Use of Whole Exome Sequencing to Diagnose Autosomal Recessive Immunodeficiency Disorder

A thesis submitted in the partial fulfillment of the requirements for the degree of Master of Philosophy in Biotechnology

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Faculty of Biological Sciences

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And mankind have not been given of knowledge except a little.

Al Qur'an

Surah Al-Israh

Ayah 85

Declaration

I hereby declare that the work presented in this thesis is my own effort, except where otherwise acknowledged, and that the thesis is my own composition. No part of this thesis has been previously published or presented for any other degree or certificate.

Yousra Wali

Reg. No. 02272013008

Signature: Yousra Wali

Certificate of Approval

This is to certify that the research work presented in this thesis, entitled "Use of Whole Exome Sequencing to Diagnose Autosomal Recessive Immunodeficiency Disorder" was conducted by Miss Yousra Wali under the supervision of Prof. Dr. Muhammad Naeem.

No part of this thesis has been submitted anywhere else for any degree. This thesis is submitted to the **Department of Biotechnology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan** in partial fulfillment of the requirements for the Degree of Master of Philosophy in the field of Biotechnology from **Department of Biotechnology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan**.

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Dedication

This work is dedicated to my beloved parents for their continuous support, care, love, and encouragement throughout my life. Their efforts and struggles have helped me to pursue my academic goals.

AKNOWLEDGEMENTS

All praises be to Allah, the lord of all worlds, the most Merciful, the most Beneficent, Who has granted man with countless blessings. Respect and regards for our Holy prophet Hazrat Muhammad (peace be upon him) whose teachings and guidance are a source of light for all humanity.

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My deepest gratitude goes to my parents for all their care, love, prayers, and sacrifices for educating me and my siblings beyond their resources. Their continuous support and appreciations have been my biggest source of motivation in life.

YOUSRA WALI

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LIST OF ABBREVIATIONS

Abbreviation	Description
%	Percentage
°C	Degree centigrade
Amps	Amperes
ACMG	American college of medical genetics and genomics
AICD	Apoptosis induced cell death
AR	Autosomal recessive
bp	Base pair
BLAST	Basic local alignment search tool
BWA	Burrows-wheeler-alignment
cDNA	Complementary deoxyribonucleic acid
CD	Cluster of differentiation
CID	Combined immunodeficiency
CV	Consensus value
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
FOXP3	Forkhead box protein 3
G	Guanine
GATK	Genome analysis toolkit
gnomAD	Genome aggregation database
HCL	Hydrochloric acid
HGMD	Human gene mutation database
HSF	Human splice finder
HSCT	Hematopoietic stem cell transplant
IEI	Inborn errors of immunity
IFNγ	Interferon gamma

Ig	Immunoglobulin
IL	Interleukin
IL2R	Interleukin-2 receptor
IL2RA	Interleukin-2 receptor alpha
IPEX	Immunedysregulation polyendocrinopathy enteropathy x-linked
IUIS	International union of immune societies
JAK	Janus kinase
Kb	kilobases
LOF	Loss of function
mL	Milliliter
mM	Millimolar
MAPK	Mitogen-activated protein kinase
MaxEnt	Maximum entropy
$MgCl_2$	Magnesium chloride
NaCl	Sodium chloride
NCBI	National center for biotechnology information
OMIM	Online mendelian inheritance in man
PCR	Polymerase chain reaction
PID	Primary immunodeficiency
PK	Proteinase K
Polyphen 2	Polymorphism phenotyping v2
RNA	Ribonucleic acid
rpm	Rotations per minute
SDS	Sodium dodecyl sulphate
SIFT	Sorting intolerant from tolerant
STAT	Signal transducer and activator of transcription
SROOGLE	Splicing regulation online graphical engine

T	Thymine
TBE	Tris base/boric acid/ethylene diamine tetra acetic acid
TE	Tris base/ ethylene diamine tetra acetic acid
Tm	Melting temperature
TF	Transcription factor
UV	Ultraviolet
VCF	Variant call format
WES	Whole exome sequencing
WHO	World health organization

ABSTRACT

Immunodeficiency type 41 with lymphoproliferation and autoimmunity, also known as CD25 deficiency is a rare genetic disease of immune dysregulation showing autosomal recessive pattern of inheritance. The disease phenotype highly resembles Immune Dysregulation Polyendocrinopathy Enteropathy X-linked (IPEX) syndrome presenting with increased susceptibility to bacterial, fungal, viral infections, lymphoproliferation, eczema, enteropathy with villous atrophy and a variety of autoimmune manifestations. The disease is caused due to mutations in the *IL2RA* gene located on chromosome 10p15.1 coding for the α-subunit (CD25) of the IL-2 receptor complex expressed on the surface of a variety of T-cells, especially the FOXP3+ CD25+ regulatory T cells. The α- subunit along with the β-subunit and γ-subunit make the high efficiency receptor for IL-2. The IL-2 is an important cytokine affecting a variety of T-cell subsets. It is crucial for the development, proliferation and regulation of regulatory T-cells. Defects in the *IL2RA* gene cause an absence or deterioration of the IL-2 receptor leading to loss of T-cell regulation and immune tolerance resulting in the paradoxical combination of immunodeficiency and autoimmunity in those affected. This study was aimed at diagnosing an autosomal recessive disease showing symptoms of immunodeficiency. Whole exome sequencing (Illumina Hiseq 4000) was performed for screening of mutations in the genomic DNA of the patient. A novel splice donor site variant c.64+1G>A was identified in the IL-2Rα gene in the proband. The in silico analysis of the variant predicted it to be pathogenic, however protein expression studies are recommended to understand the effect of variant on function and structure of the protein and its role in the disease.

Chapter 1

INTRODUCTION

1.1. Immunodeficiency diseases

Immunodeficiency is a condition in which the ability of immune system to respond to pathogens and other harmful agents such as cancer cells is either weakened or is totally absent. The immune system is a large and complex network of biological processes, molecules and cells that protects an organism from diseases. Immunity is an organism's ability to protect itself against pathogens or toxins. The immune system detects and responds to a broad variety of pathogens and differentiates them from the organisms own healthy tissues.

The human immune system has two lines of defense:

- Nonspecific/Innate immunity
- Specific/Adaptive immunity.

1.1.1. Innate immunity:

Innate immunity is the kind of immunity, that an organism is born with. It acts as the first line of defense against foreign pathogens. It gives a predetermined response to a broad variety of stimuli. The innate immune response is quick, short lived and nonspecific, which means its response to a pathogen do not change according to the specific bacteria or virus detected.

Unlike adaptive immunity, the innate immune response has no immunologic "memory" i.e., it cannot remember or initiate a more effective response upon exposure to the same pathogen in future.

It comprises of:

- Physical barriers: The physical barriers tend to protect the body from foreign invasion, by preventing foreign bodies to come in direct contact with the internal system, e.g. skin and eyelashes.
- Chemical barriers: these are the defense mechanisms that use chemical means to destroy harmful agent. Examples are mucous, tears etc.
- Cellular defenses: the cellular defenses identify harmful substances and act to destroy or neutralize it. for example, macrophages engulf and destroy any foreign entity that might have crossed the physical and chemical barriers successfully and entered the body.

1.1.2. Adaptive immune system

Although the innate immune system is efficient in rapidly sensing and eliminating pathogens, it has a little diversity for recognition of the diverse molecular patterns that pathogens can have and has a limited recognition capability. The immense variability of pathogen molecular patterns and their ability to mutate frequently to escape innate immunity has led to the gradual evolution of the adaptive immune system (Cooper & Alder, 2006).

The adaptive immunity also known as acquired immune system is specific and antigen dependent immunity. The receptors of innate immune system show lower diversity unlike the receptors of the adaptive immune system which are encoded by a large array of gene segments. These gene segments are combined in various sequences to encode for any receptor and this process of differential combination is called somatic recombination. The huge array of gene segments which enable an individual to produce an efficient immune response against a diverse set of pathogenic elements is a result of gene duplications that happened over the course of vertebrate evolution.

Unlike innate immune system, the adaptive immunity is generated only after exposure to a specific antigen from a pathogen or after vaccination. This immune response comes into play when innate immunity cannot control the invading pathogen. The unique feature of adaptive immunity is that it memorizes the antigen it is once exposed to and initiate a more efficient and rapid immune response to the pathogen upon subsequent exposure. The two

immune responses are not exclusive of each other and are quite interlinked, with defects in one system resulting in leaving the host vulnerable to pathogens (Bonilla & Oettgen, 2010).

The effectors cells of the adaptive immunity include the antibody producing B lymphocytes which originate from the bone marrow, and the T lymphocytes, named so because they mature in the thymus. The B cells once activated, give a humoral response, secreting antibodies. The T cell tend to give a cell mediated response.

The process of formation of receptors in both B and T cells is a complex process of random rearrangement of multiple DNA segments that encode the complementarity determining regions of the receptors. This rearrangement of DNA segments occurs early during the development of B and T cells before any exposure to the antigen. The process gives rise to a vast repertoire of antigen specificities, around 10^8 T receptors and 10^{10} antigen receptors which are sufficient for the number of different pathogens likely to be faced during our lifetime (Arstila et al., 1999).

1.1.3. T-cells

After being born from stem cells (hematopoietic) in the bone marrow, the developing T cells travel to the thymus where they mature and differentiate into different T cell types. The process of differentiation continues after the T cells have left the thymus.

There are three major subtypes of T cells, the **CD8+ killer/cytotoxic T cells**, the **CD4+ helper T cells and the regulatory T cells**, each with their specific immune function. The cytotoxic T cells have the ability to kill virus-infected cells and cells with malignant properties. CD8+ T cells also recruits other types of cells during an immune response by secreting small signaling molecules called "cytokines".

The CD4+ T cells perform a helping function by further activating cytotoxic T cells along with Memory B cells, thereby enhancing the immune system. The helper T cells have further many subtypes depending upon the type of cytokine they secrete, and the respective adaptive immune response generated.

