GENETIC ANALYSIS OF DOPAMINE RECEPTORS GENES AND THEIR ROLE IN CANCER

Submitted by

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IN THE NAME OF ALLAH THE MOST BENEFICIENT THE MOST MERCIFUL

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FINAL APPROVAL

It is certificate that we have read the thesis submitted by Ms. Huma Zainab and it is our judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for the M.S Degree in Biotechnology

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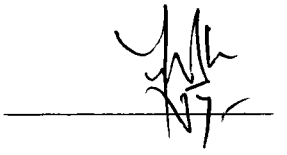

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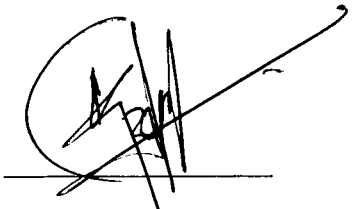

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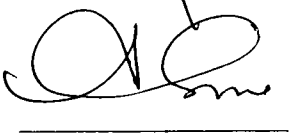

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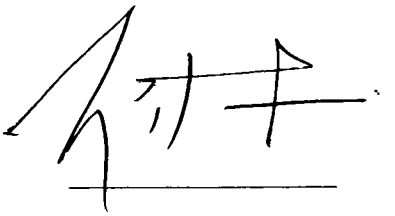

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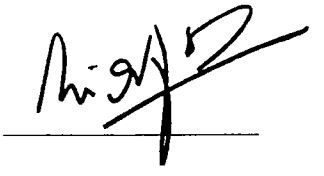

A thesis submitted to Department of Bioinformatics and Biotechnology,

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for the award of the degree of MS Biotechnology

DEDICATION

I dedicate this dissertation, with all my heart, to my beloved parents, husband and to my teachers. Without their assistance, bunch of their sincere prayers and sacrifices it would not have been possible for me to accomplish my work. I also dedicated my thesis to all those students who could get maximum benefit from my work.

DECLARATION

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

Date: _____

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Huma Zaineb

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First and above all, I praise God, the almighty for providing me this opportunity and granting me the capability to proceed successfully. This thesis appears in its current from due to the assistance and guidance of several people. I would therefore like to offer my sincere thanks to all of them.

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I pray to Allah Almighty to give me the strength and resources to serve world to the best of my efforts and wisdom not to neglect humanity. AMEEN.

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LIST OF ABBREVIATIONS

CGAP	Cancer Genome Anatomy Project
GABA	Gamma-Aminobutyric Acid
PD	Parkinson's Disease
DA	Dopamine
L-DOPA	l-3, 4-Dihydroxyphenylalanine
GPCRs	G-Protein-Coupled Receptors
DRs	DA Receptors
D1Rs	D1-Like Receptors
D2Rs	D2-Like Receptors
SNPs	Single Nucleotide Polymorphism
Nacc	Nucleus Accumbens
AC	Adenyl Cyclase
cAMP	Cyclic Adenosine Monophosphate
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
ACD	Acid Citrate Dextrose
Rpm	Revolution Per Minute
TME	Tumor Micro Environment
TBE	Tris Boric EDTA
EDTA	Ethylene Diamine Tetra Acetic Acid

SDS	Sodium Dodecyl Sulfate
Ml	Milliliter
DNA	Deoxyribonucleic Acid
µl	Microliter
Mm	Millimolar
OD	Optical Density
nm	Nanometer
ng	Nanogram
bp	Base Pair
TH	Tyrosine Hydroxylase
ICL3	Third Intracellular Loop
Ser	Serine
Cys	Cysteine
YAC	Yeast Artificial Chromosome
APOA1	Apo lipoprotein A1
NCAM	Neural Cell Adhesion Molecule
VEP	Variant Effect Predictor
SIFT	Sorting Intolerant From Tolerant
PP2A	Protein Phosphatase 2 A
TCF	T-Cell Factor
LEF	Lymphoid Enhancer-Binding Factor Proteins

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ABSTRACT

Cancer is the result of abnormal divisions of the cells. It is a group of more than 100 different diseases that can develop almost every part of the body. It is the major cause of mortality and morbidity globally. Dopamine (DA, 3- hydroxyl tyramine) has been considered as an important catecholamine neurotransmitter that shows its effect on its target organs by binding with its receptors (DRs). Recent research evidence suggests a link between dopamine and cancer. There are five different genes which encodes five different dopamine receptors. Polymorphisms which have been analyzed within the DRD2 gene were shown to be linked with different types of cancer, specifically skin, lungs, corpus uteri and colon cancers. Dopamine antagonists have been used as antitumor agents and Dopamine pathways can play their role in anticancer therapies. Many medical conditions are linked to low level of dopamine, including Parkinson's disease, Schizophrenia and depression. The purpose of present research is to analyze the association between risk of cancer and mutation in dopamine receptors genes, specifically in DRD2 in Pakistani population gene. This study included 213 sporadic samples of different cancer types and 50 healthy controls. DNA was extracted from all cancer samples and controls. PCR was performed to amplify DRD gene. Sequencing was performed for mutational analysis of DRD2 gene. Bioinformatics tools were also used to analyze the possible mutations in DRD2 gene in different types of cancer. Mutations were present in 5% of total sample size. Polyphen scores and VEP impact of reported mutations were analyzed from GDC data portal .In the reported mutations, C>T, C<A, A>G substitutions and deletion of G in DRD2 were found to have high impact and results in Cancer. The results show three polymorphic variants of DRD2 which was causing amino acid substitution in DRD2 receptor proteins. On the basis of results, we find that in our selected population, an association between DRD2 gene mutations and cancer patients was present.

CHAPTER 1

1.1 Cancer, an Uncontrolled Cell Division

Cancer is a type of disease in which there is uncontrolled division of cells that spreads to other parts of the body. It can start almost everywhere in the human body that is made up of trillions of cells. Under normal conditions, the cells of human body multiply and grow through a continuous process called cell division form which new cells are produced according to the requirement of the body. When cells become old or get damaged, they died through apoptosis, and new cells are produced to take their place. Under some circumstances this ordered process breaks down, and abnormal cell division starts and damaged and abnormal cells multiply and grow when they don't need to grow. These abnormal cells form tumors, which are the lumps of dead cells and tissues. Tumors can be cancerous which are called malignant tumors or non-cancerous which are known as benign tumors (National Cancer Institute, 2021).

1.2 Worldwide Prevalence of Cancer

Cancer stands as a prominent contributor to mortality worldwide, representing a significant obstacle to improving life expectancy in all nations. The primary and secondary cause of the death in 112 out of 183 countries is cancer before the age of 70 years. Additionally, in the remaining 23 countries, it has third and fourth rank in terms of morbidity and mortality (WHO, 2019).

In 2020, cancer posed a significant global health challenge. The data gathered related to cancer related deaths showed that there are approximately 10 million cancer-related deaths (excluding non-melanoma skin cancer) each years throughout the world. In female, breast cancer with more number of patients and lung cancer is considered as the most commonly diagnosed cancer. The estimated number of cancer patients is approximately 2.3 million (11.7%). The percentage of lung cancer is close to 11.4%, with colorectal cancer is 10.0%, and the percentage of prostate and stomach cancer is 7.3% and 5.6% respectively. Cancers also ranking high in terms of new cases. In terms of cancer-related deaths, lung cancer maintained its position as the leading cause of deaths,. Lungs cancer cause approximately 1.8 million deaths annually. Colorectal (9.4%), liver (8.3%), stomach (7.7%), and female breast (6.9%) cancers were among the other most significant contributors to cancer mortality (Hyuna et al., 2021).

Cancer can assume to affect 28.4 million people by 2040,that is approximately 47% more as compared to the number of affected cases in 2020. This surge is expected to be more pronounced

in transitioning countries that is 64% to 95% as compared to transitioned countries in which it ranges from 32% to 56% due to demographic changes.

Addressing this growing challenge requires urgent efforts to establish sustainable infrastructures for disseminating the preventive measures for cancer and providing adequate cancer treatment in transitioning countries. These endeavors are found to be crucial for effective cancer control worldwide and reducing the burden of cancer cases on a global scale (Carioli et al., 2019).

1.3 Prevalence of Cancer in Pakistan

Cancer has become a significantly growing cause of deaths in Pakistan in the recent era, so it is becoming a problem for Pakistan. There is a steady increase in the incidence of cancer in Pakistan according to the report provided by World Health Organization on the occurrence of cancer in Pakistan. According to the literature, the breast cancer (24.1%), oral cavity (9.6%), colorectal (4.9%), esophagus (4.2%), and liver cancer (3.9%) were the five most prevalent cancers in Pakistani population. Males were more prone to the cancer of oral cavity that is 14.9%. The percentages of other cancer types are colorectal cancer (6.8%), liver cancer (6.4%), prostate cancer (6.0%), and lung cancer (6.0%). In women (41.6%), breast (6.9%), oral cavity (5.5%), cervix (4.7%), and uterus cancer (4.1%) were the most common types of cancers in Pakistan. Cancer is more common in middle-aged people, the range is 43.0% that is followed by old aged people (30.0%) and then the number of adults (20.0%) come. There are more chances of cancers of the central nervous system (CNS) then leukemia (18.7%), and Hodgkin (17.3%) are common in children and adolescents. Then the number of breast, oral cavity, colorectal, and prostate cancer comes at other ages. The literature shows that the most patients were from Punjab (40.4%) and Sindh (32.2%). In approximately 30.0% of patients were diagnosed with cancer at third and fourth stage. Among the registered cases, the percentage breast cancer, oral cavity cancer, colon cancer, esophagus cancer, and liver cancer are highest (Tufail et al., 2023).

1.4 Genetics Based Cancer Research

Cancer remains the most formidable disease of our era, exhibiting a growing number of new cases annually on a global scale. Remarkable progress has been made in cancer research by leveraging deep sequencing techniques, enabling the identification of potential therapeutic targets. However, targeted therapies, which are continuously advancing may show resistance to cancer therapies

which suggests that the DNA mutations are considered as primary drivers which may not be solely responsible for initiating tumors in different parts of the body. In modern research, scientists delve into the dual nature of DNA mutations, exploring their roles as initiators of cancer and as pivotal contributors to tumor progression after initiation. To begin, it is essential to distinguish between the roles played by these DNA mutations. They can act as initiating events, triggering the onset of cancer, or they can serve as significant contributors that facilitate tumor development once initiation has occurred (Butler et al., 2015).

1.4.1. Mutational Analysis of Cancer

Mutations are consider to play a crucial role in causing cancer. It does not only serve as a hallmark of the disease but also driving its progression. Unlike normal cells, cancer cells undergo uncontrolled division, invasion, metastasis, and ultimately pose a threat to the host organism. The hereditary nature of cancer at the cellular level, combined with the presence of multiple mutations within cancer cells, strongly suggests that tumor development is primarily fueled by mutagenesis (Ames et al., 1995).

