Analyzing the Association of KPCD Gene's Non-Synonymous Variants with Breast Cancer

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A thesis submitted in partial fulfilment of the requirements for the degree of MS Healthcare Biotechnology

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DECLARATION

I certify that this research work entitled "Analyzing the Association of KPCD Gene's Non-Synonymous Variants with Breast Cancer" is my own work. The work has not been presented elsewhere for assessment. The materials that have been used from other sources have been properly acknowledged/referred.

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This thesis is dedicated to my beloved parents and my siblings.

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Somia Khan

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LIST OF ACRONYMS

KPC	Kinase Protein C
ARMS	Amplification- Refractory Mutation System
CADD	Combined Annotation Dependent Depletion
CI	Confidence Interval
DAG	Diacylglycerol
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
HOPE	Have Our Protein Explained
MD	Molecular Dynamics
OR	Odd Ratio
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
RMSD	Root Mean Square Deviation
RMSF	Root Mean Square Fluctuation
RR	Relative Risk
SASA	Solvent accessible surface area
SDS	Sodium Dodecyl Sulphate
SNP	Single Nucleotide Polymorphism
TAE	Tris Acetate EDTA
TE	Tris EDTA
UV	Ultraviolet

ATP	Adenosine Triphosphate
SIFT	Sorting Intolerant From Tolerant
ER	Estrogen Receptors
PR	Progesterone Receptors
MAPK	Mitogen activated protein kinase
ERK	Extracellular Receptor Kinase
PI3K	Phosphatidylinositol-3-kinase
STAT3	Signal transducer and activator of transcription 3

ABSTRACT

Breast cancer has the highest mortality rate among women, owing to its late diagnosis. Determining its susceptibility genes might lead to better outcomes. Several studies reported dysregulation of PKCδ in a variety of cancers. Therefore, variant rs1703863535 has also been linked to breast cancer. Thus, the study's primary purpose was to investigate the association of variant rs1703863535 with breast cancer and to analyze its impact on protein structure and function. Genotyping by ARMS PCR was done to study the variant association with breast cancer (cohort size =100). ENSEMBLE browser for data retrieval was used, and then filtering out the pathogenic variants based on scores of different tools, i.e., CADD, Mutation Accessor, Polyphen, SIFT, MetaLR, and Revel. To study the effect of missense mutation on PKCδ structure, DynaMut was used to determine the protein flexibility and interatomic interactions, HOPE and FATHMM were carried out to study the structure-functional analysis and protein stability, and in situ, mutagenesis was done by PyMOL. The molecular dynamic simulation was also done to study the impact of variant rs1703863535 on protein structure, i.e., wild and mutant. Protein stabilization, structure, and function were highly affected due to nsSNP. According to the MD results, RMSF, RMSD, and Rg values differed in wild and mutant. After the genotyping analysis, the variant showed highly significant results, which indicated that TT and TC genotypes were significantly associated with breast cancer as a significant risk factor. After accessing the results, variant rs1703863535 can be a potential diagnostic and prognostic marker for breast cancer and develop novel and successful therapeutic targets in clinical trials.

Keywords:

Breast cancer, PKCδ, variant rs1703863535, Genotyping, Molecular dynamic simulation, Diagnostic marker.

CHAPTER 1

INTRODUCTION

Globally cancer is a significant concern and a degenerative, irreversible, non-transmissible, and fatal illness caused by the abnormal growth of cells (Shewach & Kuchta, 2009). These non-communicable diseases have increased the health burden (Ghoncheh et al., 2016) effecting the patient's life (Ashiq et al., 2017). Over 2 million lives have been lost to cancer in Asia, and it is predicted that over 3 million new cancer reports are diagnosed each year in the region (Park et al., 2008). Half of all cancer patients are often found in third-world countries. That is because the lack of adequate funding and support for cancer research, treatment, and care in underdeveloped countries is a significant factor in the global cancer incidence and mortality (Parkin et al., 1993).

Cancer can be malignant (metastasize to other areas of the body) or benign (refined to one location), in which malignant tumors fall correctly under the category of cancers. Diverse types of cancers are classified depending on the type of cell or from which they arise, including sarcomas, leukemia, carcinoma, and lymphoma. More than seventy-five percent of this overall cancer incidence may be attributed to malignancies in ten specific body areas. More than half of all cases of cancer are caused by colon/rectum, lung, breast, and prostate cancers, which collectively account for more than half of all occurrences of cancer (Cooper, 2000). After analyzing the statistical data on cancer, the risk of developing breast cancer is currently the second highest among all cancers (Mahmood et al., 2011). Even though lung cancer accounts for the highest of cer-related deaths, breast cancer remains a significant public health concern (Ghoncheh et al., 2016).

In advanced and emerging countries, breast cancer incidences are very high, so a significant number of women, i.e., since breast cancer accounts for one out of every ten occurrences of the disease (Ferlay et al., 2010; Ginsburg et al., 2017). Various treatments are used for breast cancer in which most patients undergo chemotherapy harming patient's health due to renal failure that can further develop co-morbidity conditions (Tanveer et al., 2019). Progression in the etiology of breast cancer happens due to a variety of detrimental factors, including diet, environment, genetic factors, and chemicals (Banning et al., 2009), estrogen

stimuli upregulation (Cheung, 2007), leptin overexpression in adipose tissues (Majeed et al., 2014) and genetic predisposition of cancer (Hankinson, 2008). On the other hand, 5-10% of cases of breast cancer are caused by autosomal dominant gene mutations due to genetic predisposition.

Null mutations in tumor suppressor genes and proto-oncogenes with gain-of-function mutations contribute to breast cancer's onset by triggering unchecked cell growth, an impairment in DNA repair processes, and a cell cycle check point failure. If a woman has a familial loss-of-function mutation and is under the age of seventy, her lifetime risk of developing breast cancer is 70% (Loman et al., 1998) . Most common genes responsible for the development of breast cancer are BRCA 1 and 2 that account for 16% of the malignancy of breast cancer (Van der Groep et al., 2011), TP53 and Estrogen receptors (ESR) are also responsible for breast cancer development (Amir et al., 2010).

People have less awareness about lack of knowledge for early diagnosis and treatment due to less access to medical facilities, lack of screening centers, expensive cancer treatments and unavailability of trained medical practitioners. With the progression of breast cancer chances of survival and prognosis decreases. If the diagnosis is made later, the survival rate becomes lowest (Asif et al., 2014). Treatment of breast cancer involves chemotherapy, radiotherapy and endocrine therapy for underarms lymph nodes and mastectomy (Papanikolaou et al., 2019). According to the recent machinery for screening of breast cancer exposure of radiation is less which can reduce sensitivity and specificity (false positive and false negative results) and in case of women with dense breasts they will be needed another biopsies and imaging techniques for diagnosis which can be costly (Dilaveri et al., 2019). The limited number of institutions in Pakistan that offer early and multimodality procedure therapy for breast cancer is supported by studies of the disease's incidence among women.

Since this is the case, many breast cancer patients receive inadequate care. The country’s low health budget makes it difficult to provide modern infrastructure for cancer care. It also reveals how little Pakistani women are aware about breast cancer (Khan et al., 2021). Chemotherapy has many issues while treating breast cancer it can damage healthy cells, developing resistance to drug while using broad spectrum drugs, higher toxicity due to

inefficiency of anticancer drugs, influx and efflux of drugs and inability to reach the target (Selwood, 2008). Drug resistance is a difficult clinical condition that can develop in breast cancer patients as a result of a wide array of different mutations in their genes (DeMichele et al., 2015). Patients whose has an ER+ or HER-2+ subtype of breast cancer are frequently treated with chemotherapy in combination with targeted therapies, although resistance to targeted therapy can be a contributing factor in chemoresistance (Martin et al., 2014; Zhang et al., 2008). Other factor in contributing to the drug resistance is the heterogeneity of breast cancer cells (Wang et al., 2010).

Variable expression of specific macromolecules in tumor cells is a hallmark of the cancer development. These include cell surface receptor proteins, altered genes expression, micro RNAs, and others (Diaconu et al., 2013; Vidi et al., 2013). Two types of classification come under breast cancer biomarkers i.e., biomolecules based and stage dependent. For diagnostic purposes biomarkers based on biomolecules have significant role as compared to prognostic and diagnostic biomarkers (ER, HER2 and PR) (Misek & Kim, 2011). In order to make therapeutic decision-making play an important role in order to individualize treatment, they depend on the tumor type and characteristics of the patient (Weigel & Dowsett, 2010).

Wide range of tumors vary depending on their histological classification which includes morphology, behavior and presentation, all fall under the category of invasive breast cancer and according to the WHO report in histological classification there are 18 different types of breast cancer (Tavassoli & Devilee, 2003). Because earlier biomarkers have not shown promise in the treatment of breast cancer. That's why we need these new types of markers. This problem can be solved by Protein Kinase C and its isozymes as they are effective against this issue. These KPC isozymes have been demonstrated to have elevated expression in cancer, making them a valuable biomarker for diagnosing the disease. The normal and cancerous tissue expression will be compared for the diagnosis. There is a lack of research on KPC as a diagnostic marker at present (Motegi et al., 2005; Wang et al., 2013). High levels of the KPC biomarker suggest the existence of cancer and have been linked to treatment resistance, increased mortality, and difficulties in identifying the disease (Noti, 2000).

1.1.Molecular classification of breast cancer

1.1.1. Invasive ductal carcinoma

Invasive breast cancer is also known as invasive ductal carcinoma and its prevalence is 40-80% (Weigelt et al., 2008). Invasive breast cancers account 25% and they are classified into various subtypes which includes invasive lobular carcinoma, neuroendocrine, neuroendocrine, mucinous A, and mucinous B on the basis of cytological features and unique growth patterns (Erber & Hartmann, 2020).

1.1.2. Luminal Breast Cancer

70% of the breast cancers are luminal and they are ER positive tumors (Howlader et al., 2014). There are two subtypes of luminal breast cancer that are Luminal A and B that are classified depending on the two biological processes i.e., pathways for Luminal regulation and proliferation related, luminal A tumors are ER+/PR+ and HER2- (Prat et al., 2013; Weigelt et al., 2010). As a result of the increased malignancy of Luminal B lesions, the prognosis for individuals with these tumors is particularly poor. They may also be HER2+/ER+ and PR-negative (Ades et al., 2014).

1.1.3. HER2 + Breast Cancer

10–15% of all cases of breast cancer are distinguished by significant concentrations of the protein HER2. This subtype of cancer accounts for 10% of all cases. In addition, there is lack of ER and PR (Raj-Kumar et al., 2019).

1.1.4. Triple-Negative Breast Cancer

It is collection of all breast cancers constituting about 20% of the cases i.e., ER negative, HER2 negative and PR negative (Plasilova et al., 2016). As in case of BRCA1 mutations that are also included in triple negative breast cancer which is nearly 80%, and BRCA1

and 2 constitute 11 to 12% of the TNBC cases and they show poor prognosis because they are highly malignant as mentioned in figure 1 (Newman et al., 2015).

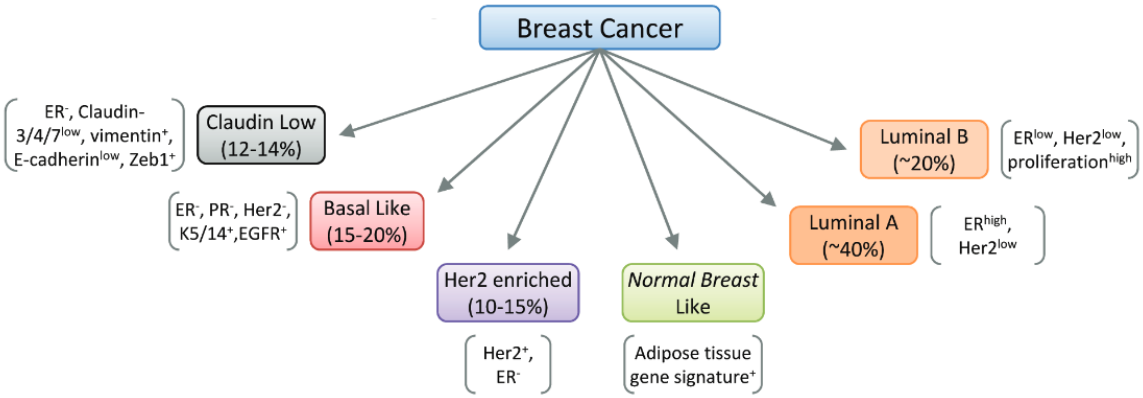


Figure 1. Subtypes of breast cancer at molecular level (Perou et al., 2000; Sørlie et al., 2001; Sørlie et al., 2003).

Previous targeted genes are present that have involved or showed high deleterious effect in breast cancer i.e., ATM, CDH1, BRCA1, BRCA2, PTEN, TP53, PABL2 and STK11. These genes after target did not show any promising results and ultimately treatment failed (Goidescu et al., 2018). Conversely, specific genes or targets have given us unsatisfactory results by increasing chemoresistance through activating alternative pathways via interaction with other genes. This is why therapeutic options have not yet been successful. The key to curing cancer is an early diagnosis; if an individual is detected with cancer at an early stage, there is a more significant possibility of recovery. Therefore, another target KPCD (KPCδ), can be used as the molecular target or solution for early cancer diagnosis, leading toward improved and rapid prognosis.

As there are many genes from same family of KPC to which KPCD belongs their genetic variants have already been identified i.e., PRKCI and PRKCE (Khan et al., 2022; Shah et al., 2022). Since the beginning of the field of genetics, one of the most important goals has been to identify genetic variations that play a role in the progression of complex diseases such as cancer, therefore in pre-diagnosis of disease and its prognosis, alleles of various risk factors have given us various advantages but it is costly and time taking (Singh et al., 2021). As 99.9% of DNA sequence of human genome globally is identical, and rest of 0.1% due to random mutations shows genomic variations (Forsberg et al., 2000). Single nucleotide changes in alleles also known as single nucleotide polymorphism is the most

common form of mutation (Collins et al., 1998). Most of the SNP occurs in the coding region of the genome and rate of change is 1 in 1000 base pairs (Forsberg et al., 2000).

And number of SNPs enlisted in the coding region is 500,000 (Collins et al., 1998). Two types of SNPs are the most common and essential, i.e., non-synonymous and synonymous SNPs; synonymous SNPs result in no alteration of amino acid sequence, whereas non-synonymous SNPs, also known as a missense mutation, will change the amino acid sequence and variations in protein (Lander, 1996), therefore due to these mutations 50% of genetic diseases (Radivojac et al., 2010) including inflammatory and autoimmune diseases occur (Azad et al., 2012).

Genomic segments or genes having SNPs or different variants playing a role in the progression of diseases can be easily screened with the help of computational analysis. These SNPs are divided into various types, such as splice sites, UTRs, missense/non-synonymous, promoter regions, nonsense, and frameshift. Non-sense SNP and nsSNP are also known as coding variants, so that they can affect proteins' structure/function and folding. Other remaining are considered regulatory variants. Changes in the expression of genes happen due to UTRs, incorrect protein translation occurs due to splice variants and a frameshift mutation (Mooney, 2005).

In silico methods for assessing the potential impact of SNPs often based on the evolutionary properties and conserved amino acids and nucleotides sequences, properties of wild and mutant type residues, and protein structure (Selga et al., 2020). Scientists have used functional bioinformatics tools for the disease-causing variants that can be further explored based on sequencing and genotyping. Other genes have also been investigated as disease-causing variants, such as Human leukocyte antigen G (HLA-G) and RASSF (tumor suppressor gene), with bioinformatics tools to study their effect on protein structure and function (Emadi et al., 2020; Hossain et al., 2020). As deleterious impacts due to variants can result in the change in sequence of protein that further cause alterations in protein charge, protein dynamics, inter protein interaction, hydrophobicity and geometry (Arshad et al., 2018; Kucukkal et al., 2015). These types of variations and molecular mechanisms behind various diseases can be studied with the help of in silico analysis on various SNPs (Rajendran et al., 2018).