The regulatory T cells are a subpopulation of T cells which tend to keep a check and downregulate the immune responses thereby maintaining homeostasis and ensuring self-tolerance.

Immunodeficiency diseases affect the normal functioning of immune system, weakening its potential to fight invading pathogens or abnormal cells thereby resulting in recurrent bacterial, viral or fungal infections, lymphomas and other cancers may also develop. The malfunction of immune system also results in infections being more severe and long lasting.

1.2. Classification of immunodeficiency diseases

The immunodeficiency diseases can be due to primary defect in genes, called primary immunodeficiency (PID) or it may be of a non-inherent nature, resulting from a secondary condition or cause (secondary immunodeficiency). The primary immunodeficiency diseases (now more commonly referred to as inborn errors of immunity) generally become evident in infancy and are relatively rare.

The secondary immunodeficiency diseases, on the other hand, normally develop later in life and may be caused by malnutrition, another infection, like HIV, or drugs that induce immune suppression.

1.2.1. Primary immunodeficiency/inborn errors of immunity (IEI)

The primary immunodeficiency diseases arise due to genetic defects which affect or alter the development, regulation, or function of the immune cells. Inborn Errors of Immunity (IEI) comprises disorders showing different clinical manifestations depending on the type of the immune defect present. Some of the most common immune impairments manifested in Immunodeficiencies include frequent and long-lasting infections, granulomatosis, autoimmunity, atopy, and malignancy.

Immune defects involving defects in B-cells show bacterial infections as a key feature, while an immune defect involving both T and B-cells exhibit infections with a variety of pathogens (e.g., viral, fungal, and bacterial).

Lymphoproliferation is observed in some of the PIDs as a characteristic feature while others show granulomas (cutaneous, GI tract related or respiratory) caused by immune dysregulation. Thus, the clinical manifestations and involvement of different systems can give an indication toward the type of the IEI present.

PIDs are relatively rare, but they provide great insights into the functioning of immune system. Studies of the genetics and functioning of PIDs led to the division of immune system into cell mediated and humoral immunity (Notarangelo et al., 2004). The clinical diagnosis and treatment of PIDs also present a great challenge.

The epidemiology of the immunodeficiency disease depends on the type of PID. For example, IgA deficiency is relatively a common disease (1 in 223 to 1 in 1000) (Yel, 2010) while SCID is a disorder of rare occurrence (1 in 5800).

1.2.2. Classification of IEI

The first inherited immunodeficiencies were reported in the 1950s (Atkinson, 2012) and since then, their number is increasing day by day. Bruton, in 1952 reported a boy with pneumococcal infections who was recovered by administration of external gamma globulins as his serum lacked gamma globulins (Castagnoli, Delmonte, & Notarangelo, 2022).

Early in the 1970s, a committee was established under the World health organization (WHO) supervision with the aim to catalog the already known and newly emerging primary immunodeficiencies. The committee, now known as International Union of Immunological Societies (IUIS) comprises of members from different immunological societies all over the world and includes clinical immunologists, researchers in immunology and scientists. According to its constitution, the main objectives of the organization are to promote cooperation in immunology at an international level and to play leading role in the progress in immunology. This umbrella organization tends to keep an up-to-date catalog of the primary immune disorders and their genetic causes being discovered around the globe.

The IUIS committee keeps clinicians and research communities around the world updated about the new discoveries being made in the field by publishing an updated report after

nearly every two years. There were more than 350 primary immune-deficiency diseases classified under nine different disease categories by the International union of immunologic societies, in the 2017 IUIS report (Picard et al., 2018).

With advances in diagnostic techniques and sequencing technologies, the discovery of new PIDs, in the last ten years has occurred at exponential rates. The IUIS committee enlisted 191 PIDs in their 2011 report and the count increased to 430 in their recent report in 2019 (Bousfiha et al., 2020).

The international union of immunological societies expert committee published their recently updated classification of Inborn errors of Immunity in January 2020 and it quickly went out of date due to the several newly reported variants associated with novel inborn errors of immunity (Tangye et al., 2021). The committee has published its latest update of the classification of inborn errors of immunity in May 2022. The latest update has made an addition of 55 novel mendelian defects related to diseases of immunity. The updated list contains 485 inborn errors of immunity (Tangye et al., 2022).

According to the most recent report of the committee, the inborn errors of immunity are classified into 10 different categories each of which is then divided into subcategories. The major categories are as listed below:

1. Cellular and Humoral immunodeficiency disorders
2. Syndromic combined Immunodeficiencies (CID)
3. Predominantly antibody deficiencies
4. Diseases related to immune dysregulation
5. Phagocytic diseases
6. Defects of innate immunity
7. Autoinflammatory diseases
8. Complement deficiencies
9. Bone marrow failure
10. Phenocopies of inborn errors of immunity

The International union of immunologic societies aims at creating awareness about PIDs and their treatment.

Mostly PID follow a monogenic mendelian inheritance, but some diseases may show a polygenic inheritance.

1.2.2.1. Diseases of immune dysregulation

The immune cells are strictly monitored and regulated during their central and peripheral development. Any error in the molecular control of these cells can result in undesirable and abnormal proliferation of immune cells leading to autoimmunity, severe inflammation and malignancy. Inborn errors of immunity have long been associated with higher susceptibility to infections due to a weakened immune response and autoimmune phenomena was previously considered inexplicable in patients with IEI. However, inborn error of autoimmunity may cause a malfunction of the control mechanism of B and T cells resulting in autoimmune manifestations (Bussone & Mouthon, 2009).

1.2.2.1.1 Inborn errors of immunity with autoimmune manifestations

As important as it is for the immune system to detect and respond to any foreign pathogen, it is also important to recognize self-antigens and remain unresponsive to them. This capability of immune system to differentiate self -antigens from foreign antigens and therefore not produce immune response against them is called immune tolerance. When the immune cells can not differentiate between self and on-self-antigens, they tend to initiate immune response against an organisms own healthy cells, called autoimmunity. The response against self, leads to different diseases called autoimmune diseases. Two of such diseases of immune dysregulation with autoimmune features are discussed below.

1.2.2.1.2. Immune dysregulation, polyendocrinopathy, enteropathy x-linked syndrome (IPEX)

Immune dysregulation, polyendocrinopathy, enteropathy, x-linked syndrome is an inborn error of immunity with X-linked inheritance and rare occurrence, characterized by autoimmunity in multiple organs. It arises due to mutations in fork head box p3 or FOXP3. FOXP3 is a transcription factor and is also known as the master regulator of regulatory T cells' development and function.

1.2.2.1.3. Clinical manifestations

The clinical symptoms appear early in life, mostly within the first month and can lead to fatality if not treated. A triad of manifestations has been observed in majority of the patients with the disease; severe diarrhea, diabetes type-1 and atopic dermatitis (Barzaghi, Passerini, & Bacchetta, 2012).

Autoimmune enteropathy is considered an important attribute of IPEX, with patients presenting with severe diarrhea. Enteropathy begins in the early days of life while breastfeeding, thus indicating to be independent of any foreign food or nutrient intolerance. This leads to severe malnutrition due to ineffective absorption of nutrients from the diet and patients show failure to thrive, often requiring intravenous administration of nutrition. Other manifestations usually observed with enteropathy are vomiting, colitis, and gastritis.

Diabetes mellitus type-1 is another manifestation present in majority of patients with IPEX. According to histological studies, immune mediated damage of pancreas may be the reason for the condition.

Skin manifestations also show an early onset where dermatitis can be of any kind, for example atopic dermatitis, psoriasiform, etc. Besides, other autoimmune manifestations have also been observed; thyroiditis, arthritis, cytopenia, nephropathy, splenomegaly, and lymphadenopathy.

Infections due to various pathogens may further worsen the clinical manifestations. Some of the most frequent infections are, pneumonia, GI infections, respiratory infections and dermal infections (Ruemmele et al., 2009). The disease shares immune manifestations with a variety of pediatric immune dysregulations and patients showing the disease symptoms may not contain mutations in the corresponding gene, FOXP3. Such diseases are clinically termed as IPEX-like diseases (Baxter et al., 2021)

1.2.2.1.4. Genetics of IPEX

The disease was first studied as an X- linked disorder with diarrhea in 1982 in a large family where 19 males were affected in 5 generations (Powell, Buist, & Stenzel, 1982).

Later it was confirmed that IPEX syndrome is caused by mutations in the FOXP3 gene, which consists of 12 exons and codes for 431 amino acids.

1.2.2.2. CD25 deficiency

Immunodeficiency type 41 also known as CD25 deficiency is a rare and complex disorder of immune dysregulation showing autosomal recessive pattern of inheritance. The clinical phenotypes include chronic infections (bacterial, viral, and fungal) and severe autoimmunity showing high resemblance IPEX syndrome.

1.2.2.2.1. History

The disease was first described in 1997 by Sharfe et al., as an immunodeficiency marked by frequent infections and reduced number of peripheral T cells and abnormal proliferation (Sharfe, Dadi, Shahar, & Roifman, 1997). The patient was a male child born to consanguineous parents suffering from cytomegalovirus infection and esophageal candidiasis. He was prone to recurrent bacterial, fungal, and viral infections, suffered from chronic diarrhea and failed to thrive.

At the age of 8 months, evident lymphadenopathy and splenomegaly was observed despite normal liver function. The patient had normal IgM level, but IgA levels were low. A notable reduction in CD3+ and CD4+ cells was observed leading to a CD4/CD8 ratio of 1:1, which normally should be 2:1. Moreover, remarkable immune dysfunction was proven as the child's immune system did not reject an allogeneic skin graft. Bone marrow transplant was done resulting in complete remission of symptoms.

A study carried out by Caudy et al. presented a child with symptoms highly overlapping with IPEX syndrome and no mutation in FOXP3 gene but having a mutated CD25 gene and lacking CD25 expression. The patient showed severe diarrhea, respiratory issues, and diabetes mellitus as clinical manifestation. Cytomegalovirus was detected in urine and blood samples.