Advancements in molecular techniques have facilitated the comprehensive exploration of the human genome, allowing researchers to dissect it from the macroscopic level of chromosomes to individual genes and even nucleotide sequences. With each deeper level of investigation, an increasing number of mutations are being cataloged in cancer cells. This emerging understanding reveals that the genomes of cancer cells exhibit an inherent instability, leading to a cascade of mutations. Some of these mutations enable cancer cells to circumvent the host's regulatory processes that govern cellular location, division, gene expression, adaptation, and programmed cell death (Gold et al., 1990).

1.5 Neurotransmitters: Emerging Targets in Cancer

Traditionally, neurotransmitters have been recognized as substances released by nerves that facilitate stimulatory or inhibitory functions within the nervous system by binding to specific receptors. However, recent scientific research has focused on the role of neurotransmitters in the regulation of physiological and pathological functions of different organs and tissues including endocrine system. These discoveries have unveiled a new perspective on the involvement of neurotransmitters in cancer (Boilly et al., 2017).

Emerging evidence suggests that cancer cells exploit signaling pathways initiated by neurotransmitters to drive uncontrolled proliferation and metastasis. Neurotransmitter also influences the immune cells and endothelial cells within the cancerous cell so they also show a positive impact in the progress of tumor to make it malignant tumor. So the understanding of the pathways and mechanisms of neurotransmitters can help the researchers to summarize their roles in the processes such as angiogenesis and tumorigenesis, and inflammation holds great potential for the development of advanced anti-cancer therapies (Hanoun et al., 2015).

Neurotransmitters also regulate the important functions of the brain through the process of transmission of neurotransmitter across synapsis. These chemicals play very important functions integral in regulating everyday life functions. Acetylcholine is involved in memory, attention, learning, and muscle control. Dopamine plays a role in reward and pleasure, motivation, movement, and mood regulation. Serotonin affects mood, appetite, sleep, and social behavior. It is often associated with feelings of well-being and happiness. One of the important neurotransmitter called Gamma-aminobutyric acid (GABA) is an inhibitory neurotransmitter that play its role in regulate anxiety, stress, and sleep. It reduces neuronal excitability. Glutamate is an excitatory neurotransmitter that is involved in learning, memory, and cognition that is present in excess amount in the brain. Norepinephrine works in the "fight or flight" response, alertness, attention, and mood regulation. Endorphins are involved in pain regulation and are associated with feelings of pleasure and euphoria. Imbalances or dysfunctions in neurotransmitter systems have been implicated in various neurological and psychiatric disorders (Jiang et al., 2020).

1.6 DOPAMINE

Dopamine (DA, 3-hydroxytyramine) is a neuro regulatory molecule that plays many important functions within the cell. Dopamine is a type of monoamine neurotransmitter which also acts as a hormone. It is one of the most important chemical of catecholamine and phenethyl amine classes. Dopamine comprises more than 80% of the catecholamine content in the brain. Dopamine is responsible for allowing you to feel pleasure, satisfaction and motivation. Dopamine works as a neurotransmitter in the brain. The level of dopamine in the brain increases as the anticipation of most types of rewards and many addictive drugs also increase the release of dopamine within brain. Dopamine, a widely acknowledged neurotransmitter in the brain, plays a vital role in regulating essential functions within various peripheral systems. Recent advancements in research

have shed light on the significant role of dopamine as a regulator of immune function. Numerous immune cells possess dopamine receptors and other dopamine-related proteins, allowing them to actively react to dopamine (Matt et al., 2020).

The synthesis and storage pathways of dopamine involve many different enzymes and cofactors which can yield more dopamine when manipulated genetically which can be used for cancer treatment .The enzyme that is involved in dopamine production is Tyrosine Hydroxylase (TH) which changes amino acid tyrosine to L- Dopa and L-Dopa is then metabolized to dopamine by another aromatic amino acid decarboxylase (Regan Webber., 2009). .

The dopamine also shows vasodilatory effect in renal tissue which provide early evidence of its role outside the CNS. Since then, DA has been found to have effects in multiple tissues, which include its effects in the tissues of vascular system, heart tissues, gastrointestinal tract, eye, kidney and pancreas (Beaulieu et al., 2011).

1.6.1 Role of Dopamine in Cancer

Neurotransmitters have been identified as influential factors in the progression of tumors by modifying the tumor microenvironment. Dopamine is a key catecholamine neurotransmitter, holds significance within the central nervous system. Abnormality in the release and pathway of dopamine can cause schizophrenia and Parkinson's disease. Several epidemiological and molecular biology investigations have explored the relationship between schizophrenia, Parkinson's disease, and various types of cancers. Modern research suggests that any abnormality in the dopamine pathway might be related to the development of cancer (JA et al., 2007).

The Human Genome Project in that was completed in 2003 revolutionized the study of cancer genomes. Advances in technology, particularly large-scale genome sequencing techniques, have enabled initiatives like the Cancer Genome Anatomy Project (CGAP) was proved to be an important source for the understanding of gene expression profiles of precancerous, cancerous cells, and normal tissues. By sequencing cancer genomes, the primary aim is to identify genetic factors, which significantly enhance the changes of mutation rate within cells and consequently accelerate tumor evolution and the formation of metastases (CGAP).

By unraveling the complex interactions between neurotransmitters and cancer, researchers aim to uncover novel therapeutic targets and strategies for combating cancer growth and metastasis. This

deeper understanding of neurotransmitter involvement in cancer biology may pave the way for the development of antitumor therapies in the next generations, offering new avenues to improve patient outcomes (American Cancer Society, 2018).

Recent research has accumulated a large number of evidences indicating that dopamine (DA) signaling pathways in peripheral tissues undergo disruptions in the association of cancer. This emerging field of study presents fresh insights into vulnerabilities of cancer cells and emphasizes the potential value of employing dopaminergic ligands and drugs, originally developed for neuro pharmacological purposes, with the aim of targeting cancer. Researchers are now focusing on understanding the role of Dopamine and its receptors to cause tumors and the tumor microenvironment (TME), within the immune system, that can be used in formulating the new therapeutic strategies for the successful treatment of cancer patients (Beaulieu et al., 2015).

By elucidating the intricate connections between DA signaling and cancer, researchers aspire to uncover new opportunities for therapeutic interventions. This includes leveraging existing libraries of dopaminergic ligands and drugs, which have been extensively studied in the field of neuropharmacology (Giovannelli et al., 2022).

Recent research has shown that in the peripheral tissues the dopamine signaling pathway is disrupted in cancer. This emerging part of study provides new insights into vulnerabilities of cancer cells and highlights the potential to utilize existing dopaminergic ligands and drugs developed for neurological conditions for cancer treatment. This suggests that the knowledge gained from neuro pharmacological drug discovery and development can be applied purposefully for cancer therapy (Allen et al., 2016).

1.6.2. Dopamine and Parkinson Disease and Schizophrenia

Many intriguing links have been analyzed between Parkinson's disease (PD) and cancer by different Physicians and epidemiologists. PD is a disorder of nervous system in which there is a loss of dopaminergic neurons in the substantia nigra pars compacta. There is also decreased release of dopamine in the striatum. On the other hand, cancer is a neoplastic disorder characterized by uncontrolled cell division and a lack of cell death in the affected tissue due to mutation in genes. So it is concluded that at a cellular level, PD and cancer represent diseases with opposite

manifestations . The literature also said that the female Schizophrenic patients using antipsychotic drugs have more chances of getting breast cancer (Bose et al., 2018).

1.7 Dopamine Receptors

Dopamine receptors (DRs) belong to a family of G protein-coupled receptors, which have seven-transmembrane domain structure. There are five main types of dopamine receptors, which are DRD1, DRD2, DRD3, DRD4, and DRD5. On the basis of their distinctive regulation of adenylyl cyclase (AC) activity, dopamine receptors are classified into two families which include 0D1-like DRs and D2-like DRs. D1-like Dopamine receptors include DRD1 and DRD5, these two stimulate the production of cAMP, while D2-like Dopamine receptors include DRD2, DRD3, and DRD4) which exert an inhibitory effect on cAMP production (Wang et al., 2019).

The D1 receptors (D1Rs) exhibit significant similarity in their amino acid sequence with D5 receptors (D5Rs) in the transmembrane domains, sharing approximately 80% homology. Dopamine receptors are abundantly present in the central nervous system. Dopamine receptors play an essential role in daily life function like movement, emotions and the reward system in the brain (Mishra et al., 2018).

Dopamine receptors are specifically express in the hippocampal dentate gyrus and sub ventricular zone of central nervous system. Dopamine receptors are also expressed more prominently in the peripheral region of the body. Their expression also affects the function of kidney and vasculature. D2, D3, and D4 receptors are prominently expressed in the striatum as well as the external Globus pallidus, core of nucleus accumbens, hippocampus, amygdala, and cerebral cortex as they show their effect in central nervous system . These receptors also affect the post synaptic receptor medicated extrapyramidal activity. D2 andD4 receptors are important in the signaling for the development and survival of human dopamine of neurons (Wang et al., 2009).

D1 and D5 receptors facilitate adenylyl cyclase activity by coupling with G stimulatory sites. After the activation of adenylyl cyclase, it leads the production of the second messenger cAMP, which in turns leads the production of protein kinase A (PKA) which involves in transcription within the nucleus (fig 1.1). D2 through D4 receptors couple to G inhibitory sites, which inhibit adenylyl cyclase and activate K+ channels (Vekshina et al., 2017)

1.7.1 Functions of Dopamine Receptors

The D1 receptor has more percentage abundance as compared to other five dopamine receptors in the central nervous system. After D1, the number of D2 comes, then D3, D5 and D4 has least abundance among all. Dopamine works by binding to its dopamine receptors and depending on the type of receptor, dopamine perform different functions within the body. D1 receptors help regulate The development of neurons is regulated by D1 receptors that will initiate when dopamine hormone binds with the dopamine receptor D1. The density of D1 and D5 receptors is high in the substantia nigra, striatum, nucleus accumbens and olfactory bulb. These receptors are involved in regulating different systems of the body like reward system, memory, learning and motor activity. D1 and D5 receptors, also activate phospholipase C, along with stimulating adenyl cyclase which leads to the a induction of intracellular calcium release and activation of kidney, these receptors show their activity when the there is an increase in excretion of electrolytes and renal vasodilation (Gilat et al., 2017).

D2, D3, and D4 receptors are expressed also expressed in the striatum. These receptors are also present in the external globus pallidus, core of nucleus accumbens, hippocampus, amygdala, and cerebral cortex. These receptors also affect the postsynaptic receptor-medicated extrapyramidal activity. D2-D4 receptors are also involved in the signaling that is important for the survival of human dopamine neurons and for the development of neurons which in turn involve in nerve transmission (Beaulieu et al., 2011).

Dopamine receptors are extensively targeted in clinical pharmacology for a wide range of abnormalities. The most targeted diseases are schizophrenia, , restless leg syndrome, Parkinson's disease, bipolar disorder, depression, hyperprolactinemia, pituitary tumors, hypertension, gastroparesis and erectile dysfunction. By modulating dopamine receptor activity, medications aim to alleviate symptoms and improve the management of these conditions (Beaulieu and Gainetdinov, 2011).