Many cancers related to endocrine, such as those of thyroid, ovary, pancreas, and breast have been linked to alterations in KPC expression, which have been shown to affect metastasis, survival and cell proliferation (Vucenik et al., 2005). In the case of the PRKCI, it has a unique role in regulation and cell cycle activities. So, the changed expression of PRKCI leads to the progression and development of various diseases. 9 SNP or variants have already been identified through in silico analysis, which includes F66Y, R130H, G581V, G34W, Y169H, G165E, G398S, and R130C; these variants have deleterious effects on protein conformation, dynamics, and stability. Variants were present in the kinase domain of the protein, which resulted in post-transcriptional modifications, and phosphorylation sites were concentrated in this domain; this means it affected the phosphorylation method of the protein. Overexpression of PRKCI can lead to fewer chances of survival; the overall connection between mutants and normal protein was studied (Shah et al., 2022).

11 SNPs (R500C, R268W, Y626C, E14K, G52V, D672H, I578N, R236Q, Y488C, D39H, and E599K) of PRKCE showed deleterious effects in different domains, and the kinase domain of PRKCE gene was concentrated with 45% of the deleterious SNP in which they were close to the active site and ATP binding site. These SNPs were investigated to determine whether or not they have the potential to influence the structure and function of the protein (Khan et al., 2022). Initial evaluation of the pathogenicity of genetic variants of KPCD by applying insilico tools and then by further proceeding them in wet lab can save a lot of time and money and could help in investing scientific efforts.

1.2. Aims and Objectives

- To study the association of KPC delta’s non-synonymous variants with Breast cancer risk factors and pathological characteristics.
- Analyzing the impact of variants on the structure and function of KPC delta.

CHAPTER 2
LITERATURE REVIEW

Nearly 8 million people were died due to cancer from all over the world in 2013, making it the second biggest cause of death globally, behind only cardiovascular disease. In 1990, cancer was the third leading cause of death (Abubakar et al., 2015; Lozano et al., 2012; Murray & Lopez, 1997). An expanding and aging global population, together with other risk factors, including smoking, eating habits, and obesity, are all contributing to an increase in the burden of cancer. It is vital to have up-to-date knowledge about the burden of cancer in each country in order to devote resources to prevention, screening, diagnosis, treatment, and palliative care in an effective manner and to assess their effectiveness (Abubakar et al., 2015).

Cancer possesses some basic features which can cause diseases of almost one hundred diverse types. Our all-body tissues can become malignant; the thirty trillion cells that make up a healthy, functioning human body are like residents of a high-rise, interconnected complex that controls its growth. These cells work in collaboration with each other, so when they have to reproduce, they are instructed to do so by the cells present nearby (Weinberg, 1996). Cancer cells is the irregular growth of cells having ability to differentiate or alter and proliferate and developing the cells into different cell types this makes the cells easier to travel to different sites and then developing tumor genesis, cells become invasive and metastasize it these left untreated then can cause morbidity and death of host (Ruddon, 2007). A cell also becomes cancerous by accumulating mutations in specific gene groups by changes in the levels of activity or the total amount of a protein product can be caused by mutations. These genes explain how human cancer develops which can then disturb a cell (Weinberg, 1996).

Mutations causing changes in the genes may be somatic or hereditary mutations that can result in either loss of function gain of function or effecting the tumor suppressor genes due to mutational effect in the proto-oncogenes (Frei & Emil). There are at least 350 gene on human that have been linked to cancer present on every chromosome except Y existing in the mutant form either through nonsense, deletion, missense, and frameshift or

translocate in one or more than one type of the cancer, these mutations may arise as a result of the epigenetic variations (Yoo & Jones, 2006). In order to understand behavior of cancer linked to mutation, knowledge of the signaling pathways is important contributing to the hallmarks of cancer (Hanahan & Weinberg, 2000). So, the hallmarks or properties of cancer cells involve angiogenesis, limitless replicative potential, invasion, resisting growth-inhibiting signals, metastasis, and avoidance of apoptosis. Some of these properties are related to cancer cells, but others depend on the communication between cancer cells and their interaction with the components of the surrounding environment (Pao & Miller, 2005). Cancer cells survive in their specific environment create by them termed as tumor microenvironment, so they have main factors that alter the pathways to maintain the viability of the cell (Castells et al., 2012; Muranen et al., 2012). The cancers most common in men in high-income countries include prostate, lung, colorectal, and bladder cancer. In contrast, the most common cancer types in women include breast, colorectal, lung, and endometrium cancer. Conversely, the types of cancer that are most common in men in low-income countries include prostate, liver, esophagus, and lung cancer, while the types of cancer that are most common in women include breast, cervical, and ovarian (Whiteman & Wilson, 2016).

2.1.Breast Cancer

Breast commonly diagnosed cause of cancer-related death in women all over the world, and it is amongst the most common types of cancer, It is responsible for 23 percent of all cancer cases in the worldwide (Jemal et al., 2011). Breast cancer is a threat that faces all women, regardless of their race, ethnicity, or country of origin or background (Naeem et al., 2008). Annually, over than 1.2 million people across the globe are given a diagnosis of breast cancer, as reported by the World Health Organization (WHO) (ZAHRA et al., 2013). It is possible for men to develop breast cancer, although it is extremely uncommon (McPherson et al., 2000). The Pakistani population has a higher rate of breast cancer than that of the West (Mahmood et al., 2006). Pakistan has one of the highest incidences of breast cancer in Asia, with one in every nine women being diagnosed (Sohail & Alam, 2007).

Emergence, grade, prognosis, and receptor activity are the four criteria for categorizing breast tumors into subtypes. The most significant risk factors for breast cancer are reproductive and menstrual factors, often known as hormonal imbalances. Research has shown that the likelihood of developing breast cancer increases with factors such as early onset of menarche, never having children, having an older age of first live delivery, and never breastfeeding.

2.2.KPC family

KPC expressed and conserved in various species belongs to the family of serine and threonine. KPC's function in the body has been studied extensively, both in healthy contexts and in the context of a wide range of diseases. Further, KPC enzymes are involved in various signal transduction networks that translate extracellular inputs into cellular responses. These investigations demonstrated that KPC enzymes are involved in numerous cellular metabolic activities across various cell types (Nishizuka, 1984). Protein kinases can phosphorylate a small number of protein substrates or their richness to control various cellular responses. KPC isozymes belong to the second group of kinases that phosphorylate serine and threonine residues on various proteins; these enzymes of KPC as kinases were discovered about 30 years before and were activated through proteolysis (Takai et al., 1977). Protein Kinase C has been classified into three forms on the basis of the DAG and calcium as follows (MELLOR & PARKER, 1998).

1. Conventional (α, β, γ)
2. Novel (δ, ε, η, θ)
3. Atypical (ζ and ι)

KPC isoforms, as mentioned in Figure 2 (A), have four conserved (C) domains separated by five variable (V) domains, including the carboxy-terminal, a catalytic domain, an ATP binding domain, and an amino-terminal region. The catalytic domain is connected to the amino-terminal region regulatory region via the hinge region of variable region 3, having a pseudo substrate sequence (House & Kemp, 1987; Nishikawa et al., 1997). C1 domain is the DAG/phorbol ester binding domain and before that is the pseudo substrate region,

calcium binds on the C2 domain, C3 is ATP binding domain and substrate binding domain is C4 (Garg et al., 2014; Newton, 1995).

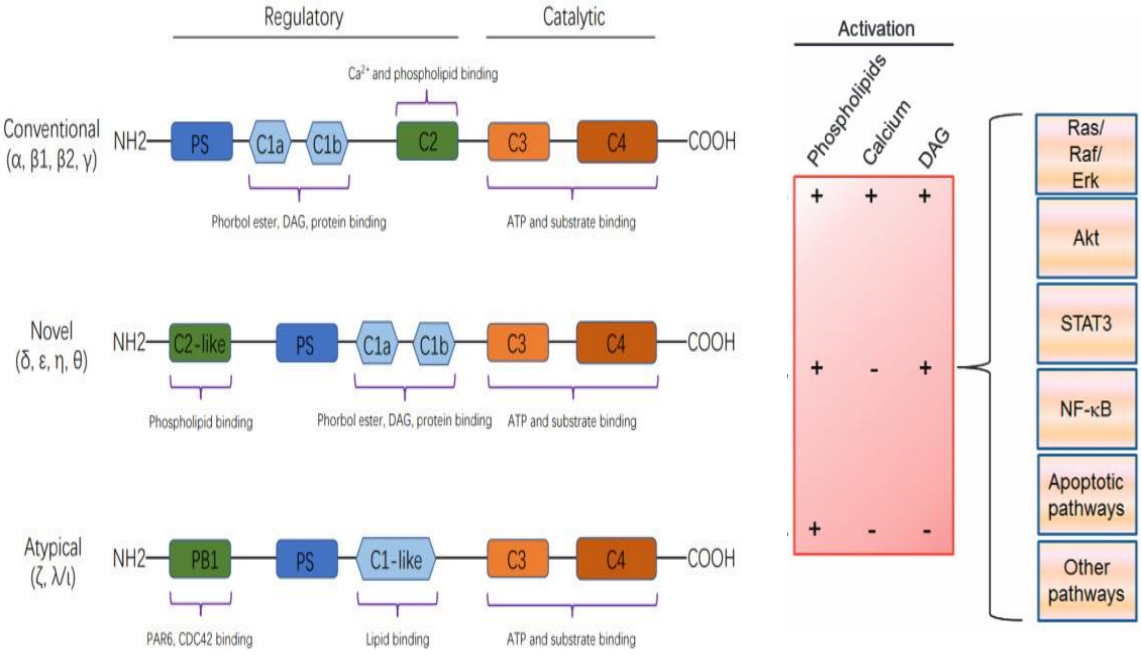


Figure 2. Illustration of the general structure of all three subfamilies or classes of KPC (Lim et al., 2015) and the activation of KPC results in activating other cellular pathways (Garg et al., 2014).

Extracellular agonists result in activation of KPC by interacting with it, these agonists include growth factors, cytokines and hormones resulting in the phosphorylation of specific substrates for the cellular functions after translocation of the agonists to different subcellular compartments (Parker, 2003). Regulation of various cellular processes by the help of KPC includes cell shape alteration, receptors and ion channel regulation, proliferation, transcription, translation, cell death and regulation of secretory products and cell to cell contact. This involve in neurodegenerative diseases, cancer, psychiatric diseases, heart failure, autoimmune diseases and stroke (Mochly-Rosen et al., 2012). But the expression of KPC varies in cancer depending on the cell type, genes of KPC family is mostly mutated in human cancer (Antal et al., 2015). Lung squamous cell carcinoma, colorectal cancer and melanoma contain 20-25% of the KPC mutations whereas breast

cancer, glioblastoma and ovarian cancer contain 5% less than mutations of KPC (Gao et al., 2013).

2.3.Target gene (KPC delta)

2.3.1. Discovery

Discovery of KPCδ was in 1986 (Gschwendt et al., 1986; Ono et al., 1987) and localized on the chromosome 3 (Huppi et al., 1994) while doing the experiment it as observed that KPCδ was obtained from three different species mouse, rat and human with amino acid residues of 673, 674 and 676 encoding protein and thus they are homologous and identical with each other with the molecular weight of 77.5KDa. According to the KPC isoforms phylogenetic tree it was observed that there was similarity between the primary structure of KPCδ and KPCθ. In one of the reviews, it was reported that KPCδ is universal so rather being specific role in cells of mammals it is widely distributed in cells and tissues (Altman & Villalba, 2002). KPCδ has catalytic and regulatory region in which catalytic domain has C3 and C4 domain just like the in other KPC forms (Hanks & Hunter, 1995). Thr-505 is the phosphorylation site in the C4 region whereas Ser-643 and Ser-662 are the two phosphorylation sites which are conserved in the carboxy terminal (Parekh et al., 2000). KPCδ has no real C2 domain but has C2 like domain instead, and it has only C1 domain, so a pseudo substrate region is located between C2 like domain and C1 domain serving as the recognition site for the substrate to keep it in inactive conformation as discussed in figure 3 (Pappa et al., 1998; Zhang et al., 1995).

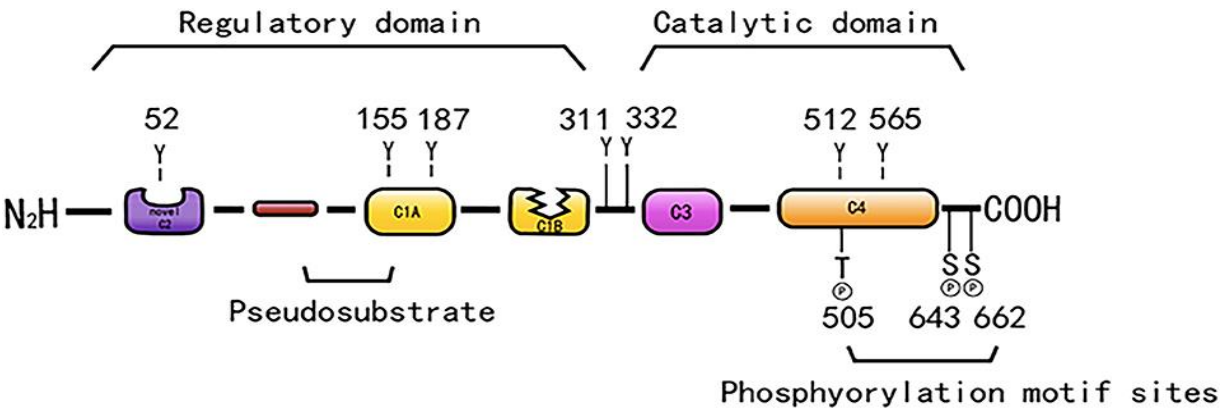


Figure 3. KPCδ structure showing regulatory domain on the NH2 terminal having C2 like domain, pseudosubstrate and C1 domain (C1A and C1B), and catalytic domain on the COOH terminal with C3 and C4 domain having phosphorylation motif sites (Miao et al., 2022).

Cell proliferation and differentiation happens due to KPCδ phosphorylation at the tyrosine, which results in the activation of KPCδ by G protein coupled receptors by ATP (Ohmori et al., 1998). KPCδ may also be activated in diverse ways by proteolytic processes and UV light. Phosphorylation at serine and threonine motif site by DAG result in KPCδ activation, which triggers enzyme activation and yields a catalytic fragment (Kikkawa et al., 2002). KPCδ phosphorylation of tyrosine residues and enzymatic activity can also be regulated by growth factor receptors, Src kinase family members and PYK2 (Basu & Pal, 2010; Kikkawa et al., 2002). Distinct isoforms interactions with the scaffolding proteins can be determined by the conformational change which happens during the inactive and active state in the KPCδ, so the functional selectivity, specific substrates accessibility and subcellular location of the various isoforms of the KPC can be determined by these interactions (Poole et al., 2004).

2.3.2. Cellular localization

When diverse substrates are phosphorylated at cellular level with different locations, so when same stimulus was applied in the same cell type KPCδ is involved directly in diverse responses (Murriel et al., 2004). Due to phosphorylation of tyrosine (Steinberg, 2004), translocation of KPCδ happen after its activation that move to plasma membrane from

cytosol in catalytically competent and mature form and then toward various subcellular compartments such as the Golgi apparatus, endoplasmic reticulum, caveolae and mitochondria (Gomel et al., 2007; Kajimoto et al., 2001; Page et al., 2003; Qi & Mochly-Rosen, 2008; Rybin et al., 1999). At nuclear level, localization of KPCδ results in the apoptotic regulation, in which the nuclear localization sequence is present in KPC delta catalytic domain (DeVries et al., 2002). As a result of genotoxic stress, apoptosis can be triggered by p53 due to phosphorylation of KPC delta at Ser46 (Yoshida et al., 2006). By raising the p53 gene's basal transcription, level of p53 is controlled by KPC delta (Abbas et al., 2004). Translocation of KPC delta to ER as result of ER stress interacting with the cAbl then the complex KPC-cAbl trigger apoptosis by further translocating to mitochondria (Qi & Mochly-Rosen, 2008).

Unphosphorylated KPC delta will be in the cytoplasm in case of normal development that depends on the regulatory domain, so phosphorylation will result in accumulation in the nucleus of the KPC delta that will help in apoptotic regulation. This will result in the accumulation of the active caspase three that will cleave KPC delta in the nucleus and activate Delta CF (δCF); this will localize in the nucleus to help regulate the cell damage response proteins as shown in Fig.3. Phosphorylation, subcellular localization, and subcellular targeting of KPCδ is affected by the increase in the level intracellular zinc, Thr505 phosphorylation site will be inhibited, which results in KPCδ translocation from cytosol to Golgi complex. His-Cys3 is the zinc-binding site or pocket in the KPCδ that sense and responds to the presence of free zinc ions concentration intracellularly in the below figure 4. There may be chances that there are other metals that can help in the regulation of ‘novel kinases’ activity to study the metal ion protein interactions by investigating cellular processes (Slepchenko et al., 2018). The C1A domain is essential for directing the localization of different KPC isoforms within the cell (Colón-González & Kazanietz, 2006; Gallegos et al., 2006).

