTNNB1 protein also fastens the actin cytoskeleton and assist in passing the adhesive reticence signal that permit cells to inhibit reproduction as the epithelial sheet is inclusive. At verge, CTNNB1 protein adheres to the *APC* gene members, which are mutant in CRC. *CTNNB1* gene mutations changes the amino-tail section of the CTNNB1 protein craft it recalcitrant to directive by APC (Morin et al., 1997, Rubinfeld et al., 1996).

1.4.1 Function of *CTNNB1* gene in Wnt/APC/β-Catenin Signaling in CRC

The Wnt/ APC/β-catenin corridor emerges as the key player in CRC carcinogenesis in sporadic as well as in hereditary CRC. APC mutations (80%) are moreover nonsense mutations or frameshift consequential in the creation of a curtailed protein (Filippo et al., 2002, Powell et al., 1992, Cottrell et al., 1992**).** The *APC* gene usually obstructs shift to S from G1 cell cycle phase. The APC/Wnt corridor sustains indigenous stem cells in their undistinguished phase in the colonic crypts, taking crucial part regarding continuation of standard stem cells. Also to the endurance of tumor stem cells. Wnt signaling pathway and β-catenin are crucial to each other. Unmutated APC encourages deprivation of β-

catenin. Consequently, function like a depressing watchdog of the Wnt signaling pathway (Polakis, 1997, Kinzler and Vogelstein, 1996)**.** Phosphorylation/degradation of β- catenin in cytosol and its check and control via Wnt being the hub of cascade related to Wnt. Axin attaches with CK1α, GSK3, and β-catenin by utilizes special domains and synchronizes chronological β-catenin phosphorylation by serine 45, serine 33 & 37, as well as threonine 41 by CK1α, and GSK3 respectively (Kimelman and Xu, 2006b). This phosphorylation at serine 33 & 37 crafts an interacting spot for the ligase β-Trcp, consequences in ubiquitination as well as deprivation of β-catenin. The discrepancies of β-catenin around or by threonine & serine residues generates mutant β- catenin that flee phosphorylation & dilapidation in colorectal cancers. Thus resulting. APC interacts with controller of G protein signaling domain of Axin forming APC-AXIN complex and this complex allocates combine goal of phosphorylation and dilapidation of β-catenin (Kimelman and Xu, 2006b). Unmutated APC encourages deprivation of β –catenin. Thus acts like a pessimistic monitor of the Wnt signaling corridor (Kinzler and Vogelstein, 1996, Morin et al., 1997). Commencement of the Wnt corridor is extended in APC mutated colorectal tumor cells through retained intensities of intacellular β-catenin. The sustained commencement of Wnt signaling corridor is allied with hypermethyalation of *APC* gene promoter in CRC subjects through normal APC gene as well as point mutation within β -catenin structure (Morin et al., 1997).

Usually stem cells exit the epithelial sepulchers in normal colonic mucosa, due to their differentiation and are consequently swamped off 72 hours post apoptosis. This drifting activity is structured by the β-catenin. In normal people, various cells attain a range of mutations through replication and segregation. Though, because they are usually discarded within a week, they have no occasion to tempt cancer. Accrual of β-catenin in enterocyte predecessors owing to Adenomatous polyposis coli inactivation results preservation related to the phenotype of a stem cell that may hampering shifting to the shell towards stand boged off. The growth of unsegregated cells within colonic crypts ultimately results in development of polyps (Samowitz et al., 2001).

1.5 Colorectal Cancer and Reproductive Hormones

The purpose of the sex variances finished decades, in countless racial mannerisms & entirely the planet exposed that sex hormones of both men and women illustrate a vibrant part in the pathogenesis of CRC & the roles are comparatively defensive (Hendifar et al., 2009, Siegel et al., 2011). Shielding function of the estrogen corridor in progression of colon cancer is revealed and recognized in various animal subjects. Numerous observations regarding estrogen therapy to rats has revealed declined pace of colon tumors (Guo et al., 2004, Smirnoff et al., 1999, Weyant et al., 2001). Hormone replacement therapy (HRT), since more than decade, granted the prospect to explore the character of this intrusion in preclusion and conclusion of CRC. Statistics from various potential and retrospective units sustain a defensive function for HRT in the progression and conclusion of CRC (Kim et al., 2005, Qi et al., 2011). It has been illustrated recently that testosterone, the important male androgen dependable for the progression of secondary sex features and spermatogenesis raises susceptibility of attaining CRC in males. Different observations hence sustain the impression that sex hormones may provide shielding effect beside colorectal cancer or enhance the progression of colorectal cancer as revealed with testosterone (Amos-Landgraf et al., 2014).

1.5.1 Effect of Estrogen in CRC

Estrogens, the associates of steroid hormone family being conventionally allied with the female reproductive expansion. Estrogens role in CRC is under consideration with bottomless attention particularly in context to the patho-physiology of breast, endometrium, prostate cancers, and CRC (Arnal et al., 2013). The accurate function mechanism of estrogen in CRC is still a controversial topic (Foster, 2013). In the natal pathway of CRC, studies have proven that endogenous estradiol have a crucial part. The flowing intensity of estradiol are allied among an elevated tumor possibilities (Gunter et al., 2008). Estrogen prejudices the mucosal retort to provocative injury in colitis. Hence encourages irritation-allied tumor progression (Heijmans et al., 2014). 17β-estradiol (E2) is the copious and in human's utmost effective estrogen. Usual recognization of esterogen by its receptor lies on its 3D pattern and charge. The estrogen receptor distinguish a

fragment as estrogen rely on its charge and 3D alignment, but E2 binding is preferred concluded E1 (3X) and E3 (2X) (RUH et al., 1973). Normally estrogen manufacturing and metabolism is oriented to tissues and is unrelated in colon in contrast to breast tissue (English et al., 2001). The discovery of ESR1 (ERα) and ESR2 (ERβ), have exposed their crucial role in different tissue types like in gastrointestinal system (Gustafsson, 1999). In normal and malignant colonic epithelium, ER β is the major expressed estrogen receptor (Elbanna et al., 2012). ER β shows reduced expression during colonic tumorigenesis, allied with grade and stage of disorder. The converse association amid its expression and tumor development has been observed in different subjects (Konstantinopoulos et al., 2003, Foley et al., 2000, Thomas and Gustafsson, 2011, Rudolph et al., 2012, Jassam et al., 2005). Thus it is presupposed that estrogen-arbitrated signaling exercise a defensive part in CRC. Additional perceptive of this may promote cancer preclusion and grant further restorative selections for ER β encouraging cancers. The confirmation for this anti-tumorigenic consequence has been revealed from a plethora of diverse studies (Hartman et al., 2009).

1.5.2 Effect of Testosterone in Colorectal Cancer

Testosterone is most crucial androgen physiologically in both sexes but have significant function in males in the maturity of reproductive organs. Recent studies have revealed that testosterone boost inclination of attaining CRC in men. These observations were strengthened by various observations which showed that orchidectomised mice encompass a minor jeopardy of mounting CRC in contrast to controls. Besides that, female mice with ovarectomy illustrated higher jeopardy of mounting colorectal cancer when compared with female mice without ovarectomy (Amos-Landgraf et al., 2014). These observations sustain the impression that sex hormones may provide shield, aligned with CRC or encourage the progression of CRC as revealed in testosterone alterations. Furthermore, the currently revealed mARs are considerable. The creation of these receptors takes place prominently in CRC and no creation in normal colonic mucosa. Additionally, commencement of mARs by testosterone-HSA conjugates activates

apoptotic corridors that tender shield, aligned with CRC. mARs may reveal significant corridors for pharmacologic analysis in CR neoplasms (Gu et al., 2009, DeLellis Henderson et al., 2010, Anagnostopoulou et al., 2013). Tumor persistentness is controlled by Akt, and controls the maneuverability of colonic cancer cells & defends respective persistentness. Often actions of Akt are triggered by HAS-testosterone, thus hampering tumor progression and metastasis (Yu and Grady, 2012).

1.5.3 Colorectal Cancer and Hormone Replacement Therapy.

An outsized meta-analysis performed by Grodstein et al.,(1999) revealed that HRT was allied with a declined danger of CRC of 35% approximately (Grodstein et al., 1999). Women's Health Initiative (WHI) Clinical Trial auxiliary strengthened their involvement (Rossouw, 2002, Chlebowski et al., 2004), where involvement with oestrogen plus progestin results a 45% decrease in colorectal cancer, whilst only oestrogen showed no concern CRC risk. Also study conducted by the California Teachers exposed the jeopardy for CRC was 37% diminished among HRT users and the consequences did not vary by formulation (DeLellis Henderson et al., 2010). Other meta analysis revealed a 20% decrease in CRC frequency among females having ever used hormone replacement therapy, and period of therapy use did not control danger approximations (Nelson et al., 2002). Consequently, though epidemiological data sustain a shielding consequence of HRT on colorectal cancer, the links connecting diverse amalgamations of hormone replacement therapy and colorectal cancer danger remain veiled.

1.6 Vitamin D Intervention with WNT/β-Catenin Cascade and association with various Signaling Pathways

Vitamin D impedes with several signaling corridors that might partially arbitrate its antitumoural bustle. An assorted interpretation has demonstrated that Vitamin D hampers the WNT/ β -catenin corridor.Also revealed its role in hindering the commencement of its candidate genes in colorectal cells, participating in inhibition of cell propagation and upholding of the distinguished phenotype(Pálmer et al., 2001, González-Sancho et al., 2011). WNT/ β-catenin pathway is hampered by vitamin D via numerous mechanisms. Primary, it swiftly raises the sum of VDR attached to β-catenin, therefore tumbling the

association connecting the transcription elements of the TCF/LEF family and β- catenin hence resulting towards the suppression of respective candidate genes (Pálmer et al., 2001). It was demonstrated the β-catenin/ vitamin D receptor association & exemplified the involved protein domains, whereas other study illustrated that wildtype APC boosts the reticence of β-catenin/TCF transcriptional effect by Vitamin D (Shah et al., 2006, Egan et al., 2010). Subsequently, allied to E-cadherin increase at the plasma crust adherens intersections, Vitamin D persuades β-catenin nuclear export (Pálmer et al., 2001). Lastly, it persuades the DKK1 expression (an extracellular WNT inhibitor) (Aguilera et al., 2007). Besides that, by THP-1 monocytic leukaemia cells, Vitamin D hampers the creation of interleukin1β. Therefore may symbolize one more system of prevention of β-catenin/TCF-reliant gene commencement in CRC cells if extrapolated to tumour-allied macrophages, as IL1 β hampers phosphorylation of β-catenin & tagging aimed at deprivation by Glycogen synthase kinase 3β (Kaler et al., 2009). Therefore, Vitamin D applies a composite match up of dogmatic activities results in prevention of the WNT/ β-catenin cascade. Meanwhile the corridor is deviantly triggered in essentially adenomas. CR tumors also, known as the chief element in respective neoplasia. Its reticence is doubtless critical with respect to anti-tumoural activity of VitaminD in CRC. Moreover, VitaminD alerts colorectal cancer cells to the anti-progression activity of TGF-β through elevating type I receptor (TGFBR1) expression (Chen et al., 2002). Furthermore, Vitamin D hampers the encouragement of cell propagation by EGF concluded the attenuation of EGFR appearance. Also hinders the stimulation of its internalization after ligand binding (Tong et al., 1999). Vitamin D might also hamper EGFR action by improving the E-cadherin expression (Pálmer et al., 2001) and by suppressing that of SPROUTY-2 (Barbáchano et al., 2010), which are uncooperative and confirmatory regulators of EGFR effect, respectively (Andl and Rustgi, 2005, Cabrita and Christofori, 2008). Similarly, Vitamin D hinder the progression-stimulatory activity of IGF2 via the reticence of insulin growth factor 2 discharge & the enunciation of the countenance of various genes encrypting IGF-binding proteins (IGFBP) (Pálmer et al., 2003, Oh et al., 2001).

1.7 Risk Factors of Colorectal Cancer.

Globally, prospective misery from colorectal carcinoma is about 5%–6%. Furthermore, numerous individual behaviors or practices are measured to be jeopardy features as they boost the possibilities of mounting polyps or CRC. Age is considered as the chief jeopardy feature for CRC. Since precedent the fifty years of life, the jeopardy of mounting CRC is patently inclined, though the initiation of CRC is rare in age group below the fifty (distant from inherited cancers (Levin et al., 2008). Besides to age, numerous innate jeopardy factors are present that are not possible to tailor. Studies revealed that patients suffering from ulcerative colitis & Crohn's disease eminenting jeopardy of mounting CRC by 2.5% and 3.7% respectively (Eaden et al., 2001, Canavan et al., 2006). In inflammatory bowel disease (IBD), the chronic inflammation give rise to dysplasia and the dysplasia have possibility of becoming anaplastic and emerging into a tumor. Familial history of CRC, particular, the relatives diagnosed CRC below age of fifty years is considered as another risk factor. An inclined jeopardy because of ancestral history may be consequent from innate mutations or the environment (Johns and Houlston, 2001).

Various jeopardy elements, allied to lifestyle, can be abridged by employing self-effacing lifestyle transforms related to dietary and physical activity habits. Such as, sedentary lifestyle is considered as increasing jeopardy of mounting CRC (Robertson, 2012). A sedentary lifestyle and obesity are allied with each other, and obesity is one of the jeopardy issues of colorectal cancer. Outstandingly, this amplified jeopardy is associated to eating and inclined intensity of visceral adipose tissue (VAT). This may encourage the growth of CRC via discharging proinflammatory cytokines, resulting to the provocative state within the rectum as well as in colon, inflection of metabolic enzymes (adiponectin or lectin), and insulin confrontation (Martinez-Useros and Garcia-Foncillas, 2016). In this perspective, nutrition is robustly allied to the jeopardy of CRC such that unhygienic dietary practices boost the possibilities of mounting CRC by almost 69.9% (Willett, 2005). In intestines, by lipoperoxidation releasing of heme group by red meat boosts the

development of carcinogenic compounds (genotoxic aldehydes and N-nitroso compounds, cytotoxic) (Bastide et al., 2011).

Moreover, the other jeopardy factors of CRC are alcohol consumption and smoking. With respect to hard drink consumption , acetaldehyde (metabolite of ethanol) is considered as carcinogenic by enhancing the jeopardy of CRC, amongst people relying on polymorphisms of enzymes related to alcohol metabolism (Pöschl and Seitz, 2004). Tobacco smoking, sequentially, can enhance the probability of misery from CRC by almost 11% because of high nicotine, the metabolites having easy access to intestine and develop polyps (Botteri et al., 2008, Cross et al., 2014). While smoking enhances CRC jeopardy, a considerable association was revealed with prolong smokers, whether they have quitted smoking or continue the smoking (Liang et al., 2009).

1.8 Dihydroquercetin (Taxifolin) and Colorectal Cancer

Polyphenols acquired from innate sources have turn up as a novel therapeutics to shield cells aligned with oxidative stress. Plants metabolites are rich source of polyphenolic compounds (Macheix and Fleuriet, 1990). The polyphenols comprehends aromatic ring attached through single or multiple hydroxyl groups (Hansen, 1995). The polyphenols comprise various types of amalgams, commencing phenolic acids to simple and complex flavonoids, and coloured anthocyanins (Shi et al., 2005). As well to anticarcinogenic activities, every polyphenols show powerful antioxidant activity, thus declining the danger of tumors (Wenzel et al., 2000).

Certainly, majority of polyphenols have been exposed to antiproliferative, estrogenic/antiestrogenic activity, antioxidative and chemoprotective in addition persuading the detoxification of enzymes and cell- apoptosis or cycle arrest. Moreover polyphenols manage the changes in cellular signalling and host's immune system (Pandey and Rizvi, 2009). Mainly flavonoids, resveratrol, anthocyanins, proanthocyanidins, gallic acid, epigallocatechin-3-gallate, tannins, and a number of plant extorts have revealed shielding bustle in a range of tumor models (Shahidi and Ambigaipalan, 2015, Jiménez et al., 2016).

The polyphenols have been classified into three types on the bases of phenol ring and its binding charcters: the flavonoids, the stilbenes, and the lignans (Nichols and Katiyar, 2010, D Archivio et al., 2007). Dihydroquercetin being an efficient flavonoid, copiously originate in olive oil,in grapes, in citrus fruits and onion (Oi et al., 2012). While a widespread bioactive component of herbs and foods, TAX illustrated remarkable assortment of pharmacological and biochemical results, including antitumor, cardioprotective, neuroprotective effects, anti-inflammatory, hepatoprotective, and anti-diabetic. It play a magnificent task in the anticipation of Alzheimer's disease (Oi et al., 2012). Furthermore, taxifolin has an effectual anti-oxidant bustle which increases apoptosis persuaded by a range of anti-cancer agents. Taxifolin is accessible under trade name of Venorutons (semisynthetic form) (Weidmann, 2012, Psahoulia et al., 2007, Russo et al., 2003). Lately it was illustrated that taxifolin involves Nrf2-dependent pathway to encourage the expression of detoxifying enzymes and phase II antioxidant and wield a decisive shielding action against DNA oxidative damage (Liang et al., 2013). Significantly, taxifolin considerably accelerates expression of Heme Oxygenase-1 by persuading expression of Nrf2 in nuclear translocation and cytoplasm (Liang et al., 2013). The curative potent of TAX in main inflammatory disease conditions like cancer was lately estimated (Weidmann, 2012). Mainly illustrating that TAX act as a forager of myeloperoxidase (MPO)-derived RNS (Weidmann, 2012). Captivatingly,TAX diminishes BSO-induced injury to dermal fibroblasts (Skaper et al., 1997). Additionally, recently structured study, emphasized that TAX was competent to downregulate the collagenase I (MMP-1) in UVB-treated skin cells (Weidmann, 2012). In addition, taxifolin illustrated inhibitory action against oxidative enzymes and the surplus assembly of reactive oxygen species, consequently resystematizing cerebral the ischemia–reperfusion injury (Voulgari et al., 2010).

1.9 Novel Treatment Diagram for CRC

The selection of first-line management for Colorectal cancer subjects presently engages a multimodal trend dependent on tumor-related distinctiveness (like, figure & localization of metastases, cancer development, existence or nonexistence of biochemical indicators)

as well as subject based problems (such as co-morbidity, diagnosis, etc.). In general, most of the features are used to categorize colorectal cancer subjects into one of four dissimilar jeopardy categories that will be used to direct the behavior policy:

Group 0: Subjects showing no malignancy ailment or through resectable lung or liver metastases and deficient of deprived predictive precursors (for example reversion for the period of adjuvant behavior). In this occasion, the suggested conduct comprises of surgical resection of the metastasis. Chemotherapy usually shown adverse benefit in the overall survival.

Group 1: Subjects with prospectively resectable metastatic syndrome. These subjects are preliminarily administrated with stimulation chemotherapy to decline the ratio of the metastases & facilitate ensuing surgical resection. Suggested chemotherapy aimed at respective subjects includes cytotoxic triplet or doublet, that might be merged through anti-VEGF/ anti-EGFR approach in KRAS wild-type tumors (Van Cutsem et al., 2010, Van Cutsem et al., 2014).

Group 2: Subjects with distributed unresectable disease. Management preferred for very category of affectives will be sedative, with the major purpose of declining the indications, destructiveness and expansion of the syndrome. As such, the first-line treatment chosen ought to encourage metastatic deterioration in a squat occasion. To that end, the chosen choices frequently includes a cytotoxic doublet in amalgamation with a aimed agent (anti-VEGF or anti-EGFR strategies) (Van Cutsem et al., 2014, Van Cutsem et al., 2010).

Group 3: Subjects with unresectable disease and deficient of exhaustive or chronological treatment. In patients missing symptoms with little jeopardy of decline, the principle of the conduct will be to avoid tumor development as well as boost conduct-unrestricted life. The furthermost frequently used approaches consists of a fluoropyrimidine as cytotoxic agent blended with a natal besieged agent (Van Cutsem et al., 2014, Van Cutsem et al., 2010)".

First-line chemotherapy with comforting functions encompasses fluoropyrimidines (e.g., 5-fluorouracil (5-FU) or capecitabine) alone or combined with leucovorin (LV) or with

other cytotoxic agents, like oxaliplatin (5-FU/LV/oxaliplatin (FOLFOX) and capecitabine/LV/oxaliplatin (CAPOX)) or irinotecan (5-FU/LV/irinotecan (FOLFIRI)). The purpose of leucovor is to reduce the injuriousness of the behavior, and accumulation of cytotoxic agents has been exposed to raise the reaction frequency and development free continued existence, even though the lethal consequences of the therapy are also exaggerated (Van Cutsem et al., 2014, Venook, 2005) .

Second-line chemotherapy will be suggested to subjects with superior organ function and is chosen according to a refractory-based regimen and subject's refractory to irinotecan is with combination of oxaliplatin such as FOLFOX or CAPOX, while subjects refractory to FOLFOX or CAPOX will be cured with irinotecan monotherapy or FOLFIRI (Venook, 2005).

The most favorable period of chemotherapy therapy be contingent on each case, with three dissimilar selections existence accessible: static conduct for 3–6 months, initiation conduct monitored through a preservation conduct, or conduct pending injuriousness or growth (Van Cutsem et al., 2014) .

1.10 Objectives of the Study

1. Mutation investigation of *β-catenin gene* and *KRAS gene* in colorectal cancer patients.
2. Modeling and simulation of Mutations.
3. Study of reproductive hormones alterations in colorectal cancer patients.
4. *In vitro* Studies

- Growth impediment and initiation of apoptosis in colorectal cancer cells induced by polyphenolic compound TAX & Nano emulsion with vitamin D (NVD). Down- regulation of Wnt/ *β-catenin* signal transduction corridor.
    - Anti-proliferative effect of treatment of TAX, β-catenin Inhibitor (FH535) and NVD in HCT116 and HT29 cells.
    - Flow cytometric analysis of colorectal cancer cells after TAX and NVD treatment for apoptosis and cell cycle

- Inhibition of colony formation in HCT116 and HT29 cells after treatment with TAX and NVD.
- Down regulation of Wnt/ *β-catenin* signal transduction pathway by treatment of TAX & NVD
- Alteration in CTNNB1 protein level after TAX and NVD administration.

5. *In vivo* Studies
   - HCT116 cells xenografts in athymic mice and intraperitoneal TAX, β-catenin Inhibitor and NVD treatment.
   - Effect of TAX, β-catenin Inhibitor and NVD treatment on proliferation of colorectal cancer tissues.
   - Validation of CTNNB1 expression in CRC tissue.
   - Study of reproductive hormones alterations in colorectal cancer after treatment.

Chapter 2
Review of Literature

2. Review of Literature

Colorectal cancer (CRC) considered all-inclusive problem with a yearly frequency of roughly 1 million reports and a yearly death of more than 520,000 (Parkin and Muir, 1992). The utter number of cases will rise over the subsequently 2 decades as a consequence of aging and growth of populations all over countries. The danger for this malignancy differs from region to region and/or inside countries. The jeopardy also fluctuates among individual people depend on diet, lifestyle, and genetic factors. In Europe, CRC being second major public malignancy and subsequent foremost source of cancer demises after lung cancer, with an predictable overall incidence of 447 per 100 000 (Arnold et al., 2015, Holleczek et al., 2015). The data obtained from GLOBOCAN 2012, which was produced by the IARC demonstrated that CRC is the fourth major prevalent melanoma following breast, cervical and prostate cancer in Pakistan (Ferlay et al., 2015). In 2012 The World Health Organization (WHO) quantified with a purpose related to age-consistent demise frequency beginning colorectal malignancy remained 5.2% in Pakistan. The frequency of colorectal cancer is inclining amongst the native people of Pakistan as observed by a three-fold growth in frequency in men from 2.3% to 6.8% within roughly four years and analogous tendency in women was seen with a boost from 2.5% to 6.7% for the similar period (Amini et al., 2013, Qayyum et al., 2016). During recognized scrutiny curriculums and ensuing initial recognition and surgeries of pre-cancerous colonic polyps, the frequency of colorectal cancer as well as its allied demises have declined since approximately last two decades in the countries with the elevated colorectal cancer rate of occurrence (Jackson-Thompson et al., 2006, Rim et al., 2009). In contrast, the rate of occurrence of CRC in Pakistan showed inclined increase because of deficiency of surveillance curriculums and deficient molecular explorations, recommends a theoretically alarming circumstances mounting in the future years (Ibrahim et al., 2008).

Captivatingly, β-catenin mutations are allied with microsatellite unstable (MSI+) sporadic CRCs usually linked with failure of functional DNA mismatch repair

(Mirabelli-Primdahl et al., 1999, Shitoh et al., 2001). Studies projected with the aim of the single base substitutions experiential within β-catenin gene are consequences commencing impaired MMR, excluding specified that MSI+ tumors are exemplified by overindulgence miniature deletions as well as insertions. It is disagreed with the purpose of the base substitutions normally initiate cutting-edge β-catenin remain nope supplementary probable to arise as compared to substitutes such as frameshift changes in Adenomatous polyposis coli (Miyaki et al., 1999, Mirabelli-Primdahl et al., 1999, Shitoh et al., 2001). Microsatellite unstable cancers orginate in HNPCC. This pattern is distinguished by mutation of germline and somatic inactivation of mis match repair genes. Studies revealed with the aim of 32% of HNPCC are with β-catenin mutations usually at nonserine/ threonine residues, therefore of tentative efficient consequence (Miyaki et al., 1999). Besides, no analogous position of sporadic tumors or adenomas has been examined. Thus assessments amid the β- catenin mutations in hereditary non-polyposis CRC melanomas and sporadic lesions, and in benign against malevolent cancers, possibly will be incompleted. Several tumors, in addition, were supposed related to hereditary non-polyposis colorectal cancer subjects on ancestor record unaided and devoid of sustaining molecular information, hence left unbolt the prospect of miscategorization (Miyaki et al., 1999). Only some observations have scrutinized that mutations of β-catenin arise in colorectal adenomas, the antecedent lacerations for the majority colorectal carcinomas. An observation revealed that mutations of β -catenin are extra frequent (6/46; 12.4%) in miniature sporadic adenomas than in outsized adenomas (2/82, 2.4%) or tumors (1.4%) (Samowitz et al., 1999). These statistics lead to proposals that mutations of β-catenin possibly may be the commencing occurrence within the expansion with a division related to sporadic colorectal tumors excluding adenomas with mutations of β-catenin progress with a reduction of as compared to APC mutations (Johnson et al., 2005).

Recently it was revealed that commencement of the canonical Wnt signalling corridor in mouse ES & in human colon cancer cells could create Chromosomal Instability (CIN)

(Aoki et al., 2007, Hadjihannas et al., 2006). Expression of stable β-catenin tempts chromosomal instability in embryonic stem cells as well as in intestinal polyps of mouse.While term of foremost adverse TCF in embryonic stem cells diminishes the anaphase bridge index, an indicator supporting chromosomal instability (Aoki et al., 2007, Gisselsson et al., 2000). Mutations in *APC* results to CIN via commencement of the canonical Wnt signaling corridor. Upon encouragement of the G2/M control stage by contact of cells to Demecolcine, Wnt beckoning cutting-edge mouse embryonic stem cells inclines the portion of existing cells. Additionally,repression of Cdc2 kinase by Wnt signaling, at the G2/M checkpoint, bustle persuading apoptosis (Aoki et al., 2007, Tan et al., 2002). Through repression of cyclin-B–Cdc2, it is hence promising, that commencement of canonical Wnt-signal reliant transcription may restrain apoptosis of cells still whilst holding chromosomal deviations. It was also revealed that appearance of conductin/axin2, an objective of TCF/β-catenin-reliant transcription, encourages chromosomal instability through conciliation of the spindle crisscross (Hadjihannas et al., 2006). Wnt signalling thus encourages CIN during boosted levels of conductin.

2.1 Signal transduction in CRC

CRC occurs owing to the steady accretion of modifications in oncogenes and cancer suppressor genes. The accrual of amendments frequently occurs due to cumulative results of numerous genetic mutations. The epigenetic changes allied with genes that are responsible for cell expansion and segregation (Chung and Fleshman, 2004). These epigenetic and genetic modifications involves dissimilar corridors that control numerous biological courses vital to malignancy progression (Hanahan and Weinberg, 2011).

2.1.1 Oncogenic commencement of β-Catenin/Wnt Signaling in CRC

Tumor maglinancy of CRC usually results due to differentiation and dysfunction of the β-catenin/WNT corridor being critical for cell propagation and migration (Clevers and Nusse, 2012, Niehrs, 2012). Reports revealed that initiating event for the preponderance of colorectal tumors are the alterations in elements related towards β-catenin/wnt corridor. The commencement of the β-catenin/wnt cascade creates creation of a free,

signaling puddle of β-catenin types. A compound in the midst of associates related to lymphoid enhancer-binding factor/ T-cell factor (TCF/LEF) after entering into nucleus, starting transcription of target genes (Morin, 1999, Barker and Clevers, 2006). Beta-catenin is main player in unit-unit union by bridging the actin cytoskeleton as well as the cytoplasmic tail of cadherin's to β-catenin. Within Wnt/Wingless beckoning cascade, β-catenin is a central downstream effector that administrates progression events such as cell providence pattern, propagation, divergence, and relocation (Miller et al., 1999). *APC* gene in large number of tumors in colon cancer comprehends defects. Thus effects in β-catenin up-regulation and constitutive signaling by the TCF-β-catenin intricate (Morin, 1999). Tumors deprived of these APC mutations, have inclined intensities of β-catenin owing to NH2 terminus mutations of β- catenin resulting GSK-3β phosphorylation inhibition and ensuing dilapidation by ubiquitin-reliant proteolysis(Sparks et al., 1998). Therefore, results in activation of missense mutations of the phosphorylation sites at codons S33, S37, S45, as well as T41 of exon3 of the *β-catenin* gene. Further may condense it unwavering equally hence, not further docketed for cellular deprivation (Aberle et al., 1997). These amino acids are recognized glycogen synthase kinase 3-B (GSK-3β) phosphorylation spots with addition to a component of chain of six-amino acids, essential aimed at ubiquitination, analogous towards I-kB (Bell, 2005, Bienz and Clevers, 2000). In these amino acid residues, the mutation can engender an alleviated sort of β-catenin that are not further phosphorylated and mortified of the *CTNNB1* gene in its Exon 3. Eventually form a organised dynamic transactivation intricates, which perform to bestow to hammering of cell growth control (Brembeck et al., 2006, Chun and Wainberg, 2009). In numerous categories of tumors, the exon 3 of *CTNNB1* gene has been screened and in these four residues mutations usually exists. Hence investigations exposed that a mutation at any phosphorylation sites is enough to form a leading constructive structure of β-catenin (Barker and Clevers, 2006, Korinek et al., 1997, Morin et al., 1997, Reya and Clevers, 2005, Valenta et al., 2012). Cre-arbitrated expression of stable β- catenin, give rise to development of abundant (700-3000/mouse) polyps morphologically

analogous polps formed in the *Apc*-knockout mouse (Harada et al., 1999, Oshima et al., 1997). By comparing, dominant-negative TCFs by repression of canonical Wnt-depedent transcription reduces the propagation of colon cancer cells to facilitate mutations in Adenomatous polyposis coli (Tetsu and McCormick, 1999, Van De Wetering et al., 2002). The commencement of the canonical Wnt signaling occurred through APC silencing remains hence adequate aimed at intestinal tumor progression. Conversely, researchers suggested that *APC* mutations results to tumor progression via events other than Wnt signaling.

Adequate outline of confirmation specifies that Adenomatous polyposis coli hampers transcription of β-catenin/ T cell transcription element dependent via numerous systems. Initially, APC offers a gallows in favor of a destruction complex mutually in the midst of axin and GSK3β, encouraging phosphorylation and subsequent ubiquitin-reliant dilapidation of β-catenin (Rubinfeld et al., 1996). Then, Adenomatous polyposis coli enhances relocation of *CTNNB1* to cytosol from nucleus, diminishing total sum of nuclear β-catenin/ T cell transcription factor (Henderson and Fagotto, 2002, Neufeld et al., 2000, Rosin-Arbesfeld et al., 2003). Further, APC attaches to β-catenin, jamming the communication with T cell transcription factor (Rosin-Arbesfeld et al., 2003, Neufeld et al., 2000). In conclusion, recent observations explored that APC hinders β- catenin/ T cell transcription factor -reliant transcription via undeviating contact with a repressor complex. Adenomatous polyposis coli fastens to transcriptional repressor C-terminal binding protein (CtBP) as well as β_TrCP, creating an unwavering intricate with supplementary co-repressors HDAC1 and TLE-1 (Hamada and Bienz, 2004, Sierra et al., 2006). Since Adenomatous polyposis coli intermingle with β_TrCP at the *MYC* enhancer, aiding C-terminal binding protein arbitrated suppression of Wnt-target gene. As the C terminal half of Adenomatous polyposis coli reconciles attaching to C-terminal binding protein, truncated Adenomatous polyposis coli mutants seen in tumor might not encourage the C-terminal binding protein arbitrated transcriptional suppression (Sierra et al., 2006, Hamada and Bienz, 2004). Such mutations consequently trigger β-

catenin/TCFdependent transcription by mounting intensity of nuclear β-catenin- TCF complexes as well as by improving CtBP-arbitrated reticence of the complex (Hamada and Bienz, 2004, Sierra et al., 2006). The other key mechanism is the amendment in β-catenin's subcellular localization, in so far as dislocalization of β-catenin commencing the cell membrane impedes amid both the cancer suppressor activity as well as cell adhesionof E-cadherin, a constructive and purposeful collaborator in the cell adhesion vehicle of the catenins (Hirohashi, 1998, Van Aken et al., 2001). Epidermal growth factor (EGF), c-erbB-(Monga et al., 2002, Taipale and Beachy, 2001), Ras (Daniel and Reynolds, 1997), and c-Met are proto-oncogenic elements to liberate beta-catenin from the cell devotion complex interceding beta-catenin phosphorylation by tyrosine. The other elements are MUC1 (Baldus et al., 2004) and peroxisome proliferator-activated receptor ά (PPARά) (Lefebvre et al., 1998) that repress β-catenin allied with the cell adhesion complex via unexplained mechanism. Studies have revealed that liberate and tyrosine-phosphorylated cytoplasmic β-catenin mount up throughout cell migration, that encourages incursion and metastasis of tumors (Müller et al., 1999). In Human as well as in rodent CRCs, significantly, mutual or synergistic activities on cancer growth and series of deficiencies in both E-cadherin-arbitrated cell union furthermore to commencement of β-catenin-arbitrated signal transduction have been seen (Smits et al., 2000). Even though widespread depiction of Wnt/ β-catenin cascade presently has indistinct the spotlight on Adenomatous polyposis coli in cell adhesion and relocation. It was revealed that Adenomatous polyposis coli displays activities arbitrated by β-catenin (Heppner Goss and Groden, 2000). In drifting cells, Adenomatous polyposis coli confined toward cells forefront is reliant on reliability of microtubules. Adenomatous polyposis coli activity in cell motility is restricted in approach is recommended by this relationship, which take place through a realm of the protein dissimilar commencing the domain with the purpose of act together with β-catenin (Näthke et al., 1996). In APC min/+ mice the intestinal tissues are distinguished via are distinguished via modifications of cell relocation beside the crypt-villus axis in histologically customary, otherwise heterozygous, intestinal

segments (Mahmoud et al., 1997). Theses sepulcher cells demonstrate inclined β-catenin intensity, signifying a relationship involving amended β-catenin dysregulation and cellular relocation (Crawford et al., 1999). An circumlocutory function of Adenomatous polyposis coli in unit-to-unit communications might be imitated in transcriptional transforms of matrix-remodeling enzymes encoded by candidate genes and E-cadherin itself, that adapt motility and cell adhesion (Mahmoud et al., 1997). Thus suggested that the potential activity of β-catenin proto-oncoprotein in tumor expansion and development is associated to the connecting cell linkage and signal transduction arbitrated by β -catenin and Adenomatous polyposis coli (Groden, 2000).

2.1.2 KRAS/BRAF/ MAP kinase signaling Pathway

The mitogen-activated protein kinase (MAPK) torrent comprises BRAF & KRAS. This cascade controls cell propagation, segregation, apoptosis and senescence. KRAS, NRAS, and HRAS are components of RAS oncogenes. The major frequently mutated RAS family associate in CRC is the KRAS gene. About 40% of sporadic colorectal cancers transduce signals from the EGFR family and show mutated KRAS. Commencing the cell facade to the core, MAPK is a main cell propagation signal transduction pathway. This commencement utilizes a sequence of transitional proteins like MEK, RAS, and RAF. Fastening of ligand to epidermal growth factor receptor give rise to receptor dimerization and phosphorylation. RAS triggers the surge via phosphoinositol kinases (PI3K) in addition to RAF. Hence functions like a core dispenser of the signal. Commencement of PI3K hampers apoptosis, while RAF commencements encourage cellular propagation. The surge is entailed in the directive of cell existence, expansion signals, and incursion in malignancy. Mutations of KRAS results to constitutive alters and encourages cell propagation and existence sovereign of the the EGFR. Therefore, salutary EGFR reticence turns into unproductive, since KRAS is positioned downstream from the epidermal growth factor receptor. RAS mutations engage exon 2 & exon 3 (codons 12 and 13, codon 61 respectively). Codon 12 of exon 2 is the main codon involved, frequently by a missense mutation. Usually in the carcinoma- adenoma sequence

exemplary subsequently mutations existing in *APC* gene, KRAS mutations exist felt to arise. Cetuximab and panitummumab, the recent constructed monoclonal antibodies aligned with the extracellular domain of EGFR are unproductive in CRC caseswith alterations in exon 2 of KRAS gene usually codons 13 & 12. A study carried by Livre et al, recognized KRAS mutations in 28% of CRC cases. A reaction frequency of zero percent to cetuximab vs. forty percent in wild-type tumors was illustrated by subjects with KRAS mutations, and a median on the whole existence of 10.1 vs. 14.3 months, correspondingly (Lievre et al., 2008). KRAS being exclusively aimed within various clinical assessments, through inconsequential bustle revealed so far. A study explored that tipifarnib, a farnesyl transferase, showed statistically insignificant largely endurance benefit contrast to most excellent encouraging concern in a phase III double blinded placebo organized assessment in cases by obstinate later CRC (Rao et al., 2004). Several observations proposed to facilitate CRC patients with KRAS mutation, EGFR monoclonal antibodies donot have effectiveness. It may have unfavorable effects with declining overall endurance (Douillard et al., 2010, Van Cutsem et al., 2011). Auxiliary observations have revealed that all KRAS mutations are dissimilar. Subjects demonstrate retort to epidermal growth factor receptor monoclonal antibodies when they show transformation within KRAS G13D codon. The reaction is unhealthy like in cases with wild-type KRAS, although subjects show enhanced in general and development-free endurance (Tejpar et al., 2011).

Serine/ threonine kinases (RAF family) contains BRAF. BRAF intercedes cellular retorts to development indicators via RAF-RAS- MAP kinase corridor. 15% of sporadic CRC demonstrate stimulated mutations in BRAF and are infrequent in familial Lynch syndrome colorectal cancer. Mutations of BRAF were recognized in 4% and 40% inMSI-low and MSI-high tumors respectively (Iacopetta et al., 2006). The huge preponderance was characterized by the V600E (Val600Glu) hot spot mutation. Since it is infrequent in favor of cases among Lynch syndrome-allied colorectal cancer having BRAF transformations.

2.2 Role of phosphorylation by AKT and CK2 in β-Catenin Transcriptional Activity

Recent studies showed that β-catenin allocation are enthusiastically synchronized. The β-Catenin phosphorylation by AKT fallout during the reconfination of β-catenin commencing cell-cell conventions hence transcriptional activity of β-Catenin is amplified (Fang et al., 2007). Stimulating alterations of Wnt modules pilot toward nuclear restriction of β-catenin. Consquently leads to cancer development and progression (Giles et al., 2003). Wnt-independent signaling, nonetheless, is in addition engaged in command of β-catenin commencement and maligancy (Lu and Hunter, 2004). Insulin as well as insulin similar growth factor I & II, EGF & hepatocyte growth elements/scatter factor triggers β- catenin -LEF-1/ TCF signaling (Lu et al., 2003, Weston and Davis, 2001). In retort to insulin stimulus, PI3K-triggered AKT phosphorylates Glycogen synthase kinase 3 at Ser9, inturns result silence of GSK-3β besides intensification of β-catenin-TCF/LEF-1 transcriptional bustle (Weston and Davis, 2001). Beside this indirect regulation, studies revealed that AKT phosphorylates β-catenin *in vitro* as well in *in vivo* (Fang et al., 2007). The mutation of Ser552 of β-catenin into Asp or Ala has no effects on its phosphorylation intensity by GSK- 3β or its half-life, in dissimilarity to WT. Demonstrating that β-catenin protein stability is not modified by phosphorylating of β-catenin by AKT ar Ser552. In its place, phosphorylation at this site permits a quantity of beta-catenin to disconnecting cell-cell association leads to mount up in the nucleus. AKT phosphorylation sites mutation abridge entirety transcriptional bustle of TCF/LEF-1 tempted by AKT (approximately 58%). This fractional result of AKT on TCF/LEF-1 transcriptional bustle is expectly due to indirect AKT involvement in amplification of transcriptional bustle of β-catenin through AKT phospohosylation & basketing Glycogen synthase kinase 3β. Consequently, AKT triggers, beta-catenin -LEF-1/ TCF transcriptional activity which may over circuitous alleviation of β-catenin through basketing of GSK-3β & through phosphorylation of beta-catenin, boosting beta-catenin nuclear accrual (Fang et al., 2007). Besides to chronological phosphorylation at β-catenin

(N terminus) by CK1 and GSK β (Amit et al., 2002, Yanagawa et al., 2002).CK2 and PKA phosphorylates β-catenin at Thr393 (Song et al., 2003) and Ser675 respectively (Taurin et al., 2006). This boosts β-catenin transactivation by alleviating β-catenin protein. Inconsistently, studies illustrated that casein kinase II-dependent phosphorylation of the ubiquitin carrier protein (E2) ubiquitin conjugating enzyme UBC3B encourages the association between UBC3B and β-transducing repeat-holding protein and boost β-catenin deprivation (Semplici et al., 2002). Also studies illustrated that AKT at Ser129, is hypertriggered by casein kinase II phosphorylation. Captivatingly, alteration at Ser129 and alanine in vivo, results in a manifested decline in catalytic bustle of AKT in addition to declined phosphorylation of Thr308 in vitro (Di Maira et al., 2005). Recently in HEK-293T cells studies, demonstrated that over-expression of the analogous AKT-S129A mutant showed parallel adverse consequence on β-Catenin -conditional bustle to that pragmatic through the central pessimistic type of casein kinase II α. These results stalwartly proposed that AKT phosphorylation at Ser129 by casein kinase II may be imperative for watchdog the transcriptional bustle allied to β-Catenin. Regardless of the consequence on transcriptional bustle of β-Catenin, endogenous protein intensity showed no alteration momentously after 12h of cycloheximide administration leading overexpression of AKTS129A mutant in contrast to DMAT-administrated CHO-K1 cells (Ponce et al., 2011). Besides that in HEK-293Tcells β-Catenin reduces in nucleus whilst AKT-S129A mutant was overexpressed only or collectively among casein kinase II α. By conclusion, these studies recommend that CK2-hyperactivated AKT phosphorylate β-Catenin, may evade the axin/APC/GSK3b pessimistic dogmatic intricate. Accordingly, might amplify the restrictions in nucleus and bustle in β-Catenin transcription. The observations working on HEK-293T cells explored that expression of casein kinase II α or the application of precise inhibitors of casein kinase II alter the β-Catenin-reliant survivin expression, which results prominent transformation in cell cycle , propagation, and apoptosis (Tapia et al., 2006). Captivatingly morphological alterations were seen in HEK-293T cells administrated with casein kinase II α. Also AKT-S129A cells, signifies

the rate of apoptosis. In reality, inferior transcriptional and protein intensity of survivin were symbolizing reduced nuclear restriction & transcriptional bustle of β-catenin in cells co-expressing casein kinase II β and AKT-S129A, which accordingly results in diminished proliferation and amplified apoptosis (Ponce et al., 2011). Recent study has provided confirmation relating the antiapoptotic activity of casein kinase II to boosted transcription of the beta-Catenin–Tcf_Lef candidate gene *survivin.* Also was revealed that casein kinase II reticence condensed survivin intensity in human cancer and embryonic kidney cells. Furthermore, it was recognized that diminished β-Catenin–Tcf_Lef-dependent transcription and allied with declined capability and amplified apoptosis is related to decrease in survivin levels. Also within G2/M segment, decreases condensed figure of cells. Mostly data recognized the system to elucidate a potential of casein kinase II to encourage endurance and prohibit apoptosis (Ponce et al., 2011).

2.3 Inhibitor therapy for Treatment of Colorectal Cancer

Recent attempts have paid attention on Ras downstream effector cascades to generate therapies against RAS. A recurrent alterational commencement of binary vital effectors with corroborated functions in RAS arbitrated tumorogensis, encoded by PIK3CA and BRAF, sustain the significance of uncharacteristic effector indicators in mutant K-Ras activity in CRC (Rajagopalan et al., 2002, Samuels et al., 2004). Particularly, the nonoverlapping frequency related to KRAS & BRAF alterations proposed that deviant B-Raf cascade is the significant system aimed at KRAS-arbitrated oncogenesis in CRC. B-Raf phosphorylates as well as triggers the MEK1&2 protein kinases, as well as stimulated MEK1/2 phosphorylate and trigger the MAPKs. Consequently, much prominence is being positioned on conduction policies to mark this protein kinase surge (Sebolt-Leopold and Herrera, 2004, Friday and Adjei, 2008, Roberts and Der, 2007). Specifically, effective and discerning inhibitors of MEK1 &2, presently in period I/II clinical checks have been constructed (XL51, ARRY-162 and AZD6244; www.clinical trials.gov). In investigational observation, revealed that ectopic expression of triggered Ras leads to ERK-reliant development alteration as well as ERK commencement (Shields

et al., 2000). Since MEK1 and MEK2 acts as only subrates for Raf, and the ERK1 and ERK2 acts as only subrate for MEK1/2. A reasonable assumption is with the aim of MEK inhibitors can remain effective opponents of Raf & RAS arbitrated commencement of ERK. Above studies support numerous operational hypothesis in favor of the purpose and efficacy of MEK inhibitors in CRC. Primarily, BRAF and KRAS mutation encouraging CRC cells are predictable to demonstrate raised ERK commencement. Subsequent, CRC cells with prominent ERK commencement should acquire ERK-reliant growth alteration, and consequently, raised ERK bustle ought to associate in the midst of compassion to growth reticence by MEK inhibitor administration (Rinehart et al., 2004, LoRusso et al., 2005). Recently, MEK inhibitor was administrated to several CRC cell lines to evaluated their reaction, and might ERK commencement and compassion to ERK reticence either *BRAF* or *KRAS* mutation condition, are precise biomarkers for MEK inhibitor administration and retort. It was shown that the preponderance of CRC cell lines revealed growth reticence using MEK inhibitors, purposely with *BRAF* or *KRAS* mutation. On the other hand, ERK activation failed to correlate consistently with *KRAS* and *BRAF* mutation category. Further, MEK inhibitor repression of ERK activity also lack correlation with repression of quay autonomous expansion. In conclusion, it was revealed that ERK is not markedly triggered in malignancy (Yeh et al., 2009).

2.4 β-Catenin for Diagnosis and Cure for Cancer

Irrespective of dissimilarities in machinery of commencement, relocation of β-catenin into the cell core being characteristic of its cancerous bustle in the midst of only some exemptions. This type of phenotype symbolizes nuclear accumulation (NA) of beta-catenin. Usually pragmatic by immunohistohchemistry in tumors of cancer (Wong and Pignatelli, 2002). Studies revealed that commencement of β-catenin resolved upon nuclear accumulation is an initial stage in CRCs (Maruyama et al., 2000). Study resolved two discrete prototypes of β-catenin commencement, symbolized by circulate NA all over the tumor (NAd) and privileged nuclear accumulation merely within tumor invasion front (NAinv), correspondingly, while initial as well as most probably delayed incidents in

colorectal cancer (Ougolkov et al., 2002). The NAinv type of beta -catenin commencement stands allied in the midst of complex tumor phase and reappearance and confirmed to be a consistent in categorizing a division of CRC subjects having vulnerablity to cancer reappearance and comprise a fewer encouraging continued existence rate (Ougolkov et al., 2002). Other study revealed that amalgamated examination of K-ras was well as the β -catenin may spot the majority clinical colorectal cancers. Also can recognize a division of patients with shoddier results (Zhang et al., 2006). Recently a study showed that mRNA intensity of β-catenin identified in blood plasma of CRC subjects is amplified than standard subjects (Wong et al., 2004). Also a recent observation revealed that β-catenin protein amount in plasma of colorectal cancer subjects is elevated than standard controls. The intensity of plasma is connected amid its NA in the relevant primary tumors. Associating existence of metastasis to secluded organs (lungs, liver). Demonstrated the worth of gauging plasma β-catenin fragments in identification of colorectal cancer (Fuchs et al., 2006). β -catenin may play manifold tasks in diverse types of cancers related to humans and function as an oncoprotein in CRC. Numerous efforts to treatment and preclusion of colorectal cancer have been illustrated by aiming and attenuating the Wnt/ β-catenin cascade. Aims of this approach comprise convinced elements of Wnt/ β-catenin corridor and its transcription bustle, the *CTNNB1* transcript (mRNA) and gene. NSAIDs and the precise COX2 inhibitors played a crucial part in dwindling of β-catenin/Tcf signaling (Dihlmann et al., 2003, Dihlmann et al., 2001) and the role of its downstream targets, PPAR d and COX-2 (Lustig and Behrens, 2003, Howe et al., 2001). The potential therapeutic efficacy of decreasing beta-catenin intensity in cancer cells is illustrated by research, in vivo as well as in in vitro, using template oligonucleotide adminstration aimed at the *CTNNB1gene* and RNA interference (RNAi) in opposition to respective transcript (mRNA) (Roh et al., 2001, Verma et al., 2003). In APC $^{Min/+}$ mice, it has been illustrated by *multidrug resistance 1(MDR1*) gene inhibitors, revealing to restrain intestinal tumor expansion (Yamada et al., 2000, Yamada et al., 2003) though, the survival of manifold effectors of this cascade. Downstream

effectors Inhibitors of Tcf/β catenin cascade remain moreover targets for molecular curatives aiming the cascade. Also specifying to facilitate hampering merely a particular downstream aim may not be satisfactory to treat or avert colorectal cancer. The substitute administration conception appears as of a approach of gene therapy by means of β-catenin/Tcf cascade equally a vector to distribute and persuade the anti-cancer molecular expression in particular cells accessible to oncogenic cascade (Chen and McCormick, 2001, Kwong et al., 2002). *Multidrug resistance 1* was primarily recognized as a gene augmented in multidrug resistance cells (Gottesman and Pastan, 1993). Afterward originate one among effector genes transactivated by Tcf/ β-catenin cascade (Yamada et al., 2000). A transmembrane protein encoded by multidrug resistance 1, vehicles structurally disimilar chemotherapeutic agents'(hydrophobic) exterior cells in an energy-reliant approach. Consequently, the *multidrug resistance 1* gene product, PGP-1, encourages CR malignancy (Yamada et al., 2003) and provides tumor cells defiant to conformist chemotherapeutic agents (Ling, 1997). An additional characteristic of Tcf/ β-catenin pathway in cancer cure is shown with recently conducted study revealing that β-catenin commencement in the initial cancer blots the colorectal cancer subjects whose endurance promotes within a particular kind of immunochemotherapy. Consequently, Tcf/ β-catenin cascade manipulates as well as concludes cancer cells' confrontation or compassion to diverse modalities of chemotherapy (Yamashita et al., 2004).

2.5 Reproductive factors and Colorectal Cancer Risk

Voguish CRC, earlier meta-analyses illustrated an converse relationship with use of contraceptives taken orally, with review comparative jeopardys of 0.81 and 0.82 (Fernandez et al., 2001, Luan et al., 2015). The studies distinguished no dissimilarities according to period of utilize, even though there were cryptogram that the defense was healthier for more topical use (Bosetti et al., 2009, Fernandez et al., 2001). In the interim, study explained a arithmeticaly momentous nonlinear converse association with period of use (Luan et al., 2015). Regarding HRT, two earlier meta-analyses observed a roughly 22% decrease in CRC jeopardy amongst always users (Grodstein et al., 1999, Akhter et

al., 2008). This defensive consequence appears to be amended during the ERβ, in the colon it is the chief receptor. In colonocytes, the expression of ERβ is vanished, throughout the tumorigenesis progression. HRT applies its activity through defending this loss (Barzi et al., 2013). Raised local meditation of estrogens diminishes existing creation of oncogenic derived bile acid, confines MSI and DNA damage. Alo hampers cell propagation of colonic cancers (Foster, 2013). The majority surveillance studies have explained that postmenopausal HRT is allied with deminished jeopardy of CRC. Females who had perpetually used hormone replacement therapy ensured a 20% lesser jeopardy, evaluated with females who had never used HRT, revealed by meta-analysis of numerous epidemiologic observations [95% confidence interval (95% CI), relative risk, 0.80; 0.74-0.86] (Grodstein et al., 1999). The jeopardy diminution was seen in potential cohort and case-control studies. Authentication of a converse alliance among HRT taken as well as jeopardy of CRC has been given by the WHI clinical trial wherein females randomized to be given estrogen + progestin HRT had a 38% lesser jeopardy of CRC than females getting placebo (Rossouw, 2002). In females who have gone through hysterectomy, were unsystematicized to be given moreover estrogen only (deprived of progestin) or a placebo, a shielding result remained not pragmatic (Anderson, 2004). This shielding result of exogenous estrogen + progestin elevates the prospect that advanced intensity of endogenous estrogen or progesterone, might be allied through a declined jeopardy of CRC. So far, barely a potential data has documented on the relationship between endogenous circulating estrogen intensity and consequent CRC growth. A topical observation of 400 CRC patients and a subgang of 800 females from the WHI Observational Study (WHIOS), containing women who were not interested or were disqualified in the medical assessment fraction of the observation, create, divergent to anticipation, an inclined jeopardy of CRC in females with elevated intensity of endogenous estradiol, regardless of the detail that females on oral hormone replacement therapy had a lower jeopardy of mounting the disease (Gunter et al., 2008).

In Caco-2 Cell line, the colonic carcinoma cell lines, an observation has revealed that estradiol was the chief commencement element amid additional steroids. Estradiol encouraged signal transduction via MAP kinase/ c-yes, c-src, cascade through triggering c-src associated serine/threonine kinases and tyrosine kinase eventually resulting toward cell expansion (Di Domenico et al., 1996). An observation revealed that by estradiol activities through mounting the intensity of c-myc (protooncogene) mRNA and ornithine decarboxylase (ODC) mRNA, and encourages CRC expansion (Narayan et al., 1992). A study accomplished that cells related to colon cancer, articulates immunoreactivity to aromatase enzyme, a crucial enzyme for estrogen creation (Sato et al., 2012). A different animal study, revealed that estradiol treated mice demonstrated amplified figures of nodules in colon. Investigators confirmed that estradiol encouraged malignancy in those mice resulting to persistent colonic adenocarcinoma development (Heijmans et al., 2014). A study recommended that estrogen independently function as a jeopardy feature for colorectal cancer in male subjects (Wu et al., 2012) and was revealed that ERα regulates the osteopontin expression, a component of extra cellular matrix in CRC. Therefore a prospect may be present that ERR-α is also allied with cancer growth (Boudjadi et al., 2013). Several observations have also demonstrated that it is estrone not esterdiol, allied to boosted jeopardy of CRC (Clendenen et al., 2009). It was hypothesized an preliminary cancer defensive role of estrogen (Foster, 2013). A study observed inclined intensity of estradiol in colorectal cancer subjects with momentous distinction between stage I and II (Stage II raised level). The precise original mechanism relics inadequately understood, specified the accessible literature and suggested that inclined intensity of serum estradiol observed in colorectal cancer subjects may act as a marker for diagnosis (BASu et al., 2015). Efficient membrane androgen obligatory positions have been formerly recognized and distinguished in human breast and prostate cancer cells (Hatzoglou et al., 2005, Heinlein and Chang, 2004, Papakonstanti et al., 2003, Kallergi et al., 2007, Kampa et al., 2006, Papadopoulou et al., 2008a). Commencement of these obligatory positions by veto porous testosterone albumin conjugates (TACs), was revealed to tempt potent anti-

tumorigenic and proapoptotic results on assorted tumors in vitro as well as in vivo (Hatzoglou et al., 2005, Kampa et al., 2006, Papadopoulou et al., 2009, Papadopoulou et al., 2008b). The molecular systems prevailing the consequences have been explicated and precise signaling surges activated through the encouragement of membrane androgen receptors (mARs) and well explained in prostate in addition to breast cancer cells (Papadopoulou et al., 2008b, Papakonstanti et al., 2003, Kallergi et al., 2007, Lieberherr and Grosse, 1994, Sun et al., 2006). Functional membrane androgen obligatory sites are also found in colon cancer cells as well in tissues (Gu et al., 2009, Gu et al., 2011). The commencement through testosterone conjugates persuaded strong pro-apoptotic reactions (Gu et al., 2009) as well as reticence of the relocation possibility (Gu et al., 2011, Schmidt et al., 2012). Furthermore, experimental confirmation was provided demonstrating that, the pro-apoptotic steroid activity as well as the reticence of the relocation capability were autonomous of the standard androgen receptors signaling (Gu et al., 2009, Gu et al., 2011, Schmidt et al., 2012). A topical study, in Caco2 colon tumor cells, in the mAR-induced testosterone reactions revealed for the foremost peroid the participation of Rac1 small GTPase. Certainly, administration of the cells with testosterone conjugates persuaded strong actin polymerization. Hence Rac1 stimulation controlled by PI3K as specified by pilot trials with a precise PI3K inhibitor (wortmannin). Testosterone-induced swift Rac1/Cdc42 commencement has been demonstrated in prostate as well as in breast tumor cells (Alkahtani, 2013). Colon tissue of Xenograft model (Caco2 and HCT116 cells) showed momentous mAR expression in contrast to control or non-transformed cells. Moreover, human Caco2 cells were nurtured with radio labeled testosterone, different doses of estradiol or dihydrotestosterone (DHT). The radio labeled testosterone was relocated by DHT. These radio ligand fastening observations propose high attraction and selectivity of mARs concerning precise androgens (Gu et al., 2009). In addition, confirmation of apoptosis through caspase-3 commencement was explored by fluorescence staining of HCT116 & Caco2 cells

nurtured through testosterone- HSA aimed at 24h. These exposed results in colon cancer cells were solitarily characterized to the stimulation of mAR expressed (Gu et al., 2009).

2.6 Dihydroquercetin (Taxifolin) as treatment for different diseases

Enormous epidemiological observations were performed to establish the shielding consequence of flavonoids aligned with cancer. Amplified expenditure of lignans & larger plasma quantities of respective consititents are allied in the midst of condensed frequency of estrogen associated tumors in several (Pietinen et al., 2001, Dai et al., 2002, Boccardo et al., 2004, McCann et al., 2002) excluding various studies (Kilkkinen et al., 2004, Zeleniuch-Jacquotte et al., 2004) and a potential research was equivocal (den Tonkelaar et al., 2001). Most studies suggested that this contradiction might have genetic roots (McCann et al., 2002). Amplified utilization of isoflavones also are allied with diminished jeopardy of estrogen associated vascular diseases and cancers (Arai et al., 2000, Birt et al., 2001). Statistics from numerous observations and many case–control studies, showed scrutinized relations of flavonoid comsumption with cancer jeopardy. Some studies exposed that flavonoids, especially quercetin and taxifolin and quercetin might decrease the jeopardy of lung cancer but a study showed nonsignificant amplified risk. Low and high utilization of quercetin, taxifolin and kaempferol were allied with 50 % decrease in jeopardy, for stomach cancer. Numerically considerable no relationship of any flavonoids with breast cancer as well as bladder cancer jeopardy was seen (Neuhouser, 2004). Within a coordination of multicentric studies (case-control) on Italian subject, which include 10,000 events, histolopathologically proved subjects of preferred cancers and over sixteen thousand standards. The involvement of flavonoids, proanthocyanidins as well as cancer jeopardy was assessed (Rossi et al., 2010). Total flavonoids, flavanols, and flavonones showed conversely linked to laryngeal and oral tumors. Flavonols and flavanones show conversely association to laryngeal cancer (OR 0.64), and esophageal cancer (OR 0.38) respectively. A condensed jeopardy of CRC was observed for elevated ingestion of flavonols (OR 0.64), anthocyanidins (OR 0.67), isoflavones (OR 0.76), and flavones (OR 0.78). Negative alliance among prostate cancer

and flavonoids appeared, while converse connection was observed between proanthocyanidins and colorectal cancer (Rossi et al., 2010).

Taxifolin usually originate in Dahurian larch, syn Larix dahurica Turoz (Pinaceae), Pseudotsuga taxifolia (Weidmann, 2012). Different observations have revealed that cytochome c through dioleyl cardiolipin showed the peroxidase action by TAX. Also condensed the lipid radical creation in a quantity-reliant approach, having significance in favor of the commencement of apoptosis. Similarly reorganized cerebral ischemia–reperfusion impairment over hampering the excess assembly of reactive oxygen species and oxidative enzymes (Vladimirov et al., 2009, Wang et al., 2006). The experiment related to diabetes showed that, taxifolin hampered the sorbitol accretion and recombinant human aldose reductase in human RBCs and also revealed that it sustained the transparency of lens of rat nurtured with glucose, signifying its efficient potential in hyperglycemia in shielding osmotic stress (Haraguchi et al., 1997). However, the potential favorable effects of TAX on diabetic cardiomyopathy (DCM) have modest to be addressed till now. The studies showed that TAX condensed the discharge of myocardial enzymes into the blood & encouraging the antioxidative behavior of CAT, SOD, and GSH-Px hence reducing MDA stuff. In H9c2 cells, the studies have proved that the implified of intracellular ROS level is hampered by combine administration of TAX and 33mM glucose. Therefore, the shielding behavior of TAX may be associated with antioxidant potential. The survival of apoptosis in the hearts diabetic subjects and mice with streptozotocin (STZ)-persuaded diabetes has been detailed in various observations (Cai et al., 2002, Fiordaliso et al., 2000, Frustaci et al., 2000). Cardiac remodeling as well as growth of heart failure results due to apoptosis of cardiac myocytes. Cardiomyocyte apoptosis is usually considered as leading transformation in diabetic cardiomyopathy (Frustaci et al., 2000). Recent study exposed that hyperglycemia encouraged apoptosis within STZ-persuaded diabetic mice as well as in H9c2 cells administrated through surplus glucose, whereas hyperglycemia-encouraged cardiomyocytes apoptosis was repressed by TAX drastically. TAX unaccompanied demonstrated no damaging activity

on H9c2 cells (Sun et al., 2014). Further in H9c2 cells administrated with 33 mM glucose, it was revealed by studying apoptosis signaling cascade that caspase-3 & 9 effects were extensively raised, demonstrating the association of mitochondrial corridor. TAX administration censored caspase-3 & 9 commencements and reinstated the depolarization of mitochondrial transmembrane potential (DWm). In addition, TAX also indicated activity on the chief controllers of mitochondrial permeability i.e, Bcl-2 family proteins. There was increase in expressions of proapoptotic proteins Bak and Bax by hyperglycemia. On the other hand, there was decline in expressions of Bcl-xLand Bcl-2 (anti-apoptotic proteins). Treatment of TAX showed amplified bcl-xL & bcl-2 expression in addition TAX reduced bak & bax expression, thus regulating the discharge of factors favoring apopotosis like mitochondrial cytochrome c. Briefly, by amending the mitochondrial pathway, TAX treatment hampers hyperglycemia-induced apoptosis. Consequently, TAX might be a prospective therapeutic treatment for diabetic cardiomyopathy (Sun et al., 2014). More recently a study explored that taxifolin shielded aligned with cardiac hypertrophy *in vitro* as well as in *in vivo*. Also it was illustrated that taxifolin controls BNP and ANP promoter activity in cardiac myocytes and restrained Ang II-temptd protein synthesis. Treatment of taxifolin in TAC mice model, thwarted the boost of fibrosis, cardiac myocyte cross sectional area, ventricular/TL, and LW/TL. Taxifolin minimizes oxidative strain & condensed the surplus assembly of ROS. The potential of taxifolin to diminish fibrosis, cardiac hypertrophy, & shield cardiac dysfunction reconciled through Smad2/3, ERK1/2, and JNK1/2 cascade (Guo et al., 2015). The study demonstrated that in *in-vitro* experiment TAX assuaged Ang II-tempted cell hypertrophy. Taxifolin hampered BNP, ANP promoter effects and protein synthesis in a dose-reliant approach. Sequentially a study on TAC model in mice confirmed that taxifolin minimized TAC-persuaded hypertrophic progress when precised by the percentage of ventricular/TL. In addition, TAX administration reduces chamber size and wall thickness. Auxiliary the hampering consequence of taxifolin on cardiac hypertrophy persuaded by TAC was explained. The markers (BNP, β-MHC and ANP) of irregular

cardiac hypertrophy remained synchronized in heart tissue tempted by pressure excess. TAX censored the overexpression of β-MHC, BNP, & ANP. Hence, taxifolin seems prospective player for the cure of heart failure and cardiac hypertrophy (Guo et al., 2015).

Cooperative studies indicated that DMH carcinogen associated with colon lead to raised incursion of permitted radicals & devastates the cellular redox homeostasis, that lead to stern oxidative stress encouraged tumor expansion (Sengottuvelan et al., 2006). These days, studies have made significant attempts on investigating plant-derived polyphenolic amalgams showingactivities against cancer for the therapeutic cure of colon cancers (Birt et al., 2001). Recent study revealed that administration of TAX amends 2 Dimethyl hydrazine induced carcinogenicity in mice equally confirmed through the results on macroscopic, antioxidant enzyme status as well as histology (Manigandan et al., 2014).