Dopamine receptors (DRs) have been identified in different subsets of immune cells, where they are involved in regulating differentiation and activation. These functions may have relevance to anticancer immunotherapeutic strategies, suggesting a potential connection between dopamine

signaling and cancer treatment, influencing factors such as tumor cell death, uncontrolled division, invasion, and migration of tumor cells (Mei et al., 2009).

Each receptor has a different function. D1 is involved in locomotion, memory, attention, regulation of renal function, locomotion and impulse control. D2 is also involved in memory, attention .learning and sleep. D3 is involved in cognition, sleep, impulse control and attention. D4 also play its role in cognition, impulse control, attention, sleep and D5 is involved in decision making, cognition, attention, renin secretion (Mishra et al., 2018)

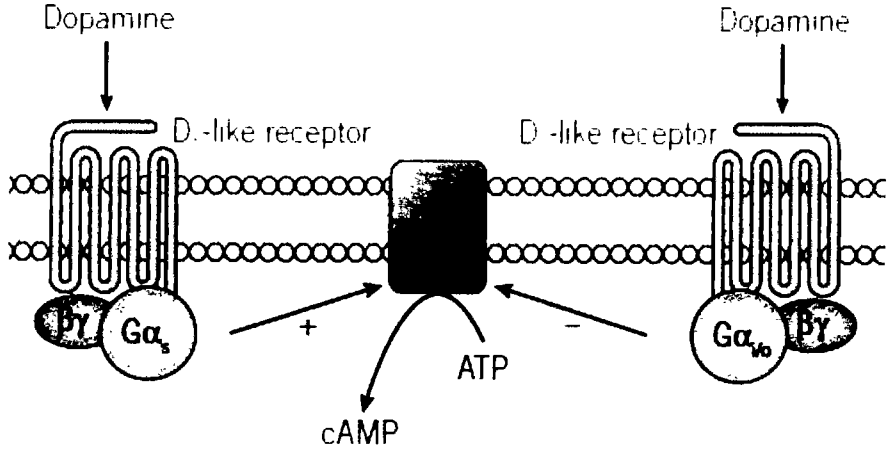

Fig 1.1 Regulation of adenylyl cyclase activity by D1-like and D2-like dopamine receptors. D1like receptor stimulates adenylyl cyclase activity while D2 like receptors inhibit adenylyl cyclase activity.

Table 1.1 DOPAMINE RECEPTORS TYPES

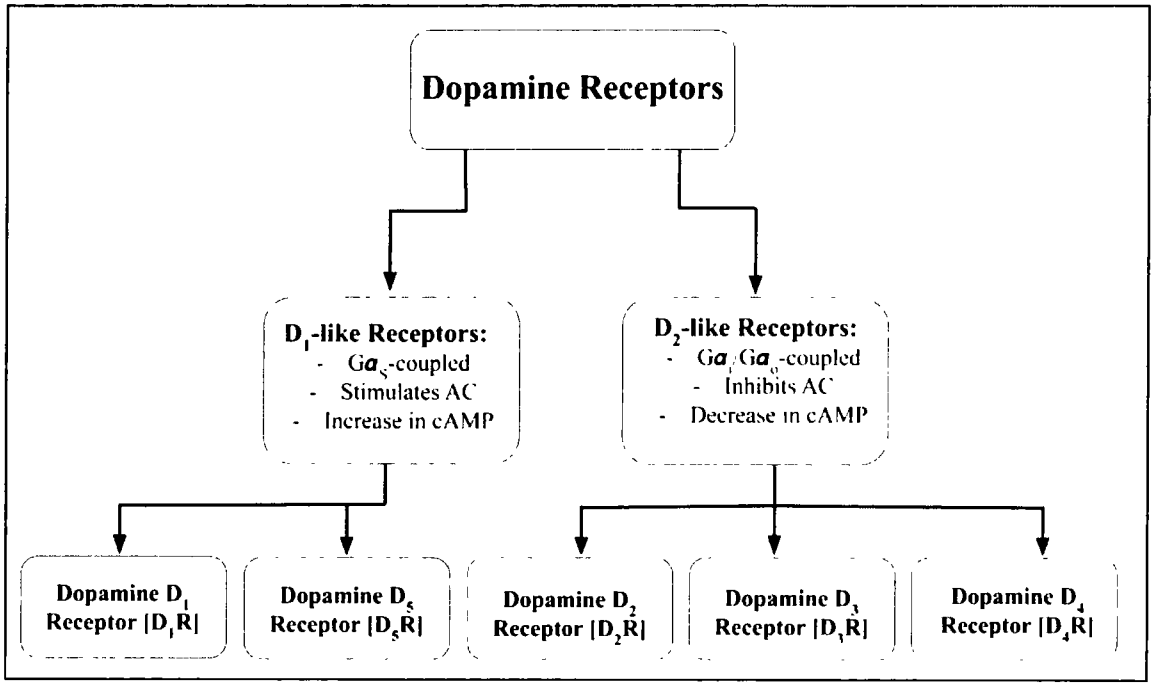

Table1.1. Schematic diagram showing dopamine receptor subtypes

1.7.2 Structural Differences between D1 and D2 like Receptors

There are also structural differences between these two types of receptors. D1-like DRs lack introns in their coding regions, whereas D2-like receptors contain many introns. Specifically, the genes of DRD2, DRD3, and DRD4 possess different numbers of introns in which six, five, and three is more prominent. As a result, D2-like receptors can variants by splicing. For example, DRD2 has two isoforms, D2S and D2L; produce an exon that contains 87-base-pairs which is located between introns 4 and 5. DRD3 can also generate splice variants; however, they encode proteins which are nonfunctional. The DRD4 genes exhibits variations which are polymorphic, including a 48 base pairs in the terminating tail that is repeated compared to D1-like DRs (Levite et al., 2016).

1.7.3 Polymorphism and Level of Dopamine Receptors in Cancer Cells

Polymorphisms in dopamine receptors (DRs) have been found to be associated with an increased risk of cancer of colon and rectum, gastric cancer cell non-small lung cancer. Elevated levels of DRD2 gene expression have been observed in various types of cancers. In patients with neuroendocrine tumors, higher DRD2 expression has been linked to longer time to progression. Interestingly, patients with gastric cancer exhibiting high levels of DRD2 expression have been found to have a longer survival duration .These findings suggest that DRD2 may play diverse roles in different types of cancer. DRD4 has been implicated in the malignant pediatric medulloblastomas. Furthermore, DRD4 gene expression is also associated with the survival rate of patients having glioblastoma (GBM), where increased levels of DRD4 gene expression is linked with poor survival compared to decreased levels of DRD4 gene expression. Accumulating evidence obtained from literature suggests that Dopamine receptors also play a significant role in progression of cancer (Akbari et al., 2016).

Mechanistic investigations have demonstrated that Glioblastoma (GBM) cells express DRD2 and are stimulated through autocrine signaling by dopamine, and mitogenic signals and leads to phenotypic alterations that promote tumor progression (Kline et al., 2018).

Table 1.2 The Polymorphisms in DRs and their Expression in Different Types of Cancer

Sr. No	Type of cancers	Relationship between dopamine receptors and cancer
1.	breast cancer	DRD1↑, DRD2↑, DRD3↑, DRD4↑
2.	cervical cancer	DRD2↑
3.	Cholangiocarcinoma	Mz-chA-1: DRD1↑, DRD2↑, DRD3↑, DRD4↓,DRD5↑ HuCCT-1: DRD1↓, DRD2↑, DRD4↓,DRD5↓ SG231: DRD2↓, DRD3↓, DRD4↓,DRD5↓ CCLP-1: DRD1↓, DRD2↑, DRD3↓, DRD4↓,DRD5↓
4.	colorectal cancer	DRD2↓; DRD2 polymorphisms -141Cdel, 957T>C, 1412A>G are associate with colorectal cancer. HCT116: DRD1↑, DRD2↑,DRD5↑ HT29: DRD1↑, DRD2↑,DRD5↑ The DRD2 rs1799732 CT, rs1800497 TT is associated with increase cancer risk.
5.	corticotrophin adenomas	DRD2↑
6.	gastric cancer	DRD2↑
7.	Glioblastoma	DRD2↑
8.	Cholangiocarcinoma	Mz-chA-1: DRD1↑, DRD2↑, DRD3↑, DRD4↓,DRD5↑

1.8 Dopamine Receptor D2 Gene (DRD2)

The DRD2 gene, found in humans, that is situated on chromosome 11q22–23. Among its significant types of variations, three notable polymorphisms have been identified. These most significant is the deletion of cytosine base at 141 position in the promoter region before the site where transcription starts that is −141CIns/Del , the second is a type of missense mutation that is present on exon 7 producing a missense variant 960C→G that results the substitution of 311Serine with Cysteine (S311C) (Itokawa et al., 1993). A TaqI A1/A2 single nucleotide polymorphism (SNP) was also analyzed that is located approximately 9.5 kb downstream from the DRD2 gene (Grandy et al. 1989).

1.8.1 Coding Region of DRD2

According to the study conducted by Grandy et al. in 1989, it was discovered that the coding sequence of the human DRD2 gene contains six introns. There is a unique exon consisting of 87 base pairs that encodes the extra amino acids that is found in the human dopamine receptor as compared to the rat receptor.

1.8.1.1 DRD2 in Cell Adhesion

Eubanks et al. (1992) conducted a study where they successfully created a physical map of DRD2 gene covers over 1.5 Mb of chromosome number 11. Their findings provided the evidences which show that the neural cell adhesion molecule gene (NCAM; 116930) is present on 150 kb downstream on the 3-prime region of the DRD2 gene on the same DNA strand. To further refine the mapping, cosmid and YAC clones were used. In this high-resolution fluorescence in situ suppression hybridization was used. This localization technique revealed that these genes are located at the center of the APOA1 and STMY genes, precisely at the junction of 11q22.3 and 11q23.1 on the chromosome.

1.8.1.2 DRD2 and Cell Death

Numerous studies have reported that dopamine exhibits the ability to diminish cell viability and trigger apoptosis in various cell lines. The role of dopamine was studied in different cells such as leukemia K562 cells, tumor cells in human oral cavity, pituitary tumor cells of rat, and human

Neuroblastoma cells SKNMC, when tested in controlled laboratory conditions in vitro. Furthermore, dopamine has been shown to reduce the occurrence of cancer stem cells in breast cancer and induce programmed cell death when studied in vitro. Additionally, the combined administration of dopamine and sunitinib has been found to augment the effectiveness of sunitinib in combating breast cancer cases in which breast cancer cells show drug resistance. (Wang et al., 2015)

1.8.1.3 DRD2 and Autophagy

Studies have provided evidence that dopamine also trigger autophagy that cause cell death so DRD2antagonists can be used to treat cancer .The cause of autophagic cell death is the generation of reactive oxygen species (ROS) which is induced by dopamine. In vitro experiments using SH-SY5Y cells have shown that Sertindole that is a DRD2 receptors antagonist that can be used to initiate autophagy. Additionally, ATG5, a protein involved in the process of autophagy, plays a crucial role in Sertindole-induced autophagy (Shin et al., 2007).