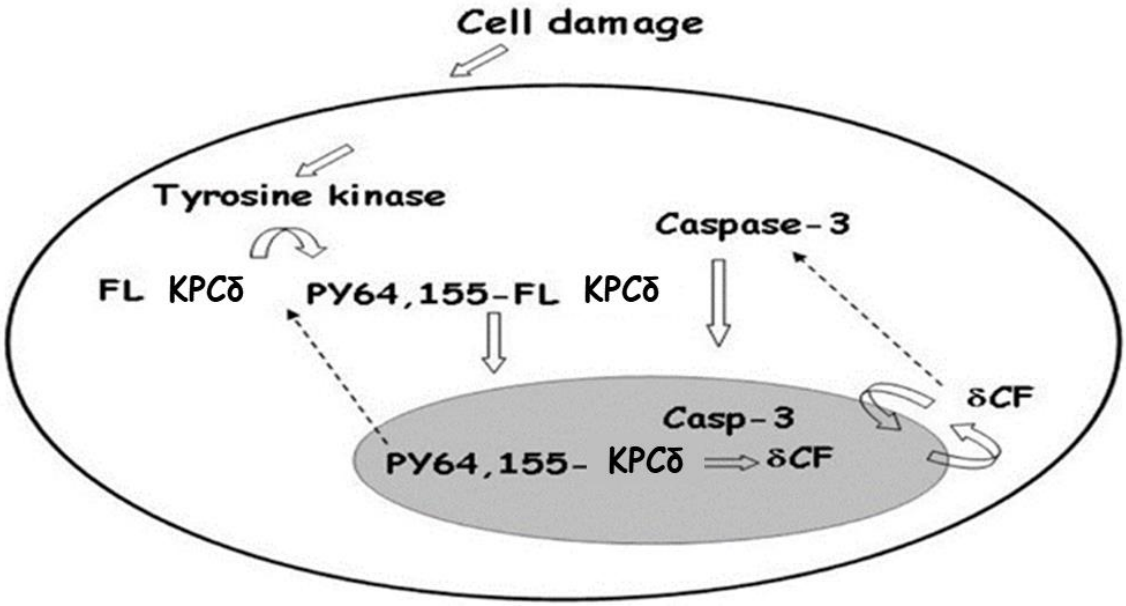


Figure 4. Phosphorylation of the KPC delta resulting in the proapoptotic regulation (Reyland, 2009).

2.3.3. KPCδ role in cell and cell cycle

In health and diseases, KPCδ has an unique and important role so in sepsis in case of the inflammatory response it act as an import regulator (Kilpatrick et al., 2002; Kilpatrick et al., 2000; Kilpatrick et al., 2011; Kilpatrick et al., 2010; Mondrinos et al., 2013). Multiple types of cells have different expression of proinflammatory mediators and KPCδ for the activation of kinases (Page et al., 2003). Proinflammatory signaling of endothelial and neutrophils is regulated by the KPCδ (Kilpatrick et al., 2010; Kilpatrick et al., 2006; Mondrinos et al., 2014). In neutrophils, regulation of production of Reactive oxidation species, transcription factor NfκB activation, secretion of the chemokines, inflammatory signaling and proinflammatory gene expression occurs by KPCδ (Kilpatrick et al., 2010; Kilpatrick et al., 2006). In endothelial cells, endothelial cell permeability regulation, expression of the adhesion molecules, transmigration of the neutrophils due to inflammatory mediators and NfκB activation is the result of KPCδ (Mondrinos et al., 2014; Soroush et al., 2016). KPCδ plays an important part as an signaling element for the regulation of many processes such as crosstalk of neutrophil- endothelial, damage of

vascular endothelial and adherence, rolling, migration of neutrophils (Kilpatrick et al., 2011; Kilpatrick et al., 2006; Mondrinos et al., 2013; Soroush et al., 2019).

As KPC delta was found to be involved in a variety of cellular processes in multiple cell types, it has also exerted its effect on proliferative and anti-apoptotic activity. So KPC delta activation has a spatiotemporal expression that is specified according to cell type and stimulus. Evidence suggests that KPC's varied subcellular localization contributes to its wide range of functional effects, much like p21Cip1/WAF1. Redistribution of the KPC delta happens between cytosol, membrane-bound organelles, and compartments associated with the cytoskeleton under the stimulus of phorbol ester and fatty acids (Knutson & Hoenig, 1994). And the nuclear accumulation is linked with KPC delta pro apoptotic effect (DeVries et al., 2002; Eitel et al., 2003). Emerging research reveals that p21Cip1/WAF1 also regulates other biological processes, such as cell differentiation and survival, in addition to controlling the cell cycle. Extended nuclear accumulation of p21Cip1/WAF1 may be what causes the poor replication and increased apoptosis seen in -cells of p21Cip1/WAF1 transgenic animals and in insulin-secreting cells with lower KPC activity (Yang et al., 2009).

As KPCδ is present or located on chromosome number 3p, therefore when it lost it results in development of many cancers. Colonic cancers were likewise linked to decreased KPCδ expression, and KPCδ overexpression inhibited the neoplastic phenotype of colon cancer cells by way of the tumor suppressor p53 (Perletti & Terrian, 2006). As with breast cancer cells, KPC has been demonstrated to reduce cell migration, whereas in mouse embryo fibroblasts, elimination of the KPC gene boosted cell migration (Jackson et al., 2005). KPC is responsible for arresting G1/S and G2/M. The expression of cyclin D1, the activity of cyclin-dependent kinase 1, and the levels of cyclin-dependent kinase inhibitors p21 and p27 may all be suppressed by KPC, suggesting that this kinase can block cell cycle progression (Jackson & Foster, 2004; Perletti & Terrian, 2006). Phoshphorylation of the CDK1of Tyr15 residue by catalytic fragment of KPC delta in response to UV exposure is important for maintaining G2/M DNA damage checkpoint (LaGory et al., 2010). According to one of the study, activation of G2/M checkpoint occurs after the initiation of

apoptosis so for the induction of apoptosis production of catalytic fragment by KPCδ is required (Basu & Pal, 2010).

2.3.4. KPCδ dysregulation in cancer

Anchorage-independent growth capabilities are conferred and the resistance to apoptosis stimuli is increased by the ectopic expression of KPCδ in mammary cells (Grossoni et al., 2007). Several forms of cancer have been shown to lack KPCδ expression (Reno et al., 2008) but this down-regulation cannot be definitively related to carcinogenesis. KPCδ is up-regulated in several forms of cancer (Tsai et al., 2000; Yu et al., 2011) in normal epithelial cells of prostate KPCδ is hardly detectable, whereas pre-cancerous lesions and carcinomas of the prostate have significant expression of KPCδ (Kharait et al., 2006; Villar et al., 2007). In specimens of breast cancer, the levels of KPCδ mRNA are noticeably greater in ER-positive tumours, and researchers have found a positive link between high levels of KPCδ mRNA and less chances of surviving the disease overall (McKiernan et al., 2008). Breast cancer cells survived by KPCδ through the activation or inhibition of many signalling pathways, including the suppression of TNF-related apoptosis-inducing ligand (TRAIL)-induced caspase activation (Yin et al., 2010; Zhang et al., 2005). KPCδ through activation of MAPKs and Akt shows increased TAM-induced MCF-7 cells and antiestrogen resistance in estrogen (Nabha et al., 2005). High levels of KPCδ expression promote cell motility and invasion through blocking the small GTPase Cdc42 in highly metastatic breast cancer (Zuo et al., 2012). In addition, KPCδ activation and MMP-9 overexpression, platelets have the ability to encourage the invasion of MCF-7 cells (Alonso-Escolano et al., 2006).

KPCδ has also been linked to a tumorigenic effect in a pancreatic cancer model. Furthermore, human ductal malignancies have shown that overexpressing KPCδ increases anchorage-independent growth and carcinogenesis in vivo (Alonso-Escolano et al., 2006). Activation of KPCδ encourages tumour growth and enhances angiogenesis in a PC-3 xenograft model through a process that involves reactive oxygen species (ROS), reduced glutathione (NADPH), and HIF-1 (Kim et al., 2011). Positive effects of KPCδ have been shown during migration and invasion (Li et al., 2013; Miyazawa et al., 2010; Razorenova

et al., 2011). KPCδ has been shown to regulate collagen release and promote invasion in prostate cancer cell lines through overexpression of PCPH oncoprotein (Villar et al., 2007). In the mouse mammary breast cancer model MTLn3, lung colonisation was inhibited due to down-regulation of KPCδ without impacting the growth of the original tumor (Kiley et al., 1999).

Increased APC phosphorylation (Hernández-Maqueda et al., 2013), $p21^{Waf1/Cip1}$ activity (Perletti et al., 2005), cyclins (Kim et al., 2007), p53 expression (Perlett et al., 2004), decrease in stabilization of β-catenin (QIN et al., 1995) by protein kinase C delta (KPC) in colon cancer cells mediates alterations in multiple cellular signalling pathways that suppress cell growth and proliferation (Hernández-Maqueda et al., 2013). It has come to light that KPCδ is a novel regulator of the progression of pancreatic cancer; however, this control appears to be driving tumour growth rather than inhibiting it. KPCδ is overexpressed in the cells that make up ductal pancreatic cancer when compared to normal tissues. In addition, KPCδ is responsible for the induction of the production of PI3K, which is a protein that controls the progression of cancer, as well as ERK, which is a crucial chemical in the mitogenic pathway. PI3K and ERK, results in a considerable rise in the development of cancer cells in conjuction with Increased expression of KPCδ, in conjunction with in a manner that is independent of anchoring, which is a characteristic of carcinoma (Mauro et al., 2010). STAT3 controls the activity of cance cells invasion and their survival as a result of this it also needs KPCδ, KPCδ increases STAT3 activity by phosphorylating it at Tyrosine 705, which helps tumour cells survive and invade (Sorescu et al., 2012). Lower levels of KPC are linked to increased cancer cell lymphovascular invasion via boosting proteases release (Jackson et al., 2005).

Depending on the context, KPCδ may either promote or inhibit tumour growth, making it a key regulator in the study of cancer's dynamic development (Jackson & Foster, 2004). Overexpression of fibroblasts due to Src acquire a malignant character when subjected to extended treatment with phorbol esters, which results in the down-regulation of KPCδ (Lu et al., 1997). KPCδ/ERK pathway is involved in two signaling pathways that have tumorigenic role sonic hedgehog and Wnt signalling (Riobo et al., 2006). Downregulation of KPCδ observed in endometrial tumors, malignant gliomas and bladder carcinoma while

overexpression was noticed in colon cancers (Griner & Kazanietz, 2007; Reno et al., 2008). Autoimmune diseases like systemic lupus erythematosus (SLE) are defined by abnormalities in the body's immune system that cause the creation of autoantibodies in an unhealthy manner (Tsokos et al., 2000), so patients with systemic lupus erythematosus have been shown to have dysfunctional T cells and monocytes/macrophages (Yang et al., 2007). Clinical research has shown that people with SLE had lower levels of KPCδ in their monocytes than the general population. Thus, it has been proposed that the disease's progression is aided by monocytes' ability to live longer and accumulate macrophages (Biro et al., 2004). Mice with defects in T cell-ERK pathway signalling develop an illness similar to lupus, and this sickness is accompanied by a reduction in the production of DNA methyltransferases, which in turn induces epigenetic alterations (Gorelik et al., 2007).

2.3.5. Molecular Targets of KPCδ in Cancer

Upregulation of the cell cycle inhibitor p21 happens when KPCδ has a detrimental effect on the G1/S and G2/M transitions of the cell cycle in vascular smooth muscle, glial and endothelial cells (Nakagawa et al., 2005). It has also been hypothesised that KPCδ can induce an autocrine apoptotic cycle by leading to the release of TNFα and TRAIL in prostate cancer cells (Griner & Kazanietz, 2007). In mammary cells, KPCδ may potentially have a prosurvival role and stimulate cell proliferation. According to reports, KPCδ stimulates a mitogenic response by activating the ERK-MAPK pathway, which increases cyclin D1 production and causes Rb to become hyperphosphorylated (Grossoni et al., 2007). KPCδ also makes cells less likely to die from apoptotic signals by turning on the Akt pathway and changing how NF-B controls gene expression (Díaz Bessone et al., 2011; Grossoni et al., 2007).

KPCδ has the ability to trigger survival signals and increase drug resistance in response to chemotherapy. Inducing cell survival via activating survival pathways such as the Akt, NF-B, and MEK pathways can be accomplished with its help. In response to treatment with TNF, KPCδ moves to the nucleus and interacts with NF-B, despite the fact that it prevents apoptosis caused by TRAIL. Hyper phosphorylation of Rb by KPCδ and the resulting elevation in cyclin D1 levels both contribute to increased cellular proliferation. Invasion and metastasis are aided by its ability to activate claudin 1 and 3 integrin. As a result of

inducing tissue transglutaminase and activating NF-B, KPCδ also plays a role in suppressing autophagy. To do this, it either degrades proapoptotic Bim or stabilises antiapoptotic Mcl-1, both of which work to prevent cell death as shown in figure 5.

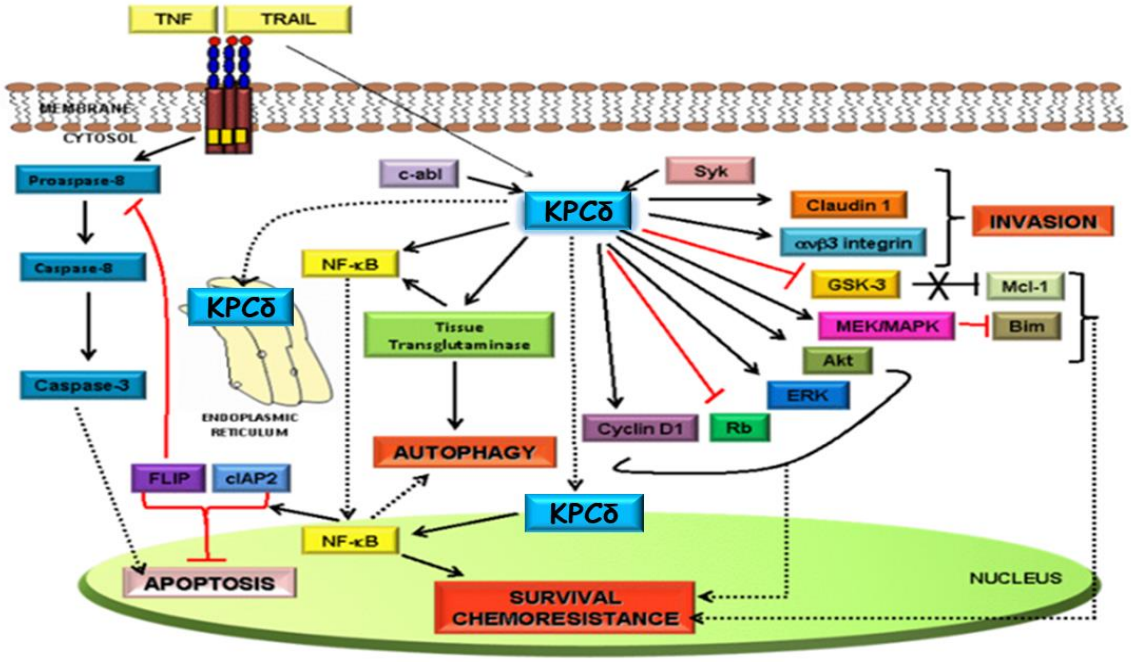


Figure 5. Function of KPCδ as prosurvival of cancer cells (Basu & Pal, 2010).

