Research Paper

Molecular and Computational Studies on Apoptotic Pathway Regulator, *Bcl-2* Gene from Breast Cancer Cell Line MCF-7

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Cancer is a dreadful disease constituting abnormal growth and proliferation of malignant cells in the body. Next to lung cancer, breast cancer is the most common form of cancer affecting women. The apoptotic pathway regulators, B cell lymphoma family of protein, play a key role in various malignancies defining cancer and their constitutive expression plays an integral role in breast cancer chemotherapy. The research work discusses the identification and molecular cloning of a B cell lymphoma like gene from human breast cancer cell line. The open reading frame of the gene consisted of 965 nucleotides, encoding a protein of 380 amino acids with a predicted molecular weight of 42.5 kilodalton. The predicted physiochemical properties of the gene were as follows: Isoelectric point - 9.49, molecular formula - $C_{1893}H_{3004}N_{534}O_{548}S_{16,}$ total number of negatively charged residues, (Aspartate+Glutamate) - 26, total number of positively charged residues, (Arginine+Lysine)-39, instability index-42.08 (unstable protein) and grand average of hydropathicity is -0.202. Additionally, phobius prediction suggested non-cytoplasmic localization of the putative protein. The presence of secondary structure in the protein was determined by Memsat program. A 3 dimensional protein homology model was generated using threading based method of protein modeling for structural and functional annotation of the putative protein. Future prospects accounts for the biochemical characterization of the enzyme including *in vitro* assays on breast cancer cell line would establish the functional characteristics of the protein and its physiological mechanisms in breast cancer development and its therapeutic-target role in future.

Key words: Apoptosis, breast cancer, *Bcl-2*, molecular cloning, MCF-7, phylogenetics, protein homology modeling

Cancer, defined as malignant neoplasm of cells and tissues, affect millions and is one of the leading causes of death worldwide. According to published report of International Agency for Research on Cancer (IARC) of the World Health Organization/ IARC GLOBOCAN database (2013), 14.1 million new cancer cases were registered and approximately 8.2 million cancer-related deaths were reported worldwide in 2013. The prevalence rate indicated that 32.6 million people were diagnosed with cancer in the past five years. The percentage statistics of the diagnosed cancer types are estimated according to the frequency of occurrence: Lung cancer (1.8 million, 13.0% of the total), breast cancer (1.7 million, 11.9%) and colorectal cancer (1.4 million, 9.7%), respectively. Additionally, the future projection rates of global occurrence of cancer are estimated to be 19.3 million new cases per year by 2025, with a higher frequency in developing countries. On the physiological platform, the disease is characterized by uncontrolled growth and proliferation of abnormal cells, gradually affecting major organs of the body. The disease may result from internal factors (mutations in genome, hormones) and external factors (chemicals, tobacco, radiation). All cancers are caused by mutations or malfunctioning of genes that control cell growth and division. About 5% of all cancers are inherited while

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most of the cancers are caused due to somatic gene mutations[1]. Furthermore, the spread of malignant cells to other organs, a process known as metastasis, is responsible for 90% cancer deaths in the world[2].