1.8.1.4 Size of DRD2 Gene

In a separate study by Eubanks et al. in 1992, it was determined that the DRD2 gene size is 270 kb approximately. Within this gene, there exists an intron, its size range is approximately 250 kb , which separates the putative first exon from the exons responsible for encoding the protein of dopamine receptor (Grandy t al., 1989).In a study that is conducted by Gelernter , the mapping of the DRD2 gene was performed by using linkage analysis in relation to several other loci on 11q . DRD2 is a protein coding gene. It contains 443 amino acids. The molecular mass of gene is 50619 Da. DRD2 gene consists of 66,087 bases. DRD2 has seven transmembrane domains. These seven-transmembrane domains lie in 51–426 positions, whereas literature also predicted the same domain to be present at the 35–437positions.

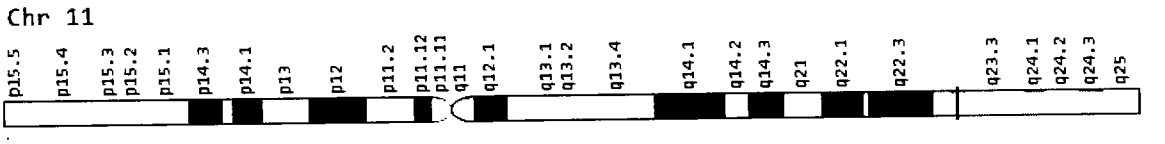

Fig 1.2 Genomic view of DRD2: chr11 gene

1.8.2 Single Nucleotide Polymorphism in DRD2

Among the variations found in DNA sequences, the most frequently occurring variations are single-nucleotide polymorphisms (SNPs) . These genetic variations can impact gene expressions and, consequently, influence susceptibility to various diseases, among these the cancers is most important. Numerous SNPs have been identified in the gene which are responsible for encoding DRD2 gene. Notably, three of these SNPs, which are named as rs1799732 (DRD2 -141C>del), rs6277 (DRD2 957C>T), and rs1800497 (DRD2 TaqIA), have been reported to significantly affect the expression and function of the DRD2 protein. Another SNP, rs1079597 (DRD2 TaqIB), is particularly noteworthy as it is located closer to the regulatory and structural coding regions of the gene including 5' region. This proximity suggests that rs1079597 may play a crucial role in the regulation of transcription. These specific DRD2 SNPs have been associated with the risk of several diseases, including Parkinson's disease and cancer (Wang et al., 2018).

Researchers have observed an allelic association between the schizophrenia and Taq1-A polymorphism of the DRD2 gene. Another significant single nucleotide polymorphism in the DRD2 gene is the C/G missense variant that was observed in exon 7, resulting in a substitution of Serine with Cysteine at position 311. These genetic variations, including the −141 C del/ins polymorphism, may be involved in schizophrenia, either directly or indirectly, possibly through dopaminergic or other pathways. Within exon 7 of DRD2, two more synonymous polymorphisms, His313 and Pro319, have been identified. Synonymous mutations are typically assumed to have no impact on the amino acid sequence of the gene product. However, the Pro319 polymorphism has demonstrated significant functional consequences thus causing the mutation because It affects mRNA stability and reduces the translation efficiency which can (Muller et al., 2003).

Over the last few decades, several polymorphisms have been discovered in this gene, including TaqIA, rs6277, and -141Cins/del polymorphisms. Among these, the TaqIA polymorphism (rs1800497) has been extensively studied. It involves a substitution of C which representsA2-allele to T which represents A1-allele. Other polymorphisms which was investigated by the researchers in the DRD2 gene include 141C Ins/Del that was due to rs1799732 polymorphism and C957T which is due to rs6277 polymorphism (Danna et al., 2010).

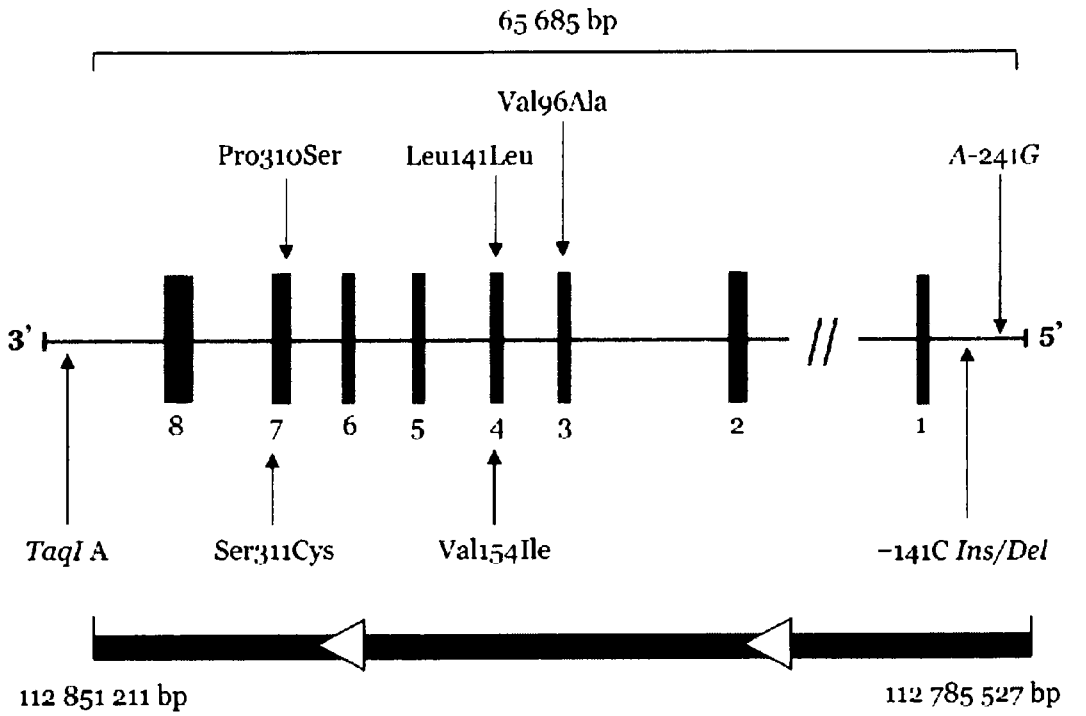

Fig 1.2 The figure represents the structure and polymorphisms lie within the dopamine D2 receptor gene (DRD2)

1.8.3 DRD2 in Canonical Wnt Signaling Pathway

D2R, belongs to G protein-coupled receptors (GPCRs) that works through two intracellular pathways The first is G protein-dependent and arrestin-independent, and the other one is G protein-independent and arrestin-dependent pathways (FreybergZ et al., 2010). In the G protein-dependent pathway, activation of D2R leads to the recruitment of Gαi/o, resulting in reduced cyclic AMP (cAMP) synthesis (Beaulieu et al., 2015).The path followed In the G protein-independent and arrestin-dependent, arrestin 3 (β-arrestin-2) causes D2R internalization which dampens D2R extracellular signaling. This pathway also binds protein phosphatase 2 A (PP2A) and serine/threonine kinase AKT to the β-arrestin-2/D2R complex. PP2A dephosphorylates AKT, leading to its inactivation. AKT, in turn, it regulates glycogen synthase kinase β (GSK3β) through phosphorylation. When GSK3β is not phosphorylated, it remains active, but AKT-induced phosphorylation inactivates GSK3β (Beaulieu et al., 2015).

The regulation of GSK3β by D2R and β-arrestin-2-dependent AKT has implications in physiology of neurons, pathophysiology and the mechanisms which are followed by antipsychotic drugs. This pathway is essential in development, cell physiology, and pathology, including cancer (Nusse et al., 2017).The Wnt/β-catenin pathway I nvolves Frizzled (FZD) Wnt receptors and the LRP5/6 co-receptor along with Dishevelled, which activates and stimulates a β-catenin destruction complex which contain Axin, Adenomatous polyposis coli (APC), and GSK3β. In the absence of stimulation, GSK3β phosphorylates β-catenin, marking it for degradation. However, when FZD/LRP5/6 are activated with the help of ligands like Wnt3a, β-catenin translocate in the nucleus and activates the transcription of Wnt target gene through binding to T-cell factor (TCF)/lymphoid enhancer-binding factor (LEF) proteins (Majidinia et al., 2018). AKT plays a crucial role in inactivating GSK3β by phosphorylation at serine 9 or 21, allowing β-catenin to evade proteosomal degradation and initiate gene transcription. Additionally, GSK3β activity can be inhibited by growth factors like insulin/insulin-like growth factor I (IGF-I) and nerve growth factor (NGF) or through inhibitory auto-phosphorylation (Fang et al., 2000).

Given AKT's prominent role in D2R's G protein-independent, arrestin-dependent pathway, the focus is on how D2R-mediated GSK3β inactivation via AKT phosphorylation affects β-catenin-driven changes in gene expressions (Porter et al., 2017)

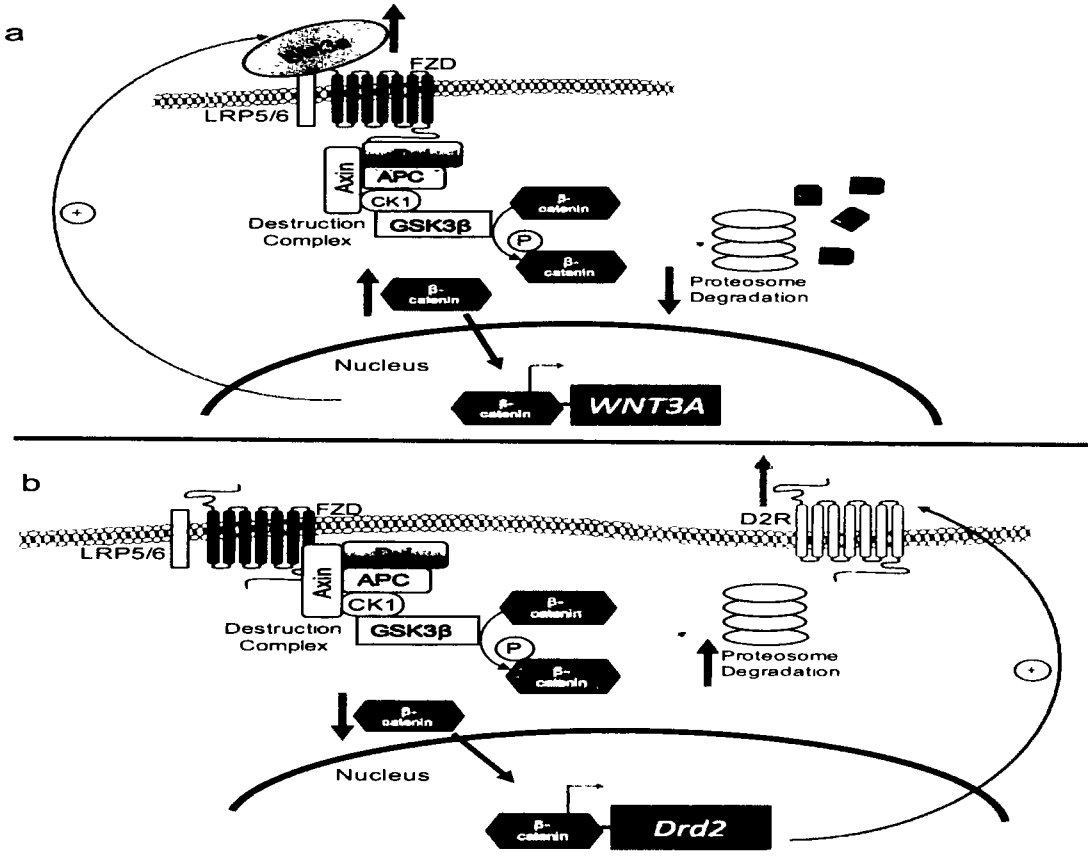

Figure1.3 Dopamine D2 receptor modulates Wnt expression and control of cell proliferation