In response to phorbol esters that promote tumour growth, such as TPA, KPCδ has the potential to serve as a tumour suppressor, It can trigger apoptosis in response to DNA damage by activating the p53 pathway or c-Abl. Contrastingly, caspase-3 Besides being a target for KPCδ-mediated phosphorylation, which can lead to its proteolytic cleavage, in which information is fed back towards itself. It is possible for the catalytic fragment (CF) of KPCδ to hasten apoptosis by stimulating the phosphorylation and subsequent destruction of the antiapoptotic Mcl-1 protein, which is a member of the Bcl-2 family. Cell cycle arrest can be induced by KPCδ via altering the amounts of cyclins, cyclin dependent kinases (cdks), and cdk inhibitors. t halts Akt from working when a cell is stressed, which makes it harder for the cell to live. It can also control autophagy through the JNK pathway by phosphorylating Bcl-2, which makes it separate from Beclin-1. Cancer can be inhibited by sending KPCδ to various organelles, where it can activate diverse signals. KPCδ modulates

mitochondrial Bcl-2 family members through its interaction with c-Abl, and it can also link with a variety of proteins in the nucleus (Basu & Pal, 2010).

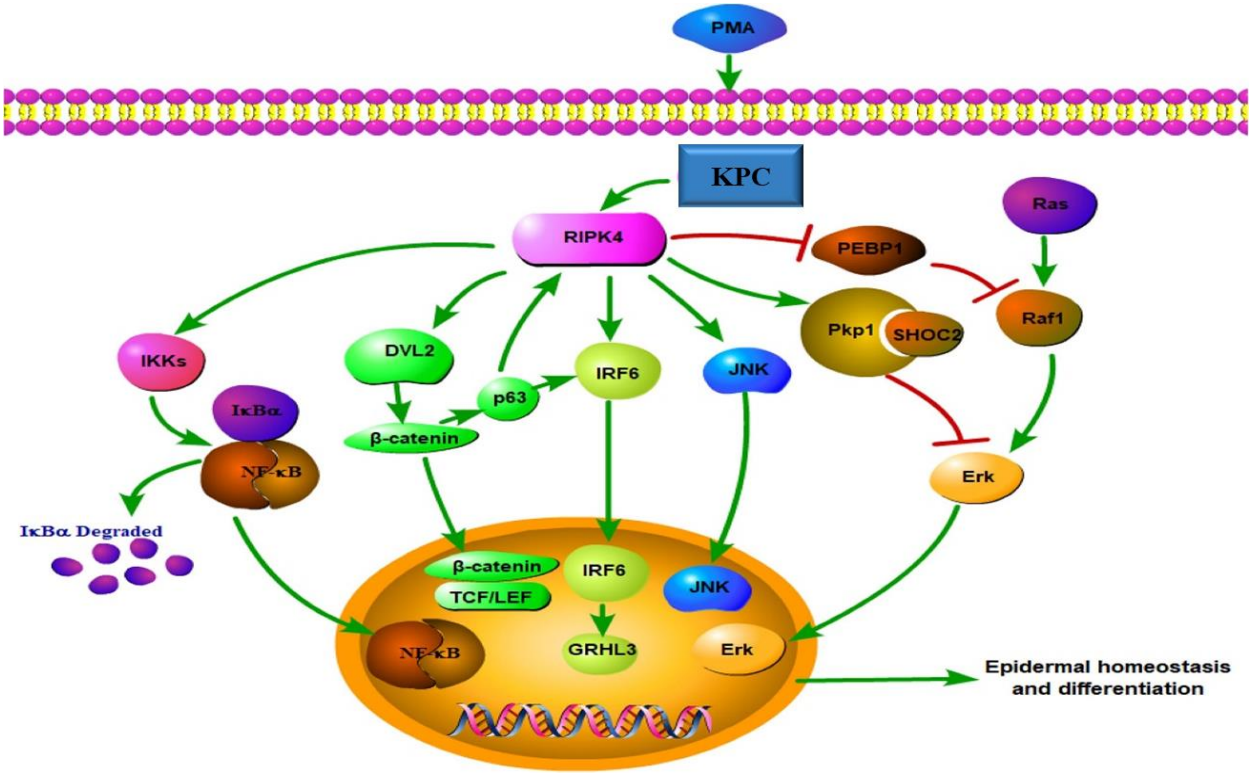


Figure 6. RIPK4-KPCδ interacting protein in differentiation of keratinocytes and Carcinogenesis (Xu et al., 2020).

One of the major factor in KPCδ signaling pathway is RIPK4 as modulator (Bähr et al., 2000) , involucrin (IVL) is the differentiation associated gene expression is increased due to Kruppel-like factor 4 (KLF4) increased transcription as a result of activation of KPCδ as shown in above pathway in figure 6 (Chew et al., 2013). It is plausible that transcription of KLF4-mediated IVL is regulated by KPCδ through the RIPK4-IRF6 regulatory module. This is a possibility due to the fact that it has been demonstrated that KLF4 is a promising target for the IRF6 protein (Botti et al., 2011; De La Garza et al., 2013).

An essential function of RIPK4 is in the differentiation of keratinocytes, the cells that make up the skin. Abnormal epidermal differentiation, brought on by RIPK4 dysregulation, has the potential to have a major impact on the emergence and progression of Squamous Cell Carcinoma (SCC) (Li et al., 2015; Pickering et al., 2014). To drive epidermal

differentiation, RIPK4 of desmosome protein plakophilin-1 (Pkp1) is phosphorylated at the N-terminal region of the). SHOC2 interaction with Pkp1 may be stimulated by the new RIPK4-Pkp1 signalling axis (Lee et al., 2017). To some extent, this suggests that RIPK4 functions as a tumour suppressor, as its knockdown increased the invasion and migratory ability of tongue cancer cells (Wang et al., 2014). NF-B signalling may be related to RIPK4's function as a tumour suppressor implicated in the development of HCC (Luedde et al., 2007), acquiring oxidative stress-induced genomic alterations and a growing pre-malignant subclone were both exacerbated by the downregulation of NF-B signalling caused by RIPK4 (Heim et al., 2015). Two kinases that are frequently elevated in lung adenocarcinoma are known as nuclear factor kappa B (NF-B) and STAT3 (Gao et al., 2007; Meylan et al., 2009). They also facilitated differentiation process, which has been associated to numerous aspects of the progression of cancer, such as invasion, metastasis, and angiogenesis of cancer cells. This was another characteristic of cancer that they stimulated (Kopparam et al., 2017).

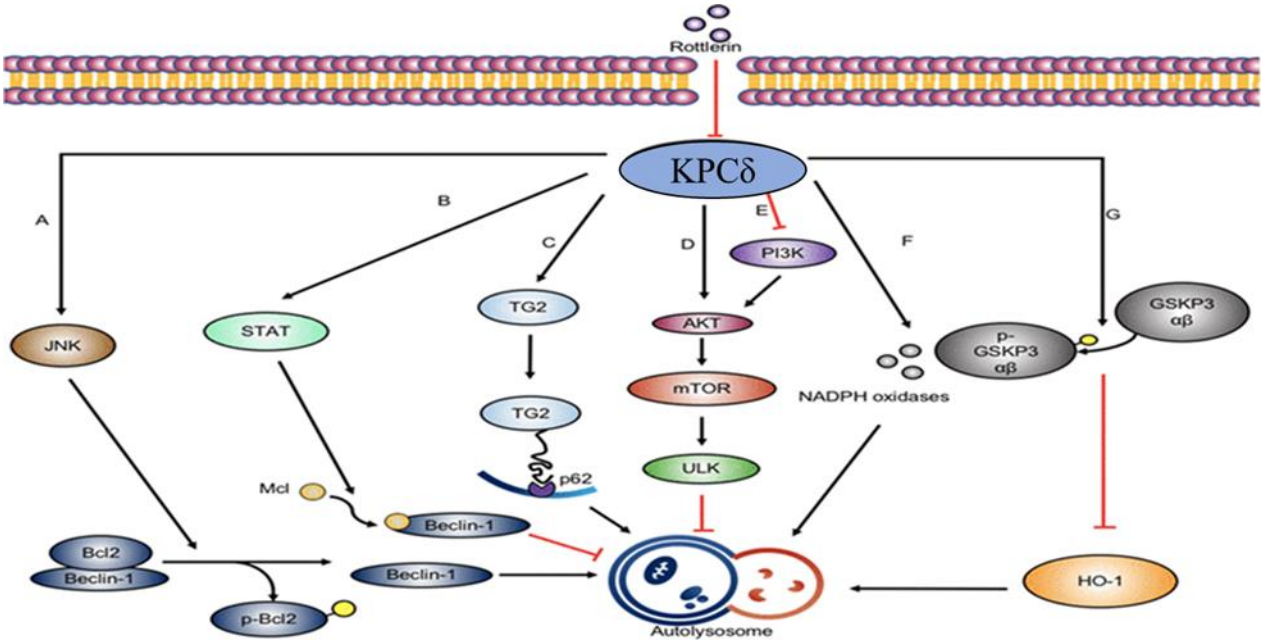


Figure 7. Mechanisms involved in the regulation of autophagy by KPCδ (Wang et al., 2018).

KPCδ activation results in the activation and upregulation of JNK epxression, which helps in the release of Beclin-1 by dissociating Bcl2 so Beclin-1 is activated thus promoting autophagy. On the other side Mcl and Belcin-1 combined due to activation of STAT so McL-Beclin-1 complex inhibit autophagy, formation or maturation of p62 and autophagosome helps in the identification of ubiquitinated protein when TG2 is activated by KPCδ. Phosphorylation and activation of Akt/mTOR results in the inhibition of autophagy, inhibiton of autophagy also gets inhibited by activation or phosphorylation of Akt/mTOR/ULK pathway. Via NADPH dependent pathway induction of autophagy and inhibition of HO-1 inhibits autophagy due to phosphorylation and activation of GSK3αβ by KPCδ (Wang et al., 2018). In case of cancer, autophagy promotes tumour growth in some cases while inhibiting it in others, and it also aids in the expansion and survival of cancer cells (Lim & Staudt, 2013; Salminen et al., 2013). To some extent, oncogene or tumour suppressor protein expression is influenced by autophagy control. Induction of autophagy and initiationi of cancer is suppressed, mTOR and AMPK are negatively regulated by tumor supressor proteins (Comel et al., 2014). Inhibition of autophagy and increase in the cancer formation happens due to activation of oncogenes by AKT, PI3K and mTOR (Choi et al., 2013). Oxygen deprivation and a lack of nutrients are two incredibly demanding conditions tumors must endure. Autophagy is a process that assists cells in overcoming the effects of stress. In the central zone of solid tumors, where cells are found to be living in an oxygen-depleted environment, autophagy becomes active. Increasing cell death is achieved by inhibiting autophagy through the inactivation of Beclin 1 in Figure 7 (Degenhardt et al., 2006; White & DiPaola, 2009)

CHAPTER 3
METHODOLOGY

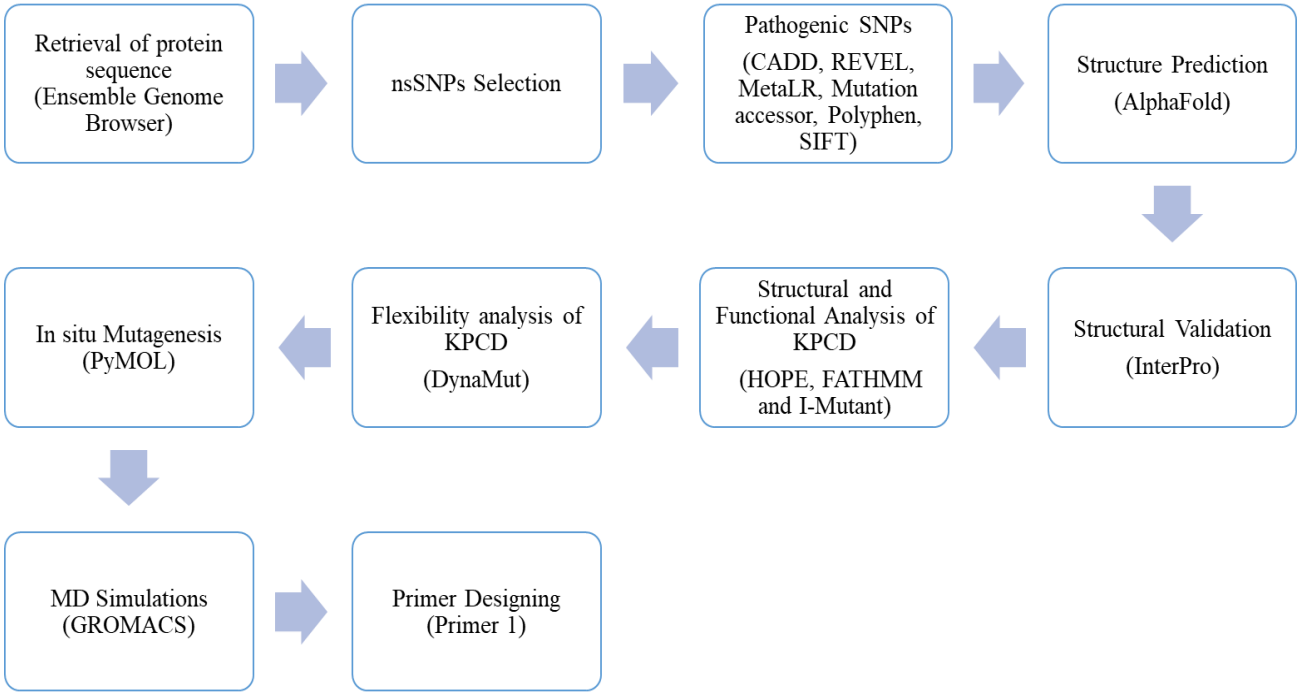


Figure 8. Schematic representation of in silico work in this study.

3.1.Retrieval of protein sequence

Protein sequence was retrieved from the ENSEMBL database with transcript ID: KPCD-201 ENST00000330452.8 in Fasta format containing 676 amino acids. Data is incorporated from different databases in ENSEMBL which includes ExAC, COSMIC, dbSNP and gnome AD (Cunningham et al., 2019). Data retrieved from the database consists of SNPs and variants related to genes, sequence retrieval and disease association.

3.2.SNPs collection and processing

SNPs data was retrieved from ENSEMBL on 2nd August, 2022 (Hubbard et al., 2002). The retrieved data gave us information about amino acid coordinates, residues, genomic coordinates, variants IDs, and mutated bases. Only missense variants were further considered, which were 2773 according to the data retrieved. They were later analyzed to predict the pathogenicity and then mapped on protein exons and domains.

3.3.Analyzing the effect of coding SNP

Missense SNPs obtained from the database were further analyzed, and pathogenicity scores were calculated through six tools, i.e., CADD, Mutation Accessor, Polyphen, MetaLR, SIFT, and REVEL. These tools helped to perform the severely accurate screening of pathogenic SNPs, and out of 2773 nsSNPs, seven non-synonymous SNPs were predicted and selected based on average and percentage through these tools that crossed the 75% threshold level. Seven SNPs were further used for the final study that can alter the structure and function of KPCD's gene.

3.4.Structural validation of KPCD delta

In order to analyze the structure of KPC delta three tools were used i.e., AlphaFold, InterPro and PyMOL. To obtain the structure first AlphaFold was used that gave the file in the pdb format that file was further analyzed by PyMOL that was used for 3D molecular visualization of structure (DeLano, 2002). InterPro that gave the detailed information about structure using amino acid sequence as input in FASTA format (Hunter et al., 2012).

3.5.Determining the stability of Protein structure

The I-Mutant tool was used to determine the stability of protein structure of KPCD gene on the basis of DDG value (free energy change values) and RI value. This tool gave the output in which 5 out 7 nsSNPs had shown decreased stability (Capriotti et al., 2005).

3.6.Point mutation's impact on the structure and function of KPCD gene

Due to the presence of nsSNPs in protein at a particular position, it induces an amino acid change. Protein structure and function can be affected depending on the accessibility of the protein surface and domain. Alterations of structure and function due to seven nsSNPs were evaluated, then, with the help of Project HOPE, estimated effects due to mutated amino acids were calculated. Three out of seven variants that brought amino acid substitution were more prominent in size than wild-type ones. Six out of seven nsSNPs had shown higher hydrophobicity (Venselaar et al., 2010).

3.7.Insilico mutagenesis

For insilico mutagenesis, PyMOL was used to analyze the protein sequence and to replace the amino acid of wild type in the original KPCδ structure with variant amino acid. So PyMOL was used for the substitution of amino acids. The variant amino acid has been

presented with different colors after a change in the amino acid residue than the wild type. From the PyMOL wizard option was selected, then mutagenesis, and finally clicked on the protein. The further mutant residue of interest was placed, and the “no mutation” option was selected. At this point, a change has been made, and now this one is saved in pdb format; later on, this mutant and the wild type of structure will be used for molecular dynamics simulations (Liu et al., 2023).

