Evaluation of FAM26F in cancer using immunohistochemistry

MS Healthcare Biotechnology

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Evaluation of FAM26F in brain cancer using immunohistochemistry

A thesis submitted in partial fulfilment of the requirement for the degree of Masters of Science in Healthcare Biotechnology

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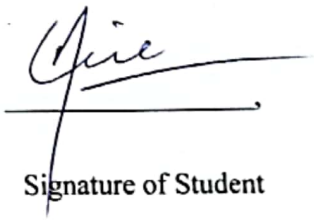

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I dedicate this thesis to my loving family for their unconditional support and encouragement

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List of Abbreviations

8-oxodG	8-oxo-2'-deoxyguanosine
ATP	Adenosine triphosphate
ASK1	Apoptosis signal regulating kinase 1
BEAST	Bayesian evolutionary analysis by sampling trees
Ca_hom_mod	Calcium Homeostasis Modulator
Ca^{+2}	Calcium
CNS	Central nervous system
DAB	3,3'-Diaminebenzidine
ER	endoplasmic reticulum
EGFR	Epidermal growth factor receptor
Ero1	ER oxidoreductases
FAM26F	Family with Sequence Similarity 26, Member F
gal-1	Galectin-1
GBM	Glioblastoma Multiforme
H2O2	Hydrogen peroxide
HIER	Heat induced epitope retrieval
HMM	Hidden Markov Model
HO^{-}	Hydroxyl Radicals
HO·	Hydroxyl radicle
HOCl	Hypochlorous acid
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
INAM	IRF-3–dependent NK-Activating Molecule
IP3	inositol 1,4,5-triphosphate
MAMs	Mitochondria associated membranes
IP3R	inositol 1,4,5-triphosphate receptors
MCC	Maximum clade credibility
MS	Mass Spectrometer

MSA	Multiple Sequence Alignment
mTOR	Mammalian target of rapamycin
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NCBI	National Centre of Bio-Informatics
NF-κB	nuclear factor-κB
NK	Natural Killer
NOX5	NADPH oxidase 5
O2	Oxygen
$O2\cdot-$	Superoxide
PAMPs	pathogen associated molecular patterns
PDB	Protein Data Bank
PDIs	Protein disulfide isomerase
Pfam	Protein family database
PRRs	Pathogen recognition receptors
Prx4	Peroxiredoxin 4
PTEN	Phosphate and tensin homolog
RACCs	Receptor activated calcium channels
ROS	Reactive oxygen species
RyR	Ryanodine receptors
SER	sarcoplasmic endoreticulum
SERCA	Sarco/endoplasmic reticulum calcium pump (SERCA)
SOD1	Superoxide dismutase 1
SOD2	Superoxide dismutase 2
STAT-1	Signal transducer and activator of transcription
TAMs	Tumor associated macrophages
TGF	Tumor growth factor
TLR	Toll like receptor
TME	tumor microenvironment
TNFα	Tumor necrosis factor alpha
tORFs	The open reading frames
Trx	Thioredoxin

VOCCs	Voltage operated calcium channels
WHO	World Health Organization

Abstract

Calcium (Ca2+) homeostasis is involved in various processes including in immune response and apoptosis. Changes in Ca2+ concentration stimulate various immune responses such as apoptosis through reactive oxygen species (ROS) production, especially in cancer. Calcium ion channels are involved in Ca2+ homeostasis and their expression may vary in cancer since calcium homeostasis and ROS production are reported to be interlinked. Tumor microenvironment studies have shown ROS to be involved in cancer promotion. International Agency for Research on Cancer (IARC) reported cancer fatalities to be 0.11 million in Pakistan hence showing their deadly nature. FAM26F (family with sequence similarity 26, member F) is one such calcium ion channel predicted to be differentially expressed in various cancers. Composed of a single domain Ca_hom_mod, FAM26F is involved in calcium regulation. Previously no attempt has been made to explore the expression of FAM26F in different types of various cancers, and its evolution. This leaves room for both, the investigation of the role of FAM26F in various tumors as well as its phylogenetic study to trace its evolution. The current study aims to answer the questions by 1) conducting immunohistochemical (IHC) analysis to observe the expression of FAM26F in different cancer samples, and 2) by employing a computational-based strategy to scan for the FAM26F domain events, integrating the data with a time-calibrated phylogenetic tree. 1) IHC on formalin fixed- paraffin embedded cancer biopsy samples displayed a varied expression of FAM26F depending on tissue type and cancer grade. A decrease in bladder and breast cancer was seen while an increase in FAM26F expression in renal, prostate, and gastric cancer was seen. 2) The evolutionary analysis identified around 4000 domains, and 14000 domain loss, duplication and loss events along with its

interacting partners. FAM26F evolution as well as the domain events of its interacting partners: Vinculin, Calpain, protein S100-A7, Thioredoxin, Peroxiredoxin and Calmodulin-like protein 5 were analyzed. Duplication events near higher eukaryotes showed its building complexity in function. Patterns of evolutionary events indicated possibility of co-evolution of CALHM6 with its interacting partner, advocating its role in calcium homeostasis and in related processes i.e cancer. Thus both IHC and the phylogenetic approach showed FAM26F to be differentially expressed in cancer.

Introduction

Tumor proliferation has been associated with the immune system for a long time and several studies give detailed insight into the function of inflammation in the tumor microenvironment (TME). There are two types of immune responses. One of the key features of innate immunity is the phenomenon of oxidative stress, which helps clear the infection by putting the cell in a highly oxidized state by the stimulation of reactive oxygen species (ROS) in a cascade of enzyme catalyzed reactions. ROS includes hydrogen peroxide, superoxide anion (O2-), hypochlorous acid (HOCl), and hydroxyl radical (HO•). Production of ROS is a strictly modulated process in the cell. Making sure the ROS are at low levels is essential for the normal execution of cellular procedures while the increase in levels takes the cell towards destruction by induction of apoptosis. This process is interlinked with calcium homeostasis. The Ca2+ levels in the intracellular matrix are vital in the induction of the ROS-producing pathway. Upon receiving the signal of stress, there is a rapid influx of Ca2+ from outside to inside of the cell through calcium channels, and from the calcium storage in the endoplasmic reticulum (ER) to plasma. Mitochondria take in the calcium in order to proceed with an oxidative burst, resulting in ROS production. Their interplay is strengthened by the studies showing the increase in permeability of endothelial membranes to calcium by enzymes of the oxidative system and the increase in ROS production by calcium.

High levels of ROS are associated with apoptosis in cells. Different concepts describe the role ROS plays in clearance or promotion of the cancer cells. It is suggested that in the presence of exogenous ROS yielding agents, their level in tumor cell increases drastically compared to that in normal cells. This may steer the cell towards death (Wang & Yi, 2008).

The second hypothesis states that if the ratio of ROS to antioxidants is disrupted and ROS level increase exceeds normal level, this will lead to either sensitivity to cancer therapy or of apoptosis and clearance of tumor cell (Kong et al., 2000). However, there is a delicate regulation carried out by cancer cells that allows them to increase their oxidative state by increased production of ROS to help immune suppression, cancer cell proliferation and angiogenesis, whilst avoiding cell death (Costa et al., 2014; Olivier et al., 2021; Reczek & Chandel, 2018).

Cancers consist of primary tumors that initiate in the organ and secondary tumors which are initiated by cancers starting in any other part of the body. Central nervous system cancer is the 10th leading cause of death around the world with 3.4% mortality in Pakistan in 2020, of which brain tumors account for 85% to 90%. Although malignant brain tumors account for 1-2% of all cancers, they are the deadliest form of cancer and the most strenous to treat. Bladder cancer is the type of cancer which originates from the cells of the bladder and is common in females while in men it is fourth most common type of cancer (Lenis et al., 2020). It may be unaggressive and non-invasive type of cancer that can transform into invasive and aggressive one cancer (Lenis et al., 2020). Gastric cancer is the cancer of stomach which is the 5^{th} most common kind of tumor worldwide while 3^{rd} in rank for cause of death (Smythe et al., 2020). Breast cancer continues to be global concern with highest incidence among females cancers in Pakistan. Cancer starting in prostate is called prostate cancer gland renal cancer and is second most common cancer found to be in males and fifth cause of death (Rawla et al., 2019). Renal cancer is found in cells in kidney tubules (Rossi et al., 2018).

Inflammation is reported to play a key role in promoting the progression of cancer. The TME of tumors is maintained by a complex signaling network between cells, immune cells, and normal tissues through cytokines (Sowers et al., 2014). Various cytokines and chemoattractants engage with immune cells to bring them to the tumor environment. The infiltrating immune cells such as eosinophils, neutrophils, macrophages, and T lymphocytes release inflammatory mediators that assist tumor angiogenesis, invasion, and proliferation and escalate oxidative damage to DNA. Cytokines namely interleukins, and tumor growth factors (TGF) among others cause the immune cells to become pro-tumor by engaging in the process like inhibition of macrophages and dendritic cells activation, apoptosis of T lymphocytes, and metastasis (Alghamri et al., 2021).

Cytokines such as interleukin-10 (IL-10), IL-6, galectin-1 (gal-1), and TGF-beta reprogram the infiltrating immune cells in such a way that they attain a pro-tumor phenotype. For example, IL-10 inhibits macrophage and dendritic cell action and inhibits the activation of T-cells while gal-1 induces T-cell apoptosis and increases the migration of cancer cells. Cytokines also stimulate the macrophages to conduct oxidative bursts resulting in ROS production. ROS interacts with lipids, nucleic acids, and proteins causing a plethora of changes and damages all contributing towards tumorigenesis. Moreover, TME has been reported to have elevated levels of epithelial growth factor receptors (EGFR) compared to healthy tissues. EGFR stimulates ROS production hence increasing oxidative stress (Jaros et al., 1992). ROS causes DNA damage leading to mutations and an increase in cytokine and chemokine production contributing to immune cell penetration. It also renders the immune cells useless against cancer and even makes them promote tumor growth.

FAM26F is a relatively new name in the world of immune system proteins. It is a transmembrane protein, with a pore-forming channel that allows the movement of ions from across the membranes (Malik & Javed, 2016). Extensive in silico and experimental analysis have led to the belief that FAM26F is actively involved in Ca2+ homeostasis, by allowing the accumulation of ions in the cell from the Golgi apparatus, ER, and outside the cell, acting as a part of innate immunity (Malik et al., 2017). It leads to an oxidative burst where the threat is cleared by the production of ROS. Hence, FAM26F helps in creating an oxidative environment for cells.

The expression of FAM26F has been found to have a link with Calcium signaling, and ROS production leading to oxidative bursts by the immune system (Malik et al., 2020). Furthermore, this ROS and Ca2+ interplay is closely regulated by the cell, and even more cleverly utilized by cancer cells to aid their proliferation and survival. This leads to the need of identifying the exact kind of role FAM26F may have in cancer, particularly in brain cancer which is yet to be studied. Preliminary studies on cancer cell lines have revealed a differential expression of FAM26F in each type of cancer; a breast cancer cell line (MCF-7), a brain tumor cell line (U87), and a non-cancerous cell line (not published). The current study aims to explore this finding in brain cancer biopsy samples by employing the technique of immunohistochemistry to observe FAM26F expression by its antibody. The data gathered from different stages of brain cancer, and also in those undergoing treatment, will enable the elucidation of FAM26F expression. Moreover, an in-depth character evolution analysis to trace the domain gain/loss/duplication events in FAM26F protein across various species will be carried out to identify the pattern of its evolution using

various bioinformatics tools. The ultimate goal is to gain a deep insight into the expression, evolution as well as the interaction of the immune modulator protein FAM26F.

1.1 1.1 Objectives:

The objectives of this research include:

- Analyzing the expression of this protein in various cancer biopsies via IHC (Immunohistochemistry).
- Evolutionary study of FAM26F over the range of species for analysis of its' domain.

Literature Review:

2.1 Calcium Homeostasis:

Calcium is an important messenger in the cellular process which is involved in the regulation of several cellular processes including metabolism, gene expression, cell death, and cell survival (Berridge, 2012).

The ions enter through calcium channels, transmembrane proteins, in the presence of any extracellular stress (Görlach et al., 2015). Different types of movements of calcium through transmembrane proteins occur depending on the requirement. The calcium that enters is through channels either receptor-operated or voltage-dependent devoid of energy requirement. Calcium pumps, on the other hand, require ATP to transport calcium. Inside the cell calcium uses Sarco/endoplasmic reticulum calcium pump (SERCA) to move to the endoplasmic reticulum (ER), while it is released through two kinds of receptors: ryanodine receptors (RyR) and inositol 1,4,5-triphosphate receptors (IP3R). The calcium uptake by the mitochondria is electrogenic in nature where voltage developed in the respiratory chain aids the movement (Berridge, 2012; Görlach et al., 2015).