The CD4/CD8 ratio in serum was nearly 1:1. Swollen lymph nodes, enlarged spleen and eczema developed at the age of 2. At 3 years of age the patient developed severe hemolytic anemia and hypothyroidism. In the next 3 years, he had several recurrent infections with

lymphadenopathy, diarrhea, enteropathy, and eczema. His clinical symptoms were suggestive of an IPEX-like syndrome, and the candidate gene for IPEX i.e., FOXP3 was sequenced, but there were no mutations observed. The IL-10 expression of the patient was compared with a FOXP3 deficient patient, to check whether expression of FOXP3 is necessary for IL-10 expression. A defective expression of IL-10 was observed in CD4 lymphocytes of the FOXP3+ patient while the FOXP3 deficient patient had normal IL-10 expression.

It was observed that the patient's CD4 lymphocytes do not express CD25, and sequence studies were carried out for CD25 gene, and a frameshift mutation was observed. Further studies confirmed the absence of CD25 on cells of the patient.

The disease was associated with IPEX-like phenotype by Caudy et al. in 2007, as the clinical manifestations of the reported patient showed high resemblance with IPEX syndrome which is caused by mutations in the FOXP3 gene (Caudy, Reddy, Chatila, Atkinson, & Verbsky, 2007).

In a study carried out in 2013, the immunological mechanisms leading to the clinical manifestations associated with CD25 deficiency were studied in a patient with a mutated CD25 gene. The patient was an 8-year-old female born to consanguineous parents who developed IPEX-like symptoms in her first month. Over the first five to six years, she suffered from severe diarrhea, eczema, recurrent infections (including CMV), autoimmune thyroiditis and dermatitis.

The sequence analysis of *IL2RA* gene of the patient revealed a substitution reaction in the 4^{th} exon of the gene. The parents were found to be heterozygous for the same mutation (Goudy et al., 2013).

1.2.2.2.2. Genetics of CD25 deficiency

The CD25 deficiency (OMIM #606367) is an inborn error of immunity caused by defects in the *IL2RA* gene (OMIM 147730; chr10p15.1) that codes for the alpha subunit (CD25) of the interleukin-2 receptor complex, resulting in severe autoimmune features and frequent infections. The disease is inherited in an autosomal recessive manner.

1.2.2.2.3. Disease prevalence

The disease is quite rare, with very few cases reported in literature. The first case was reported in 1997 by sharfe et al. The rarity may also be attributed to lack of proper diagnostic tools, early onset, and lethality of the disease.

According to Orphanet database, the prevalence is <1/1000,000. As of 2020, nine cases of CD25 deficiency have been reported from seven different families with pronounced lymphoproliferation, frequent bacterial, viral and fungal infections and autoimmune enteritis being the most common manifestations among the patients (Lai et al., 2020).

1.2.2.2.4. Disease etiology

It is a genetic disorder arising as a result of mutations in the gene, *IL2Rα* (OMIM 147730: chr10p15.1), which codes for the alpha subunit of interleukin-2 receptor complex. Along with the alpha subunit, the high affinity IL2 receptor is composed of β (122) and γ (132) subunits. The alpha subunit has been suggested to concentrate IL2 on the surface of the cell and present it to the beta and gamma subunits or that it combines with IL-2 and induces conformational changes in IL2 to stabilize IL-2Rαβγ complex (Wang, Rickert, & Garcia, 2005) .

1.3. Interleukin-2 (IL-2)

Interleukin-2 is a member of the cytokine interleukins family and the first cytokine to be cloned. At the time of discovery, it was described as an autocrine growth factor to ex-vivo T cells. Later studies in mice and humans led to the discovery of its role in the stimulation of T cell proliferation and producing memory and effector T cells. These studies resulted in studies on the capability of IL-2 to stimulate anti-tumor responses in cancer patients.

In the late 20th century, several studies created doubts on the idea that the sole function of interleukin-2 is the stimulation of T cell immune responses. When T cells from normal individuals and humans with autoimmune manifestations and autoimmune mouse models were comparatively studied, it was found that the later produced comparatively less IL-2 upon invitro stimulation. Moreover, the IL-2 knockout mice developed systemic

autoimmune features instead of immunodeficiency which was expected of them (Sadlack et al., 1993).

Further studies of inhibiting IL2 (Setoguchi, Hori, Takahashi, & Sakaguchi, 2005) and CD25 (Mchugh & Shevach, 2002) also supported the knockout mouse experiments by resulting in initiation of autoimmune responses. Moreover, later studies on rare families with CD25 deficiency also showed autoimmune diseases as an undeniable clinical manifestation. All these studies led to the suggestion that IL-2 not only stimulates T cell responses but also keeps a check on these responses and maintain self-tolerance and any defect in its production or function results not only in immunodeficiency but also autoimmunity (Rosenberg, 2014).

Now it is well established that Interleukin 2 is critical for the development, thymic selection, expansion in the periphery and survival of Treg cells. It also serves as growth factor for other T cells such as activated T cells and NK cells and has impacts on variety of immune cells (Czaja, 2021). The biological attributes of Interleukin-2 in immune cells is summarized in the table below.

Table 1.1. Effects of IL-2 cytokine on immune cells

Functions of Interleukin-2	**Biological effects**
Acts as a growth factor (for T cells)	Enhance proliferation of regulatory T-cells (Malek & Bayer, 2004). Enhance cytotoxic effector T cells and NK cells , proliferation of B-cells, CD8+ T cell subsets and CD4+ T cells (Kehrl, Muraguchi, Goldsmith, & Fauci, 1985), (Miyawaki, Suzuki, Butler, & Cooper, 1987) (Arenas-ramirez, Woytschak, & Boyman, 2015).
Promotes differentiation of Treg cells	Development of Treg cells in the thymus (Nelson, 2004). Enhance Treg expansion (Malek, Yu, Vincek, Scibelli, & Kong, 2002).

	Aids in the conversion of CD4+ T-cells to Tregs cells (Boyman & Sprent, 2012).
Activation of Treg cells	Binds to IL2R on regulatory T cells, leads to the activation of STAT5 in regulatory T-cells (Antov, Yang, Vig, Baltimore, & Van Parijs, 2003). Enhance expression of FOXP3 (Feng et al., 2014), (Chinen et al., 2016). Enhance expression of IL-10 and IL-4 (Tsuji-Takayama et al., 2008), (Cote-Sierra et al., 2004), Suppresses IFN-γ production(Seder, Paul, Davis, & Fazekas de St. Groth, 1992) (Hsieh, Heimberger, Gold, O'Garra, & Murphy, 1992). Enhance suppressor activity of Tregs (Thornton, Donovan, Piccirillo, & Shevach, 2004).
Treg cells' survival	Promotes survival in culture (Mack et al., 2020) , Crucial for the existence of regulatory T cells (Papiernik, De Moraes, Pontoux, Vasseur, & Pénit, 1998).
Self-tolerance	Deficiency of IL-2 leads to autoimmune diseases in animal models (Sadlack et al., 1993)(Sadlack et al., 1995).
Growth and apoptotic death of CD8+ T cells	Enhance natural killer cells, memory T cells and CD8+ T cells (Boyman, Cho, & Sprent, 2010). Helps in AICD of CD8+ T-cells.

1.3.1. The interleukin-2 receptor

The interleukin-2 can have its biological effects on immune cells only if it successfully binds to its corresponding receptors on the cell surface. The IL-2 receptors have three different forms based on the differential affinity of binding, a high affinity receptor, a receptor with intermediate affinity and a low affinity receptor (O'Donnell et al., 1996). The

receptors are made of glycoprotein subunits: an α-subunit made of 272 amino acids, a β-subunit weighing 75 kDa (Filion, 1984) and a γ-subunit weighing 64 kDa. They are also known as CD25, CD122 and CD132 respectively.

The trimer of all the three subunits make the high affinity receptor complex for the binding of IL-2 (Sharon, Klausner, Cullen, Chizzonite, & Leonard, 1986). The high affinity receptor is expressed constitutively on Treg cell surface. The β-subunit and γ-subunit together make the intermediate affinity receptor and the low affinity receptor is the monomer of α-subunit.

The γ-subunit is normally expressed on most lymphoid cells, however the expression for the other two subunits is strictly regulated. The IL2Rα is expressed on only 5% of lymphoid cells and in a very low level. (Morris & Waldmann, 2000). The intermediate affinity receptor is usually expressed on conventional T cells and natural killer cells, while the low affinity receptor is mostly expressed by dendritic cells.

The varying expression of the different affinity receptors on different immune cell alters the immune response in conditions with low and high levels of Interleukin-2. When IL-2 is available in low levels, the Treg cells expressing the tripartite high affinity Interleukin-2 receptor constitutively will outperform the cells expressing the intermediate affinity receptor. When Il-2 is available in high levels, the cells with intermediate affinity receptor will also be induced to perform IL-2 related activities.

This phenomena is being utilized by incorporating high Interleukin-2 doses to initiate immunity against malignancies and a low dose treatment with IL-2 is being used to treat immune tolerance related complications (Klatzmann & Abbas, 2015).

Each subunit performs a different function. The α-subunit helps in binding of the intrleukin-2 signaling molecule and enhances the sensitivity of the receptor to the cytokine.

The β subunit and γ subunit together help carrying the signal to the interior of the cell and transduction of the signal via the JAK1 and JAK3 kinase pathways (Witthuhn et al., 1994).