3.8.Analysis of protein flexibility

Seven nsSNPs resulting in the alteration in protein structure and flexibility were analyzed by DynaMut tool (Qiu et al., 2019). This tool, depending on the ENCom values, estimates the effect of mutations on molecular motions of protein and vibrational entropy resulting in the destabilizing effect.

3.9.Molecular dynamics simulations

Molecular dynamics simulations are run on supercomputers so in order to run molecular dynamics simulations of wild and mutant variant GROMACS software was used to check the effect of mutation on KPCδ structure. PuTTY, WinSCP and SFTP are used to transfer data between PC and supercomputer. 20ns simulation was run for wild and mutant variants and different matrices were observed such as root mean square deviation (RMSD), radius of gyration, solvent accessible surface area (SASA), root mean square fluctuation (RMSF) and number of hydrogen bonds.

3.10. Primer designing

Primer 1 was used to design the primers computationally for tetra ARMS PCR, genome sequence was mapped from ensemble from its chromosomal assembly in order to use it as input in Primer 1. In this tool only two parameters were changed i.e., allele difference and SNP position and other conditions were set as default.

Primers	**Sequence**
Forward inner	CCCCTTCTTCAAGACCATAAACT
Reverse inner	CGCTTTTCCAGCAGAGTCCG
Forward outer	GGATTTGCTGAAGCTCCAATTT
Reverse outer	AACATGCTATGGAGATTGCTGG

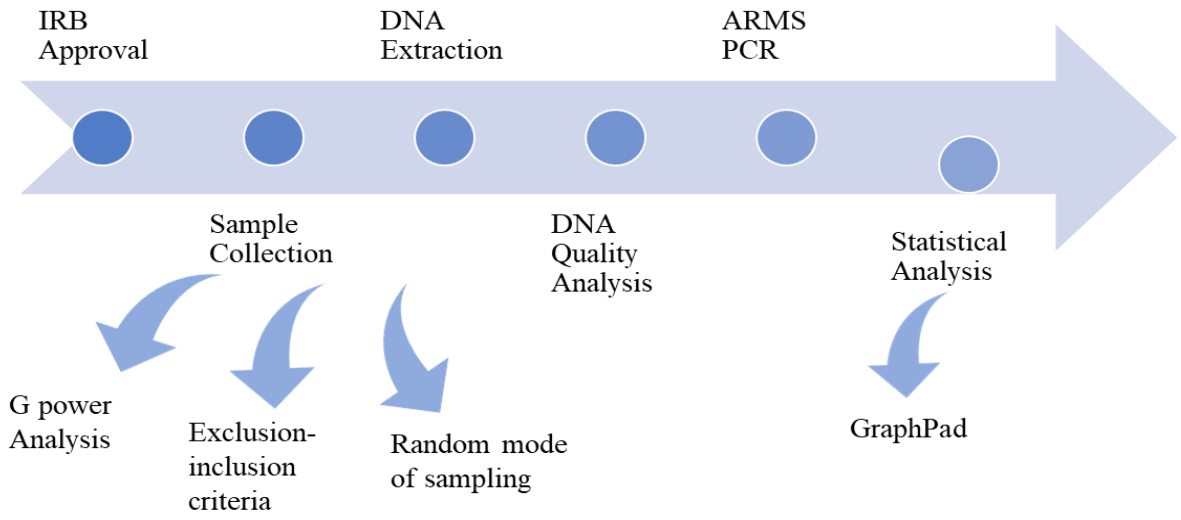


Figure 9. Schematic representation of experimental work in this study.

3.11. Sample collection

Collection of blood samples were done from the patients in Combined Military Hospital (CMH), Rawalpindi, after getting approval from Institutional Review Board of Atta ur Rahman School of Applied Biosciences and Combined Military Hospital. Patients were asked to sign the patient history form and patient consent form for their input in study. While collecting samples different criteria were kept under consideration including sample size that was nearly 200 i.e., control (n=100) and breast cancer (n=100), only females above 18 years were selected, absence of co morbidity. Then according to Declaration of Helsinki principles protocol or study was carried out.

3.12. DNA extraction

The collection of blood samples was done and from each sample 500µl of blood was extracted and added to 1.5ml of ependorf tubes. Then 500µl of solution was added to the tube containing blood of equal amount and mixed it well. The tubes were kept at room temperature for 10 minutes and after that centrifuged for 1 minute at 13000 rpm. Solution A is lysis buffer so in order to lyse the membrane properly this step was repeated twice. Supernatant was discarded and resuspension of pellet was done in 400µl of solution B, dissolved the pellet well and centrifuged it again for 1 minute at 13000 rpm. Supernatant

was discarded again, and resuspension of pellet was again done. in 400µl of solution A, 12µl of SDS and 5µl of proteinase K. Samples were then kept on incubation overnight at 37°C.

Samples were then further treated with solution C and solution D both were added of 250µl and centrifuged for 10 minutes at 13000 rpm. DNA was present in the upper layer (aqueous layer) so this layer was carefully separated into another ependorf and lower layer containing protein and debris was discarded. Later on, 55µl of sodium acetate and 500µl of ice chilled isopropanol was added in the tube having aqueous layer and tube was inverted several times to precipitate the DNA. Sample was again centrifuged for 10 minutes at 1300 rpm and after discarding the supernatant 200µl of 100% chilled ethanol was added and centrifuged it for 8 minutes at 13000rpm, ethanol was discarded from tube and in order to evaporate the ethanol completely tube sample was air dried. After this DNA was diluted in 200µl of PCR water of TE buffer.

3.13. Polymerase Chain Reaction

Point mutation was detected in DNA extracted from blood samples by using tetra amplification refractory mutation system polymerase chain reaction (ARMS-PCR). Two sets of primers i.e., two outer primers (forward and reverse) and two inner primers (forward and reverse) are used to amplify the whole gene and to detect SNP and these inner primers are allele specific. 20µl of PCR reaction was prepared with 6µl of PCR water, 1µl of all the four primers and 8µl of master mix. Samples were sort spun to mix all the reagents properly. Annealing temperature was optimized at 60°C. All samples were carried out at different temperatures simultaneously, each row has different temperature by using Gradient PCR machine. For almost 1.5 to 2 hours a total of 35 cycles was run.

3.14. Gel electrophoresis

The quality of the extracted DNA was analyzed on agarose gel electrophoresis. For this purpose, 1% agarose gel was used, to prepare 50ml of agarose gel, 5ml 10X TAE (Tris-acetate-EDTA) buffer was mixed with 45ml of distilled water. Then 0.5g of agarose was added to the above solution in a beaker. This mixture was microwaved for 1-2 minutes and 5µl of ethidium bromide was then added, the gel mixture was

poured to the gel tank and after removing the bubbles the comb was placed in the tank. The gel was placed at room temperature for 30 minutes to solidify.

The next step after the solidification of gel was to load the DNA samples in the wells. 3µl of DNA was mixed with 5µl of the loading dye. 8µl of the sample was added to the wells in the gel that was placed in the electrophoresis tank containing the 1X TAE buffer. The gel was run at 100V for 15 minutes. The gel was visualized under the UV Transilluminator.

3.15. PCR Mixture Preparation

A reaction mixture was prepared in a single tube containing all the reagents with volume of up to 20µl per reaction i.e., 6µl of PCR water, 1µl of all the four primers, 8µl of master mix and 2µl of DNA.

3.15.1. PCR steps and conditions

Step 1: In initial step PCR reaction denaturation happened at 95℃ for 5 minutes in which hydrogen bonds of all DNA molecules breaks. This step was not repeated at stage 2 instead temperature was kept at 95℃ for 30 seconds.

Step 2: In this step annealing occurs so as this PCR is gradient so multiple temperatures were set so temperature was kept at 60℃ for 30 seconds.

Step 3: In final step extension of DNA occurred at 72℃ for 30 seconds and then the final extension was kept at 72℃ for 7 minutes.

3.15.2. 2% agarose gel electrophoresis

Amplified PCR products were analyzed on 2% agarose gel. The agarose gel was prepared as described above. Analysis was carried out to search for the SNPs present or absent in the DNA samples.

3.16. Statistical Analysis

After performing genotypic analysis, genotypic data of patient and control sample was further analyzed using GraphPad Prism (Mavrevski et al., 2018). In order to analyze the distribution of genotypes Chi square test was applied. This gave the values of relative risk, odd ratio and p-value with 95% confidence intervals. The data with less than 0.005 p-value was considered statistically significant

CHAPTER 4
RESULTS

4.1. Identification of Missense SNPs of KPCδ

SNPs of KPCδ were retrieved from ENSEMBLE (Yates et al., 2020), total 2773 SNPs were obtained which contained 576 3' UTR variant, 28 frameshift variant, 748 synonymous variant, 1409 5' UTR variant and 1453 missense variant. So only non-synonymous variants or missense variants were selected and analyzed for further analysis as they were mostly associated with the disease. Following are the various types of variants in KPCδ mentioned in Figure 10.

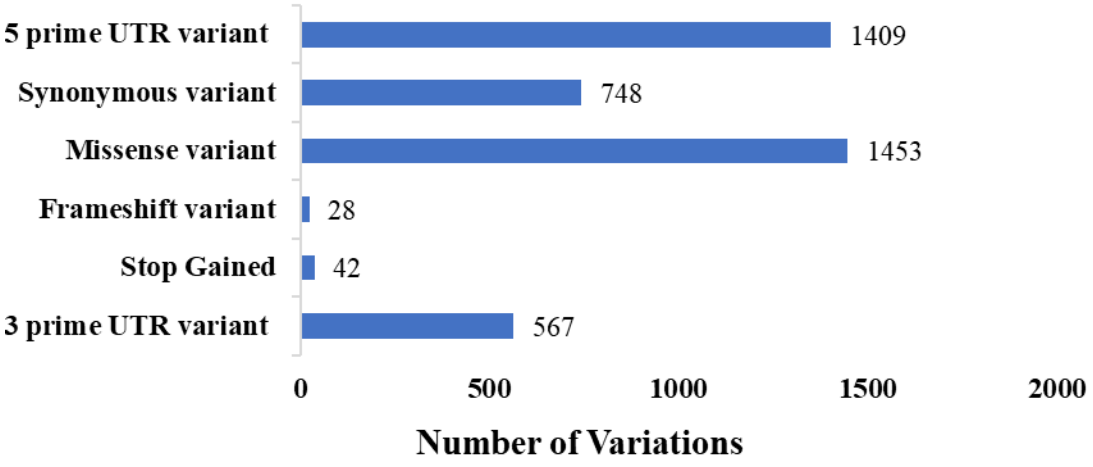


Figure 10. KPC delta SNP genomic variation analysis using ENSEMBL database.

4.2. Deleterious effect of nsSNP in KPCδ

Detailed information on missense SNPs in KPCδ was collected from the ENSMEBLE including the identities of the variants, their positions on the chromosomes, the changes in their alleles, and the amino acid changes that resulted from these variants mentioned in the table 1. Data was collected using a variety of insilico methods to find SNPs that might have the most impact on the protein's structure and function. Seven tools (CADD, Mutation Assessor, Polyphen, MetaLR, SIFT and REVEL) were used in order to identify the deleterious effect of missense variants, out of 2773 nsSNPs total seven non-synonymous SNPs were predicted and selected on the basis of average and percentage through these tools that crossed the 75% threshold level as mentioned in figure 11. Seven SNPs were

further used for final study that can alter the structure and function of KPCD's gene. All of the tools produced outcomes based on their distinct evaluation criteria, subjecting the SNP to varying levels of damaging effects. The scores of different tools i.e., SIFT; deleterious (0), Polyphen; probably and possibly damaging (0.9-1), CADD; likely deleterious (30-32), REVEL; likely disease causing (0.5-0.9), MetaLR; damaging (0.5-0.9), and Mutation Assessor; medium and high (0.5-0.9) as mentioned in table 2.

Table 1. Details of 7 non-synonymous variants with their IDs, genomic and Amino acid coordinates (coord).

	Variant ID	**Location**	**Alleles**	**AA**	**AA coord**
1	rs1201489693	3:53181721	A/G	Y/C	187
2	rs782555227	3:53181727	G/A	C/Y	189
3	rs1553668239	3:53183465	G/A	R/H	224
4	rs369078144	3:53186022	G/A	G/R	361
5	rs372295257	3:53186620	T/A	V/E	426
6	rs1703863535	3:53189951	T/C	W/R	608
7	rs1575535582	3:53181232	T/G	V/G	114

Table 2. KPCD's gene scores of seven deleterious variants through multiple tools.

Sr.	**Variant ID**	**SIFT**	**Polyphen**	**CADD**	**REVEL**	**MetaLR**	**Mutation Assessor**
1	rs1201489693	Del (0)	PD (1)	LDel (32)	LD (0.936)	D (0.876)	M (0.702)
2	rs782555227	Del (0)	PD (0.999)	LDel (32)	LD (0.963)	D (0.996)	H (0.978)
3	rs1553668239	Del (0)	PD (0.985)	LDel (32)	LD (0.663)	D (0.774)	H (0.94)
4	rs369078144	Del (0)	PD (1)	LDel (30)	LD (0.937)	D (0.814)	H (0.993)
5	rs372295257	Del (0)	PD (1)	LDel (31)	LD (0.773)	D (0.543)	M (0.825)
6	rs1703863535	Del (0)	PD (0.999)	LDel (31)	LD (0.727)	D (0.554)	H (0.995)
7	rs1575535582	(Del) (0)	PD (0.917)	LDel (31)	LD (0.908)	D (0.734)	M (0.649)

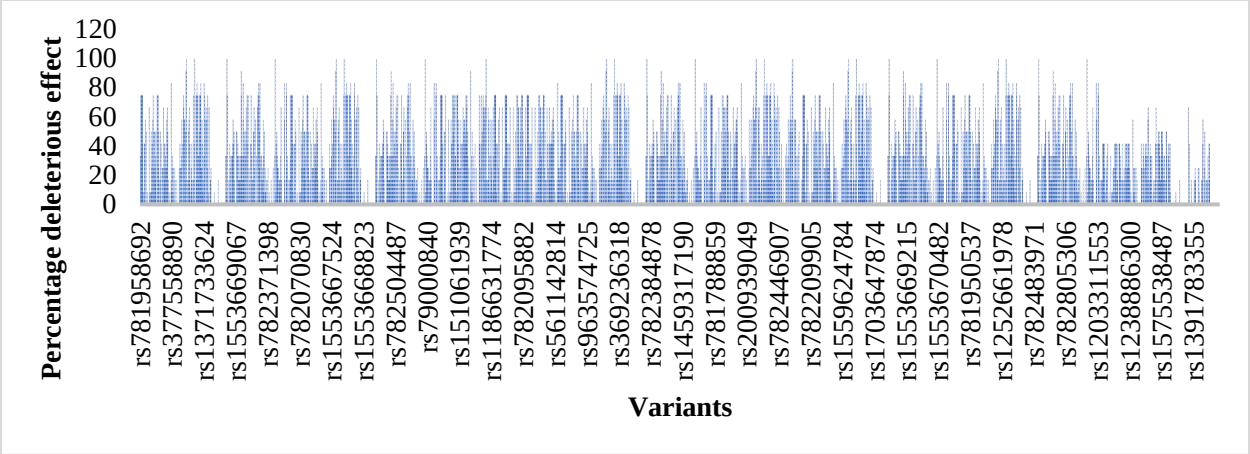
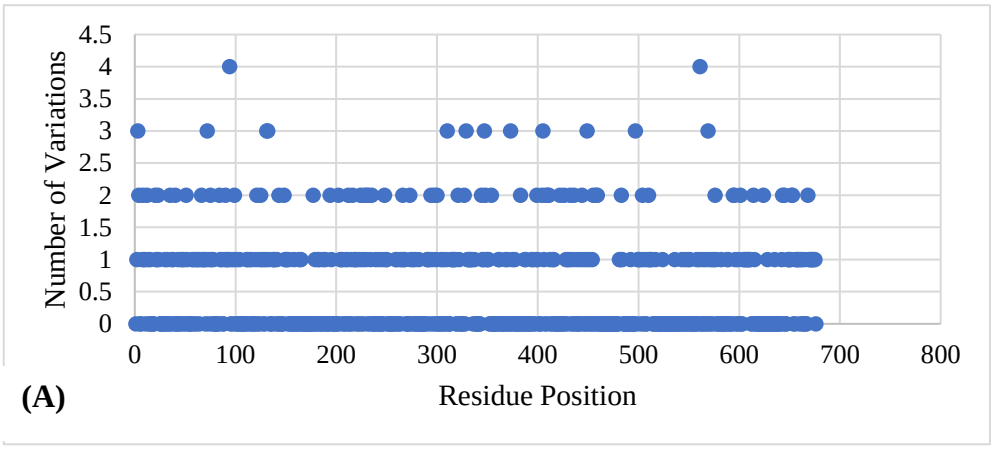


Figure 11. Prediction of 38 non-synonymous variants by in silico tools showing deleterious effect after crossing the 75% threshold.