1.8.4 Role of DRD2 in Cancer

The expression of the dopamine D2 receptor (D2R) family is elevated in many cancer types. Interestingly, certain disorders like schizophrenia and Parkinson's disease, which involve the use of dopaminergic drugs, have been linked to a reduced risk of cancer development. Studies have indicated that D2R antagonists exhibit anticancer effects in cell cultures and animal models. These effects include reducing tumor growth, inducing autophagy, influencing metabolism of lipids, and promoting apoptosis. Consequently, a prevailing hypothesis suggests that D2R ligands could serve as a novel approach for cancer treatment as these receptors can be used in chemotherapy (Schalop and Allen 2016).

In a research study, it was found that increased expression of DRD2 is linked to a lower survival rate in cancer patients. Further investigations revealed that reduced DRD2 levels resulted in the inhibition of tumor cell growth and motility, both in laboratory tests and in living organisms so these receptors can inhibit growth of cancer cells. This effect was achieved by downregulating β-catenin/ZEB signaling by the researchers. Conversely, when DRD2 was overexpressed, tumor cell progression was promoted, hence showing the link between DRD2 gene expression and cancer.To validate these findings, researchers used an antagonist called pimozide to inhibit DRD2. The inhibition of DRD2 using pimozide was shown to be effective in curbing tumor growth and preventing lymph node metastasis in live subjects. Moreover, in vitro experiments demonstrated that inhibiting DRD2 enhanced the cytotoxic effects of conventional cancer treatments. Overall, this study suggests that targeting DRD2 could be a promising strategy to inhibit cancer progression and improved treatment strategies can be used for cancer treatment (Lee et al., 2021

In addition to the neuronal functions of DRs in neurological diseases, among DRs, DRD2 especially was reported to be overexpressed in different cancer types, including cervical, lung, pituitary, colon, and gastric cancers and to be correlated with poor prognoses of different types of cancers. Moreover, blocking DRD2 was shown to suppress proliferation and induce apoptosis of both cancer cells and cancer stem cells. So an association has been found between DRD2 and several cancers. The expression and the effects of DRD2 inhibition and progression were reported in several types of cancer reported by different researchers (Cheng et al., 2018).

1.8.5 Mutations in DRD2

The dopamine receptor D2 (DRD2) , plays a very important crucial role in the therapies used for various neurological and psychiatric disorders in humans. Particularly, the third intracellular loop (ICL3) specially in DRD2 gene is necessary for the interaction of DRD2 with G proteins to activate adenylyl cyclase activity and several signaling scaffold proteins, thus facilitating proper DRD2 signaling. However, mutations in third intracellular loop (ICL3) can disrupt the binding of gene, leading to altered signaling of DRD2. The researchers investigated the detrimental effects of a specific mutation, in which serine was substituted with cysteine at position 311 (S311C) that took place within the ICL3 region of DRD2 gene. This mutation has been implicated in disorders such as schizophrenia and alcoholism. The analysts revealed a marginal impact of the mutation on the mechanism of dopamine binding and observed significant structural alterations in the mutant receptor. The findings suggest a potential mechanism exists for malfunctioning in cAMP-dependent signaling pathway and maintenance of calcium homeostasis in the brain controlled by dopaminergic system, ultimately leading to neuropsychiatric disorders in human (Mei et al., 2007).

The 'T' allele of C957T in the DRD2 gene has been responsible for decreased expression of dopamine receptor in the brain which leads to potential alterations in cognitive processes. These changes can manifest as differences in focus, stress response, and multitasking capacity. Additionally, there are indications that this allele might also be correlated with an elevated risk

of developing neurological disorders like schizophrenia. Four infrequent missense mutations (V96A, V154I, P310S, and S311C) along with a polymorphism (-141C Ins/Del) in the promoter region of the DRD2 gene have been documented (Arinami et al., 1997). Some of these missense single-nucleotide polymorphisms (SNPs) and the -141C Ins/Del polymorphism have been associated with schizophrenia in previous studies (Wong et al., 2000).

In addition to the missense mutations and the promoter polymorphism, six other DRD2 synonymous polymorphisms (C132T, G423A, T765C, C939T, C957T, and G1101A) have been identified (Gijman et al., 1994). These synonymous polymorphisms were originally believed to have no functional impact, meaning they were presumed to be functionally silent.

Statement of the Problem

The incidence of cancer is rising but very limited information is available regarding clinical presentation and genetic causes of cancer in Pakistani population. Therefore, abundant work is required to investigate the genetic risk factors of cancer. This research is planned for the genetic analysis of dopamine receptor gene, DRD2 to find its role in cancer, so that the scope of dopamine based chemotherapy can be validated.

Objectives of the Study

The aim of the study is:

- To analyze the genetic variations in dopamine receptors gene DRD2.
- To analyze the possible involvement of mutation in dopamine receptor's gene DRD2 and its role in cancer.
- In Silico analysis of Dopamine Receptor Gene DRD2 to examine the association of mutation in dopamine receptors gene with risk of cancer (GDC).

CHAPTER 2

MATERIALS AND METHODOLOGY

2.1 Sample Collection

A total of 213 fresh blood samples of cancer patients and 50 ages and sex matched negative control blood samples were collected from the hospitals of Rawalpindi and Islamabad

2.1.1 Sampling Techniques

Consecutive sampling techniques were used to recruit the participants from the study population of laboratory in both groups: cases and control. The patients were assessed by personal information and clinical investigations; all information was included in a clinical questionnaire. The patients were made well-aware about the aims of the study after which consent form was signed by each contributor. After drawing 5ml of blood from each participant, it was placed in Acid Citrate Dextrose (ACD) vacutainer (BD Franklin, Lakes NJ USA), in which 2.5 ml acidcitrate dextrose solution was present. The absolute volume was 7.5 ml. This ACD vacutainer functions as a preservative and anticoagulant for the fresh blood sample. After inverting the vacutainer tubes few times instantly, these tubes were stored at 4 °C for further use.

2.1.2Inclusion and Exclusion Criteria

All cases and controls with clinical information and consent (for fresh blood samples) available were included in the study and all those cases and control samples were excluded from the study for which the consent was not available. Therefore, after applying the exclusion criteria a total of 30 samples were excluded and the study was performed on 213 cases and 50 negative control samples.

2.2 Ethical Consideration

The study was approved by the Institutional Ethical Review Committee, International Islamic University Islamabad.

2.3 Genomic DNA Extraction from Whole Blood Samples

The extraction of DNA from all the samples was performed by following standard organic method which recruits two chief organic chemicals namely Phenol and Chloroform (Sambrook and Russell, 2001). The protocol was marginally optimized as per conditions.

2.3.1 DNA Extraction steps

The DNA extraction was performed in strict accordance to the following steps. DNA extraction procedure was completed in 2 days. The detailed procedure is as follows:

2.3.1.1 Day 1

750µl of blood samples were transferred to Eppendorf tubes and 750µl of solution A (lysis buffer) was added in each tube. The samples were mixed five times by inversion method and Eppendorf tubes were kept at room temperature for 25 minutes. Then for the duration of one minute the tube was centrifuged at 13000 rpm and then the supernatant was discarded from all the tubes. The nuclear pallets obtained at the bottom of tubes were re suspended in 400µl of solution A and centrifuged for 1 minute at maximum speed (13000 rpm). The supernatant was again discarded and the nuclear pallet was re suspended in 400µl solution B. Then the mixture was vortexed until we obtained the clear solution. After that 14µl of 20% SDS (Sodium Dodecyl Sulphate) 0r 25µl of 10% SDS was added in each tube and then 5 to 8µl Proteinase K (20µl/ml, stored at -20°C) was added in the mixture. At the end the tubes were incubated for overnight at 37°C.

2.3.1.2 Day 2

The samples were taken out from the incubator. 500µl of freshly prepared mixture of equal volume of solution C (Phenol) and solution D (480µl Chloroform and 20µl Isoamylalcohol) was added in each tube (250µl each). Then the samples were centrifuged again for the duration of 10 minutes at 13000 rpm and the upper aqueous phase was collected in the new tubes .The 500µl of solution D was added. The samples were centrifuged again for 10 minutes at 13000 rpm and the upper aqueous phase was collected in the new tubes. To the collected aqueous phase, 55µl of Sodium acetate (3M, pH 6) was added. After that 500µl of Isopropanol stored at -20°C was added in each tube. The tubes were inverted gently so that the DNA was precipitated out. Then the tubes were centrifuged for 10 minutes at 13000 rpm and the solvent phase was removed carefully without

disturbing the DNA pellet. 200µl of 70% Ethanol stored at -20°C was added in the tubes containing the pelleted DNA and then centrifuged at 13000 rpm for 7 minutes. The ethanol was removed and the DNA pellet was dried in the vacuum dryer at 45°C for 30 minutes. At the end the DNA was dissolved in 250µl Tris EDTA buffer. Then the DNA was incubated at 37°C overnight. Composition of all the solutions along with concentrations and functions used in DNA extraction are shown in table 2.1,

2.4 Quantification of Extracted DNA

After extraction, the DNA quantity was analyzed by measuring the Optical Densities of samples by Spectrophotometer at 260nm and 280nm considering the ratio 1.7-2.0 as ideal whereas ratio 1.8 the most ideal (Thermoscientific, Multiskan SKY). After taking the ODs, all samples were kept relocated in 1.5ml labeled Eppendorf tubes and were stored at -20°C till further use. For PCR amplification, 100ng dilutions of stock DNA were prepared and stored at -20°C. The ODs of all the samples are mentioned in table 2.2. The quantity of extracted DNA was also analyzed by Agarose Gel Electrophoresis.

2.5 Primer Designing

Primers for DRD2 were designed by Primer Blast and Primer 3` Free Online Primer Designing Tool and then purchased commercially from Macrogen. Oligo Analyzer Tool was used to check the stats of primers. The compatibility of primer sequences of DRD2 including Tm and GC contents of forward and reverse primers was analyzed (Insilico PCR Tool). Ensemble Genome Browser 109 was initially to find the nucleotide sequence of DRD2 primers (Table 2.3).

