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INNOVATIVE MOLECULAR SIGNATURES OF BREAST CANCER, CELLULAR PROFILING VIA PREDICTION ANALYSIS

Ambreen Aisha[1*], Esha Mustafa[2], Wardah[3], Sadia Javed[4], Ayesha Akbar[5], Iqra Qasim[6], Aruba Faiz[7], Annie Nazir[8], Shaista Nawaz[9]

*[*1]Department of Biochemistry, Punjab Medical College, Faisalabad Medical University, Faisalabad, Pakistan*
[2,3,4,5,6,7,8]College of Nursing, Allied Hospital Faisalabad, Affiliated with University of Health Sciences, Lahore, Pakistan
[9]Food and Biotechnology Research Center, Pakistan Council of Scientific and Industrial Research (PCSIR), Lahore, Pakistan

[*1]aishafmu@gmail.com

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Corresponding Author: *

Abstract

A complete understanding of diverse and intricate features pertaining to breast malignancies and the molecular etiology of multiple types of cancer stays unexplored. The work was designed to investigate breast cancer proliferative and apoptotic mediators as diagnostic tools, using spatiotemporal and all-inclusive bioinformatics approaches. Data from the Protein data bank for proteins CytochromeC (CYCc) and Granzyme B, as well as ZINC database and Swiss Docking for the ligand Cholecalciferol. Results were visualized in USF chimaera 1.15. PyMol was used to investigate protein active site interactions. Granzyme B has a single active binding site at Methionine34 aligned with Cholecalciferol through 1 hydrogen bond and a fitness energy of -3045.24 Kcal/moL and -7.38 score. Docking CYCc with Cholecalciferol revealed one active site Isoleucine82 with one hydrogen bond and a bond length of 2.092, fitness (-1425.01 Kcal/moL), and a score of 5.56. Proteoform expression has shown association of proteins like Granzyme B and CYCc, as well as Cholecalciferol, in both regulatory apoptotic mediators and functional proliferative pathways, thus emphasizing their prominence as diagnostic indicators for breast cancer. Current research will assist in the construction of chemotherapeutic mechanisms of action, producing potentially clinically significant diagnostic and prognostic information.

INTRODUCTION

Breast cancer is a multimodal disease since it entails the amalgamation of multiple types of malignancy-associated cells and mediators in a way that mimics cancer progression [1]. Proteomics can be beneficial in discriminating against breast cancer subtypes and proteins, such as gene regulating transcription factors, proteoform expressions and enzymes [2-4]. Through proteomic analysis, the efficacy of chemotherapies at the cellular and tissue levels, as well as potential target and therapeutic proteins for clinical trials may be assessed. Proteomics analysis provides valuable data for detecting and validating breast cancer diagnosis, as well as defining physiological and key of regulation. Some peptide

fingerprints of the human body tissues, and biomarkers for diagnosis and anticipating genetic mutations at the proteome level [1, 5, 6].

The capacity of the immune system to combat malignancies and viral infections is compromised by cancerous cells, which may inhibit the activity of CTL and NK cells. NK/CTL cells destroy diseased or abnormal target cells by aggressively disrupting cytosolic organelles or sequestering Granzymes to tissue necrotic components [1, 7]. Researchers have found cytosolic CYCc to be a critical biochemical predictor of apoptosis, which is associated with a pro-inflammatory response as well [8, 9]. Granzyme B is a serine protease which can be detected in the cytosol of NKC and stimulated T lymphocytes [10]. Extracellular Granzyme B was linked to rheumatoid arthritis, systemic lupus erythematosus, and other inflammatory diseases [11, 12]. Only a few of the 300 intracellular proteins on humans, were identified and verified to be related with Granzyme B -interceded the cell death [13]. In an inflammatory response, for instance, the Perforin protein promotes the exocytosis and transport of Granzyme B to the target tissue cells, where it mediates the apoptotic cascade by triggering and sequestering procaspase III, VII, and VIII. Alternatively, the truncated form of the apoptotic cleaving protein Bid releases the mitochondrial CYCc [7]. Activation of the various caspase group members including caspase3, caspase 6-9, BH3 domain cleavage of pro-apoptotic protein (Bid) [14]. In tumor cells, Granzyme B causes apoptosis through many mechanisms. Granzyme B can trigger cellular death by initiating the caspase-related suicide cycle either directly or indirectly. Direct cleavage of procaspase-3 or procaspase-8 by Granzyme B. Caspase-3 inhibits caspase-activated DNase production, allowing CAD to the reaching of the nucLeucines's interior and induce the DNA disintegration, finally leading to cell death [15]. Furthermore, Granzyme B leaves various death proteins that promote cell death, including DNA repair proteins PARP1 and DNA-PK, cell di vision proteins lamin B and NuMA protein, cytoskeletal micro tubule protein tubulin, and DNA damage proteins ICAD and DNA breakdown factor. The mitochondrial apoptotic pathway is also activated by Granzyme B, which contributes to its high ability to trigger cell death [16]. Mitochondrial apoptosis relies on caspase activation much like any other apoptotic mechanism.