Calcium homeostasis is vital in conducting various processes in the body such as the contraction and relaxation by smooth, cardiac, and skeletal cells, programmed cell death (apoptosis), exocytosis, nerve transmission by nerve cells, and in the immune system among others (Feher, 2017). In the immune system calcium, ion levels can regulate various responses including antibody secretion by B-cells, pathogen clearance by neutrophils, and apoptosis by ROS production (Feher, 2017; Grinstein & Klip, 1989; Puzianowska-Kuznicka & Kuznicki, 2009). This means that even the slightest abnormality in the Ca2+

levels in the cells can have drastic effects, hence various key players play their part in carrying a strict regulation of Ca2+ levels. These involve calcium channels, pumps, binding proteins, exchangers, and buffers which maintain a suitable Ca2+ level by its movement between plasma membrane, intracellular calcium stores e.g mitochondria and endoplasmic reticulum (ER), and cytoplasm.

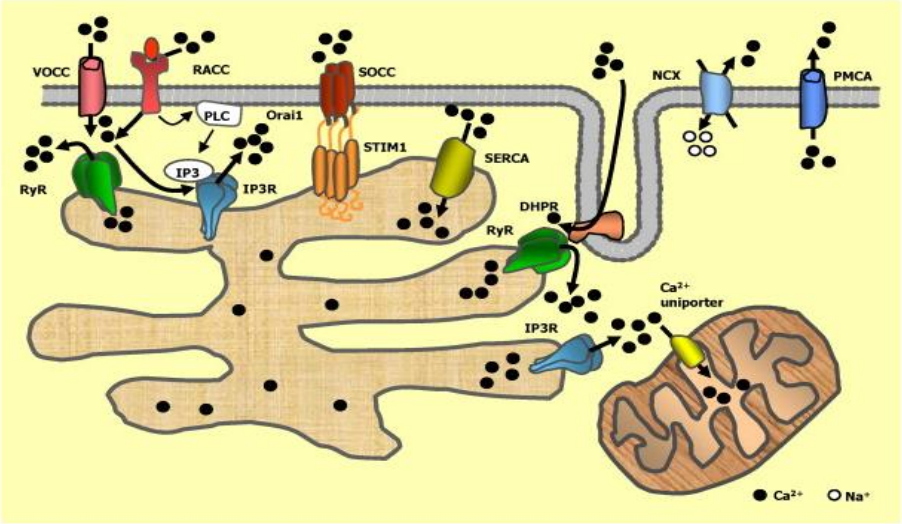


Figure 2.1 shows an overview of the endoplasmic reticulum (ER) and its proteins in regulating Ca2+ ion movement and signaling (Puzianowska-Kuznicka & Kuznicki, 2009).

Sarco-endoplasmic reticulum Ca2+ ATPases (SERCA) are examples of calcium pumps, while calcium-binding proteins include calmodulin etc. There are two main types of Ca2+ channels: receptor-activated calcium channels (RACCs) and voltage-operated calcium channels (VOCCs) (Puzianowska-Kuznicka & Kuznicki, 2009). Different types of RACCs such as inositol-1,4,5-triphosphate (IP3) receptors (IP3Rs) and ryanodine receptors (RyRs) which release calcium ions, while VOCCs have further ten members, all serving a specific function (Feher, 2017; Grinstein & Klip, 1989; Puzianowska-Kuznicka & Kuznicki, 2009).

Since all these proteins are working together, they have structural similarities, and tracing these similarities through evolutionary studies can give valuable insight into their

evolutionary past (Bordin et al., 2021). Evolutionary analysis carried out by Case et al. discussed in detail the possibility of Ca2+ ion channels being the very first types of ion channels to be developed by prokaryotes to filter ions as they tried to survive at the start of life (Case et al., 2007). This argument is strengthened by the finding that calcium ion channels are the oldest phylogenetically and are present across many prokaryotes compared to other channels (Case et al., 2007). Its evolution in eukaryotic organisms is suspected to be through the endosymbiotic relationship developed between Ca2+ ion channel-carrying protobacterium, rickettsia, and early eukaryotic cells (Andersson et al., 1998; Case et al., 2007). These evolved into calcium ion-carrying organelle, mitochondria (Andersson et al., 1998). Gradually as time progressed, these calcium channels developed according to the requirement of the environment, and more complex processes needing calcium homeostasis such as apoptosis and exocytosis also developed (Case et al., 2007).

2.2 ROS Production in The Immune Response:

The human immune system consists of a wide range of cellular compounds, cells, chemicals, and complex pathways that work in synergy to mediate immunity and provide protection against any foreign antigen that may pose a threat to the body such as viruses, bacteria, parasites, fungi, and cancer. There are two main categories of the immune system: innate immune system and adaptive immune system. The innate immune system is the first line of defense for the human body where dendritic cells and macrophages recognize the particular molecular patterns of the pathogen, unique pathogen-associated molecular patterns (PAMPs), through specific pathogen recognition receptors (PRRs) (Taganov et al., 2006). The binding of receptors with their respective antigen leads to the activation of a broad spectrum of signaling cascade which ultimately augments the inflammatory and

acquired immune system (Li et al., 2011; Taganov et al., 2006). The requirement of an adaptive immune system is a specific type of immunity that develops a targeted response to foreign antigens and is regulated by lymphocytes mainly T-cells and B-cells (Howell & Shepherd, 2021).

Immune pathways are activated in a dire situation leading to phagocytosis and clearance of pathogen, one of which is the mechanism of oxidative burst which involves the production of certain agents called “reactive oxygen species” (ROS) (Kohchi et al., 2009; Matsuzawa et al., 2005). ROS production is a potent antimicrobial defensive mechanism and a prime component of innate immunity (Nguyen et al., 2017) . Oxidative burst is the conversion of oxygen (O2) to superoxide ($O2^{\cdot -}$) and its dismutation to form ROS such as hydroxyl radicle (HO·), hypochlorous acid (HOCl), and hydrogen peroxide (H2O2) through non-enzymatic and enzymatic reactions and is thoroughly regulated in the body by antioxidative enzymes which inactivate the ROS (Babior et al., 1976; Dupré-Crochet et al., 2013). Superoxide (O2• -) is formed during respiration from Oxygen due to electron leakage, primarily through the involvement of Complex I and Complex III. From there it is likely to be converted to H2O2 which is more stable, less reactive, and diffuses more readily. This occurs either by superoxide dismutase 1 (SOD1) in the intermembrane space, spontaneously, or by matrix manganese SOD2, and the resultant H2O2 acts as a redox second messenger. These all are reactive oxygen species (ROS) that react with proteins, lipids, and nucleic acid to stimulate numerous processes which may lead to genetic mutations and the onset of cancer. If excessive ROS is produced then this can lead to damage to essential cellular processes like cell signaling and metabolism along with genomic processes. The alteration in metabolism is usually lethal to a normal cell however

neoplastic cell uses it to advantage and promote immune system suppression and ensure their own survival (Sowers et al., 2014)

Different levels of ROS in cells may induce different responses. ROS help in sustaining cellular proliferation, and stress response survival at low to moderate levels, and may also work as signal transduction molecules involved in starting pro-inflammatory response by inducing cytokines and nuclear factor-κB (NF-κB) pathway (Gorrini et al., 2013). Hence the production and regulation of reactive oxygen species is a highly regulated process and essential to maintaining the normal functions of a cell (Kohchi et al., 2009).

2.3 ROS and Calcium Signaling: An Interplay:

In ROS production, calcium uptake by the mitochondria is a vital step (Tan et al., 1998). During an immune response, the immune redox status of the cell shifts towards a more oxidized state, aided by the production of ROS. It involves a rapid movement of Calcium ions inside the cell from extracellular space, from the endoplasmic reticulum and sarcoplasmic reticulum through the respective membranes. This increased cytoplasmic concentration of Ca2+ causes nuclear and mitochondrial uptake of the ions, and subsequent pathway activation in mitochondria which leads to the production of ROS and clearance of stress (Ermak & Davies, 2002). The endothelial cells' permeability to Calcium ions may also be increased by NADPH oxidase 5 (NOX5), an important enzyme in the oxidative burst, which further indicates the synergistic relation between Calcium influx and ROS production. Calcium homeostasis and ROS production is a bidirectional interaction in which Calcium is needed for ROS production and ROS are involved in the regulation of calcium signaling (Di et al., 2016; Gordeeva et al., 2003).

2.4 Calcium Signaling and ROS In Cancer:

The association of reactive oxygen species production in cancer has long been an understudy. Increased levels of ROS in cancer cells compared to normal cells have been reported which aid cancer cells in their proliferation, survival, and growth (Szatrowski & Nathan, 1991). Despite the genomic instability and damage to DNA, lipids, and proteins brought about by high levels of ROS, they play a role as signaling molecules in cancer cells (Ames et al., 1993). Cancer cells acquire specific oncogenic mutations, increase metabolism, and get rid of a tumor suppressor to escalate the rate of ROS production to activate the protumorigenic pathways. However, this process is highly regulated as extremely elevated levels of ROS in the cell can lead to increased oxidative stress and ultimately cell death (Sabharwal & Schumacker, 2014). To balance the two processes, cancer cells increase their capacity of antioxidants to maintain the ROS levels at a scale that allows for protumorigenic processes without killing the cell (Reczek & Chandel, 2017, 2018). The interplay between Calcium and ROS has been described in various disease models. In the cardiovascular system, the dynamic between Ros and Ca2+ leads to cell death during cardiac ischemia (Webster, 2012). Furthermore, apart from apoptosis, this Ca2+ - Ros interaction plays a role in the regulation of many different cellular events, especially more so since the location of calcium storage inside the cell and the sites where ROS is produced i.e the plasma membrane and ER-mitochondrial interface, are linked closely (Giorgi et al., 2009).

Strict regulation of calcium homeostasis is done by the cell through ion pumps, ion channels, and ion exchangers. Mitochondria are actively involved in the mitochondrial matrix Ca2+ homeostasis and studies suggest that ROS production by mitochondria also

aids pro-tumorigenic redox signaling and autophagy. At the regions where mitochondria and ER are close to one another, there are domains present called mitochondria-associated membranes (MAMs). Ca2+ influx occurs in the mitochondria through these sites while ER is intrinsically source of Ca2+ storage and cellular ROS. The protein folding process which occurs in ER and causes the more oxidative environment of ER compared to cytoplasm also results in H2O2 production (Cao & Kaufman, 2014). The enzymes Protein disulfide isomerases (PDIs) and ER oxidoreductases (Ero1) exchange their disulfide bonds. Electrons are reverse shuttled to Ero1 from PDI. Molecular oxygen is reduced to make H2O2. This is one of the pathways. Another pathway that is independent of Ero1 involves Peroxiredoxin 4 (Prx4) in the endoplasmic reticulum lumen. Apart from these, one pathway involves p66shc which is a ROS-producing protein and redox sensor, and is involved in cancer progression. Proceeding a pro-apoptotic signal like apoptosis signal-regulating kinase 1 (ASK1) activating, p66shc is moved to MAMs where it interacts with cytochrome c and as a result of this ROS is produced (Giorgio et al., 2005). P66shc is actively involved in the development of cancer, with its overexpression studies, which results in increased ROS levels and proliferation of the cell. There are many drivers of ROS production by mitochondria during normal and pathophysiological conditions such as hypoxia, hyperoxia, and signaling of oncogenes and cytokines including those in the mTOR pathway and G (Bhat et al., 2015; Hempel & Trebak, 2017). Calcium dysregulation is one of main drivers of ROS production and cancer survival.

2.5 Cancer:

Cancer refers to a group of diseases characterized by the uncontrolled growth of abnormal cells in the body and is one of the major causes of mortality and morbidity around the globe.

Bladder cancer is the type of cancer which originates from the cells of the bladder and is common in females while in men it is fourth most common type of cancer (Lenis et al., 2020). It may be unaggressive and non-invasive type of cancer that can transform into invasive and aggressive one cancer (Lenis et al., 2020). Deaths due to bladder cancer ranks Pakistan at #82 across globe while death rate is 2.19 per 100,000 population (Youn et al., 2020). The bladder system, like all other systems of body, require well-regulated calcium homeostasis for proper conducting of cell proliferation etc. While a higher than normal amount of calcium ions acts as a trigger for apoptosis, disturbance in calcium regulation is recognized as a cancer feature (Zheng et al., 2022). Cancer cells can undergo apoptosis due to starvation, and calcium channels are reported to protect them from starvation by altering Ca2+ from ER (Zheng et al., 2022). It is also been reported to be involved in bladder malignancy, a cancer of the urinary tract, where calcium channels play a significant part (Ceylan et al., 2016) .