More stringent criteria were applied to obtain highly deleterious variants among these 38 nsSNPs which resulted in 7 non-synonymous variants through multiple tools. After filtration of data SNP with variant ID rs1703863535 was selected for further validation in insilico wet lab process.

Within each relative abundance analysis was performed for variants in which exon 2 has the highest number of variations whereas exon 11 has also high variations mentioned in figure 12 **(A).** Lowest number of variations were observed in exon 13 and exon 5, highest number of variations at 94 and 561 residue position were observed mentioned in figure 12 **(B).**



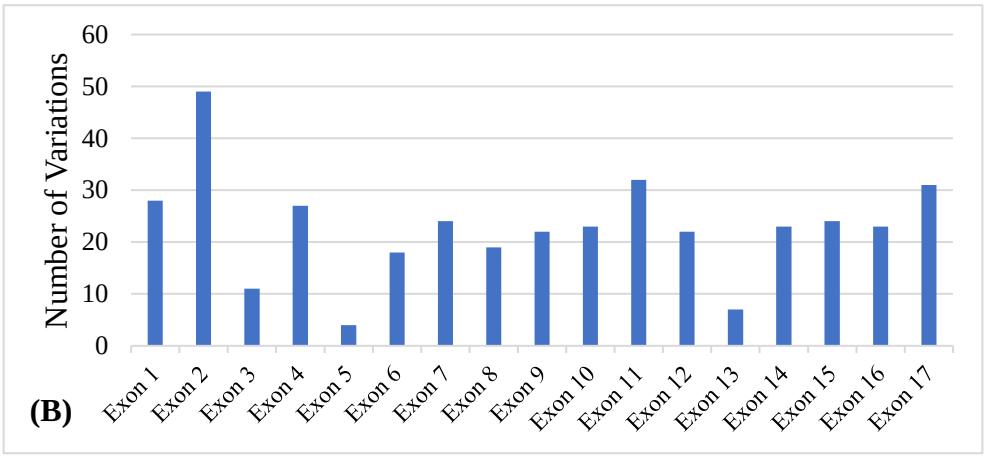


Figure 12. Mutational landscape of missense variants across protein residues and number of exons of KPCδ.

4.3. Structure of KPC Delta and mapping of variants

The protein structure of KPC delta was predicted through AlphaFold, and the 3D structure was downloaded in pdb format, which was further analyzed using PyMOL, the most efficient and reliable approach to predict the protein structure. And further information of the protein domains including regulatory and catalytic domain and hinge region was obtained from InterPro.

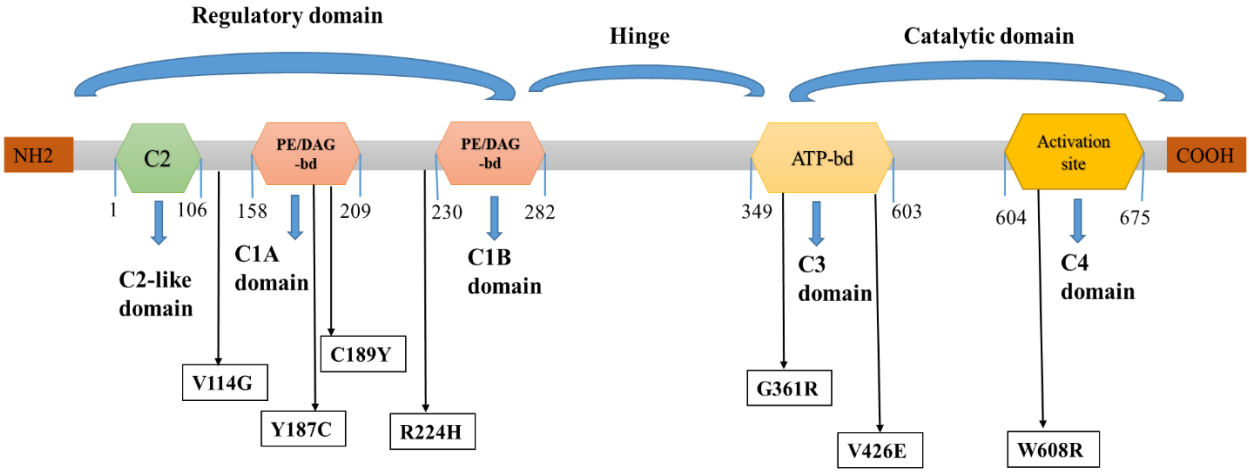


Figure 13. KPCD domains; KPCδ regulatory and catalytic domains. C1A, C1B, and C2 domains corresponding calcium and diacylglycerol (DAG) binding in the regulatory domain (RD). The catalytic domain has the ATP-binding site and substrate-binding groove. The hinge region allows conformational changes when activated. Variants were mapped on different regions and domains of KPC.

V114G resides close to the C2 like domain, two variants i.e., Y187C and C189Y resides in the C1A domain whereas fourth variant R224H is present near C1B domain. G361R and V426E is found in ATP binding domain and the one selected for the study W608R resides in activation site as mentioned in figure 13.

4.4. Structure and functional analysis

HOPE and I-Mutant were used to analyze the structure and function of SNP rs1703863535, DDG value of SNP was generated using I-Mutant showing its effect on the stability of protein structure. DDG value below zero indicates the decrease in stability of protein as mentioned in figure 14. 5 out of 7 showed decreased stability in which RI value of the SNP (W608R) is 8 with -1.4 DDG value so due to the induction of mutation it also shows the decreased stability of protein. Other variants that are bringing destabilization in protein structure are R224H, G361R, V426E, and V114G.

Table 3. Using I-Mutant to predict the stability of KPCδ variants

Variant Position	Wildtype Residue	Mutant Residue	Stability	DDG
187	Y	C	Increase	0.61
189	C	Y	Increase	0.99
224	R	H	Decrease	-2.38
361	G	R	Decrease	-0.78
426	V	E	Decrease	-2.06
608	W	R	Decrease	-1.4
114	V	G	Decrease	-3.62

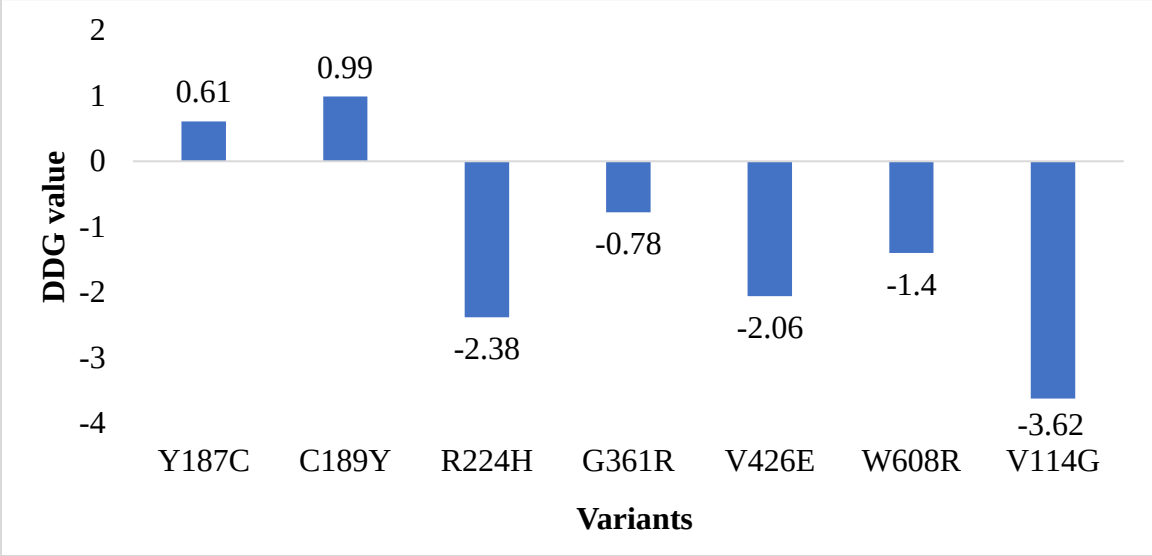


Figure 14. I-Mutant for the stability analysis of KPCδ variants. Increase or positive DDG value (Y187C and C189Y) indicates increase in stability and decrease or negative DDG value (R224H, G361R, V426E, W608R and V114G) indicates decrease in stability.

While doing analysis with the help of HOPE the following results were obtained mentioned in Table 4. Mutant residues W608R showed positive charge having higher hydrophobicity which can result in repulsion with other residues and mutation was present in AGC kinase domain present at C terminal and most importantly location of mutation is very conserved which can be damaging for protein and remaining variants have also higher hydrophobicity.

Table 4. Characterizing KPCδ nsSNPs using Project Hope

Variants	Mutant amino acid				Mutation location	
	Size	Charge		Hydrophobicity	Domain	Conservation
		Wild	Mutant			
Y187C	Smaller	-	-	More	Zinc-finger domain	Near a highly conserved position
C189Y	Bigger	-	-	More	Zinc-finger domain	Highly conserved
R224H	Smaller	Positive	Neutral	-	C1-Like domain	Very conserved
G361R	Bigger	Neutral	Positive	More	Protein Kinase	Near a highly conserved position
V426E	Bigger	Neutral	Negative	More	Protein Kinase	Near a highly conserved position
W608R	Smaller	Neutral	Positive	More	AGC-kinase C-terminal	Very conserved
V114G	Smaller	-	-	More	C2 domain	Very conserved

Variants association with cancer was observed with the help of FATHMM, results were obtained in the form of scores. 5 out of 7 variants mutations (Y187C, R224H, G361R, V426E, W608R) are predicted as passenger while the remaining two variants (C189Y and V114G) predicted oncogenicity mentioned in the table 5.

Table 5. Pathogenicity prediction of KPCδ nsSNPs using FATHMM

Variants	**FATHMM**	
	Prediction	**Score**
Y187C	Passenger	-0.41
C189Y	Cancer	-5.02
R224H	Passenger	0.63
G361R	Passenger	-0.4
V426E	Passenger	0.43
W608R	Passenger	0.21
V114G	Cancer	-1.19

4.5. Flexibility analysis

In order to analyze the change in flexibility analysis due to variants DynaMut was used. The outcome was in the form of ENCom values that tells whether there is increase or decrease in flexibility. Variants (V114G, W608R and Y187C) with increase in flexibility has increased hydrogen bonds, their bond length and type of bonding as well as their interatomic interactions. So, their vibrational entropy will also increase. On the other hand, variants (C198Y, R224H, G361R and V426E) with decrease in flexibility has decreased the vibrational entropy and their interatomic interactions have also decreased. W608R has vibrational entropy of 1.164 $kcal.mol^{-1}$. K^{-1} that indicates the increase in molecular flexibility because Arginine was more involved in interatomic interactions as to compared to the wild type of amino acid Tryptophan as mentioned in the figure 15.

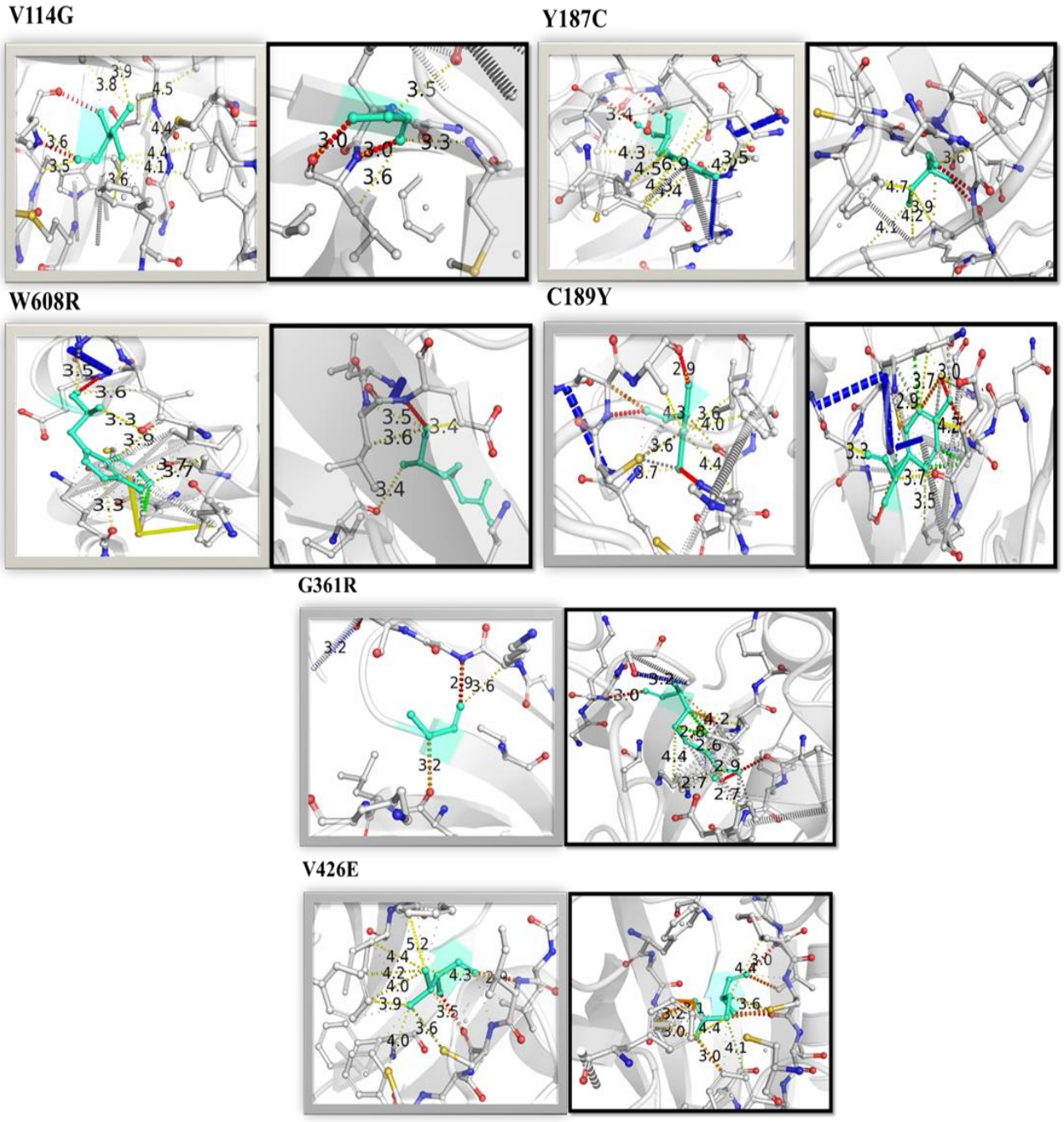


Figure 15. DynaMut analysis of the interatomic interactions of the 3D W608R and wild-type KPCδ models.