In addition, Granzyme B accumulates P53, which can communicate with Bcl-2 and prevent gtBid from binding to Bcl-2 and free Bax from the Bax-Bcl-2 complex. Free gtBid may also activate Bax, causing alterations in the mitochondrial outer membrane permeability, enabling CYCc to be released from the mitochondria, finally, render the cells to apoptosis [15].

Predicted in the involvement of the mitochondria in apoptosome generation is predicated by CYCc. The intra mitochondrial protein CYCc participates in the formation of the oxidative phosphorylation electron transport pathway and is often found in the inter membranous space [14]. The apoptosis regulator Apaf-1 interacts to the CYCc after it has been translocated into the cytosol, activating the apoptotic caspase [9]. It has been reported that the formation of CYCc permeabilized from mitochondria also exocytose from cells and may be regarded as biochemical signal of apoptosis [8, 14]. *In vivo* and *in vitro* outcomes have affirmed elevated the blood formation of cytochrome-c levels in the melanoma involving hematology and the benign diseases characterized by the systemic apoptosis [9, 17-20].

Cholecalciferol controls carcinogenesis through balancing inflammatory response and angiogenesis [21, 22]. Cholecalciferol binding protein (VDBP) a multi-functional plasma protein form intermediary role in metabolites transport and angiogenesis. As a response of maintaining Cholecalciferol levels, VDBP governs bone formation, actin sequestration, and regulation of immunological and inflammatory responses and suppression of angiogenesis [18]. Proliferation and differentiation of tumor cells *in vitro* were increased by 10.7-2.8 fold and 9.62 -1.7 fold, respectively, by the formation of cholecalciferol stimulation [19]. Furthermore, Buttigliero claims that Cholecalciferol treatment has diminutive effect in prostate cancer and may even aggravate the situation in certain cases [20]. Epithelial cancer cells and adult adipocytes may be driven to apoptosis by vitD-derived hormone 1, 25(OH)2D3 by activating the apoptotic Ca+2 signal, which acts as an apoptotic marker and recruits

directly apoptotic effectors and Ca+2-dependent proteases in adipocytes and cancer cells [21, 22]. Developing novel anti-cancer therapies that take use of vitD compounds that modulate intracellular Ca+2-dependent apoptosis proteases as prospective targets is made easier because of the connection between the cellular Ca+2 and the 1,25(OH)2D3 apoptosis in obesity and carcinoma [22]. The vast majority of the research suggested Cholecalciferol plays a crucial part in cancer cell proliferation and cellular cycle, where DNA became a focus to decode the Cholecalciferol molecular mechanisms in carcinoma [23].

Despite the fact that many of the referenced proteins play an important role in diagnosis. Proteomics, focuses on integrative study of proteins, has emerged as a powerful technique in recent years [1]. The use of molecular signatures like CYCc, Granzyme B and Cholecalciferol might add value to typical clinical and pathological characteristics. It will influence clinical treatment in a variety of cancer forms, probably most notably in breast cancer. In silico investigations may anticipate the inflammatory parameters such as the Granzyme B and CYCc, putative biomarkers in the form of Cholecalciferol for the prognosis, and tumor response to different targeted or immunological therapeutic approaches.