ACF, a sound contested pre- neoplastic marker featured by numerous crypts as compared to regular cells. Extensively employed to evaluate the occurrence of colon carcinogenesis (Khare et al., 2009). Although aberrant crypt foci can extend into polyps and eventually into colon cancer. Though, all aberrant crypt foci will not lead to tumorigenesis (Wargovich et al., 2010). Recently an observation explored that the occurrence and proportion of aberrant crypt foci on DMH induced mice showed extensively raised together with evident tumors, while, the frequency of aberrant crypt foci was extensively diminished in 5-FU control as well as TAX administrated mice (Manigandan et al., 2014)

The hampering activity of taxifolin against 1,2 Dimethly hydrazine induced aberrant crypt foci pattern is steady with earlier epidemiological observations revealing the anti-carcinogenic activity of flavonoids (Sequetto et al., 2014). Therefore it is proposed that the suppressive activity of taxifolin on aberrant crypt foci pattern might be owing to its strong antioxidant activity (Wang et al., 2009). Correspondingly administration of TAX showed no potential indications of venomousness in the body as well as liver mass prolifes (Manigandan et al., 2014).

By apoptosis, commencement of cancer cell demise remains a main characteristic in malignancy rehabilitation. Neoplastic progression interrupts regular homeostasis of colonic tissues and possibly affects the initiation of apoptosis-relevant proceedings (Sun et al., 2004). Hence, by anticancer drugs insertion of DNA segments within oligonucleosomal bases symbolizes apoptosis initiation in cancer cells (Kaufmann and Earnshaw, 2000). An observation indicated that by DMH carcinogen, encouraged the lack of DNA fragments in mice. Auxiliary, this faction reveals extraordinary molecular weight DNA deprived of breakdown within double strands, preponderating apoptotic dysregulation. On the other hand, TAX administrated extracts the incidence of DNA fragmentation by apoptosis might be owing to the elimination of mutilated cancerous cells. Therefore it is proposed that via apoptosis, TAX marks cell propagation arbitrated cancer cell death (Manigandan et al., 2014).

2.7 Antitumor activity of Vitamin D on CRC

The preliminary inherited alteration in the majority of colorectal adenomas, the preliminary phase in the tumour suppressor genes (CTNNB1/ β -catenin genes, APC, AXIN) is the somatic mutation. Every mutation exclusively progress to deviant commencement of the canonical WNT/ β -catenin signaling corridor (Clevers, 2006, Klaus and Birchmeier, 2008). Besides that, a major fraction of adenomas cart triggering mutations in BRAF and KRAS, while instimulating variations within the transforming growth factor (TGF)-b cascade which provides supplementary malevolent characteristics to adenoma cells (Markowitz and Bertagnolli, 2009). The adenoma–carcinoma conversion in approximately 55% of CRC is contested by a incommencement of the *TP53* gene (Iacopetta, 2003). The molecular systems of carcinoma succession and the possession of metastatic capability continued to be entirely explicated. Recently it was revealed that the transition to mesenchymal from epithelial bestows epithelial cells through the ability to plague the adjoining tissue & subsequently voyage towards outlying appendages (Thiery et al., 2009, Chaffer and Weinberg, 2011).

The importance of vitamin D in CRC defense remained theorized initially by Garland Cedric as well as by Frank based on ecological studies (Garland and Garland, 1980). They revealed an converse alliance amid CRCand vitamin D status (Garland et al., 1989). After that various observations have showed the allaince between CRC and vitamin D status (Platz et al., 2000, Wu et al., 2007, Gandini et al., 2011). Several observations have revealed that the anti-tumoural activity of Vitamin D, beside that colorectal cancer depends on numerous systems at the cellular level, like reticence of sensitization of apoptosis, initiation of epithelial delineation, reticence of angiogenesis and cell detoxification metabolism and cell propagation. The overall result of these systems, in a cell type- and cell-perspective-reliant approach, possibly will conclude the activity of vitamin D against tumors (Lamprecht and Lipkin, 2003, Deeb et al., 2007, Larriba and Munoz, 2005, Krishnan and Feldman, 2011, Gaschott et al., 2002, Fernandez-Garcia et al., 2005). Apoptosis predisposition by Vitamin D in carcinoma cells and colorectal adenoma engages the upregulation and downregulation of BAK1 as well as the nuclear anti-apoptotic protein (BAG1) (Díaz et al., 2000, Barnes et al., 2005). Besides that, vitamin D encourages the G0S2 expression (Pálmer et al., 2003), a mitochondrial protein that allies through Bcl2 & persuades apoptosis in colorectal cancer cells via inhibiting Bcl2 to create heterodimer with Bax, anti apopototic protein (Welch et al., 2009). Recently a study revealed that VDR gets allied with mutant p53 protein and amends the transcriptional activity of vitamin D resulting to the elevated expression of continued existence-encouraging genes and the decline of proapoptotic genes, consequently altering vitamin D equally an anti-apoptotic mediator (Stambolsky et al., 2010). Colorectal cancer cell receptiveness to vitamin D relies primarily on the VDR expression and on the bioaccessibility of vitamin D inside the cell. While within cells intensity of vitamin D being measured through the progressing intensities of $1,25(OH)_2D_3$ & 25(OH)D3 as well as by the function of CYP24A1 and CYP27B1 inside the cell. CYP27B1 expression and bustle remain increased during initial phases of CRC expansion and dramatically decline in progressed colorectal cancer, whereas CYP24A1

are raised in colorectal cancer growth. These transformations results to terminate synthesis of 1, 25$(OH)_2$ D_3 & ignited catabolism of 1, 25$(OH)_2D_3$ within latter colorectal cancer, resulting confrontation to vitamin D (Bareis et al., 2001, Cross et al., 2001, Bises et al., 2004). Various studies have exposed that vitamin D receptor is articulated by control and certain tumour colon epithelial cells (Sheinin et al., 2000, Gonzalez-Sancho et al., 2006, Modica et al., 2010). And is allied with a elevated level of cell differentiation (Shabahang et al., 1993, Zhao and Feldman, 1993). Vitamin D receptor expression is boosted in initial phases of CRC (adenomas, polyps), while it declines in latter phases (Sheinin et al., 2000, Cross et al., 2001, Larriba and Munoz, 2005, Matusiak et al., 2005, Anderson et al., 2006). Hence, increases expression of vitamin D receptor is allied with elevated absence of node association, tumour differentiation, and high-quality diagnosis in colorectal cancer (Cross et al., 1996, Evans et al., 1998). Concluded, the results accessible on the loss of vitamin D receptor appearance as well as on the modification of CYP24A1 as well as CYP27B1 intensity throughout colorectal cancer development sustain a function for vitaminD in the preclusion and/or in the therapy of initial phases more willingly as compared in the cure of proceed cases of this neoplasia (Nicholas, 2011).

Several observations stongly hightlighten that vitamin D and numerous analogues evidently decrease the expansion of colorectal xenografts (Ordonez-Moran et al., 2005, Deeb et al., 2007, Kang et al., 2011). Analogous consequences were attained using diverse compound carcinogens (N-methyl-Nnitrosourea, azoxymethane & additional) to create CRC in rats/mice. The figure showing tumors created after chronic administration of carcinogens has shown reduction with the conduct of vitamin D or numerous of its analogues (Ordonez-Moran et al., 2005). 1,25$(OH)_2D_3$ as well as fewer calcaemic derivative 1,25$(OH)_2$-16-ene-19-nor-24-oxo-D_3 decrease malignancy sum (the entirety of completely polyp regions) (Li-Kuo and Kinzler, 1992). The studies authenticated the effects and revealed that administration with vitamin D boosts the manifestation of E-

cadherin, & decreases β-catenin nuclear intensity within the colon & APC $^{min/+}$ mice small intestine (Xu et al., 2010).

The study on genetically tailored mice (Vdr-deficient) revealed that the distraction of VDR expression on colorectal tumorigenesis do not demonstrate impulsive enhance in tumor although are additional sensitive to carcinogen-induced tumors (Bouillon et al., 2008). These mice exhibit colonic crypt hyper-propagation, and raised oxidative stress and intensity of DNA breaks in the intestine (Kallay et al., 2001, Kallay et al., 2002). Recently reseachers working on $Vdr^{+/-}$ and $APC^{min/+}$ illustrated that there is boosted tumour load in $APC^{min/+}$ $Vdr^{-/-}$ mice when evaluated to $APC^{min/+}$ $Vdr^{+/+}$. Prominently, $APC^{min/+}$ and $Vdr^{-/-}$ mice illustrated advanced commencement related to WNT/ β- catenin corridor in the abrasions & a rise within the figure of colonic ACF (Larriba et al., 2010, Zheng et al., 2012).

Chapter 3
Material And Methods

3. Materials and Methods

3.1 Human Studies

3.1.1 Ethical Declaration

The observation was recommended through the Institutional Review Board (IRB) of Quaid-i-Azam University, Islamabad, Pakistan. Informed approval (written) was attained from all the applicants engaged in this observation.

3.1.2 Patient and Sample Selection

Overall, 200 colorectal tumors and blood samples of both women and men patients with sporadic or familial colorectal tumors and normal tissues were taken randomly from Department of Urology, AFIP, Rawalpindi, Pakistan. During the biopsy time, patient age ranges were 32–78 years.

Medical and demographic traits were confirmed gender, counting oldness at the period of identification, family antiquity, cell type, disease localization, stage as well as grade of tumor (Table3.1).

Table3. 1: Baseline patient characteristics

"Sex	Number of patients	Average age	locatization	Cell type	Grade	Stage	Smoker(S) Nonsmokers (NS)	HRT therapy Yes/ no	Family history of CRC"
Males (M)	136	53.67	Rectum 28(M) 08(F)	Adenocarcinoma 100(M) 58(F)	Well Differentiated 106(M) 60(F)	"T0 NO MO T1 N1 Mx T2 NO Mx T2 N1 Mx T2 Nx Mx T3 NO MO T3 N0 M1 T3 N0 Mx T3 N1 Mx T3 N2 MO T3 N2 M1 T3N2Mx T4 N0 Mx T4 N1 M1 T4 N2 Mx T4 N3 M1"	60 (S) 76(NS)	NO	130 NO 6 yes
Females(F)	64	56.03	Colon 92(M) 49(F)	Mucinous Carcinoma 30(M) 04(F)	Moderately Differentiated 27(M) 03(F)		4 (S) 60(NS)	22 yes 42 No	2 yes 62No
			Recto sigmoid 17(M) 04(F)	Signet Ring cell carcinoma 06(M) 02(F)	Poorly Differentiated 03(M) 01(F)				
			Cacum 09(M) 03(F)						

3.1.3 DNA Isolation and Purification

Before DNA extract, the occurrence of tumor tissue in paraffin-embedded (FFPE) blocks was examined and authenticated by an expert histo-pathologist. DNA was extracted after formalin-fixed and FFPE tissue cases by using, following standard protocol.

3.1.3.1 Paraffin Confiscation

1. Xylene (800µl) (VWR, CABDH6216-4) was affixed to the categorized tube restraining tissue and was tenderly shaken for 10 to 15 minutes in order to dissolve paraffin.
2. Centrifugation at 14000rpm in a microcentrifuge for 3 minutes was done.
3. Xylene supernatant was cast off exclusive of concerning the pellet.
4. Step 1 and 2 was repeated until paraffin was fully dissolved.

3.1.3.2 Ethanol Washing

5. 100% ethanol (800µl) was dispensed & vortexed, at 14000rpm centrifuged for 5 minutes.
6. Supernatant was detached carefully deprived of disturbing pellet
7. 800µl of 70% ethanol was appended and vortexed and at14000rpm centrifuged aimed at 5 minutes.
8. Supernatant was detached cautiously deprived of disturbing pellet.
9. Without disturbing pellet, supernatant was taken away carefully and pellet was dehydrated in DNA concentrator.

3.1.3.3 Tissue Digestion

10. Lysis buffer (500µL) was appended and pellet was resuspended.
11. Incubation at 56^0C in a water bath was done.
12. 20µl of Proteinase K was added twice after 12 hours.

3.1.3.4 DNA Clean up

13. Whole digestion of nuclear pellet subsequently, 500µL of unmarked blend of equivalent measurements of solution A as well as B remained appended in samples, combined & at 13,000rpm for ten minutes were centrifuged.

14. The superior layer (aqueous stage) was moved toward a novel tube & solution B (500 µl) was appended.
15. Centrifugation was formerly conceded over aimed at 10 minutes by 13,000rpm.
16. The higher deposit (aqueous stage) was reallocated to a fresh tube, & subsequent to addition of 3M sodium acetate (55 µl) & isopropanol. the conduits remained capsized numerous spells tenderly to impulsive DNA.
17. Centrifugation was subsequently performed out over then the upper layer was superfluous.
18. The DNA pellet remained rinsed through frozen & centrifuged.
19. Subsequent to desertion of scum ethanol, DNA remained disbanded in suitable quantity of TE buffer.

3.1.3.5 DNA Quantification

20. NanoDrop spectrophotometer was used to measure the A260: A280 ratio.
21. To check the quality of DNA, Horizontal Gel electrophoresis was performed.

3.1.4 Lysis Buffer

- "EDTA (100 mM), pH 8.0
- 200 µg/ml proteinase K
- Tris-HCl (10 mM), pH 8.0
- 0.5% SDS
- NaCl (50 mM)"

3.1.5 Solution A

- Phenol

3.1.6 Solution B

- 1 volume of Isoamyl alcohol
- 24 volumes of Chloroform

3.1.7 DNA Dissolving Buffer

- 10mM Tris pH 8.0
- 0.1mM EDTA or

3.2 Mutation Analysis of *CTNNB1* gene and *KRAS* gene

For amplification of all 16 and 4 exons of the *CTNNB1* and *KRAS* gene respectively, the extracted genomic DNA from normal control and tumor tissues (n=200) was used as template.

The conditions used for PCR reaction included for 6min at 94 °C, pursued through 45 cycles of 945 °C aimed at 45seconds, 60 °C aimed at 30 seconds, 70 °C aimed at 30 seconds, & final extension at 70 °C aimed at 8 min. Polymerase chain reaction artifacts were determined on 2% agarose gel. Ethidium bromide was used as the stained dye. The genotypes were assigned by visual inspection. Primers were designed by software primer3 and used reverse (3′-5′) primers and forward (5′-3′) existed as follows:

Table3. 2: List of primers of *CTNNB1* gene

Exon	Forward Primer (5′-3′)	Reverse primer (5′-3′)	Base pairs bp
Exon 2	TACAACTGTTTTGAAAATCCAGC GTGGAC	CAGCCGCTTTTCTGTCTGGTTC	264
Exon 3	CCAATCTACTAATGCTAATACTG	GCATTCTGACTTTCAGTAAGG	240
Exon 4	ATCACTGAGCTAACCCTGGC	ACCTAAGTATTTGCTATCCTAA ATG	288
Exon 5	TGTGGTGAAGAAAAGAGAGTAA TAGC	TCTGAAACTACTCCCCTTGAGC	254
Exon 6	TTGAAGTAAATGCTCAAGGGG	GAATCCACTGGTGAACTGGG	239
Exon 7	TGGTGAAAATGCTTGGGTAAG	CATGGAATGACATGACACTGG	202
Exon 8	AGGTTGGTAATATGGCTCTTCTC	TTAAAGTTCTACCACCTTTTCCT AAG	145
Exon 9	CAGATATTTAGGATTGATAGGC ACTTC	AAACAGATGGTCAGTACAAGC AC	104
Exon 10	TGCCATGGGAATAGAGTCAAG	CCATCCAACAGCTAGAGATGC	339
Exon 11	GTGTGGTGGGAATTTTAGGG	TCCTTTGGATTTATGCATTCC	159

Exon12	GGGGAACTTCGGGTATATAATG	TGGTCCCTAATTTTCTGAAATG	120
Exon 13-14	GGGCTTGCCATGTTTTAGC	ACAAGCTGCCATACCTGCTC	361
Exon 15	TTGTTCCTTTTGTAATCTGAAAG TATG	ATGAGCAAACCGGCTCTTC	61
Exon 16	TTTGGATGCCCTAACCTCAG	ACCACTCCCACCCTACCAAC	209

Table3. 3: List of primers of *CTNNB1* gene

Exon	Forward Primer (5′-3′)	Reverse primer (5′-3′)	Base pairs bp
Exon 2	TACAACTGTTTTGAAAATCCAGC GTGGAC	CAGCCGCTTTTCTGTCTGGTTC	264
Exon 3	CCAATCTACTAATGCTAATACTG	GCATTCTGACTTTCAGTAAGG	240
Exon 4	ATCACTGAGCTAACCCTGGC	ACCTAAGTATTTGCTATCCTAA ATG	288
Exon 5	TGTGGTGAAGAAAAGAGAGTAA TAGC	TCTGAAACTACTCCCCTTGAGC	254
Exon 6	TTGAAGTAAATGCTCAAGGGG	GAATCCACTGGTGAACTGGG	239
Exon 7	TGGTGAAAATGCTTGGGTAAG	CATGGAATGACATGACACTGG	202

Exon 8	AGGTTGGTAATATGGCTCTTCTC	TTAAAGTTCTACCACCTTTTCCTAAG	145
Exon 9	CAGATATTTAGGATTGATAGGCACTTC	AAACAGATGGTCAGTACAAGCAC	104
Exon 10	TGCCATGGGAATAGAGTCAAG	CCATCCAACAGCTAGAGATGC	339
Exon 11	GTGTGGTGGGAATTTTAGGG	TCCTTTGGATTTATGCATTCC	159
Exon12	GGGGAACTTCGGGTATATAATG	TGGTCCCTAATTTTCTGAAATG	120
Exon 13-14	GGGCTTGCCATGTTTTAGC	ACAAGCTGCCATACCTGCTC	361
Exon 15	TTGTTCCTTTTGTAATCTGAAAGTATG	ATGAGCAAACCGGCTCTTC	61
Exon 16	TTTGGATGCCCTAACCTCAG	ACCACTCCCACCCTACCAAC	209

Table3. 4: List of primers of *KRAS* gene

Exon	Forward Primer (5′-3′)	Reverse primer (5′-3′)	Base pairs bp
Exon2	TTAAAAGGTACTGGTGGAGTATTTG	CCTTTATCTGTATCAAAGAATGGTC	111
Exon3	TGCACAAAGATTTCAGTGTCTG	AATCCCAGCACCACCACTAC	179
Exon4	AGAAGGAAGGAAAATTTGGTG	AGAAGCAATGCCCTCTCAAG	160
Exon 5	AACTTCTTGCACATGGCTTTC	GTGGTTGCCACCTTGTTACC	120

3.3 DNA Sequencing

DNA sequence investigation of control and patients was conceded out with reference to standard DNA sequencing protocol towards monitor out all sequence variant in the *CTNNB1 and KRAS gene*. PCR conditions were kept same as used earlier in the study.

"In a 50 µl reaction blend, 2.5ul (250 ng) concentration of genomic DNA was used and 2.5 µl (20 ng/µl) concentration of primers was cast-off (5 µl 10X buffer (pH 8.3, 500 mM KCl, 100 mM Tris-HCl), 4 µl $MgCl_2$ (25 mM), 0.6 µl Taq polymerase (one unit) (MBI Fermentas, UK), 1 µl dNTPs (10 mM) (MBI Fermentas, UK), in 31.9 µl PCR water. To analyse the PCR products 2% agarose gel was used."

3.3.1 First Purification

The Rapid PCR Purification Kit (Marligen, USA) was castoff to purify the amplified products

- 45 µl of Ist PCR artifacts were shifted to eppendorf tube.
- To the amplification reaction, the binding solution (H1, 200 µl) (Tris-HCl, concentrated Guanidine HCl, Isopropanol and EDTA) was added. and then were vortexed and at 8,000 rpm for 30 seconds were centrifuged.
- Cartridge & Wash tube were fixed & beyond mixture (245µl) was dispensed and formerly tubes were vortexed & at 13,000 rpm for 1 minute were centrifuged.
- To cartridge placed in empty wash tubes, the binding solution (H2, 500 µl) was added and at 13,000 rpm for 1 minute was centrifuged.
- Once more, wash tubes were vacated and at 13,000 rpm for 1 minute were centrifuged.
- Wash tubes were redundant and cartridges were positioned in eppendorf tubes.
- At 65 ºC DNA was eluted in 25 µl Tris-EDTA buffer (0.1 mM EDTA, 10 mM Tris-HCl (pH 8.0)) and for 5minutes kept at room temperature and then for 5 minutes centrifuged at 13,000 rpm .

3.3.2 Second PCR reaction

Sanitized yields stayed allowed towards cycle sequencing by means of 1-2 µl of template, 3 µl of DTCS quick start kit, and in a 10 µl reaction mixture, 5-6 µl of PCR water with 1 µl of primer (F/R) .

3.3.3 Second Purification

The ethanol precipitation protocol (POP6 Protocol) was used to sanitize the sequencing products.

- Renewed stop solution consisting of 1 µl of 3M NaAc (PH 5.2), 1 µl of Na_2EDTA (100 mM, PH 8), & 0.5 µl of glycogen (20 mg/ml), was prepared.
- 2.5 µl and 70 µl of stop solution and ethanol (100%) respectively were added to microcentrifuge tube containing sequencing products. Tubes were vortexed and for 20 minutes at 13,000 rpm were centrifuged.
- 150 µl of ethanol (100%) was dispensed into the tubes later removal of supernatant. Aimed at 15 minutes, at 13,000 rpm, centrifugation was done.
- Supernatant was removed & the samples at 30 ºC were vacuum dried. 30 µl of sample loading solution (SLS) was used to resuspended the Pellet, then vortexed and short spinned.
- CEQ8800 DNA sequencer (Beckman Coulter, USA) was used to sequence the samples .

3.4 Mutation Analysis

Chromatograms from patients & control subjects were matched through the equivalent watchdog gene sequences.

3.5 Immunohistochemistry

The formalin fixed, paraffin embedded tissue was used to execute standard immunohistochemistry through ultraView DAB Detection Kit (Ventana, Arizona, USA), scheduled an anti β-catenin monoclonal antibody (Sigma-Aldrich) & arranged a BenchMark XT automated staining system (Ventana, Arizona, USA). Thickness of 3 micron tissue sections were sliced into a glazed slides by by means of Leica RM2235 Rotary Microtome (Leica Biosystems, Wetzlar, Germany), gestated aimed at 15 minutes in a hot air oven at 60 °C. To deparaffinized with EZ Prep (Ventana, Arizona, USA), the tissue sections were heat pretreated at 75 °C in Cell Conditioning 1 (CC1; Ventana, Arizona, USA) using standard cell conditioning for antigen reclamation at 100 °C, and

subsequently incubation at at 37 °C of 32min with the anti β-catenin primary antibody (diluted 1:50) after inactivation of the endogenous peroxidase with hydrogen peroxide for 4 min. Then the secondary antibody (Ultraview universal HRP multimer) was added. A copper-enhanced DAB reaction was used by which the immunolocalized β-catenin protein was visualized. To counterstain the slides the Hematoxylin II and Bluing Reagent (Ventana, Arizona, USA) was added for 4 min and then by the NexES Special Stains automated slide stainer, the liquid coverslip (LCS) were applied atop aqueous reagents to avoid reagent evaporation and guarantee entire slide coverage. Subsequent that, DPX was used for sample mounting (distyrene, a plasticizer, and Xylene) and were dehydrated by transient in the course of graded alcohols: 70% ethanol, 96% ethanol and absolute ethanol. Then to the specimen, a minor drop of DPX was added. After every run the slides were washed by Reaction Buffer to grant a firm aqueous environment. By Olympus BX51 light Microscope and DP72 Olympus Digital Camera, immunostained sections were reviewed (magnification 100X and 200X) (Olympus America Inc, Center Valley, PA, USA) .

3.6 3D structure prediction

In the nonexistence of a well-described or experimentally resoluted full length three dimensional protein structure, comparative modeling being the utmost precise computational methodologies to make a consistent tertiary protein structure via sequence information (Woodgett, 2001). By homology modeling methodology (http://www.rcsb.org), full length three dimensional structure of human β catenin protein was fabricated using Swiss model (https://swissmodel.expasy.org/) and fold recognition manner by means of MUSTER (Cohen and Frame, 2001). Next, the structure of mutated CTNNB1 (S33F and T44A) were predicted through the Swiss model (https://swissmodel.expasy.org/). Stereochemistry & validity of constructed wild type & mutated 3D structure of the human β-catenin protein was evaluated by Ramachandran polt (Schulman et al., 2000) ProQ (Ray et al., 2012)and Verify3D (Wall et al., 1995) and

coarse packing eminence calculated with WHAT IF (Brivanlou and Darnell, 2002). Auxiliary investigation of packing and stereochemistry was performed out and owing to several inconsistencies; refinement was prepared to the preliminary model to clasp a superior model for advance investigation. Energy minimization & structure modifications were prepared by means of GROMMACS accessible in Chimera 1.5.6 (Najdi et al., 2011) & VEGA ZZ.

3.7 Molecular docking

AutoDOCK 4.0 was used to perform molecular docking of CTNNB1^{wt} with GSK3 and TrcPB1 (Xu and Kimelman, 2007). Three-dimensional structure of GSK3 (PDB ID: 1GNG) and TrCP1 (PDB ID: 1P22) were retrieved through PDB (protein databank). The retrieved structure were subjected to geometry optimization using MMFF94 force field embedded in Chimera tool. The annealing parameters for Van der Waals association and hydrogen bonding were set respectively to 2.5 A° & 4.0 A°. Grid map on the whole protein was generated with grid parameter of 80 _ 80 _ 80 points along with spacing of 0.875 A°. For each docking experiment the number of runs was adjusted to hundred. Lamarckian genetic algorithm & empirical free energy activity remained considered using the respective variables: a maximum number of 27,000 generations, population of 150 randomly placed individuals a crossover frequency aim at 0.80 & the count of energy evaluations was 2.5 x 110 and mutation rate of 0.02, rest of the docking parameters were set to the default. Based on RMSD value of receptor ligand complex conformations cluetrs were generated. The best docked complex for CTNNB1 with GSK3 and TrcP1 were taken on the basis binding free energy value using ligplot (Coates, 2003), Discovery Studio visualizer *(http://accelrys.com/products/collaborative-science/biovia-discovery-studio)* and UCSF chimera (Najdi et al., 2011) molecular interactions were mapped.

3.8 Molecular dynamic simulations

To evaluate the stability, conformational changes and folding of CTNNB1 protein parallel molecular dynamic (MD) simulations experiments were performed with

$CTNNB1^{WT}$ and mutant S33F and T41A respectively. Using GROMACS 4.5 package (Fiol et al., 1987), running on high performance OpenSuse linux system all MD simulations were performed.. All the classifications were solvated using TIP4P water model in a periodic box (Ikeda et al., 1998), , followed by the adding of Na^+ and Cl^- counter ions to neutralize the systems. Under pressure (1 atm) and constant temperature (300K), all MD simulations were run for 20 ns time scale. To calculate electrostatic interactions PME (Particle Mesh Ewald) algorithm was used in all calculations. To analyze the stability and behavior of wild type and mutant system**,** VMD (Zhang et al., 2012), PyMol (*http://www.pymol.org*) & GROMACS tools were cast-off.

3.9 Reproductive Hormone Analysis of Colorectal cancer Patients

The experimental work was permitted by Quaid-i-Azam University, Islamabad, Pakistan. All participants were conversant about the study objectives and signed a knowledgeable agreement. The protocol of study was done in accord through the Declaration of Helsinki principles (Association, 2008).

3.9.1 Selection of case participants and collection of blood samples

Cases were recently diagnosed, histo-pathologically definited, adenocarcinoma of the colon or rectum. Venous blood of 200 colorectal patients of both female and male subjects with sporadic or familial colorectal tumors and normal tissues were taken randomly from Department of urology, AFIP, Rawalpindi, Pakistan. During the time of sample collection, the patients' age ranges from 32–78 years. Menstrual factors gathered including cause of menopause, uniformity of the menstrual cycle, menopausal eminence, age at menarche, age, hormone replacement therapy, and use of hormonal contraceptives. Reproductive history like number of miscarriages, newborns' sex, info on fertility hitches & their conduct, year of birth, children number, duration of maternal lactation, and gestational age were collected. Demographic and clinical characterists were documented, including, family history, gender, stage, grade of tumor, as well as disease localization (Table1).

3.9.2 Measurement of Testosterone by ELISA

Quantity of testosterone of controls and patients in the serum is estimated through GenWay Immunoassay Test Kit (Catalog Number GWB-472DB9).

(a) "Principle of the assay:

The Testosterone Enzyme linked immunosorbant assay (ELISA) works on the principle of competitive binding between testosterone and testosterone-horseradish peroxidase (HRP) conjugate for a constant amount of rabbit anti-Testosterone. During the incubation, a fixed amount of HRP-labeled testosterone competes with the testosterone in the standard, sample, or quality control serum for a fixed number of binding sites of the specific testosterone antibody. Thus, the amount of testosterone-HR immunologically bound to the well progressively declines as the concentration of Testosterone in the specimen inclines."

(b) Procedure:

All materials were equilibrated and reagents were prepared at room temperature prior to use.

- Standards, specimens and controls, 10 µl of all, were bestowed into proper pits.
- Testosterone-HRP conjugate component (100 µl) was assigned into every pit.
- 100 µl of rabbit anti-Testosterone reagent were bestowed in every pit. Also was assorted methodically.
- Incubation of One and half hour at 37°C was finished.
- The microtiter wells were 5 spells wash away with deionized or distilled water.
- The wells were belted severely against absorbent paper to eliminate all left over aquatic drips.
- TMB Mixture (100 µl) was distributed hooked on every well and mildly assorted aimed at 5 seconds.

- Incubation at 25^{o}C aimed at 20 minutes was done
- Stop Solution (100 µl) was appended to every well to prevent reaction.
- A microtiter well reader remained reserved to declaim at 450 nm the absorbance within 15 minutes

c) Result calculations:

To make standard curve, mean absorbance acquired from each reference standard was used via drawing the mean absorbance against respective dose in mIU/ml and the concentration of FSH in test samples is attained from the standard curve.

3.9.3 Estimation of Esterodiol by ELISA

Dose of esterdiol in the serum of controls and patients is estimated through GenWay Estradiol immunoassay test kit.

(a) Principal:

The principle is allied on the competitive binding between E2-HRP conjugate aimed at a equal quantity of rabbit anti-Estradiol and E2 with the test specimen. The level of the color seems inversely allied to the quantity of unlabeled E2 in the subject and proportional to the quantity of presence of enzyme.

***(b)Procedure*:**

Before using reagents were espoused to room temperature. Lyophilized standard was reconstituted with 1ml purified water and shake gently, for 20 min prior to use.

- 25 µl of the suitable serum reference control, or samples into the allocated well were bestowed into respective wells.
- To all wells, the Estradiol Biotin Reagent (50µl) was added.
- For 20-30 seconds, microplate was swirled tenderly to mix.
- Incubation of 30 minutes was done at room temperature.
- The Estradiol Enzyme Reagent (50µl) was added to entire wells.
- For 20-30 seconds, microplate was swirled tenderly to mix.
- Incubation at 25^{o}Celcius for 90 minutes was done.

- The stuffing of the microplate was castoff by documentation or objective.
- The wash buffer (350µl) was dispensed & this step was done two extra times washing by using an automatic washer.
- The substrate solution (100µl) was auxiliary to every pit without shaking the plate after substrate addition.
- Incubation at 25ºCelcius for 20 minutes was done.
- The stop solution (50µl) to respectively well was added and for 15-20 seconds mixed gently.
- At 450nm the absorbance in each well was read (using a reference wavelength of 620-630nm).

(c)Result calculations

Mean absorbance acquired from every reference standard was used to make standard curve via plotting the mean absorbance against respective quantity in mIU/ml and the concentration of FSH in test samples is attained from the standard curve.

3.9.4 Estimation of follicle stimulating hormone (FSH)

Amount of FSH in the serum of controls and patients were estimated through GenWay Immunoassay Test Kit (Catalog Number: GWB-44F874).

(a) Principal:

The FSH test was a solid phase ELISA. The samples were reacted concurrently with antibodies, as consequence FSH becomes sandwiched among the enzyme-linked antibodies as well as solid phase. Color intensity denotes the quantity of FSH in the control sample.

(b) Preparation of Reagents:

Before using reagents were espoused to room temperature. Lyophilized standard was reconstituted with 1ml purified water and shake gently, for 20 min prior to use.

(c) Assay procedure:

Samples, standards and controls (50 µl) were dispensed into particular wells and mixed with 100 µl enzyme conjugate reagent, and shake for 30 sec. Incubation of wells was done for 45 min at 25^{o}Celcius. Rinse the wells with deionized or distilled water at least 5 times. Tap the wells over paper towels to extract whole remaining water. Then the TMB solution (100 µl), was poured for every well dissolved gently for 10 sec and incubate in dark for 20 min at 25 ^{o}C. To stop the reaction, a stop reagent of 100 µl was drizzled per well and were shaken gently for 30 sec. Change of color was recorded at 450 NM within 15 min.

(d) ***Result calculations***

From each reference standard the mean absorbance acquired was used to make standard curve via plotting the mean absorbance against respective amounts in mIU/ml and the FSH amount in test samples is attained since the standard curve.

3.9.5 Determination of luteinizing hormone (LH)

LH hormone level in the serum of controls and patients was estimated through GenWay Immunoassay Test Kit.

(a) Principal

The LH test being solid stage direct sandwich technique. The diluted anti-LH-HRP conjugate and samples remained poured to the Mab LH β-subunit coated wells. LH adheres to anti-LH MAb on the well, which consequently adhere to the anti-LH second antibody. Wash buffer was used to wash off unbound protein and HRP conjugate. The color established after the addition of substrate indicates the LH amount in the samples. The absorbance values that proportionate the color intensity with the LH concentration was used to draw a standard curve.

(b) Reagents preparation

Wash buffer (1X) was made through dissolving the stock buffer solution (20X, 25 ml) to 475 mL of deionized or distilled H2O & kept at 25^{o}Celcius.

(c) Assay procedure

All solutions were combined gently before use. LH standards, controls and samples sera of 50 µl were taken. 100µl of the enzyme conjugate solution per well was added and the plate was sealed off and incubation of 32 minutes at 25^{o}C was done. Liquid from all wells were removed. Wash buffer (1X) was used to washed well thrice. Surplus water was detached through blotting the wells over absorbent towels. 100 µl of TMB substrate were dispensed to every well. 10 minutes Incubation at 25 ^{0}Celcius was done. Finally stop reagent (50 µl) per well was dispensed & the plate was shaken swiftly. After adding the stop reagent, at 450 nm, absorbance was recorded after15 minutes by means of ELISA Reader.

(d) Calculation of results

Absorbance of LH standards versus concentration was employed to achieve a LH standard curve that was used for calculating the concentration of LH in controls and each unknown sample.

3.9.6 "Measurement of Vitamin D

25(OH)-Vitamin D level in the serum of controls and patients is estimated through Pishtaz teb diagnostics Immunoassay Test Kit (Catalogue No. PT-25-hydroxy vitamin D -96-01).

(a) Principle:

The principle is established on competitive ELISA procedure. The procedure exploits mAb Anti-25-hydroxyvitaminD (anti-vitamin D monoclonal antibody) coated in microtiter wells. Subsequent with a convinced volume of extraction buffer, a restrained sum of standards and patient sera are dispensed to the microtiter wells to discharge Vitamin D from its binding protein (DBP-complex). The color intensity is inversely associated to the quantity of endogenous 25-hydroxyvitamin D and proportional to the amount of present biotinylated 25-hydroxy vitamin D in the specimen.

(b) Procedure:

Following protocol was used for calculation of 25-OH Vitamin D

1. Prediluted Standards 1 – 6 (200 µl), prediluted Control1 and Control2 were dispensed into the apt wells
2. Patient sample (200µl) diluted in sample/ biotin buffer was added in respective wells.
3. Incubation at 25 °Celcius for 120 minutes was done.
4. Incubation after couple of hours, the samples from the wells was aspirated and wash buffer (300 µl) was added and the step was repeated thrice and plate was inverted gently on clean dry filter paper inorder to confiscate every droplets of wash buffer.
5. 100ul of enzyme conjugate was added into each well and incubation at 25 °Celcius for about half hour was done.
6. Subsequently incubating the reagents from the wells, were aspirated and 300µl of wash buffer was done and step was repeated thrice and plate was inverted gently on clean dry filter paper inorder to remove every droplets of wash buffer.
7. 100 µl of chromogen/ substrate solution into each well was added and incubated for 15 minutes at 25 °Celcius deprived of shaking (sheltered from undeviating sunlight) was done.
8. Stop solution (100 µl) to each well was added to stop substrate reaction causing blue color to turn yellow
9. By the adding of 100 µl of Stop Solution to respectively well, the substrate reaction was stopped (turning yellow from blue color).
10. At a wavelength of 450 nm, Photometric measurement of the color intensity would be read and a reference wavelength between 620 nm and 650 nm within 30 minutes of adding the stop solution. Prior to measuring, slightly shake the microplate to ensure a homogeneous distribution of the solution.

(c) Calculations

Absorbance 25(OH)-Vitamin D of standards versus concentration was employed to obtained 25(OH)-Vitamin D standard curve that was used for calculating the concentration of 25(OH)-Vitamin D in controls and each unknown sample."

3.10 "Development and characterization of pea protein-stabilized nanoemulsions and protein-vitamin D-pectin nanocomplexes (NVD)".

Pea protein (3 gram) with 90% pea protein (dry basis) was added to 100 mL deionized water. Stirred for 30 minutes at room temperature (23 ^{0}C). Pea protein dispersion was adjusted to pH 12 with 2M NaOH. Ultrasound was applied using a laboratory scale mano-thermo-sonication (MTS) system. A VC-750 ultrasound generator at 20 kHz (Sonics & Materials) was used to deliver acoustic energy to a probe (12.5 mm diameter) placed in a specially designed sonoreactor. Different ultrasound techniques will be applied after pH-shifting to pH 12 for 1-5 min, including manosonication (MS), thermosonication (TS), and mano-thermosonication (MTS). After ultrasound treatment, samples were held 1 h (room temp) prior to the adjustment to pH 7 with 2M HCl. Protein dispersion without treatment was served as the control. Neutralized protein dispersions were centrifuged (1,200 g, 15 min, 20^{o}C). Supernatants were collected for further analyses, including protein content, mean particle size (d_{32} and d_{43}), and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) .

Nano-emulsions were prepared with vitamin D (VD) and pea protein modified with above-mentioned methods as an emulsifier. VD concentrations are 0.5, 1.0, and 2.0% (v/v) in the nano-emulsions. A set dose of soluble pea protein (2.0%, w/v) was utilized. The VD and soluble pea protein were assorted (5 min), followed by sonication (1-5 min) and/or homogenizing by IKA Labor Pilot colloidal mill to obtain the nanoemulsion. Pectin (0.01–0.5%, w/v) was added to nanoemulsion as a stabilizer. Nanoemulsion without pectin was prepared as a control. Pea protein–VD-pectin nanocomplexes were prepared at different protein: VD ratios. VD was slowly added to soluble pea protein with

strong stirring at the determined optimum protein to VD molar ratios. The pH values of the nanoemulsion and nanocomplex pH was accustomed from 3 to 7. Particle size (d_{32} and d_{43}) and stability of the nanoemulsions and nanocomplexes were calculated. Centrifugation was done to measure the Physical stability of nanoemulsions .

3.11 *In vitro* studies

3.11.1 Cell Culture

Two human HCT116 and HT29 colorectal cancer cell lines (gained from College of Pharmacy, King Saud University, KSA) were developed in a 5% CO_2 atmosphere at 37ºC in medium containing DMEM medium 1640 (GIBCO), 1% penicillin/streptomycin as well as 10% fetal bovine serum. Taxifolin (TAX), Nanoparticle with Vitamin D (NVD) and β- catenin inhibitor (FH535) dissolved in DMSO was applied for cell treatment. Cells with 70% confluency were induced with TAX, NVD and β- catenin inhibitor for 48h in cell culture medium and the dilution of DMSO applied for each treatment was 0.1%.

3.11.2 Cell Viability assay

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazoliumbromide protocol were performed to show the impact of Taxifolin (TAX), Nanoparticle with Vitamin D (NVD) and β- catenin inhibitor (FH535) on the viability of HCT116 and HT29 cell lines. 1×10^4 cells / well were plated in 1 ml of culture medium consisting of 10-200 µM dilution. Cells were gestated with humidifier aimed at 37 ºC for 48 h, 200 µl of MMT (5 mg/ml PBS) remained supplemented to respective wells & kept aimed at two hours, 200 µl of DMSO were added to each plate which were then spinned (1800 × g for 5 min at 4 ºC). The readings at 540 nm wavelength were noted on a microplate reader (Elx 800). Impact of TAX, NVD and FH535 β- catenin inhibitor on inhibition of growth was counted equally % cell viability as DMSO-administrated cells were kept as control. Absorbance numbers of media containing wells were subtracted from test sample values.

$$\text{Cell viability} = \left[\frac{\text{Absorbance of Sample - Absorbance of Blank}}{\text{Absorbance of DMSO - Absorbance of Blank}} \right] \text{X } 100$$

3.11.3 Clonogenic assay

HCT116 cells and HT29 cells were collected subsequently to treatments with active TAX and NVD for 48 h. Cells were suspended in fresh medium, cell number was determined, and 500 cells (HCT116) and 1000 cells (HT29) stayed overlaid in three experiments into 35 mm cell culture dishes. Subsequently 8 days in culture, crystal violet was used to stain colonies as described (Qaiser et al., 2014). Cell colonies stood counted beneath dark field by means of a cubic colony counter (AO scientific). In each colony respective amount of cells remained resoluted through microscope (phase contrast), Data was represented as colony number in TAX and NVD groups comparative to normal. Statistics remained expressed as mean ± SEM of separate three tests.

3.11.4 Cell cycle analysis/ Apoptosis assay

In complete medium, all HCT116 and HT29 were administrated with TAX and NVD (20-60µM, 48h) were trypsinized & fixed in 1% paraformaldehyde:1xpbs & chilled PBS was used for washing twice and spinned. Chilled 70% ethanol was used to suspend cell pellet and incubated 24hrs. The cells remained spinned aimed & Chilled PBS stayed used to wash the pellet obtained twice to eliminate ethanol then finally cells are fixed with FITC & propidiun iodide by the Apo-Direct Kit (BD Pharmagen, CA). Approximately 10,000 cells/sample remained harvested. DNA Histograms were scrutinized with ModiFitLT software (verity Software House, ME,USA).

3.11.5 Western Blot Analysis

Western blot and SDS-PAGE investigations were executed by previously described protocol withslight amendments (Trembley et al., 2011). Shortly, since 24 h & 48 h of administration with TAX and NVD at particular quantities, HCT116 & HT29 cells were lysed in DMEM buffer augmented with recently dispensed protease & phosphatase inhibitor cocktail 1:100 (Santa Cruz, CA) & protein quantity was estimated by Bradford

assay (Kruger, 1994). Same amount of proteins was loaded by means of 4-12% Tris-glycine gels (NOVAX life technologies, USA). Proteins were then relocated to nitrocellulose membrane (Millipore). Membranes were incubated in either 5% BSA (Sigma) or 5% nonfat dry milk (Bio-Rad, Cat. #170-6404) (depending on antibody instruction protocol) as blocking buffer aimed at 1 h at 25^{o}Celcius. Blots were incubated with primary antibody overnight at 4^{o}C. Immunoblotting was accomplised using the subsequent antibodies: CK2α (ab10466) and Stat 3 (SAB4300034), JAK2 (#3230), ERK1/2 (#9102), phosphoAkt (Ser473, #9271), phospho-Akt (Thr308, #9275), phospho-IκB Ser32/36 (#9246), Bcl-2 (ab32124),Bcl2-xl (ab2568),p21 (ab188224),p27 (ab137736),Cyclin B1(ab2949), Cyclin D1(DCS-6),Cyclin A (H-432),cyclin E (ab3927), Cdk-2 (ab64669), Akt (ab126811), Cdk-4 (ab137675), phospho-Stat3 (Tyr 705, #9145), Cdk-6 (ab151247),pcdc-25 (ab47322), , phosphor-ERK1/2 (Thr202/Tyr204, #9101), xIAP (#2042), PI3K (#4292) from Cell Signaling Technology; Survivin (AF886) from R&D Systems; NFκB p65 (sc8008), phospho-NFκB p65 Ser529 (sc101751), and actin (sc1616) from Santa Cruz Biotechnology. Membranes were rinsed thrice (15 min each) in Tris buffered saline containing 0.1% Tween 20 (TBS-T) before incubating with anti-rabbit or anti-goat secondary or HRP-conjugated anti-mouse antibodies (depending on the primary antibody employed) for 2 h at 25^{o}Celcius. Membranes were then bathed for 30 min with TBS-T, and HRP signal detected using super signal west Pico, Dura or Femto Chemiluminescence Reagent (Thermo scientific, USA). Protein band quantifications of was estimated through checking band density by means of Image J software. The densities of the bands (normalized to actin) relative to with respect to control (untreated, designated as 1.00) were presented as mean ± SEM of three separate experiments.

3.11.6 Preparation of lysate

- On ice the cell culture dish was positioned & by chilled PBS, the cells were washed.
- PBS was articulated as well as chilled lysis buffer (1 mL) was added.
- By expending a cold plastic cell scraper, the adherent cells were worn off the dish after that the cell suspension remained relocated gently into a pre-chilled microcentrifuge tubes.
- Continuous agitation at 4°C aimed at half an hour was maintained
- Centrifugation at 4ºC aimed at 20 minutes at 12.000rpm was done.
- On ice, the tubes were positioned, the supernatant was aloofed, retained in a novel tube & the pellet was castoff.

3.11.7 Protein extraction:

- Of BSA standard, five to eight dilutions through an assortment of 5 to 100 µg proteins were prepared
- Protein samples were adulterated to attain 5-100 µg protein/30 µl. (Ratio 5:100)
- Standard solution or protein sample (930 µl of each) was dispensed to a suitably labeled test tube.
- Bradford reagent (1.5 mL) remained dispensed to every tube & assorted properly.
- Incubation at 25ºCelcius aimed at 5 min was done.
- At 595 nm, absorbance was measured.

3.11.7.1 Lysis buffer:

RIPA buffer (radioimmunoprecipitation assay buffer)

- Sodium chloride (150 mM)
- Triton X-100

- Sodium deoxycholate (0.5%)
- sodium dodecyl sulfate
- Tris (pH 8.0, 50 mM)

Tris-HCl buffer

- Tris-HCl (pH 7.5, 20 mM)

3.11.7.2 Bradford reagent

- In 50 ml of methanol, 50 mg of Coomassie Brilliant Blue G-250 and 100 ml 85% (w/v) phosphoric acid (H_3PO_4).

3.11.8 Gene expression analysis

Whole RNA was extracted (RNeasy Mini Kit (Cat No./ID: 74104) from the cells using the following method. RNA dilution was determined by using a spectrophotometer at 260 NM. cDNA was prepared by succeeding the manufacturer protocol (BioLabs E6300) using the kit. The reaction mixture remained organized comprising 10 µl FastStart Universal SYBR Green Master (Roche, Germany), 10 µg cDNA and 6 µM reverse primers through RNAase unrestricted water dispensed to an entire bulk of 20 µl. The real time investigation & amplification were done for 35cycles with succeeding features; 95 ^{o} C (10 min) in order to stimulate of FastStart Taq DNA polymerase; 60 ^{o}C (1 min) aimed at amplification & real-time investigation. The gene expression intensities remained resoluted by means of $2^{-\Delta\Delta CT}$. Following Primer sequences were used ;

CTNNB1 Sense 5′ -TGTGAATCCCAAGTACCAGTGT-3′

CTNNB1 Antisense 5′ - CGTCAGACAAGGAGAAACATT-3′

β-Actin Sense 5′ - CCTCTTCCTCAATCTCGCTC-3′

β-Actin Antisense 5′ - GCTCAATGTCAAGGCAGGAG-3′

3.11.8.1 RNA Isolation Procedure from cells

Following protocol was followed for extraction of RNA from cells

1. 10^6 cells were taken and the media were articulated and washed once by ice chilled PBS (1–2 ml) and spinned.
2. PBS was enunciated and 1ml TRIzol was added.
3. The plate was scraped briefly and then TRIzol was pipetted out and cell lysate was relocated into a new 1.5ml eppendorf tube.
4. Incubation for 5min was done at room temperature.
5. Chloroform (250µl) was added and then for about 15 Seconds the tube was shaken vigorously.
6. Incubation for 5min at room temperature was done.
7. Centrifugation at 10,000rpm for 5minutes was done.
8. At this point, in each tube three layers were seen:
   (i) Top layer: aqueous, clear
   (ii) White precipitated DNA: Middle layer/Interphase:
   (iii) Pink organic phase: Bottom layer
9. The aqueous phase was carefully pipette out and placed in new tube.
10. Isopropanol (550 µl) was dispensed to the clear period & mixed lightly.
11. Incubation for 5min at room temperature was done.
12. Centrifugation at 14,000 rpm for 30minutes was done.
13. Samples were positioned on ice and isoproponal was pipette out and 1ml ethanol (75%) in diethyl pyrocarbonate (DEPC) administrated water was added.
14. Centrifugation at 9,500 rpm for 5 minutes was done.
15. Then ethanol was articulated & the pellet was air dried
16. Then after the mild air dye, 15-25µl of Tris EDTA (TE) was dispensed to the RNA pellet.

17. Absorbance at 260nm was taken and The 260/280 ratio was greater than 1.8

3.11.8.2 First Strand cDNA was prepared by using a kit (NEB #M0253)
All RNA was thawed and put on ice.

a) Procedure

- 4-6µl RNA sample, 1µl of 10mM dNTP and 2 µl primer $d(T)_{23}VN$ were mixed with 2 µl nuclease-free H_20 in sterilized RNase-free microfuge tubes.
- RNA for 5 minutes at 70°C was denatured then momentarily spinned & quickly positioned on frost.
- Incubation at 42^0C aimed at an hour, 20 µl cDNA synthesis retort was done
- Aimed at 5 minutes at 80°C, the enzyme was inactivated.
- Reaction was adulterated with water (30 µl) for PCR and at -20°C the cDNA product was stored.

3.11.9 Imunofluorescence Microscopy

HCT116 and HT29 cells remained cultured in a binary chamber tissue culture glass slides & were were administrated through 40µM of TAX and NVD for 24h at 75% confluence. When the chamber was removed, Phosphate buffer was used to rinse the slides, 2% paraformaldehyde was castoff to fix the cells and permeablized in methanol. Slides were rinsed with phosphate buffer and 2% serum was used as blocking agent. Overnight primary antibody incubation was tailed through incubation through applicable fluorophore tagged secondary antibody. For mounting antifade DAPI (Invitrogen NY) was used to apply & hematoxylin for counter staining. Examination was done by using Bio-Rad Radiance system (2100 MP Rainbow) aimed at imaging. The Annexin-V-fluos staining Kit (Roche, Switzerland) remained cast-off. Towards perceive necrotic & apoptotic cells, according to the kit's procedure. Zeiss 410 confocal microscopy remained cast-off to measure fluorescence. Annexin V and propidium iodide been used to stain the cells. The unstained cells in a particular arena remained premeditated to govern the necrosis level as well as apoptosis.

3.12 *In vivo* tumor xenograft model.

Athymic male mice remained assimilated since King Faisal Hospital and research center, Riyadh, KSA, were homed under contamination free environment (12 h day/12 h night schedule), and nourished with a sterilized food adlibitum. HCT116 cells were selected for evaluating the *in vivo* impact of TAX, NVD & β-catenin inhibitor (FH535), as they generate fast tumors in mice. Cells were collected, suspended in complete DMEM. Tumor xenografts HCT116 cells in mice were established by injecting cells subcutaneously (1×10^6) assorted through matrigel in a proportion of 1:1.

Forty-two mice were categorized into four categories.

Group1: Served as Control Group, consisting six mice, intra-peritoneally (i.p) received DMSO.

Group2: Divided into two subgroups; Group 2a and 2b consisting of six animals each. Received TAX (15 and 25mg/kg) intra-peritoneally (i.p) twice weekly respectively.

Group3: Divided into two subgroups; Group 3a and 3b consisting of six animals each. Received NVD (15 and 25mg/kg) intra-peritoneally (i.p) respectively, twice weekly.

Group4: Divided into two subgroups; Group 4a and 4b consisting of six animals each. Received FH535 (15 and 25mg/kg) intra-peritoneally (i.p) twice weekly, respectively.

During the observation, body weight of animals, food and water expenditure were documented twice a week. Tumor volume was measured by digital caliper and calculated using the formula L1 x L2 x H x 0.5238 (Height= height of the tumor) & tumor sizes remained chronicled twice in a week. By the finish of the experiment once tumor volume attained size to ~1200mm^3 animals were sacrificed by CO_2 inhalation was used as anesthesia. Tumors were resected, weighed and frozen at -80 ^oC for subsequent western blotting, RNA extraction and immunohistochemistry. Whole Blood was collected for hormone and Vitamin D analysis.

3.12.1 RNA Isolation Procedure for Tissue

1. 1ml TRizol was added to a sterile culture tube with frozen tissue.

2. Homogenization was done and TRIzol solution was relocated into a 1.5mL eppendorf tube.
3. Incubation for 5min was done at room temperature.
4. Chloroform (250µl) was added and then the tube for about 15 Seconds was shaken vigorously.
5. Incubation for 5min was done at room temperature.
6. Centrifugation at 10,000rpm for 5 minutes was done.
7. At this point, three layers were seen in respective tube:
   (a) Upper layer: aqueous, transparent.
   (b) Central layer/Interphase: white precipitated DNA
   (c) Lowest layer: organic phase
8. The aqueous phase was prudently detached by means of a pipette & positioned in new tube.
9. Isopropanol (550 µl) was dispensed to the upper phase & mixed mildly.
10. Incubation aimed at 5min at room temperature was done.
11. Centrifugation at 14,000 rpm for 30minutes was done.
12. Samples were positioned on ice and isoproponal was pipette out and 1ml ethanol (75%) in diethyl pyrocarbonate (DEPC) administrated water was added.
13. Centrifugation at 9,500 rpm for 5 minutes was done.
14. Then ethanol was aloofed & the pellet was dried by air.
15. Then after the mild air dye, 15-25µl of TE was dispensed to the RNA pellet.
16. Absorbance at 260nm was taken and The 260/280 ratio was greater than 1.8.

3.13 First-Strand cDNA Synthesis:

Following prodecure was used

1. 1-5µl of RNA was engaged in the effendorf tube on ice. 1 µl of 10nM dNTP Mix & 2 µl of Oligo (dT)20 (50 µM) were dispensed, mixed and spinned momentarily to collect the contents.
2. Incubation by 70^{o}C for 5minutes was done and spinned briefly to collect the condensation.
3. 5X RT Buffer were added to ice, mixed gently and spun to collect the contents .
4. The tube was transferred to thermo cycler for preheating at 50^{o}C for 50 minutes.
5. For 5minutes at 85^{o}C, the reaction was inactivated.
6. then incubation aimed at 20 minutes at 37°C was done.
7. On ice, the reactions were unflustered.
8. The prepared single stranded cDNA was used for RT-PCR.

3.14 Hormone analysis and Vitamin D estimation of Xenografts

Mandibular bleed was used to take blood samples and serum was taken out. The estimation of testosterone, LH, FSH, Estradiol and Vitamin D was done with the use of precise kits.

Concentration of testosterone, Estradiol, LH and FSH in the serum of controls and treated groups was estimated through GenWay Immunoassay Test Kit (Catalog Number GWB-472DB9, Catalog Number: GWB-AFC61F, Catalog Number: 40-052-115018 and Catalog Number: GWB-44F874 respectively).

Vitamin D in the serum of controls and treated group was estimated through My Biosource kit (Catalog Number: MBS728692).

3.15 Western Blot Analysis

a) Preparation of lysate from tissues

- The tissue was dissected on ice and placed in eppendorf tube and immersed in liquid nitrogen and kept on ice
- ~200 mg piece of tissue was taken and 12000µl of unflustered lysis buffer was dispensed rapidly to the conduit,
- Homogenization with an electric homogenizer was done and the blade was rinsed twice through 200 µL of lysis buffer, then constant agitation by orbital shaker was stabilized from 2h at 4 ^{0}C.
- Upper layer was aloofed gently & positioned in a new tube on ice & the pellet was castoff.

3.16 Immunohistochemistry

Previous illustrated method was used for immunohischemistry

3.17 Data analysis

Densitometry of western blot images was performed using an image analysis program (Image J 1.41; National Institutes of Health). Data of *in vitro* assays was analyzed GraphPad Prism 5 software to determined IC_{50} values. Intensity of consequence amid dissimilar administrated groups comparative to normal were estimated by one-way analysis of variance trailed by Tukey's multiple comparison test. Contrast between more than one parameter was accomplished using two-way analysis of variance (ANOVA) tailed by Bonferroni multiple comparison test. $p < 0.05$ remained statistically significant. Where required correlation analysis was done and R values were calculated.

Chapter 4
Results

4. Results

4.1 DNA Sequencing

Sequence analysis revealed two activating mutations (S33F and T41A) in exon 3 of *CTNNB1* gene involving the transition of C.T and A.G at amino acid position 33 and 41 respectively (p.C33T and p.A41G). This substitution resulted in replacing a hydrophilic neutral serine to a hydrophobic phenylalanine at amino acid position 33 [TCT (Ser) → TTT (Phe)] (Figure4.1) and a polar threonine was converted to non-polar alanine at amino acid position 41 [ACC (Thr) → GCC (Ala)] (Figure4.2), of exon 3 of *Beta catenin* gene. Equivalent non-tumorous tissue did not show a mutation. The adjacent normal tissue and tumor tissue was acquired from a 65-year- female and 54-year-old male subject through cecal cancer and retroperitoneal mass. The current observation revealed that β-catenin might have vital part during progression of colorectal carcinoma as well as that stimulating alterations of the *CTNNB1* gene might supernumerary bi-allelic *APC* inactivation in this tumor type in Pakistani Population.

We carried out an immune-histochemical examination on, paraffin-embedded formalin-fixed tissue using anti β-catenin monoclonal antibody exploring the protein expression intensity of β-catenin in the tumor as well as the adjacent customary tissue. Increased protein expression was shown by cancerous cells with the T41A and S33F mutation as compared to healthy adjacent cells through normal β-catenin protein (Figure 4.3). The boosted protein expression in S33F and T41A mutated cancer cells were restricted to the nucleus as well as in cytoplasm.

4.2 Mapping and characterization of mutations in Crystal Structure of β-Catenin

Due to lack of full length CTNNB1 structure, protein threading techniques and de novo loop modeling were used to model N-terminus region (1-148 amino acids). To characterize and evaluate the structural impact of mutations on CTNNB1 protein. The reported mutation in the present study were mapped upstream of the armadillo repeat

domain in the N-terminus intrinsically disordered region. The N-terminal muddled section of CTNNB1 holds a conserved squat undeviating motif phosphorylated by GSK-3 and are accountable aimed at binding of TrCP1 (β-TrCP) with phosphorylated CTNNB1 (Kimelman and Xu, 2006a, Zhang et al., 2011) (Figure 4.4). Axin helps GSK-3-mediated phosphorylation of β-Catenin by bringing them in close proximity (Mannava and Tolwinski, 2015). For the predicted $CTNNB1^{WT}$ model Ramachandran plot specified the occurrence of roughly 97.37% residues in promising region. Furthermore, additional factors including non-bonded interactions, peptide bond planarity, overall G-factor, Ca tetrahedral distortion, poor rotamers, and main chain H-bond energy for the modeled structures falls within the promising series. The $CTNNB1^{WT}$ structure was used to model mutated *CTNNB1*.

4.3 Binding orientation and Interaction mode analysis.

To evaluate the conformational modifications in CTNNB1 upon interaction with GSK3 and TrCP E3 ubiquitin–ligase, 3D structure of mutant and wild type full length CTNNB1 was investigated. Docking analysis revealed most pronounced changes in the N-terminus disordered region of *CTNNB1*. GSK3 phosphorylates β-catenin at T41,S33, and S37 residues (Zhang et al., 2011). *In-vivo* studies revealed that mutation of CTNNB1 at S/T position abolish its phosphorylating by GSK3 because it is a processive kinase that consecutively phosphorylates S/T pentad repeats from the carboxy- to amino-terminal direction (Harwood, 2001, Woodgett, 2001, Cohen and Frame, 2001). In the present study docking analyses of $CTNNB1^{WT}$-GSK3 complex indicated that the S33, S37, and T41 residues of CTNNB1 involved in an interaction with R92, R96, R180, K205, V214, I217, Y288, and E290 of GSK3 (Figure4.5). A proportional docking analysis of an interaction of GSK3 with $CTNNB1^{WT}$ and $CTNNB1^{MT(S33F\ and\ T41A)}$ revealed that due to mutations in CTNNB1 binding of GSK3 was abolished and it moved away from N-terminus disordered region containing GSK3 phosphorylation motif (Figure4.6) . Overall the total binding energy for $CTNNB1^{WT}$-GSK3 interaction was -42.789KJ/mol while the

binding interaction energy for CTNNB1$^{MT(S33F\ and\ T41A)}$-GSK3 interaction was -28.66KJ/mol and -25.89KJ/mol respectively.

TrCP1 have two domain the N-terminal domain known as F box domain (residues 139 to 186) , a C-terminal WD40-repeat domain (residues 253 to 545) and these two domain are linked by an -helical domain (residues 187 to 252) (Schulman et al., 2000). A WD40 domain structure has a narrow channel in the middle of the structure which has a narrow top face (Wall et al., 1995). Docking analysis of CTNNB1^{WT}-TrCP1 complex indicated that the destruction motif of CTNNB1 was buried at the top face of the TrCP1 narrow channel. Y271, S309, S325, R285, S448, G432, R474, Y488 of TrCP1 were involved in a number of binding interactions with N-terminus phosphorylated motif of CTNNB1^{WT}. Comparatively, docking simulation of TrCP1 with CTNNB1^{WT} destruction motif and CTNNB1$^{MT(S33F\ and\ T41A)}$ destruction motif revealed that due to mutation in phosphorylation site of destruction motif CTNNB1, its binding within the narrow channel of TrCP1 was obliterated it moved away channel of TrCP1 WD40 domain (Figure4. 5 & 4.7). Overall the total binding energy for CTNNB1^{WT}destruction motif-TrCP1 interaction was -45.789KJ/mol while the binding interaction energy for CTNNB1 $^{MT(S33F\ and\ T41A)}$-destruction motif-TrCP1 interaction was -20.66KJ/mol and -15.89KJ/mol respectively. The docking analysis showed that substitution of S33F and T41A at the N-terminus phosphorylation motif of CTNNB1 eradicated its interaction with GSK3 and TrCP1.

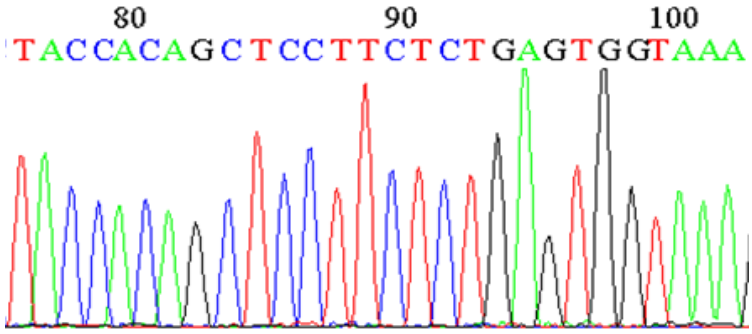
4.4 Molecular dynamics simulation analysis

The CTNNB1^{WT} and CTNNB1 $^{MT(S33F\ and\ T41A)}$ were further investigated by molecular dynamics (MD) simulation assay in order to investigate the overall stability of the scheme & to study the time-reliant behavior. The stability of secondary structure elements and conformational changes of simulated complexes were evaluated by plotting root mean square deviation (RMSD), root mean square fluctuation (RMSF), obtained throughout the trajectory. Our analysis specified that the backbone RMSD score observed over a period of 20 ns remained stable (2 Å) throughout the simulation. These data

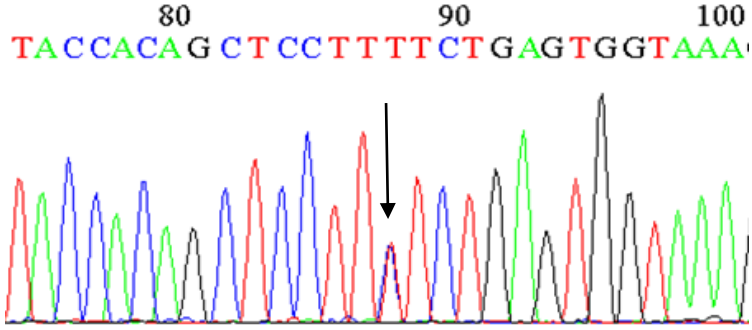
validated the stability of CTNNB1^{WT} system during MD simulation. In general, N-terminal regions of exhibited more fluctuations than the C-terminal region, which is important for the GSK3 and β-TrCP binding. In CTNNB1^{WT} the high fluctuations observe between 1-110 residues and the systems remain stable at 200-400 residues. The RMSD analysis of CTNNB1$^{MT\ (S33F)}$ indicated fluctuation in the N-terminus disordered region and extended up to 170 residues. Similarly, CTNNB1$^{MT\ (T41A)}$ showed more fluctuations in the N-terminus disordered region and extended up to 200 residues (Figure4.8a-c). The high RMSD fluctuation rate of two mutant indicated that these mutant have a negative impact on protein stability.