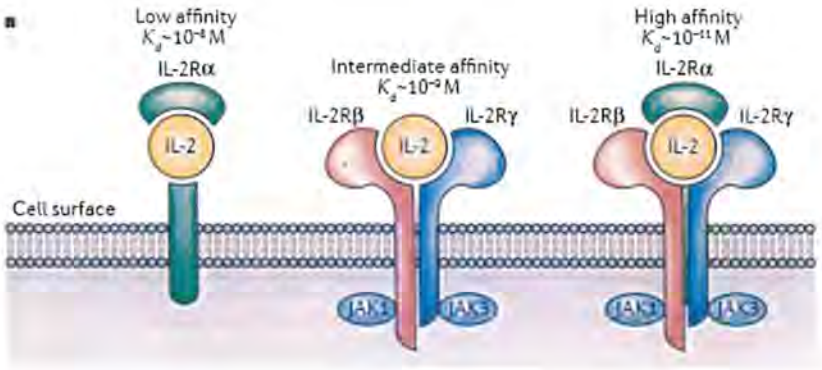

Fig1.1. Structure of IL-2 receptors (Spolski, Li, & Leonard, 2018)

The high affinity trimeric receptor is not expressed on normal/inactivated lymphocytes but shows a high expression levels on activated T cells and regulatory T cells (Robb, Munck, & Smith, 1981) (Waldmann, 1986).

1.4. The regulatory T cells

Among the different mechanism of immunologic self-tolerance, the regulatory t cell mediated tolerance, and even their existence as distinct cells remained controversial for a long time due to the absence of reliable markers and confusion in their role at the molecular level. The regulatory T cells are a subpopulation of T cells which are responsible for immune tolerance and homeostasis. These cells are also referred to as immunosuppressive cells as they tend to downregulate the activity and proliferation of effector T cells play an important role in preventing autoimmunity. Every physiologic process needs to be properly regulated and checked so that it may not turn into a pathologic event. The regulatory T cells serve this cause and prevent our defense system from causing harm to our own cells.

The basic mechanism that ensures self-antigen tolerance is the selective clonal deletion of T cells with self-reactive properties in the thymus (Sakaguchi, 2000). However, few T cells may escape this deletion and may lead to autoimmune reactions in peripheral cells. Although self-reactive T cells are present in every individual but very few people (only 5%) develop autoimmune diseases which indicates that a peripheral self-tolerance

mechanism is also present to curb the pathogenic T cells in the periphery. A failure in this regulation leads to autoimmune diseases and studies have shown that regulatory T cells downregulate the activity of these self-reactive T cells that may have escaped other mechanisms of self-tolerance (Maloy & Powrie, 2001).

Based on ontogeny, the regulatory T cells are normally subdivided into two broad categories, adaptive or induced regulatory cells (iTregs) and naturally occurring regulatory T cells. The naturally occurring regulatory T cells are presumed to originate in the thymus under normal conditions, while the induced T regulatory cells are secondarily induced upon antigenic stimulation and in tolerogenic conditions (Shalev, Schmelzle, Robson, & Levy, 2011).

Few examples of iTregs involved in curbing peripheral T cell responses are counter-regulatory IFN-γ- producing Th1 cells, Tr1 cells and IL-4 producing Th2 cells. The naturally occurring regulatory cells includes a population of CD4+ CD25+ Treg cells which play an important role in immune regulation.

Recent studies have shown that the CD4+CD25+ regulatory T cells are not only able to curb organ specific autoimmune conditions but also play a more general role in the regulation of the immune system thereby preventing immune responses against enteric microbial flora (Read et al., 2001) or even following a microbial infection (Kullberg et al., 2002) (Hesse et al., 2004). They are also involved in transplantation tolerance (Sakaguchi, 2004) and have been associated with maternal tolerance to fetus in recent studies (Aluvihare, Kallikourdis, & Betz, 2004).

These cells express the biomarkers, CD4, FOXP3 and CD25 and are believed to differentiate from the Cd4+ lineage. The transforming growth factor beta cytokine is necessary for the differentiation of these cells from naïve CD4+ cells (Chen, 2011).

1.4.1. Regulatory T cells and immune tolerance

Historically, early observations in the 1970s had led to the acceptance of T cells being linked with autoimmune diseases. Nishizuka and Sakakura observed that neonatal thymectomy in healthy mice resulted in severe autoimmune pathology in specific organs

indicating the obvious involvement of cell population originating from thymus in self-tolerance (Nishizuka Y, 1969). Similar results were observed in adult rats upon thymectomy and sublethal irradiation (Penhale, Farmer, McKenna, & Irvine, 1973). Inoculation of CD4+ t cells from healthy mice suppressed autoimmune manifestations in both the neonatal and adult irradiated experimental mice. Moreover, disease was induced in T cell deficient mice upon transfer of CD4+ T cells from the sick mice.

These studies established that CD4+ T cells not only act as inducers of autoimmune disease but also tend to inhibit such disease and that two different sub-populations of CD4+ T cells are present in the periphery of normal mice, one responsible for inducing autoimmunity and another suppressor cells which suppress self-reactive T cells to inhibit autoimmunity. These suppressor cells and their mechanism of action remained unexplained for years due to failure to find the cell markers specific to this population and ambiguity in their mechanism of suppression.

Later studies revealed that a subpopulation of CD4+ cells highly expressing the IL-2 receptor alpha chain, CD25 on the surface, was responsible for the maintenance of self-tolerance in the periphery. These cells constituted 10% of the peripheral CD4+ T cells and their absence results in a wide range of autoimmune diseases in normal mice; the restoration of CD4+ CD25+ T cells resulted in inhibition of the disease.

Further researches led to the recognition of the human CD4+ CD25+ Tregs having similar function and phenotype to those in mice. Later in 2003, it was found that the CD4+ Cd25+ Tregs both in mice and human particularly express in their nucleus, the transcription factor FOXP3 (Fontenot, Gavin, & Rudensky, 2003). Any mutation resulting in loss of function of FOXP3 causes severe autoimmune manifestations constituting the Immune dysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome caused by a dysfunction of CD25+ CD4+ regulatory T cells similar to the FOXP3 mutant scurfy mice (Powell et al., 1982).

All these studies and many more supporting evidences from in vitro and in vivo experiments over the past 50 years have now led to a firm understanding that a CD4 T cell subpopulation of naturally occurring Tregs particularly expressing the surface marker CD25 on the cell surface and the transcription factor FOXP3 in the nucleus, i.e., CD25+

FOXP3+ Treg cells are found in normal individuals and are responsible for suppression of abnormal immune responses against self, or any external antigen (Sakaguchi et al., 2020).

1.4.1.2. IL-2 and regulatory T cells homeostasis

The cytokine interleukin-2 plays a very important role in immune responses by influencing a variety of lymphocyte subsets. Studies on mice models have shown that mice lacking IL-2, CD25 or CD122 suffer from systemic autoimmunity due to lack or imbalance in regulatory T cells survival and function highlighting the essential role of interleukin-2 in Tregs homeostasis (Malek, 2008). The Tregs need a continuous stimulation via normal background levels of IL-2, for their survival and maintenance (Yu, Zhu, Altman, & Malek, 2008) (Fontenot, Rasmussen, Gavin, & Rudensky, 2006).

Besides, Interleukin-2 signals tend to enhance the expression of CD25 and FOXP3, increasing the suppressive potential of Tregs (Barron et al., 2010). Hence, a reduction in IL-2 signal following injection of IL-2 specific monoclonal antibody results in a decrease number of regulatory T cells and a decreased expression of FOXP3 (Rubtsov et al., 2010). Moreover, recent studies on in vitro and in vivo models show that IL-2 is necessary for the generation and survival of induced Tregs (Schallenberg, Tsai, Riewaldt, & Kretschmer, 2010).

1.4.1.3. IL-2 maintains homeostasis of T-cells

The CD4+ t cells are the main IL-2 producing cells, but it is also produced in small amount by CD8+ T cells, natural killer cells and dendritic cells. The Il-2 has paradoxical immune effects. On one hand, it promotes immunosuppression and ensure immune tolerance and on the other hand it is actively involved in promoting growth of effector T cells as a growth factor. It enhances the production of interleukin-4 (IL-4) resulting in the conversion of CD4+ CD- T cells into Tregs.

IL-2 binds with interleukin 2 receptor expressed on regulatory T cells and activates the STAT5, which in turn enhances the expression of Foxp3 thus stimulating proliferation of regulatory T cells.

Besides, IL-2 cause expansion of activated effector T cells and increase in effector cells increase effector t cells production of IL-2 Activated effector cells are stimulated and expanded by IL-2, and this increase in effector cells production of IL-2 cause expansion of the Treg population which in return, suppress the effector cell function creating a negative feedback loop. The secretion of proinflammatory molecules such as IFN-γ is reduced while that of anti-inflammatory molecules like IL-4, IL-10 is increased. IL-2 also causes activation induced cell death (AICD) of CD8+ effector T- cells thus suppressing the activity of effector cells. The process is summarized in the schematic diagram below (Figure 1.2). Due to the expression of CD25 on their surface, Tregs are the first cells to respond to the cytokine IL-2 during an immune response and initiate FOXP3 transcription via the IL2Ra-STAT5b pathway (Zorn et al., 2006).