Material and methods

In-silico analysis

The Swiss dock auto was used for docking of CytC and Granzyme B with the vitD and results were visualized in USF chimera 1.15 [24]. The general steps performed included, retrieval of proteins from Protein databank (PDB) and ligand from ZINC database [25], preparation of Coordinates files and removed extra protein parts, docking with Swiss dock [26], visualization of interactions compared to active sites of proteins by PyMol [27] of the structure at the center of the most populated cluster in docking CYCc with Granzyme B . The protparam was used for determination of physiochemical properties and Clus Pro was used for protein interactions [28, 29].

Auto Swiss docking

Swiss dock auto was performed for docking and results were visualized in USF chimera 1.15. The general steps were performed which include retrieval of proteins models from Protein databank (PDB) with their amino acids distribution and ligand from ZINC database [25], Preparation of Coordinates files and removed extra protein parts, docking with Swiss dock [26].

Analysis of PyMol

The interactions of anchored proteins with ligands were visualized through analysis of PyMol [27]. The structure of proteins and their active sites were analyzed. The Proteins were added into main window of PyMol then structures of proteins were analyzed by opening display menu and then selecting the sequence option. Different structural orientations were visualized to find best interaction visualization, their active sites were noted by using molecular identification to show the surface of the protein and ligand binding sites (typing show surface, protein) (typing show spheres, ligand Binding sites), the center of the most populated cluster were evident in docking CYCc with B [30].

Physicochemical properties

The protparam was used for determination of physiochemical properties and Clus Pro was used for protein interactions [28, 29]. The protparm was used for determination of physiochemical properties. Prot Param computes various physicochemical properties that can be inferred from the protein sequence. The protein can either be specified as a Swiss-Prot/TrEMBL accession number or ID, or in the form of a raw sequence. By default, the complete sequence will be analyzed.

The parameters calculated by ProtParam include the molecular weight, theoretical pI, amino acid composition, atomic composition, estimated half-life, extinction coefficient, instability index, aliphatic index, and grand average of hydropathicity (GRAVY). Molecular weight and theoretical pI are calculated as in Compute pI/Mw.

Half life

The half-life represents predicted duration required by the first half of the protein present in the cell's membrane to disintegrate after its synthesis. ProtParam calculates the half-life of a particular sequence via analysis at the N-terminal amino acid.

Extinction coefficients

The extinction coefficient of a protein reveals what amount of light it captures at a specific wavelength.

It is helpful to estimate this coefficient while isolating a protein using a spectrophotometer [31].

Results

The possible role of selected cytoplasmic proteins such as, Granzyme B with CYCc and Cholecalciferol was assessed by *in silico* molecular docking analysis. One of the most common methods for determining the possible types of interactions responsible for binding of proteins, metabolites, or vitamins with the enzyme, their binding affinities, bond distance, and the different orientations of the docked ligands at various sites of the target enzyme, specifically the active sites.

Physiochemical properties of proteins

The possible physiochemical properties of proteins were determined as shown in Fig 1. Half-life of protein is expressed in hours; Gravy of Cholecalciferol is -0.706 while Granzyme B (-0.408) depicting their instability. The positive and negatively charged amino acids of CYCc and Granzyme B shown in (Table 1).

Table 1. Physiochemical characteristics of CYCc and Granzyme B.

Protein name	CYCc	Granzyme B
Amino acids residues	105	247
Mol-weight	11748.72	27662.11
Theoretical Pi	9.59	9.56
Amino acids residues with (-ive charge i.e. Aspartate & Glutamate)	11	20
Amino acids residues with (-ive charge i.e. Arginine & Lysine)	20	34
Formula	$C_{526}H_{845}N_{143}O_{149}S_6$	$C_{1231}H_{1962}N_{352}O_{345}S_{14}$
Coefficients of Extinction (with Cystines)	13075 (M^{-1} cm^{-1})	32805 (M^{-1} cm^{-1})