Subsequent analysis of root mean square fluctuation (RMSF) per residue indicated a high fluctuation rate in the N-terminus regions upstream of armadillo repeat. However, armadillo fold were stable and exhibited minor fluctuations in CTNNB1^{WT} as compared to CTNNB1 $^{MT(S33F\ and\ T41A)}$ (Figure4.8d).

A



B



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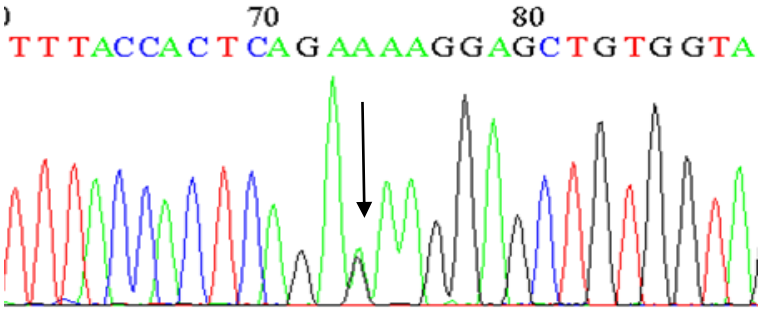


Figure4. 1: "Sequence analysis of CTNNB1 exon 3 in colorectal cancer tissue and corresponding normal tissue of the same patient. Heterozygous mutation at codon 33 (TCT to TTT) in tumor. Chromatogram of *CTNNB1* exon 3, (A): DNA from normal tissue with wild type codon TCT (S33) sequenced with forward primer and (B), (C): DNA from tumor tissue with heterozygous mutation S33F (TTT>TCT) sequenced with forward primer and with reverse primer."

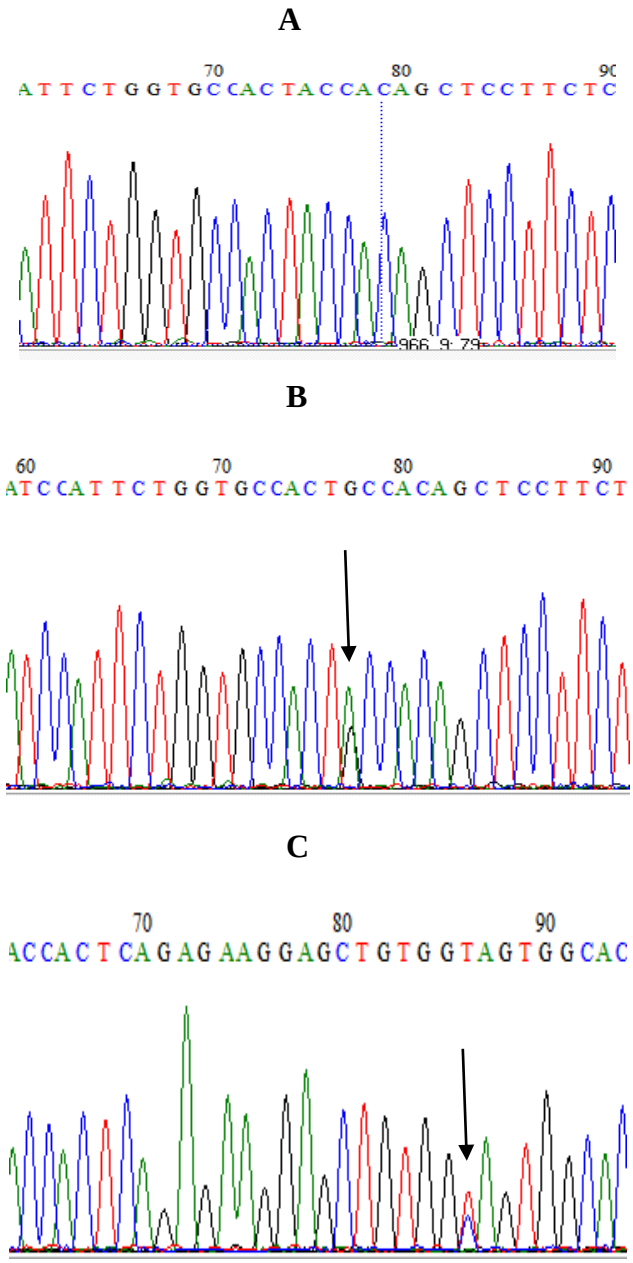


Figure4. 2: "Sequence analysis of CTNNB1 exon 3 in colorectal cancer tissue and corresponding normal tissue of the same patient. Heterozygous mutation at codon 41 (ACC to GCC) in tumor. Chromatogram of *CTNNB1* exon 3, (A): DNA from normal tissue with wild type codon ACC (T41) sequenced with forward primer and (B)and (C): DNA from tumor tissue with heterozygous mutation (ACC>GCC) sequenced with forward primer."

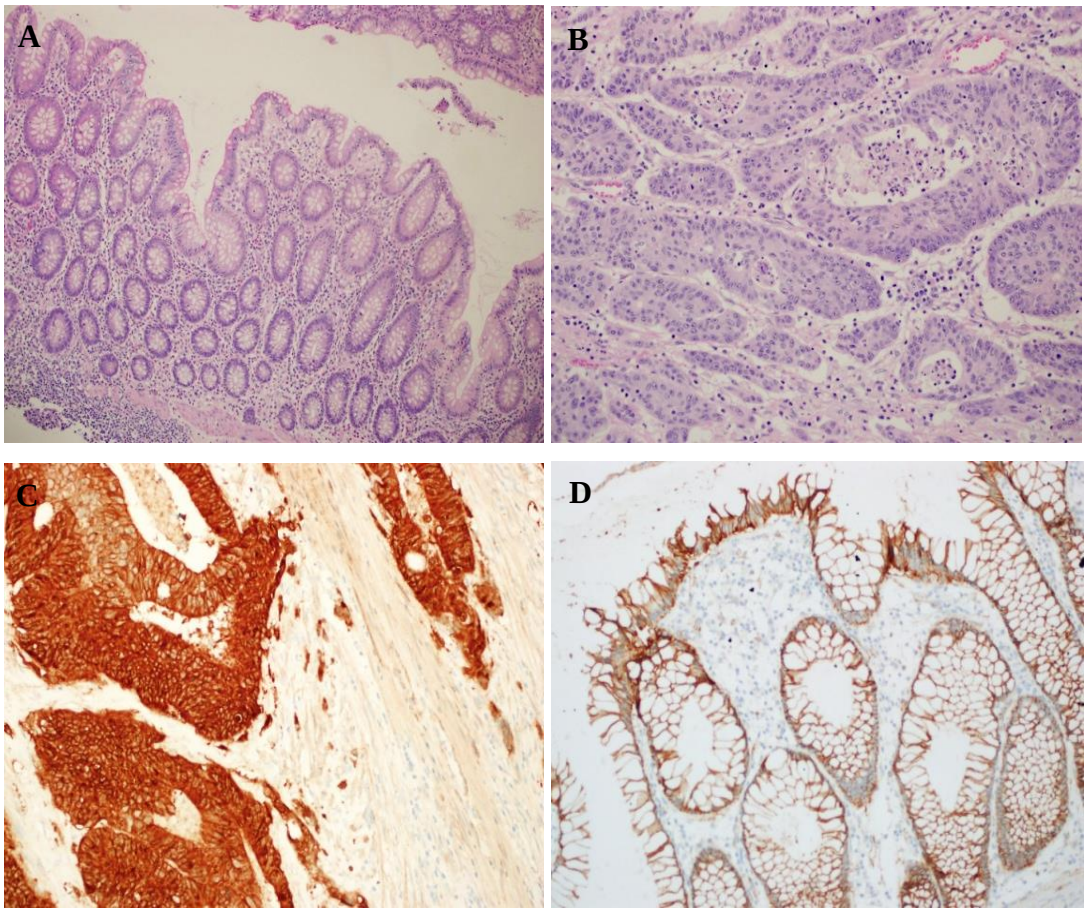


Figure4. 3: **"**Protein expression of β-catenin in normal colon and colorectal cancer tissue from the same patient. Normal colon(A) and colorectal cancer (B) stained with H & E. Slide showing strong nuclear and cytoplasmic localization of β-catenin in the tumor cells (C) and weak staining in normal tissue (D)."

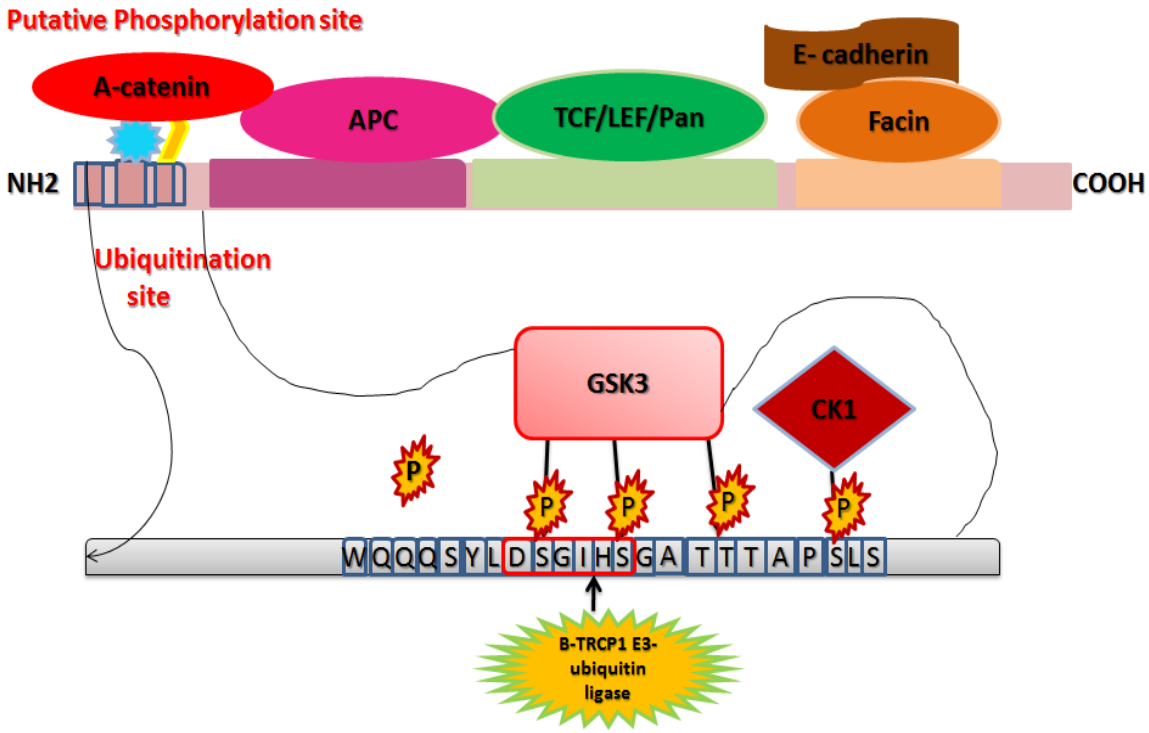


Figure4. 4: Regulation of CTNNB1 at molecular level.

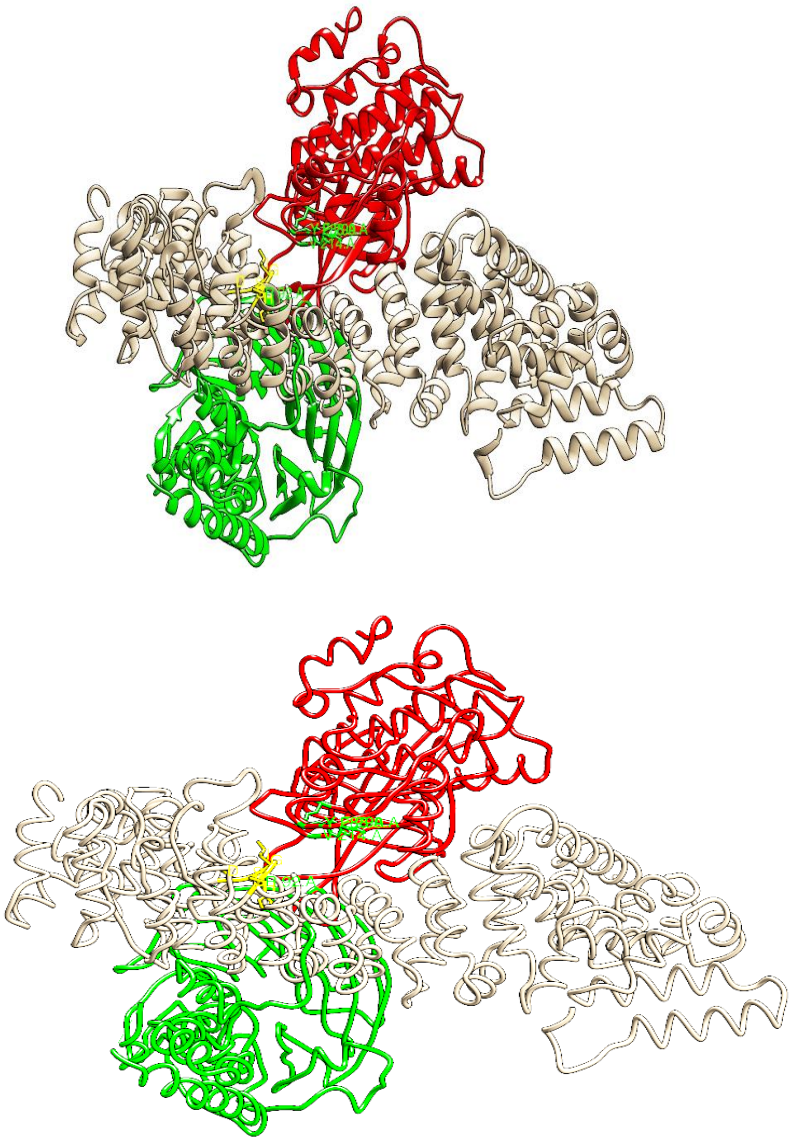


Figure4. 5: **Molecular docking complex of CTNNB1 with GSK3 and TrCP1.** Tan color ribbon represents CTNNB1 along with destruction motif shown in yellow. GSK3 is displayed in red ribbon with interacting residues shown in green sticks. TrCP1 is represented in green ribbon.

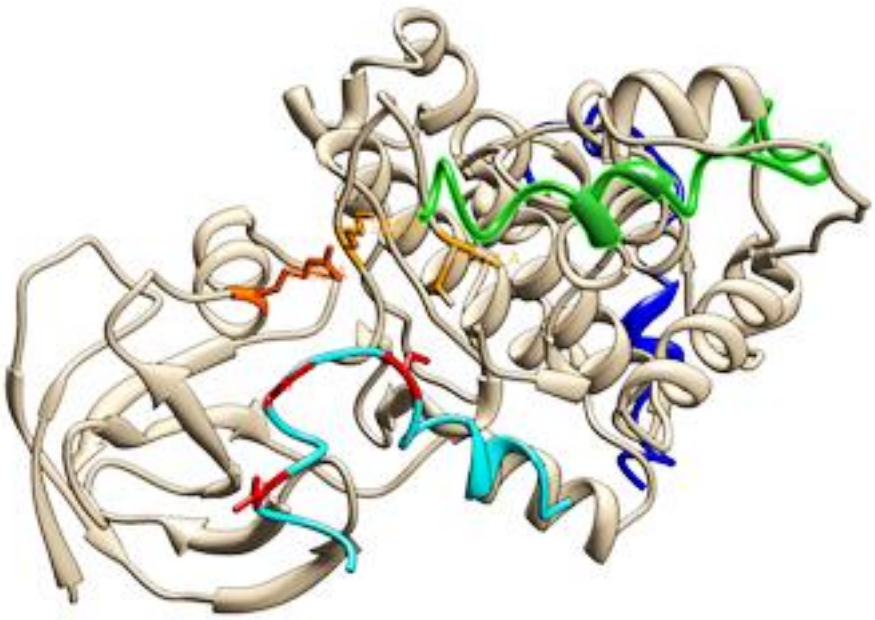


Figure4. 6: **Molecular docking complex of CTNNB1$^{MT\ (S33F\ and\ T41A)}$ with GSK3.** Interaction of GSK3 and its substrate of CTNBB1 motif. The cyan color show the wild motif, green show mutant S33F and blue showed the mutant T41A. The red color sticks represent interacting residue of CTNBB1 and interacting residue of GSK3 are shown in orange sticks.

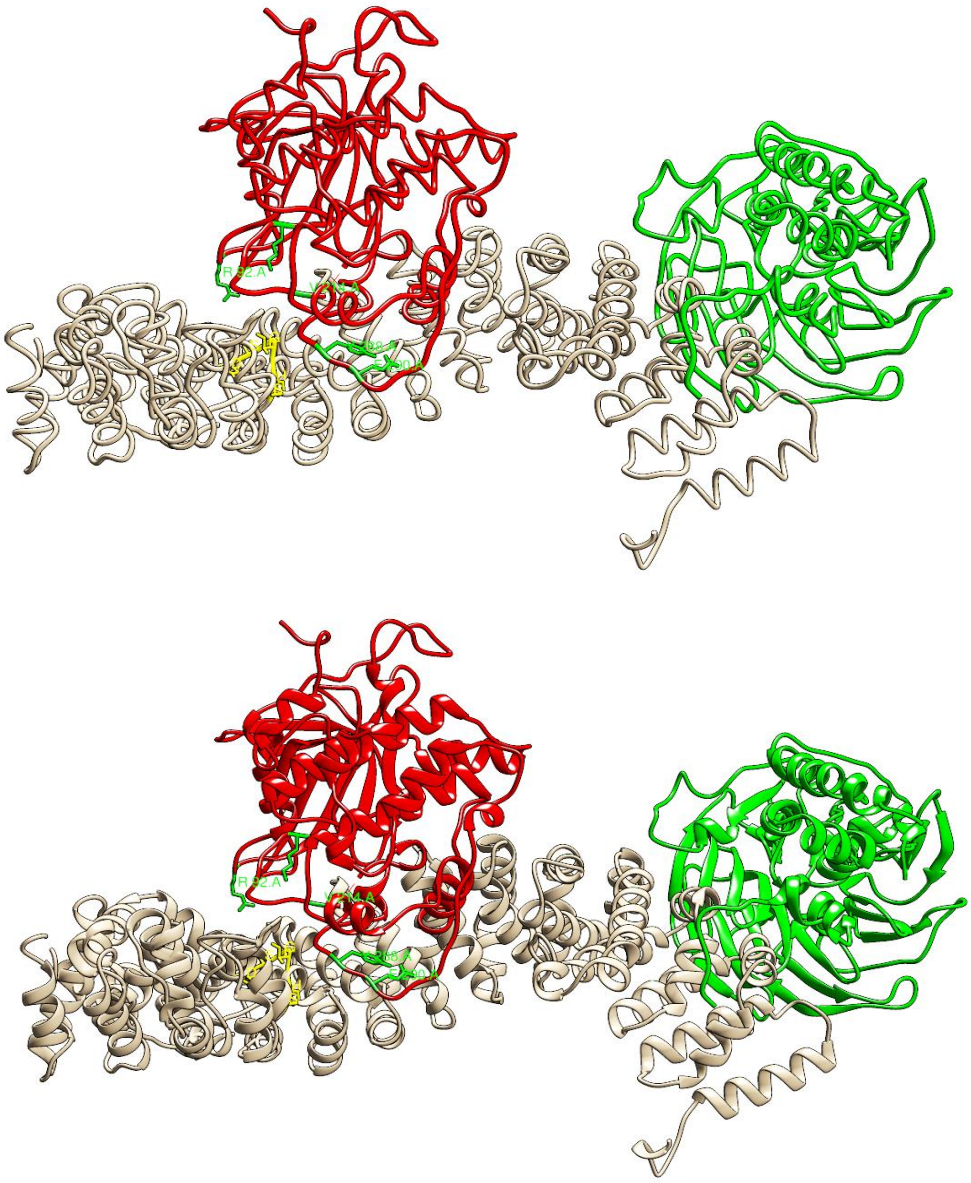


Figure4. 7: **Molecular docking complex of CTNNB1$^{MT\ (S33F\ and\ T41A)}$ with GSK3 and TrCP1**.Tan color ribbon represents CTNNB1 along with destruction motif shown in yellow. GSK3 is revealed in red ribbon with interacting residues shown in green sticks. TrCP1 is represented in green ribbon as it moves away from binding site.

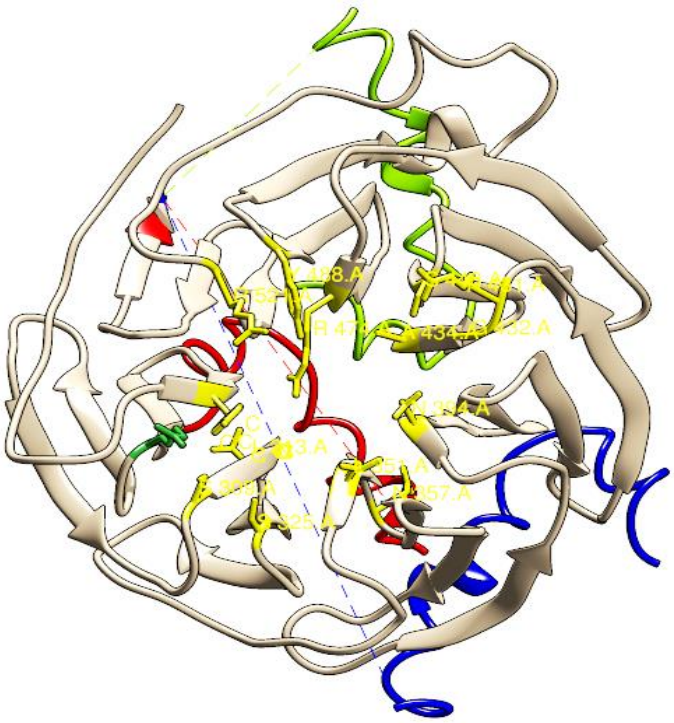


Figure4. 8: **Molecular docking complex of CTNNB1 ^{WT} and CTNNB1$^{MT\ (S33F\ and\ T41A)}$ with TrCP1**. TrCp1 is shown in tan colour ribbon with interacting residues of narrow channel shown in yellow sticks. Wild type destruction motif of CTNNB1 is shown in red ribbon and loop form. S33F mutant CTNNB1 motif is shown in blue and T41A mutant is shown in green color**.**

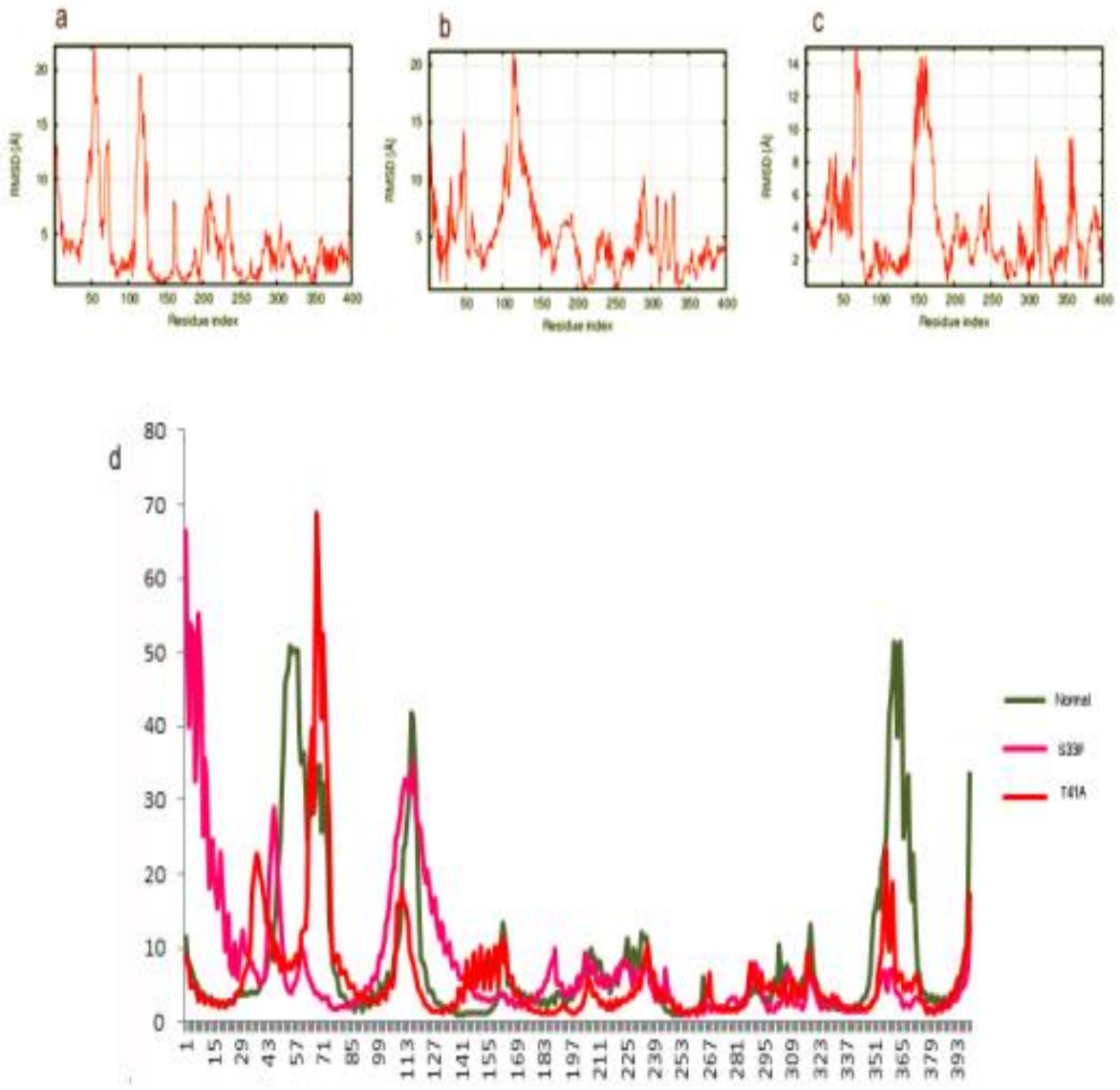


Figure4. 9: **Plots to explore the stability and fluctuation of MD trajectories for a wild type and mutant CTNNB1 systems.** RMSD Plots computed through each system trajectory (a) Wild type CTNNB1 (b) S33F mutant of CTNNB1 (c) T41A mutant of CTNNB1. (d) RMSF plot for mutant and wild type CTNNB1 systems

4.5 Demographics of male and female patients

The demographic features (Table 3.1) of CRC patients analyzed in the current study a total 200 subjects through colorectal cancer obligated a standard 25-OH vitamin D evaluated within March. The mean phase of the populace remained 55.3 years (±15.6; Assortment: 20-90 years). About 80% of the subjects obligated colon, & 20% obligated rectal cancer. All of the sample were of normal body weight (mean BMI=23.5; Range 20-24.8). Blood samples for analysis were obtained during March (before start of summer). The mean 25-OH vitamin D intensities within inclusive colorectal populace remained 18.8 (SD 9.11) ng/ml respectively.

4.6 Sex hormones concentrations in CRC patients

Table 4.1 shows serum concentrations of testosterone, estradiol, FSH and LH hormones in young male vs young female. Only estradiol concentration was considerably advanced in young female in comparsion to young male patients ($p<0.001$). Table 4.1 also shows serum concentrations of testosterone, estradiol, FSH and LH hormones in old male vs post-menopausal female CRC patients. As evident, the concentrations of these hormones were significantly lower in post-menopausal female CRC patients as compared to their male counterparts of old age (p, for all trends <0.05).

In postmenopausal cases, physiological reduction of estradiol level makes a noteworthy difference ($P<0.001$) in estradiol level amongst males and female with high levels in males of the equivalent ages. Both LH and FSH showed significant gender difference only in older ages. Furthermore, the comparison of hormone concentration between premenopausal and post-menopausal females revealed that the level of estrogen is markedly decreased in older post-menopausal CRC patients, which might be associated with CRC progression.

4.7 Impact of patients demographics on vitamin D status

Table 4.2 indicated the distribution of 25-hydroxyvitamin D [25(OH) D] intensities CRC cohort. Aimed at current observation, 25-OH VD eminence was categorized by two sorts

"very low" & "low to normal." The "very low" classification remained demarcated as ≤16 ng/ml & the "low to normal" sort remained demarcated at >16 ng/ml. Intensities beneath 16 ng/ml were factually taken as low. Additionally, ≤16 ng/ml matches towards the nethermost quartile of the populace. Parameters examined comprises gender, phase, primary site (colon vs. rectum), phase of disease (stages I–III vs. IV). BMI & date of 25-OH vitamin D assay remained unconsidered for regression analysis as all patients had normal BMI (Range 22 – 24.9) and blood was collected from all patients in the same month of the year thus excluding the possibility of significant seasonal variations in serum vitamin D concentrations (Table 4.3).

4.8 Reproductive factors and distribution of 25-OH VD concentrations in CRC patients

Table 4.4 shows data on 25-OH VD concentration in female patients according to various reproductive factors. As shown, female patients having their menarche before or after the age of 10-12 years of age had significantly lower 25-OH VD level (p=0.02). Similarly, nulliparous female had significantly developed 25-OH VD intensities in comparsion to those having children (p=0.04). Women with menopause at age <40 years had increased 25-OH VD level in comparsion to women who had their menopause after the age of 40. However, this difference didn't reach statistical significance (p=0.09). Women who never used oral contraceptive had higher 25-OH VD levels as compared to those who ever used oral contraceptives (p=0.02).

In advanced examination, we stratified the statistics of female patients by hormone therapy practice (Table 4.5), & observed statistically substantial differences in serum 25-OH VD concentrations of female patients because of differences in their reproductive health characteristics and usage of hormone therapy. In general, in the group of women who ever used hormone therapy had differences of statistical significance (p, for all trends <0.05) in their mean serum 25-OH VD doses, while group with women who never used hormone therapy had non-significant differences in their mean serum 25-OH VD

doses (p, for every trends>0.05). Furthermore, taken as single groups (ever use hormone therapy vs. never practice of hormone therapy) women with ever use of hormone therapy had significantly higher 25-OH VD concentrations as compared to women with never use of hormone therapy (p, for all trends<0.05).

Figure 4.10 and 4.11 reveals the analyzed distribution pattern of sex hormones and 25-OH vitamin D intensities allied to the stage of CRC in young and old CRC cohorts. In younger female cases, early stages have relatively higher intensities of 25-OH vitamin D and estradiol which get reduced with progression of the CRC. Furthermore, 25-OH vitamin D showed strong negative correlations with the stage of the disease in both premenopausal and postmenopausal females with CRC (r= - 0.808 and -0.434, respectively, P<0.01, Table 4.6). This was not the case in males in both age groups. Regarding estradiol level in young males, a solid positive correlation with the stage of the disease was found (r= 0.877, p<0.01), while in older ages the correlation became negative (r = - 0.643, p<0.01). Females in the reproductive ages and with CRC showed an extremely strong negative correlation with the phase of the disorder (r= - 0.976, p<0.01). In both genders and age groups, the severity of disease stage correlated positively with the age.

Table4. 1: Sex hormone Concentrations of CRC patients

	Males	Females	P-value
A. Hormone Concentrations in young male and Pre-menopause female			
Mean age (years) (n=M/F=38/25)	38.87±7.27	35.60±8.92	0.116
Testosterone (ng/dl)	1572.25±262.58	102.08±3.72	<0.001
"Estradiol (pg/ml)	42.52±16.66	50.51±23.58	0.120
FSH (mlU/ml)	31.96±4.52	31.02±3.86	0.394
LH (mlU/ml)"	33.09±4.64	33.13±2.96	0.969
B. Hormone Concentrations in old male and Post-menopause female			
Age (years) (n=M/F=74/47)	64.47±8.92	64.60±10.66	0.946
Testosterone (ng/dl)	1648.25±270.23	105.27±7.18	<0.001
"Estradiol (pg/ml)	49.72±10.91	16.86±5.30***	<0.001
FSH (mlU/ml)	32.18±4.53	34.42±3.05	0.003
LH (mlU/ml)"	31.84±4.55	34.17±3.49	0.003

*** indicate significant difference at p<0.0001 between Pre-menopause female and Post-menopause female

Table4. 2: **25-hydroxyvitamin D [25(OH) D] status in the study cohort**

Parameters	**Vit D**	**P-value**
Mean age	22.1 (9.19)	0.032
<50 years	18.8 (9.1)	
>50 years		
Gender		0.021
Male	17.1 (9.3)	
Female	21.5 (8.6)	
Site of Cancer		0.012
Rectum	22.1 (7.9)	
Colon	14.8 (5.7)	
Stage of disease	19.6 (9.8)	0.023
Stage I-III	16.4 (5.6)	
Stage IV		

Table4. 3: **Univariate and multivariate logistic regression analysis of low vs normal 25-OH VD level (≤16 ng/ml vs >16ng/ml)**

Category	Univariate Analysis OR (95% CI)	p-value	Multivariate Analysis OR (95% CI)	p-value
Age (<50 yrs vs.>50yrs)	2.42 (1.29-4.55)	0.004	1.78 (0.87-3.66)	0.110
Gender (Female vs. male)	1.87 (0.98 - 3.42)	0.038	1.97 (1.023-3.82)	0.142
Site of Cancer (Rectum vs colon)	1.67 (0.87-3.21)	0.031	1.24 (1.13-3.45)	0.150
Stage (Stage I, II, III vs. IV)	2.44 (1.22 - 4.91)	0.012	1.94 (1.07-3.56)	0.031

OR, odds ratio; CI, confidence interval

Table4. 4: **Reproductive factors and 25-OH VD concentration in female patients (*n*=54)**

Reproductive Factors	**25-OH VD level (ng/ml)**	p-value
Menarche Age		
<10	13.7 (6.4)	
10-12	18.4 (5.2)	0.02
12-14	20.6 (5.5)	
>15	18.6 (6.5)	
Parity		0.04
"Nulliparous	21.5 (5.4)	
I child	19.5 (5.4)	
2 children	18.3 (5.4)	
3 children	15.4 (5.4)	
>4 children"	13.8 (4.4)	
Menopause Age		0.09
<40	20.3 (5.9)	
40-44	20.8 (8.3)	
45-49	18.1 (6.4)	
50-54	18.2 (7.2)	
>55	16.1 (8.5)	
Oral contraceptive use		0.02
Never	21.3(5.1)	
Ever	16.2 (3.7)	

Table4. 5: Serum 25-OH VD concentrations according to reproductive and menstrual factors by hormone therapy (n=54)

Factor	Ever use of hormone therapy (n=29)	Never use of hormone therapy (n=25)	p-value
Mean age			
<50 years	22.5 (9.19)	19.5 (9.19)	0.002
>50 years	19.8 (9.1)	18.8 (8.1)	
	P=0.02	P=0.24	
Menarche Age			
<10	14.2 (5.8)	13.2 (5.3)	0.04
10-12	21.4 (5.3)	15.4 (6.3)	
12-14	26.8 (3.8)	14.4 (7.7)	
>15	22.4 (8.7)	14.8 (3.9)	
	P=0.01	P=0.26	
Parity			0.04
"Nulliparous	26.4 (6.8)	16.4 (3.9)	
I child	23.2 (4.7)	15.4 (6.9)	
2 children	21.3 (4.9)	15.2 (7.1)	
3 children	15.3 (4.2)	15.5 (3.9)	
> 4 children"	14.6 (6.8)	13.1 (2.9)	
	P=0.02	P=0.32	
Menopause Age			0.03
<40	21.3 (5.9)	19.4 (6.5)	
40-44	23.3 (12.4)	18.4 (4.5)	
45-49	18.7 (8.7)	18.3(4.9)	
50-54	18.3 (9.3)	17.9 (5.9)	
>55	16.2 (7.3)	16.8 (9.7)	
	P=0.08	P=0.09	
Oral contraceptive use			0.04
Never	26.1 (2.8)	17.3 (9.8)	
ever	16.5 (3.9)	15.8 (4.8)	
	P=0.02	P=0.21	

Table4. 6: Correlation (r values) of various investigated parameters with the stage of CRC.

	Males		Females	
Parameters	Young, <50 years (n=38)	Old, >50 years (n=74)	Premenopausal (n=25)	Postmenopausal (n=47)
Age	0.627**	0.571**	0.817**	.0764**
VD	0.163	-0.013	-0.808**	-0.434**
Testosterone	0.297	0.103	0.229	0.282
Estradiol	0.877**	-0.643**	-0.976**	0.014
FSH	-0.183	0.045	0.686**	0.001
LH	-0.013	0.030	0.045	0.063

**. "Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed)".

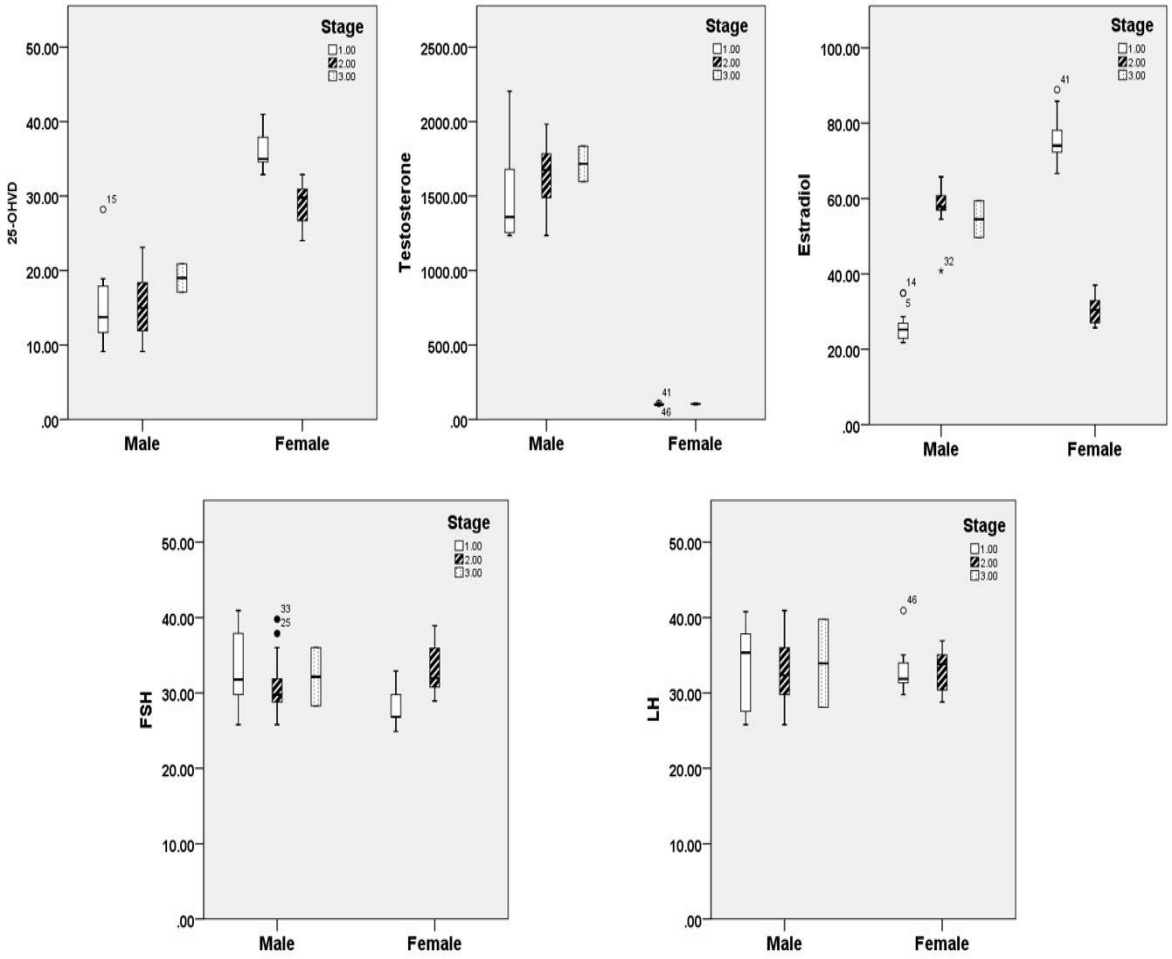


Figure 4. 10: 25 OH Vitamin D and sex hormonal patterns in relation to the stage of RCC in young participants.

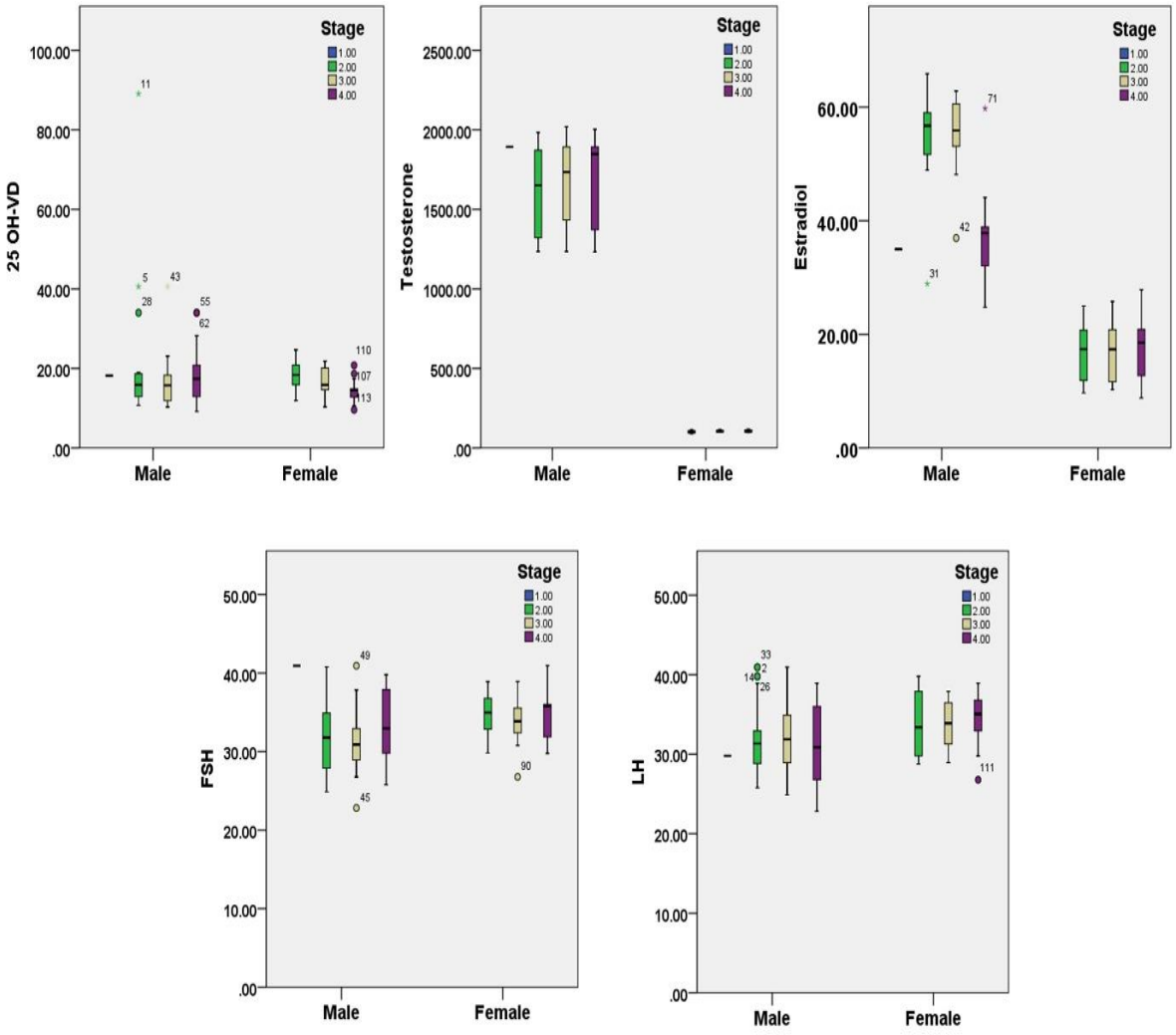
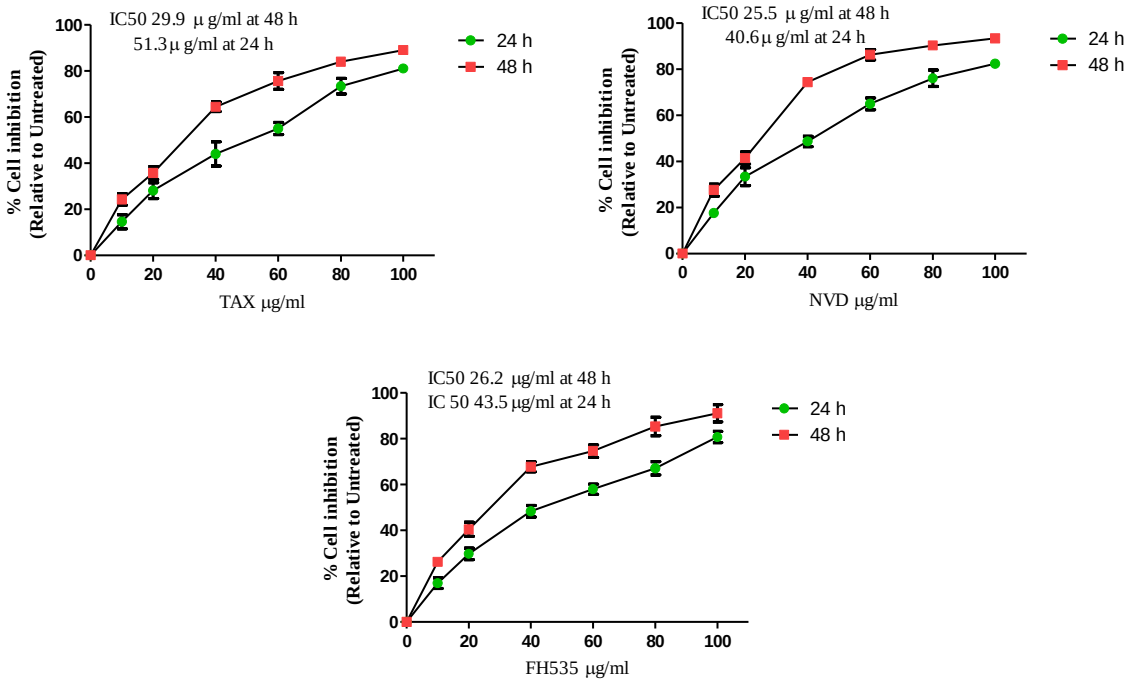


Figure4. 11: 25-OH Vitamin D and sex hormones patterns in relation to the stage of CRC in old participants.

4.9 TAX, NVD and FH535 inhibits viability and growth of colorectal cancer cells

Scrutinizing the anti-proliferative prospective of TAX, NVD and FH535, we executed MTT analysis contrary to HCT116 and HT29 colorectal cancer cells. We observed that TAX, NVD and FH535 treatment (0–100μM aimed at 24h and 48h) to colorectal tumor cells triggered reticence of cell progression in a concentration and time reliant approach. Time passage examination showed colorectal cancer cells respond to TAX, NVD and FH535 treatment within 48h. As shown in (Figure 4.12), the IC_{50} values of TAX, NVD and FH535 -treated HCT116 were 51.3, 40.6 and 40.1 and 29.9, 25.5, and 24.6 μM at 24h and 48hr respectively and IC50 values of TAX, NVD and FH535 -treated HT29 cells were 39.0, 30.3 and 26.2 and 66.1, 50.1, and 43.5μM at 24 hr and 48hr respectively. The data suggested that HCT 116 and HT29 cells showed better sensitivity to TAX, NVD and FH535 treatment.

HCT 116



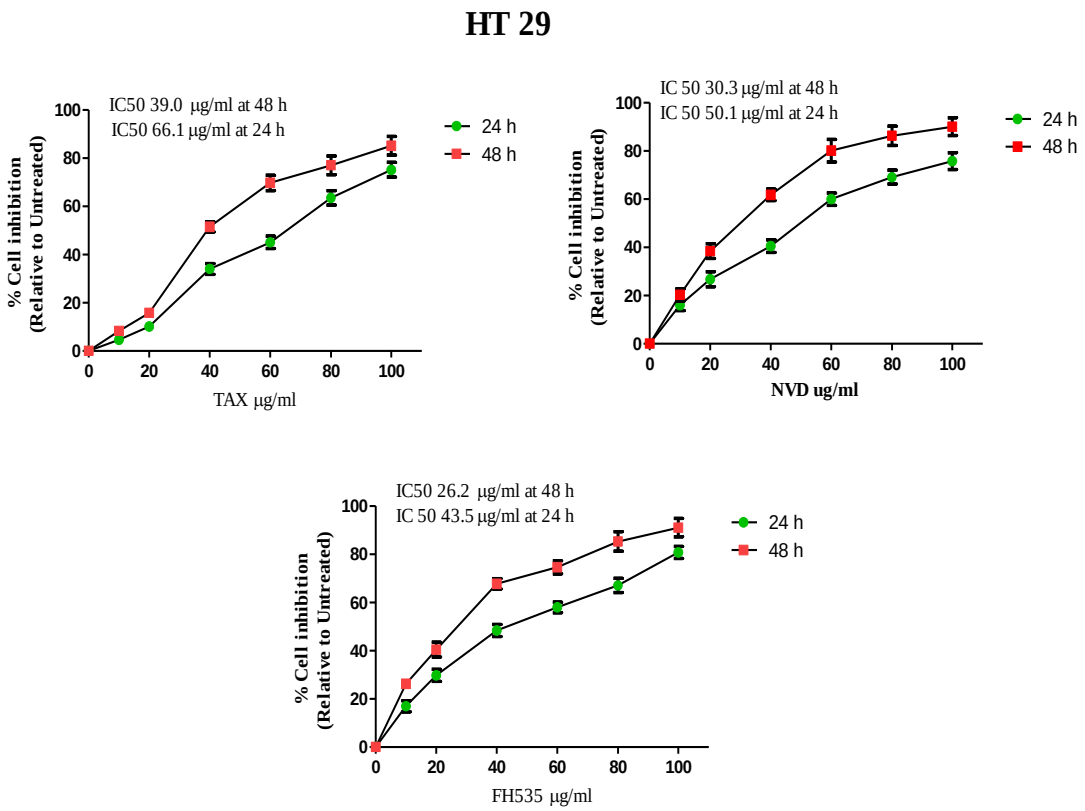


Figure4. 12: Reticence of cell growth in colorectal cancer. 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazoliumbromide assay was carried out on two colorectal cancer cell lines HCT116 and HT29 cells aiming to gauge the effect of TAX, NVD and FH535 administration on growth of these colorectal cancer cell lines ***, $P < 0.001$ and **, $P < 0.01$ vs. control. % cell viability of HCT116 and HT29 cells were determined. Each value represents a mean ± SD (n=3) .

Table4. 7 IC 50 value of TAX against HCT 116 & HCT 29 cells in cell viability assay

Treatment	**HCT 116**		**HCT 29**	
	24h	48h	24h	48h
TAX (IC50 µM)	51.3	29.9	39.0	66.1
FH535 (IC50 µM)	40.1	24.6	26.2	43.5

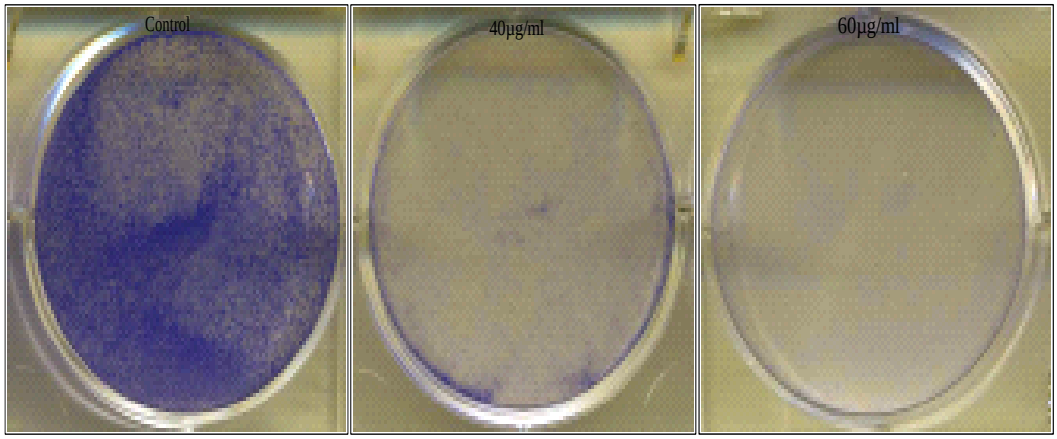
Table4. 8: IC 50 value of NVD against HCT 116 & HCT 29 cells in cell viability assay

Treatment	**HCT 116**		**HCT 29**	
	24h	48h	24h	48h
NVD (IC50 µM)	40.6	25.5	30.3	50.1
FH 535 (IC50 µM)	40.1	24.6	26.2	43.5

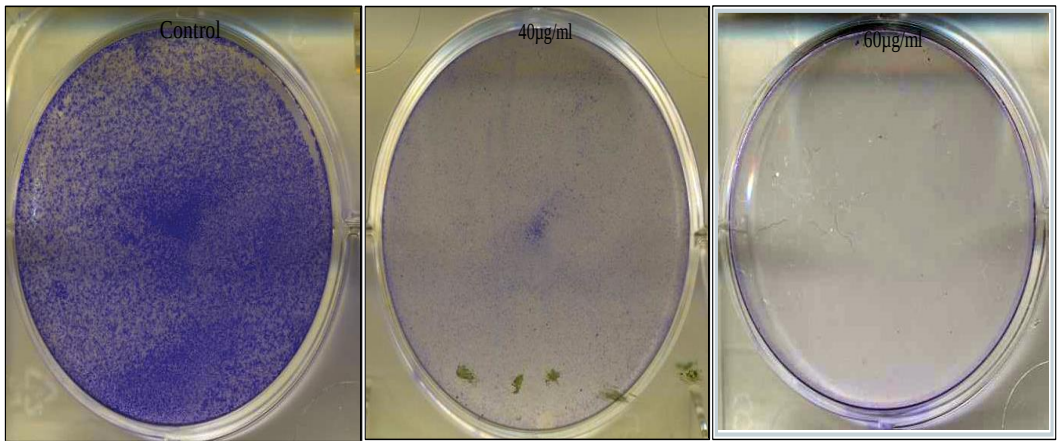
4.10 Inhibition of clonogenecity in CRC cell lines

To determine the consequence of TAX & NVD administration proceeding the clonogenecity of HCT116 and HT29 cells, clonogenecity assay was carried out. HCT116 and HT 29 cells were pre-administrated for seven days with TAX and NVD at 40 and 60 µM doses confirmed a dose dependent inhibition of colonogenecity with deference to untreated control cells. Results show that clonogenecity of HCT116 and HT29 cells after administration with TAX and NVD was markedly condensed in comparison with the control (Figure 4.13)

A) **HCT116**



B) **HT29**



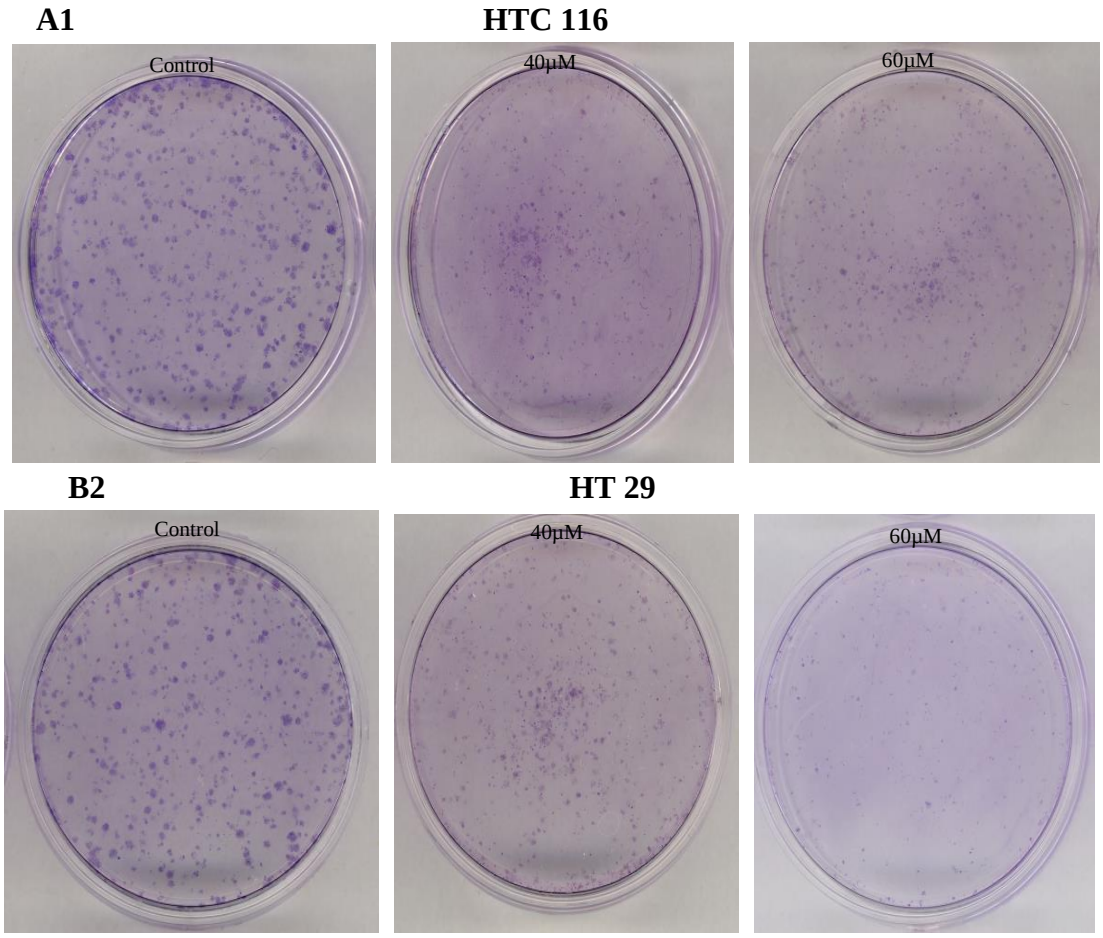


Figure4. 13: Inhibition of cologenecity in colorectal cancer cells by TAX and NVD. (Clonogenic assay; 7 days). A and B) TAX administration (40 and 60µM) of HCT116 and HT29 cells inhibiting colony formation. A1 and B1) NVD administration (40 and 60µM) of HCT116 and HT29 cells inhibiting colony formation. Each value represents a mean ± SD (n=3).

4.11 Taxifolin (TAX) behavior caused cell cycle arrest

4.11.1 Flow cytometric analysis of cell cycle arrest caused by treatment of TAX

To evaluate the cell cycle sketch of TAX administrated colorectal cancer cells, flow cytometric investigation was conceded out and pragmatic the consequence of TAX on cell cycle circulation. Investigation was performed on HCT116 and HT29 cells, a patent concentration dependent raise of cell populace of the cell cycle in the G2 state was make out by flow cytometric analysis as a result of TAX treatment. The G2-phase cell cycle circulation for HCT116 was 44.61% and 59.52% and for HT 29 was 47.72%, and 57.72% at 40 and 60µM doses of TAX respectively. This boost in G2 status of cell population was pursued through a synchronized decline in the Go/G1 & S stage cell population (Figure 4.14).

4.11.2 Flow cytometric analysis of apoptosis induced by treatment of TAX

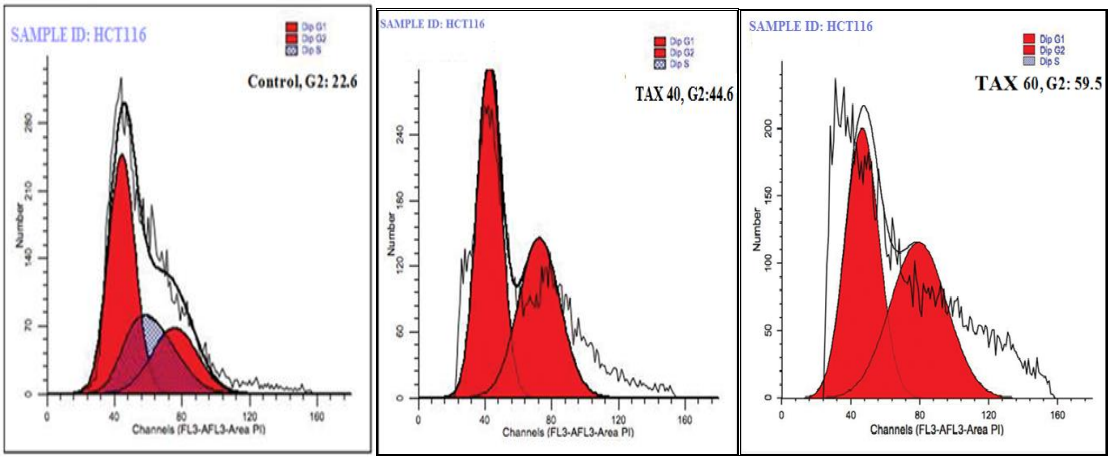
As cell cycle arrest and apoptosis is allied with each other, a manifested concentration dependent boost in population of dead cells was resoluted by flow cytometric analysis as a result of TAX administration in HCT 116 and HT29 cells. The percentage apoptosis in HCT cells observed was 34.9% and 53% and for HT 29 17.2% and 30.9% at concentration of 40 and 60 µM of TAX (Figure 4.16)

4.11.3 TAX administration induced inhibition of cyclins, cdks, & activation of KIP1/p27, WAF1/p21 in colorectal cells

Cell disunion is outgoing & cyclins/ cdks operate as check points / objectives within tumor cells. Development of cell cycle is exceedingly controlled through communication amid inhibitor proteins KIP1/p27and WAF1/p21, cyclins, & cdks. We assessed the influence of TAX in the cell cycle on molecules that control cell cycle execution in the G2 segment. The influence of TAX proceeding the activation of WAF1/p21 as well as KIP/p27 was resoluted, reputed towards standardize the pass of cells by the G1-S state transtition check point and cause cell death. The Immunoblot analysis showed that the TAX treatment to HT 29 & HCT 116 resulted in a prominent upregulation of P27 and

P21 in a concentration reliant approach (20, 40 and 60 µM) as compared to basal levels (Figure 4.15). The result of TAX administration on expression of proteins of cdks 6,2 and 4 and cyclins B1, D1, A and E was determined, which are prominently regulated by p21 and involved in G2 segment of cell cycle. Immunoblot analysis showed an amount dependent down regulation of both cyclins and cdks which support cell cycle arrest in G2 phase.

A)



B)

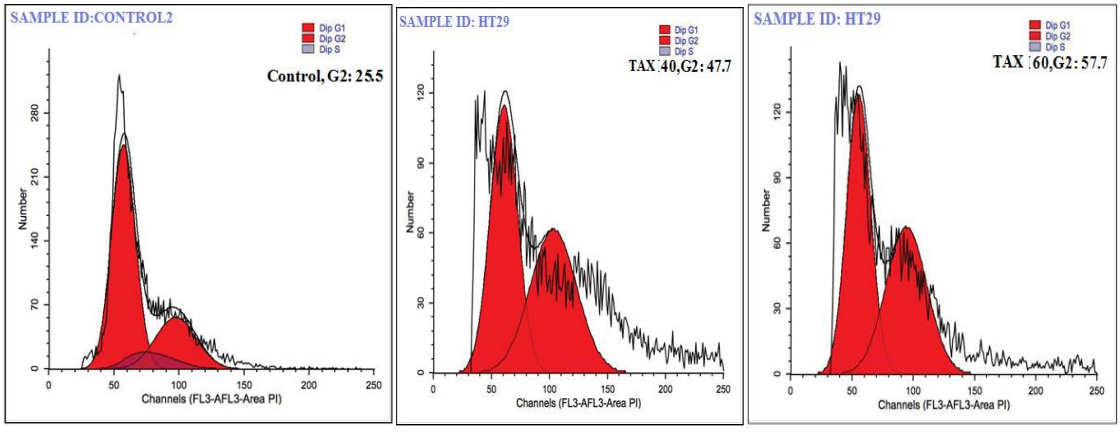


Figure4. 14: "TAX treatment on HCT116 and HT29 cells resulted in accumulation of cells at the G2-phase, (a-b). By flow cytometry, after 24h incubation of TAX treated cells and staining with propidium iodide, DNA content was examined. Percentage of cell population in G2-phase of the cell cycle is shown. Experiments were performed in triplicate."