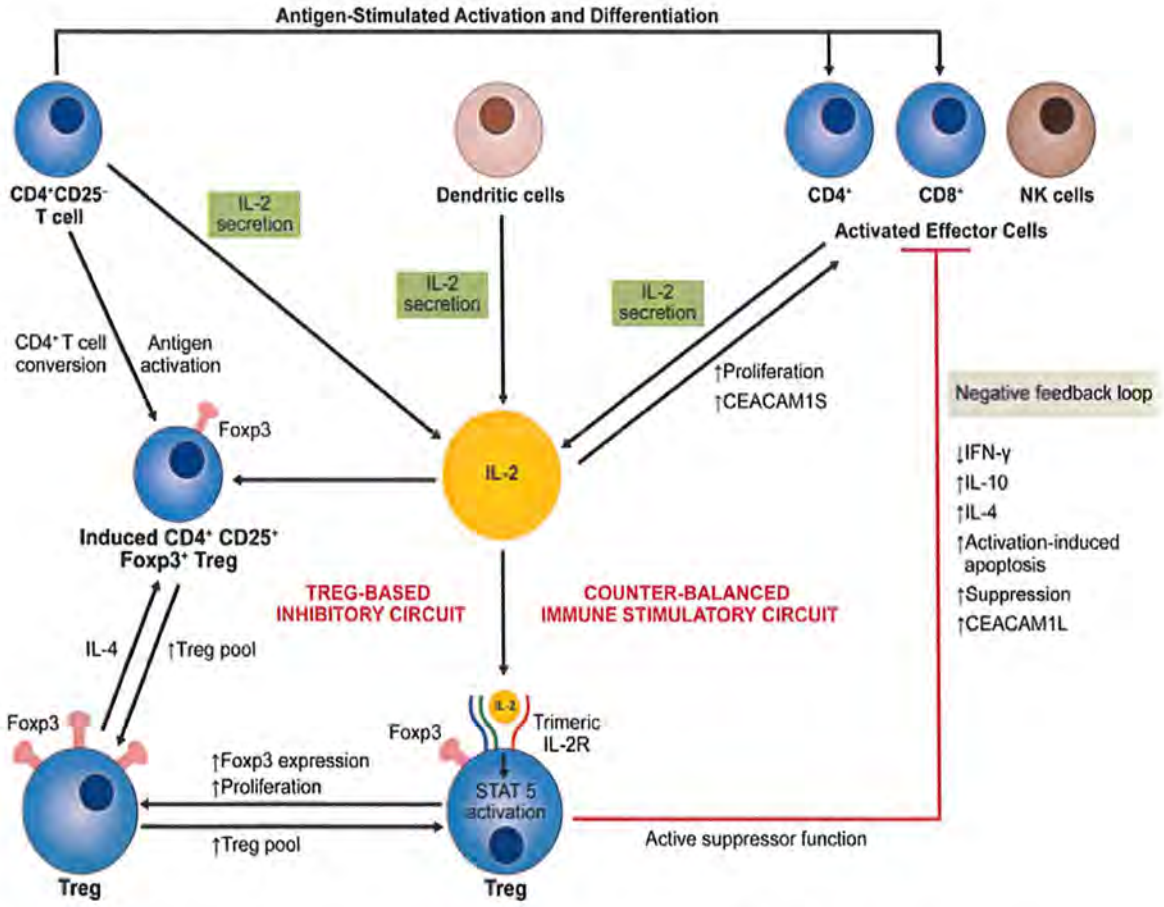

Fig. 1.2. Interaction of IL-2 with effector T cells and Tregs (Czaja, 2021).

1.5. The IL-2 signaling pathway

The signaling pathway starts when the IL2RA (CD25) captures an IL-2 molecule and bring about certain conformational changes to the molecule to enhance its affinity to the other two subunits of the receptor (β and γ). Binding of the IL-2 molecule to the receptor leads to the dimerization of the cytoplasmic tails of the IL2Rβ and IL2Rγ subunits. Unlike other signaling receptors, the cytokine receptors do not have any kinase domains in their cytoplasmic tails but are able to recruit tyrosine kinases of the Janus-kinase (JAK) family present in the cytoplasm, to their intracellular domains. Binding of JAK1 to IL2Rβ and JAK3 to IL2Rγ leads to phosphorylation of these kinases which then phosphorylate the tyrosine residues in the receptor.

From here the signaling can proceed downstream via three different pathways, the JAK-STAT pathway, the MAPK (mitogen-activated protein kinase) pathway and the PI3K (phosphoinositide3-kinase)-AKT) pathway.

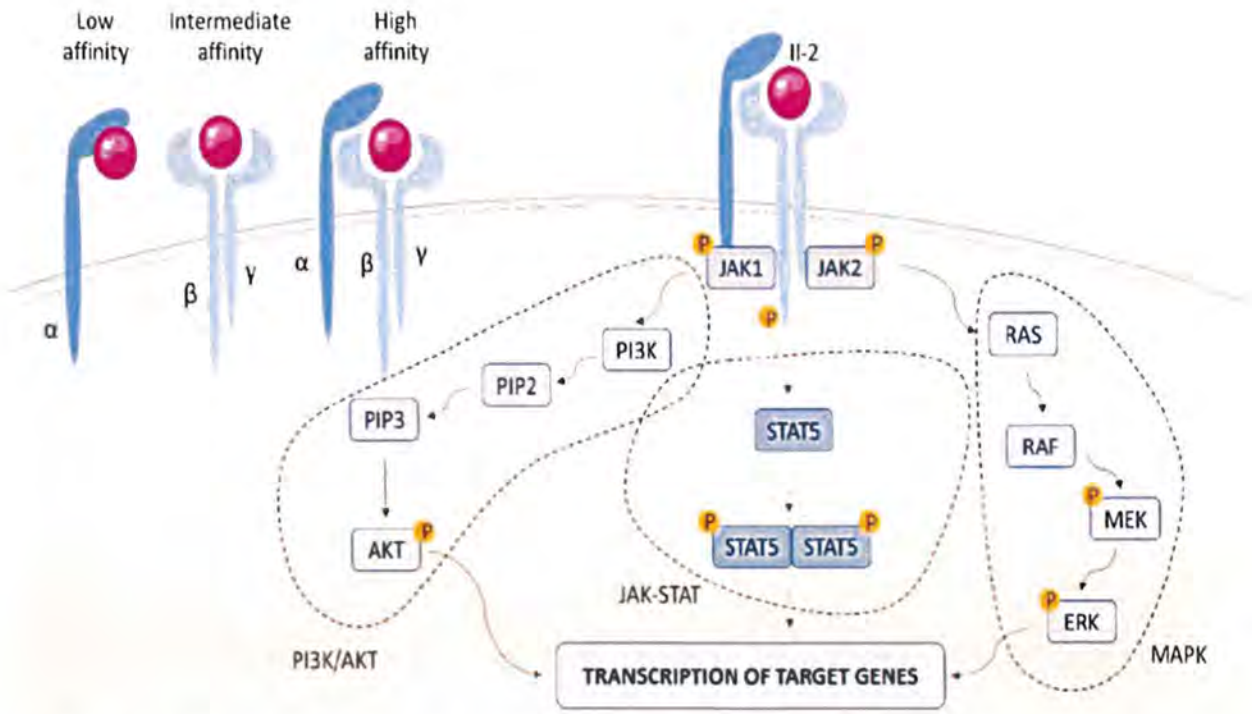

Fig.1.3. The IL-2-receptor signaling pathway (Druszczyńska, Godkowicz, Kulesza, Wawrocki, & Fol, 2022).

The three receptor forms made from different combinations of the IL2rα, IL2Rβ and IL2Rγ chains combine with their respective cytokine (IL-2) causes the cytoplasmic kinases JAK1 and JAK3 to phosphorylate. The phosphorylated JAK kinases lead to downstream signaling via STAT5. The JAK kinases also activate PI3-AKT and MAPK pathways stimulating targeted gene transcription in the nucleus. (Druszczyńska, Godkowicz, Kulesza, Wawrocki, & Fol, 2022).

The ultimate result of all these pathways is the transcription of genes which express proteins responsible to carry out the biological actions of IL-2. The adaptor protein Shc and the signal transducer and activator protein (STAT) are crucial in the IL-mediated regulatory mechanisms. The gene targets of IL-2 signaling include CD25, BCL-2, SOCS1-2, fasL, and BLIMP19.

Specifically, crucial for immune homeostasis is the negative regulator (BLIMP19) which maintains a balance between Tregs, and the effector T cells.

Although any receptor form can induce all the three downstream signaling pathways, it has been observed that mostly different cells tend to use different pathways. The regulatory T cells have been observed to use the STAT5 pathway more preferably upon induction by an IL-2 signal (Damoiseaux, 2020).

1.6. The *IL2RA* gene

The gene coding for *IL2RA* is located on the minus strand of chromosome 10 in the cytogenetic band 10p15.1 spanning 51,682 bases according to the latest chromosomal assembly (GRCh38/hg38).

Fig.1.4. Chromosomal location of *IL2RA* gene on chromosome 10.

The figure is taken from Genecards (https://www.genecards.org/).

IL2RA is a protein coding gene that encodes the alpha subunit of the cell surface receptor-alpha for interleukin-2. The IL-2 receptor is heterotrimeric protein made of the receptors IL2Rα, IL2Rβ and IL2Rγ. Together the three subunits constitute the high affinity receptor for IL-2.

The gene spans more than 25kbs of the genome and contains 8 exons. The protein coded by *IL2RA* gene is a protein with a molecular mass of 30819 Da and containing 272 amino acids.

1.6.1. Mutations in *IL2RA* gene

As of 9th August 2022, a total of 23 mutations in the *IL2RA* gene have been reported in the Human genome database (HGMD). Out of the 23 mutations reported, 19 are accessible to public while 4 new mutations are only accessible to professional users.

Among the 19 mutations, 5 mutations are related with CD25 deficiency, and the rest are involved in other diseases such as diabetes type 1, multiple sclerosis and increased risk to breast cancer.

Four out of five mutations related with CD25 deficiency are missense and one mutation is deletion.

Table 1.2. List of pathogenic variants associated with CD25 reported in HGMD

Mutation	Exon no.	Amino acid change	consequence	Reference
c.122A>C	3	Y41S	missense	(Bezrodnik, Caldirola, Seminario, Moreira, & Gaillard, 2014)
c.301C>T	4	Gln101Ter	missense	(Caudy et al., 2007)
c.497G>A	5	S166N	missense	(Goudy et al., 2013)
c.464A>C	5	N133T	missense	(Soares et al., 2016)
c.62_64+1del	1	-	deletion	(Sharfe et al., 1997)

1.6.2. Other diseases associated with mutations in *IL2RA* gene

Mutations in the *IL2RA* gene have also been associated with other diseases such as diabetes Type 1(Butter et al., 2012), multiple sclerosis (Ang et al., 2011) rheumatoid arthritis (Knevel et al., 2013) and increased risk to breast cancer (Li et al., 2013).