Gastric cancer is the cancer of stomach which is the 5th most common kind of tumor worldwide while 3rd in rank for cause of death (Smythe et al., 2020). While in Pakistan it comprises 0.28% deaths and 2.97 death rate per 100,000 of population (Smythe et al., 2020). In both genders it was found to be most common cancer (Ahmad et al., 2016). The abnormal increase in Ca2+ movement inside cancer cell has been observed in prostate,

breast and gastric cancer, due to aberrant expression of proteins of calcium homeostasis such as IP3R and SERCA (Gélébart et al., 2002; Marchi & Pinton, 2016).

Central nervous system (CNS) cancer is the heterogenous growth around CNS and brain and remains the 10th leading cause of death around the world with 3.4% mortality in Pakistan in 2020, of which brain tumors account for 85% to 90% (Quader et al., 2022). Brain cancers consist of primary tumors and secondary brain tumors. Primary tumors of the brain may be rare but they are lethal with high morbidity and mortality (Darlix et al., 2017). These may start from the neuroepithelial tissues, meninges, pituitary structure, neuroepithelial tissues, and cranial nerves among other sources. Although malignant brain tumors account for 1-2% of all cancers, they are the deadliest form of cancer and the most difficult to treat (Quader et al., 2022) .

Breast cancer continues to be global concern with highest incidence among females cancers in Pakistan. Breast cancer also has reports of calcium homeostasis being a key player in cancer proliferation. After some studies conducted which showed association between hypercalecimia and renal cancer, comparatively recent studies have reported a role of calcium homeostasis alteration in contributing to renal cancer (Carmeliet et al., 2003; Luo et al., 2020).

2.6 The Inflammatory Microenvironment of Cancer:

Tumor cells, normal cells, and cells of the immune system engage in a cross-talk by utilizing a complex cytokine network in the tumor microenvironment which aids cancer proliferation, making it immunosuppressive. Cancer was linked with calcium first in the

nineteenth century, makes other cells more liable to cancer, and it is now considered as a feature essential to cancer progression.

The cells of the immune system and its mediators act as a crucial part of the TME such as helping cancer cells evading immune system. (Guo et al., 2016). In the case of secondary cancer development, the tumor formed in another part of the body translocates from its primary tumor site to the bloodstream i.e carrying out intravasation. Here the cells of the immune system, tumor-associated macrophages (TAMs), chemokines, and cytokines, play a significant role in recruiting leukocytes to tumor site and help cancer and neovascularization (Guo et al., 2016; Keibel et al., n.d.). Cytokines are small multifunctional proteins that play a significant role in the TME by controlling proliferation, neo-angiogenesis, and immune cell penetration in autocrine and paracrine manners which cause anomalies (Zhu et al., 2012). Especially in case of brain cancer, those cancer cells enter the brain vasculature, cytokines were found to aid their site-specific behavior (Berghoff & Preusser, 2015). Cytokines stimulated macrophages to carry out oxidative burst phenomenon. ROS levels are carefully maintained in the cell, especially at the tumor site, so high amounts of ROS end up protecting the cancer cells from being destroyed by apoptosis.

The oxidative environment in cell causes Phosphate and Tensin homolog (PTEN) gene to become mutated and often loose its function which contributes in ROS levels elevation and this was reported in many cancers including brain and prostate cancers (Jamaspishvili et al., 2018; Jaros et al., 1992).). The oxidization of PTEN by ROS causes loss of PTEN tumor suppressor activity (Lee et al., 2002). High levels of epidermal growth factor receptor (EGFR) were also reported in bladder, breast, and brain cancer which aided their

progression (Ali & Wendt, 2017; Wang et al., 2020). High levels of EGFR increase ROS formed inside the cancer cell contributing to DNA damage. DNA damage by ROS includes the formation of extensively studied product, 8-oxo-2'-deoxyguanosine (8-oxodG), created due to damage inflicted on DNA by ROS and found to be twice the amount in brain tumor cells compared to normal brain tissues (Butkowski, 2020). Elevated 8-oxodG is associated with H2AX (phosphorylated histones) and induces DNA damage response that gives a signal to tumor protein (p53). A mutated version of EGFR, EGFRvIII has also been found to have elevate 8-oxodG (Sowers et al., 2014).

2.7 FAM26F: an Immune Modulator Involved In Cancer:

The immune system has a complex network operated by cells, molecules, and chemical pathways that govern the defense mechanism of the body in case of any threat. Proteins are a key player in this system through regulation of the network through a plethora of actions including their behavior as transcriptional regulators, receptors, and importantly, as signaling molecules to generate a diverse and effective immune response. One such protein is the relatively recently discovered protein named FAM26F.

Originally called IRF-3–dependent NK-activating molecule (INAM), FAM26F is from the FAM26 gene family, also known as the calcium homeostasis modulator family (Ebihara et al., 2010). The family constitutes of total six genes with 20-50% sequence similarity among themselves, and are located as two clusters on different chromosomes: chromosome 10 and chromosome 6 (Foskett, 2020; Ma et al., 2016). Found on chromosome 6, FAM26F or CALHM6 has gained much identification with having a critical immune relevance when it was studied in various infections, cancer, immune pathogenesis, and simulation studies (Malik & Javed, 2016). Functional and whole transcriptome analyses have discovered

FAM26F to be differentially expressed in various scenarios, indicating its involvement in an immune response. Zhang et al. studied the activation of macrophages by cytokines in 2010 and reported that FAM26F is induced by interferon-gamma (IFN-γ), interferon beta (IFN-B), and IL-10. In the same year, he and co-workers showed FAM26F to be up-regulated by IFN in human hepatocytes. This led to the potential of it's role in more conditions.

2.8 FAM26F Characterization:

Malik et al. carried out an elaborate study to characterize FAM26F structure as well as involvement in the body using various in-silico tools. They carried out its localization, 3D structures, phylogenetic analysis across various species and its domain and motif. FAM26F was reported to be a single domain protein, with Ca_hom_mod domain, suggested to be involved in calcium homeostasis among other biological pathways. Evolutionary analysis concluded FAM26F to be considerably conserved and closely related to that of primates (Malik et al., 2017). The group reported it to be a pore forming unit of voltage gated ion channel which was involved in bringing about oxidative stress in the cell through allowing movement of calcium ions through it and stimulating ROS production. Through co-localization studies, Malik and group shortlisted these proteins to be present with FAM26F: Thioredoxin, Calmodulin-like protein5, Vinculin, Calpain, and Protein S100-A7 (Malik et al., 2020).

2.9 Calcium Signaling and FAM26F:

FAM26F is thought to be a part of a voltage-operated ion channel that forms pores and has a calcium homeostasis modulator domain. The presence of this domain leads to FAM26F being actively involved in the essential process of oxidative burst.

As discussed earlier, Calcium homeostasis is linked to ROS production Görlach et al 2015, which in turn has an extensive role in cell survival, apoptosis, as well as tumor progression (Görlach et al., 2015). Another player of this system is thioredoxin (Trx) which is associated with decreasing the ROS levels in the cell and establishing a reduced environment in the case of a respiratory burst occurring in a normal cell (Holmgren & Lu, 2010; Jones et al., 2006; Lillig & Holmgren, 2007; Saraiva et al., 2002). An increase in Calcium and ROS levels during extracellular threat causes stress to ER, which stimulates a transfer of FAM26F from Golgi apparatus, where it is believed to be usually present, into ER as part of the innate immunity.

2.10 FAM26F and anti-Tumor Pathways:

FAM26F has been found to be involved in cancer and anti-tumor processes. Joosse et al. reported in 2009 that the regions of the genome particularly 6q22.1 which was carrying FAM26F were deleted in BRCA2-associated breast cancers (Joosse et al., 2009). FAM26F was found to have differential expression in various cancers such as breast, cervix, mammary gland, and uterus, which were found using micro-array studies and added in Atlas Data (Mosca et al., 2010). The protein was also speculated to be potentially linked to having a clinical advantage in the treatment of metastatic melanoma through immune-based MAGE-A3 therapy. FAM26F was concluded to be simulated by IFN-gamma through the STAT-1 pathway (Ulloa-Montoya et al., 2013). Subsequently, Takashi and his co-workers reported FAM26F to be a molecule derived from a toll-like receptor (TLR) which can potentially mediate the Natural Killer cells and myeloid dendritic cell interaction . This led to the theory that FAM26F expression may have a key significance against NK-

sensitive and IFN-gamma suppressible tumors. The activation and downstream anti-tumor properties of FAM26F are documented in the table.

Table 2.1 shows the expression analysis of FAM26F in various cancers.

Study Type	**Mode of study**	**Expression**	**Sample**	**Reference**
Breast		Deleted in BRCA2 associated breast tumors	Breast tumors	(Stefansson et al., 2009)
Breast, mammary gland, cervix, and uterus	*in vitro*	Differential expression	Atlas Data	(Mosca et al., 2010)
Metastatic melanoma	*in vitro*	Increased	Melanoma biopsies from 75 patients with non-resectable MAGE-A3-positive stage III or IV M1a metastatic melanoma	(Ulloa-Montoya et al., 2013)
B16D8 tumor	*in vivo*	Increased	B16D8 tumor bearing mice	(Ebihara et al., 2010)

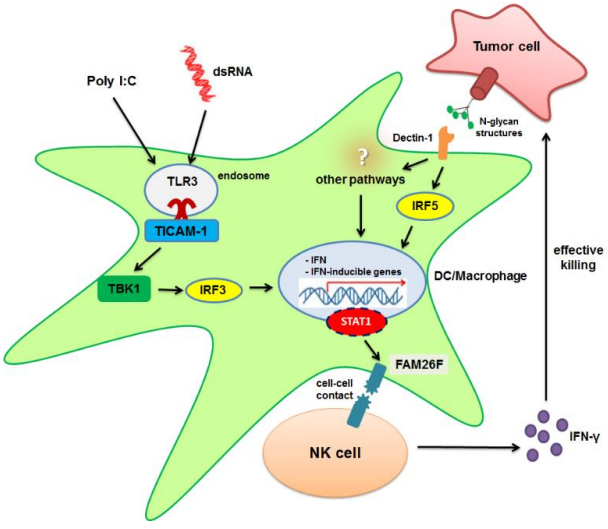
For gaining a deeper insight into the anti-tumor pathways of FAM26F, numerous mice model studies were conducted. FAM26F stimulation depends on TICAM-1 and IRF-3 as evidenced by the absence of a proper NK cytotoxicity in TICAM-1-/-or IRF3-/- knockout mDC. FAM26F was shown to be assisting the mDC-NK activation, crucial for NK cell activation, hence strengthening the argument that its expression and ability to stimulate NK cells could be vital in targeting the neoplastic cells that are NK sensitive. Moreover,

Figure 2.10 shows an overview of the predicted pathway through which FAM26F targets tumor cell through IFN gamma production (Malik et al.,2017)

Figure 3.10 shows an overview of the predicted pathway through which FAM26F targets tumor cell through IFN gamma production (Malik et al.,2017)

activated FAM26F triggers them to release IFN-γ, which could potentially be used in targeting tumors suppressible by IFN-γ (Chiba et al., 2014).

Immunohistochemistry or IHC on cancer is commonly applied method to detect specific proteins and antigens in a biopsy tissue through polyclonal or monoclonal antibodies. It yields accurate results in detecting a certain type of protein with least amount of damage to original tissue. The detection is based on interaction between antigen and antibody which is traced by fluorescent dyes, radioactive substances or enzyme substrate color changes (Duraiyan et al., 2012). It can have samples in various forms including formalin-fixed paraffin embedded biopsy tissues, and fresh tissues. For cancer detection, IHC uses antibodies against protein markers specific to certain types of cancers however this study is focused on detecting the expression of FAM26F in brain cancer samples so anti-FAM26F antibody is used. With time this technique has established itself to be essential in histopathological studies and an important tool in diagnostic and research studies.



Methodology:

3.1 Immunohistochemical (IHC) Staining on Brain Samples:

IHC is a commonly applied technique that makes use of enzymes conjugated to Antibodies to catalyze reactions and accurately locate and visualize antigens in the specific tissue samples.

3.1.1 Sample and data collection:

Tissues/biopsies of patients suffering from brain, breast, bladder, gastric, prostate and renal cancers were resected surgically and were selected to be included as part of the study. Total of 61 blocks of Formalin fixed paraffin embedded (FFPE), which included 20 blocks of brain cancer, 9 of breast cancer, 20 of bladder, 3 of gastric carcinoma, 5 of prostate and 4 of renal cancers, were provided by Northwest School of Medicine. The

MATERIALS	REAGENTS
○ Fixative coated slides	○ Ethanol concentrations (100%, 90%, 70%, 50% and 30%)
○ Microtome	○ Xylene
○ Water bath	○ Tween20
○ Slide holder	○ Distilled water
○ Microwave	○ BSA
○ 7 boxes	○ Sodium citrate buffer
○ Tweezer	○ H2O2
○ Microwavable container	○ 100% methanol
○ Cover slips	○ Primary Antibody
○ Tissue paper	○ Secondary Antibody
○ Covered tray	○ DAB substrate
	○ Organic mounting media

tissues were sectioned to thickness of 3-5um and blocks were labelled properly with their respective ID's.