Considering the rising frequency of occurrence, breast cancer is a leading cause of mortality in women leading to 5.22 lakh deaths in 2012 worldwide. According to a survey, approximately 1.7 million women were diagnosed and 6.3 million women are living with breast cancer[3]. Past decades have witnessed the prevalence of breast cancer by more than 20% and mortality rate by 14%[4]. Breast cancer is the second major cancer occurring in women (after lung cancer)[5] and is a global cause of concern. According to a survey, the global mortality rate of breast cancer was 438,000 women in 2010 and estimated to reach 747,802 women in 2030[6,7]. Several reasons can be attributed for the development of breast cancer, from genetic predispositions like obesity, genetic defects in breast cancer susceptible genes namely BRCA 1 and BRCA 2 and immunity to environmental agents like harmful radiation as well as exposures to hormonal therapies including progestin and estrogen hormones. Use of cancer cell lines is a prerequisite requirement for development of *in vitro* systems in research pertaining to breast cancer studies and drug development. MCF-7 is a breast cancer cell line isolated in 1970 from a 69 year old Caucasian woman from a pleural effusion[8] and named after Michigan Cancer Foundation; the cell line is used as cellular model for breast cancer studies. An alteration in normal course of programmed cell death mechanisms plays a critical role in the development and progression of breast cancer. The *Bcl*-2 family of proteins acts as the key regulators of apoptotic pathway in cancer. The *Bcl*-2 gene family was first identified through its involvement in B-cell lymphomas and *Bcl*-2 expression is altered by a chromosomal translocation between 14th and 18th chromosome in humans[9]. *Bcl*-2 protein family plays a key role in regulation of apoptosis including necrosis and autophagy[10,11]. The overexpression of antiapoptotic gene of the *Bcl*-2 family namely *Bcl*-2 and Bcl-xL is responsible for resistance to breast cancer chemotherapy while proapoptotic proteins like Bax promote apoptosis[12]. Studies have reported that *Bcl*-2 can prevent apoptosis initiation by a wide variety of stimuli, namely (i) neurotrophic factor withdrawal from neurons; (ii) chemotherapeutic drugs and gamma irradiation in cancer cells; (iii) cytotoxic cytokines such as tumor necrosis factor-α, Fas-ligand, and transforming growth factor-β (iv) chemicals that induce oxidative injury (v) heat shock; and (vi) calcium ionophores[13,14]. It has been suggested that *Bcl*-2 protein might regulate a final event in the common pathway involved in programmed and apoptotic cell death. *Bcl*-2 gene expression in mammary epithelial cells and in estrogen receptor (ER)-positive breast cancer cell lines is regulated by estrogens[15]. It is interesting to note that statistical analysis had revealed that breast cancer patients with positive expression of *Bcl*-2 show better survival rate as compared to *Bcl*-2 negative patients[16]. Several members of the gene family have been studied from animal species namely chicken[17], mouse[18], yeast artificial chromosome (YAC) clones, human T lymphoid cell line[19], rat[20,21] and chicken lymphoid cells[22].

The research discusses the isolation and molecular cloning of a *Bcl*-2 like gene from breast cancer cell line, MCF-7. *In silico* characterization of the putative protein predicted the physiochemical properties and protein homology modeling was employed to elucidate three dimensional structure of the putative protein. Future prospects of the study focusing on the biochemical characterization of enzyme through *in vitro* assays would help to understand the molecular mechanism underlying breast cancer and in the development of target-based therapeutic interventions against the disease.

MATERIALS AND METHODS

Cryopreservation and maintenance of cell lines:
The breast cancer cell line, MCF-7 was obtained from King George Medical University, Lucknow and maintained and stored in a cryopreservation cylinder at -196° (Nalgene). General chemicals and reagents were purchased from Sigma-Aldrich, Sisco Research Lab Ltd. and Gibco/Life Technologies. Molecular biology kits were obtained from Qiagen, Promega and Invitrogen while the restriction enzymes used were from Fermentas. Antibiotics namely penicillin and streptomycin were products of Sigma-Aldrich. The cell lines were stored in a cryopreservation cylinder at -196° and sub-cultured every week in Dulbecco modified Eagle's Medium (dMEM) according to growth of cancer cells (confluency is 50% of the culture flask) in laminar air flow chamber (Heraeus Instruments). The cells