1.7. Diagnosis of the disease

The initial diagnosis of the disease can be made by looking at the most common clinical features which can be suggestive of the disease i.e., neonatal onset of symptoms like autoimmune enteropathy with villous atrophy, frequent infections. Autoimmune cytopenia, diabetes and severe eczema have also been observed as common features. However, the occurrence of autoimmune complications is heterogenous among patients. The differences in clinical manifestations has also been a hurdle towards finding a standard treatment.

The disease cannot be exclusively diagnosed just based on clinical symptoms owing to the overlapping of the symptoms with other autoimmune disorders, especially the IPEX syndrome which shares all the autoimmune features with the disease.

Proliferative assays, cytokine production and cytofluorimetric analysis of CD25 protein expression can help to predict the disease occurrence. Cytofluorimetric analysis can be considered as an important diagnostic tool for the disease. Literature and medical reports till date backs it, the patients with mutations in *IL2RA* gene have shown marked decrease or total absence of CD25 on cell surface (Vignoli et al., 2019).

However, genetic analysis of the patient's DNA to look for any pathogenic mutations in the associated gene can lead to the ultimate diagnosis and confirmation of the disease.

1.8. Treatment and management

Unfortunately, there is no successful therapeutic option available currently, however, many drugs are in testing phases to control the complications of the disease. Timely diagnosis is mandatory so that a therapeutic intervention can be made as the disease mostly manifests in neonatal phase and is highly fatal.

Immunosuppressants such as corticosteroids and cyclosporine are mostly used to prevent autoimmune features of the disease. Various immunosuppressive drugs have been used, and none have been proven to be consistently effective to control the disease.

The immunosuppressive drug Sirolimus (also known as rapamycin) has been shown to be effective to control the autoimmune complications and without any obvious side effects in various studies (Lai et al., 2020). The ultimate proof for the drug to be effective and safe to use clinically would require a randomized placebo-controlled trial which is currently unlikely to perform due to the rarity of the disease (Yong, Russo, & Sullivan, 2008).

However, the ultimate cure available is allogeneic transplant of hematopoietic stem cells (HSCT) which promises a conclusive cure but is a scarcely available option due to lack of suitable donors and has high morbidity and mortality rates due to toxicities related to transplant.

1.9. Genetic counselling

Genetic counselling should be available to families at risk of having an affected child. Immunodeficiency type 41 with lymphoproliferation and autoimmunity is an autosomal recessive disorder and parents heterozygous for the condition have a 25% risk of giving birth to an affected child. If a patient is diagnosed with the condition, the parents should be tested for heterozygosity. If heterozygosity is confirmed the parents should be offered genetic counselling and information should be provided regarding the clinical options (like prenatal testing and pre-implantation genetic diagnosis) available for having a normal child.

1.10. Aims of the study

The study was aimed at diagnosing an immunodeficiency disorder in a Pakistani family using whole exome sequencing and to find out whether the parents are segregating, or the disease has been caused by a de-novo variant so that an effective and timely therapeutic action can be taken. Moreover, preventive measures such as genetic counselling, and prenatal diagnosis can be considered to prevent another affected child.

CHAPTER 2

MATERIALS AND METHODS

2.1. Sample description

We ascertained a family with one child manifesting symptoms suggestive of CD25 deficiency from Khyber Pakhtunkhwa (KPK), Pakistan. A verbal consent was taken from the family prior to collection of sample and data. A blood sample of 5ml was collected from the available family members. Family pedigree was constructed based on the data provided by the elders of the family and clinical data was collected.

2.2. Construction of pedigree

Microsoft (MS) power point was used for the construction of the pedigree. Males were represented via squares while females were represented via circles. Affected individuals were represented with shaded squares and circles for male and female respectively. Double horizontal marriage lines indicated consanguineous marriage. To differentiate the proband from the rest of the family members, an arrowhead was drawn below the circle representing the proband. Diagonal lines over the geometrical shapes indicated deceased members of the family.

2.3. Sample collection

Blood samples were drained in 5ml syringes from the available affected and normal members of the family. The collected samples were stored in vacutainers that contained potassium ethylene diamine tetra acetate (EDTA) for preservation. The samples were then stored at 4°C, at medical genetics research lab (MGRL) till further processing.

2.4. Genomic DNA extraction

The organic method or Phenol-chloroform extraction protocol was followed for the extraction of genomic DNA.

2.4.1. Composition of solutions used in organic method

The detailed composition of solutions used in Phenol-chloroform extraction is as given below:

Table 2.1. Composition of solutions used in chloroform-phenol method of DNA extraction.

Solutions	Composition	Quantity of ingredients
Solution A	Sucrose (0.3 M)	54.7g
	MgCl2 (5mM)	2.5ml
	Tris HCl (5ml)	5ml
	Triton X-10	5ml
	Distilled water	400ml
Solution B	NaCl (400Mm)	40ml
	Tris HCl (10mM)	5ml
	EDTA (2mM)	1ml
	Distilled water	Make total volume 500ml
Solution C	Phenol	100ml
	Hydroxyquiniline	10mg
	Tris HCl	10ml
Solution D	Chloroform	24ml
	Isoamylalcohol	1ml

2.4.2. Organic/phenol chloroform method

The blood sample to be used for extraction was kept at room temperature for one hour prior to performing the phenol-chloroform protocol.

- 750 microliters of Blood and solution A each were mixed in a 1.5mL Eppendorf tube and then kept at room temperature for 10-15 minutes. Next, the mixture was centrifuged for one minute at 13,000 rpm.
- After centrifugation, the pellet obtained was kept while the supernatant was discarded. The pellet obtained was then dissolved in 400 microliters of solution A followed by another centrifugation for a minute at the same rpm.
- The supernatant was again removed, and pellet was kept. The pellet was dissolved in 400 microliters of solution B followed by the addition of 12 microliters of SDS(20%) and 5-8 microliters of Proteinase K (stored at 20 °C).
- The pellet was completely dissolved (via tapping) and then incubated at 37°C overnight.
- 500 microliters each of solution C and solution D was added to the incubated tube (Eppendorf) followed by centrifugation for 10 minutes at 13000 rpm. This time the upper layer formed after centrifugation was transferred to a new Eppendorf tube and pellet was discarded.
- 500 microliters of solution D was added to the pellet in the new Eppendorf tube and centrifuged for 10 minutes at 13000 rpm. The upper layer was then transferred to another Eppendorf tube and pellet discarded.
- 55 microliters of sodium acetate and 500 microliters of chilled isopropanol (-20°C) and the tube was inverted several times to precipitate out DNA. The tube was then centrifuged for 10 minutes at 13000 rpm.
- The DNA pellet was kept, and supernatant discarded carefully. 200microliters of 70% ethanol was added for washing followed by centrifugation for 7 minutes at 13000 rpm.
- After that, the ethanol was removed and the Eppendorf containing DNA pellet was placed in an incubator at 45°C for 10 minutes to make sure the residual ethanol evaporates completely.
- The DNA pellet was suspended in 80 microliters of deionized water or TE buffer and incubated at 37°C.

2.5. Molecular analysis of extracted DNA

Agarose gel electrophoresis was performed to analyze the extracted DNA. The following protocol was followed:

- 0.5 gram of agarose was added to 5 ml distilled water and 5ml of 10X TBE was added to the mixture to make 1% agarose gel. The mixture was heated for 2-3 minutes in a microwave to ensure the complete dissolution of agarose. The solution was then allowed to cool at room temperature for about 5-7 minutes.
- 5µl of ethidium bromide was added to the solution.
- Combs were inserted in the gel casting tray and the solution was carefully poured into it.
- The solution was allowed to solidify at room temperature for about 15-20 minutes.
- After the solidification of the gel, the casting tray containing the solidified gel was placed in a gel tank carefully without disturbing the gel. 10X TBE buffer (to be used as an electrolyte) was added to the gel tank such that the gel gets submerged in it.
- Equal volume (3µl each) of Bromophenol blue and DNA sample were mixed using a micropipette and parafilm and loaded into the wells. The bromophenol serves as a loading dye and helps to check the movement of DNA in the gel.
- The Bio-Rad power supply connected with the gel tank was run after setting it at a voltage of 120 volts, current of 400A and 30 minutes' time.
- After 30 minutes, the power supply was disconnected, the gel was carefully taken out from the tank and visualized under UV-Transilluminator to check for bands and their intensity.
- 60µl of PCR water was added to 20µl of extracted DNA to dilute the DNA to a final concentration of 40ng/µl.

2.6. Gene sequencing

2.6.1. Whole exome sequencing

To sequence the DNA of the proband, whole exome sequencing (WES) was carried out. Exome libraries were captured using SureSelect V5-post kit (Agilent Technologies, Santa Clare, CA, USA). To perform sequencing, illumina Hiseq 4000 (illumine San Diego, CA,

USA) with an average sequencing depth of 142x of the targeted regions for 150bp reads was utilized.

Burrows-Wheeler alignment tool (accessible at http:// bio-bwa.sourceforge.net/bwa.shtml) was utilized to map the reads against reference human genome. Any duplicated reads upon analysis with Pi-card tools were removed. Variant calling was carried out using the Genome Analysis Toolkit (GATK). For the annotation of the variant alleles, SnpEff (SnpEff_v4.1) was used.

The following criteria was followed to filter out the most relevant variant to the disease among the thousands of variants in WES.

- The variants in the candidate genes for the disease were prioritized.
- Variants including splice site, non-synonymous, gain/loss of start/stop codon and frameshift were selected.
- Variants having minor allele frequency <1% in the gnomAD (genome aggregation database) were kept and those having higher frequency were excluded.
- Different tools as Polyphen2, splice predictor, Mutation Taster and SIFT were used for scoring and prediction of variant.
- According to 1000 genome project (http://www.1000genomes.org/), the minor allele frequency of the variant should be less than 0.01 for it to be pathogenic, hence variants with frequencies greater than 0.01 were excluded.
- Sanger sequencing is yet to be performed for the confirmation of the respective disease-causing variant.
- The affected individual is most likely to be homozygous for the variant.
- Obligate carriers should show heterozygous peaks for the variant.