Table 2 General biopsies samples of cancers

Biopsies Type	Sample number
Brain cancer	20
Bladder cancer	20
Breast cancer	9
Renal cancer	4
Prostate Cancer	5
Gastric Cancer	3

3.1.2 Study design:

The cancer samples and control tissues were obtained from Northwest School of Medicine, Peshawar and were grouped according to the organ they were from. Immunohistochemistry was performed on all the formalin fixed paraffin embedded tissues by sectioning them with a microtome, subjecting them to heat induced antigen retrieval to expose the proteins and incubating them with anti-human anti-FAM26F antibody following with an appropriate detection system. The results were observed under a microscope and were analyzed through statistics to infer the relation between FAM26F expression and cancer.

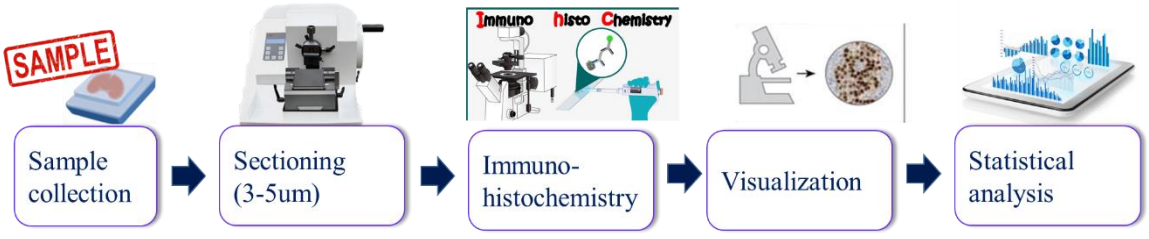


Figure 4.1.2 shows diagrammatic overview of methodology

3.1.3 Immunohistochemistry

3.1.3.1 IHC pre requisites

The reagents and materials needed for IHC are mentioned in the table below:

3.1.3.2 Microtome sectioning and fixation:

The process starts with careful sectioning of tissue about 3-5um thick using a rotatory microtome. The thin section is floated on tap water prior to floating it on 42 C water bath and mounted on glass slides. Slides were coated with albumin-glycerol fixative before mounting to adhere tissue sections properly on the slide and prevent it from detaching during the IHC procedure. The tissue is fixed onto the slide by incubating it on a hotplate at 65 degrees for 20 minutes. This makes sure tissue maintains its structure and antigenicity is retained.

3.1.3.3 Deparaffinization and Rehydration

The tissue fixated on slides was deparaffinized by placing it in 2 changes of xylene solution, 10 minutes each. It is a common deparaffinized agent used in IHC as it completely removes paraffin wax that has penetrated deep into the tissues. The xylene is further removed by incubating the slides in a decreasing concentration of alcohol as follows:

Percentage of ethanol (%)	**Time (minutes)**
100% ethanol	5 minutes
90% ethanol	2 minutes
70% ethanol	1 minute
50% ethanol	1 minute

30% ethanol	1 minute

The slides were then washed by placing the container with a slide holder containing slides under running tap water, in a way that water doesn't directly hit the tissue, for 1-2 minutes. The tissue will seem like white discs on the slide. Now the tissue is ready for antigen retrieval. From this point onwards the tissue should not be allowed to dry out as it will cause increased non-specific binding of the antibody.

3.1.3.4 Antigen retrieval and washing:

Antigen or epitope retrieval is one of the most critical steps in ensuring proper IHC staining. One factor that can lead to the loss of immunoreactivity of antigens is the fixation process. Crosslinking of proteins and masking of epitope sites can cause epitopes to become unreactive and hence no antibody-specific staining. This necessitates the epitope retrieval process to reverse the process of masking.

The process of heat-induced epitope retrieval (HIER) was carried out. The antigen retrieval buffer prepared was sodium citrate buffer with pH 6.0. A microwavable container was taken and half-filled with buffer in a way that slides were completely covered and loosely caped before microwaving it for 8-10 minutes and letting it rest for 30-35 minutes at room temperature. It was washed with distilled water thoroughly.

3.1.3.5 Blocking and washing:

The slides are marked by a hydrophobic marker around the edges to make sure the liquid covers the tissue. The slides proceeded to the Blocking step to remove any chances of false positive staining. The blocking solution (2% H2O2, 20% Methanol) is prepared with 6ul of 35% H2O2, 80ul H2O, and 20ul Methanol to make 100ul solution per slide. The slides are placed in a dark humidity chamber (created with water at the bottom of a tray and

placing slides above it to minimize evaporation of buffer) for 3-5 minutes. After washing slides, BSA Blocking for 10 minutes was done and washed off.

3.1.3.6 Primary antibody incubation and washing:

The slides are now ready for primary antibody binding. Anti-FAM26F anti-rabbit polyclonal antibody was used in a 1:100 ratio of dilution. Diluent was prepared with BSA and PBS. 100ul of dilution was applied and incubated for 1 hour at room temperature in a humidity chamber. The slides were washed afterward.

3.1.3.7 Secondary antibody incubation and washing:

After the primary antibody, horseradish peroxidase HRP labeled secondary antibody dilution was applied to each section. It was incubated for 40 minutes in a dark humidity chamber at room temperature. The same diluent was used in the mentioned ratio. It was washed afterwards thoroughly.

3.1.3.8 Detection:

Visualization of the antigen expression in the microscope requires proper staining which is achieved by Diaminobenzidine (DAB) substrate from a DAB staining kit. Fresh dilution of DAB was prepared every time with no more than 10 minutes before staining (50ul chromogen in 1000ul substrate buffer). It was incubated for 5-10 minutes at room temperature in a humidity chamber and washed afterward.

3.1.3.9 Counterstain and dehydration

The counterstain with hematoxylin to stain the nucleus for clear visualization of cells was carried out with subsequent dehydration with alcohol in the manner mentioned below. The

slide was completely dried after ethanol dips and then dipped in xylene before mounting the coverslip with help of mounting media.

Reagent	Time (minutes)
Hematoxylin	1-2 minutes
Tap water	Till clear
1% acid-alcohol	1 dip only
Tap water	Till clear
50% ethanol	1-2 minutes
90% ethanol	1-2 minutes
100% ethanol	1-2 minutes
Xylene	1-5 minutes

3.1.3.10 Visualization:

The slides were visualized by using a Labomed TCM400 inverted microscope (Optika Microbiology Italy) and the pictures of stained sections were captured using Optika version light 2.1. Lastly, the stained sections were scored high, moderate, or low protein expression based on the intensity of the stain.

3.1.4 Scoring scheme

Based on the level of expression of FAM26F in cancer samples, following scoring scheme mentioned in table was applied (Fedchenko et al., 2014).

Table 3 Immunohistochemical scoring is adopted from Allred Score

Proportion score A	Positive cells, %	Intensity	Intensity score B
0	0	None	0
1	<1	Weak	1
2	1 to 10	Intermediate	2
3	11 to 33	Strong	3
4	34 to 66	Final score range (A + B): 0-8	
5	≥67		

3.2 3.2 In-Silico Phylogenetic Analysis of FAM26F

The first objective of this study focused on tracing the evolution of Ca_hom_mod domain of FAM26F through a in-silico approach which integrated a python pipeline and bio-informatics tool to generate a phylogenetic tree which displayed the evolutionary events and reconstruct its ancestral states. The evolutionary events in this case are the gain, loss, and duplication occurring in protein domains over time which impact its structure and complexity.All information regarding protein domains have been placed in various databases, of which pfam will be focused on in this study (Fong et al., 2007; Zmasek & Godzik, 2011).

To identify FAM26F across various organisms, FAM26F gene id's and sequences were retrieved from NCBI gene databank for 20 organisms. A python pipeline was used for domain matrix generation of FAM26F protein through an open-source repository on GitHub. This domain matric was proceeded to create phylogenetic tree (Brito & Pinney, 2020; OpenData/Brito_2019_HVsDomains/4_charEvol at Master · Andersonbrito/OpenData, n.d.).

3.2.1 Identifying protein domain through Python pipeline:

3.2.1.1 Retrieval of tORFs of organisms from NCBI Accession Ids':

The following organisms were selected based on their diversity to include a wide range for evolutionary analysis: *Austrofundulus limnaeus* (Killifish), *Bos mutus* (Wild yak), *Camelus dromedarius* (Camel), *Canis lupus familiaris (*Dog), *Columba livia* (Rock dove), *Danio rerio* (Zebrafish), *Exaiptasia diaphana* (Sea anemone), *Falco cherrug* (Saker Falon), *Felis catus* (Cat), *Ficedula albicollis* (collared flycatcher), *Gallus gallus* (Red jungle fowl), *Homo sapiens* (Human), *Lates calcarifer* (Barramundi), *Maylandia zebra* (Zebra Mbuna), *Microtus ochrogaster* (Prairie vole), *Pogona vitticeps* (Central Bearded Dragon), *Python bivittatus* (Burmese python), *Rattus norvegicus* (Brown rat), *Salmo salar* (Atlantic salmon), *and Stylophora pistilliata* (Hood coral). The python pipelines started by retrieving all of their Open Reading Framed (ORFs) once their accession id's from NCBI were given as input as a single text file.

3.2.1.2 Retrieval of Protein Domains by Scanning Pfam Database:

Hmmerrep2 was ran in the second step. It's a part of HMMER software which requires running hmmscan before (Eddy, 1992). The fasta files given as output by tORFs step was given as input to run hmmscan. It scanned for protein domain hits across HMM protein database, pfam-A in this case. (Finn et al., 2014; Mistry et al., 2021). To shortlist only the reliable hits and rule out any false positive and negative hits, an inclusion criterion for per-sequence e-value and per-domain conditional value was defined to be 0.001. It is a time-taking software to run and only accessible on LINUS or MAC system for which reason, it was accessed through supercomputer.

3.2.1.3 Retrieval of Domain Repertoires:

Domain repertoires were searched and retrieved by using the same above-mentioned thresholds. A domblout file generated by hmmscan was given as input to hmmerrep2.py and result was repertoires for each organism.

3.2.1.4 Generation of Domain Matrix:

The result of hmmerrep2 was given as input to run domMatrix.py and the domain repertoires was used to generate a domain matrix (Eddy, 1992). The protein domain matrix contained details of any presence and absence in/of domains in genome of each organism. This domain matrix was critical in conducting the phylogenetic analysis via bio-informatics tools further.

3.2.2 Phylogenetic analysis and protein substitution model prediction using ProtTest:

In the second part of evolutionary analysis, the amino acid sequence of FAM26F in each organism was taken from NCBI. In order to carry out the analysis, an amino-acid substitution model which was suitable for protein sequence being studies had to be selected. Alignment was carried through MAAFT (Katoh & Standley, 2013). ProtTest, a tool for describing amino-acid probability of change, was used to select best suited evolutionary model to reconstruct protein phylogeny (Abascal et al., 2005; Darriba et al., 2011).

3.2.3 Phylogenetic Analysis:

For phylogenetic analysis, Byesian evolutionary analysis was used. The divergence dating function in BEAST 2 software was used and Markov Chain Monte Carlo Bayesian approach was used to create a time-calibrated maximum clade credibility (MCC) tree (Jin & Brown, 2018). As a partition the multiple alignment file generated using MAAFT was imported with JTT amino acid substitution model as selected by ProtTest under Relaxed Clock Log Normal with Yule speciation process as tree prior. It was analyzed for 6 million generations for its running feasibility sampled every 10,000 generations. To annotate the

generated tree, Tree annotator v2.6.6 was used with burn in percentage set as 10% to generate a MCC tree (Jin & Brown, 2018).

3.2.3.1 Ancestral state reconstruction:

By conducting the ancestral state reconstruction, the result of python pipeline and BEAST will merge to give information about domain events in a visual manner. The time calibrated tree created in BEAST was opened in Mesquite as well as domain matrix generated through python was imported (Maddison, 2009). A linear parsimony reconstruction model was applied by Mesquite software to reconstruct the ancestral states. The domains were treated as meristic (additive) characters while the model used maximum parsimony principle. It reconstructed domains with minimum evolutionary events by using phylogenetic tree and domain distribution matrix provided as input.

3.2.3.2 Character evolution:

At the last step, python pipeline was used again. CharEvol.py was used to reconstruct the domain evolution by giving Mesquite output as input along a given phylogeny. The character states in this

case were protein domain counts per genome matrix. The result was visualized on an online tool, iTOL.