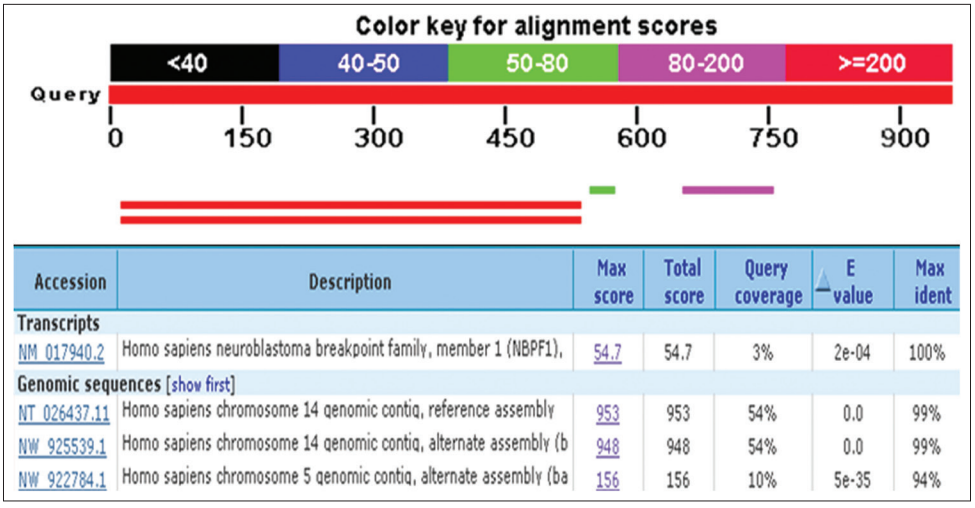
were given a quick washing with phosphate buffer saline (PBS) and proteinized with trypsin. Cells were counted in haemocytometer slide and cell density (1×10^5 cells/25 cm^2 flask approximately) were inoculated in fresh dMEM media. The culture flask was incubated in CO_2 incubator at 37°.

Genomic DNA isolation and PCR amplification:
Genomic DNA was isolated from breast cancer cells, MCF-7 by Axygen DNA Isolation Kit as per manufacturer's instructions. Nanodrop spectrophotometer was used for the qualitative and quantitative estimation of the genomic DNA by measuring relative absorbance values at 260 and 280 nm (A_{260}/A_{280}) and agarose gel (1.2%) electrophoresis. The PCR conditions were initial denaturation at 94° for 5 min, followed by 45 cycles at 94° for 1 min, annealing at 59.8° for 1 min, extension at 72° for 2 min and final extension at 72° for 5 min. The RT-PCR products were subjected to gel electrophoresis on 1.2% agarose gel.

Molecular cloning of *Bcl-2* gene:
The resolved fragments were purified through Qiagen columns and cloned in pGEMT vector (Promega) according to manufacturer's instructions[23]. The recombinant plasmid containing the cloned gene was subjected to automated sequencing using universal sequencing primers (M-13F and M-13R). The purified PCR products were then analyzed on automated capillary based DNA sequencer (3130 excel applied biosystems, USA) for validation of the respective gene.

Phylogenetic studies:
Phylogenetic analysis of the *Bcl-2* like gene was performed utilizing NCBI database (http://www.ncbi.nlm.nih.gov) and Expasy proteomics server (http://www.expasy.org). Phylogenetic tree was constructed by Phylogeny.fr at Expasy bioinformatics resource portal to establish the evolutionary relationship between *Bcl*-2 like gene and members of *Bcl*-2 gene family from different animal species[24]. Protparam and Protscale tools at Expasy Proteomics server were employed to predict the various physiochemical properties of the cloned gene such as molecular weight, molecular formula, isoelectric point (PI), amino acid composition, instability index, molar extinction coefficient, estimated half-life, aliphatic index, total number of positively and negatively charged residues and grand average of hydropathicity (GRAVY) respectively.

Protein homology modeling:
Protein modeling of the putative protein was performed through I-TASSER online program (zhanglab.ccmb.med.umich.edu/I-TASSER/)[25] for structural and functional predictions on default parameters. Structural genomics studies have revealed that many proteins have disordered regions which may interfere with their purification and crystallization. Thus, it is important to determine such regions in proteins from their amino acid sequences. The prediction of disordered residues in protein and presence of transmembrane segment was performed employing memsat program (www.sacs.ucsf.edu/cgi-bin/memsat.py) for functional annotation of



Accession	Description	Max score	Total score	Query coverage	E value	Max ident
Transcripts						
NM_017940.2	Homo sapiens neuroblastoma breakpoint family, member 1 (NBPF1),	54.7	54.7	3%	2e-04	100%
Genomic sequences [show first]						
NT_026437.11	Homo sapiens chromosome 14 genomic contig, reference assembly	953	953	54%	0.0	99%
NW_925539.1	Homo sapiens chromosome 14 genomic contig, alternate assembly (b	948	948	54%	0.0	99%
NW_922784.1	Homo sapiens chromosome 5 genomic contig, alternate assembly (ba	156	156	10%	5e-35	94%

Fig. 1: BLASTn analysis of the *Bcl-2* like gene with those reported in NCBI Database.

protein. Studies had reported that such regions may be involved in many biological processes namely cell signaling, regulation and control of cell cycle[26]. Such factors play a key role in the determination of functional aspects of protein.