2.6 Polymerase Chain Reaction (PCR)

Polymerase chain reactions were carried out to amplify the DRD2 gene for genetic analysis. PCR was performed for all the samples including control. PCR conditions were altered for the optimization pf results. DNA sample was optimized in a total reaction volume of 25µl .Detail of reagents and their volumes are given in table 2.4.

The thermal cycler program for the mutational analysis of DRD2 consisted of an initial melting step at 95°C for the duration of three minutes, proceeding with 30 cycles encompassing three steps

each, denaturation at 95°C for 30s, primer annealing at Tm-5°C for 30s and primer extension at 72°C foe 1 min. The final extension was carried out at 72°C for 10 mins in one cycle.

2.7Agarose Gel Electrophoresis

Electrophoresis was performed after extraction on 1% gel for analyzing DNA and to check the quantity of bands. The PCR amplified fragments were electrophoretically separated for about 60 min at a voltage of 100V on a 1.5% agarose gel. 100bp ladder was used as a marker. The 1.5% agarose gel was prepared by dissolving 1.5g of Agarose in 100ml 10X TBE buffer in a microwave oven. The mixture was cooled and then 10µl of ethidium bromide was added during the preparation of gel which aided in the visualization of DNA band UV transilluminator. After 60 min an image of the gel was captured by gel documentation system.

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2.8 Purification of amplified PCR Products.

PCR Purification Kit (Ferments, USA) was used to purify the PCR products before sending it for sequencing. PCR products were amplified with 135-145ul binding solution following centrifugation for 1-2 minutes at 4000 rpm and after that vortex were done for 1-2 minutes. Purification column was used and reaction mixture was shifted to column coated with silica membrane and then centrifugation was done for 1 minute at 13000 rpm. After centrifugation flow through was discarded. Ethanol (600 µl) was used as washing buffer. After washing, centrifugation was done for 1 minute at 13000rpm. Flow through was completed and then it was discarded. And again washing process was done. Flow through was again discard repeating the washing step with 400 µl of washing buffer and a minute of centrifugation at 13,000 rpm. Discarded the flow-through, empty the spin and remaining product were shifted to other tube. Elution of purified DNA was performed with elution buffer of the amount 20-30 µl which was preheated with 65°C, and then after his the incubation was done for 10 minutes in silica tube. At the end the purified product was checked on 2% agarose gel.

2.8.1 Sanger Sequencing

Sequencing by Sanger process was done to find out the variants of DRD2 gene. Sequencing was out sourced. The automated sequencer was used to check the sequence.

2.8.2 Sequenced Data Analysis

The sequenced data was compared with normal sequences downloaded from Ensemble Genome Browser database (http://www.ensemble.org/index.html) to identify any nucleotide change presents in bands. Finch TV was used to identify any sequence variation among the affected individuals and alignment of sequencing results was performed by "Cluster W Multiple Alignment" tool. GDC data portal was also used to analyze mutations within DRD2 gene.

2.9 Statistical And Computational Analysis

The data was also analyzed statistically. The data of cancer patients was sorted out according to age, gender and type of cancer and the percentage of each category were calculated. Bioinformatics techniques were used to analyze data collected regarding DRD2 gene mutations.

Table 2.1: Composition of Solutions Used in Genomic DNA Extraction

SOLUTIONS	COMPOSITION
Solution A	Tris (pH 7.5) 10Mm 1% (v/v), Triton X-100, Sucrose 0.32M, $MgCl_2$ 5mM
Solution B	EDTA (pH 8.0), Tris (pH 7.5) 10Mm, NaCl 400 mM
Solution C	Phenol
Solution D	1 volume of Isoamylalcoho:24 volume of chloroform i.e Chloroform ($CHCl_3$) 480µl Isoamylalcohol ($C_5H_{12}O$)20µl
10% SDS	50ml water containing 10g of SDS
DNA dissolving buffer	0.1 Mm EDTA, 10Mm Tris (pH 8.0)

Table 2.2: Primer Sequence of DRD2 Gene

EXONS	PRIMERS	Tm °C	GC CONTENTS%	SELF DIMER Kcal/mol	PRODUCT LENGTH(bp)
Exon 1 Forward	TGGAAGCCTCAAGCAGCA	57.2	55.6	-3.14	150
Reverse	CAAACTTCTGGTCCTGGCCT	57.4	55	-9.2	
Exon 2 forward	TCCAGCACTTTCTCCAGCAG	57.3	55	-3.14	102
reverse	AAAAACCACCTGGGGAAGCA	57.4	50	-5.05	
Exon 3 forward	GTGTGCCATCAGCATCGACA	58	55	-6.2	219
Reverse	GCTTCTCTTTAGGGAGTGTGTGC	57.7	52.2	-3.17	
Exon4 Forward	CACCGTCATGATCTCCATCGT	56.8	52.4	-8.53	202
Reverse	GTAGTGATAGGCCTCCATTGGG	57	54.5	-9.4	
Exon5 Forward	TGTACTCACCCCGAGGACAT	57.6	55	-3.65	172
Reverse	ATTGGCCAGCTTCTGAGGTC	57.5	55	-13.1	
Exon6 Forward	CCCACCCTCCTGATCTCTAA	55.4	55	-4.62	251
Reverse	CAGAAAGACCTGGCTCTGGG	57.7	59.5	-3.16	
Exon7 Forward	GGAGCTGGAGATGGAGATGC	57.6	60	-6.34	374
Reverse	AAGGACATGGCAGGGAATGG	57.6	55	-5.38	
Exon8 Forward	CCACCGTCTTGGCATACGAG ACTGCCTCTGCCTTAGAGGA	58 57.8	59.5 60	-5.02 -4.67	269

Table 2.3: Detail of Reagents used in Polymerase Chain Reaction

S. No	Reagents	Stock Concentration	Final Volume
1	Taq Buffer	10x	2µl
2	$MgCl_2$	25mM	2µl
3	dNTPs	50Mm	1µl
4	Forward Primer	10µM	1µl
5	Reverse Primer	10µM	1µl
6	Taq DNA Polymerase	5µM	0.25µl
7	DNA	100ng	1µl
8	dH_2O		16.75µl
	Total Volume		25µl

Table 2.4: Thermal Cycling Optimized Conditions for PCR

Step	Temperature °C	Time	No. of Cycles
Initial Denaturation	95	1-3 mins	1
Denaturation	95	30s	25-40
Annealing	Tm-5	30s	25-40
Extension	75	1min	30
Final Extension	72	10mins	1

CHAPTER 3

3.1 Genetic Analysis of DRD2 Gene

The data was categorized on the basis of sex of patients, types of cancer, types of mutation, genetic history of disease, survival rate and impact of genetic mutation in DRD2 gene. The data consists of 59.2% females and 40.8% males (Table 3.1).

3.2 Age Distribution among Cancer Patients

Distribution of cancer patients according to different age groups have been shown in table 3.2. The cancer patients were categorized into different age groups to evaluate the most susceptible age for the diagnosis of cancer. The diagnosis of tumor was observed to be highest between the fifth and sixth decade of life. This age group had both genders. The calculated mean and standard deviation for age was 49.17±12.96 (Table 3.2).

3.3 Distribution of Cancer Patients According to Type of Cancer

The data was also categorized on the basis of types of cancer. Among the different types of cancer, there was more number of female patients having cancer of corpus uteri (24.9%), skin cancer patients (21.1%) and bronchus and lungs cancer patients (17.8%). The 5.6% cases of colon cancer were reported. There were 2 cases in which the site of cancer was ill defined (Table 3.3).

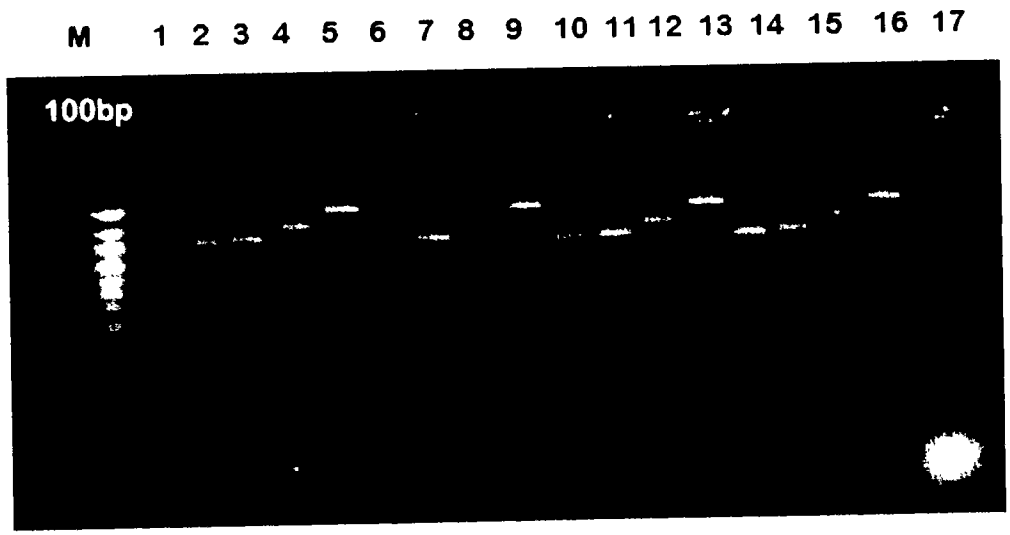

Figure. 3.1(b) Agarose gel electrophoresis for amplified DRD2 Gene using primers of Exon 7.Bands were fractionated by gel electrophoresis on 2% agarose gel and visualized under UV light after staining with ethidium bromide, 100 bp ladder was used.