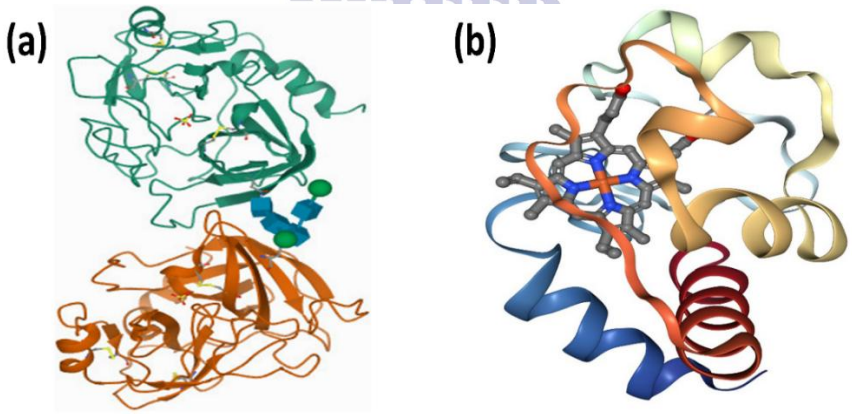

Fig. 1. (a) Human Granzyme B as 3D model (PDB: IFQ3) (b) Human CYCc protein 3 D structure (PDB: G41S).

***In silico* assay of Cholecalciferol with Granzyme B**

The possible role of Cholecalciferol was evaluated through *in silico* molecular docking with Granzyme B. This method is most commonly used to assess possible variety of interactions responsible for binding of ligand (Cholecalciferol in our case) with proteins. The binding affinity, bonds distance and different orientations of docked ligand at different sites of target, specifically the active site was determined. The amino acid residue involved in binding with Cholecalciferol at target site of Granzyme B is methionine, one hydrogen bond (bond length 2.75) participated in bonding of Cholecalciferol with Granzyme B. Fitness (-3045.24 Kcal/mol) and (-7.38 Kcal/mol) score are presented by Cholecalciferol and Granzyme B (Table 2). Rest of target sites not actively participating details are in (Table 2).

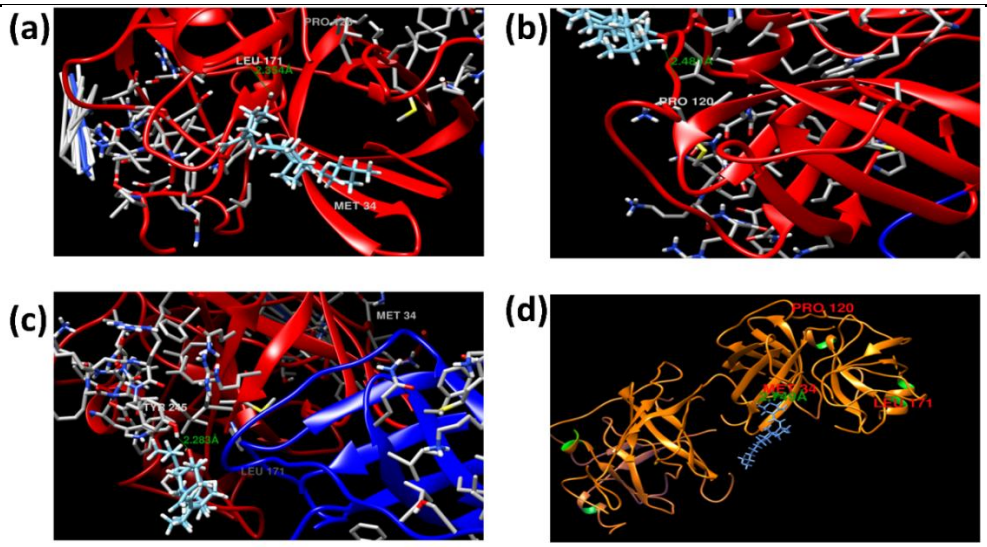

Fig. 2. Granzyme B docked with Cholecalciferol at receptor site (a) Leucine171, (b) Proline120, (c) Tyrosine245, and (d) Methionine34. Cholecalciferol shown in blue, white sticks.