RESULTS

A PCR based homology screening approach was used for the identification and isolation of *Bcl*-2 gene from human breast cancer cell line, MCF-7. PCR amplification resulted in *Bcl*-2 amplicon (965 bp) from an enriched library of MCF-7 cancer cell line. Molecular cloning of the gene in pGEMT vector and automated sequencing of the cloned fragment (965 bp) established its homology with Homo sapiens neuroblastoma breakpoint family and Homo sapiens chromosome 14 genomic contig through BLAST search (BLASTn and BLASTp) and related member of *Bcl*-2 gene family (fig. 1). The *Bcl-2* gene consisted of 965 nucleotides encoding a protein of 380 amino acids with a predicted molecular weight of 42.5 KDa. The various physiochemical properties predicted through protparam and protscale servers at expasy proteomics server (http://www.expasy.org) are listed in Table 1. The evolutionary relationship of *Bcl-2* like gene and those reported in NCBI database was elucidated by maximum likelihood method of tree construction employing Phylogeny.Fr at Expasy proteomics server (http://www.expasy.org, fig. 2).

For structural elucidation of the putative protein, threading based protein modeling approach was followed for construction of a 3D protein model via I-TASSER online tool. The active binding sites in the *Bcl-2* protein model were predicted and analyzed on default parameters following standard protocol. The secondary structure of the protein was determined by Raptor X prediction method (raptorx.uchicago.edu/, fig. 3). Furthermore, phobius prediction suggested the non-cytoplasmic localization of *Bcl*-2 like gene (fig. 4).

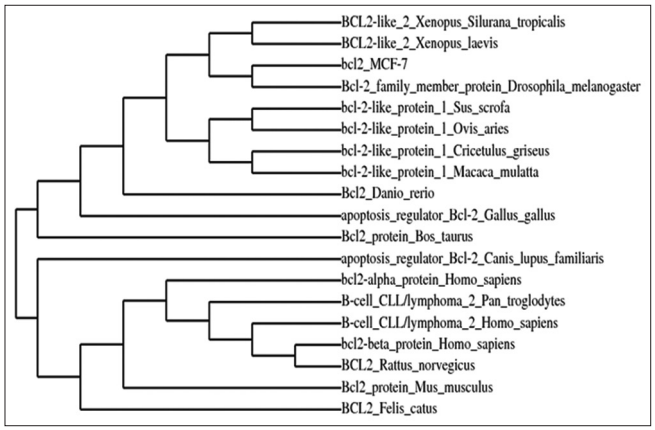


Fig. 2: Phylogenetic tree was constructed using *Phylogeny. fr* tool at Expasy Proteomics sever.
The evolutionary relationship between *Bcl-2* like protein and different members of *Bcl-2* gene family was established by maximum likelihood method of tree construction (http://www.expasy.org).