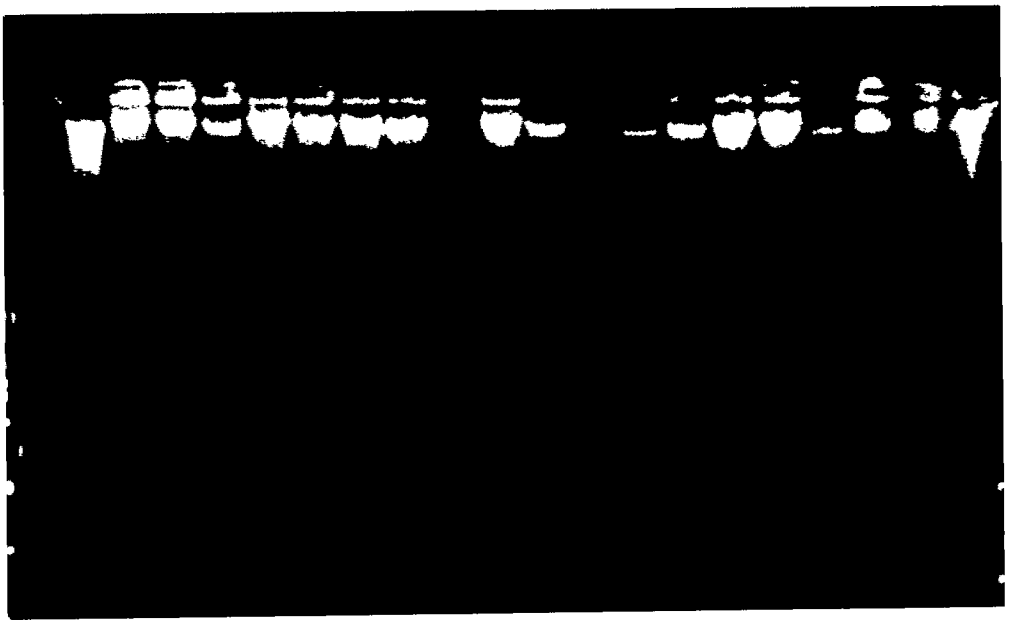

Figure 3.1(a) Bands of Extracted DNA by Agarose Gel Electrophoresis on UV Illuminator

Table 3.1 Distribution of cancer patients according to gender

Gender	No. of Individuals	Percentage (%)
Female	126	59.2
Male	87	40.8
Total	213	100

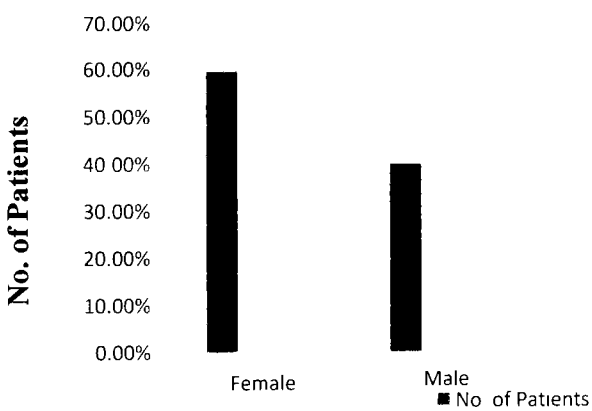

Figure 3.3 Representing no. of female and male patients. The percentage of female patients is 59.2% and the male patients is 40.8%

Table 3.2 Distribution of Cancer Patients According to the Age Group

Age Groups	Percentage (%)
<10	0
10-24	5.5
25-39	16.6
40-54	40.2
55-69	34.7
70-84	2.7
Total	100

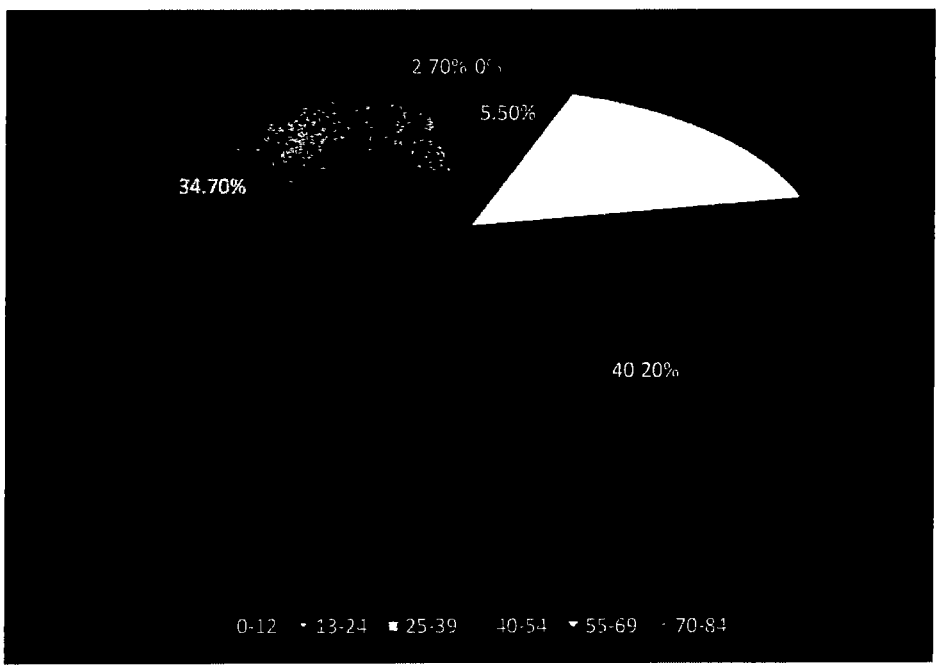

Figure 3.1: Pie chart representing distribution of cancer patients according to the age group

Table 3.3 Types of Cancer and the Number of Patients Reside with Each Type

Types of Cancer	Percentage (%)
Skin Cancer	21.1
Hematopoietic reticuloendothelial	3.3
Corpus Uteri	24.9
Bladder	3.3
Bronchus and lungs	17.8
Kidney	0.9
Liver	0.9
Colon	5.6
Stomach	5.2
Ovary	2.3
Esophagus	1.9
Pancreas	0.9
Brain	2.3
Breast	1.9
Rectum	0.9
Connective and subcutaneous	0.9
Uterus	4.7
Ill-defined sites	0.9
Total	100

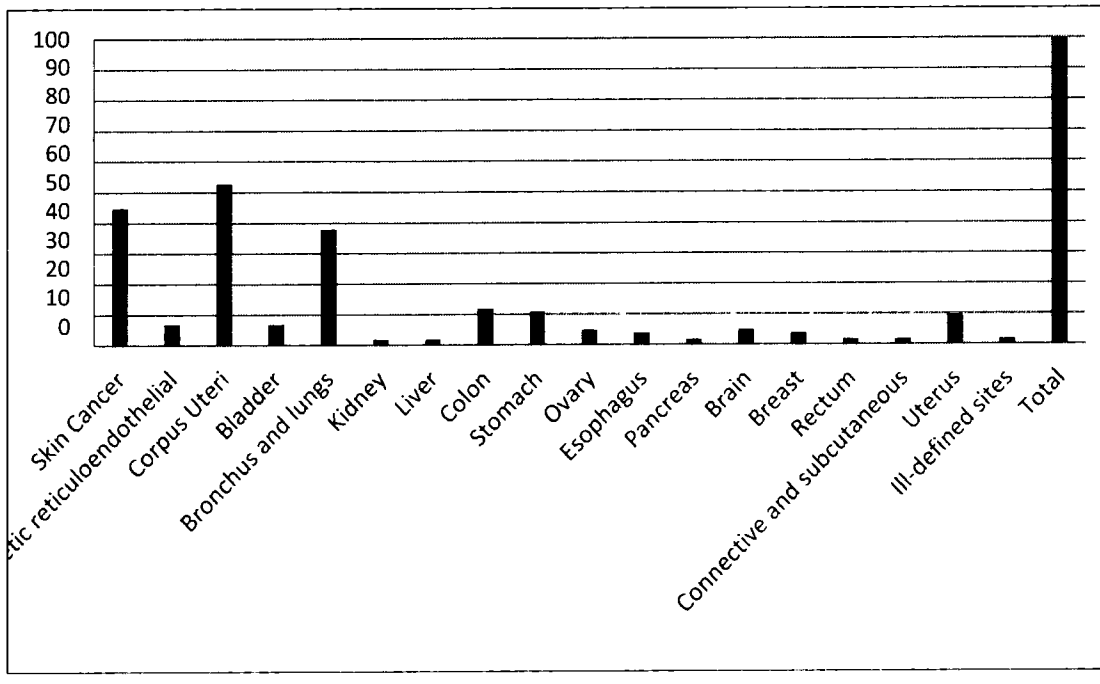

Figure 3.4 Types of cancer and the number of patients reside with each type, represents maximum no. of female cancer patients having cancer of corpus uteri then the no. of skin cancer and bronchus and lungs patients comes

3.4 Mutational Analysis of DRD2 GENE

The data was also analyzed on the basis of types of mutations reported in 213 cases. The type of mutation that is most prominent among the reported cases is Single Base Substitution. There are 98.6% cases in which single base substitutions were reported and deletion was reported only in 1.4% affected cases. Among the 26.2% cases, the Guanine was substituted by Adenine and in 24.7% cases, the cytosine was substituted by thymine nucleotide base. There are 3.7% cases in which cytosine was substituted by Guanine nucleotide base (Table 3.6). G>A, C>T, T>C and C>G, these four types of base substitutions were found to be more prominent among the mutational data provided by Genomic Data Common for DRD2 mutations.

For the genetic analysis of DRD2, three bioinformatics tools were used. The Ensemble Variant Effect Predictor (VEP) impact was used to determine the variant effects which can be SNPs, insertions and deletions on gene transcripts. Protein sequence as well as regulatory region can also be analyzed. Second bioinformatics tool that was used to find the impact of mutation was SIFT (Sorting Intolerant from Tolerant. SIFT scores predict whether a protein function is changed or effected by amino acid substitution or not. Polyphen (Polymorphism Phenotyping) tool was used for the analysis of the effect of an amino acid substitution on the structure of a human protein.

There were 26.7% affected cases in which synonymous mutations in DRD2 gene were observed. For synonymous mutations, the VEP impact was "Low", that predict the mutations were either harmless or not change DRD2 gene sequence. SIFT and Polyphen scores were not found for synonymous mutations as these were considered to be harmless.

There were 64.7% affected cases in which missense mutations were analyzed. The VEP impact for all missense mutations was moderate. Moderate impact represents a variant of DRD2 was not disruptive that might change the structure and effectiveness of protein. In the cases of missense mutations, the SIFT impact was found to be deleterious with SIFT score: 0 and tolerated with SIFT score ranges from 0.05-1.00. The variants with scores nearer to 0.0 were more accurately consider to deleterious. The SIFT score of 30% cases was 0.0, so these mutations had deleteriouseffect on DRD2 gene and SIFT score of 68 cases of missense mutation was between the range of

0.05-1.00, so these mutations in DRD2 gene were found to be tolerated. The Polyphen score of missense mutations was analyzed. Out of 64.7% cases of missense mutations, the Polyphen impact of 29.5% cases was Benign (BE) with Polyphen score ranges from 0.0-0.15.The Polyphen impact of 39.8% cases of missense mutation Probably damaging (PR) impact with the Polyphen score ranges from 0.85-1.0 and 20 cases had Polyphen score of 0.15-1.0, so variants with this scores were possibly damaging (PO).

There were only nine consequences of mutations other than synonymous and missense mutations in which the VEP impact was high. Out of total 213 affected case, only in three cases, there was deletion of single nucleotide base, in Chr11:g 113412659delG as a result of Frameshift mutation at DRD2 M348cfs*(case of Bronchus and Lungs cancer in female patient), Chr11:g 113412770delG as a result of Frameshift mutation at DRD2 D311Tfs*50 (case of Corpus Uteri female patient), Chr11:g 113414428delAG as a result of Frameshift mutation at DRD2 C253Hfs*7 (case of colon cancer female patient). So Frame shift mutation in which there was deletion of G has deleterious effect which can change the DRD2 gene structure and function. Out of 9 affected cases with High VEP impact, in four cases, Stop gained mutations were analyzed and in two cases Splice Donor mutations in Chr:11g 113416861 A>G Substitution andChr:11g 113415480G>A Substitution were analyzed (GDC).