Table 2. Behavior of spiking amino acids of Granzyme B as target sites of ligand Cholecalciferol

Ligand docking	Protein docking	Energy Fitness	Scoring	H-bonds	bond length	Target sites
Cholecalciferol	Granzyme B	-3047.11	-7.39	1	2.354	Leucine171
Cholecalciferol	Granzyme B	-3045.24	-7.38	1	2.75	Methionine34
Cholecalciferol	Granzyme B	-3043.62	-7.06	1	2.283	Tyrosine245
Cholecalciferol	Granzyme B	-3050.72	-6.83	1	2.481	Proline120

Granzyme B docked with Cholecalciferol at receptor sites

Granzyme B had interaction with Cholecalciferol through hydrogen bonding (1 H-bond with 2.354 Å bond length) (Fig. 2a).

Granzyme B docked with Cholecalciferol

Receptor site Proline120 of Granzyme B docked with Cholecalciferol with 1 H-bond comprising of 2.481 Å bond length) (Fig. 2b).

Granzyme B docking with Cholecalciferol at Receptor site TYR245

One H-bond with 2.283 Å bond length had les binding energy (Fig. 2c).

Granzyme B docking at the MET34 with Cholecalciferol

Binding of MET34 with 1 H-bond of 2.750 Å bond length proved to be the active site of Granzyme B with binding energy (-3045.24 kcal /moL) (Fig. 2d)

CYCc interactions with Cholecalciferol through Swiss docking

Among the findings provided by Swiss docking, those were selected which had at least one hydrogen bond of the ligand (Cholecalciferol) with the receptor protein CYCc (Table 3). The average number of hydrogen bonds secured between the amino acid residues (Aspartate62, Glycine 56, Homosystein33) of CYCc and Cholecalciferol falls to four.

Table 3. Behavior of spiking amino acids of Cytochrome C

Ligand docking	docking	length	H-bond	Scoring	Target sites	Site activation
Cholecalciferol	CYCc	2.092	1	-5.56	Isoleucine82	Site is active
Cholecalciferol	CYCc	2. 023	1	-5.06	Aspartate62	Not actively participating
Cholecalciferol	CYCc	2.60	1	-5.56	Glycine 56	Not actively participating
Cholecalciferol	CYCc	2.49	1	-5.45	Homosystein33	Not actively participating

ILE81 active site of CYCc protein

Amino acid Isoleucine of CYCc acts as an active site subsequently bonded with Cholecalciferol with 1 H-bond (bond length 2.092), with binding free energy -1425.01Kcal/ moL and (-5.56 Kcal/mol) score as presented in (Fig. 3). Rest of binding sites not actively participating due to their higher bonding free energy

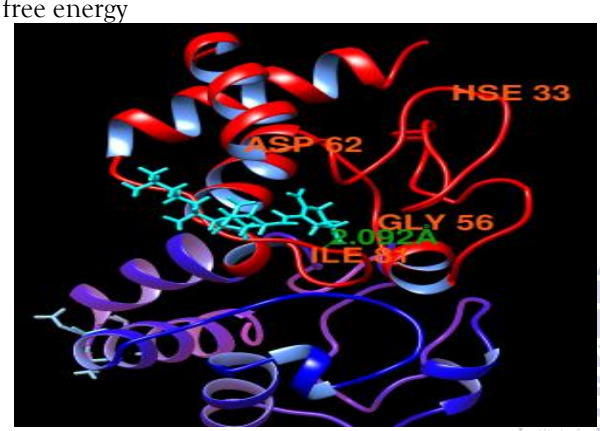

Fig. 3. CYCc docked with Cholecalciferol, the binding pocket at Isoleucine81 (active site) with 1 hydrogen bond of 2.092 Å length**.** Cholecalciferol in blue sticks, while rest of binding pockets Glycine56, Aspartate62, Homocysteine33 are also highlighted.

Interactive proteins CYCc and Granzyme B, docking via Cluster Pro

The proteins interactions were studied via online tool ClusPro and best fitted model is shown in (Fig. 4a). The Interactive docking produced many models but top 10 models which showed energy are represented as Red cartoon is CYCc and green is Granzyme B. The receptor sites are in blue and cyan color which non-specific interactions. Ligand Granzyme B bound with the CYCc, 3D structure is visualized through PyMol, showing the center of the most populated cluster in docking. The x-ray determined in native form of Granzyme B aligned with receptor CYCc is in (Fig. 4b). For the native form, the data exhibits reduced radius of gyration as compared.