2.7. Bioinformatic analysis

The selected variant was then analyzed and evaluated for pathogenicity under the light of ACMG guidelines and by using relevant in silico tools. The variant was studied for its pathogenicity from the variant data and from previous studies on the gene, the associated

diseases and how mutations in the gene have previously been reported as a known mechanism of disease in the literature.

To predict the disease-causing potential of the variant, the in-silico tool, Mutation taster (https://www.mutationtaster.org/) was accessed on August 5, 2022.

The possible changes to spliceogenicity of the variant were checked via the MaxEntScan accessible at (http://hollywood.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html).

The change in the splice site strength due to the variant was analyzed using the in-silico freely accessible online tools NNSplice (https://www.fruitfly.org/seq_tools/splice.html), SROOGLE (https://bio.tools/sroogle), NetGene 2 (https://www.healthtech.dtu.dk/english).

The human splicing finder (HSF) tool (https://hsf.genomnis.com/mutation/analysis) was used to check for the potential of the variant to affect the wild-type splice site. The data was recruited in the form of HSF score.

To predict for the possibility of exon skipping or cryptic site activation, CRYP-SKIP tool (https://cryp-skip.img.cas.cz/) was used which gives a probability score for splice site variants between 0 and 1, where a value close to zero indicates chances of exon skipping while a value close to 1 indicates activation of cryptic splice sites in the vicinity of the exon.

2.8. Primer designing

For the PCR amplification of the desired gene, the corresponding primers for the targeted region were manually designed. The gene sequence was downloaded using Ensembl genome browser. The primers were checked for self-complementarity and hairpin loop formation and general characteristics both manually and cross checked using in silico tool Oligo Calc. Ensembl BLAST was performed to check for homology of the primers with reference human genome and single hit primers were selected. To avoid the formation of primer dimers, the forward and reverse primers were checked for complementarity.

For the conformation of PCR suitability of the primers and expected product size, in silico PCR was run. The primers with all the desired characteristics were selected and ordered

from MACROGEN, (South Korea). The primers and their properties are as listed in the table below.

Table 2.2. List of primers for the PCR amplification of Exon 1 of *IL2RA* gene.

Exon #	Type Forward /reverse	Primer sequence 5' to 3'	Primer length (bp)	Tm °C	GC content (%)	Product size (bp)
1	Forward	GGGCGTAGCTGAAGAAAGG	19	60	57%	601
1	Reverse	GCAAGAGGTGGAACCCAAG	19	60	57%	601

CHAPTER 3

RESULTS

3.1. Pedigree analysis of IEI-1 family

IEI-1 family was recruited from Bannu, Khyber Pakhtunkhwa, Pakistan. A four-generation pedigree was constructed with two consanguineous marriages at third generation (fig 3.1). Four members of the family in the fourth generation had the disease but at the time of sampling only the proband was alive. The parents in both the cases were phenotypically normal. The proband is a one year four months old female born to consanguineous parents with clinical features highly resembling with typical features of CD25 deficiency. The onset of the symptoms was observed within the first week of birth. The proband is the third child in the family and the first two children were also affected with the same disease and deceased at an early age.

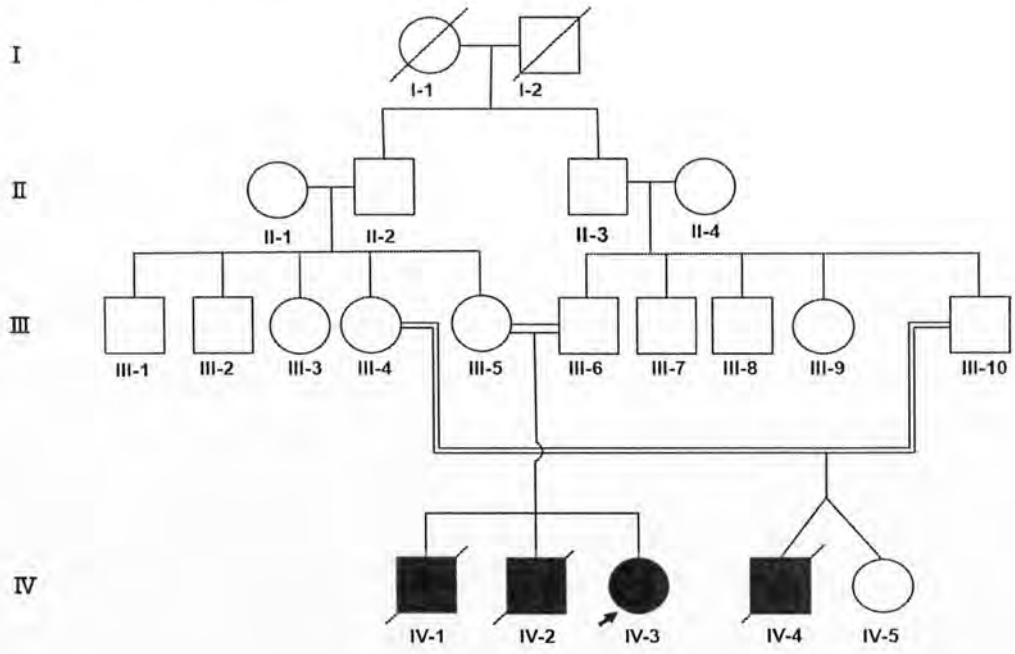

Fig 3.1. Pedigree of family IEI-1. Consanguinity shown in third generation, disease expressed in four members in fourth generation. Circle represents female, square represent male, dark shapes represent affected individuals and shape with arrowhead represents proband.

3.2. Gel electrophoresis

To check the quality and concentration of the DNA extracted from the patient's DNA, it was analyzed using gel electrophoresis. The bands shown on the gel were indicating a good concentration of DNA to be used for PCR and were free from protein contamination (fig. 3.2).

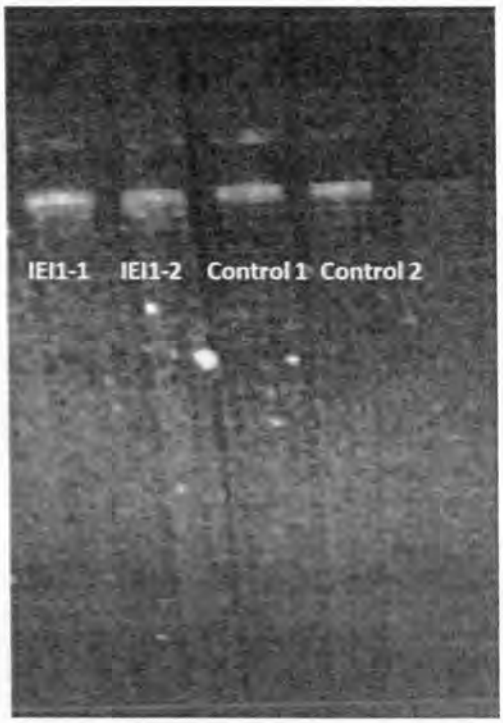

Fig. 3.2. Gel-doc image of extracted DNA of the proband, mother and controls.

3.3. DNA sequencing

DNA of the affected individual was sequenced using Whole Exome Sequencing while confirmation of allele segregation is yet to be done via sanger sequencing.

3.3.1. Whole exome sequencing

Whole Exome Sequencing of the patient's DNA disclosed 91,253 variants. The variants were narrowed down to one variant most likely to be causing the disease by using various filters to cut off variants which were synonymous or irrelevant to the disease of interest.

3.3.1.1. Variant filtration

The total variants reduced to 12,103 upon applying filter for synonymous and intronic region variants. Several variants were excluded due to their heterozygous nature. The number reduced to 67 variants after excluding all the variants which had a minor allele frequency (MAF) greater than 0.01 in the general human population.

The number reduced to three after excluding variants with lower likeliness of being pathogenic. The variant was finally narrowed down to a single variant showing higher chances of pathogenicity upon in silico analysis.

After applying various filters to the Whole Exome data, the variants were narrowed down to a novel splice site mutation c.64+1G>A in *IL2RA* gene. The mutation occurs in the canonical donor splice site of the first exon of the gene. The variant is absent in HGMD, Clinvar and gnomAD.

Table 3.1 Variant filtration data

Filter applied	**No. of variants**
Initial variants	78, 253
Variants potentially affecting protein were kept	12,103
Homozygous/compound heterozygous variants were kept	321
Variants with MAF >0.01 in the 1000 genome, ExAC or gnomAD were excluded	67
variants predicted to be deleterious >50% by software	3
1 variant was identified pathogenic	1

Table 3.2 Mutation identified in the patient

Exon	Mutation	Sequence change	Mutation type	Protein variation
1 (Splice site)	c.64+1G>A	GT/AT	5' splice site	N/A

3.3.1.2. Bioinformatic/in silico analysis of the variant

The bioinformatic analysis of the *IL2RA* variant c.64+1G>A, predicted the variant to be likely pathogenic. The results from bioinformatic tools used to analyze the variant were consistent with the manual ACMG guidelines-based classification of the variant. we predicted and classified the variant to be a pathogenic variant of PVS1 category owing to the variant falling in the highly conserved splice site of a gene where LOF has been reported as a known disease mechanism.

The output data from all the bioinformatics tool were consistent with the probable pathogenic effects of the variant. The web tool Mutation taster predicted the variant to be disease causing owing to the possible splice site abolition.

The MaxEntScan score for wild type splice site and variant splice site indicated clear differences of splice site strength. The ideal MaxEnt value for 5' splice site is 11.81. The MaxEnt score for wild type had dropped from a nearly ideal value of 11.08 to a negligible value of 2.90 indicating significant loss of splice site strength.

Moreover, the NNSplice, NetGene 2 and SROOGLE scores for the wild-type splice site were maximum for the splice site strength while the scores were below the threshold value for the variant.

The human splice finder (HSF) values were consistent with the other predictive algorithms. The HSF score for the wild type was 97.41 and that for the variant was 70.27, with a delta CV value of -27% indicating a broken WT donor site.