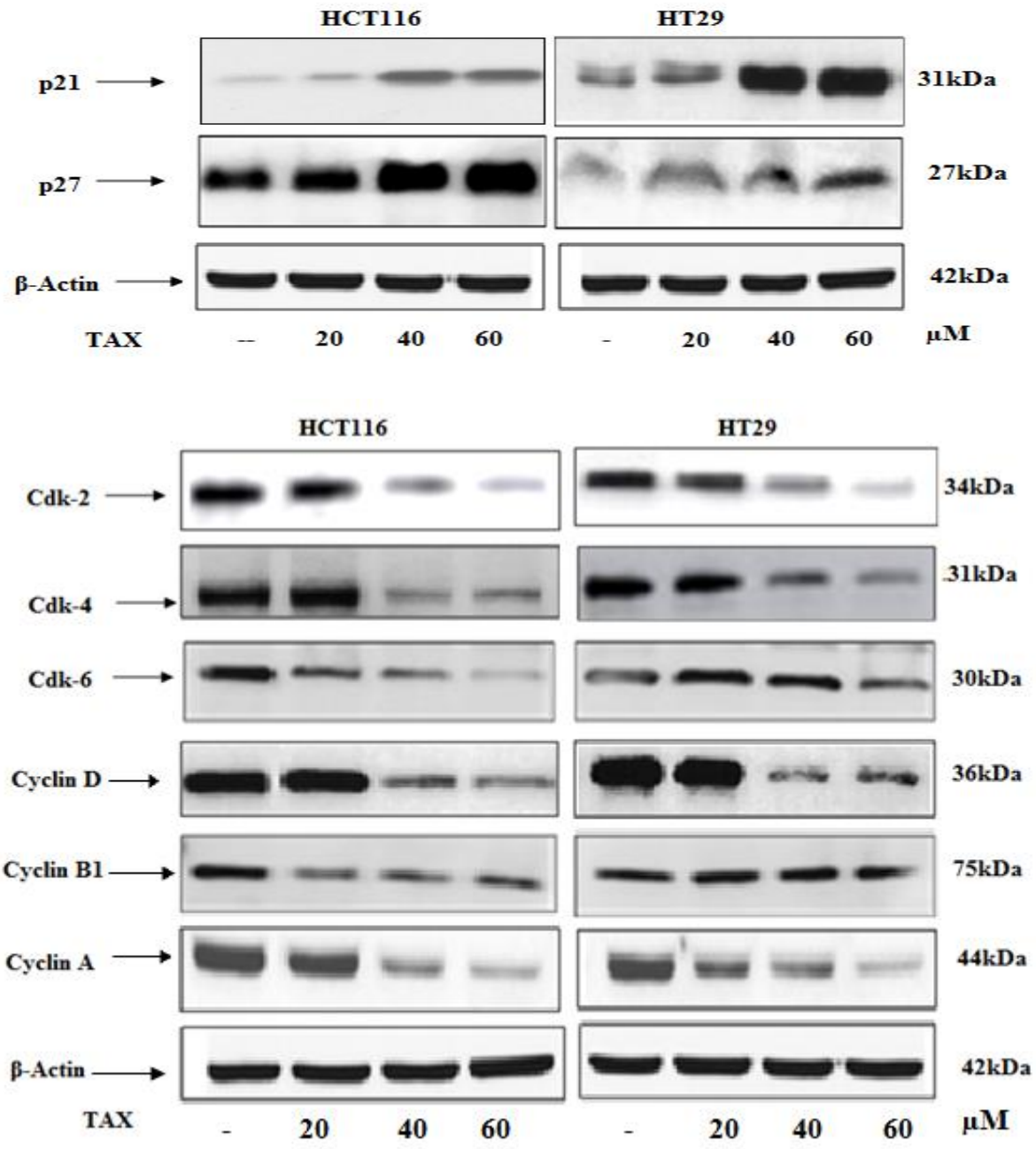


Figure4. 15: Effect of TAX treatment of HCT116 and HT 29 cells on protein expression of WAF1/p21 & KIP1/p27, cdk 2, 4 and 6, cyclin D, B1 and A. Experiments were performed in triplicate.

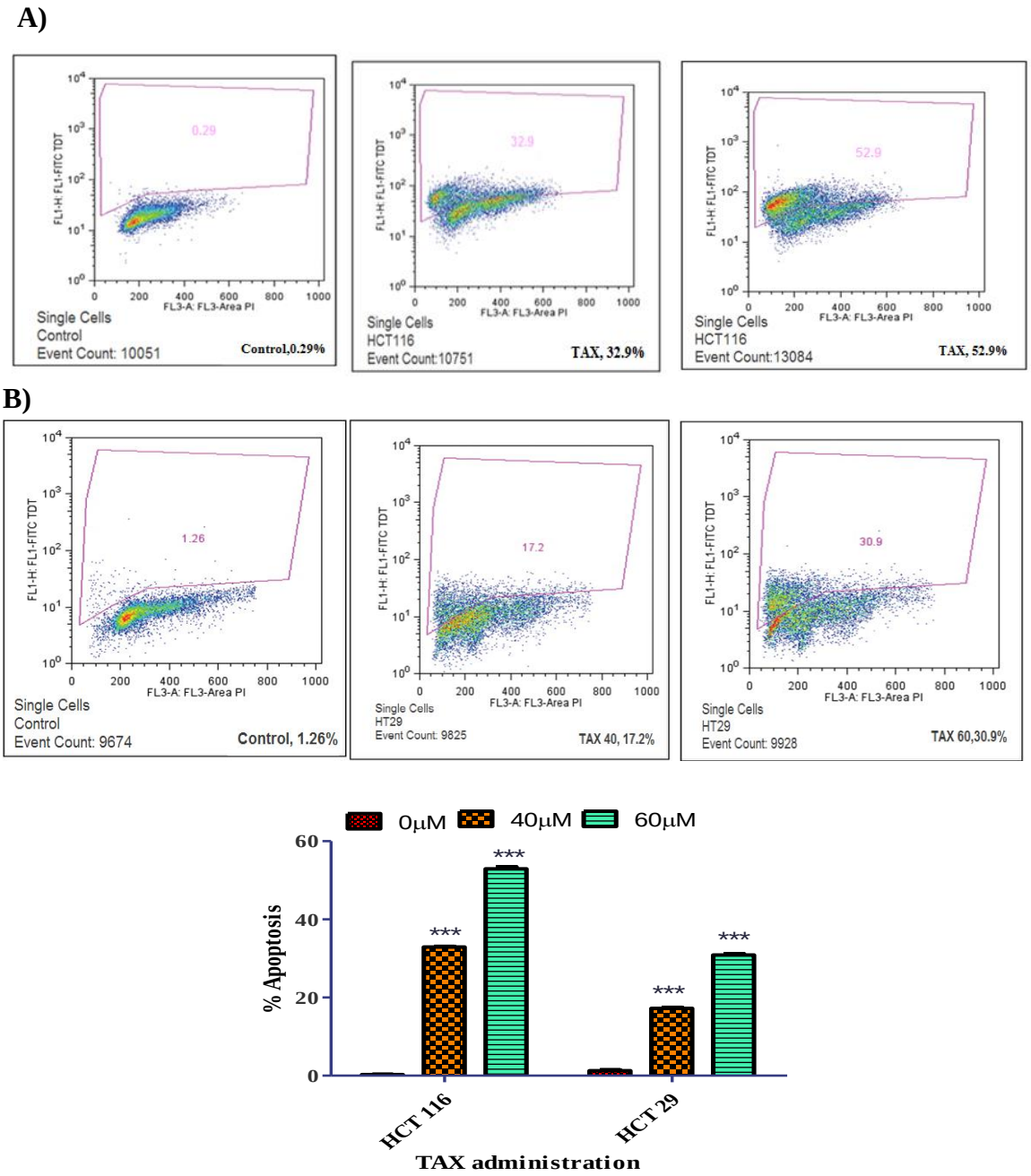


Figure4. 16: TAX treatment on HCT116 and HT29 cells resulted in growth inhibition and apoptosis, after 24h incubation of TAX treated cells and staining with propidium iodide, Cells were evaluated through flow cytometry. Proportion of apoptotic cells is shown. Experiments were performed in triplicate.

Table4. 9: Proportion of cell populace within several stages of the cell cycle after administration of Taxifolin

Taxifolin (µg/ml)	% Apoptosis	
	HCT116	HT29
0	0.29±0.01	1.26±0.11
40	32.9±0.08	17.2±0.14
60	52.9±0.02	30.9±0.12

Mean ± SD of experiments performed in triplicate is shown.

4.11.4 TAX administration tempts apoptosis via commencement of intrinsic and extrinsic pathway

4.11.4.1 TAX administration caused initiation of active Caspase 3, 8 and 9 in colorectal cancer cells

Caspases are cysteine proteases that have a vital role in various forms of apoptosis. Usually they occur in an inactive proenymes which are triggered to active form by oligomerization to form a large multimeric complex, which inturn trigger caspases 7 and 9 and caspase 3 are triggered by proteolytic cleavage. The caspase once active, are responsible for cleavage of various substrates, both in nucleus or cytoplasm which in turn forms distinguishing morphological features of cell death by apoptosis. To explore the TAX induced apototosis through the trigerring of intrinsic or extrinsic pathway the expression of cleaved caspases 3/7 and 9 were analyzed by western blotting. A prominent increased induction of cleaved caspase 3/7 and 9 after administration of TAX in a concentration dependent approach to HCT116 and HT29 cells was seen. Also truncation of Bid which further supports apoptosis. (Figure4.17)

4.11.5 TAX induces apoptosis through cleavage of poly (ADP-ribose) polymerase (PARP) expression in colorectal cancer

PARP is well known hallmark of initial occurrences in apoptosis. On administration of taxifolin to HCT116 and HT29cells, an induction in 89 kDa fragment of PARP protein expression, a reduction in complete length PARP (116 KD) protein expression remained seen (Figure 4.18).

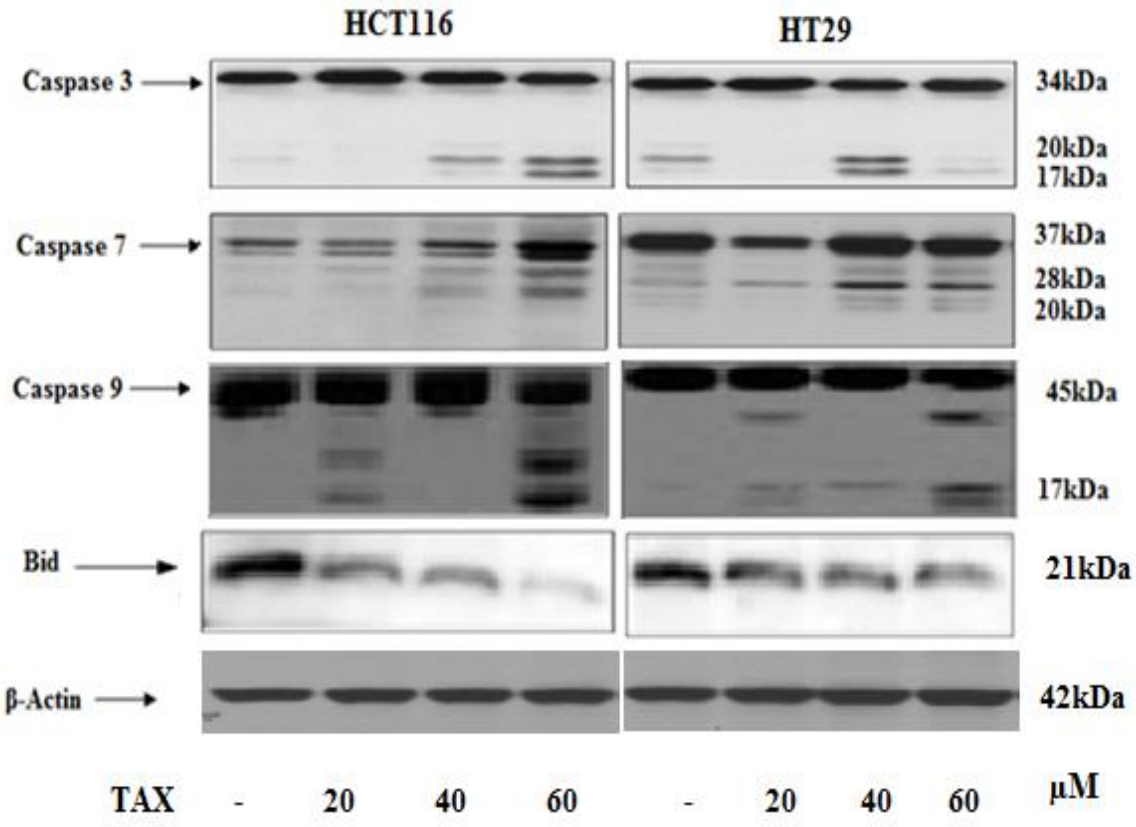


Figure4. 17: "Effect of TAX on induction of apoptosis. Result of TAX on protein expression of active Caspase 3, 7, 9 and truncation of Bid in HCT116 and HT29 cells. Cells were administrated with TAX (20, 40 and 60 µM), total cell lysates were prepared and 40 µg proteins was exposed to SDS page tailed by immunoblot analysis and chemiluminescence detection. Identical loading of protein was verified by stripping the Immunoblot and again probing it for Actin. The immunoblots shown here are representative of three individual experiments with alike results".

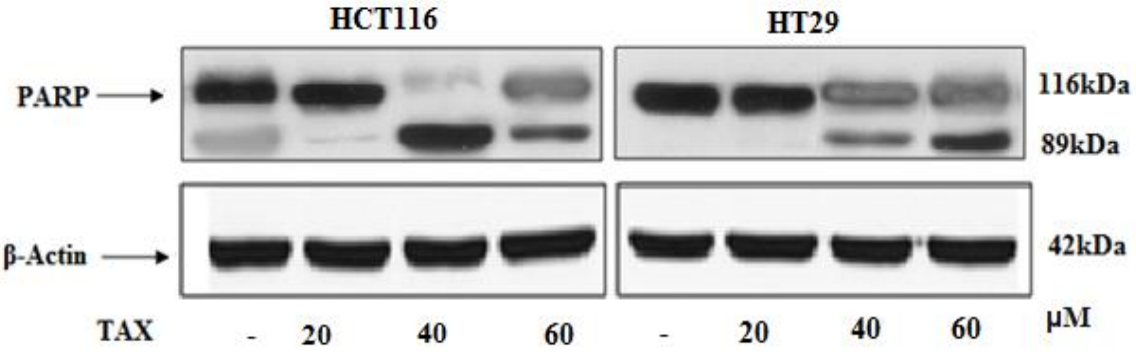


Figure4. 18: "Effect of TAX treatment on cleavage of PARP. The cells were administrated with DMSO only or quantified doses of TAX for 48h and harvested and cell lysates were prepared, the data are representative of three independent experiments with similar results".

4.11.6 TAX treatment amends Bax, Bcl2, Bak and Bcl-XL protein expression in HT 29 & HCT116 cell lines

Bax and Bcl2 are vital in apoptosis; their expression gives prominent picture of deregulation of apoptotic process. Bax and Bcl2 working antagonistly, Bax act as apoptotic promoting marker and Bcl2 works as antiapoptotic element. A prominent intensification in bax protein expression through a concentration reliant decline in Bcl2 protein expression on administration of TAX on HCT116 and HT29 cells was seen. Also Bak, a proapoptotic protein and Bcl2-$X_{L,}$ a antiapoptotic showed prominent increase and decrease respectively in protein expression on treatment with TAX in a concentration dependent approach in both HCT116 as well as in HT 29, which effectively confirm generation of apoptosis (Figure 4.19)

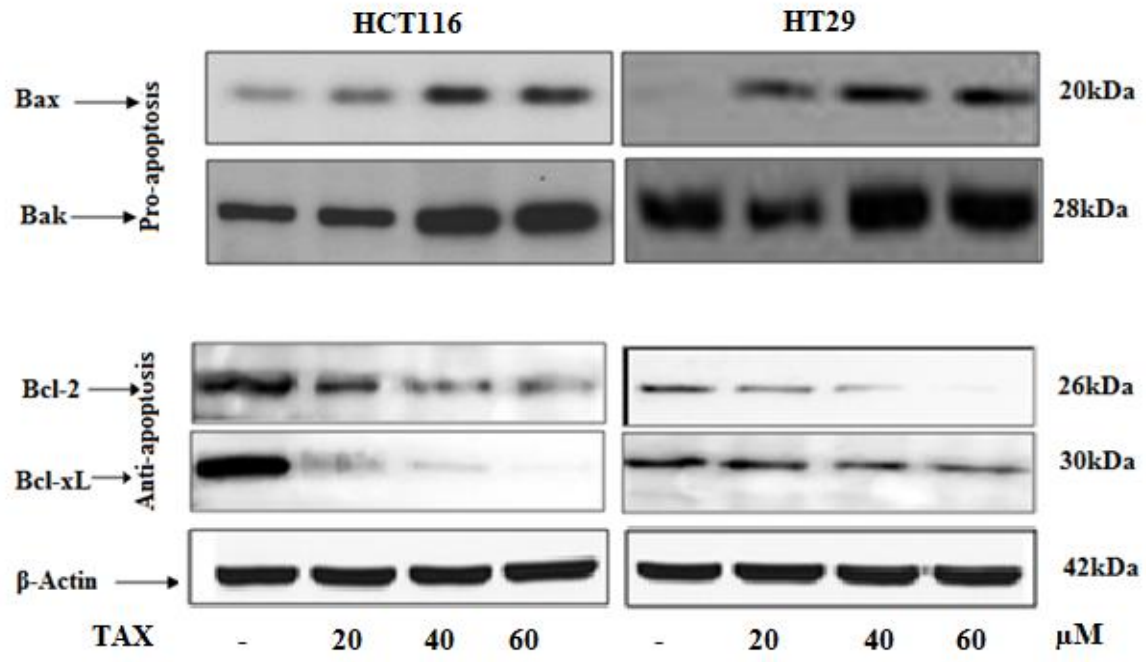


Figure4. 19: Effect of treatment of TAX on HCT116 and HT29 cell lines on protein expression of Bax, Bcl2, Bak & Bcl-X_L. The cells were administrated with TAX for 48h.

4.11.7 TAX reduces the β-catenin expression in CRC.

Results revealed that β-catenin have a crucial task in succession and propagation of colorectal cancer. Decrease in β-catenin expression in concentration dependent fashion was observed on colorectal cancer cells (HCT116 and HT29) after administration of TAX for 24h by employing immunoblotting (Figure4.20a). The HCT116 and HT29 cells show high protein content of β-catenin. The concentration dependent effect of TAX on HCT116 and HT29 cells demonstrated a noticeable diminution in β-catenin protein levels at 20, 40 and 60 µM doses.

To elevate, either the observed decline in β-catenin protein was owing to the condensed transcription of β-catenin gene, alteration of β-catenin expression by TAX induction in HCT116 and HT29 cells, a prominent decline in mRNA expression by employing RT-

PCR, was seen to be in concentration dependent manner. At 20, 40 and 60 µM of TAX a momentous reduced in β-catenin expression was seen (Figure 4.20 b and c)

Immunofluorescence staining of HCT116 and HT29 cells illustrated reduction in β-catenin expression at concentration of 40µM of TAX administrated as compared to control (untreated). As our study evidently illustrated that TAX administration induced apoptosis & cell cycle detention by modulation of appearance of β-catenin in HT29 & HCT116 cells as revealed earlier by cleavage of Caspase 3 & PARP, also through variation of cyclin dependent kinases in HCT116 and HT29 cells by administration of TAX in a dose dependent fashion. Significant Alexa fluor staining of β-catenin (nucleus) of both cell lines (green fluorescence) (counter stain used was DAPI, blue fluorescence) were observed in control, although the expression of β-catenin as indicated by staining was markedly reduced in TAX administrated cells as revealed in the Figure 4.21

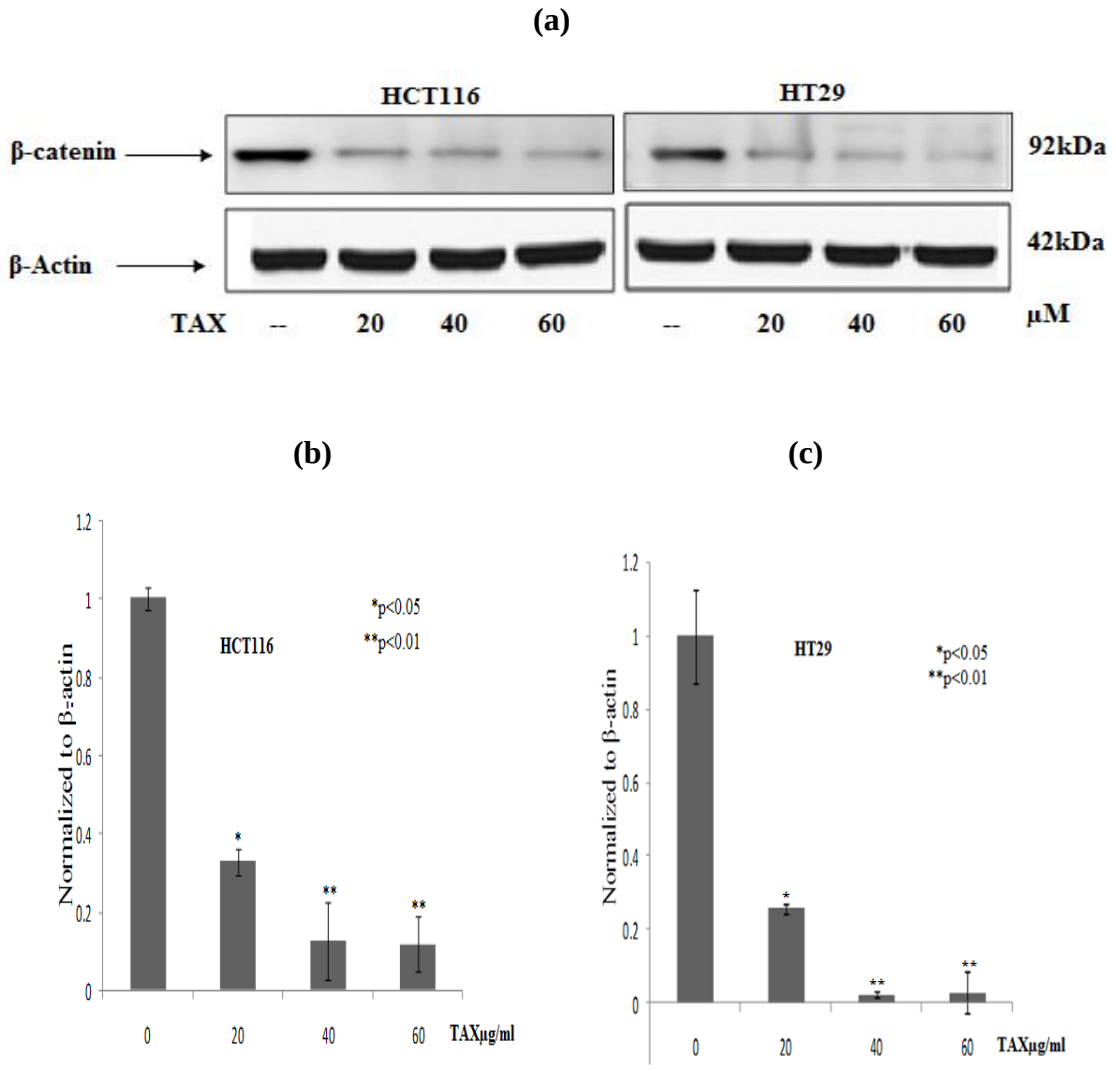
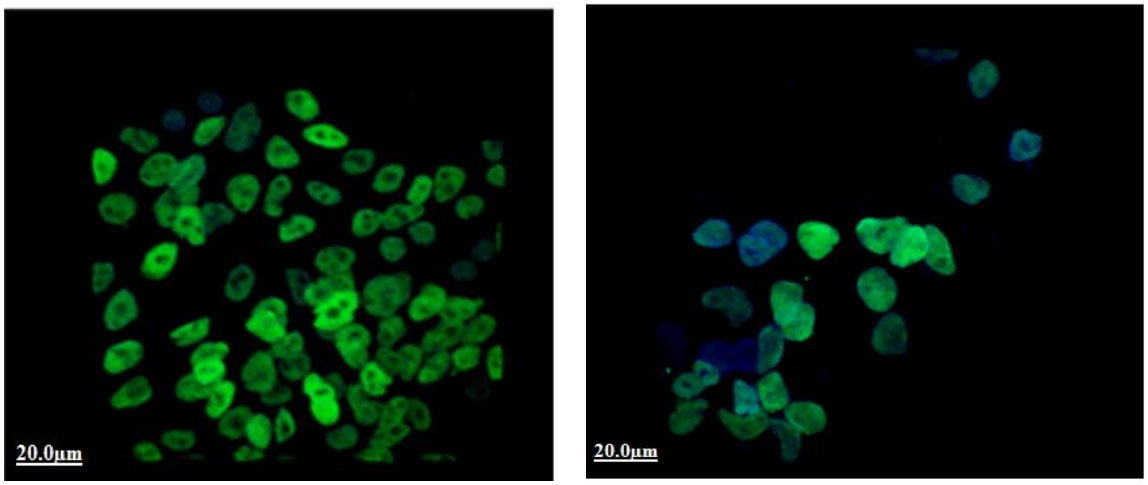


Figure4. 20: a) "Immunoblot analysis of β-catenin expression of HCT116 and HT29 in TAX administrated group as compared to control group b) and c) qPCR analysis of TAX administrated HCT116 and HT29 cells for deviations in β-catenin mRNA levels. The data expressed as fold variation signify the mean± standard errors experiment carried in triplicate where*p<0.05, **p<0.01 was taken significant vs control."

a) Control (HCT116) b) TAX: 40 μM (Treated)



c) Control (HT29) d) TAX: 40 μM (Treated)

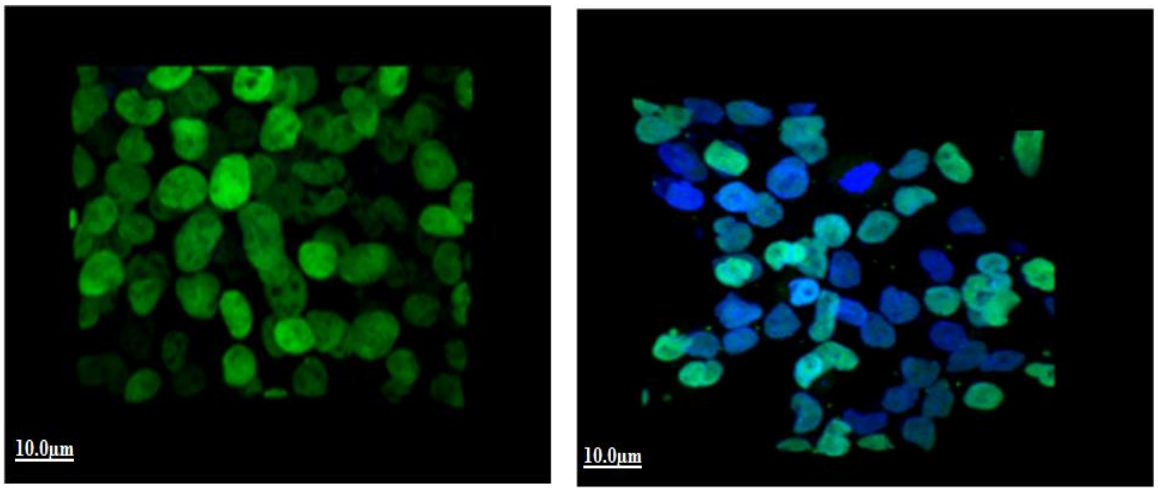


Figure4. 21: Imunofluorescence staining of HCT116 (b) and HT29 cells (d) demonstrating expression of β-catenin in both TAX treated (40mol/L) as compared to control HCT116 (a) and HT29 (c) cells (untreated). Alexa fluor staining of TAX of both cell lines (green fluorescence) and counter stained with DAPI (blue fluorescence) were seen.

4.11.8 TAX administration induced inhibition of PI3K as well as phosphorylation of protein expression of AKT in colorectal cells.

The PI3K commands major signing networks in malignancy cells which results to cell succession, survival, apoptosis and angiogenesis. PI3Ks transduce signals from assorted cytokines and growth factors into intracellular messages by engendering phospholipids, which consecutively ignite the serine/threonine kinase AKT and further downstream effector corridors. Administration of HCT116 and HT29 cells with TAX induced an amount dependent inhibition in the expression of PI3K. Akt is crucial key of signaling cascades for progression and cell survival throughout carcinogenesis and growth. It maintains growth and cell cycle by tortuously shifting intensities of cyclin D1 and activating cdks inhibitors WAF1/p21 as well as KIP1/p27. As reduction in the intensities of cyclin D1 as well as cdks inhibitors on induction of cells with TAX was seen, the effect on phosphorylation of Akt was elevated. The administration of TAX to HCT116 and HT29 cells, a concentartion reliant inhibition in the Akt phosphorylation was seen.

Immunofluorescence staining of HCT116 as well as HT29 cells showed reduction in p-Akt expression at concentration of 40 μM of TAX treated as compared to control (untreated). Significant Alexa fluor staining of p-akt (cytoplasm) of both cell lines (green fluorescence) (counter stain used was DAPI, blue fluorescence) were observed in control, although the expression of p-Akt as indicated by staining was markedly reduced in TAX administrated cells as revealed in the Figure 4.23.

The above results indicated that TAX administration of HCT116 and HT29 cells resulted in apoptosis via inhibition of β-Catenin, survivin and p-Akt. Since FH535 β-catenin inhibitor inhibits Akt phosphorylation which stemmed in decline in protein expression of β-catenin as well as survivin. Compared through TAX treatment (40 μM), pre-incubation of HCT116 and HT29 cells with FH535 β-catenin inhibitor (20 μM) for 2h before TAX administration caused noteworthy lessening in the portein appearance of p-Akt, Survivin & β-catenin as observed by Immunoblot analysis. TAX administration (40 μM) to β-

catenin inhibitor administrated cells further augmented the decrease of β-catenin expression & survivin, suggesting the consequences remain arbitrated within part concluding Akt (Figure 4.24).

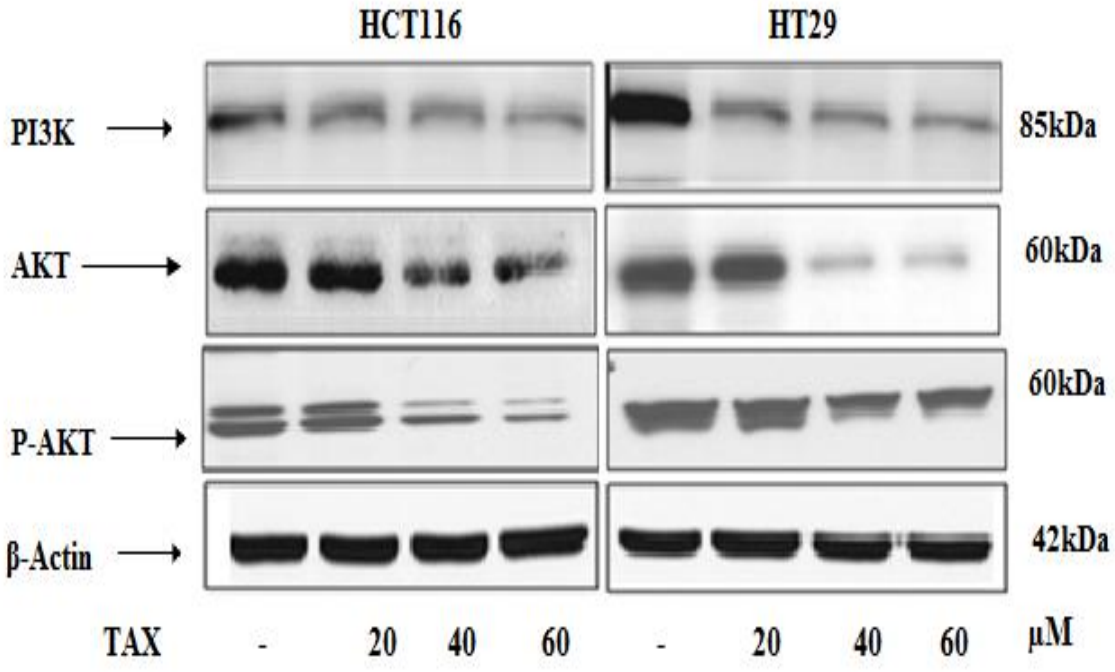
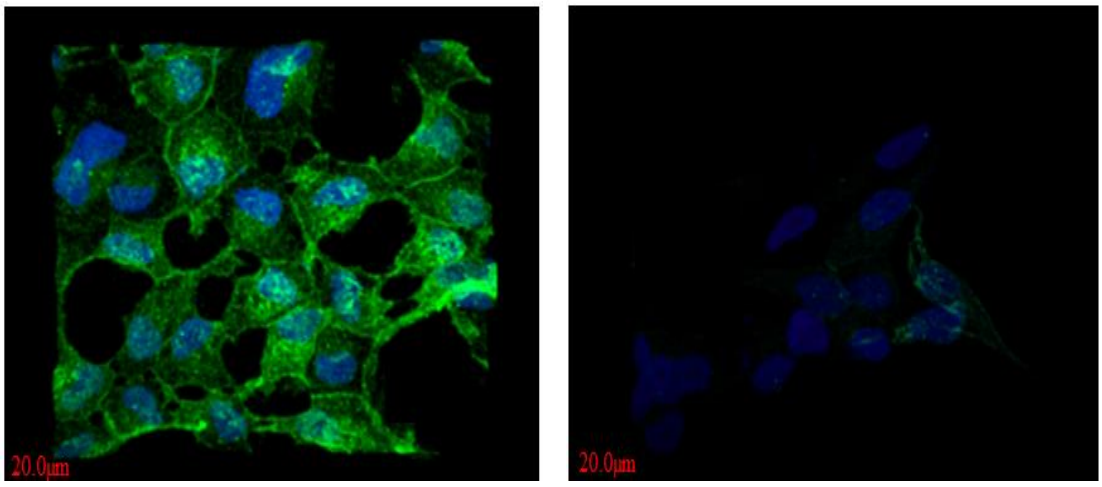


Figure4. 22: TAX triggered reticence of PI3K & phosphorylation of Akt protein expression in colorectal cancer a) Effect of TAX on protein expression of PI3K and phosphorylation of Akt at Ser473 in HCT116 and HT29 cells. Total cell lysate was prepared and 40µM protein was subjected to SDS-page followed by Immunoblot analysis and chemiluminescence detection. Equal loading of protein was confirmed by stripping the immunoblot and reprobing it for Actin .

A) Control(HCT116) **A1) TAX: (60µM)**



B) Control (HT29) **B1) TAX: (60µM)**

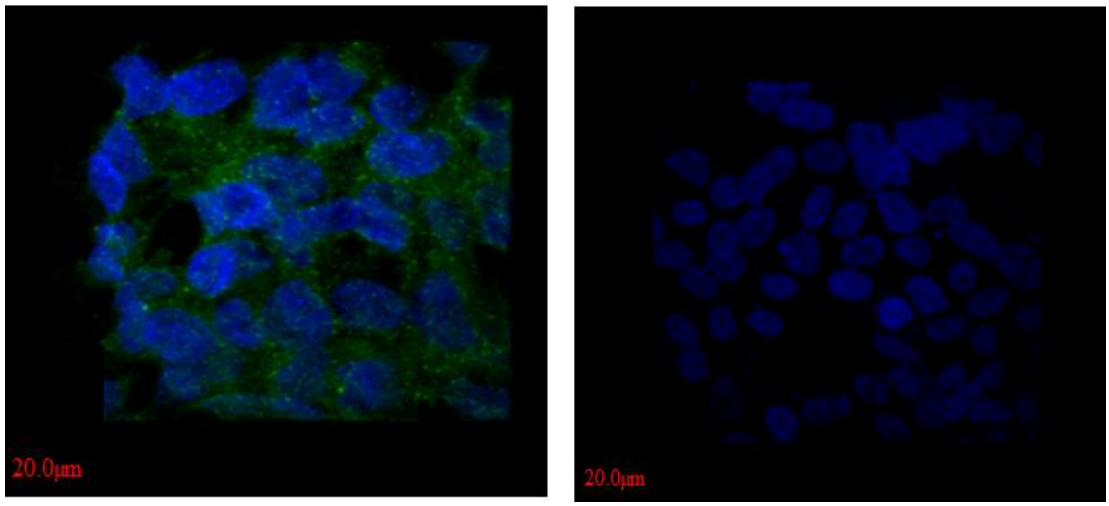


Figure4.23: Imunofluorescence staining of HCT116 (A1) and HT29 cells (B1) demonstrating expression of p-Akt in both TAX treated (60 µM) as compared to control HCT116 (A) and HT29 (B) cells (untreated). Alexa fluor staining of p-Akt of both cell lines (green fluorescence) and counter stained with DAPI (blue fluorescence) were observed.

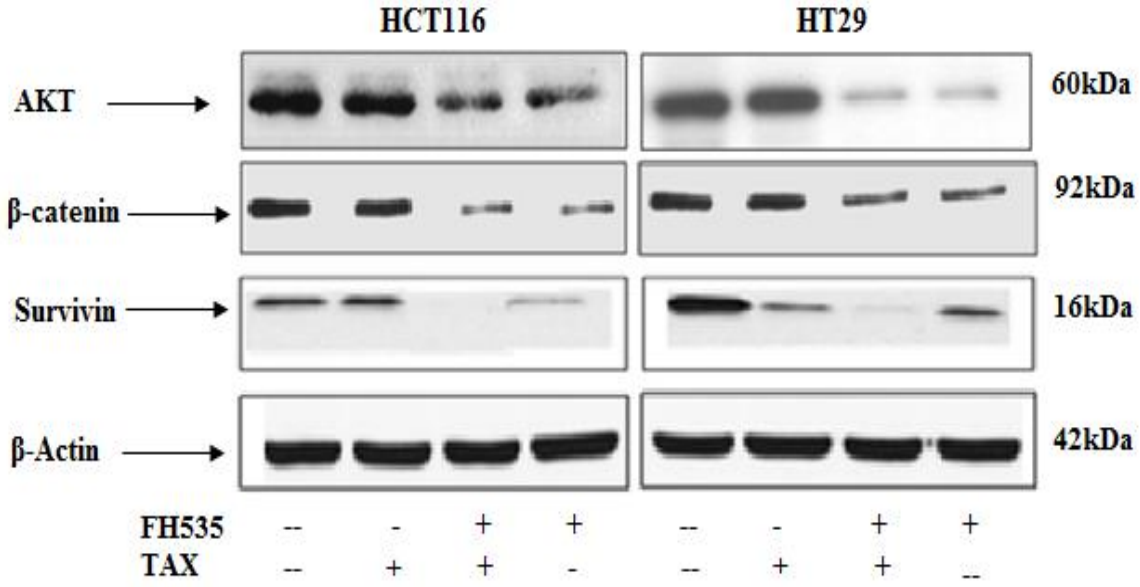


Figure4. 24: Reticence of β-catenin causes down regulation of β-catenin, Akt and survivin protein expression by immune blot analysis. Experiments were performed in triplicate.

4.12 Nano-particle plus Vitamin D (NVD) administration caused cell cycle arrest

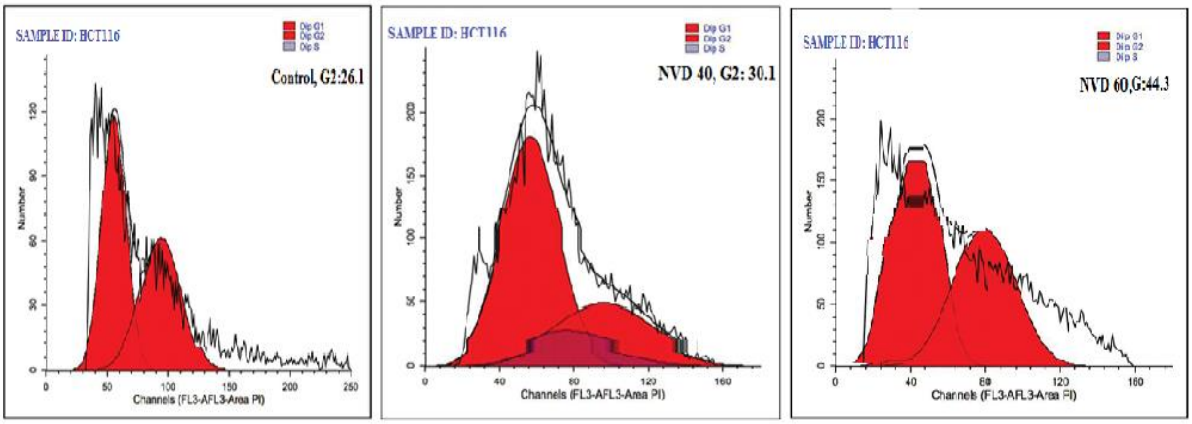
4.12.1 Flow cytometric analysis of cell cycle arrest caused by administration of NVD

To investigate whether NVD administration will cause the cell arrest, the flow cytometric analysis carried on HCT116 and HT29 cells administrated with NVD showed a noticeable concentration dependent boost of cell populace of the cell cycle in the G2 state. The G2-period cell cycle circulation for HCT116 was 30.1% & 56.3% and for HT29 was 40.5%, and 54.3% at 40 and 60µM doses of NVD respectively. This rise in G2 state of cell populace was followed through a synchronized decline in the Go/G1 & S phase cell populace (Figure 4.25).

4.12.2 NVD administration promotes apoptosis ----Flow cytometric Analysis

Apoptosis and cell cycle arrest are associated. To quantitatively examine whether NVD induces apoptosis of the HCT116 and HT29 cells, analysis was carried out by flow cytometry. Results showed prominent dose dependent increase in population of dead cells, as a result of NVD administration in HCT116 and HT29 cells**.** The percent apoptosis in HCT116 cells observed was 26.7% and 54% and for HT29 was 17.8% and 32.8% at concentration of 40 and 60µM of NVD respectively (Figure 4.26).

A) **HCT116**



B) **HT29**

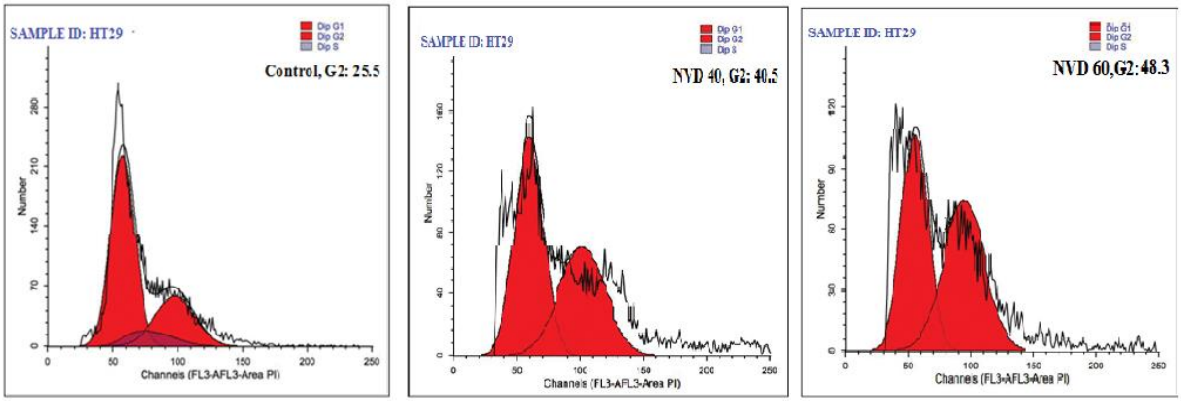
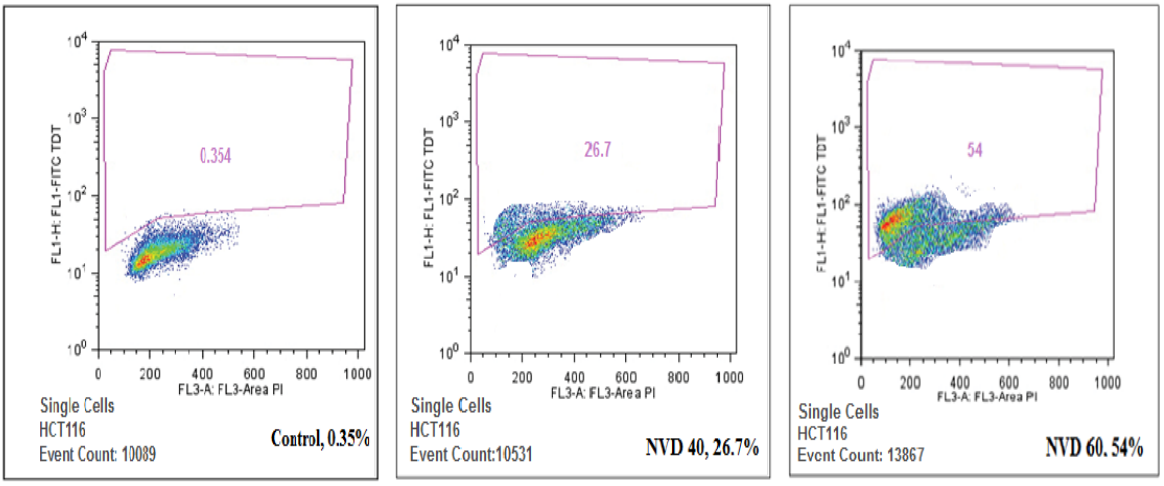


Figure4. 25: NVD treatment on HCT116 and HT29 cells resulted in accretion of cells at the G2-phase. By flow cytometry, after 24h incubation of NVD treated cells and staining with propidium iodide, DNA content was investigated. Experiments were performed in triplicate.

A)



B)

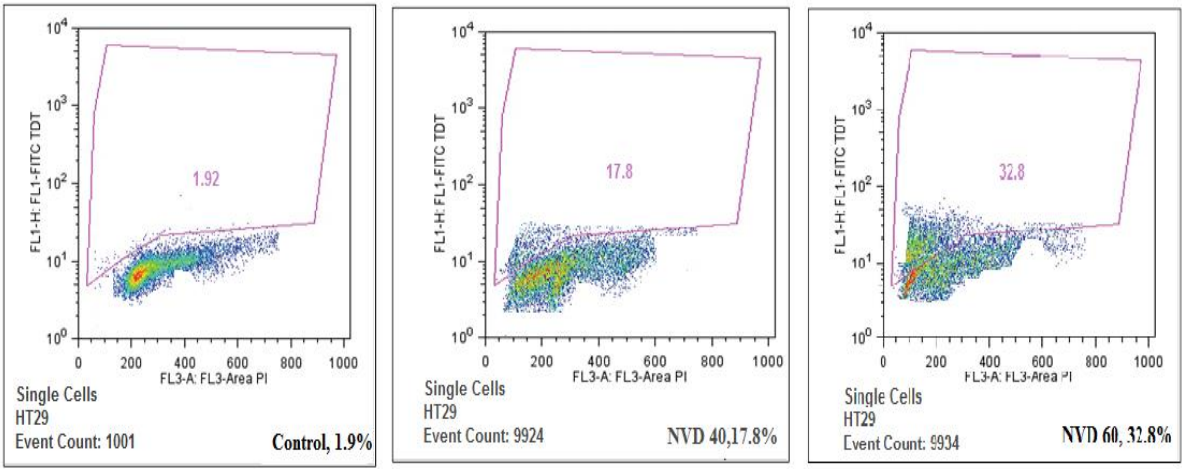


Figure4. 26: NVD treatment on HCT116 and HT29 cells resulted in growth inhibition and apoptosis, after 24h incubation of NVD treated cells and staining with propidium iodide, Cells were scrutinized by flow cytometry. Proportion of apoptotic cells is shown. Experiments were performed in triplicate.

Table4. 10: Percentage of cell population after administration of NVD

NVD (µM)	% Apoptosis	
	HCT116	HT29
0	0.35±0.02	1.92±0.01
40	26.7±0.03	17.8±0.21
60	54±0.14	32.8±0.16

Mean ± SD of experiments executed in triplicate is shown.

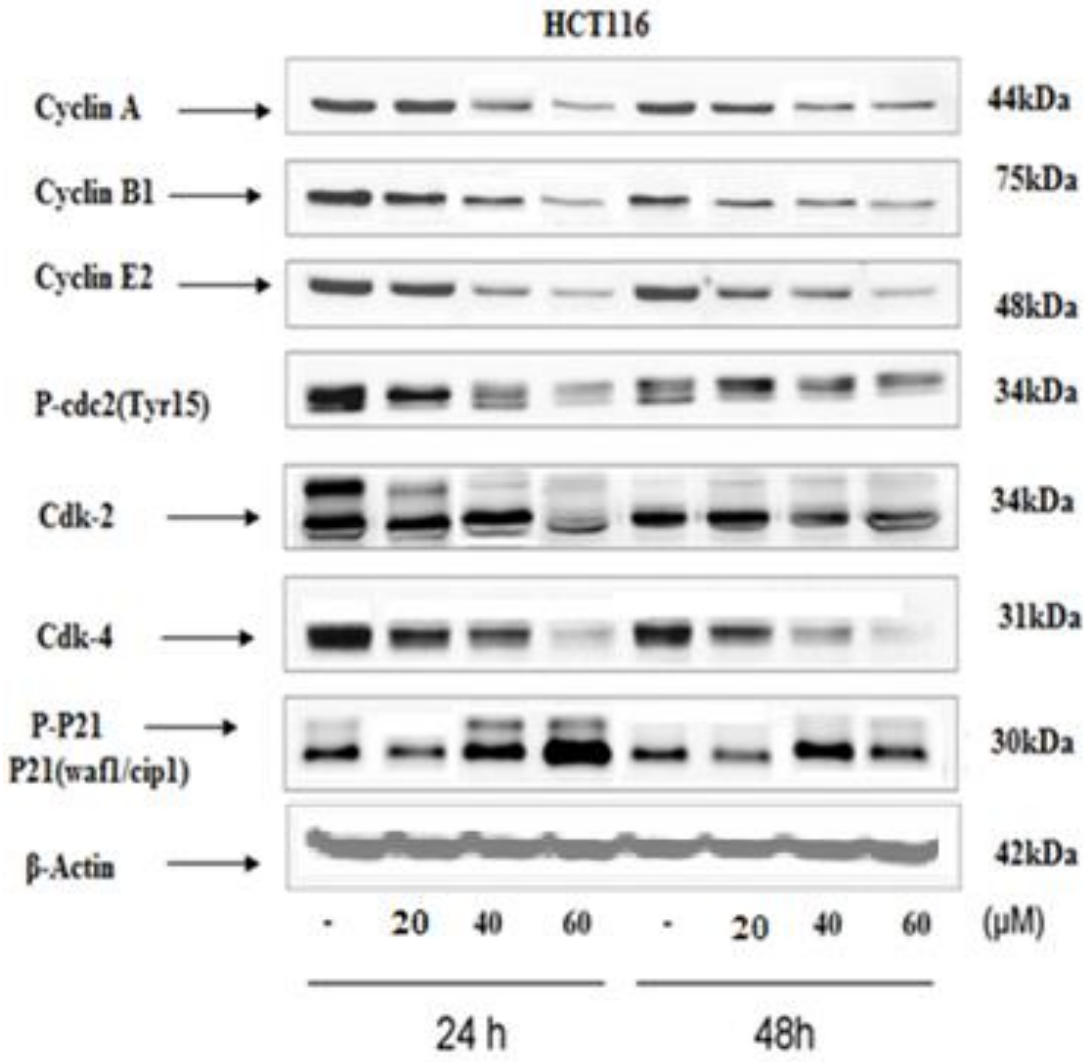
4.12.3 Effect of NVD on the expression of caspases, Bcl-2 family, & cell cycle regulators

To discover the molecular mechanisms of growth arrest induced by NVD, the cell cycle monitors were analyzed by western blotting for their expression levels. Results showed decreased protein expression of Cyclins A, B1, E2 and also decrease in Cdc25c levels after administration of NVD in a concentration dependent approach in HCT116 and HT29. P21 showed increased expression levels (Figure 4.27A and B).

The protein expression intensities allied to apoptosis were analyzed by immune- blotting. Bax and Bak, the pro-apoptotic protein showed increase in treated as well as in control. Apoptosis in HCT116 and HT293 cells was accompanied by loss of Bcl-xL protein expression, and results indicated that NVD induced noteworthy ($p < 0.001$) inhibition of Bcl-xL protein after 24 h of administration. The inhibitory effect of NVD against Bcl-2 protein expression was also decreased as like as Bcl-xL (Figure 4.29). In the NVD-administrated cells, we restrained the seperation of Caspases & PARP. PARP is crucial symbol of initial events in apopotosis and caspases are important proteases in

mitochondria-mediated apoptosis. Results showed dose and time dependent cleavage of PARP (Poly (ADP-ribose) polymerase, caspase 7, 9 & 3 after administration of NVD in HT29 and HCT116 cells for 24h and 48h. Enhanced cleavage of these proteins was seen at 48h treatment. The enhanced cleavage seemed to related well with the amplified apoptotic rate (Fig4.28A and B).

A)



B)

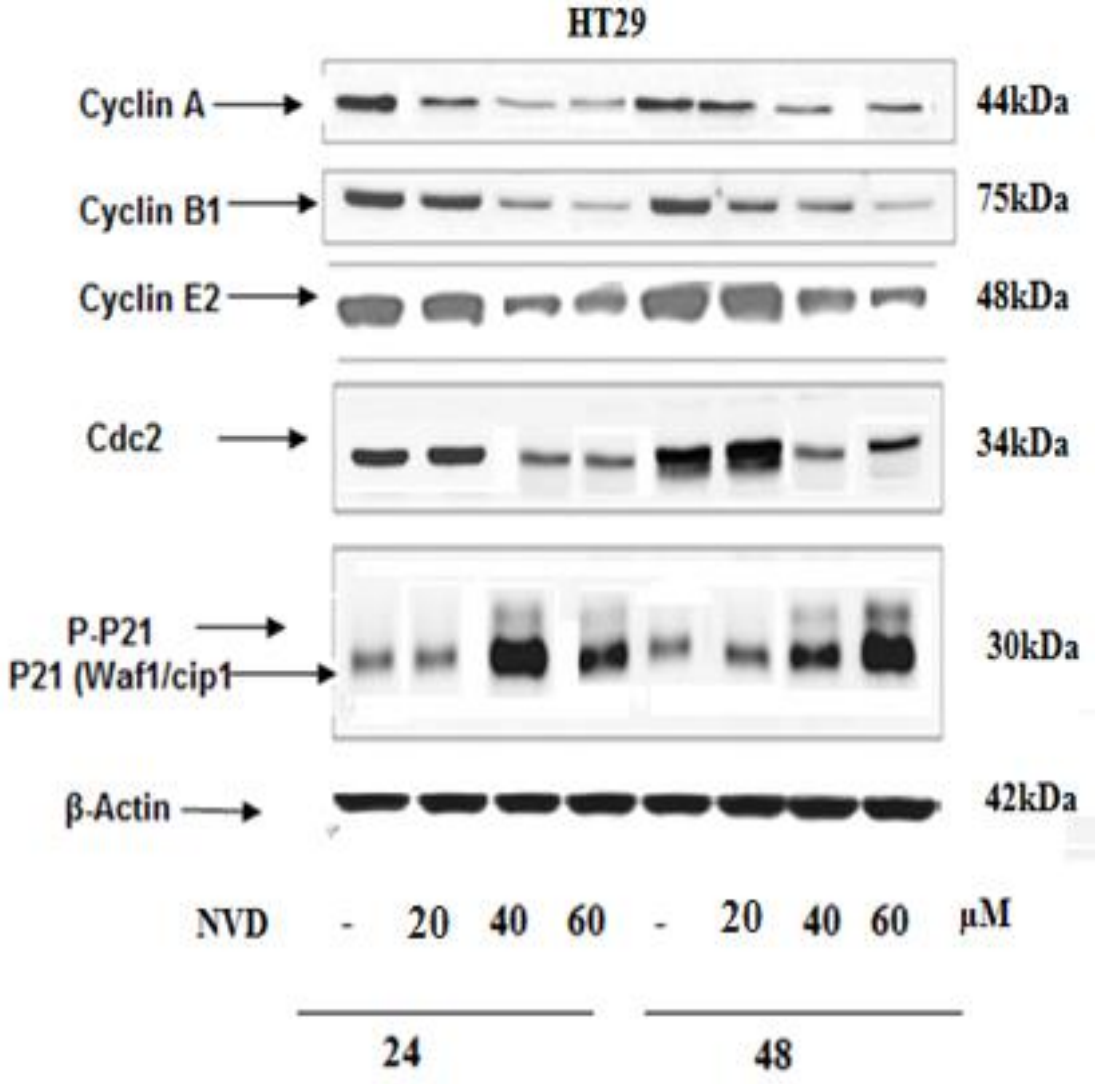
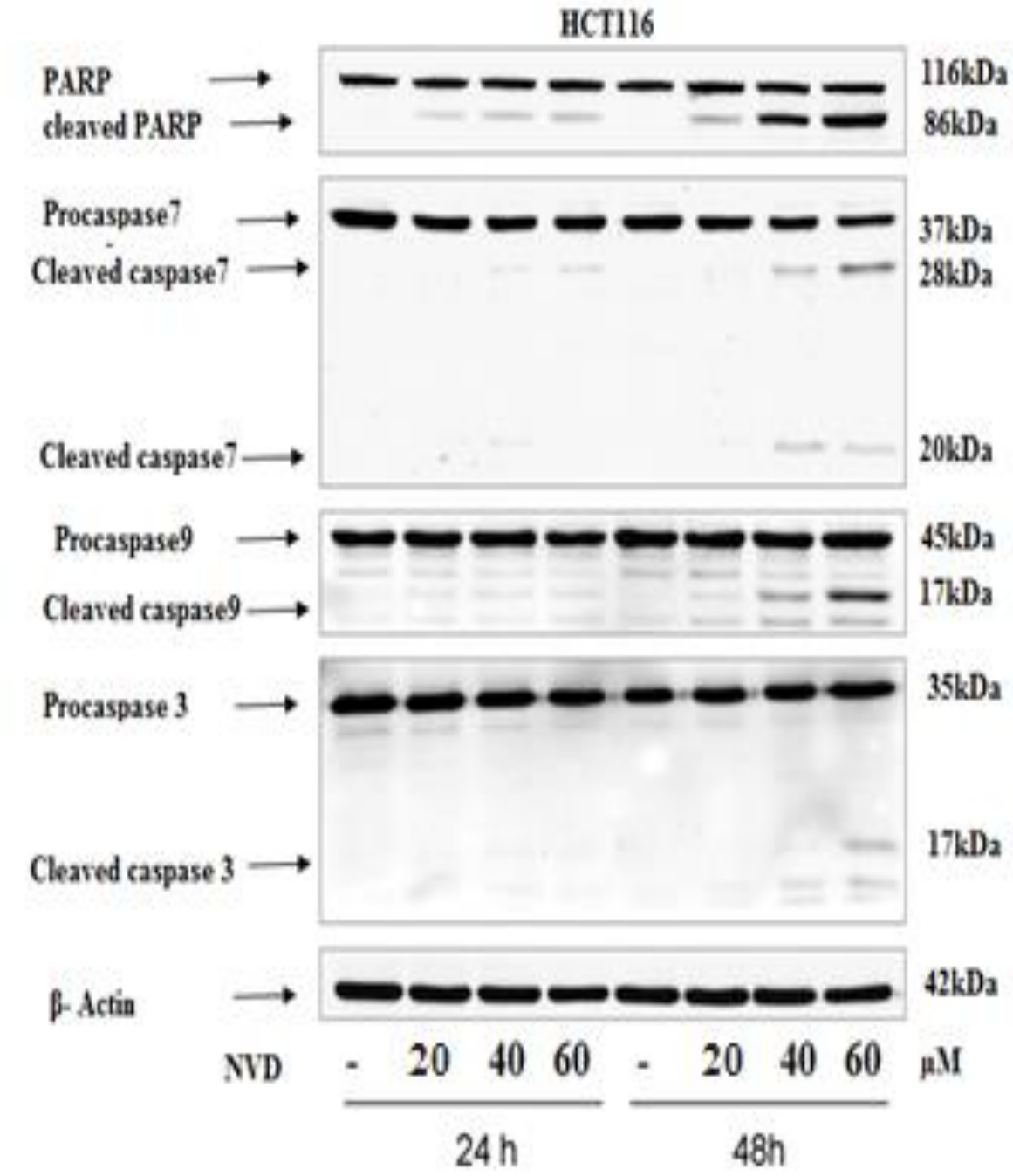


Figure4. 27: Effect of NVD treatment of HCT116 and HT29 (A and B) cells on protein expression of cdc2, WAF1/p21 and cdk 2, 4 cyclin A, B1 and E. Cells were administrated with NVD (20, 40, and 60 µM) for 24h and 48h and harvested. "Total cell lysates were prepared and 40µg proteins was subjected to SDS page followed by immunoblot analysis and chemiluminescence detection. Equal loading of protein was verified by stripping the Immunoblot and again probing it for Actin. The immunoblots shown here are representative of three individual experiments with similar results".

A)


HCT116
PARP
cleaved PARP
116kDa
86kDa
Procaspase7
Cleaved caspase7
37kDa
28kDa
Cleaved caspase7
20kDa
Procaspase9
Cleaved caspase9
45kDa
17kDa
Procaspase 3
Cleaved caspase 3
35kDa
17kDa
β- Actin
42kDa
NVD - 20 40 60 - 20 40 60 µM
24 h
48h

B)

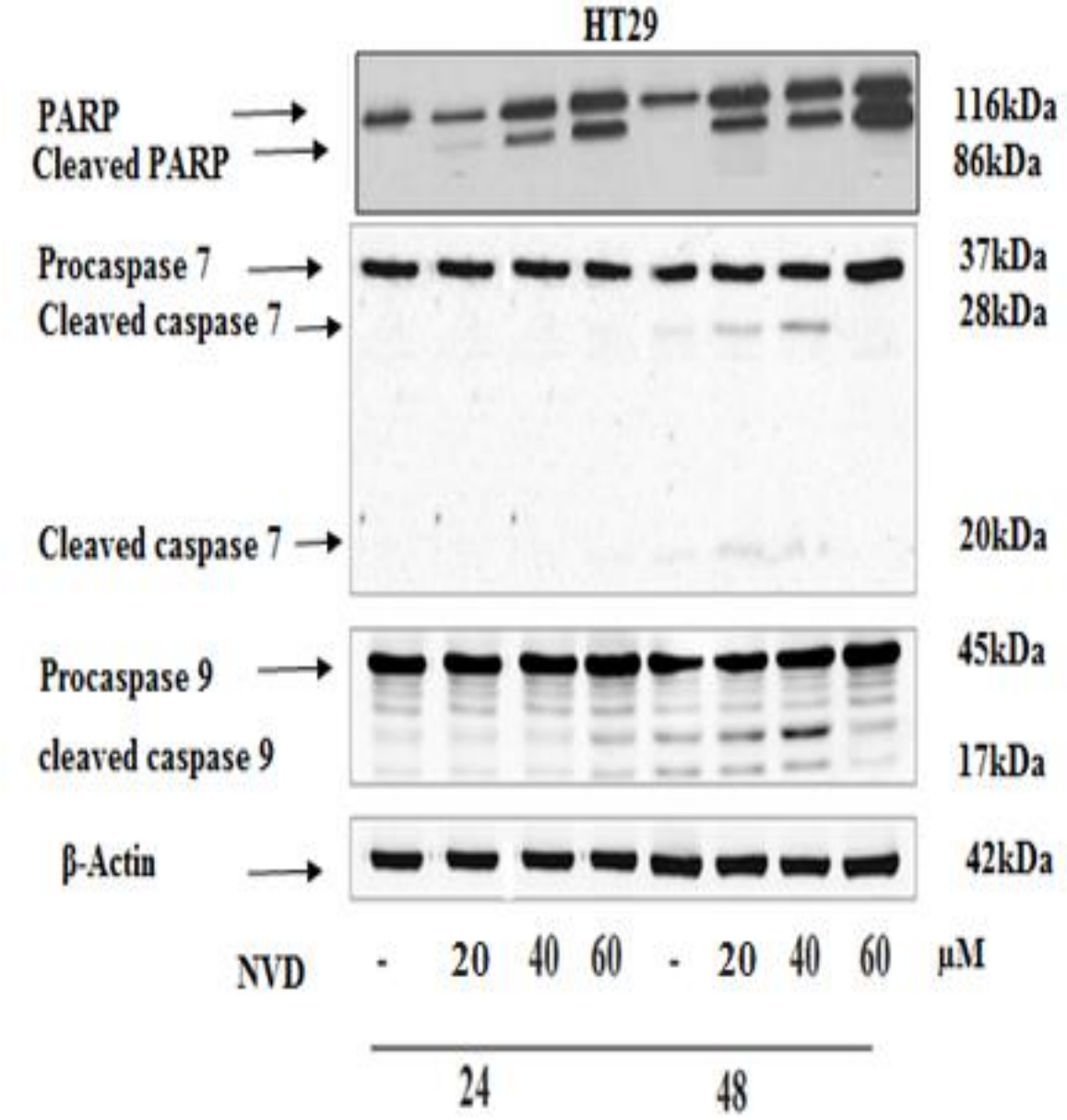


Figure4. 28: Effect of NVD on induction of apoptosis. Effect of NVD on protein expression of active Caspase 3, 7, 9 and PARP in HCT116 and HT29 cells (A and B). Cells were administrated with NVD (20, 40, and 60 µM) for 24h and 48h and harvested. Total cell lysates were prepared and 40 µg proteins was exposed to SDS page tailed by immunoblot examination & chemiluminescence recognition.