Figure 3.1 Figure depicting the pipeline followed for phylogenetic analysis

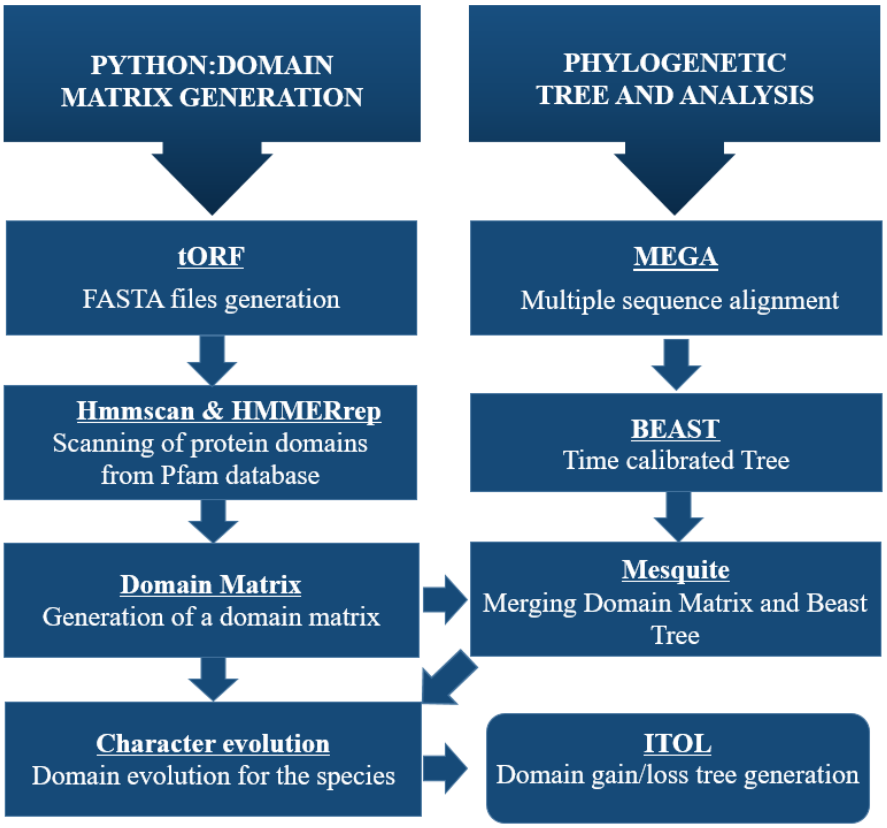


Figure 3.2.3 Depicting the pipeline followed for phylogenetic analysis

Results

4.1 FAM26F expression in various cancers:

The expression of FAM26F in various cancer biopsies were explored through immunohistochemistry to observe its involvement in the tumorigenic pathways. Total of 58 samples were obtained from Northwest school of Medicine. The collected samples included cancers of several regions such as brain, prostate, renal, bladder, breast, bladder, and gastric region. The samples were fixed and then sectioned by using a microtome on poly-L-lysine slides and further subjected to Immunohistochemical analysis. This technique is widely recognized as being a process that helps in detection of the presence of any desirable or protein of choice in the sample on the basis of antigen interaction with its respective antibody. It has been established as a key tool to analyze the protein expression in tissues of our choice.

4.1.1 Expression of FAM26F in Brain cancer Tissues:

At the start of brain cancer there is inflammation that tries to control cancer progression but this inflammation turns chronic which causes an oxidative environment in the astrocyte cells and microglia, the immune cells residing in the brain. Cytokines stimulated macrophages to carry out oxidative burst phenomenon. There is an influx of calcium ions which result in high amounts of ROS and they end up protecting the cancer cells from being destroyed by apoptosis (Jaros et al., 1992). Calcium ion channels are predicted to play a significant role in calcium influx and since FAM26F is a calcium ion channel, it can be important in cancer progression.

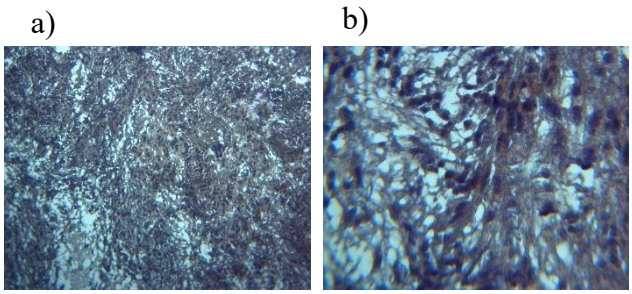


Figure 7 Brain cancer tissues. a) IHC of brain tumor at 10X. b) IHC of brain tumor at 40X.

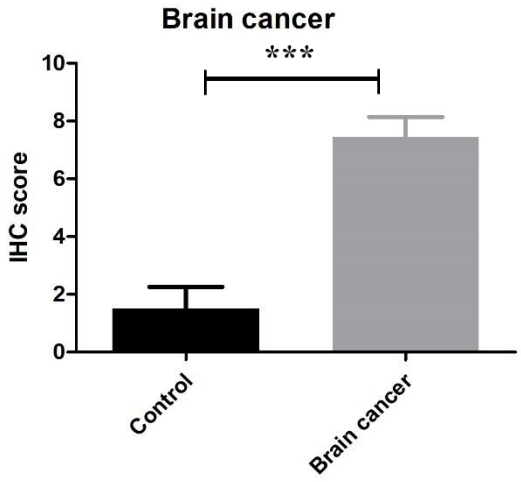


Figure 6 Graph shows FAM26F expression in brain cancer. It is higher in cancer positive samples compared to control tissues.

FAM26F expression was high in cancer positive samples showing FAM26F to be high in cancer.

4.1.2 Expression of FAM26F in Bladder carcinoma Tissues

Bladder cancer is the type of cancer which originates from the cells of the bladder. Like all body cells, cells of the urinary bladder also need a well-regulated calcium homeostasis system to conduct cell proliferation and other processes. A higher than normal amount of calcium ions acts as a trigger for apoptosis, however, this disturbance in calcium regulation is being recognized as a cancer feature (Zheng et al., 2022). The calcium channels have been found to protect these cancerous cells from starvation by altering Ca2+ from ER

(Zheng et al., 2022). It is also been reported to be involved in bladder malignancy, a cancer of the urinary tract, where calcium channels play a significant part (Ceylan et al., 2016) .

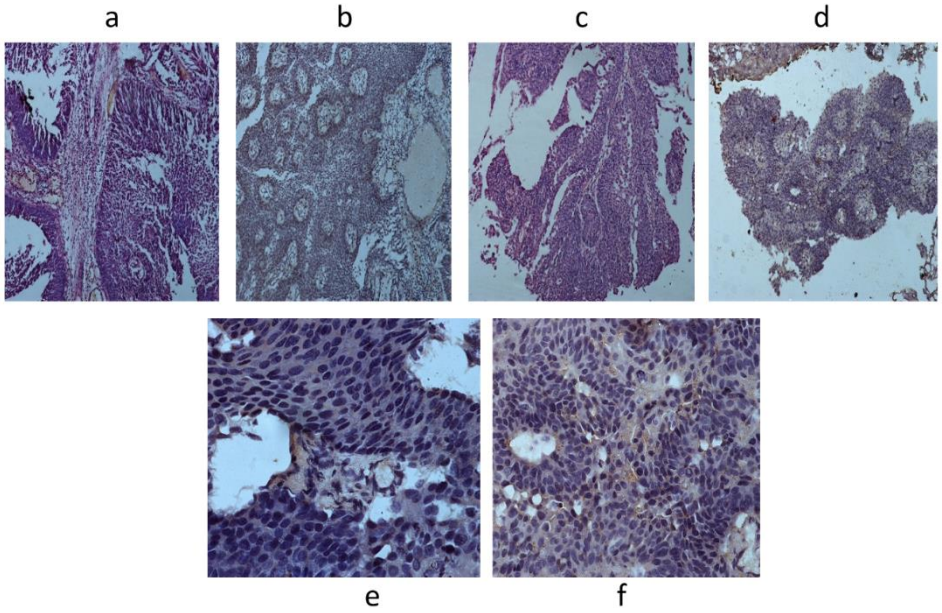


Figure 9 Bladder carcinoma tissues. HE stained slide of bladder tumour taken at 10X whereas (b) IHC stained slide of bladder tumour at 10x, (c) HE slide at 10 X (d) IHC slide at 40X , (e) IHC at 40X, (f) IHC at 40X.

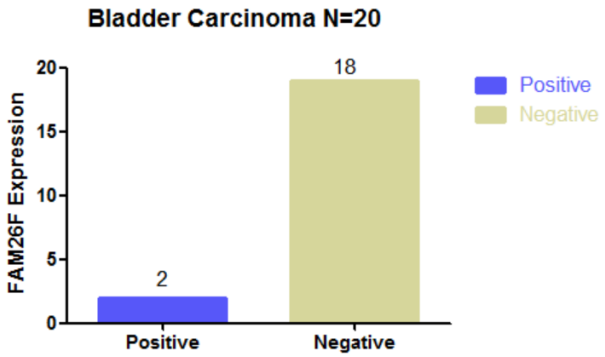


Figure 8 Graph shows expression of FAM26F in cancer positive samples. 2 samples had detectable expression while 18 were negative.

Since FAM26F is a calcium ion channel, its role in bladder cancer could contribute to cancer pathology.

No detectable expression of FAM26F was observed in bladder carcinoma as out 0f 20 only 2 samples were positive and 18 were negative. This explains that there's no FAM26F expression seen in bladder carcinoma.

Age and gender has shown no effect on the expression of FAM26F in bladder cancer while its association with necrotic factors was checked (Table 4). The expression of FAM26F in necrotic vs non necrotic tissue is compared. Out of 20 bladder cancer samples. Necrosis was present in 8 i.e. 40 % of the samples, 75% of the necrotic tissues show low expression of FAM26F. Whereas 100 % of the non-necrotic tissues show no expression of FAM26F. 93% of the low grade tumor show low or no expression, only 7% shows positive expression. Same is the case with high grade tumors, 80% of the high grade tumor shows no expression of FAM26F whereas 20% show low expression. This means tumor grade has no effect on FAM26F expression.

Table 4 Clinicopathological significance of FAM26F expression in bladder carcinoma

Clinicopathological features		FAM26F expression		
Bladder carcinoma	Null	Low	Moderate	High
Gender				
Male	13	1	0	0
Female	5	0	0	1
Age				
Below 50	3	0	0	0
Above 50	15	1	0	1
Malignant	6	1	0	1
Benign	12	0	0	0

Grade				
Low	14	0	0	1
High	4	1	0	0
Necrosis				
Present	6	1	0	1
Absent	12	0	0	0

4.1.3 Expression of FAM26F in GIT carcinoma Tissues

After Carruthers and Suntzeff, in 1944, described the link between cancer and Ca2+, various studies have dived to explore it (Marchi & Pinton, 2016) . After more than 70 years

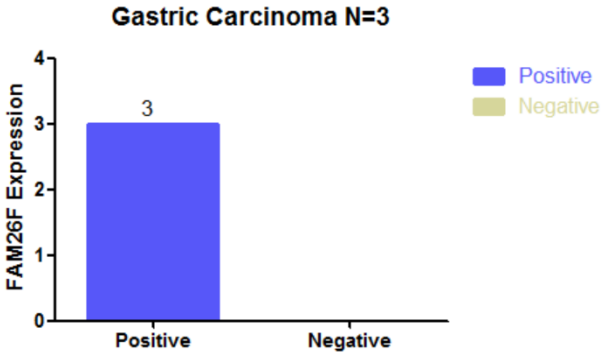


Figure 10 Graph shows expression of FAM26F in cancer positive samples. 3 samples had detectable expression while none were negative.

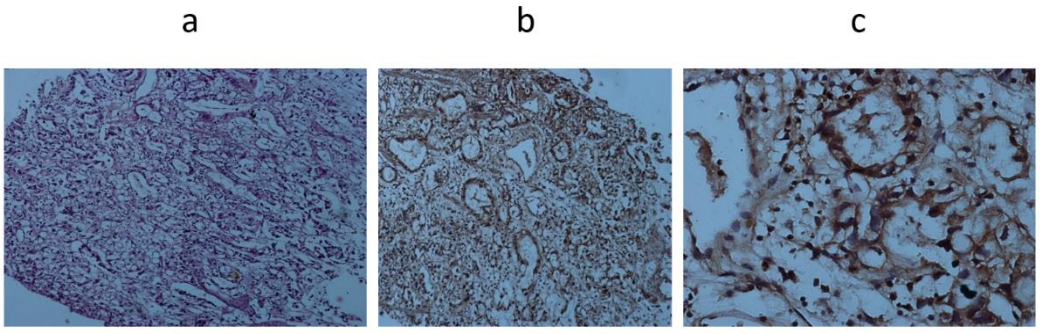


Figure 11 Gastric Carcinoma tissues (a) HNE at 10X, b) IHC at 10X, c) IHC at 40x of GIT Carcinomas. Darkly stained color shows the expression of FAM26F whereas blue stain is attained by nuclei. HE slides are used to visualize the structure of tissue

calcium homeostasis dysregulation has been established to be a vital feature of cancer. The abnormal increase in Ca2+ movement inside cancer cell has been observed in prostate, breast and gastric cancer, due to aberrant expression of proteins of calcium homeostasis such as IP3R and SERCA (Gélébart et al., 2002; Marchi & Pinton, 2016). The expression of FAM26F is high in necrotic and malignant tissues of gastric carcinoma, indicating that high grade tumors have more expression of FAM26F and is present in glandular tumors. Out of 3 tissues, all were positive for the expression of FAM26F, depicting that the expression of FAM26F is gastric tissues.