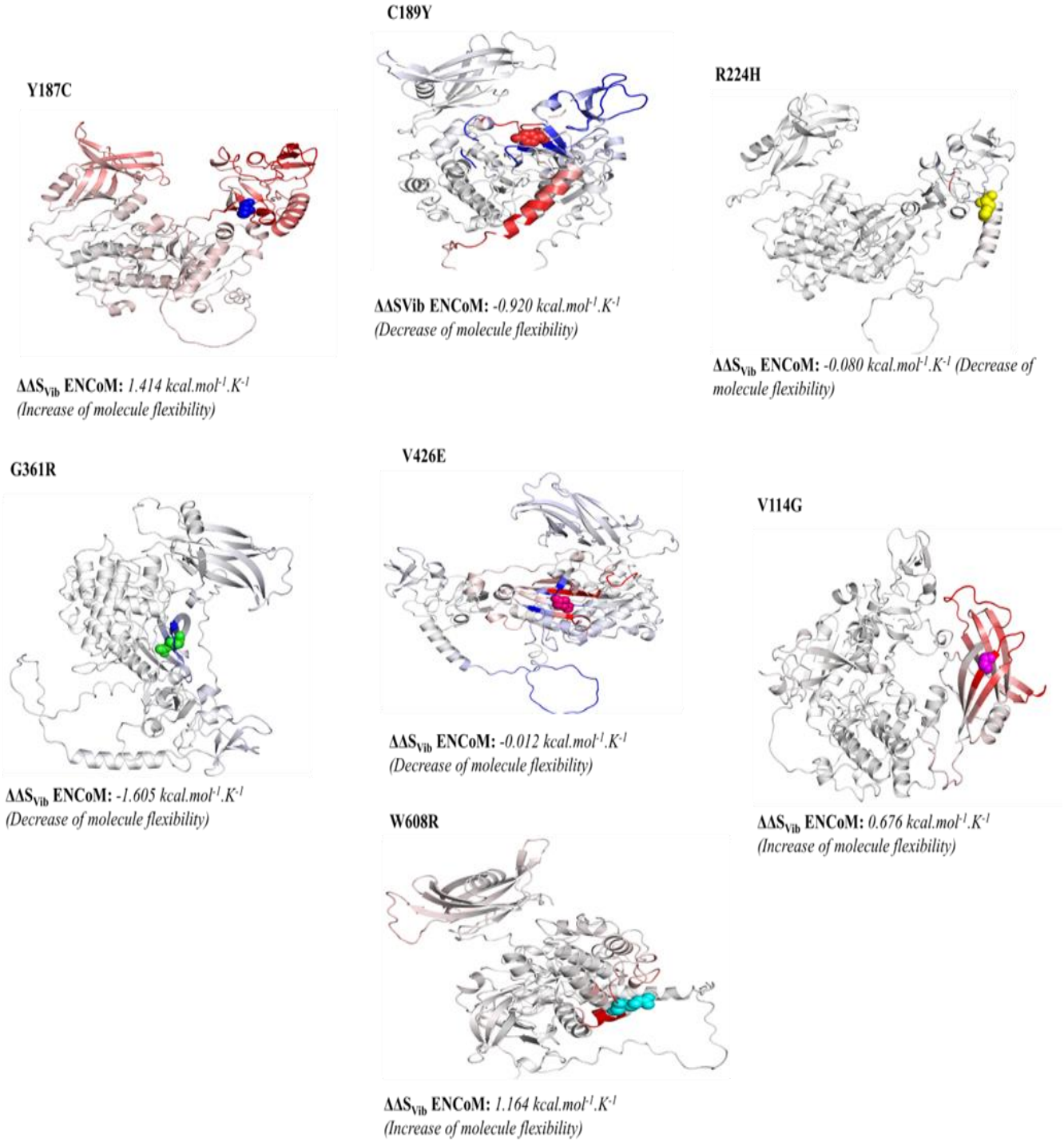


Figure 16. Illustration of molecular flexibility of wild and mutant type PKCδ through DynaMut.

4.6. In situ mutagenesis

PyMOL was used to create the mutated model of KPCδ by inducing the mutation at 608 positions in amino acid sequence in which Tryptophan (W) was replaced by Arginine (R) and this was the mutagenesis done by the PyMOL in figure 16, later on it was saved in the pdb format for further in silico analysis.

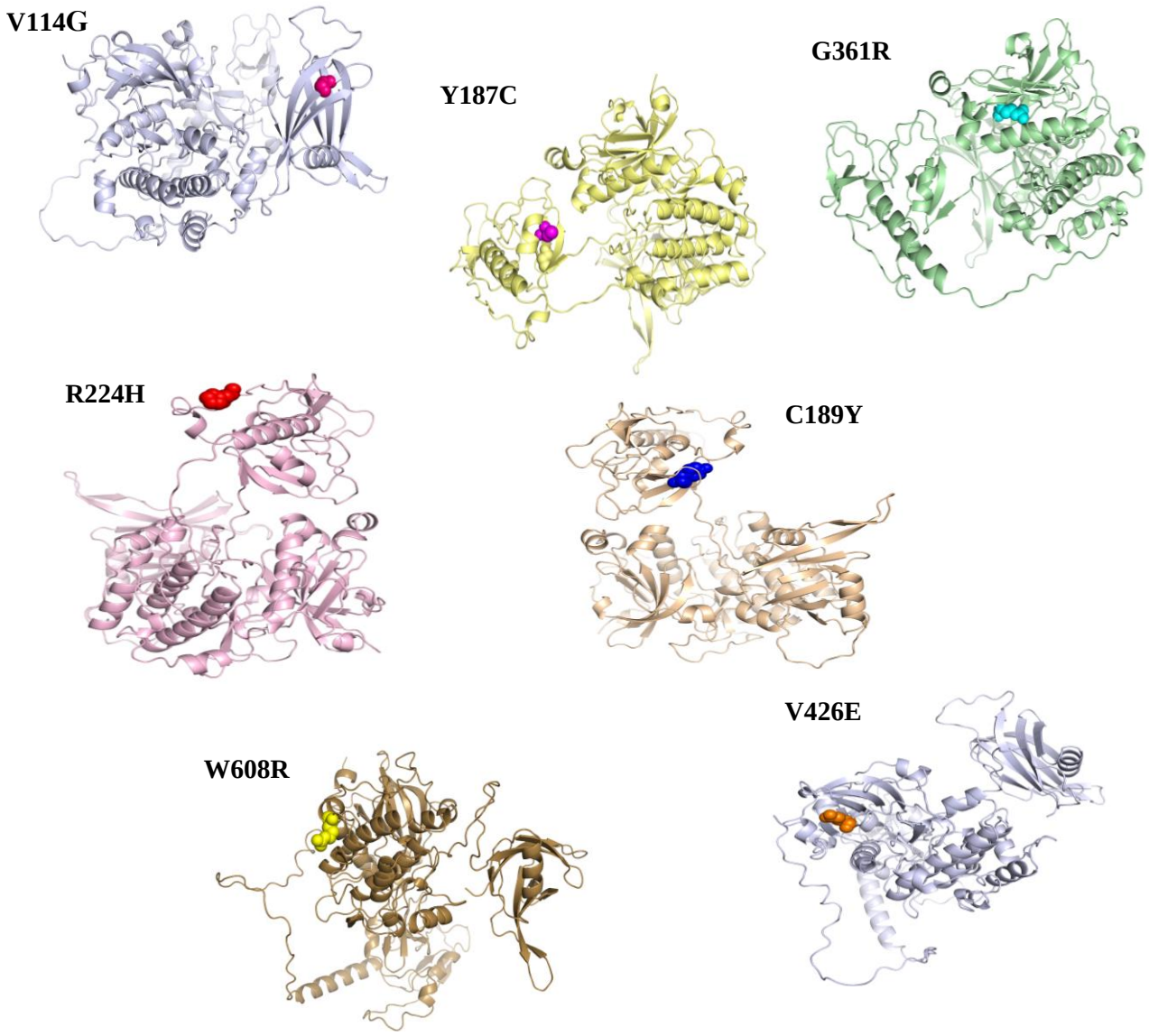


Figure 17. PyMOL-based in situ mutagenesis of KPCδ variants. Highlighted spherical shaped regions indicate structural changes induced by the mutation.

4.7. Molecular dynamics simulations

After 20ns molecular dynamics simulations has been run for wild and mutant variant i.e., W608R, data was generated in the form of several files. This data is further inferred in the form of graphs, so to differentiate between wild and mutant four different matrices were used i.e., root mean square deviation (RMSD), root mean square fluctuation (RMSF), radius of gyration, number of hydrogen bonds and solvent accessible surface area (SASA).

4.7.1. Root Mean Square Fluctuation (RMSF)

It is the difference or fluctuation of amino acid residues of wild and mutant protein. It tells us the individual difference or fluctuation in amino acid, so it does not give the positional difference of entire structure. Graph against RMSF and residues were plotted of wild and mutant structure. Highest RMSF value i.e., 1.2nm was observed at 317 residue position, overall fluctuation from mean point was observed from 300-330 residue position as shown in figure 17 (A).

4.7.2. Root Mean Square Deviation (RMSD)

Root Mean Square Deviation is the quantitative representation of the deviations between two structures i.e., wild and mutant. Higher RMSD value indicates greater deviation or difference between two structures or lower the RMSD value indicates less deviation and therefore both structures will show greater similarities. According to RMSD graph, mutant protein gradually showing increase in deviation from 0ns, first highest RMSD value was 0.7nm at 5ns, other structural changes or deviations were observed at 7.2ns, 9.5ns, 16ns and 20ns. This indicates that mutant structure is less stability as compared to wild structure as shown in figure 17 (B).

4.7.3. Radius of Gyration (Rg)

The degree of compactness or how tightly a protein structure packed, is calculated on the basis of radius of gyration. Protein started to become extended, and the highest Rg value was 3.15nm at 2.4ns and 3.12nm at 3.6ns. Afterwards, the Rg value went down to 2.9nm at 5.6ns and then back up to 3.1nm at 6.6ns. But after, there was a big drop until 16ns, and then it went back up until the end, which was 20ns. Overall, by observing the trend, protein mutant structure was affected as compared to wild structure as mentioned in figure 17 (C).

4.7.4. Number of Hydrogen Bonds

Stability of protein structure also depends on the number of hydrogen bonds. Increase or decrease in the number of hydrogen bonds make the protein unstable. According to the graph there was no significant difference in the increase or decrease in number of hydrogen bonds, as there was only one amino acid change due which there was overlapping between wild and mutant KPCδ so there was no significant change observed shown in figure 17 (D).

4.7.5. Solvent Accessible Surface Area (SASA)

SASA means how much surface area of protein is accessible to the solvent molecules. According to the graph obtained after running simulation of about 20ns, there was not any significant trend observed as mutant protein peaks was consistently below peaks of wild protein in SASA graph which means mutant protein structure was not exposed, structure was compact and buried. Overall, the structure was stable as shown in graph (E) of figure 17.

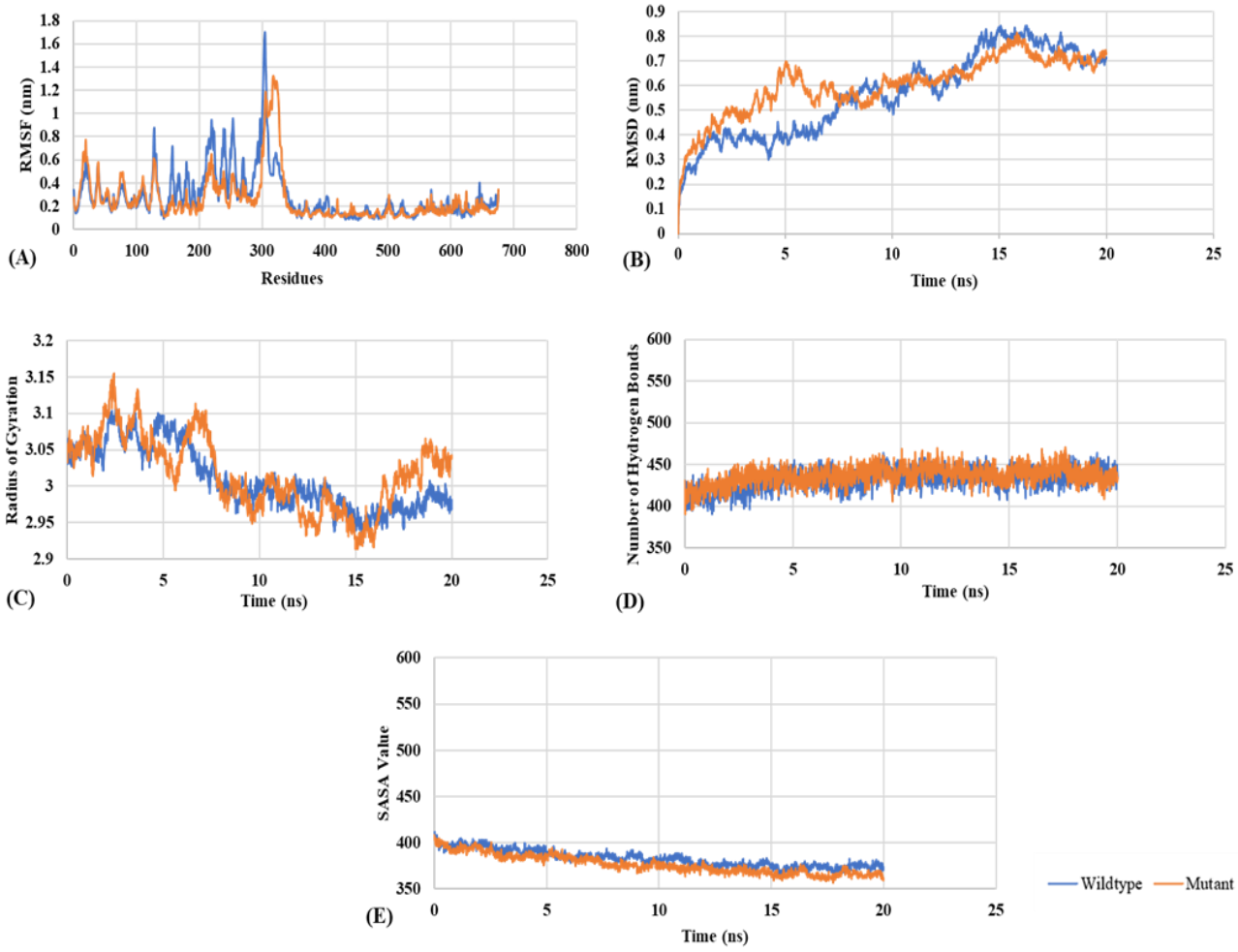


Figure 18. KPCδ Molecular dynamics simulation recorded at 0ns and 20ns. **(A)** Root Mean Square Fluctuation **(B)** Root Mean Square Deviation **(C)** Radius of Gyration **(D)** Number of Hydrogen Bonds **(E)** Solvent Accessible Surface Area.

4.8. Wet Lab Analysis for Pathogenic SNP

4.8.1. KPCD variants association with Breast Cancer

The Phenol chloroform DNA extraction method was used in order to study the association of KPCD rs1703863535 with breast cancer. rs1703863535 was further analyzed by Genotyping assay that is responsible for the substitution of Tryptophan with Arginine at 608 positions. Genotyping analysis was done by Tetra ARMS PCR, so to amplify the target gene sequence four primers were used giving outer band of control and inner band of genotype.

Genotype frequency distribution was calculated in control and breast cancer samples of KPCδ variant rs1703863535. Frequency distribution, odd ratio, relative risk and p value of patient and control are mentioned in the table. Frequency distribution of heterozygous genotype TC with 0.0202 p-value indicated that it is also statistically significant and is related with breast cancer risk factor whereas TT and CC genotype showed non-significant results according to the p-value mentioned in table 6.

Table 6. Association of patient and control genotypic data of KPCδ variant rs1703863535 with breast cancer.