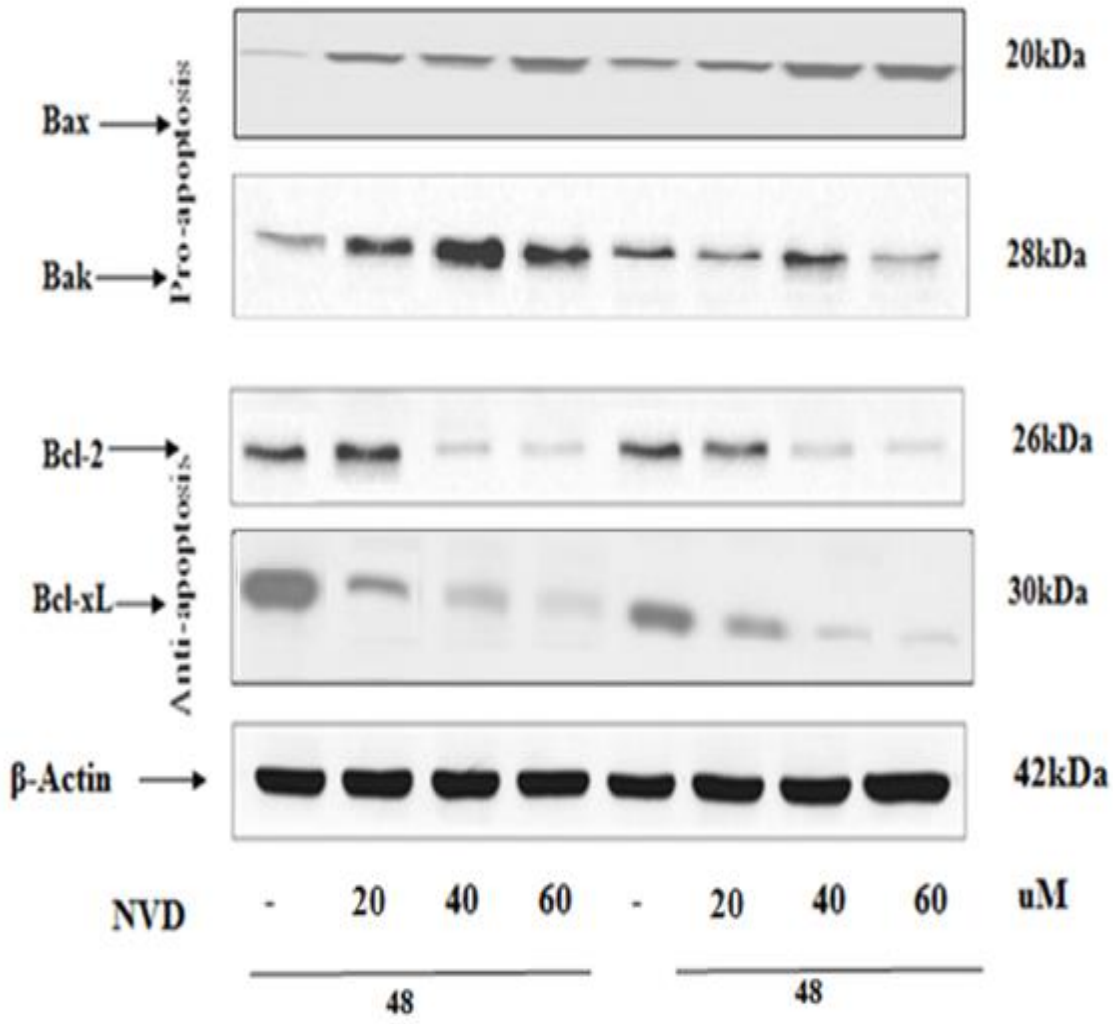


Figure4. 29: Effect of treatment of NVD to HCT116 and HT29 cell lines on protein expression of Bax, Bcl2, Bak & Bcl-X_L. The cells were administrated with NVD for 48h and harvested & cell lysates prepared. The data are demonstrative of three independent experiments with alike results .

4.12.4 NVD administration induces a decrease in β- catenin expression

In humans, genetic, clinical, and molecular data are scant nonetheless they recommended that vitamin D is having shielding effect against colon cancer (Pereira et al., 2012). Results displayed a discernible decline in β-catenin protein expression in a amount dependent appproach on HT29 and HCT116 cells after administration with NVD (20, 40, and 60 μM) for 48h by immunobloting (Figure4. 30). To investigate whether the observed drop off in β-catenin protein was consequence of diminished transcription of β-catenin gene, the mRNA appearance of *CTNNB1* gene was evaluated by administration of NVD in HT29 and HCT116 cells. A noticeable decline was observed in mRNA expression in a dose dependent approach (Figure 4.31)

Immunofluorescence staining of HCT116 and HT29 cells demonstrated reduction in β-Catenin expression at concentration of 60μM of NVD (treated) as compared to control (untreated). Significant Alexa fluor staining of β-catenin (nucleus) of both cell lines (green fluorescence) (counter stain used was DAPI, blue fluorescence) were seen in control, although the expression of β-catenin as indicated by staining was noticeably decreased in NVD administrated cells as shown in the Figure 4.32

FH535 β-catenin inhibitor inhibits survivin and Akt phosphorylation which initiated in reduction in protein expression of β-catenin. Compared with NVD treatment (60 μM), pre-incubation of HCT116 and HT29 cells with FH535 β-catenin inhibitor (20 μM) for 2h before NVD administration caused in noteworthy reduction in the β-catenin protein expression, p-Akt and survivin as pragmatic by Immunoblot analysis (Figure 4.33)

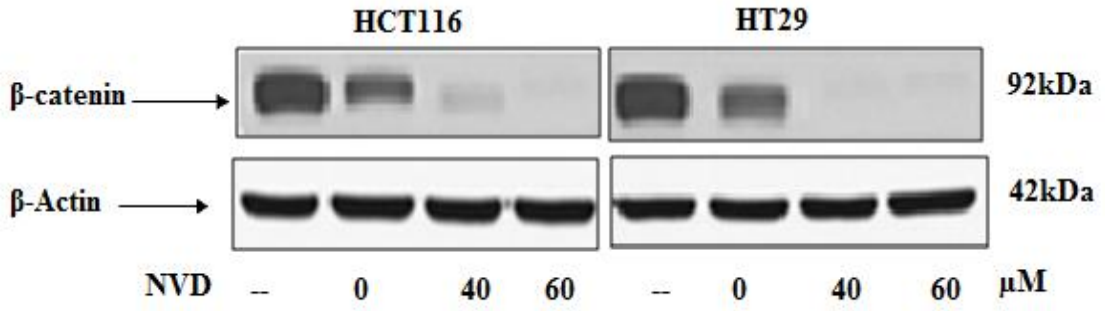


Figure4.30: Effect of NVD treatment on β-catenin expression in HCT116 and HT29 cells lines. Immunoblot analysis of β-catenin expression of HCT116 (B) and HT29 (D) in NVD treated group in comparsion to control group.

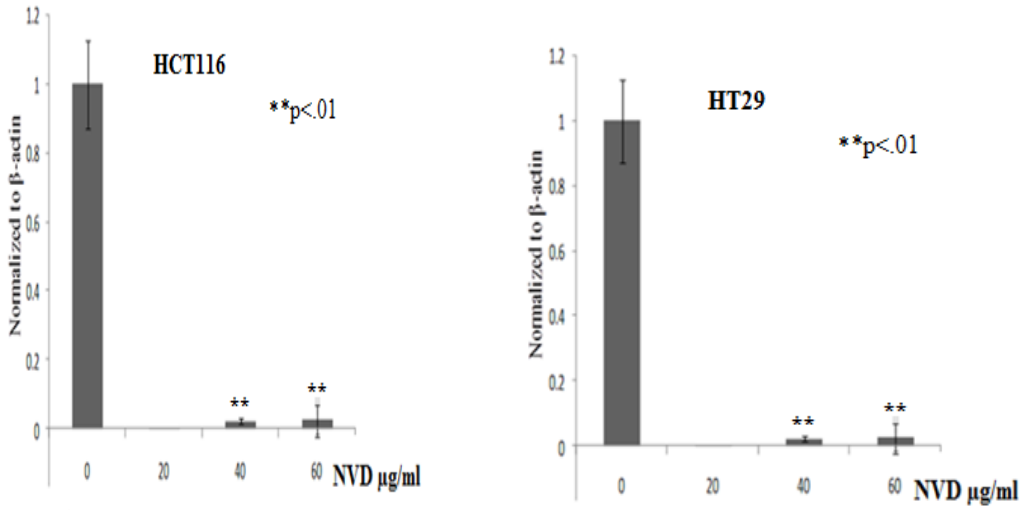
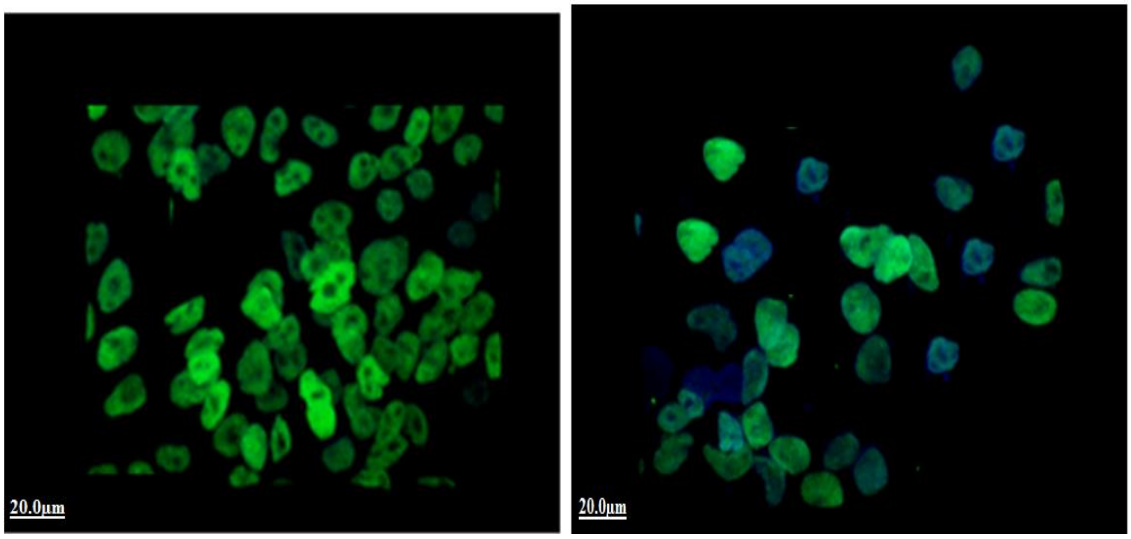


Figure 4.31: Modulation of β-catenin expression by NVD treatment in HCT116 (a) and HT29 (b) cells. mRNA expression of β-catenin in NVD treated HCT116 and HT29 cells (RT-PCR), experiment performed in triplicate (mean±SD), **, $p < 0.01$

A) CONTROL (HCT116) **B) NVD (60µM)**



C) CONTROL (HT29) **D) NVD (60µM)**

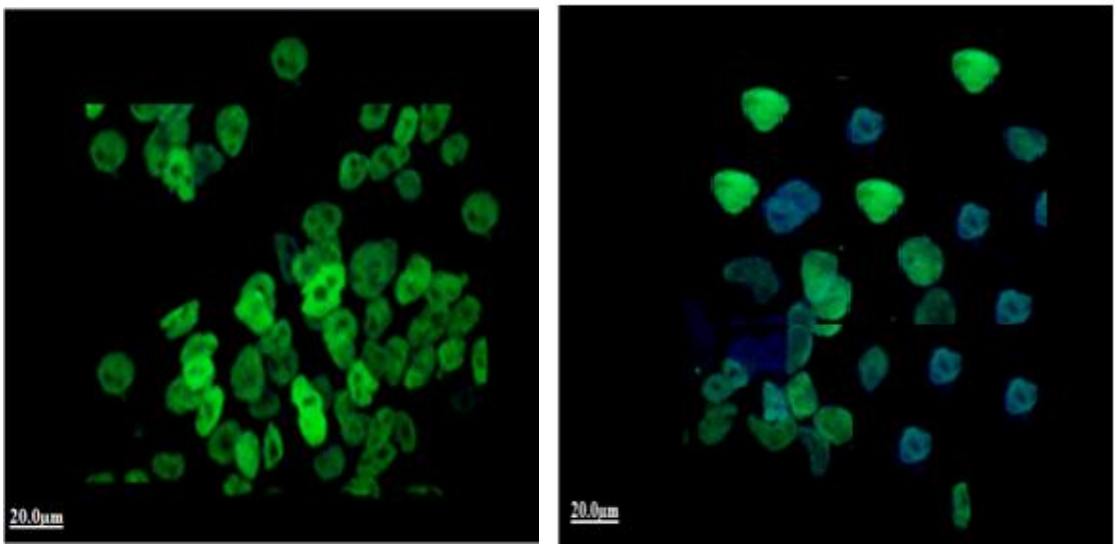


Figure4. 32: Imunofluorescence staining of HCT116 (b) and HT29 (d) demonstrating expression of β-catenin in both NVD treated (60µM) as compared to control HCT116 (a) and HT29 (c) cells (untreated). Alexa fluor staining of β-catenin of both cell lines (green fluorescence) and counter stained with DAPI (blue fluorescence) were observed.

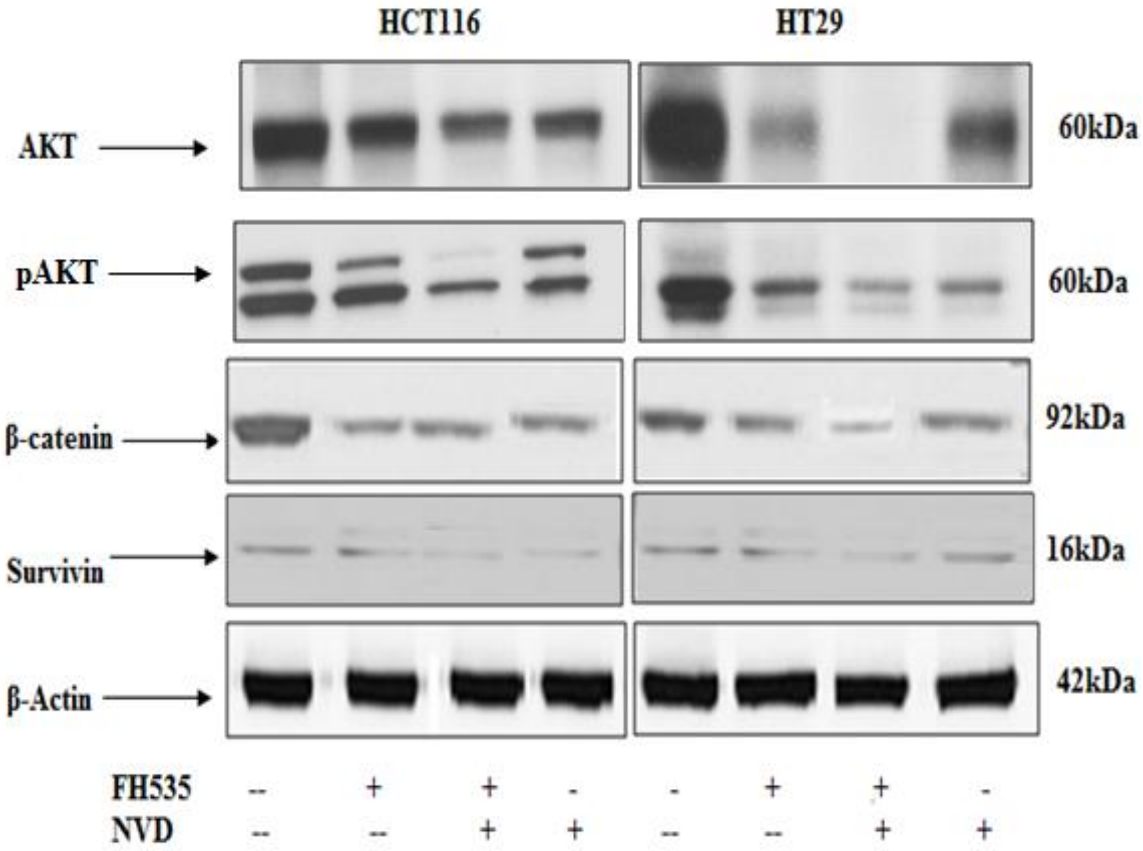


Figure4. 33: "Effect of NVD on protein expression of β-catenin, Survivin and phosphorylation of Akt at Ser473 in HCT116 and HT29 cells., Total cell lysate were prepared and 40μM proteins was subjected to SDS-page followed by Immunoblot analysis and chemiluminescence detection. Equal loading of protein was confirmed by stripping the immunoblot and reprobing it for β-Actin".

4.12.5 Signaling pathways and endurance proteins amended by NVD administration

Western blot analyses were executed to assess the consequence of NVD on signaling pathways engaged in cancer cell endurance and propagation. HCT116 and HT29 cells were administrated with 20, 40, and 60 μM of NVD. The effects of NVD on various signaling pathways in colorectal cancer cells were studied.

4.12.6 NVD effect on CK2 and NFκB p65

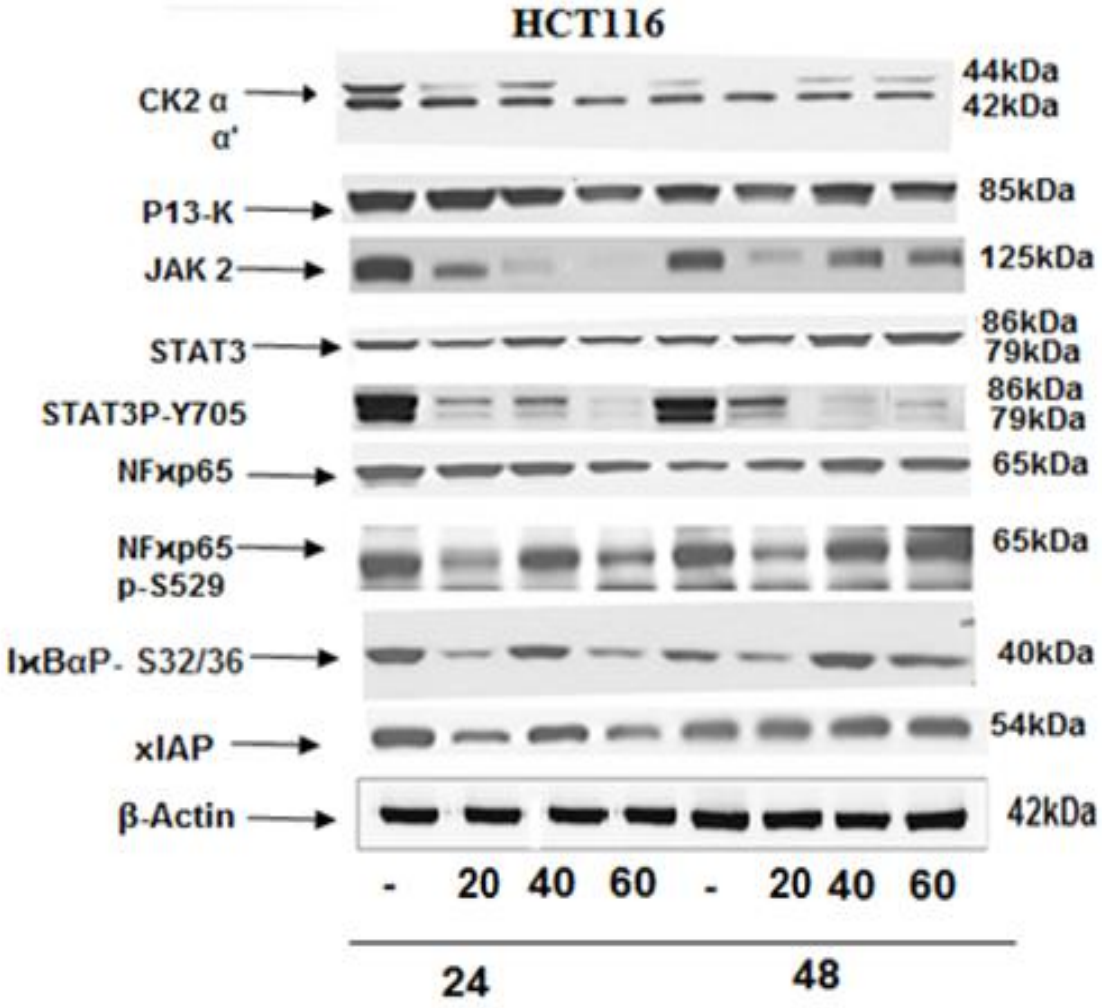
An evolutionary conserved Protein kinase CK2 being a ubiquitous protein kinase, exceedingly pleiotropic with multi- substrates controlling a wide range of cellular processes. CK2 α expression perform a crucial part in cellular as well as in organismal endurance. HCT116 and HT29 cells administrated with NVD for 24 h drastically diminished expression of CK2α protein in a dose-dependent approach (Figure4.34). Nuclear factor-κB (NF-κB) includes family of transcription factors that perform significant part in immunity, inflammation, survival, differentiation, and cell proliferation. Aberrant NFκB regulation leads to constitutive cell survival by avoiding program cell death in various malignancies. In HCT116 and HT29 cells administrated with NVD inhibited the expression of both NFκB p65 P-Ser529 and total NFκB p65 protein in a dose and time dependent approach. Moreover protein expression of phospho-IκBα Ser32/Ser36 was significantly declined in a time and dose reliant approach following NVD administration (Figure 4.34 A and B).

4.12.7 Impact of NVD on further signaling pathways

Numerous actions of the Ras/PI3-K/Akt/mTOR as well as Ras/Raf/MEK/ERK corridors on apoptosis are transitionally by ERK or Akt phosphorylation of key apoptotic effector molecules. The figure of ERK 1/2 phosphorylation targets are more than hundreds, thus suppression of MEK and ERK activities will have reflective influence on cell growth. Results showed that NVD administration appreciably restrained of ERK1/2 and Akt phosphorylation within HCT116 & HT29 cells (Figure 4.34B). The NVD adminstration

on the expression of JAK-STAT signaling showed prominent loss of JAK2 protein expression in a time dependent approach with significant ($p < 0.001$) suppression of protein expression was exhibited after adminstration for 48 h. Prominent STAT3 has been allied with growth, progression, and maintenance of many human tumors. Results showed that treatment with NVD for 48h significantly declined phospho-STAT3 Tyr705. Also NVD led to noteworthy inhibition of total STAT3 protein expression (Figure4.34A).

A)



B)

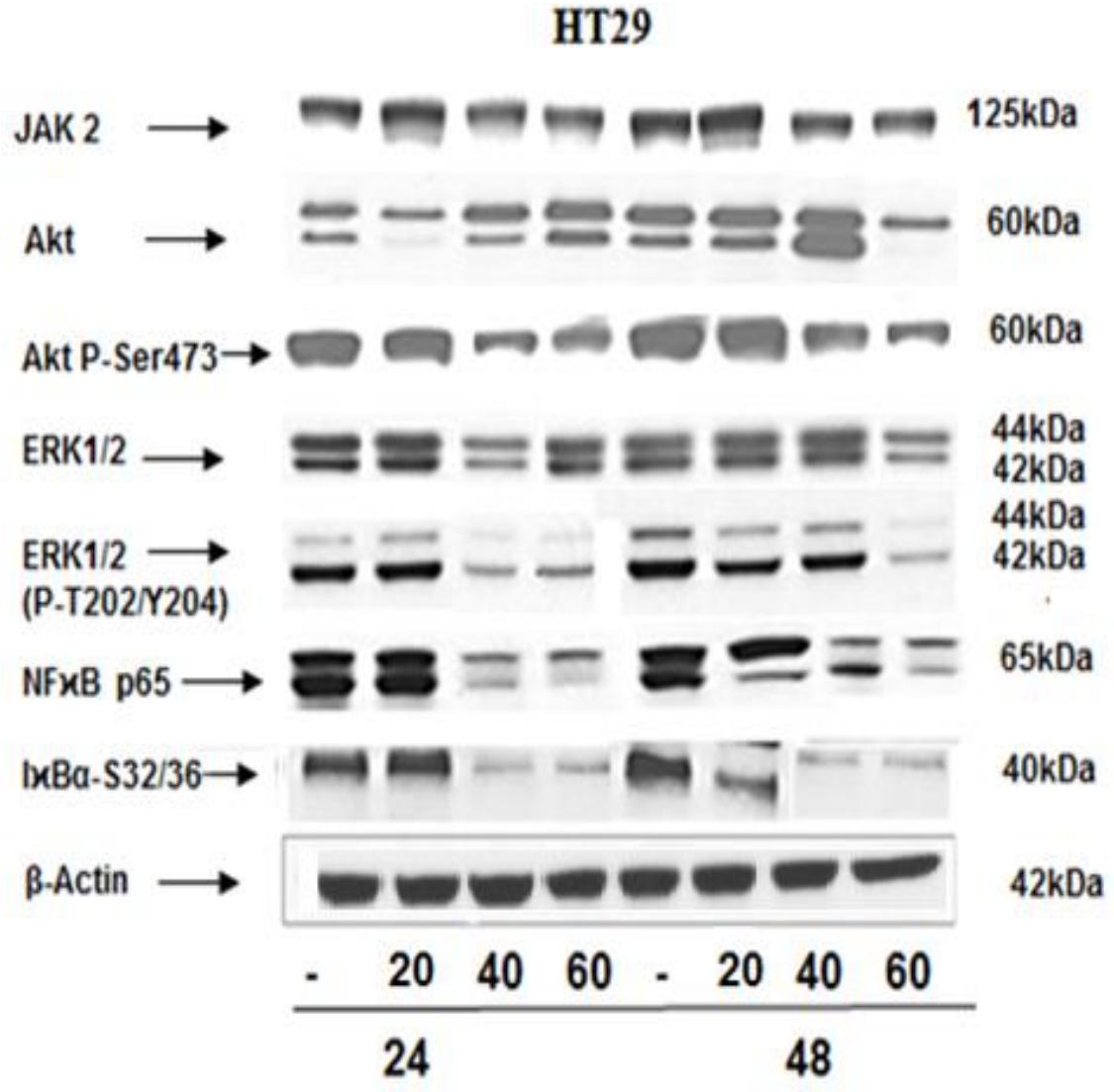


Figure4. 34: Immuno blot examination of cellular lysates primed from HCT116 & HT29 cells. Lysates prepared from HT29 & HCT116 cells, initially seeded at density of 1.5 $\times 10^4$ cells were administratedwith 20, 40, and 60 µM concentrations of Nano-particle with Vitamin D. The blots probed with CK2 αα', PI3-K, STAT3, STAT P-Try705, NFκB p65, NFκB p65 P-S529, IκBα P-S32/36 and xIAP antibodies were shown. Actin blots are shown as loading controls. Data based on three different experiments, each carried out in triplicate.

4.13 *In vivo* studies

TAX restrains the tumorigenic ability in vivo in alliance with inhibition of Wnt signaling

4.13.1 TAX hampers growth of HCT116 Xenografts in athymic nude mice

TAX infused intraperitoneally to nude mice results in reticence of colorectal carcinoma HCT116 tumor xenografts growth. In nude mice, presence of little solid tumors was witnessed after eighth day of cell inoculation, getting water as drinking fluid. This incubation phase was comprehensive to 14-18 days in animals administrated with taxifolin intraperitonially. Taxifolin was infused intraperitoneally *ad libitum* to these mice a week after tumor implantation. Tumor growth inhibition was momentous in mice receiving 15 and 25mg/kg of TAX. More prominent inhibition was witnessed in animals administrated with 25mg/kg of TAX. At the culmination of experiment, all mice were sacrificied when the tumor implanted attained ~ 1200 mm^3 in volume. The mean lump size of 800mm^3 was seen after ~ 35±4 days succeeding to tumor cell inoculation (Figure 4.34a). Meanwhile, the mean tumor volumes of 15 and 25mg/kg TAX administrated groups were 68 and 48mm^3, respectively. The prominent tumor hampering reaction was withnessed in the 25mg/kg administrated group. The endurance probability of tumor volume data was investigated by Kaplan-Meier analysis, specifying that constant TAX injection to athymic mice confirmed amplified existence through a norm existence of 38 & 39 days (15 & 25mg/kg of TAX, respectively) as compared with vehicle group ($P < 0.0001$, log-rank test).

H & E staining of xenograft tumors illustrated that TAX administration results in contraction of nucleus, apoptosis and inflammatory cell infiltration in TAX administrated groups in comparsion to control group (Figure 4.35 b and c).

4.13.2 No difference in body weight was observed in TAX administrated mice

There was no observed difference in body weight between vehicle and TAX treated group throughout the experiment. The animals were monitored daily; no signs of stress throughout the experiment were witnessed. Before sacrificing, all mice were observed for

gross pathology and nothing was seen adverse, there was no edema or atypical organ size enlargement in non-candidate regions.

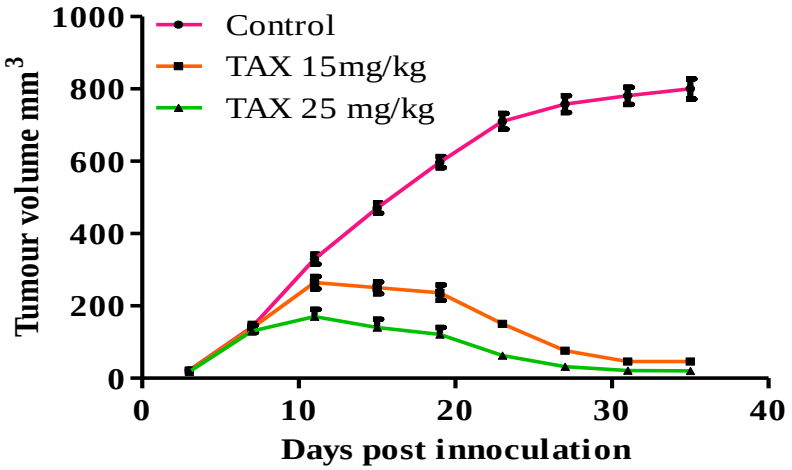
4.13.3 Cyclin D1 and Caspases arbitrates resistance to apoptosis by administration of TAX

4.13.3.1 TAX induces apoptosis in xenograft tumors

Caspases are an exceedingly conserved cysteine proteases family that functions as widespread effector molecules in apoptotic progression. Caspases are typically synthesized as inactive proenzymes and gets triggered either by oligomerization to form a large multimeric complex or by proteolytic cleavage that pertains for effector caspases like Caspase 3. Numerous substrates are hewed once caspases get triggered in the cytoplasm or nucleus, causing several discrete transformations for apoptotic process.

The result of TAX on cleavage of Caspase 3 was appraised in order to recognize whether the growth inhibition of cells is due to initiation of apoptosis (Figure 4.36A). The morphology of TAX administrated cells also showed encouragement of cell death when observed under fluorescence microscope after staining (immunohistochemistry) the xenografts tumors with cleaved Caspase 3 (Figure 4.36 B). This method was used to recognize the brown stained cells for apoptosis. Results demonstrated that administration with TAX induced apoptosis as the dead cells number amplified in a dose dependent approach.

a)



b) Control **c) TAX treated**

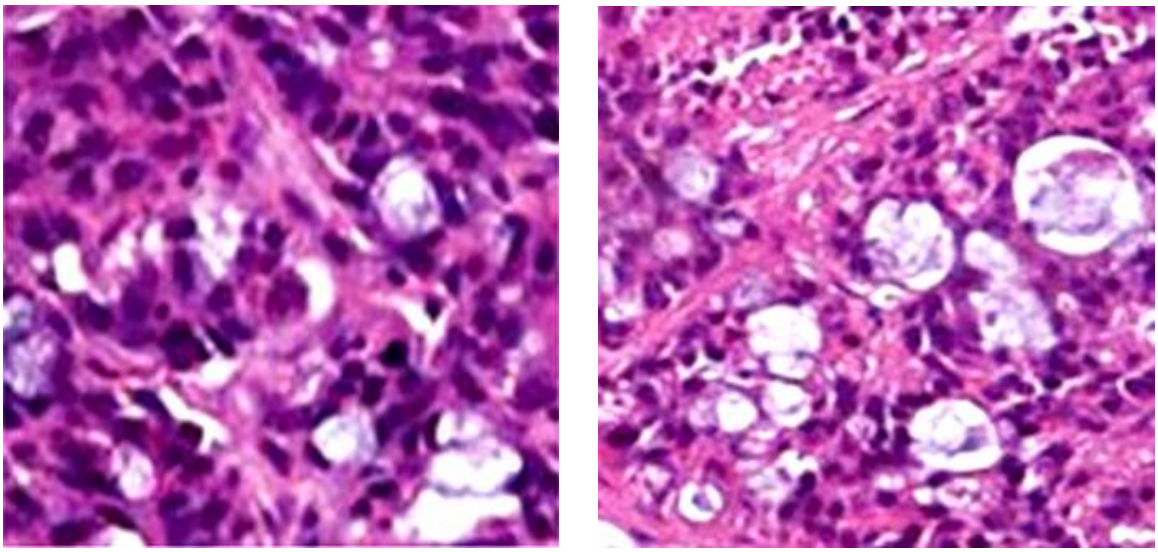
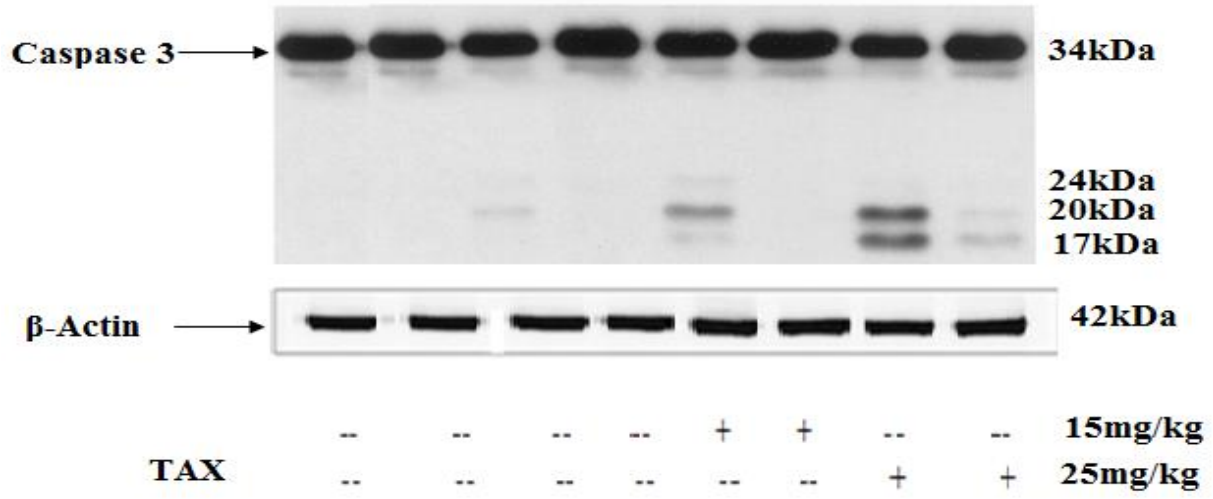


Figure4. 35: "Consequence of TAX administration on HCT116 tumor growth in athymic nude mice. (a) Average tumor volume of water fed, 15 & 25kg/mg TAX injected mice plotted over days after tumor cell inoculation. Values represent mean±SD of six mice. *, $p < 0.01$ (25 mg/kg); **, $p < 0.05$ (15 mg/kg) vs water fed normal control mice ***, $p < 0.001$. (b) H&E staining of TAX administrated xenograft tumors (b) vs control (c)".

A)



B)

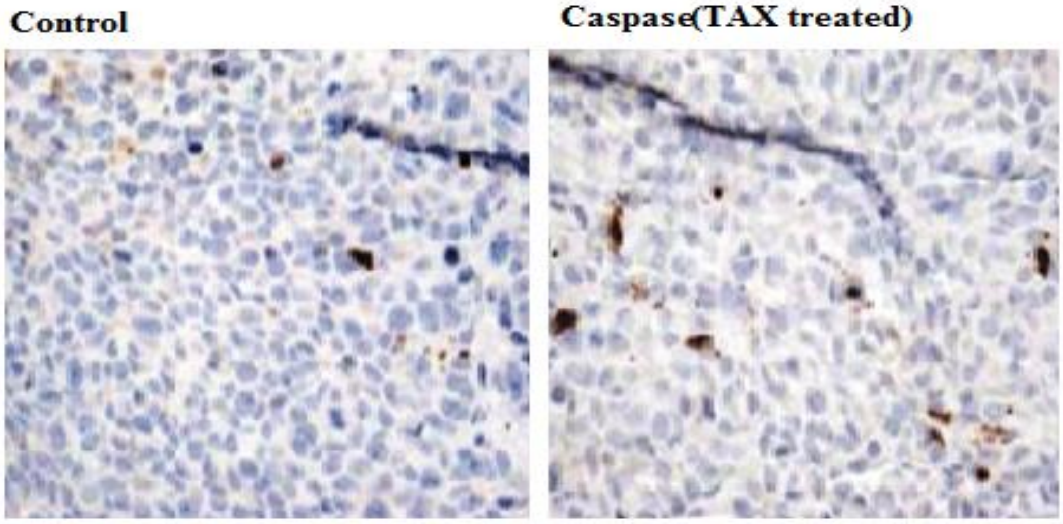


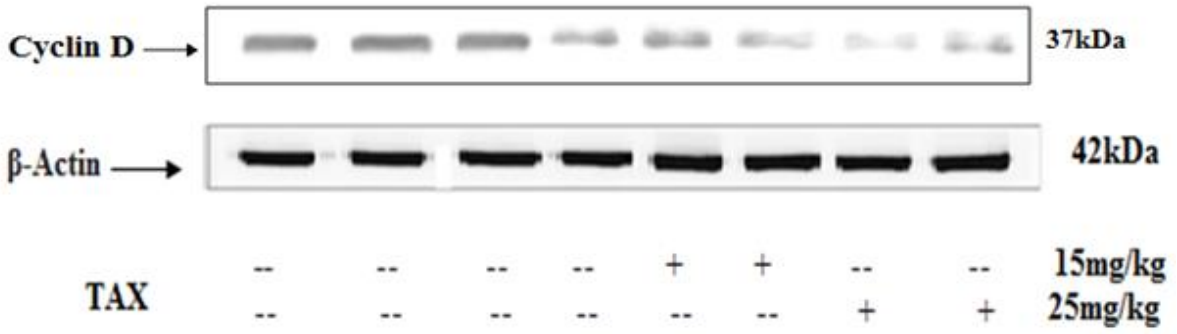
Figure4. 36: TAX administration induces apoptosis in HCT116 xenograft tumors in athymic nude mice. a) Effect of TAX treatment (15 and 25mg/kg) on protein expression of cleaved Caspase 3 of HCT116 implanted xenografts tumors in athymic nude mice. b) Immunohistochemistry of cleaved Caspase 3 of TAX administrated tumor xenograft vs untreated control tumor xenografts.

4.13.4 TAX hampers proliferation in xenograft tumors

Cyclin is a class of proteins that organize the development of cells during the cell cycle by activating cyclin-dependent kinase (Cdk) enzymes. Cyclin D is one of the chief cyclins formed in terms of its functional importance and is a vital watchdog of cell proliferation, apoptosis and tumourigenesis.

Protein expression of cyclin D was assessed in both TAX administrated and vehicle group. Results demonstrated induction of Cyclin D protein expression in control group as compared to groups TAX administrated group (25 and 15 mg/kg). Down regulation of Cyclin D protein expression in TAX administrated group, favors inhibition of proliferation (Figure 4.37A). The expression of cyclin D in staining (immunohistochemistry) of group administrated with taxifolin and vehicle group was also investigated. A prominent difference was seen between TAX administrated and vehicle. The expression of cyclin D was found to be high in vehicle group while TAX administrated show low expression of cyclin D (Figure 4.37B)

A)



B)

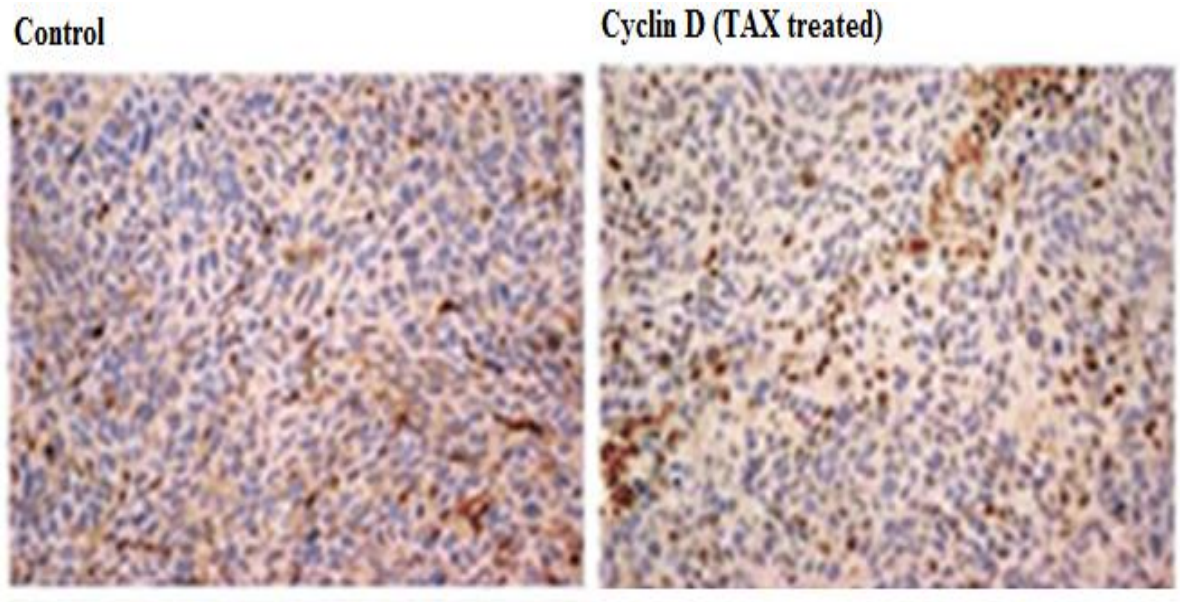


Figure4. 37: TAX hampers proliferation in xenograft tumors in athymic nude mice. a) Effect of TAX administration (15 and 25 mg/kg) on protein expression of cyclin D of HCT116 implanted xenograft tumors in athymic nude mice. b) Immunohistochemistry of cyclin D tumor xenograft vs untreated control tumor xenografts.

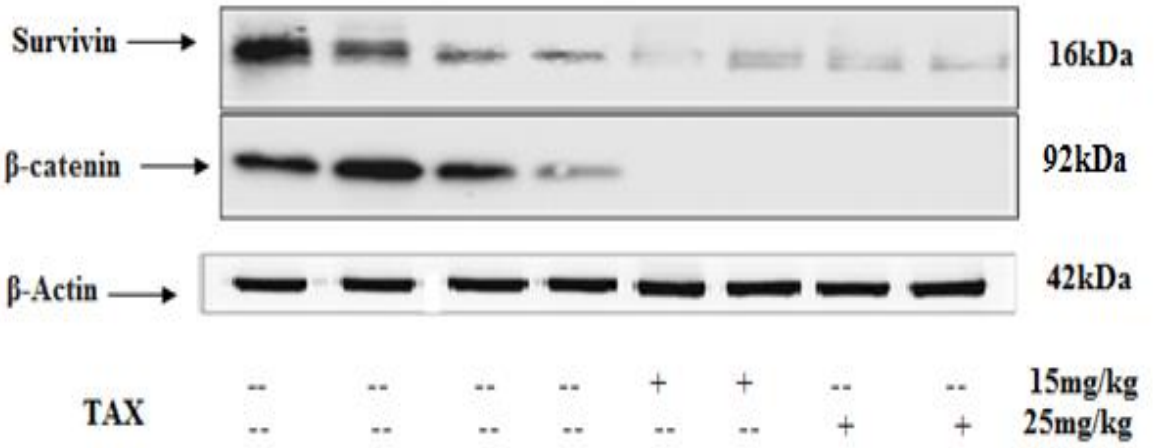
4.13.5 TAX administration decreases β-catenin and survivin expression in xenograft tumors

Effect of TAX (15 and 25mg/kg) on β-catenin and survivin expression in HCT116 xenograft tumors was cofirmed by immunobloting, immunohistochemical staining and RT-PCR.

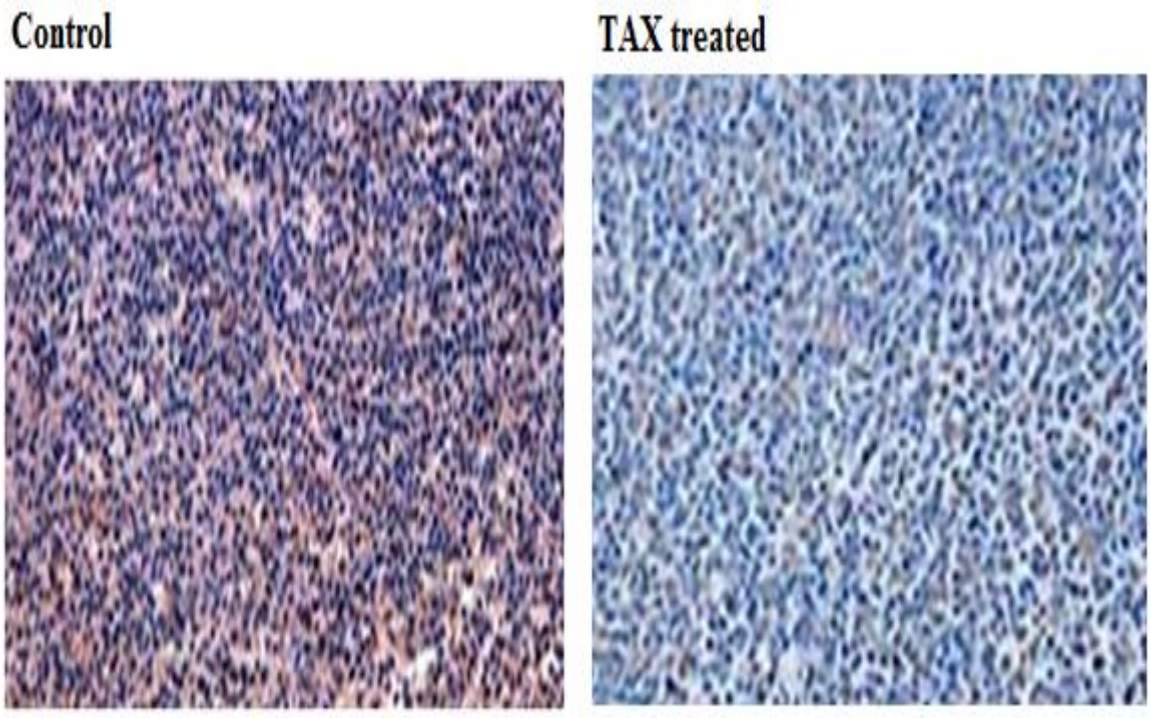
A prominent decline in expression was observed (Figure 4.38A). The concentration dependent impact of TAX on HCT116 xenograft tumors demonstrated a prominent decline in β-catenin and survivin protein levels at 25mg/Kg and 15mg/kg concentrations (Figure 4.38A.). Immunohistochemical staining of HCT116 xenograft tumors in athymic nude mice illustrated decline in β-catenin expression in TAX adminstrated as compared to vehicle ones (Figure 4.38 B).

To elevate, either the observed reduction in β-catenin protein was owing to declined transcription of *β-catenin* gene, alteration of β-catenin expression by TAX induction in HCT116 xenograft, a prominent decline in mRNA expression by employing RT-PCR, was seen to be in concentration dependent approach. At 15 and 25mg/kg of TAX a momentous reduced in β-catenin expression was seen (Figure 4.38C)

A)


Survivin
β-catenin
β-Actin
16kDa
92kDa
42kDa
TAX
15mg/kg
25mg/kg

B)


Control
TAX treated

C)

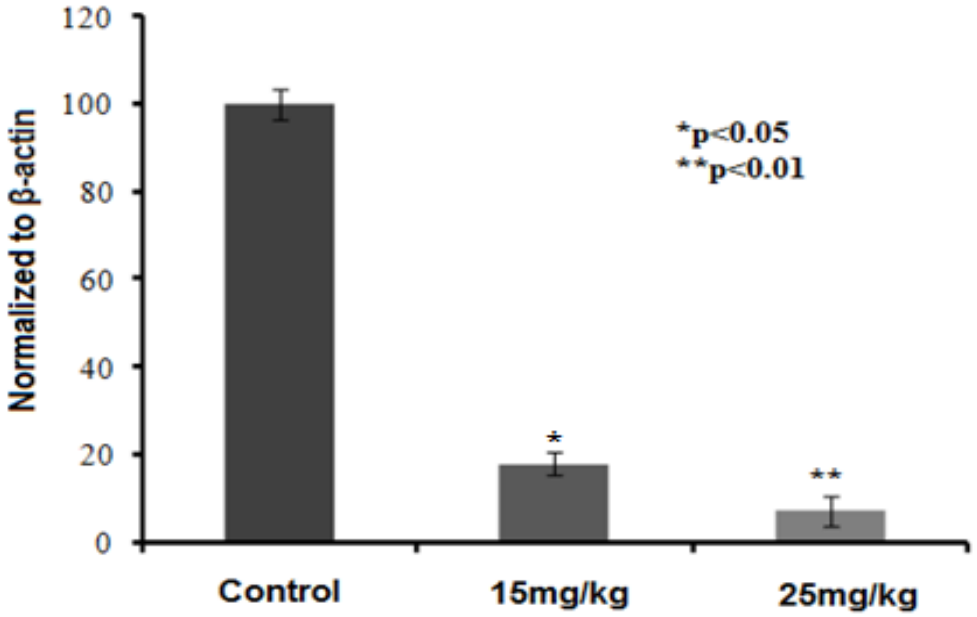
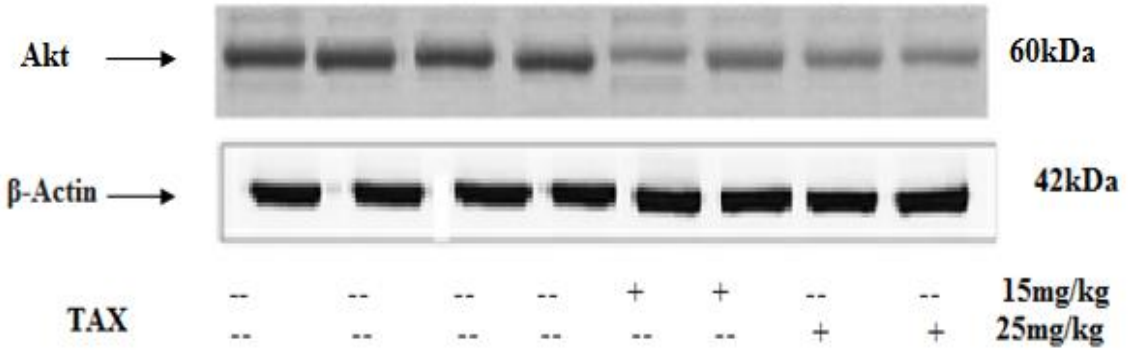


Figure4. 38: "Effect of TAX admininstrated treatment on HCT116 xenograft tumors for protein expression of β-catenin. Expression of β-catenin and survivin protein by immunobloting in TAX administrated and control group, experiment executed in triplicate. b). Effect of TAX administrated as detected by immunohistochemical staining. DAB staining of β-catenin (brown) and counter stained with hematoxylin (blue) qPCR analysis of TAX administrated for changes in *β-catenin* mRNA levels. The data expressed as fold modification represent the mean± standard errors experiment executed in triplicate where*p<0.05, **p<0.01 was taken significant v/s control".

4.13.6 TAX induced inhibition of Akt phosphorylation in HCT116 xenografts in athymic nude mice

AKT Phosphorylationof β-catenin boosts its transcriptional bustle as well as encourages cancer cell incursion, demonstrating that AKT-reliant control of β-catenin displays a crucial part in tumor incursion and progression. Animals implanted with HCT116 xenografts with TAX administration induced a concentration dependent reticence of Akt phosphorylation. Akt is essential player of signaling cascades for cell growth and survival during formation and cancer. The protein expression of Akt was investigated by immunohistochemical staining and immune blotting. The down regulation of Akt protein expression by TAX (15 and 25mg/kg) administration, favors the inhibition of proliferation and there was prominent difference between expressions of vehicle and treated group. The Akt expression was observed diminished in TAX administrated group as compared to vehicle group. Also p-Akt expression was investigated but no expression of p-Akt protein was seen (Figure 4.39)

a)



b) Control c) TAX treated

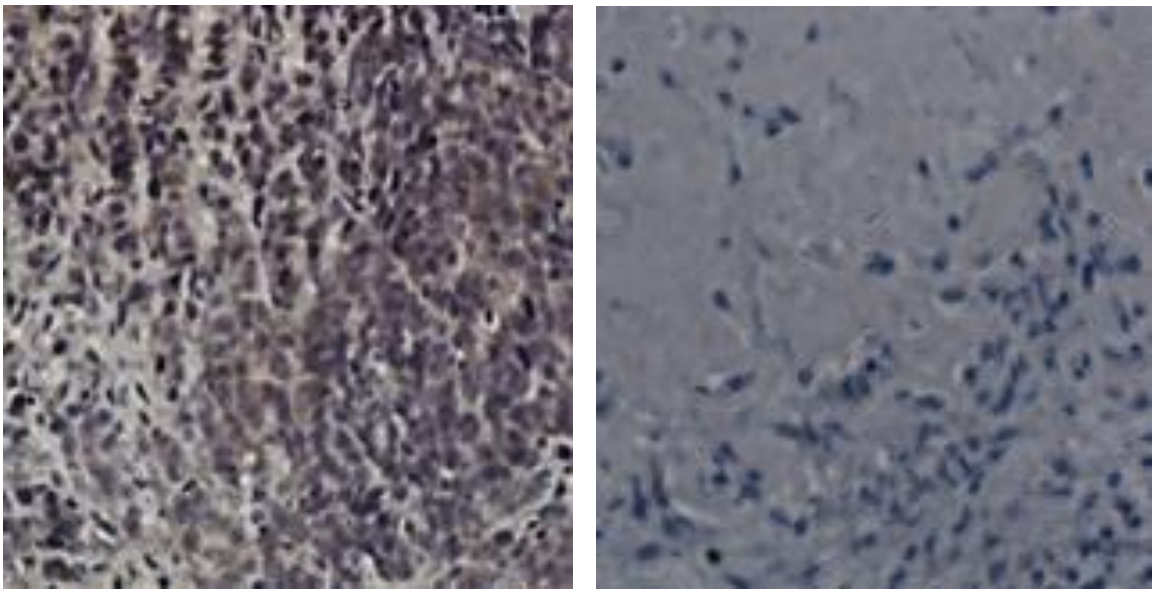


Figure4. 39: "Effect of TAX administrated on HCT116 xenograft tumors for protein expression of Akt a). Expression of Akt protein by immunobloting in TAX administrated and control group, experiment performed in triplicate. (b and c). Effect of TAX administrated on protein expression of Akt and control as detected by immunohistochemical staining. DAB staining of Akt (brown) and counter stained with hematoxylin (blue)."

4.14 Vitamin D-Nanoemulsion (NVD) and FH535 β-catenin inhibitor administration results to momentous diminution in tumor volume.

Colorectal cancer developed in athymic (nu/nu) male nude mice was used for appraising antitumor effects of NVD. On 8th day of injection of HCT116 cells, when evident tumor was noticeable, nude mice were administered NVD (15mg/kg and 25mg/kg), intra-peritoneally (i.p), twice weekly. Based on a pilot study the particular doses of compounds were chosen. when administrated with NVD, result illustrated a notable diminution in tumor volume in comparsion to untreated control animals having tumor after 15-17th day of administration. Though, the decrease in tumor size was more noteworthy in case of 25mg/kg NVD administrated mice.

4.14.1 Administration of NVD perks up the lifespan of developed xenografts, with no side effects.

Rise in life span was assessed in case of NVD and FH535 administrated (15 and 25mg/kg) tumor bearing mice as defined in Materials and Methods. Whilst untreated control nude mice subsisted for a maximum of only ~50 days, the NVD and FH535 administrated groups led to ~ 4-fold amplification in the life span for atleast 43% of mice (Fig.). This corroborated that treatment of NVD and FH535 results veto side effects. Thus, our statistics illustrated that NVD and FH535 administration in nude mice, decreases the tumor load, boost survival and results no side effects. Histopathological studies were carried on tumor section of control, NVD and FH535 administrated nude mice at 38th days of administration, by H&E staining (Figure4.40). In control tumor animals, dense nuclear staining by hematoxylin was pragmatic consequently presenting the occurrence of huge figure of proliferating cells in comaprsion to administrated tumor (Figure 4.40).

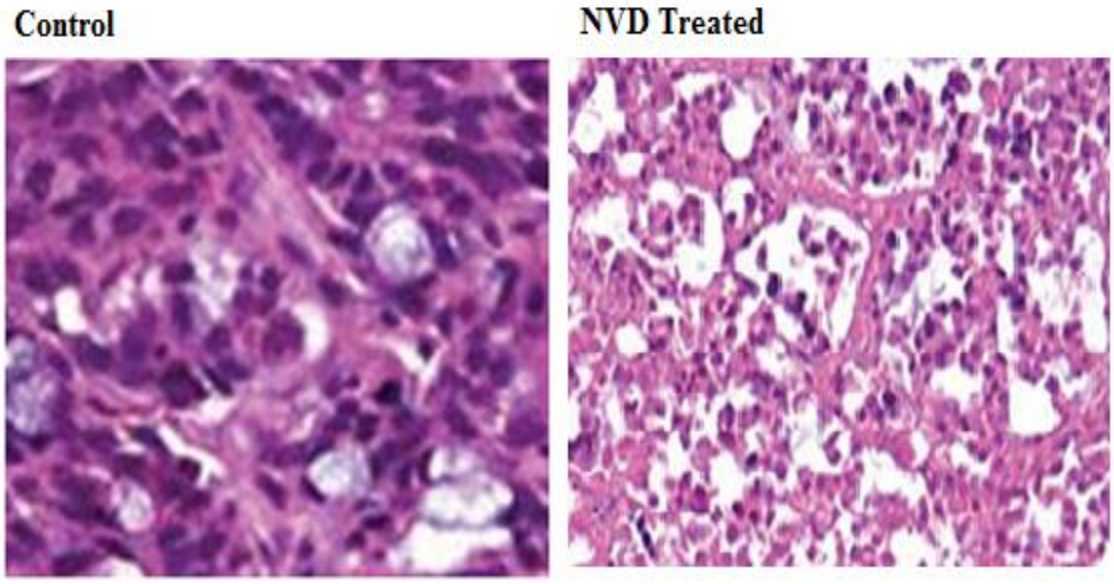
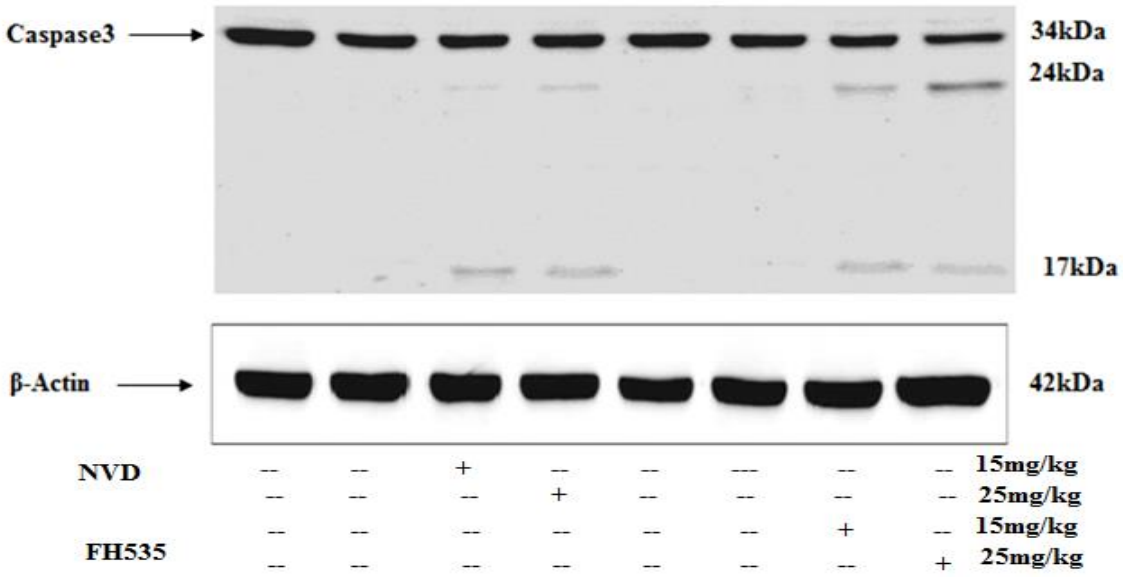


Figure4. 40: Consequence of NVD administration on HCT116 tumor growth in athymic nude mice. (a) Average tumor volume of water fed, 15 & 25mg/kg NVD injected mice plotted over days after tumor cell inoculation. Values represent mean±SD of six mice. *, $p < 0.01$ (25 mg/kg); **, $p < 0.05$ (15 mg/kg) vs water fed normal control mice ***, $p < 0.001$. (b) H&E staining of NVD administrated xenograft tumors (b) vs control (c) .

4.14.2 Caspases mediate confrontation to apoptosis by administration of NVD and FH535 in xenograft tumors

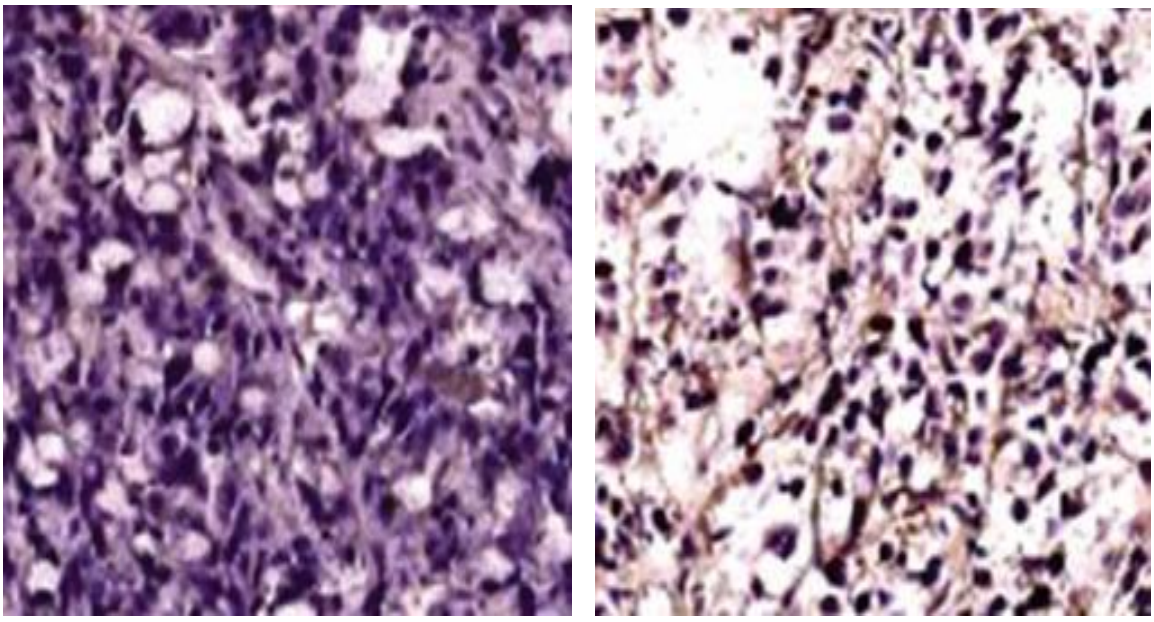
The result of NVD and FH535 on cleavage of Caspase 3 was reviewed in order to distinguish whether the growth inhibition of cells is due to initiation of apoptosis (Figure4.41). The morphology of NVD and FH535administrated cells also illustrated support of cell death. Immunostaining of tumor sections from NVD and FH535 administrated groups exhibited an intensification in cleaved Caspase-3 staining. Results demonstrated that administration with NVD induced apoptosis as the dead cells number amplified in a concentration dependent approach (Figure 4.41 B and C. Western blot data demonstrated more cleavage in NVD administrated group in comparsion to control (Figure 4.41A).

A)



B)

Control NVD treated Caspase 3



C)

Control FH535 treated caspase 3

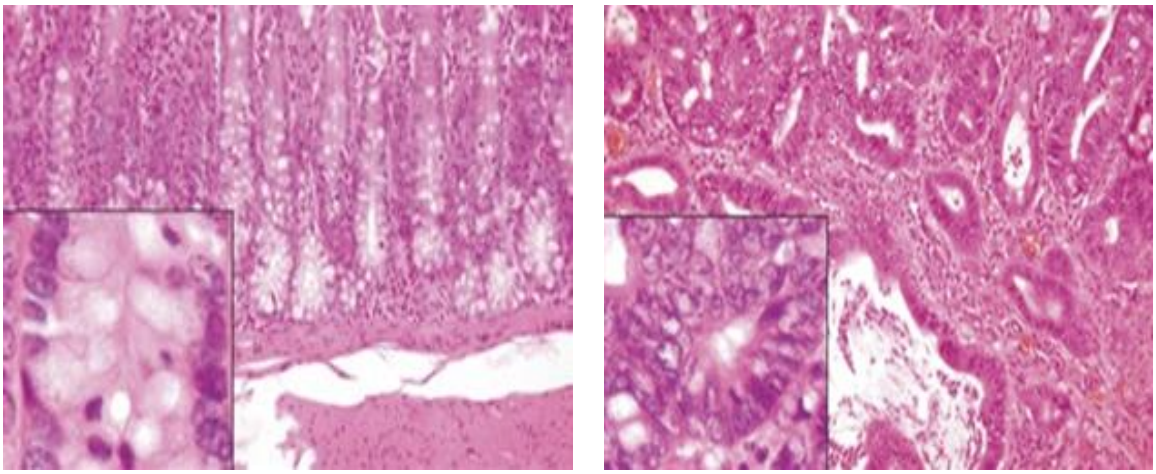
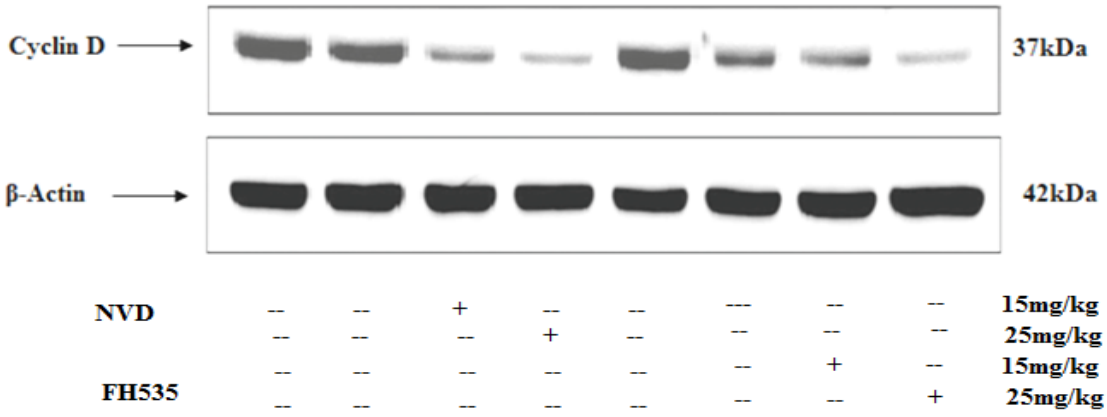


Figure4. 41: NVD and FH535 administration induces apoptosis in HCT116 xenograft tumors in athymic nude mice. a) Effect of NVD and FH535 treatment (15 and 25mg/kg) on protein expression of cleaved Caspase 3 of HCT116 implanted xenografts tumors in athymic nude mice. b) and c) Immunohistochemistry of cleaved Caspase 3 of NVD and FH535 administrated tumor xenograft vs untreated control tumor xenografts.