TABLE: 1 PHYSIOCHEMICAL PROPERTIES OF THE *BCL-2* LIKE GENE GENERATED BY PROTPARAM

Physiochemical properties	Values
Number of amino acids	380
Molecular weight	42.5 kDa
Theoretical pI (isoelectric point)	9.49
Amino acid composition (%)	Ala (A) 34 (8.9)
	Arg (R) 26 (6.8)
	Asn (N) 11 (2.9)
	Asp (D) 8 (2.1)
	Cys (C) 9 (2.4)
	Gln (Q) 10 (2.6)
	Glu (E) 18 (4.7)
	Gly (G) 19 (5.0)
	His (H) 10 (2.6)
	Ile (I) 14 (3.7)
	Leu (L) 34 (8.9)
	Lys (K) 13 (3.4)
	Met (M) 7 (1.8)
	Phe (F) 14 (3.7)
	Pro (P) 33 (8.7)
	Ser (S) 45 (11.8)
	Thr (T) 31 (8.2)
	Trp (W) 4 (1.1)
	Tyr (Y) 9 (2.4)
	Val (V) 22 (5.8)
	Pyl (O) 9 (2.4)
	Sec (U) 0 (0.0)
Total number of negatively charged residues, (Asp + Glu)	26
Total number of positively charged residues, (Arg + Lys)	39
Formula	$C_{1893}H_{3004}N_{534}O_{548}S_{16}$
Total number of atoms	5995
Extinction coefficients (units of M/ cm, at 280 nm measured in water)	35,910, 0.844, assuming all pairs of Cys residues form cystinesm
Extinction coefficient Abs 0.1% (=1 g/L)	
Extinction coefficient abs 0.1% (=1 g/L)	35,410, 0.833, assuming all Cys residues are reduced
Estimated half-life	1.1 h (mammalian reticulocytes, *in vitro*)
The N-terminal of the sequence considered is D (Asp)	3 min (yeast, *in vivo*)
	>10 h (*Escherichia coli*, *in vivo)*
Instability index	42.08 (unstable)
Aliphatic index	75.00
Grand average of hydropathicity	−0.202

Physiochemical properties of the *Bcl-2* like gene generated by ProtParam online server at Expasy Proteomics server (http://www.expasy.org)

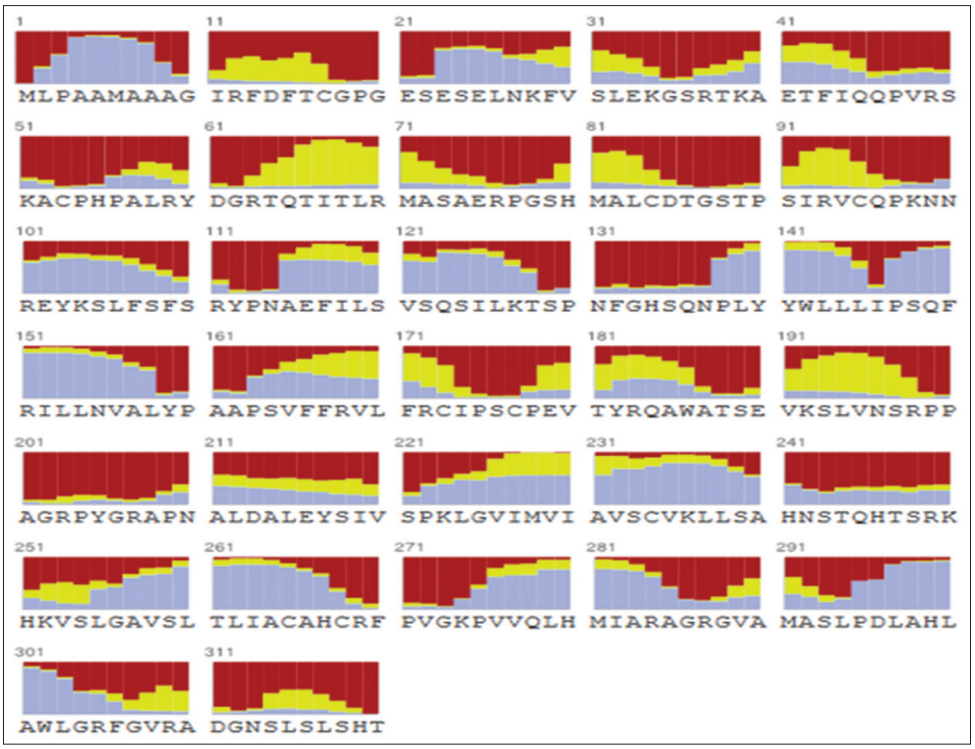


Fig. 3: Secondary structure of the *Bcl-2* was determined by Raptor X prediction method.
Secondary structure of the *Bcl-2* was determined by Raptor X prediction method from raptorx.uchicago.edu/. The color diagram shows the distribution of secondary structures and represented by various color combination as follows: Alpha helix (blue), Beta (yellow) and Loop/irregular region (red), respectively.