Table 5 Clinicopathological significance of gastric carcinomas

Clinicopathological features		**FAM26F expression**		
Gastric carcinoma N=3	Null	Low	Moderate	High
Gender				
Male	0	2	0	0
Female	0	0	0	1
Age				
Below 50	0	1	0	0
Above 50	0	1	0	1
Malignant	0	2	0	1
Benign	0	0	0	0
Grade				
Low	0	0	0	0
high	0	2	0	1
Necrosis				
Present	0	2	0	1
Absent	0	0	0	0

4.1.4 Expression of FAM26F in Breast cancer Tissues

Breast cancer also has reports of calcium homeostasis being a key player in cancer proliferation. In breast cancer we had a range of normal breast tissues, breast cancer tissues, post chemo breast cancer tissues from a variety of patients. The IHC and HE staining was

performed on all of them. Later the slides were observed under microscope at different magnifications..

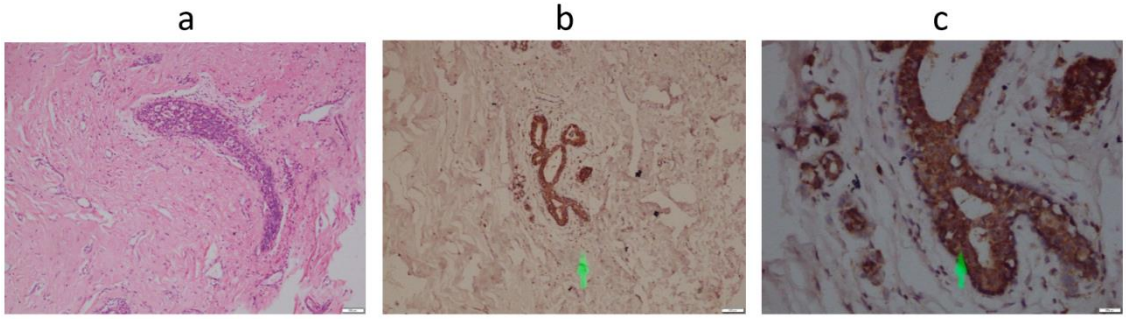


Figure 14 Normal Breast tissues (a) 10X HE slide of normal breast tissue. (b) 10X IHC of normal breast tissue, (c) 40X IHC slide of normal breast showing the expression of FAM26F. In normal breast tissues, expression of FAM26F is shown with dark brown stain

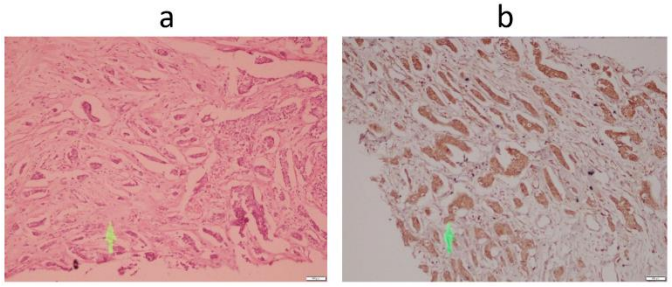


Figure 13 Breast Cancer Tissues (a) HE at 10x of breast cancer tissues, (b) IHC of breast cancer tissues showing no expression at 10x. It is clearly observed that the IHC staining score in breast cancer tissue is zero or very low. Which means that Fam26F is suppressed in cancer as compared to normal tissues.

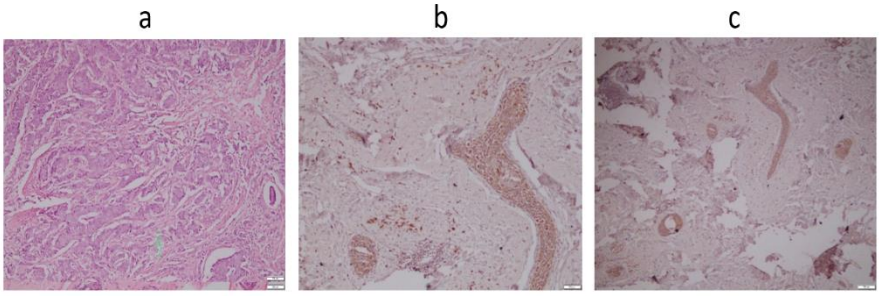


Figure 12 Post chemo Breast Cancer tissues HE slide at 10X of post chemo breast tissue, (b) IHC of post chemo breast tissues at 10 X and (c) IHC of post chemo breast tissues at 4x.

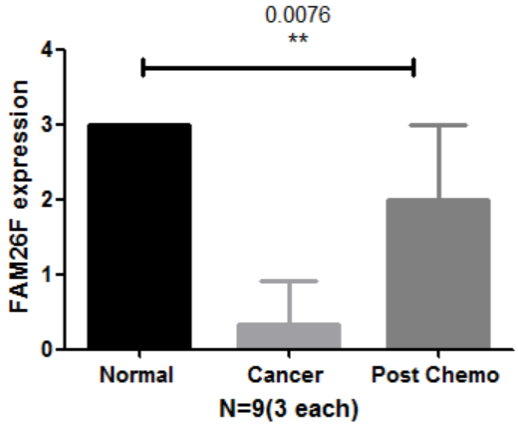


Figure 15 FAM26F expression in normal vs cancer vs post chemo breast tissues.

In post chemo breast tissues the expression of FAM26F is restored. It means when infection or inflammation is suppressed by, Fam26F is enhanced like the expression of FAM6F in normal tissues. In cancer its expression is suppressed. The statistical evaluation of the relative levels of FAM26F in three groups i.e. normal, Breast and post chemo was performed by One-Way ANOVA. By applying the test a p-value of 0.0076 was obtained which is below 0.05. From these results, we have deduced that the correlation amongst these groups is highly significant.

Table 6 Clinicopathological significance of FAM26F expression in breast tissues.

Clinicopathological features	FAM26F expression			
Breast carcinoma N=9	Null	Low	Moderate	High
Gender				
Male	0	0	0	0
Female	2	2	1	4
Age				
Below 50	2	0	1	2
Above 50	1	1	0	2
Malignant	1	1	0	1
Benign	1	1	1	3
Grade				
Grade I	1	1	1	0
Grade II	0	0	0	1
Grade III	1	1	0	1
None	0	0	0	3
Ki67				
<5	0	1	1	3
>5	2	1	0	1
Necrosis				
Present	1	1	0	1
Absent	1	1	1	3

Breast cancer is majorly present in females. The effect sent in females. The females with age more than 50 are at more risk for breast carcinoma. The expression of FAM26F in non-necrotic and benign samples is more in co-relation to necrotic and malignant.

4.1.5 Expression of FAM26F Prostate cancer tissues:

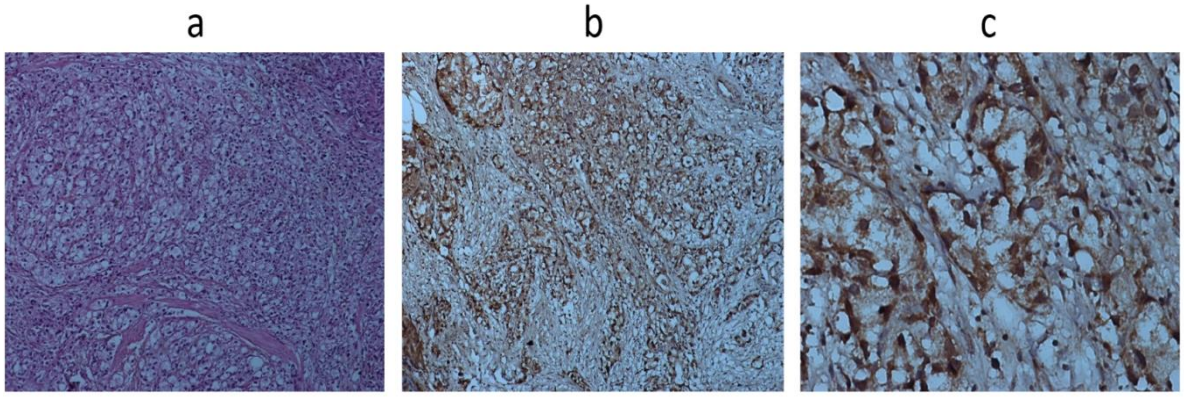


Figure 3.21. Prostate cancer tissues

(a) HE slides of prostate cancer at 10X, (b) IHC of prostate cancer tissues is seen at 10X, (c) IHC at 40X of prostate cancer tissues

Table 11. Clinicopathological significance of FAM26F expression in prostate cancer

Clinicopathological features		FAM26F expression			
Prostate carcinoma	Null	Low	Moderate	High	
Gender					
Male	1	0	0	3	
Female	0	0	0	0	
Age					
Below 50	0	0	0	0	
Above 50	1	0	0	3	
Malignant	0	0	0	3	
Benign	1	0	0	0	
Grade					
Low(GS-06)	1	0	0	1	
High(GS-07)	0	0	0	2	
Necrosis					
Present	0	0	0	3	
Absent	1	0	0	0	

As prostate gland is found in males, therefore there's no case of prostate found in females. High expression of FAM26F is seen in males above 50. GS-07 Gleason score

tumor has more expression of FAM26F in relation to GS-06. Malignant and necrotic tissues show high expression of FAM26F in comparison with benign and non-necrotic. The data interprets that FAM26F could be used as a prognostic marker to predict the likelihood of prostate cancer. As its expression is seen high in prostate.

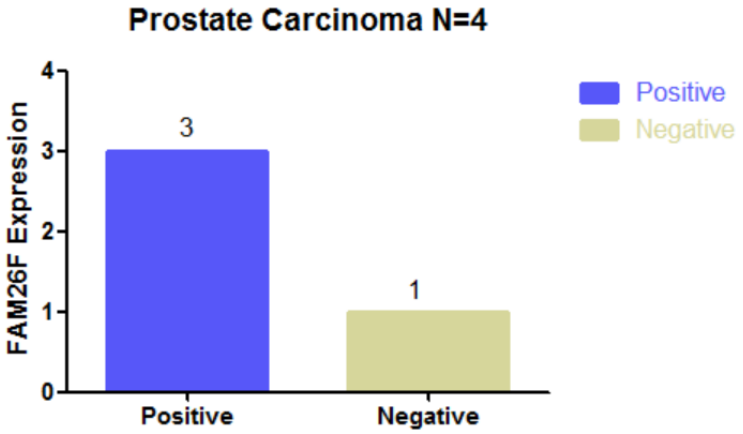


Figure 16 Prostate cancer tissues Positive vs Negative samples

This shows the expression of FAM26F is high in prostate as out of 4, 3 samples had positive result. This outcome follows our observation that the presence of fam26f is more in glandular tissues.

4.1.6 Expression of FAM26F Renal cancer tissues:

After some studies conducted which showed association between hypercalecimia and renal cancer, comparatively recent studies have reported a role of calcium homeostasis alteration in contributing to renal cancer (Carmeliet et al., 2003; Luo et al., 2020). The expression of FAM26F is not dependent on the age and gender type. 80% of the malignant and necrotic cells have high expression of FAM26. The nuclear grading has no effect on the FAM26F

expression. In 5 samples, the expression of FAM26F is positive. The overall expression profile of FAM26F in renal cancer is high.

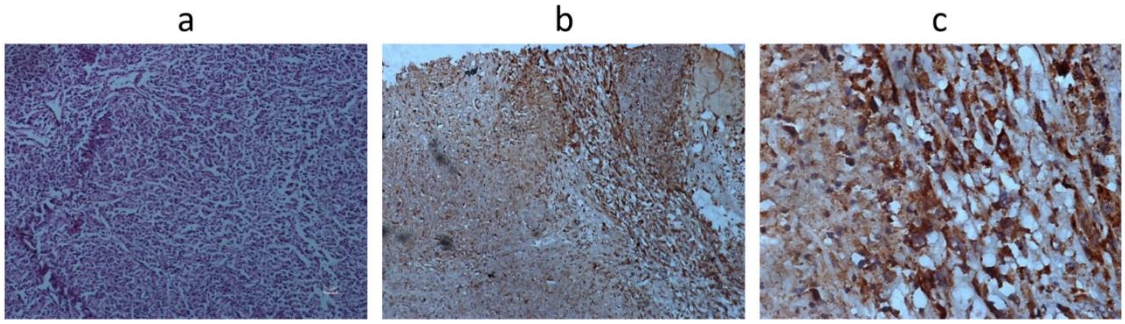


Figure 17 Renal cancer Tissues. (a) HE slide of renal carcinoma at 10X, (b) IHC slide of renal carcinoma at 10X, (c) 40X IHC slide of Renal carcinoma The expression of our marker is high in renal carcinoma. Approximately the Immuno-staining score is 3.