4.14.3 NVD and FH535 baskets propagation in xenograft tumors

Cyclins are a category of dogmatic proteins that organize the development of the cell cycle. *Cyclins* make active *cyclin* dependent kinases (CDKs), which manage cell cycle processes through phosphorylation. Dysregulation of cyclin D1 gene expression or function contributes to the loss of normal cell cycle control during tumorigenesis. Immunoblot analysis illustrated down regulation of cyclin D in NVD and FH535 administrated group (25 and 15 mg/kg) as compared to control group. A prominent difference was seen between NVD, FH535 administrated (25 and 15 mg/kg) and control in expression of cyclin D in immunohistochemistry analysis (Figure 4.42 A and B)

A)



B)

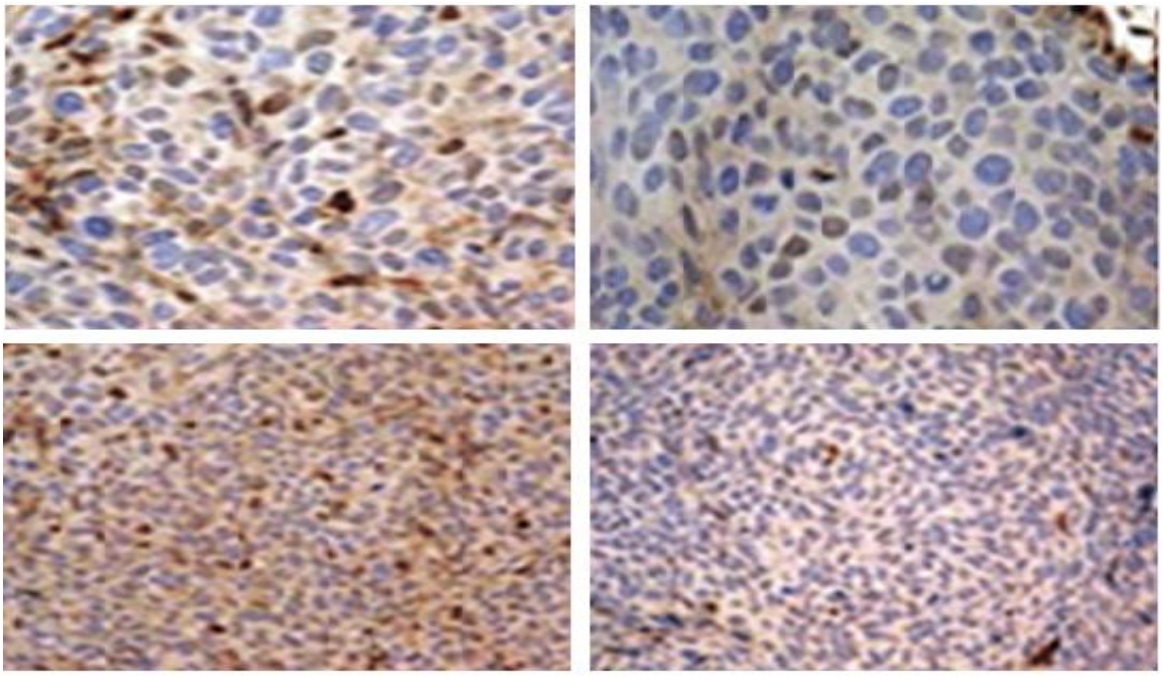


Figure4. 42: NVD and FH535 hamper proliferation in xenograft tumors in athymic nude mice. a) Effect of NVD and FH535 administration (15 and 25 mg/kg) on protein expression of cyclin D of HCT116 implanted xenograft tumors in athymic nude mice. b) Immunohistochemistry of cyclin D tumor xenograft vs untreated control tumor xenografts.

4.14.4 NVD and FH535 administration dwindles β-catenin and survivin expression in xenograft tumors

Effect of NVD and FH535 (15 and 25mg/kg) on β-catenin and survivin expression in HCT116 xenograft tumors was corroborated by immunobloting, immunohistochemical staining and RT-PCR. An outstanding sink in expression was seen. The xenograft tumors (HCT116) revealed inclined levels of β-catenin protein. The dose dependent effect of NVD and FH535 on HCT116 xenograft tumors demonstrated an outstanding sink in β-catenin and survivin protein levels at 25 and 15 mg/kg doses (Figure 4.43A). Immunohistochemical staining of HCT116 xenograft tumors in athymic nude mice illustrated decline in β-catenin expression in NVD and FH535 administrated as compared to control ones (Figure 4.43B). To explore, either the observed decline in β-catenin protein was owing to decreased transcription of β-*catenin* gene, alteration of *β-catenin* expression by NVD induction in HCT116 xenograft, a prominent decline in mRNA expression by employing RT-PCR, was seen to be in concentration dependent approach. At 15 and 25mg/kg of NVD a momentous reduction in *β- catenin* expression was seen (Figure4.43C).

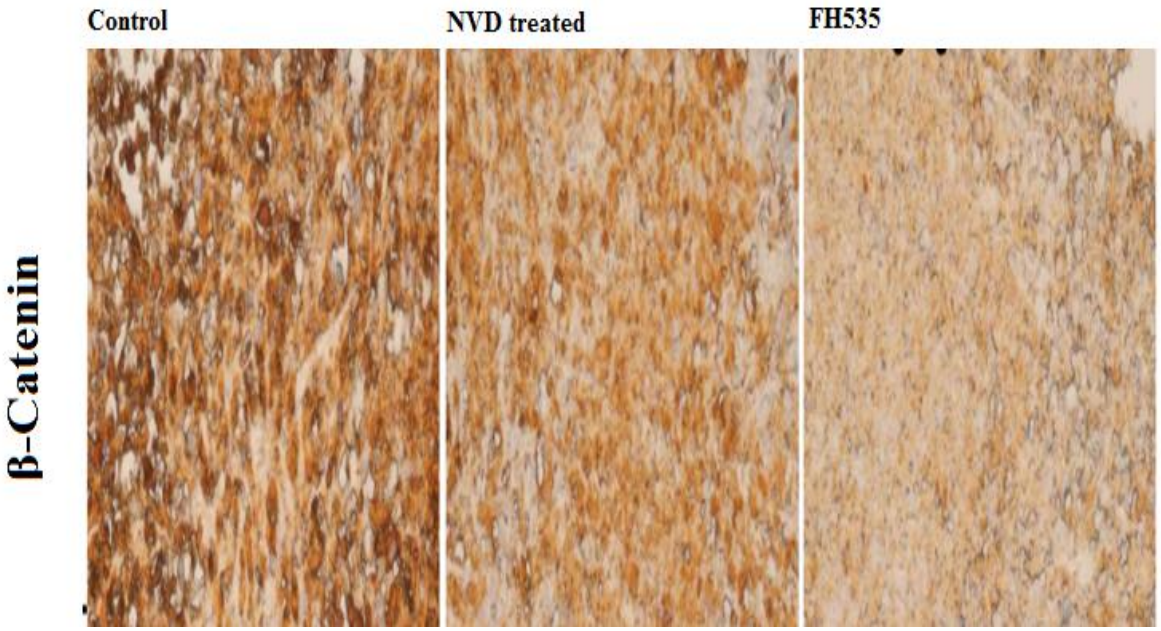
4.14.5 NVD and FH535 inhibits Akt phosphorylation in HCT116 xenografts in athymic nude mice

β-Catenin and Akt signaling corridors are vital watchdog in cell proliferation, differentiation and growth. AKT phosphorylation encourages *β-catenin* transcriptional activity. Therefore, AKT and β-Catenin association reveals a dynamic role in tumor progression and invasion. The HCT116 xenografts administrated with NVD and FH535 in a dose dependent approach buckets AKT phosphoryalation. Immunoblotting showed down regulation of AKT protein expression by administration of NVD and FH535 (15 and 25mg/kg), which inturn hampers proliferation. The control and treated groups illustrated prominent differences in AKT protein expression (Figure4.43A).

A)


AKT
60kDa
Survivin
16kDa
β-catenin
92kDa
β-Actin
42kDa
NVD
FH535
15mg/kg
25mg/kg
15mg/kg
25mg/kg

B)


Control
NVD treated
FH535
β-Catenin

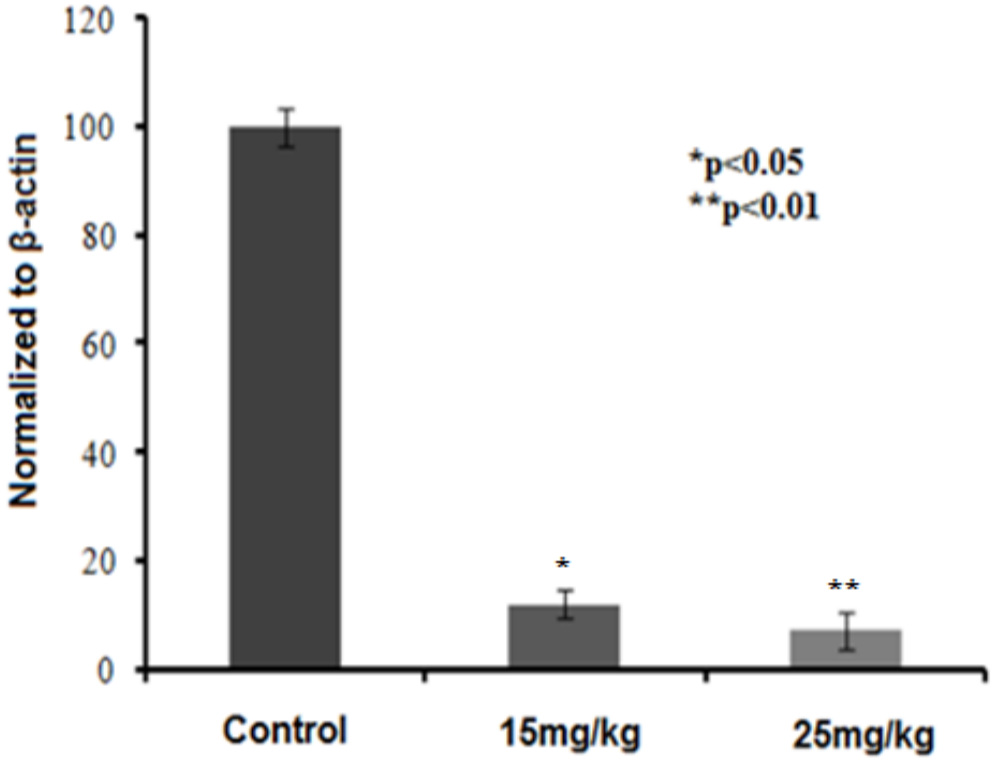


Figure4. 43: Effect of NVD and FH535 administrated on HCT116 xenograft tumors for protein expression a). Expression of Akt, β-Catenin and Survivin protein by immunobloting in taxifolin administrated & control group, experiment performed in triplicate. (b). Effect of NVD and FH535 administrated on protein expression of β-Catenin and control as detected by immunohistochemical staining.c). Effect of NVD on expression of mRNA of β-catenin byRT-PCR.

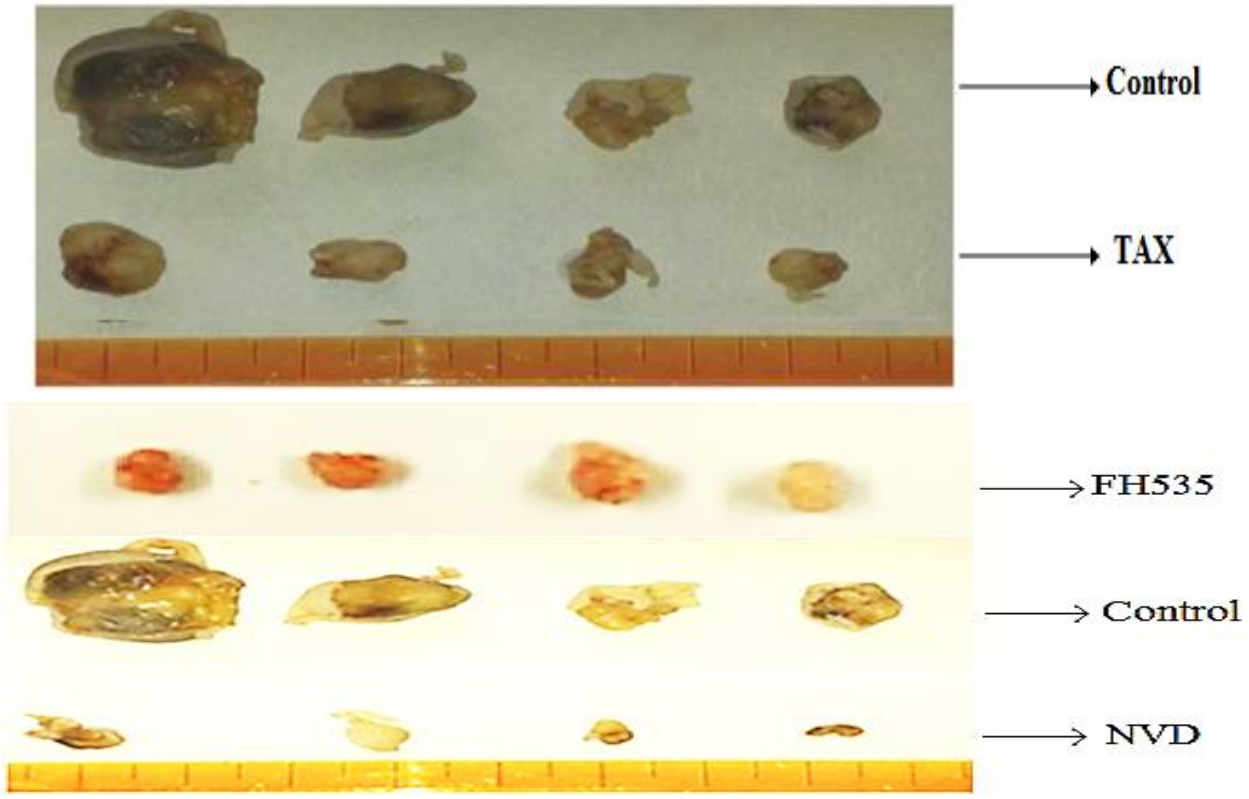


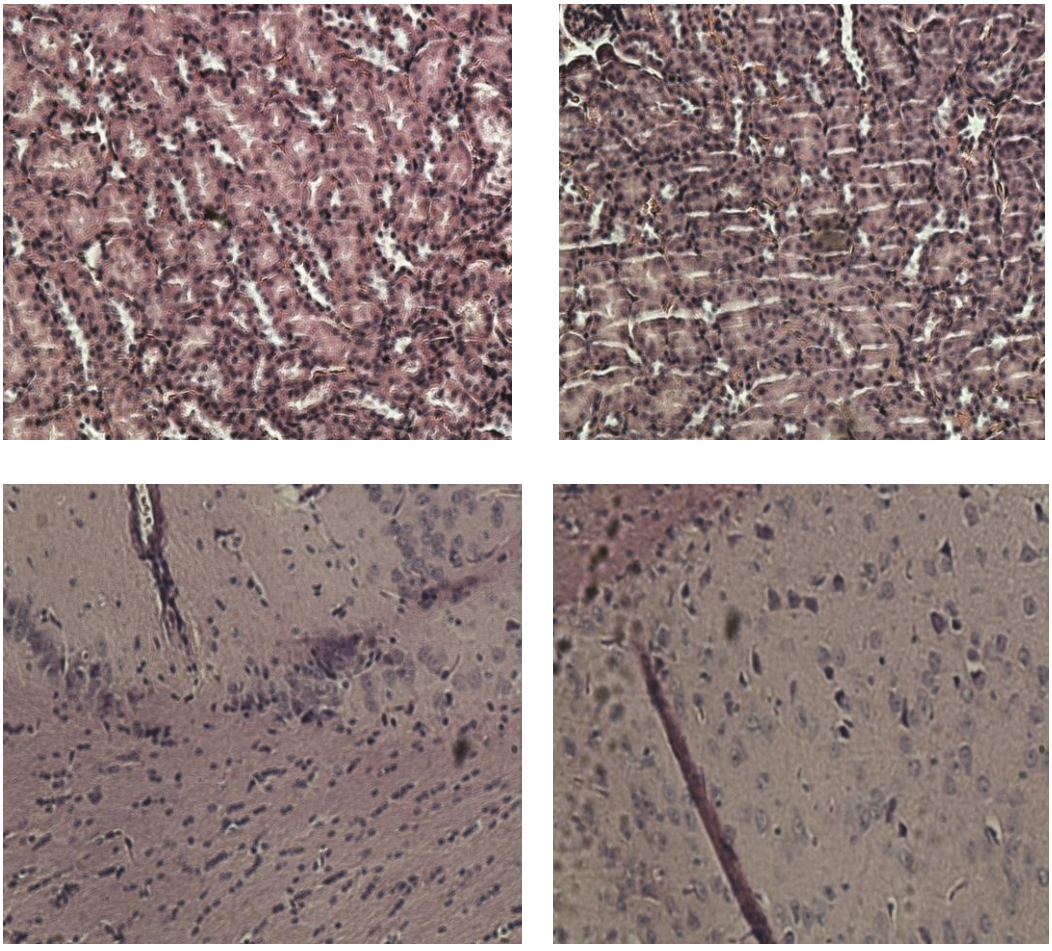
Figure4. 44: The inhibition of xenograft tumor growth of human HCT116 by FH535, TAX and NVD.

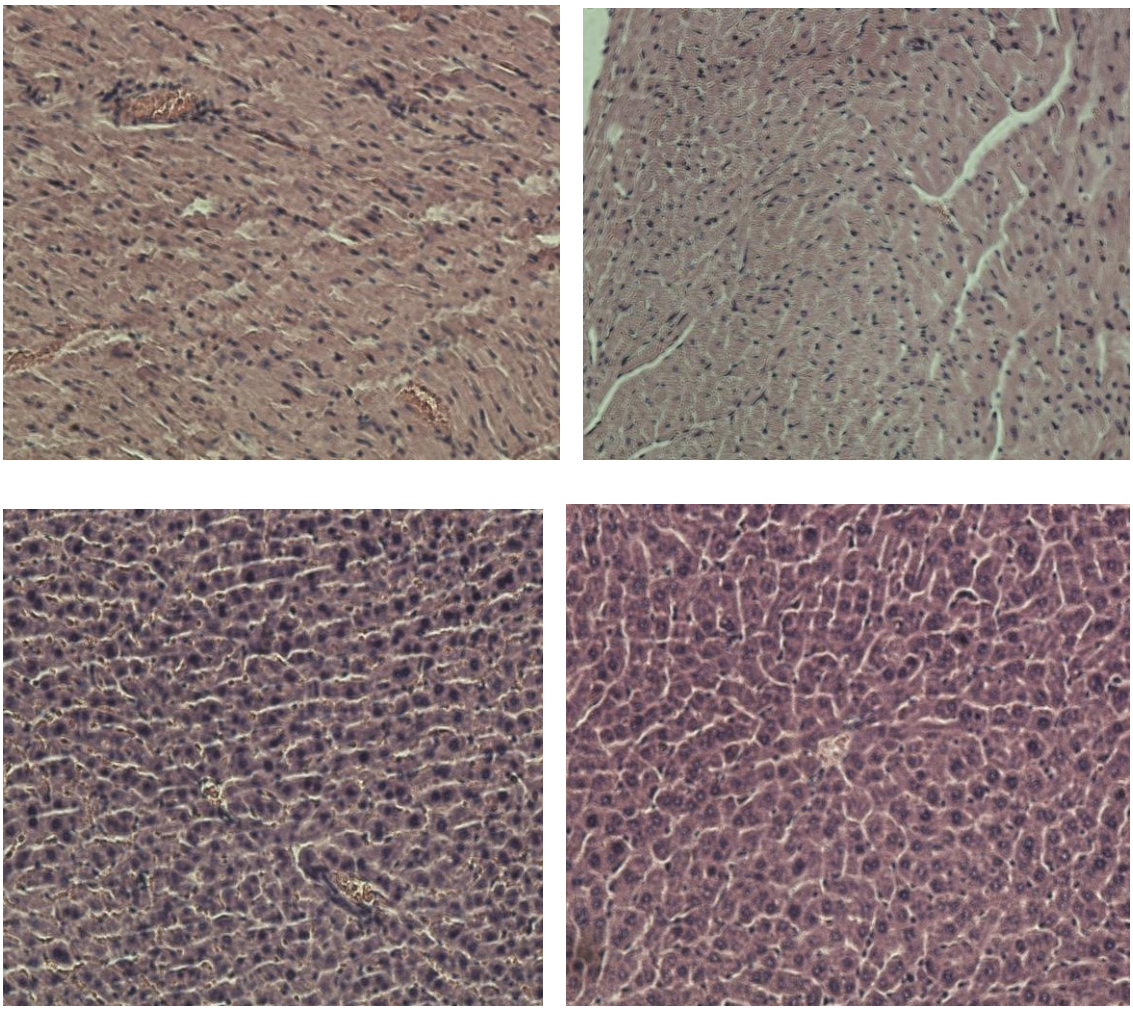
4.14.6 TAX and NVD administration results no adverse effect on other tissues

In view of the fact that harmfulness of the administrated compounds was a chief reflection, body weights were documented two times a week to appraise the overall wellbeing and safety of mice during administration. (Figure 4.45) shows no major weight transformations in the administrated in comparsion to the normal groups. Furthermore, the subjects exhibited zero signs of unrest throughout the administration routine. The histopathological assessment of the tissues of kidneys, brain, heart, and lung from TAX, & NVD administrated mice and vehicle group shown no detectable differences in approach (Figure 4.45). Veto signs of toxicity, precise to taxifolin and NVD were spotted

in the appendages by the pathologist (Pathologist Report). Conversely, in liver, mild inflammation suggestive of peritonitis was seen in some mice from both control and compound administrated groups. Cooperatively, the statistics engendered since xenograft studies stalwartly proposed initiation of vigorous apoptosis allied with malignant growth reticence as well as repressed wnt/β-Catenin cascade in TAX and NVD administrated mice with no contrary effects allied with the administrated.

Control **Treated**





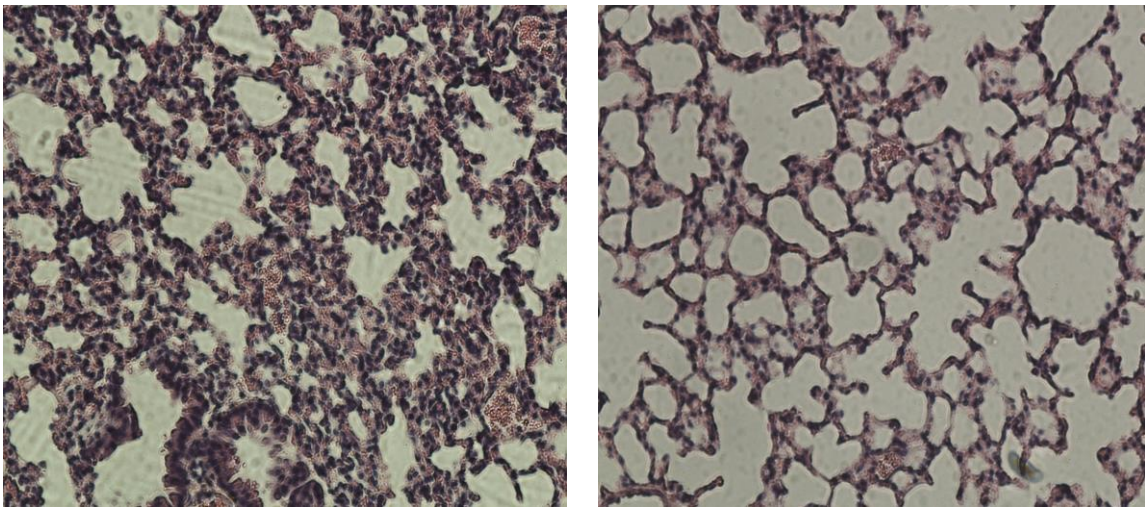


Figure4. 45: "Mice weight was taken twice weekly and values represent mean±SD of six mice. **, $p < 0.02$ (25 mg/kg),*, $p < 0.01$ (15mg/kg) vs control group;**, $p < 0.001$. (6C) H&E staining of kidney brain, heart, liver and lung of TAX and NVD treated mice vs. control for toxicity studies."

4.15 Report generated by pathologist

Dr. Abdul Malik Al Sheikh (Pathologist), MD, FRCPC

Table4. 11: Slide review: Preliminary observations

Organ	Control	Taxifolin and NVD treated
Liver	1. Minimal randomly scattered mononuclear and supportive hepatitis 2. Minimal lymphoplasmacytic and histolytic portal hepatitis. 3. Sinusoidal brown pigment accumulation interpreted as probable artifact of red blood cell staining.	1. Capsular fibrosis and mild chronic mononuclear and mildly suppurative inflammation (suggestive of peritonitis). 2. Minimal mononuclear portal hepatitis. 3. Mildly enhanced hepatocellular mitotic rate, presumptive. 4. Locally extensive moderate accumulation of pigment laden macrophages/Kupffer cells 6. Minimal extra-medullary hematopoiesis
Brain	Extensive dark neuron artifact interpreted as an artifact of dissection. There are extremely rare mild extravasations of blood into Virchow-Robbins' space. The habenular nuclei have a mesh-work of cells (presumptive neurons) with smudged nuclear features. Diagnoses: 1. Locally extensive nuclear smudging in the habenular nuclei (a finding of uncertain significance). 2. Minimal extravasations of blood into Virchow-Robbins' space.	There is fairly extensive dark neuron artifact (presumptive secondary to dissection).
Heart	There is rare individual cardiac myocytes with increased cytoplasmic eosinophilia and bland darkly staining contracted nuclei. Diagnoses: 1. Minimal individual myocytes change, interpreted as probable degerative change.	There are rare individual cardiac myocytes with slightly more darkly eosinophilic cytoplasm than neighboring cells and with more homogenous and darkly eosinophilic chromatin staining in contracted and shrunken nuclei. Diagnoses: 1. Minimal individual myocytes change, interpreted as probable degerative change.

Kidney	No significant histological lesions are noted.	The renal capsule is segmentally broadened with fibrous connective tissue that is occasionally infiltrated with small numbers of mononuclear leukocytes and neutrophils. Diagnoses: 1. Capsular fibrosis and mild chronic mononuclear and mildly suppurative inflammation (suggestive of peritonitis)
Lung	Neutrophils are moderately numerous percolating through alveolar septal walls and occasionally within alveolar air spaces. Occasional alveolar pneumocytes have expansive cytoplasm. The pulmonary parenchyma is multifocally collapsed, presumed secondary to dissection technique. Diagnoses: 1. Pneumonitis, suppurative, moderate	Alveolar air spaces in some areas are mildly collapsed presumed secondary to dissection technique. There are rare megakaryocytes in the pulmonary parenchyma (EMH). There is scant alveolar hemorrhage presumed secondary to euthanasia. Neutrophils are in mildly enhanced numbers in alveolar air spaces and septal walls in a few scattered regions. The tip of the lung lobe has a focal accumulation of slightly increased numbers of foamy macrophages on alveolar septal walls and occasionally in alveoli. Uncommonly, pneumocytes lining alveolar septal walls have expanded cytoplasm (pneumocyte hypertrophy). There is one focus of perivascular lymphoid cuffing at the tip of one lung lobe. Diagnoses: 1. Pneumonitis, mild, suppurative 2. Extramedullary hematopoiesis (EMH), mild

Chapter 5
Discussion

5. Discussion

5.1 Screening and computational analysis of colorectal cancer associated non-synonymous polymorphism in Pakistani population

Colorectal cancer (CRC) is recognized to be the accumulative consequence of numerous mutations in the cell that permit it to outflow progress regulation as well as monitoring appliances (Xue et al., 2012, Humphries et al., 2013). Studies have proven within the accrual of gene transformations cutting-edge clonal cell effects in the alteration of colorectal carcinoma from the normal colon epithelial cell (van Veelen et al., 2011). In humans, *CTNNBI* gene mapped at 3p22 encodes the beta-catenin protein (van Veelen et al., 2011, Kanczuga-Koda et al., 2014). This protein coordinates gene transcription and cell-cell adhesion (White et al., 2012). The cascade (canonical Wnt signaling) alleviates β-catenin transcription whereas the Wnt effector β-catenin is a transcriptional co-activator that may correspondingly transform towards an effective cancer causing gene. (White et al., 2012, Kanczuga-Koda et al., 2014). The mutations & the overexpression of beta-catenin both are allied with different malignancies, containing ovarian and endometrial carcinomas, lung cancer, colorectal carcinoma, and malevolent breast cancers (Hao et al., 2001).

To our acquaintance, our study is first comprehensive connotation study of *CTNNB1* gene with colorectal cancer patients in the Pakistani populace. In very observation, the incidence of transformations in the *CTNNB1* genes as well as also manifestation of the CTNNB1 protein in tumor tissue of 200 CRC subjects, remained examined. The manifestation of transformations in the *CTNNB1* gene, remained infrequent, merely two of 200 tumors analyzed stayed having a alteration in exon 3 at codon 33 and 41 in colorectal cancer tissues. This substitution resulted in replacing a hydrophilic neutral serine to hydrophobic phenylalanine at amino acid position 33 [TCT (Ser) → TTT (Phe)] and a polar threonine was converted to non-polar alanine at amino acid position 41 [ACC (Thr) → GCC (Ala)] of exon 3 of *CTNNB1* gene. No alteration was seen in

corresponding non-tumorous tissue. Our finding comprehends the previous study carried out by Alomar and colleagues which screened *CTNNB1* gene from Kingdom of Saudi Arabia (KSA) in 60 CRC patients and) in one of the tumor samples, showing an activating mutation (S33F (Alomar et al., 2016). Our observations also investigated the grade of β-catenin protein of the *CTNNB1* gene in CRC in Pakistani population. Strong increases of cytoplasmic as well as nuclear β-catenin quantity in the malicious cells of two of the 200 examined subjects were seen when compared with normal adjacent tissue. Our results proposed a conceivable part of β-catenin accumulation due to the S33F and T41A mutations in the pathogenesis of colorectal cancer in two of the subjects involved within current study. Similar observation was reported by Michiko and colleagues revealing the accretion of β-catenin in nucleus in CRC (Iwamoto et al., 2000).

CTNNB1 is phosphorylated by GSK3 involves a priming kinase that performs on a four threonine (T)/serine(S) (S33, S37, T41, and S45) amino acid C-terminal towards the phosphorylation site of GSK3, & allow additional phosphorylation by GSK3 (ter Haar et al., 2001). These phospho-S/T residues are vital for β -catenin detection concluded the F box protein β-Trcp, which is most important player of ubiquitination device (Winston et al., 1999, Kitagawa et al., 1999, Hart et al., 1999, Latres et al., 1999, Liu et al., 1999). The importance of S33 S37, T41, and S45 phosphorylation in β-catenin deprivation is emphasized by the surveillance that mutations at these S/T residues recurrently arise in human CRC as well as numerous additional menaces, which are allied with as well as utmost prospectively occurred by the decontrolled accrual of β–catenin (Morin et al., 1997, Korinek et al., 1997, Rubinfeld et al., 1997, Polakis, 2000). Through our *in silico* deep structural analysis, we mapped docking sites of GSK3 and TrCP1 with N-terminus of CTNNB1 which clearly revealed interaction of GSK3 and TrCP1 and putative phosphorylation motif of CTNNB1. We are tempting to speculate that our findings open a room for cancer researcher through a functional interplay between CTNNB1, GSK3, and TrCP1. Molecular docking and dynamic simulation analysis of an interaction of

GSK3 with CTNNB1^{WT} and CTNNB1$^{MT(S33F\ and\ T41A)}$ revealed that because of mutation in CTNNB1 binding of GSK3 was eliminated. Comparatively, docking simulation of TrCP1 with CTNNB1^{WT} destruction motif and CTNNB1$^{MT(S33F\ and\ T41A)}$ destruction motif revealed that owing to a mutation in the phosphorylation point of destruction motif CTNNB1, its binding within the narrow channel of TrCP1 was abolished. The intertwined relationship of CTNNB1, GSK3, and TrCP1 could be a novel and interesting area for cancer therapeutic development.

5.2 Reproductive factors and Vitamin D Analysis

The existing observation assessed Vitamin D concentrations in serum of 200 colorectal cancer (CRC) patients. The sample comprised of 122 males & 78 females with mean age of 55.8±6.9 and 54.5±14.6 years, respectively. The present study reports main three findings:

5.2.1 Age and gender as determinant of hormone levels:

When subjects were divided in young males and female groups, in the serum concentrations of hormone levels no significant difference was pragmatic (p, for all trends>0.05; Table 2). However, substantial variances were observed in the serum meditations of old male and female (p, for all trends <0.05; Table 2).

5.2.2 Age, gender, site of cancer, as determines of serum 25-OH Vitamin D oncentrations:

Findings of the present study show that mean 25-OH vitamin D intensities within overall patients were 18.8±9.11 ng/ml. 25-OH VD grade was classified into two classes “very low” and “low to normal.” The “very low” category was characterized as ≤16 ng/ml and the “low to normal” category was described at >16 ng/ml (Fakih et al., 2009). The other purpose for selecting this threshold was that 25-OH VD intensities of our subjects were within these ranges. Regression analysis using univariate model show age, gender, site of cancer, and stage of disease were found to be associated with the disease. Age, gender, site of cancer, and stage of disease were found to be 2.42. 1.87, 1.67 and 2.44 times more

likely to be associated with low 25-OH VD (p, for all trends<0.05; Table 3). However, multivariate logistic regression results show that only stage of disease (OR: 1.94; CI: 1.07-3.56) was significantly allied with low 25-OH VD (p=0.031; Table 4)."

5.2.3 Prominent relation among serum hormones & 25 (OH) D Intensity:

Some previous reports have shown that amplified intensity of testosterone and estrogen, in males and females are confidently related with serum 25 (OH) D intensities (Parikh et al., 2010, Wehr et al., 2010, Pilz et al., 2011). We, thus, analyzed distribution pattern of sex hormones and 25-OH vitamin D intensities comparative to the stage of CRC (Figure 1 and 2). Serum 25 (OH) D levels exhibited optimisticly allied through inclined intensity of estrogen and testosterone in males as well as females (Parikh et al., 2010, Wehr et al., 2010, Pilz et al., 2011). Estrogen unswervingly controls hepatic hepcidin expression via a functional estrogen retort constituent of the hepcidin gene (in the promoter region) (Hou et al., 2012). In pre-menopausal females, particularly, 17β-estradiol boosts iron absorption to recompense for iron deficiency throughout menstruation (Yang et al., 2012). Nevertheless, postmenopausal females show an iginited decline of estrogens occured due to menopause. Consequently, regardless of the raise in vitamin D concentrations in serum, the ferritin intensity might be not expressively diverse in postmenopausal females. Though, an observation proposed that testosterone may boost ferritin by hampering hepcidin in males (Bachman et al., 2010).

5.2.4 Effects of reproductive characteristics on 25-OH VD concentrations

We also analyzed some selected reproductive factors as predictors of 25-OH VD quantities (Table 6). High 25-OH VD concentrations were observed in females who had their menarche at the age 15 years or more. Nulliparous women had the highest mean 25-OH VD concentrations as compared to unparious or multiparious women. Women having their menopause at 40-44 years of age had the highest 25-OH VD concentrations, even if the variation was not noteworthy (p=0.08). Women who never used any oral contraceptive had higher 25-OH VD concentrations as compared to those who ever used

oral contraceptives. Furthermore, it is interesting that these differences were more profound when the female patients were stratified into two classed based on their hormone therapy i.e. ever use hormone therapy and never use hormone therapy (Table 6). So for example, women having menarche at age 15 years or more and who ever used hormone therapy had significantly higher 25-OH VD concentrations as compared to women who had menarche <15 years of age with either ever use hormone therapy or with never use hormone therapy (p, for all trends<0.05) in the same way being nulliparous and parity (Table 6).

Initial menarche is allied to amplified jeopardy of unfavorable health consequences throughout adulthood including type 2 diabetes (Van Lenthe et al., 1996), obesity (He et al., 2009), breast and endometrial cancers (Lakshman et al., 2009), and (Hsieh et al., 1990) cardiovascular disease (Dossus et al., 2010). Women with later onset of menarche demonstrate significantly decreased peak bone mass (Armamento-Villareal et al., 1992), and bone mass and bone density at skeletal maturity exhibit an inverse relationship with pubertal timing in healthy adolescents (Gilsanz et al., 2011). Advanced age of menarche has also been allied with increased fracture risk (Cooper and Sandler, 1997). In this study, we observed mean lower 25-OH VD concentrations in women having their menarche age <10 years or >15 years (Table 5). For example phase at menopause and menarche, being gauge of the period of revelation towards cyclic ovarian utility, & several (Apter et al., 1989, Macmahon et al., 1982), although not the entire (Trichopoulos et al., 1987) observations have illustrated an contrary association related to the circulating estrogen level and age at menarche. Pregnant women and women breast-feeding for long periods of time are at elevated jeopardy of hypovitaminosis D (Arya et al., 2004, van der Meer et al., 2006, Eagleton and Judkins, 2006). Advanced age at menopause is an recognized jeopardy aspect for the elevated figure of ovulatory cycles as well as amplified estrogen revelation allied with advanced menopause has been theorized to constrain respective association (Chavez-MacGregor et al., 2005, Kelsey et al., 1993).

The mean age of menopause in Pakistan has been reported that varied greatly i.e. 44.5 years (Adhi et al., 2007) to 47-49 years (Yahya and Rehan, 2002, Wasti et al., 1993). Similarly, age of menarche in Pakistani women is also variable (Asif et al., 2017) and has profound health outcomes. These are important factors and may have significant effects on 25-OH VD concentrations and overall health of women both healthy and those suffering from chronic diseases like CRC.

5.3 In vitro Analysis

The assorted molecular outlines of colorectal cancer and the necessity to categorize subjects which could efficiently receive medical benefit from merged chemotherapies ignited the categorization of the systems accountable for compassion and confrontation to therapies. The preponderance of sporadic types of colorectal cancer is distinguished through dysfunction of Wnt/*β*-catenincascade effecting in amplified transcriptional bustle of the *β-catenin*. Regardless of the complexities towards scrutinizing the association of Wnt/*β*-Catenin cascade in the commencement & succession of colorectal carcimona, the exploration of these systems is at the present rising as a capable platform to recognize budding objectives of intrusion for colorectal cancer therapy.

HCT116 and HT29 cells consequent from human colorectal adenocarcinoma at the metastatic stage are a universally used cell model for advanced metastatic colorectal cancer, which presently have no efficient alleviate. A perfect therapeutic loom is to develop drugs that are confined to aim tumors while scanting normal tissues.

Innumerable innate compounds have been proposed to show activity against different tumors or minimize the adverse consequences of supplementary anti-cancer therapeutic drugs by escalating the salutary consequences of the imperative anti-cancer agent or act as "enhancers" (Boik, 1995, Borchers, 2002). Flavonoids have been authenticated to wield a multiplicity of therapeutic effectiveness via hampering the cell cycle, receding oxidative trauma, supplementing the competence of enzymes detoxification, persuading exciting the immune functions and apoptosis. These intrinsic possessions of flavonoids

arrange them as a category of expensive amalgams which clutch physical conditions and ailment-thwarting nutritional value, together with usefulness in cancer prevention (Thiery-Vuillemin et al., 2005, Yang et al., 2001).

TAX is a flavanonol imitative of flavonoids, bountiful in herbs as well as foods (Drobek-Słowik et al., 2007). It flaunts a broad assortment of bioactivities, among which the antioxidant bustle is quite idiosyncratic (Topal et al., 2016). Frequent observations have substantiated taxifolin exhibits valuable chemopreventive function on colon cancer malagnacy , however the detailed mechanism against anti cancer activity is still curtained (Manigandan et al., 2014) . Several observation have illustrated the the salutary performances of taxifolin cramped on in vivo as well as in silico-arbitrated tone of Nrf2, wnt/β-catenin and inflammatory signaling cascade (Manigandan et al., 2015). It has been revealed that taxifolin defend RPE cells aligned with oxidative strain by hampering the H_2O_2- tempted reduction in cell apoptosis, cell viability, & the intracellular production of reactive oxygen species. The effective system emerges to engage the commencement of NRF2 as well as the upsurge of the stage second antioxidant enzyme classification (Xie et al., 2017). Vitamin D has been scrutinized preclinically for its salutary prospective in anticancer activity and chemopreventive. Earlier an observation on *Nkx3-1;Pten* mutant mice revealed reiterate prostate metastatis. Also revealed that vitamin D treatment deferred the commencement of prostate intraepithelial neoplasias (PIN). Further it has been illustrtaed that vitamin D had potential anti-tumour function while treated to mice with premature-phase (PIN) relatively compared to complex-phase prostate disease (Banach-Petrosky et al., 2006).

Moreover, the observations working with prostate cancer, breast cancer squamous cell carcinoma (SCC), & lung tumor illustrated that vitamin D & its vitamin D analogues, showed a prominent activity against cancer. This effect of vitamin D along with its derviatives, acts in the course of the VDR to control propagation as well as apoptosis. The study related to SCC cells revealed that treatment of vitamin D encourages G0/G1

cell-cycle arrest because of the transcriptional commencement of CDKN1B subsequent pRb hypophosphorylation (McElwain et al., 1995, Zhang et al., 2005, Nakagawa et al., 2005, Wang and Studzinski, 2001, Hershberger et al., 1999).

Reports revealed that treatment of vitamin D decreases BCL2 expression in breastcancer cell lines (MCF7) and HL-60 leukaemia cells and increase protein expression of bak and bax in CRC, prostate cancer, and carcinoma cells. Also vitamin D has shown to manage protein expression of BCL2 family, illustrating that Vitamin D, may trigger caspase effector fragments (Ylikomi et al., 2002). Keeping all effects of VD in view we formulated protein-vitamin D-pectin nanocomplexes (NVD) to explore its anticancer activity.

Cellular propagation consequential in tumor pattern may occur owing to alteration in cell cycle control (Gupta et al., 2002, Adhami et al., 2003). A central principal source of cancer succession is ascribed to speedy and unsubdued propagation consequences to series and expansion of tissue accrual. Data of MMT assay specified that taxifolin and NVD are definite in their activity. Also are efficient against cell cancer cell lines from different derivation. Cell growth in both HT29 and HCT116 cells is distorted by taxifolin as well as by NVD. Treatment with taxifolin and NVD consequences in detain of cellular proliferation in a concentration reliant mode, magnification in trouncing of cell viability was experiential with amplify in the meditation of dose.

Apoptosis is a sovereign incident to apposite unambiguous constituents of cells while shunning inflammatory effect typically shepherding necrosis, hence no reproachful result to the contiguous healthy cells will take place in the interim the cells undergoes apoptosis. Therefore apoptosis is an experience ratifying chemotherapy competence, alleged as an endpoint of anticancer drug therapy (Enari et al., 1998, van Loo et al., 2002).

In eukaryotes, channel during the cell cycle is synchronized through the members of protein kinase intricates, encompassing of a catalytic subunit, the cyclin reliant kinase

plus of triggering correlate, cyclin (Baillon and Basler, 2014, Sánchez and Dynlacht, 2005). Alliance of cyclins D & B1 & cdks 2, 4 & 6 in an authorized cell expansion situation results RB phosphorylation and its discharge commencing E2F while consequences within series of the cell cycle as well as cellular expansion (Baillon and Basler, 2014, Solá et al., 2013, Santamaria and Ortega, 2006). As soon as DNA damage is sustained in propagating cells, by cyclin kinase inhibitors, cell cycle succession is impeded via the reticence of CDKs. The cyclin E–Cdk2 and cyclin kinase inhibitor p21 get allied to intiate G1/S arrest, with cyclin A–CDK2 to cause S phase impedence and with cyclin A–CDC2 to begin G2/M arrest (Morgan, 1997, Roberts, 1999). The in vitro study illustrated that the N-terminal of p21 contains 1–82 amino acids, is vital on behalf of the discretion of cyclin–CDK, and participation with cyclin E, cyclin A, or CDK2 in an detached appearance or an complicated structure (Fotedar et al., 1996). The studies demonstrated that p21 cronies with cyclin E via the Cy motif while there is no association with cyclin A (17) and also observations signifying that the p21 treated with anti-Cy motif antibody coprecipitated with cyclin A–CDK2 and that Cy-deleted mutant-p21 precipitated the cyclin A–CDK2. The communiqué of the p21-linked CDK inhibitor, cyclin A, as well as p27 is arbitrated by an RNLFG series in p27 that attaches with a hydrophobic groove on the exterior of cyclin A (Schulman et al., 1998). While p27 and p21, as affiliates of the Kip/Cip family, have mutual configurations and activities, may vary in mechanisms for cell cycle inhibition. In the present study, appraisal of apoptosis encouraged in HT 29 & HCT116 cells exemplified that taxifolin as well as NVD are incredibly competent inducers of apoptosis in dose reliant approach. Results of our study illustrated down regulated expression of Cyclin A, B1, D1, & E & Cdk-2, 4 and 6 and escort through an upregulation in expression of cdk inhibitors WAF1/p21 as well as KIP1/p27 within taxifolin and NVD administrated cells. In addition, taxifolin and NVD administrated CRC cells demonstrated apprehend in the cell cycle (G2 phase) These results are momentous since cell cycle directive is a fundamental intention for expectancy

beside CRC. Zhang et al., (2013) exemplified that taxifolin boosts the anti-proliferation and apoptotic effects of Andrographolide. Also recommended that taxifolin might perform as an activator in cell death of DU145 cells and andro-induced cell cycle arrest (Zhang et al., 2013), but our dataillustrated that taxifolin unaided persuaded cell death of HT29 and HCT116 cells and cell-cycle arrest.

Dysregulated cell cycle, through consequent effect on players of propagative regulator for instance cell cycle checkpoints plus the retort to DNA mutilation being merely element of the predicament within malignancy management (Kasibhatla and Tseng, 2003). Modern explorations in the class of cancer biology have expanded our consideration to cover deviant cellular continued existence and malfunction to tempt cell deathor apoptosis, equally a most important element towards the converted state. In favor of that purpose, numerous modern chemotherapeutic policy are intended to prompt tumor-selective cell death with partial negative consequence to standard cell activity (Khan et al., 2014).

Poly ADP ribose polymerase, an emblematic caspase substrate, is a decisive participant of DNA repair against conservational strain plus in the persistence of cell viability. Cleavage of PARP is measured as a promise of apoptosis (Oliver et al., 1998). PARP cleavage ensues synchronizely through cleavage of procaspase 3, 7, & 9 in a dose-reliant approach, signifying that taxifolin and NVD persuaded apoptosis in HT29 and HCT116 cells is mediated in the course of a fundamental apoptosis cascade.

Several observations have demonstrated that Poly ADP ribose polymerase-1 is overexpressed in diverse human tumors (Bièche et al., 1996, Shimizu et al., 2004, Ghabreau et al., 2004). Additionally, it was exhibited that PARP-1 take crucial part in colon malignancy (Idogawa et al., 2005, Nosho et al., 2006), subsequently respective expression was noticeably elevated in colon cancer hence was allied through tumor amount as well as histopathology (Nosho et al., 2006). Above data reinforced our results

screening elevated PARP expression in taxifolin as well as in NVD treated cells than control.

Bcl-2 family associates are the chief watchdogs of apoptosis. Their overexpression stalwartly hampers apoptosis due to cytotoxic injuries by abolition of free radicals, deterrence of mitochondrial canal formation, and the liberation of c cytochrome (Borner, 2003, Kroemer and Reed, 2000). Bcl-2 known as an suppressor of apoptosis which is upstream effector in the cell death network are exceedingly showing expression in a preponderance of human cancers. A heterodimer intricate is formed by Bcl-2 and Bax thus neutralizing the proapoptotic effects of the latter (Oltersdorf et al., 2005). Consequently, the expression intensity of these apoptotic proteins was assessed in this study. In the anti-apoptotic subfamily, Bcl-xL and Bcl-2, showed decreased expression in TAX and NVD administrated cells as compared to control cells in concentration dependent approach. In contrast, the expression of Bax and Bak, pro-apoptotic proteins, showed increased expression in TAX as well as NVD treated HCT116 and HT29 cells as compared to control cells. Our results proposed that TAX and NVD arbitrated amplify in the expression of Bax and decrease of Bcl2 expression might be a promising direction through which TAX and NVD encourages apoptosis in CRC. Additionally, TAX and NVD have showed potential to aim Bak and Bcl-xl signifying its pleiotropic consequence on the apoptotic beckoning corridor.

The canonical Wnt/ β-catenin, in progression, stem cell continuance & tissue revival, beckoning corridor is considered crucial. Similarly, improved signaling through this corridor is allied with the succession of multiple malignances and expansion (Beachy et al., 2004). Commencement of the corridor is allied to cytosolic alleviation, hypophosphorylation, and growth of β-catenin in the nucleus pursued through amplified candidate gene expression (Heeg-Truesdell and LaBonne, 2006, Kimelman and Xu, 2006b). Increased cytosolic β-catenin intensities as well as consequent nuclear import authorize its union with Tcf/Lef family, organizing the genes expression, together with

surviving, cyclin D1, and vegf, entirely contributing to tumor development (Altieri, 2004, Neri and Bicknell, 2005, Nagy et al., 2007, Kerbel, 2008). CK2 was proposed the component of a multifaceted protein that certainly standardizes the Wnt/ β-catenin corridor as well as play a vital role in phosphorylation of β-catenin, consequentially boosts its transcriptional activity owing possibly to an improved cytoplasmic stability (Song et al., 2000, Song et al., 2003). Catalytic subunit of CK2α has been illustrated by studies that it only appears to be accountable of the β-catenin standardization, subsequently CK2b showed pragmatic effect with β-catenin association. Additionally, β-catenin shows increased expression in malignant transfected merely through CK2α, as wellas the carcinomic latent of CK2α declines while co-transfected through CK2b in 3T3 and CHO cells (Li et al., 1999, Pinna, 2002). Our results showed decreased expression of Ck2 after administration of NVD to HT29 cells in a concentration and time reliant approach, whereas, in HCT116 cells, NVD did not showed down regulation of CK2 protein expression representing that the targeting of CK2 signal seemed to be different in both cell lines. No direct inhibition of CK2α protein expression in HCT16 and HT29 cells by administration of TAX in a time dose reliant behaviour. Studies illustrated that phosphorylation of β-catenin through AKT at Ser552, results to its amplified transcriptional bustle (Fang et al., 2007) and also revealed that over expression of constitutively dynamic as well as a central disparaging type of AKT obligated analogous consequences to originate with the dominant negative types of CK2α as well as wild-type, correspondingly (Ponce et al., 2011). In this study AKT and β-catenin showed down regulated expression by administration of TAX and NVD in HCT116 and HT29 colorectal cancer cells as compared to control cells. Immunofluorescence staining of HCT116 and HT29 cells demonstrated decline in p-Akt expression and β-catenin at dose of 40 µg/ml of TAX and NVD treated as compared to control. Momentous Alexa fluor staining of p-akt (cytoplasm) of both cell lines (green fluorescence) were pragmatic in control, while the expression of p-Akt as specified by staining was prominently

diminished in TAX and NVD treated cells. Further our findings illustrated that TAX and NVD induction of HCT116 and HT29 cells resulted in apoptosis through inhibition of β-catenin and p-Akt. While FH535 β-catenin inhibitor baskets Akt phosphorylation which inturn reduces β-catenin protein expression. TAX and NVD administration to β-catenin inhibitor induced cells showed the decreased β-catenin expression, sustaining the fact that these alterations are arbitrated by protein kinase B (Akt). Our results sustenance the involvement of AKT in encouraging the β-catenin's transcriptional bustle as well as proposed that CK2a may be involved in this event. Sustaining this involvement, studies have illustrated that CK2 at Ser129 hyper activates AKT by phosphorylation. Captivatingly, transformation of Ser129 to alanine results in a prominent decline in AKT's catalytic activity in vivo in addition, in vitro showed declined phosphorylation of Thr308 (Di Maira et al., 2005).

The results obtained in our study sustenance an appliance of CK2α -reliant control of β-catenin transcriptional bustle that may circumvent the deleterious multifaceted shaped by APC/ GSK3b/ axin, nonetheless it static deems numerous stages of phosphorylation. Phosporylation of β-catenin by CK2 ensues at residue Thr393 with the section of the ARM realm wherever APC interrelates towards encourage deprivation of β-catenin (Song et al., 2000, Song et al., 2003). The phosphorylation may be carried out by CK2α subunit alone and probably in nucleus somewhere the CK2α have been identified and also, remarkably, where β-catenin and APC interrelates to encourage its nuclear export (Fabbro and Henderson, 2003). Our study also supports an appliance of CK2a reliant control of β-catenin cell viability and transcriptional bustle arbitrated by phosphorylation of AKT. Conversely this is an essential nonetheless not satisfactory occurrence for such regulation, since further phosphorylations might be also crucial. Akt being the most crucial downstream effector of CK2 and phosphatidylinositol 3 kinase (PI3-K). Once triggered by phosphorylation, AKT encourages cell endurance through hampering pro-apoptotic proteins (Zhou et al., 2001). The commencement of PI3K/Akt may trigger the

canonical Wnt beckoning via the phosphorylation of GSK-3β by the phosphorylated Akt1/2, jamming the configuration of β-catenin abolishing complex (28). Therefore, the upregulation of PTEN may hamper the canonical Wnt signaling by encouraging the deprivation of β-catenin . While we demonstrated that administration of TAX and NVD to HCT116 and HT29 colon cells reduces the phosphorylation of PI3k, AKT and diminished the protein expression of β-catenin. The recent study revealed that it might not occur commencing the reduced phosphorylation of GSK-3β by PTEN/PI3K/Akt signaling. Since appear prone to alteration of β-catenin in HCT116 cells, the β-catenin could negatively tarnished in this colon cancer cells through the destruction complex (Liu et al., 2014). Further we investigated that administration of TAX and NVD hampered the mRNA expression of β-catenin and consequently we strongly illustrated that TAX and NVD has compelling anti-proliferation bustle in human colon cancer cells, promoting apoptosis and the anti-propagation consequence of TAX and NVD might be arbitrated by PI3K/Akt corridor by jamming Wnt/β-catenin beckoning transduction, by hampering the β-catenin expression respectively.

Because of critical part in crucial cellular courses, comprising the homeostasis, restoration of cells, and growth control. The two signaling pathways, Wnt/β- catenin and RAS-ERK must be firmly synchronized (van Amerongen and Nusse, 2009, Pinto and Clevers, 2005, Anastas and Moon, 2013). Unusual activations may lead to types of cancer including CRC. The communication between RAS/ERK andWnt/β-catenin pathways has been confirmed. Observations illustrated that RAF-1-MEK-ERK pathway is instantaneously triggered by recombinant Wnt3a administration in NIH3T3 and L cells, specifies undeviating relations of the RAF-1-MEK-ERK as well as Wnt/β-catenin pathways (Yun et al., 2005). The Wnt3a triggers ERK and PI3K also. Wnt3a is allied with the cellular proliferation (Kim and Choi, 2007, Kim et al., 2007). Auxiliary numerous observation have been confirmed control of the RAF-1-MEK-ERK signaling torrent by the Wnt/β-catenin beckoning (Jeon et al., 2007a, Jeon et al., 2007b, Park et al.,

2006). Additionally, the cross-talk amongst GSK3β as well as PI3K/AKT/mTOR signaling corridors is allied in the pathogenesis of pancreatic cancer as well as HCC and GSK3β, chief player of Wnt/β-catenin corridor, is controled by PI3K-Akt beckoning cascades (Cervello et al., 2017, Hermida et al., 2017). This observation supports our results illustrating patent reticence of MAPK i.e ERK ½ phosphorylation, that might remain a probable method aimed at NVD mediated cell death and apoptosis in HCT116 and HT29 cells, while TAX showed no effect on MAPK i.e ERK ½ phosphorylation.

Earlier study has proved that overexpressed beta-catenin could actually interrelate with NF-κB indirectly as well as hamper its bustle, signifying a narrative mechanism for β-catenin-mediated oncogenesis; namely, β-catenin hampers NF-κB activity, which may permit cancer cells to flee immune scrutiny and also the study stalwartly proposed that β-catenin is a chief mediator for the crossregulation of NF-κB through the GSK-3β corridor (Deng et al., 2002). The commencement of NFκB entails phosphorylation; results demonstrated that the transcription factor was not triggered due to inhibition of its phosphorylation by NVD which inturn results in hampering of cancer cell endurance. These observations recommended that inactivation of NFκB via inhibition of its phosphorylation at p65 P-Ser529 might among the systems by which NVD induce growth arrest in HCT116 and HT29 cells while no effect was seen with the administration of TAX.

Recently studies confirmed that in colorectal cancer cells, phosphatidylinositide 3-kinase (PI3K) is essential aimed at the substantial collaboration as well as efficient reticence of NF-κB through β-catenin (Liu et al., 2013). Obstruction of PI3K via chemical inhibitors rescinds the configuration of NF-κB and β-catenin protein complexes. Cutting-edge quiescent CRC cells, NF-κB & β-catenin restricted within cytoplasm. The administration through PI3K inhibitor give rise to nuclear transfer of NF-κB. Also membrane retaining of β-catenin. Conversely, remaining veiled even if PI3K unswervingly aids as an association player amongst β-catenin & NF-κB or instead take part in β-catenin-arbitrated

suppression of NF-κB triggered by diverse spurs. Our studies proposed that inhibition of overexpression of β-catenin by administration of TAX and NVD in HCT116 and HT29 results in reduced protein expression of NF-κB and PI3K, hence this may be one of the possible mechanism cell death and apoptosis.

Genetic deviation in the JAK/STAT/SOCS-signaling pathway emerges to be allied with colon as well as rectal cancer risk. The JAK/STAT/SOCS-signaling pathway plays a vital part in immune defense and control of inflammation specified its indispensable association with cytokine signaling. Furthermore, machinery of the pathway, such as STAT3, is engaged in promoting uninhibited cell growth and continued existence in the course of dysregulation of gene expression responsible for apoptosis, cell-cycle regulation, and angiogenesis (Hsieh et al., 2005). JAK1, JAK2, and STAT3 have been allied with colorectal cancer development (Xiong et al., 2008).In addition of commencement of NFκB, there is incentive of assorted other pro-survival pathways during series of cancer, including MAPK (ERK, JNK) and STAT3, usually not hampered by proteasome inhibitor therapy in tumors or cell lines. Any drug affecting numerous pathways might be used as an efficient chemotherapeutic (Steelman et al., 2011). CK2 is also compulsory for cytokine and growth hormone prompted incentive of the JAK-STAT signaling pathway so we assume that inhibition of CK2 catalytic subunit may be accountable for hampering JAK-STAT signaling pathway in colorectal cancer. Conversely, in HT29 cells, JAK2 protein expression was appreciably restrained with prominent alteration in STAT3 phosphorylation, hence we assume that may be a budding system for NVD arbitrated cell death and apoptosis in HT29 cells.

Survivin (BIRC5), an associate of the inhibitor of apoptosis (IAP) protein class, act as delegate anti-apoptotic protein which enhance cancer cell expansion (Lee et al., 2013). Survivin in humans is copiously articulated throughout fetal growth, nevertheless is infrequently there in adult tissues (Ambrosini et al., 1997), though, the majority human cancer cells articulate survivin, including colorectal cancer cells and CRC cells express

upto 68% as reported (Sarela et al., 2000). Studies also revealed that expression of survivin allies with progressive disease, shoddier endurance, and radiation and chemotherapy confrontation (Altieri, 2003, Zaffaroni et al., 2002). Hence, survivin is of growing curiosity equally a probable beneficial target to hamper cancer intensification (Altieri, 2008). Survivin enhances tumor propagation by renovating various significant cell signaling pathways. Our results clearly confirmed a noteworthy inhibition of an anti-apoptotic protein survivin expression in both HT29 and HCT116 cells by administration of TAX as well as NVD. Therefore, TAX and NVD is predictable to offer more influential prospective to restrain metastasisa and tumor incursion, demonstrating as a novel tool for potential future colorectal cancer treatment.

5.4 In vivo validation

We confirmed related finding in *in vivo* system, Athymic mice implanted with HCT116 xenografts with TAX, NVD and FH535 β-catenin inhibitor, administration induced a dose dependent downregulation of β-catenin, survivin and Akt phosphorylation. Akt is crucial element of signaling cascades for cell strength and propagation during growth and series of cancer. Currently, protein appearance of β-catenin, Survivin and FH535 β-catenin inhibitor p-Akt was assessed in TAX, NVD and FH535 administrated against untreated control group.

Our statistics displayed, for the foremost time, the *in vivo* potential of TAX, NVD and FH535 in plummeting the HCT116 xenograft expansion. In tissue samples from xenografts, the decreased β-Catenin expression, and down-regulation of Cyclin D1 and caspase 3 cleavage symbolize an encouraging validation of the TAX, NVD and FH535 potential *in vivo* in tumors inhibition and present adequate direct confirmation aimed at the reticence of the canonical Wnt/*β*-Catenin corridor & β-Catenin candidate genes by TAX, NVD and FH535 in human CRC. Results demonstrated stimulation of p-Akt protein and cyclin D expression in control group as compared to both groups encouraged with TAX, NVD and FH535 administration. The decline in protein expression is in

support of reticence of propagation in groups by TAX, NVD and FH535 administration. Manifest discrepancy was present between expressions of p-Akt and cyclin D in control verses TAX, NVD and FH535 administrated group when checked by staining (Immunohistochemistry). The expression of Cyclin D and p-Akt was downregulated in TAX, NVD and FH535 administrated group than control group. The decline in tumor growth and volume with simultaneous reduction in β-catenin and Survivin proteins expression levels was seen in the TAX, NVD and FH535 administrated groups by western blot analysis, may have various therapeutic significance. Further mRNA expression was checked by RT-PCR, revealed decline in β-Catenin gene expression in TAX, NVD and FH535 group as compared to control. The conclusion of the current observation can have a constructive suggestion and translational impact to colorectal cancer subjects as it demonstrates that TAX and NVD can effect tumor development, which could amplify the continued existence and superiority lifespan of the subjects' affliction with colorectal carcinoma.

Furthermore, conclusion of current study is that the TAX and NVD hold ability for maturity as a possible therapeutic and chemopreventive agent against colorectal cancer. While auxiliary experiments are considered necessary to entirely dissect the association of TAX and NVD with Wnt/*β*-Catenin pathway, we suppose that to attain significant clinical effects within conduct of colorectal cancer harboring stabilizing mutation of *β*-catenin, TAX and NVD could symbolize a superior prospect to amplify the effectiveness of therapies.

The present study proposed that TAX and NVD act equally Wnt/β-catenin corridor inhibitors. Also has the prospective to restrain tumor incursion as well as metastasis in both *in vitro* (HT 29 as well as HCT116 cells) and *in vivo* (Xenograft) CRC models: TAX and NVD demonstrated evident and consistent inhibition of CRC cell proliferation. This prospective to restrain CRC invasion and metastasis were evident by cell cycle regulators and apoptotic proteins (Pro apoptotic and ant apoptotic proteins). The anticancer effect of

TAX and NVD has been recognized to the inhibition of β-catenin and AKT protein expression. Additional to this, we observed that TAX and NVD diminish the expression of surviving, known as an anti-apoptotic protein. Survivin encourages malignancy propagation by approach of amending several crucial cell signaling corridor. Consequently, TAX and NVD is predictable to offer noval potent prospective to restrain tumor incursion and malignancies, demonstrating as an encouraging approach for future CRC behavior.

Based on our finding, we propose a mechanism by which TAX and NVD elicits its effect on CRC. We cheifly took under consideration different pathways like, Wnt/β-catenin, Erk, PI3K/Akt. As reported, TAX and NVD decrease protein expression of β-catenin, AKT & also reduces protein expression of survivin, the downstream mark of Wnt/β-catenin signaling therefore, we proposed that TAX and NVD may act as a β-catenin inhibitor.

5.5 Conclusion:

- In summary, screening of Pakistani population revealed association of two non-synonymous polymorphisms in *CTNNB1* gene with colorectal cancer. These genetic variants led to the accumulation of *CTNNB1*, a hallmark of tumor development. Further molecular modeling, docking and simulation approaches revealed significant conformational changes in the N-terminus region of normal to mutant *CTNNB1* gene critical for binding with GSK3 and TrCp1. Analysis of structure to function alterations in *CTNNB1* gene is crucial in understanding downstream biological events. Our interpretations need to be deep-rooted on a superior figure of tumors in Pakistani population and we suggest that the documentation of β-catenin/ *CTNNB1*genes articulated in colon cancer cells might suggest novel prospects for emerging therapeutics against candidates with crucial part in colon cancer.