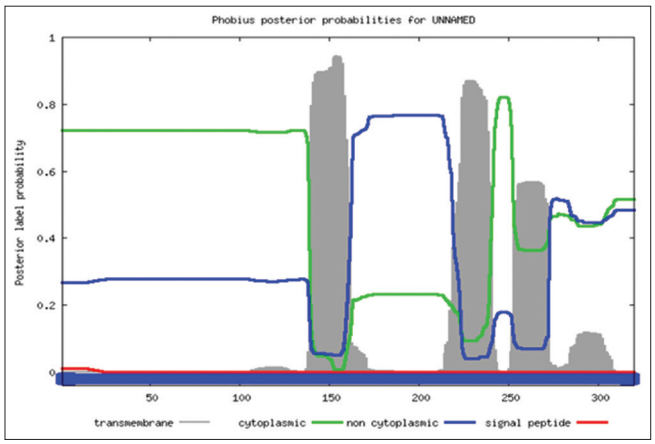


Fig. 4: Plot of phobius prediction method.
The localization of *Bcl-2* like gene and the presence of transmembrane segment were determined by Phobius prediction method (phobius.sbc.su.se/).

The presence of transmembrane segment and disordered state of *Bcl-2* gene was determined by memsat tool (www.sacs.ucsf.edu/cgi-bin/memsat.py, fig. 5).

DISCUSSION

The *Bcl*-2 families of proteins are the key regulators of apoptotic pathway in cancer. These are involved in various forms of malignancies and play a crucial role in breast cancer chemotherapy. The overexpression of antiapoptotic *Bcl-2* members namely *Bcl-2* and Bcl-xL has chemoresistance implications in cancer whereas pro-apoptotic proteins such as Bax enhance tumor cells sensitization to anticancer therapies. Moreover, the *Bcl-2* family acts diversely in apoptotic regulation, governing the key conclusive step of activation or silencing of the caspases for cell survival.

The research investigation discusses the identification, molecular cloning and bioinformatics analysis of a member of the apoptotic pathway regulator, *Bcl*-2 gene family. A *Bcl*-2 homology was isolated from MCF-7 cancer cell line employing PCR based screening approach. Automated sequencing of the cloned gene revealed it to be closely related to Homo sapiens neuroblastoma breakpoint family and Homo sapiens chromosome 14 genomic contig, respectively. Phylogenetic analysis predicted the physiochemical properties of *Bcl*-2 like gene namely molecular weight of 42.5 KDa, isoelectric point (pI)-9.49, molecular formula- $C_{1893}H_{3004}N_{534}O_{548}S_{16,}$ total number of negatively charged residues, (Asp+Glu)-26, total number of positively charged residues, (Arg+Lys)-39, instability index- 42.08 (unstable protein), total number of atoms-5995, extinction