Genotype	Frequency distribution		Odd ratio		Relative risk		P value
	Control	Patient	Value	CI 95%	Value	CI 95%	
TT	42%	48%	0.5833	0.3381 to 0.9933	0.7778	0.5920 to 1.001	0.0551
TC	38%	22%	2.173	1.153 to 4.118	1.43	1.081 to 1.856	0.0202
CC	20%	30%	0.5833	0.3088 to 1.112	0.75	0.5045 to 1.054	0.1412

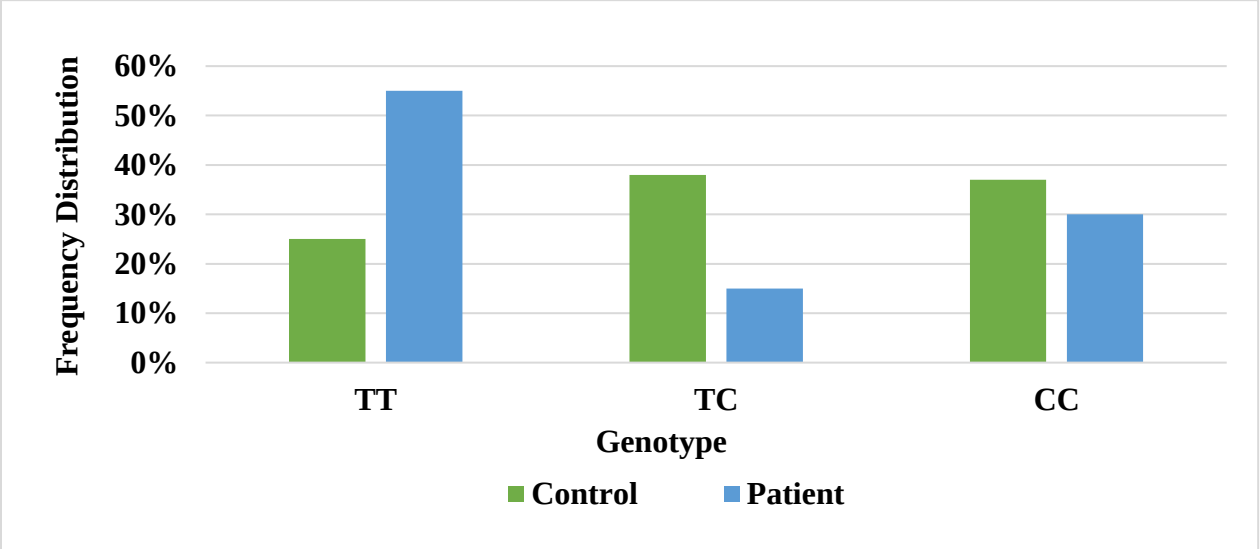


Figure 19. Genotypic data association of KPCD variant rs1703863535 with breast cancer.

4.8.2. Association of KPCD variant rs1703863535 with treatment

The data shown in table 7 concluded that the heterozygous genotype TC is highly significant with p-value of <0.0001, high significant results were also obtained from homozygous genotypes TT with 0.0024 p-value and CC have also shown deleterious role in treated state in breast cancer with p-value of 0.097.

Table 7. Genotypic data association of rs1703863535 with treated breast cancer patients.

Genotype	Frequency distribution		Odd ratio		Relative risk		P value
	Treated	**Untreated**	**Value**	**CI 95%**	**Value**	**CI 95%**	
TT	29%	11%	3.305	1.554 to 7.350	1.634	1.230 to 2.084	0.0024
TC	32%	8%	5.412	2.313 to 11.67	1.882	1.453 to 2.370	<0.0001
CC	14%	6%	2.55	1.000 to 6.793	1.465	0.9826 to 1.918	0.097

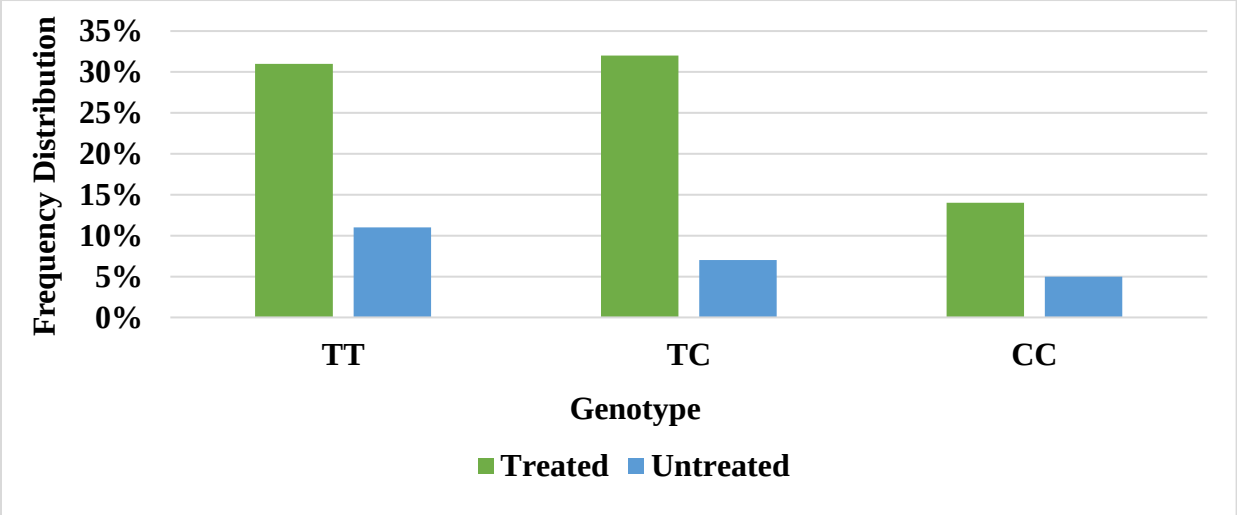


Figure 20. Genotypic association analysis of variant with treatment.

4.8.3. Association of KPCD variant rs1703863535 with metastatic breast cancer patients.

In case of genotypic data for metastatic and non-metastatic and family history data none of the genotypes were found to be related to metastatic breast cancer.

Table 8. Genotypic data association of rs1703863535 with metastasis and family history.

Metastasis						**Family history**				
Genotype	**Odd ratio**		**Relative risk**		**P value**	**Odd ratio**		**Relative risk**		**P value**
	Value	**CI 95%**	**Value**	**CI 95%**		**Value**	**CI 95%**	**Value**	**CI 95%**	
TT	0.7783	0.3929 to 1.554	0.878	0.5842 to 1.229	0.5963	1.285	0.6434 to 2.545	1.128	0.7914 to 1.512	0.5963
TC	0.8824	0.4323 to 1.769	0.9383	0.6333 to 1.297	0.8599	1.285	0.6434 to 2.545	1.128	0.7914 to 1.512	0.5963
CC	1.25	0.5013 to 2.987	1.112	0.6787 to 1.573	0.8143	1	0.3873 to 2.582	1	0.5881 to 1.461	>0.9999

CHAPTER 5
DISCUSSION

It is estimated that there are approximately 2.26 million new cases of breast cancer diagnosed around the world in the year 2020. Additionally, breast cancer is the leading cause of cancer death among females. these make breast cancer a significant threat to the health of people all over the world (Wilkinson & Gathani, 2022). According to the GLOBCAN data, breast cancer with 2.3 million cases and 6.9% mortality rate was reported from the 185 countries (Bray et al., 2018). Breast cancer has various subtypes depending on its pathological features and molecular profiling i.e., HER2, luminal A and luminal B, basal like receptor and triple negative breast cancer (Eliyatkın et al., 2015). High rates of cases of breast cancer and fatalities in underdeveloped nations are mostly attributable to a lack of education about the illness, ineffective screening procedures, late diagnosis, and inadequate healthcare infrastructure (da Costa Vieira et al., 2017).

Various factors are responsible for the late diagnosis of breast cancer as there is not any proper awareness, there are not enough proper treatments and if available, yet they are still expensive ones and not everyone can afford such expensive treatments. And there are more difficulties in diagnosing breast cancer, most of the time it is diagnosed at the later stage. There are many diagnostic procedures and they are not successfully operational making it impossible to treat breast cancer at early stages (Jafari et al., 2018). Many genes are involved for the development of breast cancer i.e., BRCA1 and BRCA2, CHECK, PTEN, ATM, p53 but when these genes are targeted, they have not given any successful results. Instead, they have developed chemoresistance or resistance to other therapies (Goidescu et al., 2018). So, another target have been identified i.e., KPCδ belongs to the KPC family also responsible factor for breast cancer. KPCδ involved in the multiple pathways in cell with proliferative and anti-apoptotic properties in case of dysregulation. In case of dysregulation KPCδ expression is upregulated in cellular pathways, its expression also depends on the cell type, subcellular localization and stimulus (Knutson & Hoenig, 1994). Various studies have been done on the genetic association with high risk factor genetic variants with breast cancer, but study on the genetic association on KPCδ variants with breast cancer development still remains unclear.

So, the purpose of the study is to study the association of KPC delta's non-synonymous variants with breast cancer risk factors and pathological characteristics through genotyping analysis to investigate the KPCδ variants as prognostic marker for breast cancer of control and patients' samples to analyze the impact of variants on the structure and function of KPC delta. After the selection of SNP, the effect of the variant on protein function and structure was evaluated and studied by molecular dynamics simulations.

SNP through various modifications and regulations can affect the different characteristics of genome and then contributing their role in complex diseases (Zhang et al., 2019). Because of their important role in determining disease pathogenicity and therapeutic strategy, only missense variations were selected for this detailed analysis (Ferrer-Costa et al., 2002). To examine the process and approach for these modifications, bioinformatics methods is used to analyze a large group of functionally significant SNPs in a disease (Chitrala & Yeguvapalli, 2014). A 3-dimensional structure was obtained from PDB database in order to study the effect the of missense variant on KPCδ shape and composition. This structure was further predicted and visualized by the AlphaFold and PyMOL (DeLano, 2002). Ensemble genome browser was used to retrieve total 1453 missense variants and then one nsSNP rs1703863535 was further used in order to determine its role against breast cancer, either it is protective or pathogenic. In this study mutation discussed was present in activation site or C4 domain at 608 position, in which tryptophan was converted to arginine. Characterization of KPCδ was done and then mutational hotspots of nsSNPs were found in KPCδ protein. Exon 2 had the highest number of mutations while exon 3, 5 and 13 had the lowest number of mutations or variations. Protein effected by the SNP depends on the domain or region in which it is located. According to the tools, that checked the deleteriousness of the SNPs with threshold greater than 75% filtered the total 7 nsSNPs i.e., V114G, W608R, Y187C C198Y, R224H, G361R and V426E. 4 out seven SNPs fall under the regulatory domain while the remaining 3 SNPs fall under the catalytic domain. Activity of C1 domain of KPCδ is enhanced by the binding of the DAG and phorbol esters (Dashzeveg & Yoshida, 2016; Livneh & Fishman, 1997; Watanabe et al., 1992). Alterations to protein functionality may come from the SNPs' potential effects on activation. W608R radius of gyration has increased in overall trend in the mutant structure of protein that cause the gain of compactness in structure of protein.

The greater the radius of gyration the more the structure will fold and therefore lose compactness. Therefore, protein compactness has indirect relationship to the folding of protein (MIu et al., 2008). Interaction between domains also seemed to be disturbed while doing the analysis from Project HOPE and I-Mutant, so with the one amino acid change in protein has affected the hydrophobicity, charge and sizes of residues making it bigger and smaller. The selected variant for the study was very conserved located in the AGC kinase domain which reduces the stability along with the entire protein as well as its flexibility based on its DDG value according to the I-Mutant analysis. The size of the mutant protein was smaller with positive charge and higher hydrophobicity. Kinase domain present in every member of the KPC family is identical to the each other (Ono et al., 1989). FATHMM was used to validate the pathogenic association of variants with cancer. And 2 variants (C189Y and V114G) were predicted to be cancer while other 5 variants were predicted as passenger.

Prediction of molecular dynamics were done after performing molecular dynamics simulations on KPCδ wild and mutant variants. MD simulations gave us the idea of the protein behaviour when mutation is exposed to it (Chitrala & Yeguvapalli, 2014). MD analysis gave the RMSD, RMSF, radius of gyration, number of hydrogen bonds and SASA. RMSD results gave insight on the deviation of mutant structure from the wild type. 0.7nm highest RMSD value was observed indicating the decreased stability of mutant structure. Analysis on the difference in amino acid residues and their fluctuations was recorded during 20ns simulations and gave the RMSF graph. 300-330 residue positions seemed to be highly fluctuated and high fluctuation was observed at 317 amino acid residue. According to the given results, fluctuation rate was higher in the hinge region due to the mutation as compared to the wild type. Graph was plotted on the basis of radius of gyration determining the compactness and folding of the protein by comparing the wild and mutant variant. According to the results, a high radius of gyration was recorded from 3.1nm to 3.15nm at this Rg value structure was extended, overall results indicated that mutant structure had gained compactness toward 20ns simulation as compared to wild type. Intermolecular interactions were also analyzed to determine their number of hydrogen bonds in mutant and wildtype structure. A small change was observed in the number of hydrogen bonds that can be considered not a significant one, because one amino acid

change did not affect the intermolecular interactions. Surface accessibility surface area was also recorded during 20ns simulation, according to the results the mutant trend was below from wild type throughout the simulation, surface area was not affected from the mutation.

Genotyping analysis was done for rs1703863535 in vivo to identify their pathogenic role. Variant against the breast cancer as a risk factor was genotyped using Tetra ARMS PCR. Genotype frequency distribution was calculated in control and breast cancer samples of KPCδ variant rs1703863535 according to the calculated frequency, heterozygous genotype TC genotype was associated with breast cancer and homozygous genotypes TT and CC showed non-significant results. These outcomes were similar to the findings of previously published studies, which indicated that modified genotypes had a correlation with the disease development, whereas reference genotypes were not associated with the disease against disease (Khan et al., 2022; Wang et al., 2010).

Genotyping analysis was also done in treated and non-treated patients, and none of the genotypes in case of treatment. Genetic variants associated with family history and metastasis were also studied and according to the analysis none of the genotypes showed association.

5.1. Future prospectives

Novel pharmacological strategies can be developed by identifying the new genetic variants and then helping develop treatments that can reduce the recurrence and occurrence of cancer. Identified SNP can be used as a potential therapeutic target, a prognostic marker for early breast cancer diagnosis. After identifying the damaging effect of the SNP, expression analysis can be done to explore more strategies for cancer therapy. Besides cancer, this study can also help treat and diagnose various metabolic and neurological diseases. This study can be further explored at the clinical level and for a comprehensive study of interactions of various molecules in breast cancer. By studying the SNPs, the development of drugs can be more effectively explored with less toxicity and fewer side effects. SNP identification can help in understanding the genetic profile of a person, which can help in developing personalized treatments related to cancer. Knowing the person’s genetic makeup can identify a person’s response to a specific drug.

5.2.Conclusion

Single nucleotide polymorphisms have been linked to various diseases' progression and development, including malignancies, according to the results of different experimentations. In silico identification of SNPs was retrieved in order of their association with diseases, and potentially damaging ones were selected. In this study, rs1703863535 (W608R) of KPCδ, the harmful genetic variant, was selected. Further analysis was done to investigate the effect of nsSNPs on protein structure, flexibility, and function, which confirmed that mutation had disrupted the regular activity of the protein. MD simulations showed a change in mutant protein compared to the wild type depending on the hydrogen bonds, RMSF, the radius of gyration, and RMSF. To evaluate or reveal its association with the incidence of breast cancer as a risk factor, genotyping analysis with the help of Tetra ARMS PCR was done; this confirmed the deleteriousness of the variant. Further validation needs to be done through in vitro and in vivo means to check the expression of the protein and then intermolecular interactions with other proteins so it can help in the development of a new approach for cancer therapy. In-depth, studies must be done to study the effect of the variant and other variants related to breast cancer in multiple populations and different ethnic groups because, in this study, data collection was from one specific area.

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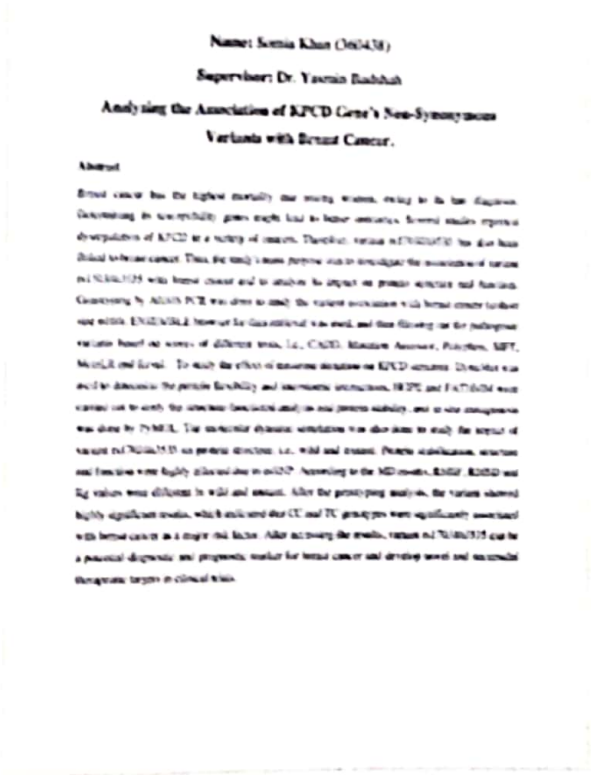

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