- In supposition, the conclusions of this examination sustenance a part for hormones in 25-OH VD concentrations. Auxiliary potential studies that unswervingly evaluate intensities of circulating hormones and hormone therapy in women allied to 25-OH VD concentrations as well as their possible role in colorectal cancer jeopardy would be exceedingly explanatory.
- In an *in vitro* study, TAX and NVD administrated HCT116 and HT29 cells revealed reduced β-catenin expression at protein & mRNA levels, allied through reticence of AKT and Survivin.
- Decline in the intensities of cyclin A, B1 & E2 and cyclin dependent kinase inhibitors on induction of HT29 & HCT116 cells with TAX & NVD was observed. We determined that HT29 & HCT116 cells administrated with TAX and NVD induced inhibition in the phosphorylation of Akt in a dose dependent approach.
- Immunofluorescence staining of HCT116 and HT29 cells showed diminish in p-Akt expression further supported the fact that TAX and NVD administration inhibits Akt phosphorylation. These findings indicated that HCT116 andHT29 cells administration with TAX and NVD resulted in apoptosis through inhibition of β-catenin and p-Akt.
- Since β-catenin inhibitor inhibits Akt phosphorylation which caused downregulation of β-catenin protein expression. TAX and NVD administration of cells administrated with β-catenin inhibitor further amplified the decline of β-catenin expression, suggestive of that Akt in part is responsible for these effects.
- Crucial components of different signaling pathways like wnt/ β-catenin, CK2, NF-κB, & PI3k were considered & showed decrease in protein expression after administration of TAX and NVD in HCT116 and HT29 cells.
- We demonstrated similar finding in *in-vivo* system, Athymic nude mice implanted with HCT116 xenografts in athymic nude mice administrated with TAX and

NVD triggered reticence of phosphorylation of Akt in a dose reliant fashion. The expression of p-Akt (Immunohistochemical staining) was high in control as compared to TAX and NVD treated group which as almost showing no expression of p-Akt protein.

- Results revealed the effect of TAX and NVD in inhibiting cell growth in colorectal carcinoma in equally *in vitro* as well as *in vivo* model. The observed decrease in progress and volume of tumor with simultaneous decline in the intensities of β-catenin and Survivin (*in vitro* model) may have some therapeutic importance.
- Similarly, *in vitro* data demonstrates that intraperitoneal injection of TAX and NVD significantly decelerated tumor progression in athymic mice with considerable reticent β-catenin and survivin expression. No momentous variation in the tissue design, reduction in body weights as well as other allied contrary effects in mice administrated through TAX and NVD further supported the notion that the TAX and NVD might be a potentially explored for the treatment as well as cure of colorectal cancer.

Chapter 6
References

6. "References:

ABERLE, H., BAUER, A., STAPPERT, J., KISPERT, A. & KEMLER, R. 1997. β-catenin is a target for the ubiquitin–proteasome pathway. *The EMBO journal,* 16**,** 3797-3804.

ADHAMI, V. M., AHMAD, N. & MUKHTAR, H. 2003. Molecular targets for green tea in prostate cancer prevention. *The journal of nutrition,* 133**,** 2417S-2424S.

ADHI, M., HASAN, R., SHOAIB, S. & TAUHEED, S. 2007. Age and symptomatology of menopause in Karachi, Pakistan. *Pak J Physiol,* 3**,** 41-4.

AGUILERA, O., PEÑA, C., GARCÍA, J. M., LARRIBA, M. J., ORDÓÑEZ-MORÁN, P., NAVARRO, D., BARBÁCHANO, A., LÓPEZ DE SILANES, I., BALLESTAR, E. & FRAGA, M. F. 2007. The Wnt antagonist DICKKOPF-1 gene is induced by 1α, 25-dihydroxyvitamin D 3 associated to the differentiation of human colon cancer cells. *Carcinogenesis,* 28**,** 1877-1884.

AKHTER, M., INOUE, M., KURAHASHI, N., IWASAKI, M., SASAZUKI, S., TSUGANE, S. & GROUP, J. S. 2008. Reproductive factors, exogenous female hormone use and colorectal cancer risk: the Japan Public Health Center-based Prospective Study. *European Journal of Cancer Prevention,* 17**,** 515-524.

ALKAHTANI, S. 2013. Testosterone induced apoptosis in colon cancer cells is regulated by PI3K/Rac1 signaling. *Asian journal of andrology,* 15**,** 831.

ALOMAR, S. Y., MANSOUR, L., ABUDERMAN, A., ALKHURIJI, A., ARAFAH, M., ALWASEL, S., HARRATH, A. H., ALMUTAIRI, M., TRAYHYRN, P. & DAR, J. A. 2016. β-Catenin accumulation and S33F mutation of CTNNB1 gene in colorectal cancer in Saudi Arabia. *Polish Journal of Pathology,* 67**,** 156-162.

ALTIERI, D. C. 2003. Validating survivin as a cancer therapeutic target. *Nature Reviews Cancer,* 3**,** 46.

ALTIERI, D. C. 2004. Molecular circuits of apoptosis regulation and cell division control: the survivin paradigm. *Journal of cellular biochemistry,* 92**,** 656-663.

ALTIERI, D. C. 2008. Survivin, cancer networks and pathway-directed drug discovery. *Nature Reviews Cancer,* 8**,** 61.

AMBROSINI, G., ADIDA, C. & ALTIERI, D. C. 1997. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nature medicine,* 3**,** 917.

AMINI, A. Q., SAMO, K. A. & MEMON, A. S. 2013. Colorectal cancer in younger population: our experience. *J Pak Med Assoc,* 63**,** 1275-1277.

AMIT, S., HATZUBAI, A., BIRMAN, Y., ANDERSEN, J. S., BEN-SHUSHAN, E., MANN, M., BEN-NERIAH, Y. & ALKALAY, I. 2002. Axin-mediated CKI phosphorylation of β-catenin at Ser 45: a molecular switch for the Wnt pathway. *Genes & development,* 16**,** 1066-1076.

AMOS-LANDGRAF, J. M., HEIJMANS, J., WIELENGA, M. C., DUNKIN, E., KRENTZ, K. J., CLIPSON, L., EDERVEEN, A. G., GROOTHUIS, P. G., MOSSELMAN, S. & MUNCAN, V. 2014. Sex disparity in colonic

adenomagenesis involves promotion by male hormones, not protection by female hormones. *Proceedings of the National Academy of Sciences,* 111**,** 16514-16519.

ANAGNOSTOPOULOU, V., PEDIADITAKIS, I., ALKAHTANI, S., ALARIFI, S. A., SCHMIDT, E.-M., LANG, F., GRAVANIS, A., CHARALAMPOPOULOS, I. & STOURNARAS, C. 2013. Differential effects of dehydroepiandrosterone and testosterone in prostate and colon cancer cell apoptosis: the role of nerve growth factor (NGF) receptors. *Endocrinology,* 154**,** 2446-2456.

ANASTAS, J. N. & MOON, R. T. 2013. WNT signalling pathways as therapeutic targets in cancer. *Nature Reviews Cancer,* 13**,** 11-26.

ANDERSON, G. 2004. Women's Health Initiative Steering Committee. Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial. *Jama,* 291**,** 1701-1712.

ANDERSON, M. G., NAKANE, M., RUAN, X., KROEGER, P. E. & WU-WONG, J. R. 2006. Expression of VDR and CYP24A1 mRNA in human tumors. *Cancer chemotherapy and pharmacology,* 57**,** 234-240.

ANDL, C. D. & RUSTGI, A. K. 2005. No one-way street: Cross-talk between E-cadherin and receptor tyrosine kinase (RTK) signaling—A mechanism to regulate RTK activity. *Cancer biology & therapy,* 4**,** 35-38.

AOKI, K., AOKI, M., SUGAI, M., HARADA, N., MIYOSHI, H., TSUKAMOTO, T., MIZOSHITA, T., TATEMATSU, M., SENO, H. & CHIBA, T. 2007. Chromosomal instability by β-catenin/TCF transcription in APC or β-catenin mutant cells. *Oncogene,* 26**,** 3511-3520.

APTER, D., REINILÄ, M. & VIHKO, R. 1989. Some endocrine characteristics of early menarche, a risk factor for breast cancer, are preserved into adulthood. *International journal of cancer,* 44**,** 783-787.

ARAI, Y., WATANABE, S., KIMIRA, M., SHIMOI, K., MOCHIZUKI, R. & KINAE, N. 2000. Dietary intakes of flavonols, flavones and isoflavones by Japanese women and the inverse correlation between quercetin intake and plasma LDL cholesterol concentration. *The Journal of nutrition,* 130**,** 2243-2250.

ARMAGHANY, T., WILSON, J. D., CHU, Q. & MILLS, G. 2012. Genetic alterations in colorectal cancer. *Gastrointestinal cancer research: GCR,* 5**,** 19.

ARMAMENTO-VILLAREAL, R., VILLAREAL, D. T., AVIOLI, L. V. & CIVITELLI, R. 1992. Estrogen status and heredity are major determinants of premenopausal bone mass. *The Journal of clinical investigation,* 90**,** 2464-2471.

ARNAL, J.-F., GOURDY, P. & LENFANT, F. In vivo dissection of the estrogen receptor alpha: uncoupling of its physiological effects and medical perspectives. Annales d'endocrinologie, 2013. Elsevier, 82-89.

ARNOLD, M., KARIM-KOS, H. E., COEBERGH, J. W., BYRNES, G., ANTILLA, A., FERLAY, J., RENEHAN, A. G., FORMAN, D. & SOERJOMATARAM, I. 2015. Recent trends in incidence of five common cancers in 26 European

countries since 1988: Analysis of the European Cancer Observatory. *European journal of cancer,* 51**,** 1164-1187.

ARYA, V., BHAMBRI, R., GODBOLE, M. M. & MITHAL, A. 2004. Vitamin D status and its relationship with bone mineral density in healthy Asian Indians. *Osteoporosis international,* 15**,** 56-61.

ASIF, H. M., SULTANA, S., SHAFIQUEE, S. & AKHTAR, N. 2017. Age at Menarche, Menstrual Pattern and Premenstrual Symptoms of Female Students of Islamia University Bahawalpur. *RADS Journal of Pharmacy and Pharmaceutical Sciences,* 5**,** 44-48.

ASSOCIATION, W. M. 2008. World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. *http://www. wma. net/e/policy/b3. htm*.

BACHMAN, E., FENG, R., TRAVISON, T., LI, M., OLBINA, G., OSTLAND, V., ULLOOR, J., ZHANG, A., BASARIA, S. & GANZ, T. 2010. Testosterone suppresses hepcidin in men: a potential mechanism for testosterone-induced erythrocytosis. *The Journal of Clinical Endocrinology & Metabolism,* 95**,** 4743-4747.

BAILLON, L. & BASLER, K. Reflections on cell competition. Seminars in cell & developmental biology, 2014. Elsevier, 137-144.

BALDUS, S. E., MÖNIG, S. P., HUXEL, S., LANDSBERG, S., HANISCH, F.-G., ENGELMANN, K., SCHNEIDER, P. M., THIELE, J., HÖLSCHER, A. H. & DIENES, H. P. 2004. MUC1 and nuclear β-catenin are coexpressed at the invasion front of colorectal carcinomas and are both correlated with tumor prognosis. *Clinical cancer research,* 10**,** 2790-2796.

BANACH-PETROSKY, W., OUYANG, X., GAO, H., NADER, K., JI, Y., SUH, N., DIPAOLA, R. S. & ABATE-SHEN, C. 2006. Vitamin D inhibits the formation of prostatic intraepithelial neoplasia in Nkx3. 1; Pten mutant mice. *Clinical Cancer Research,* 12**,** 5895-5901.

BARBÁCHANO, A., ORDÓÑEZ-MORÁN, P., GARCÍA, J. M., SÁNCHEZ, A., PEREIRA, F., LARRIBA, M. J., MARTÍNEZ, N., HERNÁNDEZ, J., LANDOLFI, S. & BONILLA, F. 2010. SPROUTY-2 and E-cadherin regulate reciprocally and dictate colon cancer cell tumourigenicity. *Oncogene,* 29**,** 4800-4813.

BAREIS, P., BISES, G., BISCHOF, M. G., CROSS, H. S. & PETERLIK, M. 2001. 25-hydroxy-vitamin d metabolism in human colon cancer cells during tumor progression. *Biochemical and biophysical research communications,* 285**,** 1012-1017.

BARKER, N. & CLEVERS, H. 2006. Mining the Wnt pathway for cancer therapeutics. *Nature reviews Drug discovery,* 5**,** 997-1014.

BARNES, J., ARHEL, N., LEE, S., SHARP, A., AL-OKAIL, M., PACKHAM, G., HAGUE, A., PARASKEVA, C. & WILLIAMS, A. 2005. Nuclear BAG-1

expression inhibits apoptosis in colorectal adenoma-derived epithelial cells. *Apoptosis,* 10**,** 301-311.

BARZI, A., LENZ, A. M., LABONTE, M. J. & LENZ, H.-J. 2013. Molecular pathways: estrogen pathway in colorectal cancer. *Clinical Cancer Research,* 19**,** 5842-5848.

BASTIDE, N. M., PIERRE, F. H. & CORPET, D. E. 2011. Heme iron from meat and risk of colorectal cancer: a meta-analysis and a review of the mechanisms involved. *Cancer prevention research,* 4**,** 177-184.

BASU, A., SETH, S., ARORA, K. & VERMA, M. 2015. Evaluating Estradiol Levels in Male Patients with Colorectal Carcinoma. *Journal of clinical and diagnostic research: JCDR,* 9**,** BC08.

BEACHY, P. A., KARHADKAR, S. S. & BERMAN, D. M. 2004. Tissue repair and stem cell renewal in carcinogenesis. *Nature,* 432**,** 324.

BELL, D. A. 2005. Origins and molecular pathology of ovarian cancer. *Modern Pathology,* 18**,** S19-S32.

BIÈCHE, I., DE MURCIA, G. & LIDEREAU, R. 1996. Poly (ADP-ribose) polymerase gene expression status and genomic instability in human breast cancer. *Clinical cancer research,* 2**,** 1163-1167.

BIENZ, M. & CLEVERS, H. 2000. Linking colorectal cancer to Wnt signaling. *Cell,* 103**,** 311-320.

BIRT, D. F., HENDRICH, S. & WANG, W. 2001. Dietary agents in cancer prevention: flavonoids and isoflavonoids. *Pharmacology & therapeutics,* 90**,** 157-177.

BISES, G., KÁLLAY, E., WEILAND, T., WRBA, F., WENZL, E., BONNER, E., KRIWANEK, S., OBRIST, P. & CROSS, H. S. 2004. 25-hydroxyvitamin D3-1α-hydroxylase expression in normal and malignant human colon. *Journal of Histochemistry & Cytochemistry,* 52**,** 985-989.

BOCCARDO, F., LUNARDI, G., GUGLIELMINI, P., PARODI, M., MURIALDO, R., SCHETTINI, G. & RUBAGOTTI, A. 2004. Serum enterolactone levels and the risk of breast cancer in women with palpable cysts. *European journal of cancer,* 40**,** 84-89.

BOIK, J. 1995. Cancer and natural medicine: a textbook of basic science and clinical research. *Princeton, Minnesota: Oregon Medical Press xiv, 315p. ISBN,* 964828006.

BOLAND, C. R. & GOEL, A. 2010. Microsatellite instability in colorectal cancer. *Gastroenterology,* 138**,** 2073-2087. e3.

BORCHERS, A. T. 2002. Natural Compounds in Cancer Therapy—Promising Nontoxic Antitumor Agents from Plants & Other Natural Sources, by John Boik, 2001, 521 pages, softcover, $32. Oregon Medical Press, LLC, Princeton, MN. Am Soc Nutrition.

BORNER, C. 2003. The Bcl-2 protein family: sensors and checkpoints for life-or-death decisions. *Molecular immunology,* 39**,** 615-647.

References

BOSETTI, C., BRAVI, F., NEGRI, E. & LA VECCHIA, C. 2009. Oral contraceptives and colorectal cancer risk: a systematic review and meta-analysis. *Human reproduction update,* 15**,** 489-498.

BOTTERI, E., IODICE, S., BAGNARDI, V., RAIMONDI, S., LOWENFELS, A. B. & MAISONNEUVE, P. 2008. Smoking and colorectal cancer: a meta-analysis. *Jama,* 300**,** 2765-2778.

BOUDJADI, S., BERNATCHEZ, G., BEAULIEU, J.-F. & CARRIER, J. C. 2013. Control of the human osteopontin promoter by ERRα in colorectal cancer. *The American journal of pathology,* 183**,** 266-276.

BOUILLON, R., CARMELIET, G., VERLINDEN, L., VAN ETTEN, E., VERSTUYF, A., LUDERER, H. F., LIEBEN, L., MATHIEU, C. & DEMAY, M. 2008. Vitamin D and human health: lessons from vitamin D receptor null mice. *Endocrine reviews,* 29**,** 726-776.

BREMBECK, F. H., ROSÁRIO, M. & BIRCHMEIER, W. 2006. Balancing cell adhesion and Wnt signaling, the key role of β-catenin. *Current opinion in genetics & development,* 16**,** 51-59.

BRIVANLOU, A. H. & DARNELL, J. E. 2002. Signal transduction and the control of gene expression. *Science,* 295**,** 813-818.

CABRITA, M. A. & CHRISTOFORI, G. 2008. Sprouty proteins, masterminds of receptor tyrosine kinase signaling. *Angiogenesis,* 11**,** 53-62.

CAI, L., LI, W., WANG, G., GUO, L., JIANG, Y. & KANG, Y. J. 2002. Hyperglycemia-induced apoptosis in mouse myocardium. *Diabetes,* 51**,** 1938-1948.

CANAVAN, C., ABRAMS, K. & MAYBERRY, J. 2006. Meta-analysis: colorectal and small bowel cancer risk in patients with Crohn's disease. *Alimentary pharmacology & therapeutics,* 23**,** 1097-1104.

CERVELLO, M., AUGELLO, G., CUSIMANO, A., EMMA, M. R., BALASUS, D., AZZOLINA, A., MCCUBREY, J. A. & MONTALTO, G. 2017. Pivotal roles of glycogen synthase-3 in hepatocellular carcinoma. *Advances in biological regulation,* 65**,** 59-76.

CHAFFER, C. L. & WEINBERG, R. A. 2011. A perspective on cancer cell metastasis. *Science,* 331**,** 1559-1564.

CHAVEZ-MACGREGOR, M., ELIAS, S. G., ONLAND-MORET, N. C., VAN DER SCHOUW, Y. T., VAN GILS, C. H., MONNINKHOF, E., GROBBEE, D. E. & PEETERS, P. H. 2005. Postmenopausal breast cancer risk and cumulative number of menstrual cycles. *Cancer Epidemiology and Prevention Biomarkers,* 14**,** 799-804.

CHEN, A., DAVIS, B. H., SITRIN, M. D., BRASITUS, T. A. & BISSONNETTE, M. 2002. Transforming growth factor-β1 signaling contributes to Caco-2 cell growth inhibition induced by 1, 25 (OH) 2 D 3. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 283**,** G864-G874.

References

CHEN, R.-H. & MCCORMICK, F. 2001. Selective targeting to the hyperactive β-catenin/T-cell factor pathway in colon cancer cells. *Cancer research,* 61**,** 4445-4449.

CHLEBOWSKI, R. T., WACTAWSKI-WENDE, J., RITENBAUGH, C., HUBBELL, F. A., ASCENSAO, J., RODABOUGH, R. J., ROSENBERG, C. A., TAYLOR, V. M., HARRIS, R. & CHEN, C. 2004. Estrogen plus progestin and colorectal cancer in postmenopausal women. *New England Journal of Medicine,* 350**,** 991-1004.

CHUN, P. & WAINBERG, Z. A. 2009. Adjuvant chemotherapy for stage II colon cancer: the role of molecular markers in choosing therapy. *Gastrointestinal cancer research: GCR,* 3**,** 191.

CHUNG, T. P. & FLESHMAN, J. W. The Genetics of Sporadic Colon Cancer. Seminars in Colon and Rectal Surgery, 2004. Elsevier, 128-135.

CLENDENEN, T. V., KOENIG, K. L., SHORE, R. E., LEVITZ, M., ARSLAN, A. A. & ZELENIUCH-JACQUOTTE, A. 2009. Postmenopausal levels of endogenous sex hormones and risk of colorectal cancer. *Cancer Epidemiology and Prevention Biomarkers,* 18**,** 275-281.

CLEVERS, H. 2006. Wnt/β-catenin signaling in development and disease. *Cell,* 127**,** 469-480.

CLEVERS, H. & NUSSE, R. 2012. Wnt/β-catenin signaling and disease. *Cell,* 149**,** 1192-1205.

COATES, J. C. 2003. Armadillo repeat proteins: beyond the animal kingdom. *Trends in cell biology,* 13**,** 463-471.

COHEN, P. & FRAME, S. 2001. The renaissance of GSK3. *Nature reviews. Molecular cell biology,* 2**,** 769.

COOPER, G. & SANDLER, D. 1997. Long-term effects of reproductive-age menstrual cycle patterns on peri-and postmenopausal fracture risk. *American journal of epidemiology,* 145**,** 804-809.

COTTRELL, S., BODMER, W., BICKNELL, D. & KAKLAMANIS, L. 1992. Molecular analysis of APC mutations in familial adenomatous polyposis and sporadic colon carcinomas. *The Lancet,* 340**,** 626-630.

CRAWFORD, H. C., FINGLETON, B. M., RUDOLPH-OWEN, L. A., GOSS, K. J. H., RUBINFELD, B., POLAKIS, P. & MATRISIAN, L. M. 1999. The metalloproteinase matrilysin is a target of β-catenin transactivation in intestinal tumors. *Oncogene,* 18.

CROSS, A. J., BOCA, S., FREEDMAN, N. D., CAPORASO, N. E., HUANG, W.-Y., SINHA, R., SAMPSON, J. N. & MOORE, S. C. 2014. Metabolites of tobacco smoking and colorectal cancer risk. *Carcinogenesis,* 35**,** 1516-1522.

CROSS, H., BAJNA, E., BISES, G., GENSER, D., KALLAY, E., PÖTZI, R., WENZL, E., WRBA, F., ROKA, R. & PETERLIK, M. 1996. Vitamin D receptor and

cytokeratin expression may be progression indicators in human colon cancer. *Anticancer research,* 16**,** 2333-2337.

CROSS, H. S., BAREIS, P., HOFER, H., BISCHOF, M. G., BAJNA, E., KRIWANEK, S., BONNER, E. & PETERLIK, M. 2001. 25-Hydroxyvitamin D 3-1α-hydroxylase and vitamin D receptor gene expression in human colonic mucosa is elevated during early cancerogenesis. *Steroids,* 66**,** 287-292.

D ARCHIVIO, M., FILESI, C., DI BENEDETTO, R., GARGIULO, R., GIOVANNINI, C. & MASELLA, R. 2007. Polyphenols, dietary sources and bioavailability. *Annali-Istituto Superiore di Sanita,* 43**,** 348.

DAI, Q., FRANKE, A. A., JIN, F., SHU, X.-O., HEBERT, J. R., CUSTER, L. J., CHENG, J., GAO, Y.-T. & ZHENG, W. 2002. Urinary excretion of phytoestrogens and risk of breast cancer among Chinese women in Shanghai. *Cancer Epidemiology and Prevention Biomarkers,* 11**,** 815-821.

DALERBA, P., DYLLA, S. J., PARK, I.-K., LIU, R., WANG, X., CHO, R. W., HOEY, T., GURNEY, A., HUANG, E. H. & SIMEONE, D. M. 2007. Phenotypic characterization of human colorectal cancer stem cells. *Proceedings of the National Academy of Sciences,* 104**,** 10158-10163.

DANIEL, J. M. & REYNOLDS, A. B. 1997. Tyrosine phosphorylation and cadherin/catenin function. *Bioessays,* 19**,** 883-891.

DEEB, K. K., TRUMP, D. L. & JOHNSON, C. S. 2007. Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. *Nature reviews cancer,* 7**,** 684-700.

DELELLIS HENDERSON, K., DUAN, L., SULLIVAN-HALLEY, J., MA, H., CLARKE, C. A., NEUHAUSEN, S. L., TEMPLEMAN, C. & BERNSTEIN, L. 2010. Menopausal hormone therapy use and risk of invasive colon cancer: the California Teachers Study. *American journal of epidemiology,* 171**,** 415-425.

DEN TONKELAAR, I., KEINAN-BOKER, L., VAN'T VEER, P., ARTS, C. J., ADLERCREUTZ, H., THIJSSEN, J. H. & PEETERS, P. H. 2001. Urinary phytoestrogens and postmenopausal breast cancer risk. *Cancer Epidemiology and Prevention Biomarkers,* 10**,** 223-228.

DENG, J., MILLER, S. A., WANG, H.-Y., XIA, W., WEN, Y., ZHOU, B. P., LI, Y., LIN, S.-Y. & HUNG, M.-C. 2002. β-catenin interacts with and inhibits NF-κB in human colon and breast cancer. *Cancer cell,* 2**,** 323-334.

DI DOMENICO, M., CASTORIA, G., BILANCIO, A., MIGLIACCIO, A. & AURICCHIO, F. 1996. Estradiol activation of human colon carcinoma-derived Caco-2 cell growth. *Cancer Research,* 56**,** 4516-4521.

DI MAIRA, G., SALVI, M., ARRIGONI, G., MARIN, O., SARNO, S., BRUSTOLON, F., PINNA, L. & RUZZENE, M. 2005. Protein kinase CK2 phosphorylates and upregulates Akt/PKB. *Cell death and differentiation,* 12**,** 668.

DÍAZ, G. D., PARASKEVA, C., THOMAS, M. G., BINDERUP, L. & HAGUE, A. 2000. Apoptosis is induced by the active metabolite of vitamin D3 and its

analogue EB1089 in colorectal adenoma and carcinoma cells: possible implications for prevention and therapy. *Cancer Research,* 60**,** 2304-2312.

DIHLMANN, S., KLEIN, S. & VON KNEBEL DOEBERITZ, M. 2003. Reduction of β-catenin/T-cell transcription factor signaling by aspirin and indomethacin is caused by an increased stabilization of phosphorylated β-catenin. *Molecular cancer therapeutics,* 2**,** 509-516.

DIHLMANN, S., SIERMANN, A. & VON KNEBEL DOEBERITZ, M. 2001. The nonsteroidal anti-inflammatory drugs aspirin and indomethacin attenuate [beta]-catenin/TCF-4 signaling. *Oncogene,* 20**,** 645.

DOSSUS, L., ALLEN, N., KAAKS, R., BAKKEN, K., LUND, E., TJONNELAND, A., OLSEN, A., OVERVAD, K., CLAVEL-CHAPELON, F. & FOURNIER, A. 2010. Reproductive risk factors and endometrial cancer: the European Prospective Investigation into Cancer and Nutrition. *International journal of cancer,* 127**,** 442-451.

DOUILLARD, J.-Y., SIENA, S., CASSIDY, J., TABERNERO, J., BURKES, R., BARUGEL, M., HUMBLET, Y., BODOKY, G., CUNNINGHAM, D. & JASSEM, J. 2010. Randomized, phase III trial of panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) versus FOLFOX4 alone as first-line treatment in patients with previously untreated metastatic colorectal cancer: the PRIME study. *Journal of clinical oncology,* 28**,** 4697-4705.

DROBEK-SŁOWIK, M., KARCZEWICZ, D. & SAFRANOW, K. 2007. The potential role of oxidative stress in the pathogenesis of the age-related macular degeneration (AMD). *Postepy higieny i medycyny doswiadczalnej (Online),* 61**,** 28-37.

EADEN, J., ABRAMS, K. & MAYBERRY, J. 2001. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut,* 48**,** 526-535.

EAGLETON, C. & JUDKINS, A. 2006. Vitamin D deficiency in pregnant New Zealand women. *The New Zealand Medical Journal (Online),* 119.

EGAN, J. B., THOMPSON, P. A., VITANOV, M. V., BARTIK, L., JACOBS, E. T., HAUSSLER, M. R., GERNER, E. W. & JURUTKA, P. W. 2010. Vitamin D receptor ligands, adenomatous polyposis coli, and the vitamin D receptor FokI polymorphism collectively modulate β-catenin activity in colon cancer cells. *Molecular carcinogenesis,* 49**,** 337-352.

ELBANNA, H. G., EBRAHIM, M. A., ABBAS, A. M., ZALATA, K. & HASHIM, M. A. 2012. Potential value of estrogen receptor beta expression in colorectal carcinoma: interaction with apoptotic index. *Journal of gastrointestinal cancer,* 43**,** 56-62.

ENARI, M., SAKAHIRA, H., YOKOYAMA, H., OKAWA, K., IWAMATSU, A. & NAGATA, S. 1998. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature,* 391**,** 43-50.

References

ENGLISH, M., STEWART, P. & HEWISON, M. 2001. Estrogen metabolism and malignancy: analysis of the expression and function of 17β-hydroxysteroid dehydrogenases in colonic cancer. *Molecular and cellular endocrinology,* 171**,** 53-60.

EVANS, S., NOLLA, J., HANFELT, J., SHABAHANG, M., NAUTA, R. J. & SHCHEPOTIN, I. B. 1998. Vitamin D receptor expression as a predictive marker of biological behavior in human colorectal cancer. *Clinical Cancer Research,* 4**,** 1591-1595.

FABBRO, M. & HENDERSON, B. R. 2003. Regulation of tumor suppressors by nuclear-cytoplasmic shuttling. *Experimental cell research,* 282**,** 59-69.

FAKIH, M. G., TRUMP, D. L., JOHNSON, C. S., TIAN, L., MUINDI, J. & SUNGA, A. Y. 2009. Chemotherapy is linked to severe vitamin D deficiency in patients with colorectal cancer. *International journal of colorectal disease,* 24**,** 219.

FANG, D., HAWKE, D., ZHENG, Y., XIA, Y., MEISENHELDER, J., NIKA, H., MILLS, G. B., KOBAYASHI, R., HUNTER, T. & LU, Z. 2007. Phosphorylation of β-catenin by AKT promotes β-catenin transcriptional activity. *Journal of Biological Chemistry,* 282**,** 11221-11229.

FEARON, E. R. & VOGELSTEIN, B. 1990. A genetic model for colorectal tumorigenesis. *Cell,* 61**,** 759-767.

FERLAY, J., SOERJOMATARAM, I., DIKSHIT, R., ESER, S., MATHERS, C., REBELO, M., PARKIN, D. M., FORMAN, D. & BRAY, F. 2015. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *International journal of cancer,* 136.

FERNANDEZ-GARCIA, N. I., PALMER, H. G., GARCIA, M., GONZALEZ-MARTIN, A., DEL RIO, M., BARETTINO, D., VOLPERT, O., MUNOZ, A. & JIMENEZ, B. 2005. 1α, 25-Dihydroxyvitamin D3 regulates the expression of Id1 and Id2 genes and the angiogenic phenotype of human colon carcinoma cells. *Oncogene,* 24**,** 6533-6544.

FERNANDEZ, E., LA VECCHIA, C., BALDUCCI, A., CHATENOUD, L., FRANCESCHI, S. & NEGRI, E. 2001. Oral contraceptives and colorectal cancer risk: a meta-analysis. *British journal of cancer,* 84**,** 722-727.

FILIPPO, C. D., LUCERI, C., CADERNI, G., PACINI, M., MESSERINI, L., BIGGERI, A., MINI, E., TONELLI, F., CIANCHI, F. & DOLARA, P. 2002. Mutations of the APC gene in human sporadic colorectal cancers. *Scandinavian journal of gastroenterology,* 37**,** 1048-1053.

FIOL, C., MAHRENHOLZ, A., WANG, Y., ROESKE, R. & ROACH, P. 1987. Formation of protein kinase recognition sites by covalent modification of the substrate. Molecular mechanism for the synergistic action of casein kinase II and glycogen synthase kinase 3. *Journal of Biological Chemistry,* 262**,** 14042-14048.

References

FIORDALISO, F., LI, B., LATINI, R., SONNENBLICK, E. H., ANVERSA, P., LERI, A. & KAJSTURA, J. 2000. Myocyte death in streptozotocin-induced diabetes in rats is angiotensin II-dependent. *Laboratory investigation,* 80**,** 513-527.

FOLEY, E. F., JAZAERI, A. A., SHUPNIK, M. A., JAZAERI, O. & RICE, L. W. 2000. Selective loss of estrogen receptor β in malignant human colon. *Cancer Research,* 60**,** 245-248.

FOSTER, P. A. 2013. Oestrogen and colorectal cancer: mechanisms and controversies. *International journal of colorectal disease,* 28**,** 737-749.

FOTEDAR, R., FITZGERALD, P., ROUSSELLE, T., CANNELLA, D., DORÉE, M., MESSIER, H. & FOTEDAR, A. 1996. p21 contains independent binding sites for cyclin and cdk2: both sites are required to inhibit cdk2 kinase activity. *Oncogene,* 12**,** 2155-2164.

FRIDAY, B. B. & ADJEI, A. A. 2008. Advances in targeting the Ras/Raf/MEK/Erk mitogen-activated protein kinase cascade with MEK inhibitors for cancer therapy. *Clinical cancer research,* 14**,** 342-346.

FRUSTACI, A., KAJSTURA, J., CHIMENTI, C., JAKONIUK, I., LERI, A., MASERI, A., NADAL-GINARD, B. & ANVERSA, P. 2000. Myocardial cell death in human diabetes. *Circulation research,* 87**,** 1123-1132.

FUCHS, S., OUGOLKOV, A., SPIEGELMAN, V. & MINAMOTO, T. 2006. Oncogenic β-catenin signaling networks in colorectal cancer. *Cancer Research Institute report,* 2003**,** 105.

GANDINI, S., BONIOL, M., HAUKKA, J., BYRNES, G., COX, B., SNEYD, M. J., MULLIE, P. & AUTIER, P. 2011. Meta-analysis of observational studies of serum 25-hydroxyvitamin D levels and colorectal, breast and prostate cancer and colorectal adenoma. *International Journal of Cancer,* 128**,** 1414-1424.

GARLAND, C., GARLAND, F., SHAW, E., COMSTOCK, G., HELSING, K. & GORHAM, E. 1989. Serum 25-hydroxyvitamin D and colon cancer: eight-year prospective study. *The Lancet,* 334**,** 1176-1178.

GARLAND, C. F. & GARLAND, F. C. 1980. Do sunlight and vitamin D reduce the likelihood of colon cancer? *International journal of epidemiology,* 9**,** 227-231.

GASCHOTT, T., STEINMEYER, A., STEINHILBER, D. & STEIN, J. 2002. ZK 156718, a low calcemic, antiproliferative, and prodifferentiating vitamin D analog. *Biochemical and biophysical research communications,* 290**,** 504-509.

GHABREAU, L., ROUX, J. P., FRAPPART, P. O., MATHEVET, P., PATRICOT, L. M., MOKNI, M., KORBI, S., WANG, Z. Q., TONG, W. M. & FRAPPART, L. 2004. Poly (ADP-ribose) polymerase-1, a novel partner of progesterone receptors in endometrial cancer and its precursors. *International journal of cancer,* 109**,** 317-321.

GILES, R. H., VAN ES, J. H. & CLEVERS, H. 2003. Caught up in a Wnt storm: Wnt signaling in cancer. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer,* 1653**,** 1-24.

References

GILSANZ, V., CHALFANT, J., KALKWARF, H., ZEMEL, B., LAPPE, J., OBERFIELD, S., SHEPHERD, J., WREN, T. & WINER, K. 2011. Age at onset of puberty predicts bone mass in young adulthood. *The Journal of pediatrics,* 158**,** 100-105. e2.

GISSELSSON, D., PETTERSSON, L., HÖGLUND, M., HEIDENBLAD, M., GORUNOVA, L., WIEGANT, J., MERTENS, F., DAL CIN, P., MITELMAN, F. & MANDAHL, N. 2000. Chromosomal breakage-fusion-bridge events cause genetic intratumor heterogeneity. *Proceedings of the National Academy of Sciences,* 97**,** 5357-5362.

GONZÁLEZ-SANCHO, J. M., LARRIBA, M. J. & MUNOZ, A. 2011. Vitamin D and Wnt/b-catenin signaling. *Vitamin D***,** 235-250.

GONZALEZ-SANCHO, J. M., LARRIBA, M. J., ORDONEZ-MORAN, P., PALMER, H. G. & MUNOZ, A. 2006. Effects of 1α, 25-dihydroxyvitamin D3 in human colon cancer cells. *Anticancer research,* 26**,** 2669-2681.

GOTTESMAN, M. M. & PASTAN, I. 1993. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annual review of biochemistry,* 62**,** 385-427.

GRADY, W. M. & CARETHERS, J. M. 2008. Genomic and epigenetic instability in colorectal cancer pathogenesis. *Gastroenterology,* 135**,** 1079-1099.

GRODEN, J. 2000. Touch and go: mediating cell-to-cell interactions and Wnt signaling in gastrointestinal tumor formation. *Gastroenterology,* 119**,** 1161-1164.

GRODSTEIN, F., NEWCOMB, P. A. & STAMPFER, M. J. 1999. Postmenopausal hormone therapy and the risk of colorectal cancer: a review and meta-analysis. *The American journal of medicine,* 106**,** 574-582.

GU, S., PAPADOPOULOU, N., GEHRING, E.-M., NASIR, O., DIMAS, K., BHAVSAR, S. K., FÖLLER, M., ALEVIZOPOULOS, K., LANG, F. & STOURNARAS, C. 2009. Functional membrane androgen receptors in colon tumors trigger pro-apoptotic responses in vitro and reduce drastically tumor incidence in vivo. *Molecular cancer,* 8**,** 114.

GU, S., PAPADOPOULOU, N., NASIR, O., FÖLLER, M., ALEVIZOPOULOS, K., LANG, F. & STOURNARAS, C. 2011. Activation of membrane androgen receptors in colon cancer inhibits the prosurvival signals Akt/bad in vitro and in vivo and blocks migration via vinculin/actin signaling. *Molecular Medicine,* 17**,** 48.

GUNTER, M. J., HOOVER, D. R., YU, H., WASSERTHEIL-SMOLLER, S., ROHAN, T. E., MANSON, J. E., HOWARD, B. V., WYLIE-ROSETT, J., ANDERSON, G. L. & HO, G. Y. 2008. Insulin, insulin-like growth factor-I, endogenous estradiol, and risk of colorectal cancer in postmenopausal women. *Cancer research,* 68**,** 329-337.

GUO, H., ZHANG, X., CUI, Y., ZHOU, H., XU, D., SHAN, T., ZHANG, F., GUO, Y., CHEN, Y. & WU, D. 2015. Taxifolin protects against cardiac hypertrophy and

fibrosis during biomechanical stress of pressure overload. *Toxicology and applied pharmacology,* 287**,** 168-177.

GUO, J.-Y., LI, X., BROWNING, J. D., ROTTINGHAUS, G. E., LUBAHN, D. B., CONSTANTINOU, A., BENNINK, M. & MACDONALD, R. S. 2004. Dietary soy isoflavones and estrone protect ovariectomized ERαKO and wild-type mice from carcinogen-induced colon cancer. *The Journal of nutrition,* 134**,** 179-182.

GUPTA, S., AFAQ, F. & MUKHTAR, H. 2002. Involvement of nuclear factor-kappa B, Bax and Bcl-2 in induction of cell cycle arrest and apoptosis by apigenin in human prostate carcinoma cells. *Oncogene,* 21**,** 3727-3738.

GUSTAFSSON, J.-A. 1999. Estrogen receptor beta--a new dimension in estrogen mechanism of action. *Journal of Endocrinology,* 163**,** 379-383.

HADJIHANNAS, M. V., BRÜCKNER, M., JERCHOW, B., BIRCHMEIER, W., DIETMAIER, W. & BEHRENS, J. 2006. Aberrant Wnt/β-catenin signaling can induce chromosomal instability in colon cancer. *Proceedings of the National Academy of Sciences,* 103**,** 10747-10752.

HAMADA, F. & BIENZ, M. 2004. The APC tumor suppressor binds to C-terminal binding protein to divert nuclear β-catenin from TCF. *Developmental cell,* 7**,** 677-685.

HANAHAN, D. & WEINBERG, R. A. 2011. Hallmarks of cancer: the next generation. *cell,* 144**,** 646-674.

HANSEN, C. 1995. *Grape Seed Extract: Procyanidolic Oligomers (PCO),* Healing Wisdom Publications.

HAO, X. P., PRETLOW, T. G., RAO, J. S. & PRETLOW, T. P. 2001. β-Catenin expression is altered in human colonic aberrant crypt foci. *Cancer Research,* 61**,** 8085-8088.

HARADA, N., TAMAI, Y., ISHIKAWA, T. O., SAUER, B., TAKAKU, K., OSHIMA, M. & TAKETO, M. M. 1999. Intestinal polyposis in mice with a dominant stable mutation of the β-catenin gene. *The EMBO journal,* 18**,** 5931-5942.

HARAGUCHI, H., OHMI, I., FUKUDA, A., TAMURA, Y., MIZUTANI, K., TANAKA, O. & CHOU, W.-H. 1997. Inhibition of aldose reductase and sorbitol accumulation by astilbin and taxifolin dihydroflavonols in Engelhardtia chrysolepis. *Bioscience, biotechnology, and biochemistry,* 61**,** 651-654.

HART, M., CONCORDET, J., LASSOT, I., ALBERT, I., DEL LOS SANTOS, R., DURAND, H., PERRET, C., RUBINFELD, B., MARGOTTIN, F. & BENAROUS, R. 1999. The F-box protein β-TrCP associates with phosphorylated β-catenin and regulates its activity in the cell. *Current Biology,* 9**,** 207-211.

HARTMAN, J., EDVARDSSON, K., LINDBERG, K., ZHAO, C., WILLIAMS, C., STRÖM, A. & GUSTAFSSON, J.-Å. 2009. Tumor repressive functions of estrogen receptor β in SW480 colon cancer cells. *Cancer research,* 69**,** 6100-6106.

References

HARWOOD, A. J. 2001. Regulation of GSK-3: a cellular multiprocessor. *Cell,* 105**,** 821-824.

HATZOGLOU, A., KAMPA, M., KOGIA, C., CHARALAMPOPOULOS, I., THEODOROPOULOS, P. A., ANEZINIS, P., DAMBAKI, C., PAPAKONSTANTI, E. A., STATHOPOULOS, E. N. & STOURNARAS, C. 2005. Membrane androgen receptor activation induces apoptotic regression of human prostate cancer cells in vitro and in vivo. *The Journal of Clinical Endocrinology & Metabolism,* 90**,** 893-903.

HE, C., ZHANG, C., HUNTER, D. J., HANKINSON, S. E., BUCK LOUIS, G. M., HEDIGER, M. L. & HU, F. B. 2009. Age at menarche and risk of type 2 diabetes: results from 2 large prospective cohort studies. *American journal of epidemiology,* 171**,** 334-344.

HEEG-TRUESDELL, E. & LABONNE, C. 2006. Wnt signaling: a shaggy dogma tale. *Current biology,* 16**,** R62-R64.

HEIJMANS, J., WIELENGA, M. C., ROSEKRANS, S. L., DE JEUDE, J. F. V. L., ROELOFS, J., GROOTHUIS, P., EDERVEEN, A., DE JONGE-MULLER, E. S., BIEMOND, I. & HARDWICK, J. C. 2014. Oestrogens promote tumorigenesis in a mouse model for colitis-associated cancer. *Gut,* 63**,** 310-316.

HEINLEIN, C. A. & CHANG, C. 2004. Androgen receptor in prostate cancer. *Endocrine reviews,* 25**,** 276-308.

HENDERSON, B. R. & FAGOTTO, F. 2002. The ins and outs of APC and β-catenin nuclear transport. *EMBO reports,* 3**,** 834-839.

HENDIFAR, A., YANG, D., LENZ, F., LURJE, G., POHL, A., LENZ, C., NING, Y., ZHANG, W. & LENZ, H.-J. 2009. Gender disparities in metastatic colorectal cancer survival. *Clinical Cancer Research,* 15**,** 6391-6397.

HEPPNER GOSS, K. & GRODEN, J. 2000. Biology of the APC tumor suppressor. *J Clin Oncol,* 18**,** 1967-79.

HERMIDA, M. A., KUMAR, J. D. & LESLIE, N. R. 2017. GSK3 and its interactions with the PI3K/AKT/mTOR signalling network. *Advances in biological regulation,* 65**,** 5-15.

HERSHBERGER, P. A., MODZELEWSKI, R. A., SHURIN, Z. R., RUEGER, R. M., TRUMP, D. L. & JOHNSON, C. S. 1999. 1, 25-Dihydroxycholecalciferol (1, 25-D3) inhibits the growth of squamous cell carcinoma and down-modulates p21Waf1/Cip1 in vitro and in vivo. *Cancer research,* 59**,** 2644-2649.

HIROHASHI, S. 1998. Inactivation of the E-cadherin-mediated cell adhesion system in human cancers. *The American journal of pathology,* 153**,** 333-339.

HOLLECZEK, B., ROSSI, S., DOMENIC, A., INNOS, K., MINICOZZI, P., FRANCISCI, S., HACKL, M., EISEMANN, N., BRENNER, H. & GROUP, E.-W. 2015. On-going improvement and persistent differences in the survival for patients with colon and rectum cancer across Europe 1999–2007–Results from the EUROCARE-5 study. *European Journal of Cancer,* 51**,** 2158-2168.

References

HOU, Y., ZHANG, S., WANG, L., LI, J., QU, G., HE, J., RONG, H., JI, H. & LIU, S. 2012. Estrogen regulates iron homeostasis through governing hepatic hepcidin expression via an estrogen response element. *Gene,* 511**,** 398-403.

HOWE, L. R., CRAWFORD, H. C., SUBBARAMAIAH, K., HASSELL, J. A., DANNENBERG, A. J. & BROWN, A. M. 2001. PEA3 is up-regulated in response to Wnt1 and activates the expression of cyclooxygenase-2. *Journal of Biological Chemistry,* 276**,** 20108-20115.

HSIEH, C. C., TRICHOPOULOS, D., KATSOUYANNI, K. & YUASA, S. 1990. Age at menarche, age at menopause, height and obesity as risk factors for breast cancer: Associations and interactions in an international case-control study. *International journal of cancer,* 46**,** 796-800.

HSIEH, F.-C., CHENG, G. & LIN, J. 2005. Evaluation of potential Stat3-regulated genes in human breast cancer. *Biochemical and biophysical research communications,* 335**,** 292-299.

HUMPHRIES, A., CERESER, B., GAY, L. J., MILLER, D. S., DAS, B., GUTTERIDGE, A., ELIA, G., NYE, E., JEFFERY, R. & POULSOM, R. 2013. Lineage tracing reveals multipotent stem cells maintain human adenomas and the pattern of clonal expansion in tumor evolution. *Proceedings of the National Academy of Sciences,* 110**,** E2490-E2499.

IACOPETTA, B. 2003. TP53 mutation in colorectal cancer. *Human mutation,* 21**,** 271-276.

IACOPETTA, B., LI, W., GRIEU, F., RUSZKIEWICZ, A. & KAWAKAMI, K. 2006. BRAF mutation and gene methylation frequencies of colorectal tumours with microsatellite instability increase markedly with patient age. *Gut,* 55**,** 1213-1213.

IBRAHIM, E. M., ZEENELDIN, A. A., EL-KHODARY, T. R., AL-GAHMI, A. M. & SADIQ, B. M. B. 2008. Past, present and future of colorectal cancer in the Kingdom of Saudi Arabia. *Saudi journal of gastroenterology: official journal of the Saudi Gastroenterology Association,* 14**,** 178.

IDOGAWA, M., YAMADA, T., HONDA, K., SATO, S., IMAI, K. & HIROHASHI, S. 2005. Poly (ADP-ribose) polymerase-1 is a component of the oncogenic T-cell factor-4/β-catenin complex. *Gastroenterology,* 128**,** 1919-1936.

IKEDA, S., KISHIDA, S., YAMAMOTO, H., MURAI, H., KOYAMA, S. & KIKUCHI, A. 1998. Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3β and β-catenin and promotes GSK-3β-dependent phosphorylation of β-catenin. *The EMBO journal,* 17**,** 1371-1384.

IWAMOTO, M., AHNEN, D. J., FRANKLIN, W. A. & MALTZMAN, T. H. 2000. Expression of β-catenin and full-length APC protein in normal and neoplastic colonic tissues. *Carcinogenesis,* 21**,** 1935-1940.

References

JACKSON-THOMPSON, J., AHMED, F., GERMAN, R. R., LAI, S. M. & FRIEDMAN, C. 2006. Descriptive epidemiology of colorectal cancer in the United States, 1998–2001. *Cancer,* 107**,** 1103-1111.

JASSAM, N., BELL, S., SPEIRS, V. & QUIRKE, P. 2005. Loss of expression of oestrogen receptor β in colon cancer and its association with Dukes' staging. *Oncology reports,* 14**,** 17-21.

JEMAL, A., CENTER, M. M., DESANTIS, C. & WARD, E. M. 2010. Global patterns of cancer incidence and mortality rates and trends. *Cancer Epidemiology and Prevention Biomarkers,* 19**,** 1893-1907.

JEON, S. H., JEONG, W.-J., CHO, J.-Y., LEE, K.-H. & CHOI, K.-Y. 2007a. Akt is involved in the inhibition of cell proliferation by EGF. *Experimental & molecular medicine,* 39**,** 491.

JEON, S. H., YOON, J.-Y., PARK, Y.-N., JEONG, W.-J., KIM, S., JHO, E.-H., SURH, Y.-J. & CHOI, K.-Y. 2007b. Axin inhibits extracellular signal-regulated kinase pathway by Ras degradation via β-catenin. *Journal of Biological Chemistry,* 282**,** 14482-14492.

JIMÉNEZ, S., GASCÓN, S., LUQUIN, A., LAGUNA, M., ANCIN-AZPILICUETA, C. & RODRÍGUEZ-YOLDI, M. J. 2016. Rosa canina extracts have antiproliferative and antioxidant effects on caco-2 human colon cancer. *PloS one,* 11**,** e0159136.

JOHNS, L. E. & HOULSTON, R. S. 2001. A systematic review and meta-analysis of familial colorectal cancer risk. *The American journal of gastroenterology,* 96**,** 2992-3003.

JOHNSON, V., VOLIKOS, E., HALFORD, S., SADAT, E. E., POPAT, S., TALBOT, I., TRUNINGER, K., MARTIN, J., JASS, J. & HOULSTON, R. 2005. Exon 3 β-catenin mutations are specifically associated with colorectal carcinomas in hereditary non-polyposis colorectal cancer syndrome. *Gut,* 54**,** 264-267.

KALER, P., AUGENLICHT, L. & KLAMPFER, L. 2009. Macrophage-derived IL-1β stimulates Wnt signaling and growth of colon cancer cells: a crosstalk interrupted by vitamin D3. *Oncogene,* 28**,** 3892-3902.

KALLAY, E., BAREIS, P., BAJNA, E., KRIWANEK, S., BONNER, E., TOYOKUNI, S. & CROSS, H. 2002. Vitamin D receptor activity and prevention of colonic hyperproliferation and oxidative stress. *Food and chemical toxicology,* 40**,** 1191-1196.

KALLAY, E., PIETSCHMANN, P., TOYOKUNI, S., BAJNA, E., HAHN, P., MAZZUCCO, K., BIEGLMAYER, C., KATO, S. & CROSS, H. S. 2001. Characterization of a vitamin D receptor knockout mouse as a model of colorectal hyperproliferation and DNA damage. *Carcinogenesis,* 22**,** 1429-1435.

KALLERGI, G., AGELAKI, S., MARKOMANOLAKI, H., GEORGOULIAS, V. & STOURNARAS, C. 2007. Activation of FAK/PI3K/Rac1 signaling controls

actin reorganization and inhibits cell motility in human cancer cells. *Cellular Physiology and Biochemistry,* 20**,** 977-986.

KAMPA, M., KOGIA, C., THEODOROPOULOS, P. A., ANEZINIS, P., CHARALAMPOPOULOS, I., PAPAKONSTANTI, E. A., STATHOPOULOS, E. N., HATZOGLOU, A., STOURNARAS, C. & GRAVANIS, A. 2006. Activation of membrane androgen receptors potentiates the antiproliferative effects of paclitaxel on human prostate cancer cells. *Molecular cancer therapeutics,* 5**,** 1342-1351.

KANCZUGA-KODA, L., WINCEWICZ, A., FUDALA, A., ABRYCKI, T., FAMULSKI, W., BALTAZIAK, M., SULKOWSKI, S. & KODA, M. 2014. E-cadherin and β-catenin adhesion proteins correlate positively with connexins in colorectal cancer. *Oncol Lett,* 7**,** 1863-1870.

KANG, W., LEE, S., JEON, E., YUN, Y.-R., KIM, K.-H. & JANG, J.-H. 2011. Emerging role of vitamin D in colorectal cancer. *World journal of gastrointestinal oncology,* 3**,** 123.

KASIBHATLA, S. & TSENG, B. 2003. Why target apoptosis in cancer treatment? *Molecular cancer therapeutics,* 2**,** 573-580.

KAUFMANN, S. H. & EARNSHAW, W. C. 2000. Induction of apoptosis by cancer chemotherapy. *Experimental cell research,* 256**,** 42-49.

KELSEY, J. L., GAMMON, M. D. & JOHN, E. M. 1993. Reproductive factors and breast cancer. *Epidemiologic reviews,* 15**,** 36.

KERBEL, R. S. 2008. Tumor angiogenesis. *New England Journal of Medicine,* 358**,** 2039-2049.

KHAN, K. H., BLANCO-CODESIDO, M. & MOLIFE, L. R. 2014. Cancer therapeutics: Targeting the apoptotic pathway. *Critical reviews in oncology/hematology,* 90**,** 200-219.

KHARE, S., CHAUDHARY, K., BISSONNETTE, M. & CARROLL, R. 2009. Aberrant crypt foci in colon cancer epidemiology. *Cancer Epidemiology: Modifiable Factors***,** 373-386.

KILKKINEN, A., VIRTAMO, J., VARTIAINEN, E., SANKILA, R., VIRTANEN, M. J., ADLERCREUTZ, H. & PIETINEN, P. 2004. Serum enterolactone concentration is not associated with breast cancer risk in a nested case-control study. *International journal of cancer,* 108**,** 277-280.

KIM, E. J., SHIN, H.-K. & PARK, J. H. Y. 2005. Genistein inhibits insulin-like growth factor-I receptor signaling in HT-29 human colon cancer cells: a possible mechanism of the growth inhibitory effect of Genistein. *Journal of medicinal food,* 8**,** 431-438.

KIM, S.-E. & CHOI, K.-Y. 2007. EGF receptor is involved in WNT3a-mediated proliferation and motility of NIH3T3 cells via ERK pathway activation. *Cellular signalling,* 19**,** 1554-1564.

References

KIM, S.-E., LEE, W.-J. & CHOI, K.-Y. 2007. The PI3 kinase-Akt pathway mediates Wnt3a-induced proliferation. *Cellular signalling,* 19**,** 511-518.

KIMELMAN, D. & XU, W. 2006a. [beta]-Catenin destruction complex: insights and questions from a structural perspective. *Oncogene,* 25**,** 7482.

KIMELMAN, D. & XU, W. 2006b. β-Catenin destruction complex: insights and questions from a structural perspective. *Oncogene,* 25**,** 7482-7491.

KINZLER, K. W. & VOGELSTEIN, B. 1996. Lessons from hereditary colorectal cancer. *Cell,* 87**,** 159-170.

KITAGAWA, M., HATAKEYAMA, S., SHIRANE, M., MATSUMOTO, M., ISHIDA, N., HATTORI, K., NAKAMICHI, I., KIKUCHI, A., NAKAYAMA, K. I. & NAKAYAMA, K. 1999. An F-box protein, FWD1, mediates ubiquitin-dependent proteolysis of β-catenin. *The EMBO journal,* 18**,** 2401-2410.

KLAUS, A. & BIRCHMEIER, W. 2008. Wnt signalling and its impact on development and cancer. *Nature Reviews Cancer,* 8**,** 387-398.

KONSTANTINOPOULOS, P., KOMINEA, A., VANDOROS, G., SYKIOTIS, G., ANDRICOPOULOS, P., VARAKIS, I., SOTIROPOULOU-BONIKOU, G. & PAPAVASSILIOU, A. 2003. Oestrogen receptor beta (ERβ) is abundantly expressed in normal colonic mucosa, but declines in colon adenocarcinoma paralleling the tumour's dedifferentiation. *European journal of cancer,* 39**,** 1251-1258.

KORINEK, V., BARKER, N., MORIN, P. J., VAN WICHEN, D., DE WEGER, R., KINZLER, K. W., VOGELSTEIN, B. & CLEVERS, H. 1997. Constitutive transcriptional activation by a β-catenin-Tcf complex in APC−/− colon carcinoma. *Science,* 275**,** 1784-1787.

KRAUS, C., LIEHR, T., HÜLSKEN, J., BEHRENS, J., BIRCHMEIER, W., GRZESCHIK, K.-H. & BALLHAUSEN, W. G. 1994. Localization of the human β-catenin gene (CTNNB1) to 3p21: a region implicated in tumor development. *Genomics,* 23**,** 272-274.

KRISHNAN, A. V. & FELDMAN, D. 2011. Mechanisms of the anti-cancer and anti-inflammatory actions of vitamin D. *Annual review of pharmacology and toxicology,* 51**,** 311-336.

KROEMER, G. & REED, J. C. 2000. Mitochondrial control of cell death. *Nature medicine,* 6**,** 513.

KRUGER, N. J. 1994. The Bradford method for protein quantitation. *Basic protein and peptide protocols.* Springer.

KWONG, K. Y., ZOU, Y., DAY, C.-P. & HUNG, M.-C. 2002. The suppression of colon cancer cell growth in nude mice by targeting [beta]-catenin/TCF pathway. *Oncogene,* 21**,** 8340.

LAKSHMAN, R., FOROUHI, N. G., SHARP, S. J., LUBEN, R., BINGHAM, S. A., KHAW, K.-T., WAREHAM, N. J. & ONG, K. K. 2009. Early age at menarche

associated with cardiovascular disease and mortality. *The Journal of Clinical Endocrinology & Metabolism,* 94**,** 4953-4960.

LAMPRECHT, S. A. & LIPKIN, M. 2003. Chemoprevention of colon cancer by calcium, vitamin D and folate: molecular mechanisms. *Nature reviews cancer,* 3**,** 601-614.

LAO, V. V. & GRADY, W. M. 2011. Epigenetics and colorectal cancer. *Nature Reviews Gastroenterology and Hepatology,* 8**,** 686-700.

LARRIBA, M. & MUNOZ, A. 2005. SNAIL vs vitamin D receptor expression in colon cancer: therapeutics implications. *British journal of cancer,* 92**,** 985-989.

LARRIBA, M. J., BONILLA, F. & MUÑOZ, A. 2010. The transcription factors Snail1 and Snail2 repress vitamin D receptor during colon cancer progression. *The Journal of steroid biochemistry and molecular biology,* 121**,** 106-109.

LATRES, E., CHIAUR, D. & PAGANO, M. 1999. The human F box protein -Trcp associates with the Cul1/Skp1 complex and regulates the stability of -catenin. *Oncogene,* 18**,** 849-854.

LEE, J., CHOI, J. & JOO, C. 2013. TGF-β1 regulates cell fate during epithelial–mesenchymal transition by upregulating survivin. *Cell death & disease,* 4**,** e714.

LEFEBVRE, A.-M., CHEN, I., DESREUMAUX, P., NAJIB, J., FRUCHART, J.-C., GEBOES, K., BRIGGS, M., HEYMAN, R. & AUWERX, J. 1998. Activation of the peroxisome proliferator-activated receptor γ promotes the development of colon tumors in C57BL/6J-APCMin/+ mice. *Nature medicine,* 4**,** 1053-1057.

LEVIN, B., LIEBERMAN, D. A., MCFARLAND, B., SMITH, R. A., BROOKS, D., ANDREWS, K. S., DASH, C., GIARDIELLO, F. M., GLICK, S. & LEVIN, T. R. 2008. Screening and surveillance for the early detection of colorectal cancer and adenomatous polyps, 2008: a joint guideline from the American Cancer Society, the US Multi-Society Task Force on Colorectal Cancer, and the American College of Radiology. *CA: a cancer journal for clinicians,* 58**,** 130-160.

LI-KUO, S. & KINZLER, K. W. 1992. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. *Science,* 256**,** 668.

LI, D., DOBROWOLSKA, G., AICHER, L. D., CHEN, M., WRIGHT, J. H., DRUECKES, P., DUNPHY, E. L., MUNAR, E. S. & KREBS, E. G. 1999. Expression of the casein kinase 2 subunits in Chinese hamster ovary and 3T3 L1 cells provides information on the role of the enzyme in cell proliferation and the cell cycle. *Journal of Biological Chemistry,* 274**,** 32988-32996.

LIANG, L., GAO, C., LUO, M., WANG, W., ZHAO, C., ZU, Y., EFFERTH, T. & FU, Y. 2013. Dihydroquercetin (DHQ) induced HO-1 and NQO1 expression against oxidative stress through the Nrf2-dependent antioxidant pathway. *Journal of agricultural and food chemistry,* 61**,** 2755-2761.

References

LIANG, P. S., CHEN, T. Y. & GIOVANNUCCI, E. 2009. Cigarette smoking and colorectal cancer incidence and mortality: Systematic review and meta-analysis. *International Journal of Cancer,* 124**,** 2406-2415.

LIEBERHERR, M. & GROSSE, B. 1994. Androgens increase intracellular calcium concentration and inositol 1, 4, 5-trisphosphate and diacylglycerol formation via a pertussis toxin-sensitive G-protein. *Journal of Biological Chemistry,* 269**,** 7217-7223.

LIEN, W.-H. & FUCHS, E. 2014. Wnt some lose some: transcriptional governance of stem cells by Wnt/β-catenin signaling. *Genes & development,* 28**,** 1517-1532.

LIEVRE, A., BACHET, J.-B., BOIGE, V., CAYRE, A., LE CORRE, D., BUC, E., YCHOU, M., BOUCHÉ, O., LANDI, B. & LOUVET, C. 2008. KRAS mutations as an independent prognostic factor in patients with advanced colorectal cancer treated with cetuximab. *Journal of clinical oncology,* 26**,** 374-379.

LING, V. 1997. Multidrug resistance: molecular mechanisms and clinical relevance. *Cancer chemotherapy and pharmacology,* 40**,** S3-S8.

LIU, C., KATO, Y., ZHANG, Z., DO, V. M., YANKNER, B. A. & HE, X. 1999. β-Trcp couples β-catenin phosphorylation-degradation and regulates Xenopus axis formation. *Proceedings of the National Academy of Sciences,* 96**,** 6273-6278.

LIU, J., LIAO, Y., MA, K., WANG, Y., ZHANG, G., YANG, R. & DENG, J. 2013. PI3K is required for the physical interaction and functional inhibition of NF-κB by β-catenin in colorectal cancer cells. *Biochemical and biophysical research communications,* 434**,** 760-766.

LIU, Y.-Z., WU, K., HUANG, J., LIU, Y., WANG, X., MENG, Z.-J., YUAN, S.-X., WANG, D.-X., LUO, J.-Y. & ZUO, G.-W. 2014. The PTEN/PI3K/Akt and Wnt/β-catenin signaling pathways are involved in the inhibitory effect of resveratrol on human colon cancer cell proliferation. *International journal of oncology,* 45**,** 104-112.

LORUSSO, P. M., ADJEI, A. A., VARTERASIAN, M., GADGEEL, S., REID, J., MITCHELL, D. Y., HANSON, L., DELUCA, P., BRUZEK, L. & PIENS, J. 2005. Phase I and pharmacodynamic study of the oral MEK inhibitor CI-1040 in patients with advanced malignancies. *Journal of clinical oncology,* 23**,** 5281-5293.

LU, Z., GHOSH, S., WANG, Z. & HUNTER, T. 2003. Downregulation of caveolin-1 function by EGF leads to the loss of E-cadherin, increased transcriptional activity of β-catenin, and enhanced tumor cell invasion. *Cancer cell,* 4**,** 499-515.

LU, Z. & HUNTER, T. 2004. Wnt-Independent?-catenin Transactivation in Tumor Developement. *Cell cycle,* 3**,** 569-571.

LUAN, N.-N., WU, L., GONG, T.-T., WANG, Y.-L., LIN, B. & WU, Q.-J. 2015. Nonlinear reduction in risk for colorectal cancer by oral contraceptive use: a meta-analysis of epidemiological studies. *Cancer Causes & Control,* 26**,** 65-78.

LUSTIG, B. & BEHRENS, J. 2003. The Wnt signaling pathway and its role in tumor development. *Journal of cancer research and clinical oncology,* 129**,** 199-221.

LYNCH, H. T. & DE LA CHAPELLE, A. 2003. Hereditary colorectal cancer. *New England Journal of Medicine,* 348**,** 919-932.

MACDONALD, B. T., TAMAI, K. & HE, X. 2009. Wnt/β-catenin signaling: components, mechanisms, and diseases. *Developmental cell,* 17**,** 9-26.

MACHEIX, J.-J. & FLEURIET, A. 1990. *Fruit phenolics*, CRC press.

MACMAHON, B., TRICHOPOULOS, D., BROWN, J., ANDERSEN, A., COLE, P., DEWAARD, F., KAURANIEMI, T., POLYCHRONOPOULOU, A., RAVNIHAR, B. & STORMBY, N. 1982. Age at menarche, urine estrogens and breast cancer risk. *International journal of cancer,* 30**,** 427-431.

MAHMOUD, N. N., BOOLBOL, S. K., BILINSKI, R. T., MARTUCCI, C., CHADBURN, A. & BERTAGNOLLI, M. M. 1997. Apc gene mutation is associated with a dominant-negative effect upon intestinal cell migration. *Cancer research,* 57**,** 5045-5050.