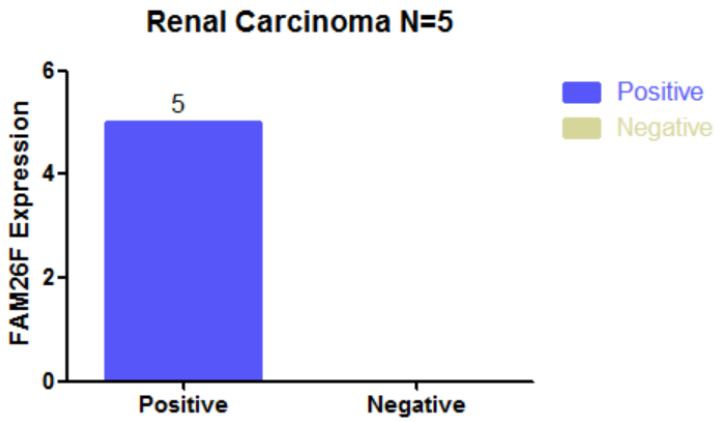


Figure 18 Positive vs negative in Renal carcinoma tissues

Table 12. Clinicopathological significance of FAM26F expression in Renal Carcinoma

Clinicopathological features		FAM26F expression		
kidney carcinoma N=5	Null	Low	Moderate	High
Gender				
Male	0	1	2	2
Female	0	0	0	0
Age				
Below 50	0	0	1	1
Above 50		1	1	1
Malignant	0	0	2	2
Benign	0	1	0	0
Fuhrmans nuclear Grade				
Low(1/4)	0	1	1	1
High(3/4)	0	0	1	1
Necrosis				
Present	0	0	2	2
Absent	0	1	0	0

Table 7 The intensity scoring of FAM26F expression in different cancer type according to the allred scoring scheme

Cancer types	Intensity Score			
	Null (0)	Low (1)	Moderate (2)	High (3)
Bladder (N=20)	18	1	0	3
Prostate (N=4)	1	0	0	3
Renal (N=5)	0	1	3	1
Gastric (N=3)	0	2	0	1
Breast (N=9)	2	2	1	4

4.2 In-Silico Phylogenetic Analysis of FAM26F

4.2.1 Protein domain identification:

4.2.1.1 tORFs retrieval:

Feeding the NCBI gene id's of FAM26F in each organism resulted in individual FASTA files. They contained all of the protein sequences in FASTA format against each open reading frame (ORF).

4.2.1.2 Protein Domains from Pfam:

Domain hits of the protein was scanned by Hmmscan using HMMER and was recorded in a text file named **domtblout** which contained all the scanned domains. The hits were scanned from pfam database. The domtblout file had per domain data. The individual data line per homologous domain is identified in a query sequence for each homologous model.

4.2.1.3 Domain Repertoires retrieval:

The domain repertoires were retrieved from previous result which had all the scanned domains. These domains were filtered for any unreliable hits by using *Hmmerrep2.py* and as a result the final result only contained domains with protein E values and domain E values smaller than or equal to *1 x 10 ^ -3.*

4.2.1.4 Generation of Domain Matrix:

A protein domain matrix was generated from python pipeline which gave in a numerical form the details of absence or presence of domain in each genome. It was formed with domain counts per taxa data.

4.2.2 4.2.2 Phylogenetic analysis:

ProtTest predicted the amino acid substitution model of our sequences to be JTT+G+ (Darriba et al., 2011). This model was used along with MAAFT multiple alignment to generate a maximum clade credibility tree by employing a Markov Chain Monte Carlo Bayesian approach (Bouckaert et al., 2014)

4.2.2.1 Ancestral state reconstruction and character evolution:

The liner parsimony model method reconstructs the domains at the mesquite tree internal node. It is effective in detecting domain loss, gain, and duplication events of domains. The final results were analyzed using python pipeline i.e character evolution. The output was the main determinant of domain evolution dynamics. It had the list of gain, duplication, and loss events reconstructed at every branch of the time-calibrated tree. The final tree with all the events was visualized using iTOL. The evolutionary events for FAM26F domain Ca_hom_mod repertoires, were mapped along the branches of the tree (Letunic & Bork, 2016).

For all 20 organisms, a total of 4410 non-redundant sets of domains were achieved through character evolution. Around 14000 domain loss, gain, and duplication events were recorded for these domains. All of these are listed below.

4.2.2.2 Evolution of FAM26F domain ca_hom_mod:

The phylogenetic tree obtained from iTOL was a rooted one having a common ancestor on its root for all the organisms. The main root was further divided into two branches. At one end of the branch aka node was *Stylophora pistillata* i.e. coral reef, the simplest of all organisms. There was no event of domain gain, loss or duplication observed for *S.pistillata*

indicating that it has the original CALHM6 from the history/common ancestor millions of years ago. Whereas upon dividing into node 3, there was an event of domain gain (shown as a green box) on the node branch.

Node (n3) from the common ancestor was further divided into two branches (containing 19 leaves), *Exaiptasia diaphana* on one end and node 4 (n4) on the other end. There was no domain event detected for *Exaiptasia diaphana* but at node 4 there was a duplication event (shown as a purple box) occurred before dividing into node 5 and node 15. Node 5 contains 5 leaves at its end. *Lates calcarifer*, *Maylandia zebra*, and *Austrofundulus* inherited the same CALHM6 domain as of node 5 (duplicated one) but *Danio rerio* gained another duplication while *Salmo salar* observed domain loss (shown as a white box) of CALHM6 in it.

Node 14 is divided into two branches node 28 and node 15. Node 28 is further subdivided into node 36 and node 29. Node 28 observed domain loss before being divided into *Pogona vitticeps* and *Python bivittatus*. *Gallus gallus* inherited the same domain as node 14. *Ficedula albicollis* observed two duplication events while *Falco* and *Columba* observed one duplication each.

Node 15 first observed domain duplication and was divided into node 16 and node 25. Node 25 is further divided into *Rattus Norvegicus* and *Microtus ochrogaster* but with another domain duplication.

Node 16 is divided into two clades. One containing *Camelus* with domain duplication, and *Bos* with domain loss. On the other clade, they have *Canis* with domain duplication while *Felis* along with *Homo sapiens* with a domain loss event.

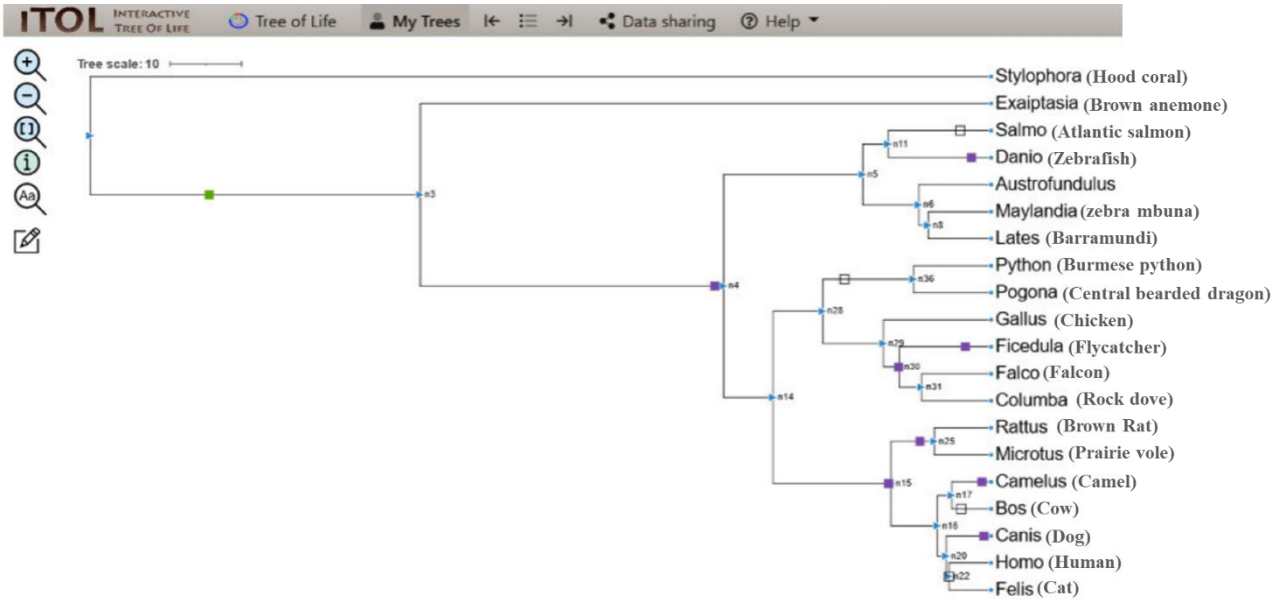


Figure 19 iTOL tree showing domain gain (green box), loss (white), and duplication (purple) events of CALHM6 only

4.2.2.3 Domain evolution of interacting partners of FAM26F:

The results for domain evolution of interacting partners of CALHM6 are as follows. For *S.pistillata,* there was no event of domain gain, loss, or duplication for any of the interacting partners observed. For *Exaiptasia diaphana,* there were events of domain gain for CALHM6 (at node 3) and for peroxiredoxin (at node *exaiptasia*) observed. For *Salmosalar*, there were events of cal_hom_mod domain duplication (at node 4) and loss (at node *Salmo*), and a gain of S_100 (at node 11) domain was detected.

For *Danio,* along with duplication of cal_hom_mod (node 4) and gain of S_100 (node 11), there was gain followed by duplication of EF-hand 7, duplication of Calpain III, and duplication of Thioredoxin observed.

Austrofundulus and *Lates calcarifer* did not observe any specific event after cal hom mod duplication (node 4) and s_100 gain (node 11). While *Maylandia zebra* observed gain of TNF, EF HAND 7, S_100, CALPAIN III, and Thioredoxin along with duplication of TNF and d S_100 further.

At node 36, there was a cal hom mod domain loss observed and then there was no domain event for *Python bivittatus* and *Pogona vitticeps* further. At node 29, there was domain gain and then duplication for proteins ef hand 7 and Thioredoxin each, and domain gain for Calpain III. Then for Gallus, there was domain duplication for Calpain III along with gain and duplication for the TGF beta as well.

A duplication of the Cal_hom_mod domain was observed at node 30. For *Ficedula albicollis*, there was a gain and duplication of TGF beta, a gain of Vinculin, and duplications of Ef hand7, Calpain III, and Thioredoxin. Before the division of node 30 into *Falco cherrug* and *Columba livia*, there were domain losses observed for Calpain III, Ef hand7, and Thioredoxin at node 31.

At nodes 15 and 25 domain gain of cal hom mod was detected. For *Rattus Norvegicus*, domain gain and duplications were observed for TNF and TGF beta, and gain of peroxiredoxin was observed. There was no domain event for the *Microtus ochrogaster*.

For Camelus, domain gains of ef hand7 and Thioredoxin along with duplication of cal hom mod domain. For bos, loss of cal hom mod domain. For Canis, there were gains and duplications for EF hand7, Calpain III, Thioredoxin, and TGF beta was observed. The only gain of peroxiredoxin and only duplication of cal hom mod were detected. Before the division of node 22 into *felis* and homo, there was a domain loss of cal hom mod. Then

there was no event detected for *felis* and there were domain gains for TGF beta and Thioredoxin.