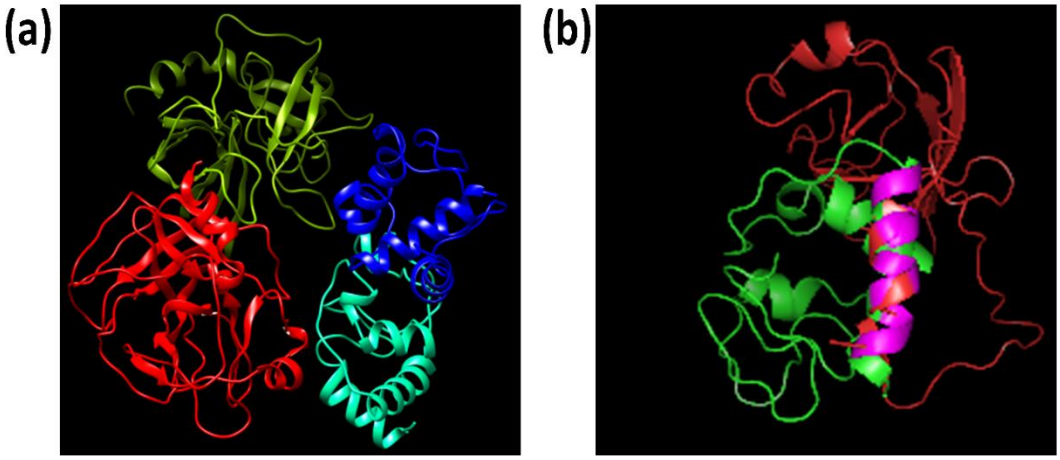

Fig. 4. (a) The docked ligand structure (lig.000.00.pdb) of CYCc with Granzyme B is shown in red and green, whereas the receptor is represented in blue and cyan (b). PyMol depiction shows the arrangement in the heart of the highly packed clustering in docking; X-Ray depicts the natural positioning of the properly aligned region containing Granzyme B and CYCc as a fuchsia pink.

Discussion

Computational prediction of substrates in apoptotic cascades, is quite useful in developing preliminary hypothesis and executing experimental evaluation. To investigate specific role of the functional and regulatory proteins, emphasizing on peptide profiles in the pathways, Pymol and Zinc data sources were employed. Purpose was served by *in silico* molecular docking followed by four main stages; Recovery of desirable metabolites and enzymes, assembling of grid settings and coordinated files, docking and conception of interfaces, and analysis of results [32]. Recently, novel protein markers are additionally focused, as researchers have explored the interaction between CYCc (mitochondrial component) with apoptotic protein (Apaf-1) [15, 33]. Individual role of caspases with Granzymes are reported by combining several modeling approaches including nucleotide variations, multiple mutations & transcript levels of various enzymes involved in regulations of CYCc through dATP [34, 35]. Several hypotheses have been proposed, yet no ideal approach was found to be reported emphasizing Cholecalciferol and CYCc role, likewise, the Granzyme B approach remained enigmatic in chemotherapy for breast cancer. Protein-protein docking was employed for CYCc, Granzyme B with the formation of metabolic mediator Cholecalciferol along with their energy balance estimation. The physicochemical properties like number of amino acids comprised negative and positive charges, half-life instability index and aliphatic chain representing index, were depicted in (Table1). These properties draw a schematic picture of interactive protein molecules.

Apoptosis, a cell death process, is tightly linked to the pro-survival protein BCL2, apoptosis is driven under the expression of *BAX* [36] and down streaming of *BCL2* [37]. The anti-apoptotic BCl-2 protein (proliferation signal) binds and penetrates into the mitochondrial membrane for the further release of CYCc [38, 39], which is cursor for the further carcinogenesis also. The lysine residue (K72) of CYCc is critical in the CYCc-Apaf-1 interaction, many lysine residues are discovered, including K72, which are known to interact with ATP and play an important role in the CYCc-Apaf-1 interaction. To date, the participation of amino acid residues ASP 62, GLY 56, HSE 33, and ILE8 of CYCc as active binding site has not been documented. Nevertheless, the mutation of these lysine residues prevents the development of apoptosomes and inhibits caspase activation, alterations in nucleotides and their expression of ASP 62, GLY 56, HSE 33, and ILE8 of CYCc may interrelate in proliferative pathways of human disorders such as cancer.