MANIGANDAN, K., JAYARAJ, R. L. & ELANGOVAN, N. 2014. Taxifolin ameliorates 1, 2-dimethylhydrazine induced cell proliferation and redox avulsions in mice colon carcinogenesis. *Biomedicine & Preventive Nutrition,* 4**,** 499-509.

MANIGANDAN, K., MANIMARAN, D., JAYARAJ, R. L., ELANGOVAN, N., DHIVYA, V. & KAPHLE, A. 2015. Taxifolin curbs NF-κB-mediated Wnt/β-catenin signaling via up-regulating Nrf2 pathway in experimental colon carcinogenesis. *Biochimie,* 119**,** 103-112.

MANNAVA, A. G. & TOLWINSKI, N. S. 2015. Membrane bound GSK-3 activates Wnt signaling through Disheveled and Arrow. *PloS one,* 10**,** e0121879.

MARKOWITZ, S. D. & BERTAGNOLLI, M. M. 2009. Molecular basis of colorectal cancer. *New England Journal of Medicine,* 361**,** 2449-2460.

MARTINEZ-USEROS, J. & GARCIA-FONCILLAS, J. 2016. Obesity and colorectal cancer: molecular features of adipose tissue. *Journal of translational medicine,* 14**,** 21.

MARUYAMA, K., OCHIAI, A., AKIMOTO, S., NAKAMURA, S., BABA, S., MORIYA, Y. & HIROHASHI, S. 2000. Cytoplasmic beta-catenin accumulation as a predictor of hematogenous metastasis in human colorectal cancer. *Oncology,* 59**,** 302-309.

MATUSIAK, D., MURILLO, G., CARROLL, R. E., MEHTA, R. G. & BENYA, R. V. 2005. Expression of vitamin D receptor and 25-hydroxyvitamin D3-1α-hydroxylase in normal and malignant human colon. *Cancer Epidemiology and Prevention Biomarkers,* 14**,** 2370-2376.

MCCANN, S. E., MOYSICH, K. B., FREUDENHEIM, J. L., AMBROSONE, C. B. & SHIELDS, P. G. 2002. The risk of breast cancer associated with dietary lignans

differs by CYP17 genotype in women. *The Journal of nutrition,* 132**,** 3036-3041.

MCELWAIN, M. C., DETTELBACH, M., MODZELEWSKI, R., RUSSELL, D., USKOKOVIC, M., SMITH, D., TRUMP, D. & JOHNSON, C. 1995. Antiproliferative effects in vitro and in vivo of 1, 25-dihydroxyvitamin D3 and a vitamin D3 analog in a squamous cell carcinoma model system. *Mol. Cell. Differ,* 3**,** 31-50.

MILLER, J. R., HOCKING, A. M., BROWN, J. D. & MOON, R. T. 1999. Mechanism and function of signal transduction by the Wnt/beta-catenin and Wnt/Ca2+ pathways. *Oncogene,* 18**,** 7860-7872.

MIMEAULT, M., HAUKE, R., MEHTA, P. P. & BATRA, S. K. 2007. Recent advances in cancer stem/progenitor cell research: therapeutic implications for overcoming resistance to the most aggressive cancers. *Journal of cellular and molecular medicine,* 11**,** 981-1011.

MIRABELLI-PRIMDAHL, L., GRYFE, R., KIM, H., MILLAR, A., LUCERI, C., DALE, D., HOLOWATY, E., BAPAT, B., GALLINGER, S. & REDSTON, M. 1999. β-Catenin mutations are specific for colorectal carcinomas with microsatellite instability but occur in endometrial carcinomas irrespective of mutator pathway. *Cancer research,* 59**,** 3346-3351.

MIYAKI, M., IIJIMA, T., KIMURA, J., YASUNO, M., MORI, T., HAYASHI, Y., KOIKE, M., SHITARA, N., IWAMA, T. & KUROKI, T. 1999. Frequent mutation of β-catenin and APC genes in primary colorectal tumors from patients with hereditary nonpolyposis colorectal cancer. *Cancer research,* 59**,** 4506-4509.

MODICA, S., GOFFLOT, F., MURZILLI, S., D'ORAZIO, A., SALVATORE, L., PELLEGRINI, F., NICOLUCCI, A., TOGNONI, G., COPETTI, M. & VALANZANO, R. 2010. The intestinal nuclear receptor signature with epithelial localization patterns and expression modulation in tumors. *Gastroenterology,* 138**,** 636-648. e12.

MONGA, S. P., MARS, W. M., PEDIADITAKIS, P., BELL, A., MULÉ, K., BOWEN, W. C., WANG, X., ZARNEGAR, R. & MICHALOPOULOS, G. K. 2002. Hepatocyte growth factor induces Wnt-independent nuclear translocation of β-catenin after Met-β-catenin dissociation in hepatocytes. *Cancer research,* 62**,** 2064-2071.

MORGAN, D. O. 1997. Cyclin-dependent kinases: engines, clocks, and microprocessors. *Annual review of cell and developmental biology,* 13**,** 261-291.

MORIN, P. J. 1999. β-catenin signaling and cancer. *Bioessays,* 21**,** 1021-1030.

MORIN, P. J., SPARKS, A. B., KORINEK, V., BARKER, N., CLEVERS, H., VOGELSTEIN, B. & KINZLER, K. W. 1997. Activation of β-catenin-Tcf signaling in colon cancer by mutations in β-catenin or APC. *Science,* 275**,** 1787-1790.

References

MÜLLER, T., CHOIDAS, A., REICHMANN, E. & ULLRICH, A. 1999. Phosphorylation and free pool of β-catenin are regulated by tyrosine kinases and tyrosine phosphatases during epithelial cell migration. *Journal of Biological Chemistry,* 274**,** 10173-10183.

NAGY, J. A., DVORAK, A. M. & DVORAK, H. F. 2007. VEGF-A and the induction of pathological angiogenesis. *Annu. Rev. Pathol. Mech. Dis.,* 2**,** 251-275.

NAJDI, R., HOLCOMBE, R. F. & WATERMAN, M. L. 2011. Wnt signaling and colon carcinogenesis: beyond APC. *Journal of carcinogenesis,* 10.

NAKAGAWA, K., KAWAURA, A., KATO, S., TAKEDA, E. & OKANO, T. 2005. 1α, 25-Dihydroxyvitamin D 3 is a preventive factor in the metastasis of lung cancer. *Carcinogenesis,* 26**,** 429-440.

NARAYAN, S., RAJAKUMAR, G., PROUIX, H. & SINGH, P. 1992. Estradiol is trophic for colon cancer in mice: effect on ornithine decarboxylase and c-myc messenger RNA. *Gastroenterology,* 103**,** 1823-1832.

NÄTHKE, I. S., ADAMS, C. L., POLAKIS, P., SELLIN, J. H. & NELSON, W. J. 1996. The adenomatous polyposis coli tumor suppressor protein localizes to plasma membrane sites involved in active cell migration. *The Journal of cell biology,* 134**,** 165-179.

NELSON, H. D., HUMPHREY, L. L., NYGREN, P., TEUTSCH, S. M. & ALLAN, J. D. 2002. Postmenopausal hormone replacement therapy: scientific review. *Jama,* 288**,** 872-881.

NERI, D. & BICKNELL, R. 2005. Tumour vascular targeting. *Nature Reviews Cancer,* 5**,** 436.

NEUFELD, K. L., ZHANG, F., CULLEN, B. R. & WHITE, R. L. 2000. APC-mediated downregulation of β-catenin activity involves nuclear sequestration and nuclear export. *EMBO reports,* 1**,** 519-523.

NEUHOUSER, M. L. 2004. Dietary flavonoids and cancer risk: evidence from human population studies. *Nutrition and cancer,* 50**,** 1-7.

NICHOLAS, J. 2011. Vitamin D and cancer: uncertainty persists; research continues. Oxford University Press.

NICHOLS, J. A. & KATIYAR, S. K. 2010. Skin photoprotection by natural polyphenols: anti-inflammatory, antioxidant and DNA repair mechanisms. *Archives of dermatological research,* 302**,** 71-83.

NIEHRS, C. 2012. The complex world of WNT receptor signalling. *Nature reviews Molecular cell biology,* 13**,** 767-779.

NOSHO, K., YAMAMOTO, H., MIKAMI, M., TANIGUCHI, H., TAKAHASHI, T., ADACHI, Y., IMAMURA, A., IMAI, K. & SHINOMURA, Y. 2006. Overexpression of poly (ADP-ribose) polymerase-1 (PARP-1) in the early stage of colorectal carcinogenesis. *European journal of cancer,* 42**,** 2374-2381.

OH, Y. S., KIM, E. J., SCHAFFER, B. S., KANG, Y. H., BINDERUP, L., MACDONALD, R. G. & PARK, J. H. 2001. Synthetic low-calcaemic vitamin D

3 analogues inhibit secretion of insulin-like growth factor II and stimulate production of insulin-like growth factor-binding protein-6 in conjunction with growth suppression of HT-29 colon cancer cells. *Molecular and cellular endocrinology,* 183**,** 141-149.

OI, N., CHEN, H., KIM, M. O., LUBET, R. A., BODE, A. M. & DONG, Z. 2012. Taxifolin suppresses UV-induced skin carcinogenesis by targeting EGFR and PI3K. *Cancer Prevention Research,* 5**,** 1103-1114.

OLIVER, F. J., DE LA RUBIA, G., ROLLI, V., RUIZ-RUIZ, M. C., DE MURCIA, G. & MÉNISSIER-DE MURCIA, J. 1998. Importance of poly (ADP-ribose) polymerase and its cleavage in apoptosis Lesson from an uncleavable mutant. *Journal of Biological Chemistry,* 273**,** 33533-33539.

OLTERSDORF, T., ELMORE, S. W., SHOEMAKER, A. R., ARMSTRONG, R. C., AUGERI, D. J., BELLI, B. A., BRUNCKO, M., DECKWERTH, T. L., DINGES, J. & HAJDUK, P. J. 2005. An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature,* 435**,** 677.

ORDONEZ-MORAN, P., LARRIBA, M. J., PENDAS-FRANCO, N., AGUILERA, O., GONZALEZ-SANCHO, J. M. & MUNOZ, A. 2005. Vitamin D and cancer: an update of in vitro and in vivo data. *Front Biosci,* 10**,** 2723-2749.

OSHIMA, H., OSHIMA, M., KOBAYASHI, M., TSUTSUMI, M. & TAKETO, M. M. 1997. Morphological and molecular processes of polyp formation in ApcΔ716 knockout mice. *Cancer research,* 57**,** 1644-1649.

OUGOLKOV, A. V., YAMASHITA, K., MAI, M. & MINAMOTO, T. 2002. Oncogenic β-catenin and MMP-7 (matrilysin) cosegregate in late-stage clinical colon cancer. *Gastroenterology,* 122**,** 60-71.

PÁLMER, H. G., GONZÁLEZ-SANCHO, J. M., ESPADA, J., BERCIANO, M. T., PUIG, I., BAULIDA, J., QUINTANILLA, M., CANO, A., DE HERREROS, A. G. & LAFARGA, M. 2001. Vitamin D3 promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of β-catenin signaling. *J Cell Biol,* 154**,** 369-388.

PÁLMER, H. G., SÁNCHEZ-CARBAYO, M., ORDÓÑEZ-MORÁN, P., LARRIBA, M. J., CORDÓN-CARDÓ, C. & MUÑOZ, A. 2003. Genetic signatures of differentiation induced by 1α, 25-dihydroxyvitamin D3 in human colon cancer cells. *Cancer research,* 63**,** 7799-7806.

PANDEY, K. B. & RIZVI, S. I. 2009. Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative medicine and cellular longevity,* 2**,** 270-278.

PAPADOPOULOU, N., CHARALAMPOPOULOS, I., ALEVIZOPOULOS, K., GRAVANIS, A. & STOURNARAS, C. 2008a. Rho/ROCK/actin signaling regulates membrane androgen receptor induced apoptosis in prostate cancer cells. *Experimental cell research,* 314**,** 3162-3174.

References

PAPADOPOULOU, N., CHARALAMPOPOULOS, I., ANAGNOSTOPOULOU, V., KONSTANTINIDIS, G., FÖLLER, M., GRAVANIS, A., ALEVIZOPOULOS, K., LANG, F. & STOURNARAS, C. 2008b. Membrane androgen receptor activation triggers down-regulation of PI-3K/Akt/NF-kappaB activity and induces apoptotic responses via Bad, FasL and caspase-3 in DU145 prostate cancer cells. *Molecular cancer,* 7**,** 88.

PAPADOPOULOU, N., PAPAKONSTANTI, E. A., KALLERGI, G., ALEVIZOPOULOS, K. & STOURNARAS, C. 2009. Membrane androgen receptor activation in prostate and breast tumor cells: molecular signaling and clinical impact. *IUBMB life,* 61**,** 56-61.

PAPAKONSTANTI, E. A., KAMPA, M., CASTANAS, E. & STOURNARAS, C. 2003. A rapid, nongenomic, signaling pathway regulates the actin reorganization induced by activation of membrane testosterone receptors. *Molecular Endocrinology,* 17**,** 870-881.

PARIKH, G., VARADINOVA, M., SUWANDHI, P., ARAKI, T., ROSENWAKS, Z., PORETSKY, L. & SETO-YOUNG, D. 2010. Vitamin D regulates steroidogenesis and insulin-like growth factor binding protein-1 (IGFBP-1) production in human ovarian cells. *Hormone and metabolic research,* 42**,** 754-757.

PARK, K.-S., JEON, S. H., KIM, S.-E., BAHK, Y.-Y., HOLMEN, S. L., WILLIAMS, B. O., CHUNG, K.-C., SURH, Y.-J. & CHOI, K.-Y. 2006. APC inhibits ERK pathway activation and cellular proliferation induced by RAS. *Journal of cell science,* 119**,** 819-827.

PARKIN, D. M., BRAY, F., FERLAY, J. & PISANI, P. 2005. Global cancer statistics, 2002. *CA: a cancer journal for clinicians,* 55**,** 74-108.

PARKIN, D. M. & MUIR, C. S. 1992. Cancer Incidence in Five Continents. Comparability and quality of data. *IARC scientific publications***,** 45-173.

PEIFER, M., RAUSKOLB, C., WILLIAMS, M., RIGGLEMAN, B. & WIESCHAUS, E. 1991. The segment polarity gene armadillo interacts with the wingless signaling pathway in both embryonic and adult pattern formation. *Development,* 111**,** 1029-1043.

PEREIRA, F., LARRIBA, M. J. & MUÑOZ, A. 2012. Vitamin D and colon cancer. *Endocrine-related cancer,* 19**,** R51-R71.

PIETINEN, P., STUMPF, K., MÄNNISTÖ, S., KATAJA, V., UUSITUPA, M. & ADLERCREUTZ, H. 2001. Serum enterolactone and risk of breast cancer. *Cancer Epidemiology and Prevention Biomarkers,* 10**,** 339-344.

PILZ, S., FRISCH, S., KOERTKE, H., KUHN, J., DREIER, J., OBERMAYER-PIETSCH, B., WEHR, E. & ZITTERMANN, A. 2011. Effect of vitamin D supplementation on testosterone levels in men. *Hormone and Metabolic Research,* 43**,** 223-225.

References

PINNA, L. A. 2002. Protein kinase CK2: a challenge to canons. *Journal of cell science,* 115**,** 3873-3878.

PINO, M. S. & CHUNG, D. C. 2010. The chromosomal instability pathway in colon cancer. *Gastroenterology,* 138**,** 2059-2072.

PINTO, D. & CLEVERS, H. 2005. Wnt control of stem cells and differentiation in the intestinal epithelium. *Experimental cell research,* 306**,** 357-363.

PLATZ, E. A., HANKINSON, S. E., HOLLIS, B. W., COLDITZ, G. A., HUNTER, D. J., SPEIZER, F. E. & GIOVANNUCCI, E. 2000. Plasma 1, 25-dihydroxy-and 25-hydroxyvitamin D and adenomatous polyps of the distal colorectum. *Cancer Epidemiology and Prevention Biomarkers,* 9**,** 1059-1065.

POLAKIS, P. 1997. The adenomatous polyposis coli (APC) tumor suppressor. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer,* 1332**,** F127-F147.

POLAKIS, P. 2000. Wnt signaling and cancer. *Genes & development,* 14**,** 1837-1851.

PONCE, D. P., MATURANA, J. L., CABELLO, P., YEFI, R., NIECHI, I., SILVA, E., ARMISEN, R., GALINDO, M., ANTONELLI, M. & TAPIA, J. C. 2011. Phosphorylation of AKT/PKB by CK2 is necessary for the AKT-dependent up-regulation of β-catenin transcriptional activity. *Journal of cellular physiology,* 226**,** 1953-1959.

PÖSCHL, G. & SEITZ, H. K. 2004. Alcohol and cancer. *Alcohol and alcoholism,* 39**,** 155-165.

POWELL, S. M., ZILZ, N., BEAZER-BARCLAY, Y., BRYAN, T. M., HAMILTON, S. R., THIBODEAU, S. N., VOGELSTEIN, B. & KINZLER, K. W. 1992. APC mutations occur early during colorectal tumorigenesis. *Nature,* 359**,** 235-237.

PSAHOULIA, F. H., DROSOPOULOS, K. G., DOUBRAVSKA, L., ANDERA, L. & PINTZAS, A. 2007. Quercetin enhances TRAIL-mediated apoptosis in colon cancer cells by inducing the accumulation of death receptors in lipid rafts. *Molecular cancer therapeutics,* 6**,** 2591-2599.

QAISER, F., TREMBLEY, J. H., KREN, B. T., WU, J. J., NAVEED, A. K. & AHMED, K. 2014. Protein Kinase CK2 Inhibition Induces Cell Death via Early Impact on Mitochondrial Function. *Journal of cellular biochemistry,* 115**,** 2103-2115.

QAYYUM, A., NAGI, A. H., MURTAZA, B. N. & SHAKOORI, A. R. 2016. Prevalence of Colorectal Adenocarcinoma in Northern Regions of Pakistan: A Cross Sectional Study. *Pakistan Journal of Zoology,* 48.

QI, W., WEBER, C. R., WASLAND, K. & SAVKOVIC, S. D. 2011. Genistein inhibits proliferation of colon cancer cells by attenuating a negative effect of epidermal growth factor on tumor suppressor FOXO3 activity. *BMC cancer,* 11**,** 219.

RAJAGOPALAN, H., BARDELLI, A., LENGAUER, C., KINZLER, K. W., VOGELSTEIN, B. & VELCULESCU, V. E. 2002. Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. *Nature,* 418**,** 934-934.

References

RAO, S., CUNNINGHAM, D., DE GRAMONT, A., SCHEITHAUER, W., SMAKAL, M., HUMBLET, Y., KOURTEVA, G., IVESON, T., ANDRE, T. & DOSTALOVA, J. 2004. Phase III double-blind placebo-controlled study of farnesyl transferase inhibitor R115777 in patients with refractory advanced colorectal cancer. *Journal of Clinical Oncology,* 22**,** 3950-3957.

RAY, A., LINDAHL, E. & WALLNER, B. 2012. Improved model quality assessment using ProQ2. *BMC bioinformatics,* 13**,** 224.

REYA, T. & CLEVERS, H. 2005. Wnt signalling in stem cells and cancer. *Nature,* 434**,** 843-850.

RIM, S. H., SEEFF, L., AHMED, F., KING, J. B. & COUGHLIN, S. S. 2009. Colorectal cancer incidence in the United States, 1999-2004. *Cancer,* 115**,** 1967-1976.

RINEHART, J., ADJEI, A. A., LORUSSO, P. M., WATERHOUSE, D., HECHT, J. R., NATALE, R. B., HAMID, O., VARTERASIAN, M., ASBURY, P. & KALDJIAN, E. P. 2004. Multicenter phase II study of the oral MEK inhibitor, CI-1040, in patients with advanced non-small-cell lung, breast, colon, and pancreatic cancer. *Journal of clinical oncology,* 22**,** 4456-4462.

ROBERTS, J. M. 1999. Evolving ideas about cyclins. *Cell,* 98**,** 129-132.

ROBERTS, P. J. & DER, C. J. 2007. Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. *Oncogene,* 26**,** 3291-3310.

ROBERTSON, D. J. 2012. ABC of colorectal cancer. *Gastroenterology,* 143**,** 868-869.

ROH, H., GREEN, D. W., BOSWELL, C. B., PIPPIN, J. A. & DREBIN, J. A. 2001. Suppression of β-catenin inhibits the neoplastic growth of APC-mutant colon cancer cells. *Cancer research,* 61**,** 6563-6568.

ROSIN-ARBESFELD, R., CLIFFE, A., BRABLETZ, T. & BIENZ, M. 2003. Nuclear export of the APC tumour suppressor controls β-catenin function in transcription. *The EMBO journal,* 22**,** 1101-1113.

ROSSI, M., BOSETTI, C., NEGRI, E., LAGIOU, P. & VECCHIA, C. L. 2010. Flavonoids, proanthocyanidins, and cancer risk: a network of case-control studies from Italy. *Nutrition and cancer,* 62**,** 871-877.

ROSSOUW, J. E. 2002. Writing Group for the Women's Health Initiative Investigators. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. *Jama,* 288**,** 321-333.

RUBINFELD, B., ALBERT, I., PORFIRI, E. & FIOL, C. 1996. Binding of GSK3beta to teh APC-beta-catenin complex and regulation of complex assembly. *Science,* 272**,** 1023.

RUBINFELD, B., ROBBINS, P., EL-GAMIL, M., ALBERT, I., PORFIRI, E. & POLAKIS, P. 1997. Stabilization of β-catenin by genetic defects in melanoma cell lines. *Science,* 275**,** 1790-1792.

References

RUDOLPH, A., TOTH, C., HOFFMEISTER, M., ROTH, W., HERPEL, E., JANSEN, L., MARX, A., BRENNER, H. & CHANG-CLAUDE, J. 2012. Expression of oestrogen receptor β and prognosis of colorectal cancer. *British journal of cancer,* 107**,** 831-839.

RUH, T. S., KATZENELLENBOGEN, B. S., KATZENELLENBOGEN, J. A. & GORSKI, J. 1973. Estrone interaction with the rat uterus: in vitro response and nuclear uptake. *Endocrinology,* 92**,** 125-134.

RUSSO, M., PALUMBO, R., MUPO, A., TOSTO, M., IACOMINO, G., SCOGNAMIGLIO, A., TEDESCO, I., GALANO, G. & RUSSO, G. L. 2003. Flavonoid quercetin sensitizes a CD95-resistant cell line to apoptosis by activating protein kinase Cα. *Oncogene,* 22**,** 3330-3342.

SAMOWITZ, W. S., CURTIN, K., MA, K.-N., SCHAFFER, D., COLEMAN, L. W., LEPPERT, M. & SLATTERY, M. L. 2001. Microsatellite instability in sporadic colon cancer is associated with an improved prognosis at the population level. *Cancer Epidemiology and Prevention Biomarkers,* 10**,** 917-923.

SAMOWITZ, W. S., POWERS, M. D., SPIRIO, L. N., NOLLET, F., VAN ROY, F. & SLATTERY, M. L. 1999. β-catenin mutations are more frequent in small colorectal adenomas than in larger adenomas and invasive carcinomas. *Cancer research,* 59**,** 1442-1444.

SAMUELS, Y., WANG, Z., BARDELLI, A., SILLIMAN, N., PTAK, J., SZABO, S., YAN, H., GAZDAR, A., POWELL, S. M. & RIGGINS, G. J. 2004. High frequency of mutations of the PIK3CA gene in human cancers. *Science,* 304**,** 554-554.

SÁNCHEZ, I. & DYNLACHT, B. D. New insights into cyclins, CDKs, and cell cycle control. Seminars in cell & developmental biology, 2005. Elsevier, 311-321.

SANTAMARIA, D. & ORTEGA, S. 2006. Cyclins and CDKS in development and cancer: lessons from genetically modified mice. *Frontiers in bioscience: a journal and virtual library,* 11**,** 1164-1188.

SARELA, A., MACADAM, R., FARMERY, S., MARKHAM, A. & GUILLOU, P. 2000. Expression of the antiapoptosis gene, survivin, predicts death from recurrent colorectal carcinoma. *Gut,* 46**,** 645-650.

SATO, R., SUZUKI, T., KATAYOSE, Y., MIURA, K., SHIIBA, K., MIKI, Y., KAMOGAWA, Y., YAMAMOTO, K., EGAWA, S. & UNNO, M. 2012. Aromatase in colon carcinoma. *Anticancer research,* 32**,** 3069-3075.

SCHMIDT, E. M., GU, S., ANAGNOSTOPOULOU, V., ALEVIZOPOULOS, K., FÖLLER, M., LANG, F. & STOURNARAS, C. 2012. Serum-and glucocorticoid-dependent kinase-1-induced cell migration is dependent on vinculin and regulated by the membrane androgen receptor. *The FEBS journal,* 279**,** 1231-1242.

References

SCHULMAN, B. A., CARRANO, A. C., JEFFREY, P. D. & BOWEN, Z. 2000. Insights into SCF ubiquitin ligases from the structure of the Skp1-Skp2 complex. *Nature,* 408**,** 381.

SCHULMAN, B. A., LINDSTROM, D. L. & HARLOW, E. 1998. Substrate recruitment to cyclin-dependent kinase 2 by a multipurpose docking site on cyclin A. *Proceedings of the National Academy of Sciences,* 95**,** 10453-10458.

SEBOLT-LEOPOLD, J. S. & HERRERA, R. 2004. Targeting the mitogen-activated protein kinase cascade to treat cancer. *Nature Reviews Cancer,* 4**,** 937-947.

SEMPLICI, F., MEGGIO, F., PINNA, L. A. & OLIVIERO, S. 2002. CK2-dependent phosphorylation of the E2 ubiquitin conjugating enzyme UBC3B induces its interaction with β-TrCP and enhances β-catenin degradation. *Oncogene,* 21**,** 3978.

SENGOTTUVELAN, M., SENTHILKUMAR, R. & NALINI, N. 2006. Modulatory influence of dietary resveratrol during different phases of 1, 2-dimethylhydrazine induced mucosal lipid-peroxidation, antioxidant status and aberrant crypt foci development in rat colon carcinogenesis. *Biochimica et Biophysica Acta (BBA)-General Subjects,* 1760**,** 1175-1183.

SEQUETTO, P. L., OLIVEIRA, T. T., MALDONADO, I. R., AUGUSTO, L. E. F., MELLO, V. J., PIZZIOLO, V. R., ALMEIDA, M. R., SILVA, M. E. & NOVAES, R. D. 2014. Naringin accelerates the regression of pre-neoplastic lesions and the colorectal structural reorganization in a murine model of chemical carcinogenesis. *Food and chemical toxicology,* 64**,** 200-209.

SHABAHANG, M., BURAS, R. R., DAVOODI, F., SCHUMAKER, L. M., NAUTA, R. J. & EVANS, S. R. 1993. 1, 25-Dihydroxyvitamin D3 receptor as a marker of human colon carcinoma cell line differentiation and growth inhibition. *Cancer Research,* 53**,** 3712-3718.

SHAH, S., ISLAM, M. N., DAKSHANAMURTHY, S., RIZVI, I., RAO, M., HERRELL, R., ZINSER, G., VALRANCE, M., ARANDA, A. & MORAS, D. 2006. The molecular basis of vitamin D receptor and β-catenin crossregulation. *Molecular cell,* 21**,** 799-809.

SHAHIDI, F. & AMBIGAIPALAN, P. 2015. Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects–A review. *Journal of functional foods,* 18**,** 820-897.

SHEININ, Y., KASERER, K., WRBA, F., WENZL, E., KRIWANEK, S., PETERLIK, M. & CROSS, H. S. 2000. In situ mRNA hybridization analysis and immunolocalization of the vitamin D receptor in normal and carcinomatous human colonic mucosa: relation to epidermal growth factor receptor expression. *Virchows Archiv,* 437**,** 501-507.

SHI, J., NAWAZ, H., POHORLY, J., MITTAL, G., KAKUDA, Y. & JIANG, Y. 2005. Extraction of polyphenolics from plant material for functional foods—Engineering and technology. *Food reviews international,* 21**,** 139-166.

References

SHIELDS, J. M., PRUITT, K., MCFALL, A., SHAUB, A. & DER, C. J. 2000. Understanding Ras:'it ain't over'til it's over'. *Trends in cell biology,* 10**,** 147-154.

SHIMIZU, S., NOMURA, F., TOMONAGA, T., SUNAGA, M., NODA, M., EBARA, M. & SAISHO, H. 2004. Expression of poly (ADP-ribose) polymerase in human hepatocellular carcinoma and analysis of biopsy specimens obtained under sonographic guidance. *Oncology reports,* 12**,** 821-825.

SHITOH, K., FURUKAWA, T., KOJIMA, M., KONISHI, F., MIYAKI, M., TSUKAMOTO, T. & NAGAI, H. 2001. Frequent activation of the β-catenin-Tcf signaling pathway in nonfamilial colorectal carcinomas with microsatellite instability. *Genes, Chromosomes and Cancer,* 30**,** 32-37.

SIEGEL, R., WARD, E., BRAWLEY, O. & JEMAL, A. 2011. Cancer statistics, 2011. *CA: a cancer journal for clinicians,* 61**,** 212-236.

SIERRA, J., YOSHIDA, T., JOAZEIRO, C. A. & JONES, K. A. 2006. The APC tumor suppressor counteracts β-catenin activation and H3K4 methylation at Wnt target genes. *Genes & development,* 20**,** 586-600.

SKAPER, S. D., FABRIS, M., FERRARI, V., DALLE CARBONARE, M. & LEON, A. 1997. Quercetin protects cutaneous tissue-associated cell types including sensory neurons from oxidative stress induced by glutathione depletion: cooperative effects of ascorbic acid. *Free Radical Biology and Medicine,* 22**,** 669-678.

SMIRNOFF, P., LIEL, Y., GNAINSKY, J., SHANY, S. & SCHWARTZ, B. 1999. The protective effect of estrogen against chemically induced murine colon carcinogenesis is associated with decreased CpG island methylation and increased mRNA and protein expression of the colonic vitamin D receptor. *Oncology Research Featuring Preclinical and Clinical Cancer Therapeutics,* 11**,** 255-264.

SMITS, R., RUIZ, P., DIAZ–CANO, S., LUZ, A., JAGMOHAN–CHANGUR, S., BREUKEL, C., BIRCHMEIER, C., BIRCHMEIER, W. & FODDE, R. 2000. E-cadherin and adenomatous polyposis coli mutations are synergistic in intestinal tumor initiation in mice. *Gastroenterology,* 119**,** 1045-1053.

SOLÁ, S., MORGADO, A. L. & RODRIGUES, C. M. 2013. Death receptors and mitochondria: two prime triggers of neural apoptosis and differentiation. *Biochimica et Biophysica Acta (BBA)-General Subjects,* 1830**,** 2160-2166.

SONG, D. H., DOMINGUEZ, I., MIZUNO, J., KAUT, M., MOHR, S. C. & SELDIN, D. C. 2003. CK2 phosphorylation of the armadillo repeat region of β-catenin potentiates Wnt signaling. *Journal of Biological Chemistry,* 278**,** 24018-24025.

SONG, D. H., SUSSMAN, D. J. & SELDIN, D. C. 2000. Endogenous protein kinase CK2 participates in Wnt signaling in mammary epithelial cells. *Journal of Biological Chemistry,* 275**,** 23790-23797.

References

SPARKS, A. B., MORIN, P. J., VOGELSTEIN, B. & KINZLER, K. W. 1998. Mutational analysis of the APC/β-catenin/Tcf pathway in colorectal cancer. *Cancer Research,* 58**,** 1130-1134.

STAMBOLSKY, P., TABACH, Y., FONTEMAGGI, G., WEISZ, L., MAOR-ALONI, R., SIGFRIED, Z., SHIFF, I., KOGAN, I., SHAY, M. & KALO, E. 2010. Modulation of the vitamin D3 response by cancer-associated mutant p53. *Cancer cell,* 17**,** 273-285.

STEELMAN, L. S., CHAPPELL, W. H., ABRAMS, S. L., KEMPF, R. C., LONG, J., LAIDLER, P., MIJATOVIC, S., MAKSIMOVIC-IVANIC, D., STIVALA, F., MAZZARINO, M. C., DONIA, M., FAGONE, P., MALAPONTE, G., NICOLETTI, F., LIBRA, M., MILELLA, M., TAFURI, A., BONATI, A., BASECKE, J., COCCO, L., EVANGELISTI, C., MARTELLI, A. M., MONTALTO, G., CERVELLO, M. & MCCUBREY, J. A. 2011. Roles of the Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways in controlling growth and sensitivity to therapy-implications for cancer and aging. *Aging (Albany NY),* 3**,** 192-222.

STOFFEL, E. M. & KASTRINOS, F. 2014. Familial colorectal cancer, beyond Lynch syndrome. *Clinical Gastroenterology and Hepatology,* 12**,** 1059-1068.

SUN, S.-Y., HAIL JR, N. & LOTAN, R. 2004. Apoptosis as a novel target for cancer chemoprevention. *Journal of the National Cancer Institute,* 96**,** 662-672.

SUN, X., CHEN, R.-C., YANG, Z.-H., SUN, G.-B., WANG, M., MA, X.-J., YANG, L.-J. & SUN, X.-B. 2014. Taxifolin prevents diabetic cardiomyopathy in vivo and in vitro by inhibition of oxidative stress and cell apoptosis. *Food and chemical toxicology,* 63**,** 221-232.

SUN, Y. H., GAO, X., TANG, Y. J., XU, C. L. & WANG, L. H. 2006. Androgens Induce Increases in Intracellular Calcium Via a G Protein—Coupled Receptor in LNCaP Prostate Cancer Cells. *Journal of andrology,* 27**,** 671-678.

TAIPALE, J. & BEACHY, P. A. 2001. The Hedgehog and Wnt signalling pathways in cancer. *nature,* 411**,** 349-354.

TAN, M., JING, T., LAN, K.-H., NEAL, C. L., LI, P., LEE, S., FANG, D., NAGATA, Y., LIU, J. & ARLINGHAUS, R. 2002. Phosphorylation on tyrosine-15 of p34 Cdc2 by ErbB2 inhibits p34 Cdc2 activation and is involved in resistance to taxol-induced apoptosis. *Molecular cell,* 9**,** 993-1004.

TAPIA, J., TORRES, V., RODRIGUEZ, D., LEYTON, L. & QUEST, A. 2006. Casein kinase 2 (CK2) increases survivin expression via enhanced β-catenin–T cell factor/lymphoid enhancer binding factor-dependent transcription. *Proceedings of the National Academy of Sciences,* 103**,** 15079-15084.

TAURIN, S., SANDBO, N., QIN, Y., BROWNING, D. & DULIN, N. O. 2006. Phosphorylation of β-catenin by cyclic AMP-dependent protein kinase. *Journal of Biological Chemistry,* 281**,** 9971-9976.

TEJPAR, S., BOKEMEYER, C., CELIK, I., SCHLICHTING, M., SARTORIUS, U. & VAN CUTSEM, E. 2011. Influence of KRAS G13D mutations on outcome in patients with metastatic colorectal cancer (mCRC) treated with first-line chemotherapy with or without cetuximab. *Journal of Clinical Oncology,* 29**,** 3511-3511.

TER HAAR, E., COLL, J. T., AUSTEN, D. A., HSIAO, H.-M., SWENSON, L. & JAIN, J. 2001. Structure of GSK3 [beta] reveals a primed phosphorylation mechanism. *Nature Structural & Molecular Biology,* 8**,** 593.

TETSU, O. & MCCORMICK, F. 1999. β-Catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature,* 398**,** 422-426.

THIERY-VUILLEMIN, A., NGUYEN, T., PIVOT, X., SPANO, J. P., DUFRESNNE, A. & SORIA, J. C. 2005. Molecularly targeted agents: their promise as cancer chemopreventive interventions. *Eur J Cancer,* 41**,** 2003-15.

THIERY, J. P., ACLOQUE, H., HUANG, R. Y. & NIETO, M. A. 2009. Epithelial-mesenchymal transitions in development and disease. *cell,* 139**,** 871-890.

THOMAS, C. & GUSTAFSSON, J.-Å. 2011. The different roles of ER subtypes in cancer biology and therapy. *Nature Reviews Cancer,* 11**,** 597-608.

TONG, W.-M., HOFER, H., ELLINGER, A., PETERLIK, M. & CROSS, H. S. 1999. Mechanism of antimitogenic action of vitamin D in human colon carcinoma cells: relevance for suppression of epidermal growth factor-stimulated cell growth. *Oncology Research Featuring Preclinical and Clinical Cancer Therapeutics,* 11**,** 77-84.

TOPAL, F., NAR, M., GOCER, H., KALIN, P., KOCYIGIT, U. M., GÜLÇIN, İ. & ALWASEL, S. H. 2016. Antioxidant activity of taxifolin: an activity–structure relationship. *Journal of enzyme inhibition and medicinal chemistry,* 31**,** 674-683.

TREMBLEY, J. H., UNGER, G. M., TOBOLT, D. K., KORMAN, V. L., WANG, G., AHMAD, K. A., SLATON, J. W., KREN, B. T. & AHMED, K. 2011. Systemic administration of antisense oligonucleotides simultaneously targeting CK2α and α′ subunits reduces orthotopic xenograft prostate tumors in mice. *Molecular and cellular biochemistry,* 356**,** 21-35.

TRICHOPOULOS, D., BROWN, J. & MACMAHON, B. 1987. Urine estrogens and breast cancer risk factors among post-menopausal women. *International journal of cancer,* 40**,** 721-725.

TROMPOUKI, E., BOWMAN, T. V., LAWTON, L. N., FAN, Z. P., WU, D.-C., DIBIASE, A., MARTIN, C. S., CECH, J. N., SESSA, A. K. & LEBLANC, J. L. 2011. Lineage regulators direct BMP and Wnt pathways to cell-specific programs during differentiation and regeneration. *Cell,* 147**,** 577-589.

UMAR, A., BOLAND, C. R., TERDIMAN, J. P., SYNGAL, S., CHAPELLE, A. D. L., RÜSCHOFF, J., FISHEL, R., LINDOR, N. M., BURGART, L. J. & HAMELIN, R. 2004. Revised Bethesda Guidelines for hereditary nonpolyposis

colorectal cancer (Lynch syndrome) and microsatellite instability. *Journal of the National Cancer Institute,* 96**,** 261-268.

VALENTA, T., HAUSMANN, G. & BASLER, K. 2012. The many faces and functions of β-catenin. *The EMBO journal,* 31**,** 2714-2736.

VAN AKEN, E., DE WEVER, O., DA ROCHA, C. A. & MAREEL, M. 2001. Defective E-cadherin/catenin complexes in human cancer. *Virchows Archiv,* 439**,** 725-751.

VAN AMERONGEN, R. & NUSSE, R. 2009. Towards an integrated view of Wnt signaling in development. *Development,* 136**,** 3205-3214.

VAN CUTSEM, E., CERVANTES, A., NORDLINGER, B. & ARNOLD, D. 2014. Metastatic colorectal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Annals of oncology,* 25**,** iii1-iii9.

VAN CUTSEM, E., KÖHNE, C.-H., LÁNG, I., FOLPRECHT, G., NOWACKI, M. P., CASCINU, S., SHCHEPOTIN, I., MAUREL, J., CUNNINGHAM, D. & TEJPAR, S. 2011. Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor KRAS and BRAF mutation status. *Journal of Clinical Oncology,* 29**,** 2011-2019.

VAN CUTSEM, E., NORDLINGER, B., CERVANTES, A. & GROUP, E. G. W. 2010. Advanced colorectal cancer: ESMO Clinical Practice Guidelines for treatment. *Annals of oncology,* 21**,** v93-v97.

VAN DE WETERING, M., SANCHO, E., VERWEIJ, C., DE LAU, W., OVING, I., HURLSTONE, A., VAN DER HORN, K., BATLLE, E., COUDREUSE, D. & HARAMIS, A.-P. 2002. The β-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell,* 111**,** 241-250.

VAN DER MEER, I. M., KARAMALI, N. S., BOEKE, A. J. P., LIPS, P., MIDDELKOOP, B. J., VERHOEVEN, I. & WUISTER, J. D. 2006. High prevalence of vitamin D deficiency in pregnant non-Western women in The Hague, Netherlands. *The American journal of clinical nutrition,* 84**,** 350-353.

VAN LENTHE, F. J., KEMPER, C. & VAN MECHELEN, W. 1996. Rapid maturation in adolescence results in greater obesity in adulthood: the Amsterdam Growth and Health Study. *The American journal of clinical nutrition,* 64**,** 18-24.

VAN LOO, G., SAELENS, X., MATTHIJSSENS, F., SCHOTTE, P., BEYAERT, R., DECLERCQ, W. & VANDENABEELE, P. 2002. Caspases are not localized in mitochondria during life or death. *Cell Death Differ,* 9**,** 1207-1211.

VAN VEELEN, W., LE, N. H., HELVENSTEIJN, W., BLONDEN, L., THEEUWES, M., BAKKER, E. R., FRANKEN, P. F., VAN GURP, L., MEIJLINK, F. & VAN DER VALK, M. A. 2011. β-catenin tyrosine 654 phosphorylation increases Wnt signalling and intestinal tumorigenesis. *Gut***,** gut. 2010.233460.

VENOOK, A. 2005. Critical evaluation of current treatments in metastatic colorectal cancer. *The Oncologist,* 10**,** 250-261.

References

VERMA, U. N., SURABHI, R. M., SCHMALTIEG, A., BECERRA, C. & GAYNOR, R. B. 2003. Small interfering RNAs directed against β-catenin inhibit the in vitro and in vivo growth of colon cancer cells. *Clinical Cancer Research,* 9**,** 1291-1300.

VLADIMIROV, Y. A., PROSKURNINA, E., DEMIN, E., MATVEEVA, N., LUBITSKIY, O., NOVIKOV, A., IZMAILOV, D. Y., OSIPOV, A., TIKHONOV, V. & KAGAN, V. 2009. Dihydroquercetin (taxifolin) and other flavonoids as inhibitors of free radical formation at key stages of apoptosis. *Biochemistry (Moscow),* 74**,** 301-307.

VOULGARI, C., PAPADOGIANNIS, D. & TENTOLOURIS, N. 2010. Diabetic cardiomyopathy: from the pathophysiology of the cardiac myocytes to current diagnosis and management strategies. *Vascular health and risk management,* 6**,** 883.

WALL, M. A., COLEMAN, D. E., LEE, E., IÑIGUEZ-LLUHI, J. A., POSNER, B. A., GILMAN, A. G. & SPRANG, S. R. 1995. The structure of the G protein heterotrimer Giα1β1γ2. *Cell,* 83**,** 1047-1058.

WANG, X.-D., MENG, M.-X., GAO, L.-B., LIU, T., XU, Q. & ZENG, S. 2009. Permeation of astilbin and taxifolin in Caco-2 cell and their effects on the P-gp. *International journal of pharmaceutics,* 378**,** 1-8.

WANG, X. & STUDZINSKI, G. P. 2001. Activation of extracellular signal-regulated kinases (ERKs) defines the first phase of 1, 25-dihydroxyvitamin D3-induced differentiation of HL60 cells. *Journal of cellular biochemistry,* 80**,** 471-482.

WANG, Y.-H., WANG, W.-Y., CHANG, C.-C., LIOU, K.-T., SUNG, Y.-J., LIAO, J.-F., CHEN, C.-F., CHANG, S., HOU, Y.-C. & CHOU, Y.-C. 2006. Taxifolin ameliorates cerebral ischemia-reperfusion injury in rats through its anti-oxidative effect and modulation of NF-kappa B activation. *Journal of biomedical science,* 13**,** 127-141.

WARGOVICH, M. J., BROWN, V. R. & MORRIS, J. 2010. Aberrant crypt foci: the case for inclusion as a biomarker for colon cancer. *Cancers,* 2**,** 1705-1716.

WASTI, S., ROBINSON, S., AKHTAR, Y., KHAN, S. & BADARUDDIN, N. 1993. Characteristics of menopause in three socioeconomic urban groups in Karachi, Pakistan. *Maturitas,* 16**,** 61-69.

WEHR, E., PILZ, S., BOEHM, B. O., MÄRZ, W. & OBERMAYER-PIETSCH, B. 2010. Association of vitamin D status with serum androgen levels in men. *Clinical endocrinology,* 73**,** 243-248.

WEIDMANN, A. E. 2012. Dihydroquercetin: More than just an impurity? *European journal of pharmacology,* 684**,** 19-26.

WEISENBERGER, D. J., SIEGMUND, K. D., CAMPAN, M., YOUNG, J., LONG, T. I., FAASSE, M. A., KANG, G. H., WIDSCHWENDTER, M., WEENER, D. & BUCHANAN, D. 2006. CpG island methylator phenotype underlies sporadic

microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. *Nature genetics,* 38**,** 787-793.

WELCH, C., SANTRA, M. K., EL-ASSAAD, W., ZHU, X., HUBER, W. E., KEYS, R. A., TEODORO, J. G. & GREEN, M. R. 2009. Identification of a protein, G0S2, that lacks Bcl-2 homology domains and interacts with and antagonizes Bcl-2. *Cancer research,* 69**,** 6782-6789.

WENZEL, U., KUNTZ, S., BRENDEL, M. D. & DANIEL, H. 2000. Dietary flavone is a potent apoptosis inducer in human colon carcinoma cells. *Cancer research,* 60**,** 3823-3831.

WESTON, C. R. & DAVIS, R. J. 2001. Signaling specificity--a complex affair. *Science,* 292**,** 2439-2440.

WEYANT, M. J., CAROTHERS, A. M., MAHMOUD, N. N., BRADLOW, H. L., REMOTTI, H., BILINSKI, R. T. & BERTAGNOLLI, M. M. 2001. Reciprocal expression of ERα and ERβ is associated with estrogen-mediated modulation of intestinal tumorigenesis. *Cancer Research,* 61**,** 2547-2551.

WHITE, B. D., CHIEN, A. J. & DAWSON, D. W. 2012. Dysregulation of Wnt/β-catenin signaling in gastrointestinal cancers. *Gastroenterology,* 142**,** 219-232.

WILLETT, W. C. 2005. Diet and cancer: an evolving picture. *Jama,* 293**,** 233-234.

WINSTON, J. T., STRACK, P., BEER-ROMERO, P., CHU, C. Y., ELLEDGE, S. J. & HARPER, J. W. 1999. The SCFβ-TRCP–ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IκBα and β-catenin and stimulates IκBα ubiquitination in vitro. *Genes & development,* 13**,** 270-283.

WONG, N. A. C. S. & PIGNATELLI, M. 2002. β-catenin—a linchpin in colorectal carcinogenesis? *The American journal of pathology,* 160**,** 389-401.

WONG, S. C. C., LO, S. F. E., CHEUNG, M. T., NG, K. O. E., TSE, C. W., LAI, B. S. P., LEE, K. C. & LO, Y. D. 2004. Quantification of plasma β-catenin mRNA in colorectal cancer and adenoma patients. *Clinical Cancer Research,* 10**,** 1613-1617.

WOODGETT, J. R. 2001. Judging a protein by more than its name: GSK-3. *Sci Stke,* 100**,** 1-12.

WU, H., XU, L., CHEN, J., HU, J., YU, S., HU, G., HUANG, L., CHEN, X., YUAN, X. & LI, G. 2012. Association of estrogen receptor beta variants and serum levels of estradiol with risk of colorectal cancer: a case control study. *BMC cancer,* 12**,** 276.

WU, K., FESKANICH, D., FUCHS, C. S., WILLETT, W. C., HOLLIS, B. W. & GIOVANNUCCI, E. L. 2007. A nested case–control study of plasma 25-hydroxyvitamin D concentrations and risk of colorectal cancer. *Journal of the National Cancer Institute,* 99**,** 1120-1129.

XIE, X., FENG, J., KANG, Z., ZHANG, S., ZHANG, L., ZHANG, Y., LI, X. & TANG, Y. 2017. Taxifolin protects RPE cells against oxidative stress-induced apoptosis. *Molecular vision,* 23**,** 520.

References

XIONG, H., ZHANG, Z.-G., TIAN, X.-Q., SUN, D.-F., LIANG, Q.-C., ZHANG, Y.-J., LU, R., CHEN, Y.-X. & FANG, J.-Y. 2008. Inhibition of JAK1, 2/STAT3 signaling induces apoptosis, cell cycle arrest, and reduces tumor cell invasion in colorectal cancer cells. *Neoplasia,* 10**,** 287-297.

XU, H., POSNER, G. H., STEVENSON, M. & CAMPBELL, F. C. 2010. Apc MIN modulation of vitamin D secosteroid growth control. *Carcinogenesis,* 31**,** 1434-1441.

XU, W. & KIMELMAN, D. 2007. Mechanistic insights from structural studies of β-catenin and its binding partners. *Journal of cell science,* 120**,** 3337-3344.

XUE, B., DUNKER, A. K. & UVERSKY, V. N. 2012. The roles of intrinsic disorder in orchestrating the Wnt-pathway. *Journal of Biomolecular Structure and Dynamics,* 29**,** 843-861.

YAHYA, S. & REHAN, N. 2002. Age, pattern and symptoms of menopause among rural women of Lahore. *Age (Years),* 42**,** 51-60.

YAMADA, T., MORI, Y., HAYASHI, R., TAKADA, M., INO, Y., NAISHIRO, Y., KONDO, T. & HIROHASHI, S. 2003. Suppression of intestinal polyposis in Mdr1-deficient ApcMin/+ mice. *Cancer research,* 63**,** 895-901.

YAMADA, T., TAKAOKA, A. S., NAISHIRO, Y., HAYASHI, R., MARUYAMA, K., MAESAWA, C., OCHIAI, A. & HIROHASHI, S. 2000. Transactivation of the multidrug resistance 1 gene by T-cell factor 4/β-catenin complex in early colorectal carcinogenesis. *Cancer research,* 60**,** 4761-4766.

YAMASHITA, K., NAKAZATO, H., ITO, K., OUGOLKOV, A., TAKAHASHI, Y., MAI, M. & MINAMOTO, T. 2004. Effect of adjuvant immunochemotherapy with PSK for colon cancer patients showing oncogenic β-catenin activation in primary tumor. *Journal of Clinical Oncology,* 22**,** 3650-3650.

YANAGAWA, S. I., MATSUDA, Y., LEE, J. S., MATSUBAYASHI, H., SESE, S., KADOWAKI, T. & ISHIMOTO, A. 2002. Casein kinase I phosphorylates the Armadillo protein and induces its degradation in Drosophila. *The EMBO journal,* 21**,** 1733-1742.

YANG, C. S., LANDAU, J. M., HUANG, M.-T. & NEWMARK, H. L. 2001. Inhibition of carcinogenesis by dietary polyphenolic compounds. *Annu Rev Nutr,* 21**,** 381-406.

YANG, Q., JIAN, J., KATZ, S., ABRAMSON, S. B. & HUANG, X. 2012. 17β-Estradiol inhibits iron hormone hepcidin through an estrogen responsive element half-site. *Endocrinology,* 153**,** 3170-3178.

YEH, J. J., ROUTH, E. D., RUBINAS, T., PEACOCK, J., MARTIN, T. D., SHEN, X. J., SANDLER, R. S., KIM, H. J., KEKU, T. O. & DER, C. J. 2009. KRAS/BRAF mutation status and ERK1/2 activation as biomarkers for MEK1/2 inhibitor therapy in colorectal cancer. *Molecular cancer therapeutics,* 8**,** 834-843.

References

YLIKOMI, T., LAAKSI, I., LOU, Y.-R., MARTIKAINEN, P., MIETTINEN, S., PENNANEN, P., PURMONEN, S., SYVÄLÄ, H., VIENONEN, A. & TUOHIMAAM, P. 2002. Antiproliferative action of vitamin D.

YU, M. & GRADY, W. M. 2012. Therapeutic targeting of the phosphatidylinositol 3-kinase signaling pathway: novel targeted therapies and advances in the treatment of colorectal cancer. *Therapeutic advances in gastroenterology,* 5**,** 319-337.

YUN, M.-S., KIM, S.-E., JEON, S. H., LEE, J.-S. & CHOI, K.-Y. 2005. Both ERK and Wnt/β-catenin pathways are involved in Wnt3a-induced proliferation. *Journal of cell science,* 118**,** 313-322.

ZAFFARONI, N., PENNATI, M., COLELLA, G., PEREGO, P., SUPINO, R., GATTI, L., PILOTTI, S., ZUNINO, F. & DAIDONE, M. 2002. Expression of the anti-apoptotic gene survivin correlates with taxol resistance in human ovarian cancer. *Cellular and Molecular Life Sciences CMLS,* 59**,** 1406-1412.

ZELENIUCH-JACQUOTTE, A., ADLERCREUTZ, H., SHORE, R., KOENIG, K., KATO, I., ARSLAN, A. & TONIOLO, P. 2004. Circulating enterolactone and risk of breast cancer: a prospective study in New York. *British journal of cancer,* 91**,** 99-105.

ZHANG, B., OUGOLKOV, A., YAMASHITA, K., TAKAHASHI, Y., MAI, M. & MINAMOTO, T. 2006. β-catenin and ras oncogenes detect most human colorectal cancer. *Cancer Research Institute report,* 2003**,** 102.

ZHANG, X., JIANG, F., LI, P., LI, C., MA, Q., NICOSIA, S. V. & BAI, W. 2005. Growth suppression of ovarian cancer xenografts in nude mice by vitamin D analogue EB1089. *Clinical Cancer Research,* 11**,** 323-328.

ZHANG, Y., HAN, Y., ZHENG, R., YU, J.-H., MIAO, Y., WANG, L. & WANG, E.-H. 2012. Expression of Frat1 correlates with expression of β-catenin and is associated with a poor clinical outcome in human SCC and AC. *Tumor Biology,* 33**,** 1437-1444.

ZHANG, Y., LIU, S., MICKANIN, C., FENG, Y., CHARLAT, O., MICHAUD, G. A., SCHIRLE, M., SHI, X., HILD, M. & BAUER, A. 2011. RNF146 is a poly (ADP-ribose)-directed E3 ligase that regulates axin degradation and Wnt signalling. *Nature cell biology,* 13**,** 623.

ZHANG, Z. R., AL ZAHARNA, M., WONG, M. M.-K., CHIU, S.-K. & CHEUNG, H.-Y. 2013. Taxifolin enhances andrographolide-induced mitotic arrest and apoptosis in human prostate cancer cells via spindle assembly checkpoint activation. *PLoS One,* 8**,** e54577.

ZHAO, X. & FELDMAN, D. 1993. Regulation of vitamin D receptor abundance and responsiveness during differentiation of HT-29 human colon cancer cells. *Endocrinology,* 132**,** 1808-1814.

ZHENG, W., WONG, K. E., ZHANG, Z., DOUGHERTY, U., MUSTAFI, R., KONG, J., DEB, D. K., ZHENG, H., BISSONNETTE, M. & LI, Y. C. 2012. Inactivation of the vitamin D receptor in APCmin/+ mice reveals a critical role

for the vitamin D receptor in intestinal tumor growth. *International journal of cancer,* 130**,** 10-19.

ZHOU, G., MYERS, R., LI, Y., CHEN, Y., SHEN, X., FENYK-MELODY, J., WU, M., VENTRE, J., DOEBBER, T., FUJII, N., MUSI, N., HIRSHMAN, M. F., GOODYEAR, L. J. & MOLLER, D. E. 2001. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest,* 108**,** 1167-74.

Tausat Jahan

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RESEARCH Open Access

Growth inhibition and apoptosis in colorectal cancer cells induced by Vitamin D-Nanoemulsion (NVD): involvement of Wnt/β-catenin and other signal transduction pathways

Suhail Razak[1,2*], Tayyaba Afsar[2], Ali Almajwal[2], Iftikhar Alam[2] and Sarwat Jahan[1]

Abstract

Background: More than the two decades, the question of whether vitamin D has a role in cancer frequency, development, and death has been premeditated in detail. Colorectal, breast, and prostate cancers have been a scrupulous spot of center, altogether, these three malignancies report for approximately 35% of cancer cases and 20% of cancer demises in the United States, and as such are a chief public health apprehension. The aim was to evaluate antitumor activity of Vitamin D-Nanoemulsion (NVD) in colorectal cancer cell lines and HCT116 xenograft model in a comprehensive approach.

Methods: Two human colorectal cancer cell lines HCT116 and HT29 (gained from College of Pharmacy, King Saud University, KSA were grown. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazoliumbromide protocol were performed to show the impact of NVD and β-catenin inhibitor (FH535) on the viability of HCT116 and HT29 cell lines. Apoptosis/cell cycle assay was performed. Analysis was done with a FACScan (Becton–Dickinson, NJ). About 10,000 cells per sample were harvested and Histograms of DNA were analyzed with ModiFitLT software (verity Software House, ME, USA). Western blotting and RT-PCR were performed for protein and gene expression respectively in in vitro and in vivo.

Results: We found that NVD induced cytotoxicity in colorectal cells in a dose-dependent manner and time dependent approach. Further, our data validated that NVD administration of human colorectal cancer HCT116 and HT29 cells resulted in cell growth arrest, alteration in molecules regulating cell cycle operative in the G2 phase of the cell cycle and apoptosis in a dose dependent approach. Further our results concluded that NVD administration decreases expression of β-*catenin* gene, *AKT* gene *and Survivin* gene and protein expression in in vitro and in vivo.

Conclusion: Our findings suggest that targeting β-catenin gene may encourage the alterations of cell cycle and cell cycle regulators. Wnt/*β-catenin* signaling pathway possibly takes part in the genesis and progression of colorectal cancer cells through regulating cell cycle and the expression of cell cycle regulators.

Keywords: Vitamin D, CRC, Wnt/β-catenin, Cell cycle, NVD

*Correspondence: ruhail12345@yahoo.com
[1] Department of Animal Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan
Full list of author information is available at the end of the article

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A Prospective Evaluation of Serum Vitamin D (1, 25(OH)$_2$ D$_3$) and Endogenous Sex Hormone Levels in Colorectal Cancer Patients

Suhail Razak[1,2]*, *Iftikhar Alam*[2], *Tayyaba Afsar*[2], *Mahmoud M. A. Abulmeaty*[2], *Ali Almajwal*[2] *and Sarwat Jahan*[1]

[1] Department of Animal Sciences, Quaid-i-Azam University, Islamabad, Pakistan, [2] Department of Community Health Sciences, College of Applied Medical Sciences, King Saud University, Riyadh, Saudi Arabia

Background: Data on 25-OH VD concentrations and the associated factors in colorectal cancer (CRC) patients are scarce and need to be investigated.

Methods: A total of 200 CRC patients participated in this cross-sectional study conducted in Pakistan. Socio-demographic and other health data were collected in a pretested questionnaire. Serum measurements of Vitamin D (1, 25(OH)$_2$ D$_3$) levels and hormones were performed. Association of age, sex, primary site, effects of hormone therapy and stage of disease and selected reproductive health indicators on vitamin D status were primarily scrutinized by univariate analysis.

Results: Mean age of the population was 55.3 years (±15.6; Range: 20–90 years). Estradiol concentration was considerably elevated in young females compared to young male patients ($p < 0.001$). The concentrations of FSH, LH testosterone and estradiol were significantly lower in post-menopausal female CRC patients as compared to their male counterparts of old age (p, for all trends < 0.05). Both LH and FSH showed significant gender difference but only in older patients. Level of estrogen was markedly decreased in older post-menopausal CRC patients compared to premenopausal CRC patients, which might be associated with CRC progression. In the group of women, who "ever used hormone therapy" had differences of statistical significance (p, for all trends < 0.05) in their mean serum 25-OH VD concentrations, while in the group of women who "never used hormone therapy" had non-significant differences in their mean serum 25-OH VD concentrations (p, for all trends > 0.05). High 25-OH VD concentrations were observed in women who had their menarche at the age of 15 years or more. Nulliparous women had the highest mean 25-OH VD concentrations as compared to unparious or multiparious women. In addition, women having their menopause at 40–44 years of age had the highest 25-OH VD concentrations, although the difference was not significant ($p = 0.08$). Women who "never used any oral contraceptive" had higher 25-OH VD concentrations as compared to those "whoever used oral contraceptives."

Conclusion: Our findings suggest that vitamin D has a positive effect on the development of CRC through the mediation of hormones. Other health and reproductive traits that affect hormone levels may have an indirect effect on the development of CRC.

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All India Institute of Medical Sciences, Rishikesh, India
Francesco Caiazza,
University of California, San Francisco, United States

***Correspondence:**
Suhail Razak
ruhail12345@yahoo.com

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BMC Cancer

RESEARCH ARTICLE Open Access

Taxifolin, a natural flavonoid interacts with cell cycle regulators causes cell cycle arrest and causes tumor regression by activating Wnt/ β -*catenin* signaling pathway

Suhail Razak[1,2*], Tayyaba Afsar[2], Asad Ullah[1], Ali Almajwal[2], Musaed Alkholief[3], Aws Alshamsan[3] and Sarwat Jahan[1]

Abstract

Background: New approaches for the prevention of colon cancer perseveres an essential necessity. Though, resistance to existing chemo-preventive drugs is moderately predominant in colon carcinogenesis. Taxifolin (dihydroquercetin) is a flavononol, have shown virile biological activities against few cancers. The current study was designed to investigate and equate antitumor activity of Taxifolin (TAX) in colorectal cancer cell lines and in HCT116 xenograft model in a comprehensive approach.

Methods: Two human colorectal cancer cell lines HCT116 and HT29, were used. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazoliumbromide (MMT) protocol was performed to elucidate the impact of TAX and β- catenin inhibitor (FH535) on the viability of HCT116 and HT29 cell lines. Apoptosis /cell cycle assay was performed. Data interpretation was done with a FACScan (Becton Dickinson, NJ). About 1×10^4 cells per sample were harvested. Histograms of DNA were analyzed with ModiFitLT software (verity Software House, ME, USA). Western blotting and RT-PCR were performed for protein and gene expression respectively in in vitro and in vivo.

Results: We found that TAX induced cytotoxicity in colorectal cells in a dose-dependent manner and time dependent approach. Further, our data validated that administration of TAX to human colorectal cancer HCT116 and HT29 cells resulted in cell growth arrest, variation in molecules controlling cell cycle operative in the G2 phase of the cell cycle and apoptosis in a concentration dependent approach. Further our results concluded that TAX administration decreases expression of *β-catenin* gene, *AKT* gene *and Survivin* gene and protein expression in in vitro and in vivo.

Conclusion: Our findings proposed that targeting *β-catenin* gene may encourage the alterations of cell cycle and cell cycle regulators. Wnt/*β-catenin* signaling pathway possibly takes part in the genesis and progression of colorectal cancer cells through regulating cell cycle and the expression of cell cycle regulators.

Keywords: Colorectal cancer, Wnt/ β-catenin, Taxifolin and cell cycle

* Correspondence: ruhail12345@yahoo.com
[1]Department of Animal Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan
[2]Department of Community Health Sciences, College of Applied Medical Sciences, King Saud University, Riyadh, Saudi Arabia
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BMC Medical Genetics

RESEARCH ARTICLE Open Access

Screening and computational analysis of colorectal associated non-synonymous polymorphism in *CTNNB1* gene in Pakistani population

Suhail Razak[1,2*], Nousheen Bibi[3], Javid Ahmad Dar[4], Tayyaba Afsar[2], Ali Almajwal[2], Zahida Parveen[5] and Sarwat Jahan[1]

Abstract

Background: Colorectal cancer (CRC) is categorized by alteration of vital pathways such as β-catenin *(CTNNB1)* mutations, *WNT* signaling activation, tumor protein 53 (*TP53*) inactivation, *BRAF*, Adenomatous polyposis coli (*APC*) inactivation, *KRAS*, dysregulation of epithelial to mesenchymal transition (*EMT*) genes, *MYC* amplification, etc. In the present study an attempt was made to screen *CTNNB1* gene in colorectal cancer samples from Pakistani population and investigated the association of *CTNNB1* gene mutations in the development of colorectal cancer.

Methods: 200 colorectal tumors approximately of male and female patients with sporadic or familial colorectal tumors and normal tissues were included. DNA was extracted and amplified through polymerase chain reaction (PCR) and subjected to exome sequence analysis. Immunohistochemistry was done to study protein expression. Molecular dynamic (MD) simulations of $CTNNB1^{WT}$ and mutant S33F and T41A were performed to evaluate the stability, folding, conformational changes and dynamic behaviors of CTNNB1 protein.

Results: Sequence analysis revealed two activating mutations (S33F and T41A) in exon 3 of *CTNNB1* gene involving the transition of C.T and A.G at amino acid position 33 and 41 respectively (p.C33T and p.A41G). Immuno-histochemical staining showed the accumulation of β-catenin protein both in cytoplasm as well as in the nuclei of cancer cells when compared with normal tissue. Further molecular modeling, docking and simulation approaches revealed significant conformational changes in the N-terminus region of normal to mutant *CTNNB1* gene critical for binding with Glycogen synthase kinase 3-B (GSK3) and transducin containing protein1 (TrCp1).

Conclusion: Present study on Pakistani population revealed an association of two non-synonymous polymorphisms in the *CTNNB1* gene with colorectal cancer. These genetic variants led to the accumulation of the *CTNNB1*, a hallmark of tumor development. Also, analysis of structure to function alterations in *CTNNB1* gene is crucial in understanding downstream biological events.

Keywords: CTNNB1, Colorectal cancer, Immunohistochemistry, DNA, Molecular modeling, And protein expression

* Correspondence: ruhail12345@yahoo.com; smarazi@ksu.edu.sa
[1]Reproductive physiology lab, Department of Animal Sciences Quaid-i-Azam University, Islamabad, Pakistan
[2]Department of Community Health Sciences, College of Applied Medical Sciences, King Saud University, Riyadh, KSA
Full list of author information is available at the end of the article