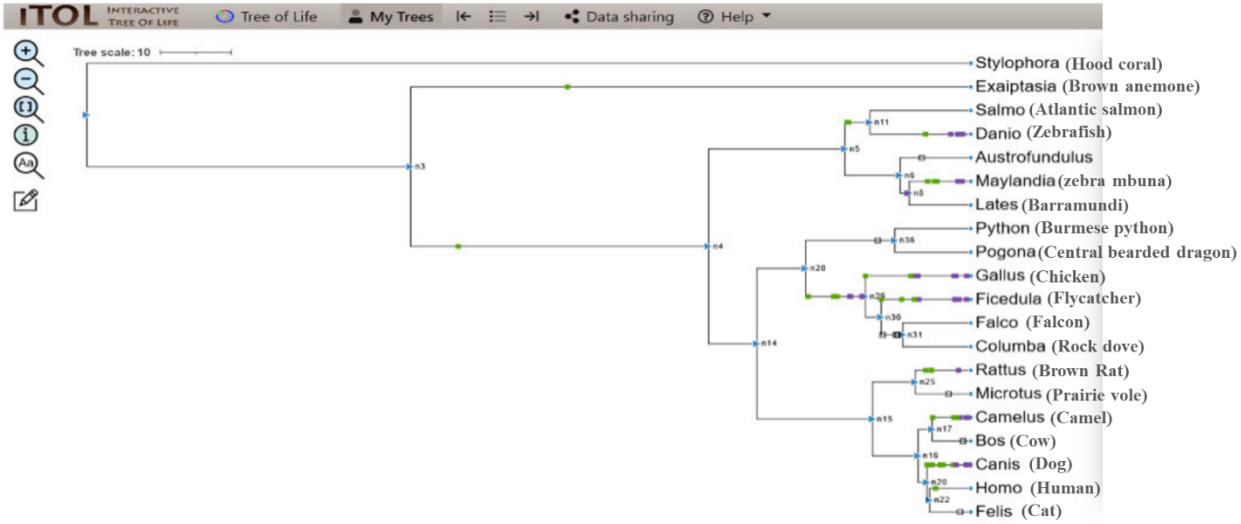


Figure 20 iTOL tree showing domain gain (green box), loss (white), and duplication (purple) events of CALHM6 along with its interacting partners.

4.2.2.4 The Domain Repertoire Events for CALHM6/FAM26F

FAM26F is reported to have only one domain i.e ca_hom_mod. The evolutionary events centered around this domain were screened from the events file generated through python and given as input in iTOL to visualize the iTOL tree. The loss, gain, and duplication events recorded for FAM26F are listed in Table 1. There is a total of 14 domain loss events, and 6 duplication events, while only one event was recorded where the domain had a gain, as shown in figure 2. The duplication and gain of domain events increased progressively in the higher organisms, indicating that the increase in size and complexity of protein led to its functionality in higher mammals, especially humans.

4.2.2.5 The Domain Repertoire Events for CALHM6 and its Calcium Interacting Partners

Along with CALHM6, the python pipeline yielded many protein domains. Through reported literature, major interacting protein partners of CALHM6 were shortlisted which

were majorly the calcium homeostasis proteins involved in the immunological phenomenon, and were searched for in the results (Malik et al., 2020). The presence of those proteins was also a positive indicator of CALHM6 function in calcium homeostasis and respiratory burst based on the similarity of specific motifs present in the protein.

The co-evolution of those proteins along with CALHM6 on the phylogenetic tree was determined. The partners included Thioredoxin, Calpain, Vinculin, Protein Kinase C, Tetraspanin, SLC35F, SLC3A2, USP, IRF3, TPX2, NMT, INFGR, and TGF. A total of 118 evolutionary events were recorded for these proteins along with CALHM6.

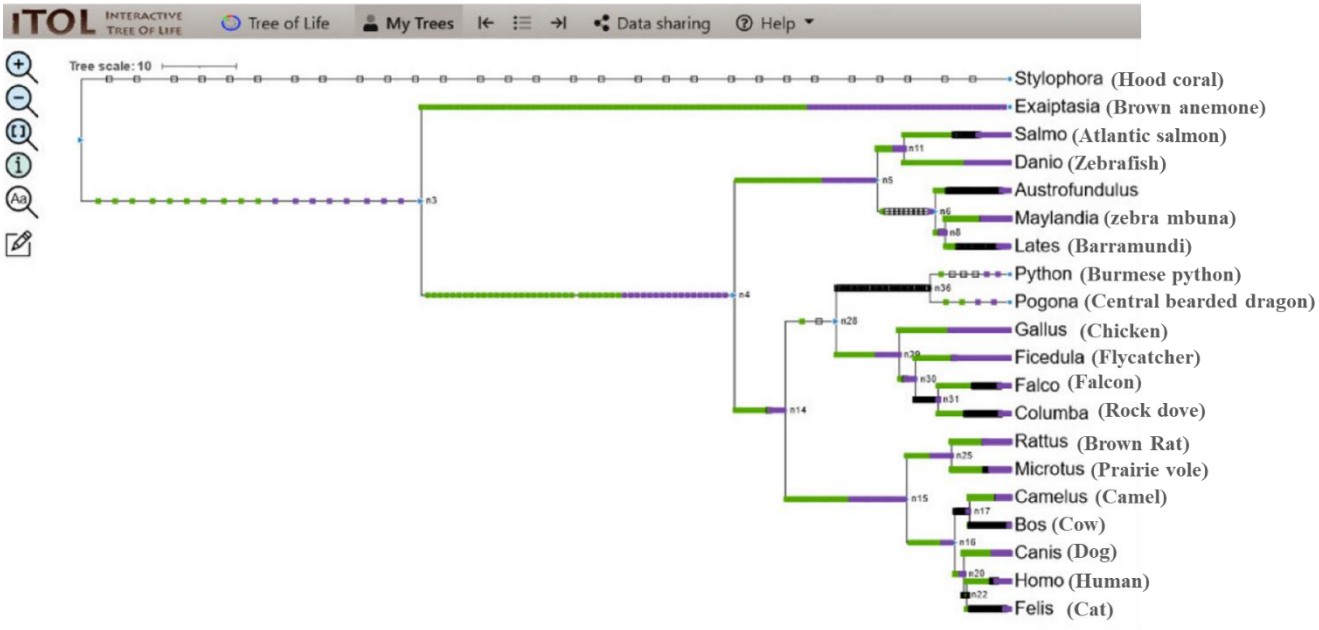


Figure 21 iTOL tree showing domain gain (green box), loss (white), and duplication (purple) events of all the domains detected at the end of python pipeline

Discussion:

FAM26F is a significant immune protein that has started gaining attention as being a protein of immunological importance, while still having room for exploration in various aspects. One aspect was the investigation of the role FAM26F, a calcium ion channel protein, has to offer in various cancers. Main focus was on brain cancer since the brain tumor microenvironment has an evident involvement of calcium regulation and ROS in tumor progression, however, samples were obtained for renal, bladder, gastric, prostate, and breast cancers which helped in giving us additional insight of the expression of FAM26F in various cancers. The involvement of FAM26F is predicted due to the involvement of calcium homeostasis and subsequently of ROS in cancer progression. Since FAM26F is an ion channel protein, it is hypothised to aid in calcium ion movement inside the cancer cell. A decrease in FAM26F expression was observed in breast and bladder cancer as compared to expression in normal tissues. While an increase in FAM26F expression was observed in gastric carcinoma, renal cancer, brain cancer, and prostate cancer. This differential expression of FAM26F in different cancer biopsies indicate the possible involvement of this protein in cancer. Whether this involvement is for cancer progression or cancer clearance is yet to be explored in detail in each type of cancer.

Moreover, one approach was conducting phylogenetic analysis across a range of species to have a clear insight in its evolutionary pattern. Phylogenetic analysis is an important tool throughout biological science for conducting comparison on historical relationships and information about proteins, genes, individuals, species, and population. This information is depicted in the form of behavioral, morphological, or molecular data, and visualized as

a branching diagram, known as a phylogenetic tree. In 2017, Malik et al. conducted an evolutionary analysis on FAM26F, showing it to be relatively conserved with the most homology shown with the chimpanzee. Further in-silico studies conducted by them showed that FAM26F was located on human chromosome 6, position 6q22.1, which translated to make a 315 amino acids long protein. However, no previous attempt to trace the exact domains and the evolution of FAM26F had been made. This study is the first study designed to explore the evolution of protein domain repertoires of FAM26F. It aims to trace the specific events of gain, loss, and duplication in the FAM26F protein domain, concerning the evolution of its interacting partners across 20 different eukaryotic organisms as listed: *Austrofundulus limnaeus* (Killifish), *Camelus dromedarius* (Camel), *Bos mutus* (Wild yak), *Columba livia* (Rock dove), *Canis lupus familiaris (*Dog), *Falco cherrug* (Saker Falon), *Danio rerio* (Zebrafish), *Exaiptasia diaphana* (Sea anemone), *Felis catus* (Cat), *Gallus gallus* (Red jungle fowl), *Maylandia zebra* (Zebra Mbuna), *Pogona vitticeps* (Central Bearded Dragon), *Microtus ochrogaster* (Prairie vole), *Lates calcarifer* (Barramundi), *Homo sapiens* (Human), *Python bivittatus* (Burmese python), *Salmo salar* (Atlantic salmon), *Stylophora pistilliata* (Hood coral), *Ficedula albicollis* (collared flycatcher), and *Rattus norvegicus* (Brown rat).

The genomic id's from NCBI database were submitted which gave the open reading frames which were submitted further in the pipeline to get the domain matrix. The end result was a phylogenetic tree with domain events presented on each branch. These domain shuffling events may occur during evolution and these include the domain losses, duplications and gains which help to increase the domain function versatility and occurrence and eventually in protein function. Additionally the idea of proteins being co-evolved involved in similar

pathways has also been reported as an important feature of the evolution to ensure the proteins remain in relationship throughout the course of evolution (Holzerlandt et al., 2002). Malik et. Al has reported the possibility of FAM26F being in interaction with calcium homeostasis proteins (Malik et al., 2020). This study confirmed that idea by further strengthening that association through possibility of co-evolution of interacting partners with FAM26F.

Through the combined approach of python and bio-informatics tools, a deep insight in the domain contents and events of proteins in relation to their phylogenetic history was effectively achieved. Apart from generation of domain matrix which was time consuming, the overall pipeline was rather user friendly. Ancestral character reconstruction proved to be an invaluable way in yielding exact domain events pattern of FAM26F, Calpain, protein S100-A7, Vinculin, Peroxiredoxin, Thioredoxin, and Calmodulin-like protein 5. Phylogenetic approach alongside the immunohistochemistry on cancer biopsies testified the role of FAM26F as a protein of calcium homeostasis.

Conclusion and Future Prospects

Overall, the findings of our study highlight the importance of FAM26F as an innate immune modulator, and it is suggested that FAM26F expression could be used as an early cancer predictor. Whether FAM26F is involved early in cancer formation or later in immune resistance, our research has indicated that it is an important molecule with obvious intrinsic value that deserves further examination. CALHM6 is still a relatively new protein with much potential for exploration in various aspects.

The current study applies the computational approach to trace the evolution of CALHM6s' domain involved in calcium homeostasis and compare the events with the proteins involved in calcium homeostasis and which have shown an association with it in previous studies. The genomic id of CALHM6 across a wide range of species, coinciding with the protein id of CALHM6, was selected and translated. Their open reading frames were recruited and domain events were traced using a python pipeline to generate a domain matrix which was then used as input in bioinformatics tools. A time-calibrated tree of CALHM6 was generated with amino acid sequence and suitable amino acid substitution model and used as input in Mesquite which combined the domain matrix and tree to generate a phylogenetic tree. It was visualized in iTOL. It was easy to visualize, read, and further analyzed. Further analysis of the remaining domains given by the software is needed for an even clearer understanding of CALHM6 evolution.

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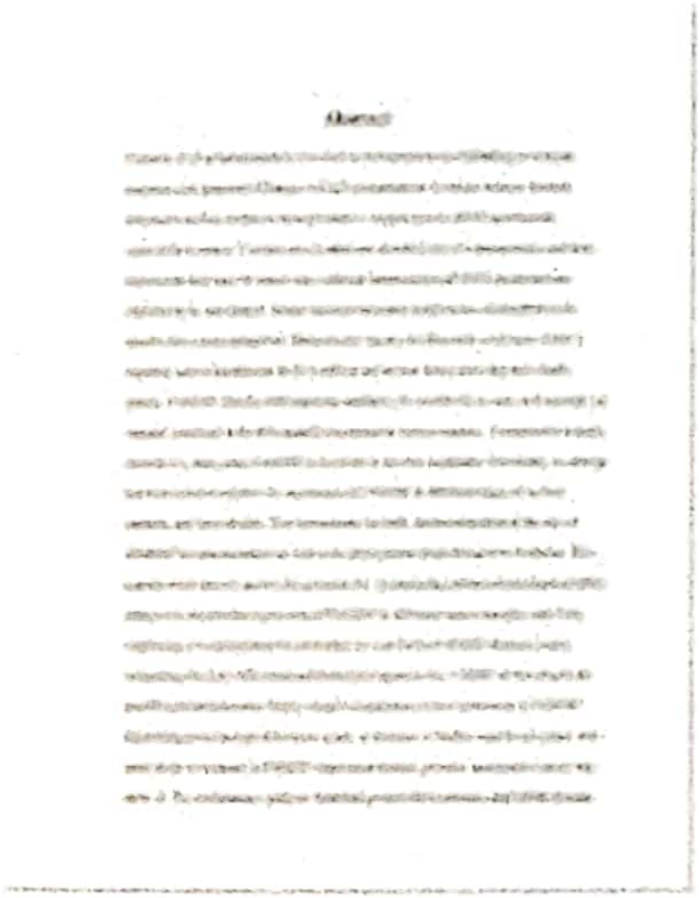

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