The Swiss dock interaction Hydrogen bond analysis between Cholecalciferol and CYCc exhibited a substantially four number of contacts: ASP 62, GLY 56, HSE 33, and ILE8 suggesting binding stability of the interaction between CYCc and cholecalciferol. When evaluated of the four target sites, one binding site ILE8 with Cholecalciferol in the blue sticks had most stable and shortest bond length of the 2.092 A∘ (Fig 5 a-d).

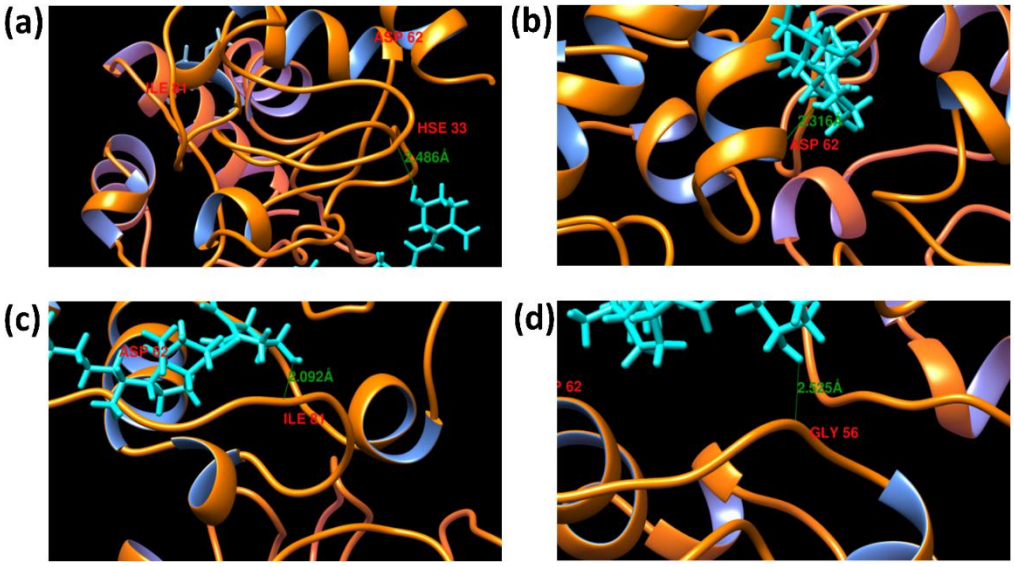

Fig. 5. Docking of Cholecalciferol with CYCc protein at receptor site (a) ASP62, (b) GLY56, (c) HSE33, and (d) ILE81.

As the model 0 is best fitted model (the lowest energy means best fit) [29], Anterior embedded region of the CYCc subunit interacts with Cholecalciferol at the anterior middle face. The notion of interactive stability of CYCc with other proteins also articulated with Yadav, who also explained the hydrogen bond analysis of neighboring subunits UQCRC1 and UQCR10 [34]. As CYCc translocates into the cytoplasm, initiates binding to the apoptotic regulator Apaf-1 coupled by dATP, resulting in the creation of apoptosomes and the completion of the death cascade [8, 9, 14], which supports our hypothesis of Cholecalciferol involvement along with CYCc.