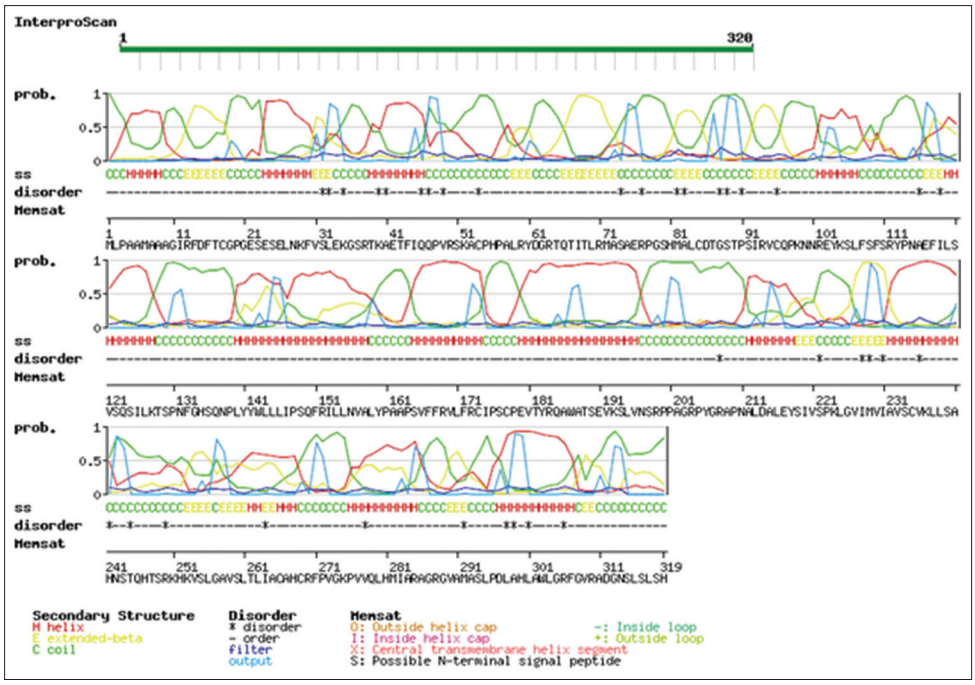


Fig. 5: Putative protein prediction through Memsat online tool.
The state of disorderliness in the *Bcl-2* like putative protein was predicted through Memsat online tool (www.sacs.ucsf.edu/cgi-bin/memsat.py). The presence of secondary structures was represented in various colors as follows: Alpha helix (red), extended beta (yellow) and coil (green) respectively. The disorderliness state in the protein is represented by a star (black) in the diagram.

Fig. 6: The 3 dimensional structure of the putative *Bcl-2* protein.
The 3 dimensional structure of the putative *Bcl-2* protein was determined *via* protein homology modeling (I-TASSER protein modeling suite) (zhanglab.ccmb.med.umich.edu/I-TASSER/).

coefficients-35910, 0.844, assuming all pairs of cysteine residues form cystines and 35410, 0.833, assuming all cysteine residues are reduced, aliphatic index-75.00 and grand average of hydropathicity (GRAVY) is -0.202 (Table 1). Further, phobius prediction suggested non cytoplasmic localization of *Bcl*-2 like gene (fig. 4) in agreement with the literature which suggest the intracellular localization of the *Bcl-2* gene including the nuclear membrane and outer mitochondrial membrane and nuclear pore complexes, mitochondrial complexes, and some parts of endoplasmic reticulum[27]. The expression of *Bcl-2* gene in breast cancer patients is controlled by estrogen in mammary epithelial cells and estrogen receptor positive breast cancer cell lines[28,29]. Studies have suggested better survival and prognosis rate in *Bcl-2* positive patients compared to the *Bcl-2* negative patients[30,31]. Although the exact mechanism is unknown, the antiproliferative effect of the gene is responsible for this phenomenon, is a prospective hypothesis. Moreover, the prediction of protein homology model of *Bcl-2* gene is significant for structural and functional annotation of the protein (fig. 6). The presence of disordered region and transmembrane segment in *Bcl-2* gene suggests its role in biological processes namely cell signaling, regulation and control of cell cycle as reported in literature[26].

The future prospects in the study highlight the requirement of biochemical characterization of the cloned gene through *in vitro* assays for defining the functional characteristics of the putative *Bcl*-2 like protein. Furthermore, protein docking studies would decipher the role of key amino acids in the protein's active site and would help to establish the functional

properties of the protein. Such studies in future would help in target based approach for drug discovery and management in breast cancer studies. Moreover, the estrogen receptor regulated expression of the *Bcl-2* gene in mammary epithelial cell suggests the expression of *Bcl-2* gene as a prospective biomarker for tumors, showing the functional aspects of hormone receptors in breast cancer patients. Another significant area correlates with *Bcl-2* expression and higher survival rate suggesting better treatment possibilities in breast cancer patients showing positive expression. The future studies should focus on the research on apoptotic proteins and their prognostic effect on a larger group of patients.

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Conflicts of interest:
No conflict of interest exists with any individual or organization.

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