Table 3.4 Distribution of Affected Cases According to The Types and Consequences of Single Base Substitutional Mutations

Type of mutation	Percentage (%) Occurrence
Missense	63.8
Synonymous	24.2
Frame Shift	2.8
Intron DRD2	1.87
3' Prime UTR	1.4
Stop gained	1.4
Splice Donor	0.9
Splice Region DRD2	0.9
Down Stream	0.5
Total	100

Table 3.5 Distribution of Affected Cases According to the Types and Consequences of Single Base Substitutional Mutations

Type of mutation	Percentage (%) Occurrence
Missense	63.8
Synonymous	24.2
Frame Shift	2.8
Intron DRD2	1.87
3` Prime UTR	1.4
Stop gained	1.4
Splice Donor	0.9
Splice Region DRD2	0.9
Down Stream	0.5
Total	100

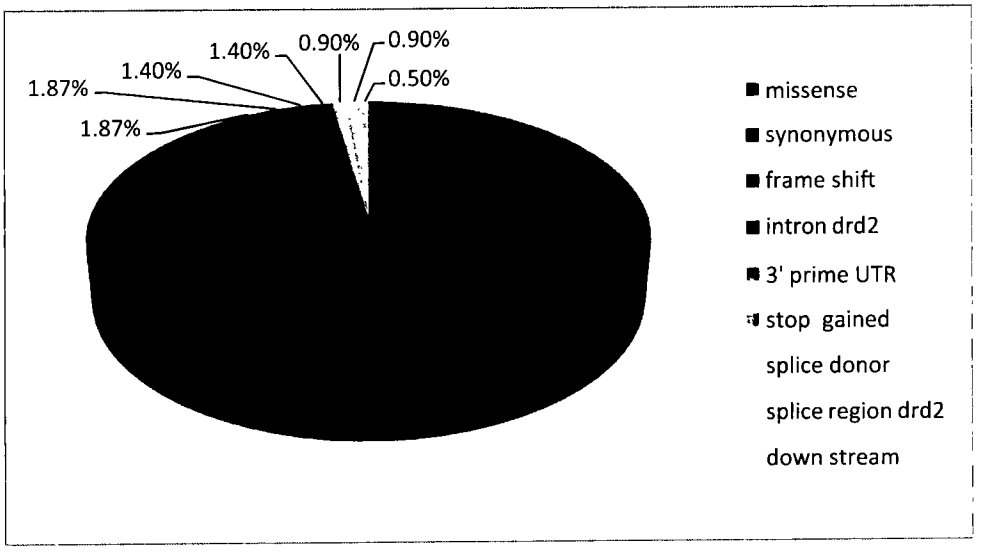

Figure 3.4 Representing the consequences of mutations within DRD2

Table 3.5 Representation of Polyphen Impact of missense mutations in DRD2Gene

Polyphen Impact	**Polyphen score**	**Percentage (%) Occurrence**
Possibly Damaging (PO)	0.15-1.0	9.4
Benign (BE)	0.0-0.15	29.6
Probably Damaging (PR)	0.85-1.0	25.8
Nonspecific	----	35.2
Total	----	100

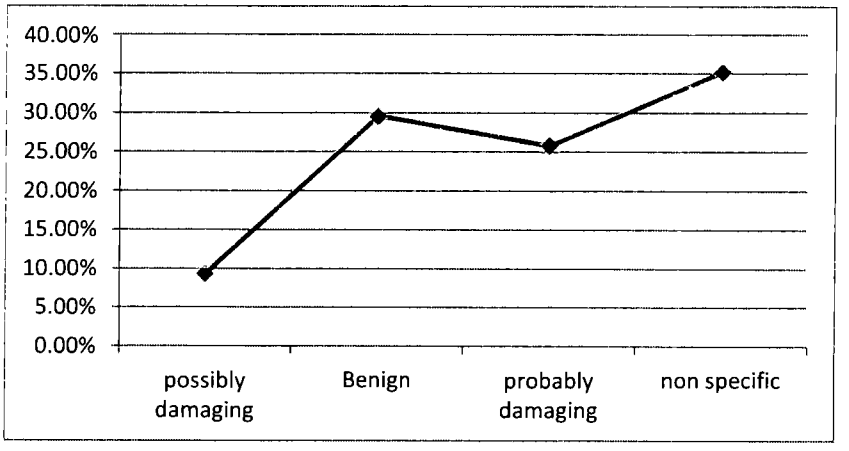

Figure 3.5 representing Polyphen Impact of missense mutations in DRD2 Gene

Table 3.6 Representing the types of mutations in which the VEP impact was HIGH

Type of Mutations	Position	Consequence	Exon	Cancer Site	Gender
Stop gained (Substitution)	DRD2W 160	C>T	7	Skin	Male
Splice Donor (Substitution)	DRD2 X 95	C>T	7	Kidney	Female
Stop gained (Substitution)	DRD2 E280	C>A	7	Lungs	Male
Splice Donor (Substitution)	DRD2 X 178	A>G	1	Uterus	Female
Stop gained (Substitution)	DRD2 R222	G>A	1	Hematopo-ietic Cellular	Female
Frame Shift (Deletion)	DRD2 M348Cfs*13	del G	7	Lungs and Bronchus	Female
Frame Shift (Deletion)	DRD2 D311Tfs*50	del G	4	Corpus Uteri	Female
Frame Shift (Deletion)	DRD2 C253Hfs*47	del G	7	Colon	Female

CHAPTER 4

Discussion

Cancer poses a significant global health challenge, affecting millions of people annually. It remains a major cause of mortality worldwide, with over half of diagnosed patients succumbing to the disease. In some nations, cancer ranks as the second leading cause of death, trailing only cardiovascular diseases (WHO).

Recent findings indicate that cancer cells exploit neurotransmitter-initiated signaling pathways to trigger unchecked growth and spread. Moreover, these neurotransmitters has their role in influencing immune cells and endothelial cells which are present within the tumor, further fueling tumor advancement. Consequently, gaining a deeper comprehension of how neurotransmitters function in tumorigenesis, angiogenesis, and inflammation holds great importance for the advancement of novel and more effective antitumor therapies. By focusing these mechanisms, researchers aim to develop the next generation of treatments to combat cancer more efficiently (Jiang et al., 2020)

The purpose of the present study was to analyze the role of mutations in DRD2 with cancer in selected population. Total 213 samples were collected to conduct the study along with 50 controls. Some of samples were degraded and some of them were containing the minute quantity of DNA which was insufficient for amplification.

Single-nucleotide polymorphisms (SNPs) are found to be more prevalent among different variations within DNA sequence, and they can influence gene expressions, thereby affecting susceptibility to diseases like cancer (Sripichai et al., 2007). In the case of the gene encoding DRD2, several SNPs have been identified. Notably, the following threeSNPs, representing rs1799732 (DRD2 -141C>del), rs6277 (DRD2 957C>T), and rs1800497 (DRD2 TaqIA), have been found to impact the expression and functioning of the DRD2 gene. Additionally, rs1079597 (DRD2 TaqIB) which is located near the regulatory and structural 5'coding regions of the gene, that play its role in transcriptional regulation of DRD2 (Zhang et al., 2016). Literature shows that DRD2 SNPs have been linked with the risk of various diseases, including Parkinson's disease, schizophrenia, colon cancer, and lung cancer. Furthermore, rs1800497 has been observed in adenoma recurrence and an increased risk of smoking among cancer patients was observed. Researchers have delved into the contributions of four specific DRD2 SNPs, named as rs1799732, rs1079597, rs6277, and rs1800497, which were located in the promoter, intron 1, exon 7, and 3'

untranslated region (UTR) downstream regions, respectively, which was found to be responsible for urothelial carcinoma (Peculis et al., 2016).

In the present study, age was one of the parameter. This study showed that most of the patients with the age group above 40 and below 69 were found to have more mutations than the age group below 30. The calculated mean and standard deviation for age was 49.17±12.96. It is reported with this age, risk of cancer and mortality rate gets higher (Cancer Facts and Figures 2016-2018). Among cancer patients, median age was 59 in males and 63 in female (Howlader et al., 2013).

In our study the frequency of Corpus Uteri Cancer in females was 24.9%. This finding is quite closer to the data reported in other studies from West (Wang et al., 2019) i.e. 70% but does not correlate with the information reported in other researches. This dissimilarity may be because of difference in the sample size or might be environmental and genetic factors are involved. The data shows more number of skin cancer patients (21.1%) and Bronchus and lungs cancer patients (17.8%) those had mutations in DRD2.

Current study showed that the most patients had synonymous mutations and missense mutation in DRD2 gene (GDC) but the impact of synonymous mutations are very low that mean the synonymous mutations in DRD2 are not responsible for cancer. In case of missense mutations in DRD2 gene, the impact is moderate that may or may not cause cancer. In the cases of Frame shift mutations in DRD2 which were responsible for the deletion of G, those mutations were responsible for cancer. In this study there are only 5% affected cases in which DRD2 gene mutations are responsible for causing cancer. Out of 5% cases, in 1.4% cases frame shift mutations, in 1.87% cases stop gained mutations and in 0.93% cases splice donor mutations were observed. These mutations were found to involve in cancer.

Conclusion

Cancer is more prevalent in developed countries and incidence of cancer is increasing over the past two or three decades. In 2020, an estimated 18.1 million new cases of cancer were reported globally. Among all cancer types, the four most common ones were female breast cancer, lung cancer, bowel (colorectal) cancer, and prostate cancer. These four types combined accounted for over 40% of all cancer diagnoses worldwide. They represent a significant portion of the cancer burden and continue to be major health concerns on a global scale. Efforts to prevent, diagnose,

and treat these common cancers remain crucial in addressing the overall impact of cancer on individuals and public health.

DRD2 (D2 dopamine receptor) is one of the member of dopamine receptors family, and is a member of D2-like receptors. Dopamine signaling pathways play very important roles in memory, reward, learning and other important functions. Evidences gathered from literature also reveal the role of dopamine in immune system and the development of cancer.

The exon 7 of DRD2 is the largest exon and contains 70% of gene coding sequence and also most of the mutations of DRD2 occur on this exon. Mutations found on this exon are either frameshift or stop gained mutations. A specific missense mutation in this gene leads to myoclonus dystonia, while other mutations have been linked to schizophrenia. Moreover, the gene undergoes alternative splicing, resulting in the production of two transcript variants that encode different isoforms. The two missense variants at exon 7 had been reported. A DRD2 missense mutation was reported which was resulted in a missense variant C/G in exon 7 of DRD2 that is responsible for ser/cys substitution on chromosome 11 at position 311.

Current study was formulated to analyze the role of DRD2 mutations in cancer. By viewing at computational results and discussion mentioned above. It can be concluded that DRD2 variants are involved in developing risk of cancer. To accurately determine how family history and other genetics and environmental factors can influence the risk of DRD2 gene mutations in cancer patients further studies are required.

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