Docking confirms that Cholecalciferol delivers efficient role in apoptotic cascade as it interacts closely in the spatial proximity of the active site of cytC. The bonding between active site of CYCc and Cholecalciferol, with the scoring values of -5.66, and-1425.01Kcal/moL fitness refers to the apoptotic activity underlying the breast cancer. Yadav reported that *in silico* the interaction between CYCc and Apaf-1 is primarily electrostatic. Side chains K13, K72, and K86 of Apaf-1 are arranged in such a way, to promote electrostatic interactions with negatively charged amino acid 's surfaces in a manner that minimizes energy loss. i.e., ASP (1147, 1106, 1064, 1945) [38]. Contrastingly, in our study the interaction between CYCc and Cholecalciferol is, majorly hydrogen bonding. In this context, it appears that alignment of both facilitate the apoptotic caspases, rationalizing the structural model of CYCc-Apaf-1 assembly suggested by Yadav [34]. According to Ismail et al [40], Breast cancer patients with Cholecalciferol deficiency had a reduced overall and progression-free survival. Low serum vitD levels were found to have a negative prognostic effect in various cohort studies, including prostate cancer, colon cancer, melanoma and breast cancer,[41]. Karkeni and colleagues concluded a study on Cholecalciferol, which plays a in reducing tumor development in the breast cancer., whereas hypovitaminosis D was related to the worse prognosis in few cancers, as supplementation with Cholecalciferol failed to subside prostate cancer among patients [20]. Abdel-Razeq indicated the Cholecalciferol status in his study, that patients presented at an advanced stage of illness manifested with low Cholecalciferol levels (46.7 % vs. 2.9 %) had larger tumor sizes [42], signifying the Cholecalciferol and Granzyme B involvement in proliferation and carcinogenesis. As variety of computational approaches (Pymol & swiss dock) have been used to anticipate the substrates of related proteases [43]. The Swiss dock interaction Hydrogen bond analysis between Cholecalciferol and Granzyme B exhibited extensively four amino acid residues: Leucine171, proline120, Tyrosine24 and Methionine34 responsible for interaction between Cholecalciferol and Granzyme B. Anterior embedded region of the Granzyme B subunit

interacts with Cholecalciferol at the anterior middle face.

Above 500 Granzyme B substrates are elucidated so far, and many more are predicted. Only direct heavy atom such as carbon, oxygen and nitrogen involved in in interactions within the range of 0.45 nm are taken into account. Comprehensive experimental identification and confirmation of the genuine substrates is required to elucidate the Granzyme B degradome [48]. In contrast, the X-ray structure of the Granzyme B revealed through the PyMol, which matched with the receptor molecule of (CYCc), as loaded, whereas the native pose of Granzyme B and CYCc is presented. The number of connections was stabilized in relation to the number of CYCc residues included in the alignment [44]. Granzyme B with proteolytic activity sequentially combines effectively with caspases and functions with specifically cleaving aspartate residues of their substrates at the carboxyl terminus [44]. Caspases and Granzyme B, like CYCc, are essentially engaged in cellular activities in apoptosis.

Apoptosome development requires BCL-2 and pro-apoptotic proteins, mitochondrial components such as CYCc. (Apoptotic events). Barczyk and workers published Granzyme B and C as a bioinformatics tool that enables score-based prediction of possible cleavage sites for caspases 1–9 and Granzyme B [9]. In addition to cleaving proteins that regulate apoptotic cell death, We detected caspases, Granzyme B specific tetrapeptide sequence patterns (P4-P3-P2-P1), and cleave proteins following the aspartate residue at P1 [43]. Granzyme B manifestation was classified as high in 46.5 percent of breast cancer patients and low in 53.5 percent of them [45]. Thus, the Granzyme B, CYCc, and Cholecalciferol as biomarkers of prediction and breast cancer-promoting mediators are reinforced by molecular docking data, based on the previously reported experimental data. It is imperative that future plans must include in silico modeling research on the interconnections between CYCc, Granzyme B, calmodulin, and calcium ions.

Conclusions

Protein interactions with the ligands provide a prognostic and diagnostic tool to assess the breast cancer. It is proposed that Granzyme B and CYCc are involved in progressive and proliferative breast cancer. Most likely the prevalence of carcinogenesis termed hypovitaminosis D condition. With USF chimaera 1.15 & Py Mol, new active sites & amino acid residues were discovered for the development of targeted cancer therapeutics. The mutual role of the Granzyme B, CYCc and interactions with Cholecalciferol revealed them as bimolecular mediators and biomarkers of Breast cancer, which may assess the efficacy of cancer therapies at cellular and tissue level and can even identify new therapeutic target proteins in clinical studies.

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