Since all the in-silico tools were predictive of the abolition of the WT splice site, the in-silico tool CRYP-SKIP was used to check for the prediction of the probability of skipping of exon or activation of cryptic splice site. The results from CRYP-SKIP were showing higher probability of exon skipping as compared to cryptic splice site activation.

Table 3.3. In silico pathogenicity evaluation

Variant program	**Wild Type splice site data output/score**	**Mutant (c.64+1G>A) Splice site data output/score**	**Interpretation**
Mutation taster	Normal	Disease causing	Splice site changes can lead to changes in protein features
MaxEntScan	MaxEnt: 11.08 MDD: 14.98 MM: 11.06 WMM: 12.14	MaxEnt: 2.90 MDD: 6.80 MM: 2.87 WMM: 3.96	The mutation has caused significant loss in strength of donor splice junction and may result in recognition of another site either in the exonic or intronic region.
NNSplice	0.99 Values/range (0-1)	Below threshold	The NNSplice score is below the threshold value to be considered a splice site.
NetGene 2	1 Values (0-1)	No score/below threshold	The mutation has caused the NetGene 2 score from the maximum value

			(i.e., 1) for the wild type to below threshold.
SROOGLE	1	No score Values (0-1)	The percentile value of SROOGLE has dropped from maximum for wild type to below threshold for variant.
HSF	97.41	70.27	Broken WT donor site. (delta CV=-27%)
CRYP-SKIP	N/A	0.34 Values/range (0-1)	Values closer to 0 imply a higher probability of exon skipping as opposed to cryptic splice site activation.

Chapter 4

DISCUSSION

Immunodeficiency type 41 with lymphoproliferation and autoimmunity or CD25 deficiency is an autosomal recessive disorder that arises due to mutations in the *IL2RA* gene. The *IL2RA* gene has been mapped on the short arm of chromosome 10 and codes for a 272 amino acid long protein, alpha subunit of the high efficiency tripartite IL-2 receptor, also called CD25.

The patients with defects in the gene normally lack effective IL2RA receptor on their cell surface. IL2RA receptor is crucial for the lymphocytes to normally mature, function and survive. It is also critical for the regulation and maintenance of these cells (Leonard et al. 1982). The patients therefore, manifest both immunodeficiency symptoms as well as autoimmune symptoms at the same time (Cepika et al., 2018).

CD25 is expressed in low quantities on the surface of several types of immune cells, but the regulatory T(Tregs) cells show a higher expression level. The regulatory T cells are responsible for the suppression of immune responses and maintenance of tolerance to self-antigens and immune homeostasis (Sakaguchi, 2004). The high expression of CD25 on their cell surface enable regulatory T cells to give a quick response to the interleukin-2 cytokine during an immune response (O'Gorman et al., 2009). IL-2 is a crucial cytokine acting as a growth factor for T cells, necessary for survival, differentiation, and activation of Treg cells, and AICD of CD8+ T cells.

CD25 deficiency is a disease of rare occurrence with only few cases reported in literature. The first case was reported in 1997 by Sharfe et.al, in an infant with recurrent infections and lymphoproliferation. The underlying cause of the disease was found to be a deletion mutation in the *IL2RA* gene (Sharfe et al., 1997).

The characterizing clinical features of the disease include early onset of chronic infections, diarrhea with enteropathy, lymphoproliferation, and autoimmunity. However, the type of

infections and other autoimmune manifestations largely vary among patients. The clinical manifestations are attributed to an impaired T-regulatory cell function.

The disease shares a high similarity of clinical features with Immune dysregulation Polyendocrinopathy, enteropathy X-linked (IPEX), an X-linked disease caused by mutations in the *FOXP3* gene. Owing to the similar manifestations, CD25 deficiency has also been referred to as IPEX-like disease in literature (Caudy et al., 2007).

In this study, a patient from a Pakistani family IEI-1, was investigated to pinpoint the underlying cause of the clinical manifestations and to help in the proper diagnosis of the disorder. The proband is a one-year-old female born to consanguineous parents presenting characteristic features of the disease. The symptoms started within the first week of birth with severe diarrhea, vomiting and continued with frequent infections and failure to thrive. The family history revealed that the proband had two brothers who were deceased with the same disease manifestations due to lack of timely diagnosis and management of symptoms. The parents are phenotypically normal. The proband's cousin also died of the same disease condition, who was also a child of consanguineous parents.

Whole exome sequencing of the patient's DNA revealed several variants in the exome. These variants were then filtered using various web-based filtration tools to find the variants most likely to be pathogenic and relevant to the disease conditions. The possibility of IPEX diseases was ruled out by the family pedigree not following an X-linked inheritance and it was further confirmed with whole exome results showing no mutations in the candidate gene, *FOXP3* for the disease.

A novel mutation, c.64+1G>A, was detected in the canonical splice donor site of the first exon of *IL2RA* gene. The variant lies in the highly conserved and almost invariant GT site of the 5' splice donor predicting a high probability of deleterious changes to the protein features. The variant is absent in GnomAD and HGMD. The variant has not been reported in ClinVar yet.

We carried out a bioinformatic analysis of the novel variant to confirm the pathogenicity of the variant. The web-based tool Mutation Taster predicted the variant to be disease causing due to a high likeliness of the abolition of the splice site.

The splice prediction tool Human Splice Finder (HSF) predicted a broken WT donor site for the variant. The HSF score or consensus value (CV) for the wild type was 97.41 and that for the variant was 70.27. Strong splice sites tend to give a CV score above 80. The mean consensus value for 5' splice site is 87.53 with a standard deviation of 8.34. Not only the CV but also the delta value of the CV values for WT splice site and variant is critical in determining the effects of the variant to the splice site. Results from previous mutation studies have indicated that a delta CV value of at least 10% is predictive of having significant impact on splicing and needs further investigation (Desmet et al., 2009). The Delta CV value for the variant in this study is -27% indicating a high likeliness of splicing errors.

For further confirmation of splice site abolition, the splice site prediction tools MaxEnt, NNSplice, NetGene 2 and SROOGLE were accessed. These prediction tools also indicated a significant loss of splice site strength for the variant. The splice site strength score for the variant were below the threshold value indicating a clear loss of splice site in the variant.

Moreover, CRYP-SKIP tool was used to check for the probability of exon skipping or cryptic site activation for the given variant. it a comprehensive in silico tool that distinguishes between exons that may get skipped and exons that may activate cryptic splice sites due to the corresponding mutation. The tool uses the corresponding exonic sequence and about 100 nucleotides of the intronic region following the exon in FASTA format and gives output in the form of a probability score (P_{CR-E}, probability for cryptic splice site activation) ranging from 0 (Exon skipping) to 1 (activation of cryptic splice site). The tool predicted the correct results in 72% of the cases and was introduced as a web-based tool for splicing errors prediction (Divina, Kvitkovicova, Buratti, & Vorechovsky, 2009). The tool predicted a higher probability of exon skipping as compared to cryptic splice site activation for the variant.

The variant was studied for its possible effects in silico, and literature was studied to better understand the pathogenicity of the variant and to classify the variant under the five-tier classification system in the light of the guidelines provided by ACMG. We predicted the variant to be a pathogenic variant of the PVS1 category as the variant lies in the canonical

splice site of the *IL2RA* gene, and LOF is a known mechanism of the associated disease (Richards et al., 2015).

The bioinformatic analysis for the variant is highly supportive of splice site abolition and exon skipping however the confirmation of the studies can only be made by RNA and protein-based studies.

Conclusion

According to our research findings mutations in the *IL2RA* gene are responsible for the occurrence of immunodeficiency type 41 with lymphoproliferation and autoimmunity or CD25 deficiency. The family history and pedigree used in this study is suggestive of autosomal recessive pattern of inheritance which will further be confirmed using sanger sequencing to check for the heterozygosity of the variant in the parents.

Whole exome sequencing (WES) has proven to be an effective way to diagnose and confirm the disease occurrence. Moreover, genetic analysis of patients and other family members (healthy) including parents of the proband is considered necessary for the exact diagnosis of the disease and genetic counseling to be offered for carriers.

Additionally, protein expression studies should be carried out to understand the possible effects of the novel variant and the region where it lies, on the protein structure and function. The study can be helpful in a better understanding of the protein, the associated disease and hence help in finding better therapeutic options.

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LIST OF DATABASES USED IN THE STUDY

1. ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/)
2. CRYP-SKIP (https://cryp-skip.img.cas.cz/)
3. Ensembl genome browser (https://asia.ensembl.org/index.html)
4. Genecards (http://umd.be/Redirect.html)
5. gnomAD (https://gnomad.broadinstitute.org/)
6. HGMD (https://www.hgmd.cf.ac.uk/ac/all.php)
7. Human Splicing Finder (http://umd.be/Redirect.html)
8. MaxEntScan (http://hollywood.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html)
9. Mutation taster (https://www.mutationtaster.org/)
10. NCBI (https://www.ncbi.nlm.nih.gov/)
11. NetGene 2 (https://services.healthtech.dtu.dk/service.php?NetGene2-2.42)
12. NNSplice (https://www.fruitfly.org/seq_tools/splice.html)
13. OligoCalc (https://www.bioinformatics.org/JaMBW/3/1/9/index.html)
14. OMIM (https://www.omim.org/)
15. SROOGLE (https://bio.tools/sroogle)
16. UCSC In-Silico PCR (https://genome.ucsc.edu/cgi-bin/hgPcr)

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Thesis

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PRIMARY SOURCES

1	Albert J. Czaja. "Exploring the Pathogenic Role and Therapeutic Implications of Interleukin 2 in Autoimmune Hepatitis", Digestive Diseases and Sciences, 2020 Publication	1%
2	www.science.gov Internet Source	1%
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5	Clint A. Coleman. "T regulatory cells: aid or hindrance in the clearance of disease?", Journal of Cellular and Molecular Medicine, 11/2007 Publication	<1%
6	"Primary Immunodeficiency Diseases", Springer Science and Business Media LLC, 2017 Publication	<1%