**Original Research Article**

**Cloning, sequence analysis and structure prediction of B cell Lymphoma-2 of Canis familiaris**

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**ABSTRACT**

Programmed cell death (apoptosis) is used by multicellular organisms during development and to maintain homeostasis within mature tissues. One of the first genes shown to regulate apoptosis was Bcl-2. Despite overwhelming evidence that Bcl-2 proteins are evolutionarily conserved regulators of apoptosis, their precise biochemical function remains controversial. Bcl-2 has been implicated in the resistance of many cancers to treatment with radiation and chemotherapeutic agents, therefore, it represents a potential target for the treatment of cancers. The design of molecules that bind to Bcl-2 and inhibit its antiapoptotic activity could be aided greatly by the three-dimensional structure of Bcl-2 and Bcl-2–ligand complexes. The present study describes cloning, sequence analysis and molecular modelling / 3D-structure prediction of partial canine Bcl-2. The cloned sequence consists of an open reading frame of 153 amino acid residues. Sequence alignment of canine Bcl-2 with other species revealed a number of conserved regions across the species.

**Keywords**

Bcl-2, apoptosis, sequence analysis, structure prediction

**Introduction**

Programmed cell death plays a significant role in a variety of physiological processes, including for example, elimination of self-reactive lymphocytes, removal of redundant cells during development, and eradication of old differentiated cells in most adult tissues with self-renewal capacity (1,2). One of the major controllers of programmed cell death and its morphological equivalent “apoptosis”³ is the Bcl-2 gene. Bcl-2 is the founding member of a family of proteins involved in apoptosis that was identified originally at the breakpoint of t (14;18) translocation in a lymphocytic leukemia cell line(4,5). Bcl-2 and other members of the family play an important role in embryogenesis, tissue remodelling, and the immune response through their actions as either inhibitors or promoters of...
apoptosis (6,7). There are at least 16 Bcl-2 homologues found in humans (8). These include Bcl-2, Bcl-xl, Bcl-w, and Mcl-1, which are inhibitors of cell death, and Bad, Bak, Bax, Bid, Bim, and Bcl-xS, which are cell-death promoters. The antagonistic interaction of these antiapoptotic and proapoptotic proteins maintains homeostasis in normal tissues (9).

In addition to their normal function, aberrant expression of Bcl-2 proteins has been linked to many diseases such as autoimmunity and neurodegenerative disorders and cancer (10,11). Indeed, Bcl-2 has been found to be overexpressed in many cancer cells, including most B cell-derived lymphomas, colorectal adenocarcinomas, and undifferentiated nasopharyngeal cancers (12). Bcl-2 has also been implicated in the resistance of many cancers to treatment with radiation and chemotherapeutic agents (13). Therefore, Bcl-2 represents a target for the treatment of cancers, especially those in which Bcl-2 is over expressed and for which traditional therapy has failed (12,14,15). The design of molecules that bind to Bcl-2 and inhibit its antiapoptotic activity could be aided greatly by the three-dimensional structure of Bcl-2 and Bcl-2 ligand complexes. This present study was carried out in order to sequence analysis of canine Bcl-2 orf region and further to locate conserved areas on canine Bcl-2, phylogenetic analysis, deduce its biochemical properties along with structure prediction and its validation.

Materials and Methods

Bacterial Strains and Plasmids

E. coli Top10 cells were obtained from Invitrogen, Life Technologies and were made competent by RbCl (Rubidium Chloride) method. LB broth and LB agar were procured and prepared as per the manufacturer’s recommendations (HiMedia, Mumbai). For cloning experiment pCR®2.1-TOPO® cloning vector (Invitrogen, Life Technologies) was used.

Sample collection and RNA extraction

Tissue sample (mammary gland tissue) for RNA extraction was obtained from dog (Spitz breed) who had been admitted to the Department of Veterinary Surgery and Radiology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana and was stored in ‘RNA later’ at -80°C for RNA extraction. RNA was extracted with TRI Reagent-Ambion (1ml/100mg tissue) as per manufacturer’s instructions. RNA concentration (quantification) was measured using Qubit® 2.0 Fluorometer (Invitrogen- Life Technologies).

cDNA Synthesis and designing of primers

For preparation of cDNA from RNA template, ‘First strand cDNA synthesis Kit (Fermentas, USA)’ was used. In order to amplify a part of Bcl-2 orf, a degenerate primer set was designed after thorough clustal analysis (ClustalW) of sequences of Canis familiaris, Homo sapiens, Felis catus, Bos taurus, Sus scrofa, Mus musculus available from NCBI (National Center for Biotechnology Information, USA) database. Primers were custom synthesized from IDT, India. The sequence of the primer pair used was:

Bcl2-KF 5’CRCAGMGGGGCTACGAGTGG 3’
Bcl2-KR 5’TGCCGGGTTACGATCTCRGTCATC 3’

Where R= A/G and M= A/C
Second set of primer pair was selected to amplify another fragment of Bcl-2 gene whose forward primer (Bcl2-RF) overlapped with the reverse degenerate primer (Bcl2-KR) which was used to amplify the first Bcl-2 orf fragment. The sequence of primer pair was as follows:

Bcl2-RF 5’ TGGATGACTGAGTACCTGAA 3’
Bcl2-RR 5’ GGCCTACTGACTTTAT 3’

**Polymerase chain reaction**

**PCR conditions using first set of primers (Bcl2-KF/Bcl2-KR)**

The PCR assay was optimized using 25µl reaction mixture to get a single specific amplicon of desired size. Final standardized PCR reaction contained 1X PCR buffer, 2.5mM MgCl2, 200µM dNTP mix, 2µM each of forward and reverse primers, 1.5U of Taq DNA polymerase and 100 ng of template cDNA. Cycling conditions standardized for PCR amplification included one cycle of initial denaturation at 94°C for 5 min followed by 35 cycles each of denaturation (94°C for 1 min), annealing (56°C for 1 min) and extension (72°C for 1.5 min) followed by final extension at 72°C for 10 min.

**PCR Conditions Using Second Set of Primers (Bcl2-RF/Bcl2-RR)**

Final 25µl reaction mixture contained 1X PCR buffer, 2.5mM MgCl2, 200 µM dNTP mix, 0.8 µM each of forward and reverse primers, 1.25U of Taq DNA polymerase and 100 ng of template cDNA. The PCR cycling conditions were: one cycle of initial denaturation at 95°C for 5 min followed by 40 cycles each of denaturation (95°C for 30 sec), annealing (52°C for 20 sec) and extension (72°C for 35 sec) followed by final extension at 72°C for 10 min. The PCR products were visualized after electrophoresis at 80 V for approx. 1 hour. Electrophoresis was carried out on 1.5% agarose gel prepared in 0.5X TBE buffer and containing 0.5µg/ml ethidium bromide and photographed on ChemiDoc XRS gel documentation system (Biorad, USA).

**Cloning of canine Bcl-2 orf fragments**

After PCR standardization, Bcl-2 orf fragments were amplified in bulk (100µl each) under similar conditions as described earlier. For cloning purpose, extraction and purification of amplified PCR product was done using Qiagen gel extraction kit as per manufacturer’s protocol (Qiagen, Germany). Purified Bcl-2 fragments were ligated into linearized pCR™2.1-TOPO® TA vector. Reaction was set up on ice and after mixing, ligation mixture was incubated for 30 minutes at room temperature (22–23°C). Competent E. coli Top10 cells prepared by rubidium chloride (RbCl) method were transformed with ligated plasmid. Heat shock was given at 42°C for 60 sec followed by immediate cooling of the tubes on ice for 5-10 min. The micro centrifuge tubes (MCTs) were tightly capped and were shaken horizontally (200 rpm) at 37°C for 1 hr after adding 250 µl of LB. The cells were plated on to LB agar plates containing ampicillin (100µg/ml) and X-gal (80µg/ml) for blue white selection and incubated at 37°C for 12-16 hrs. Next day, white recombinant colonies were picked and inoculated in LB containing ampicillin. Plasmid was isolated by alkaline lysis method and restriction digestion of the isolated plasmids was carried out with EcoRI enzyme at 37°C for overnight. Resultant inserts were analyzed on 1.5% agarose gel. Clone with the specific insert was selected and stabbed in buffered semisolid agar with ampicillin.
After the overnight growth, stab culture was stored at 4°C.

Commercial Sequencing and Submission of Bcl-2 gene fragments’ Sequence to NCBI Stab-cultured clones were sent for sequencing to “DNA sequencing facility” at Department of Biochemistry, Delhi University, New Delhi (India). The two sequences obtained were overlapped and submitted to NCBI GenBank.

Sequence Analysis, Phylogenetic Analysis and Structure Prediction

The amino acid sequence was deduced with the program EditSeq (DNA star, Madison, WI, USA). The above sequence was subjected to BLAST search using the blastp program against the SWISS-PROT database and from the top 50 hits, representative protein sequences of dog, cat, pig, rat, mouse, horse, cattle, sheep, fish, rabbit and human were chosen for the for alignment study. The MegAlign (DNA star, Madison, WI, USA) program using Clustal W was used for the generation of the multiple sequence alignments.

The nucleotide sequence was analyzed and compared to the gene sequences for the Bcl-2 protein available at GenBank (www.ncbi.nlm.nih.gov) using NCBI online Nucleotide BLAST program and from the top 50 hits, representative nucleotide sequences viz. Canis familiaris (NM_001002949.1 and AY509563.1), Homo sapiens (NG_009361.1, NM_000633.2 and NM_000657.2), Felis catus (NM_001009340.1), Bos taurus (NM_001166486.1), Sus scrofa (AB271960.1), Mus musculus (BC095964.1) were chosen for the alignment study. The UPGMA, Neighbor-Joining and Minimum evolution programs in the Mega 4.119 package were used for the phylogenetic analysis and a phylogenetic tree based on the evolutionary distances was constructed. The Bootstrapping option with 1000 replicates was used with the p-distance model and with pair-wise deletion of the gaps/missing data. Canis familiaris Bcl-2 protein structure was predicted by SWISS-MODEL Workspace (http://swissmodel.expasy.org/workspace/) using PDB viewer software.

Results and Discussion

PCR Amplification and Cloning

The yield of RNA obtained varied from 220–550 ng/µL. The quantity of total RNA taken for the cDNA synthesis was 2000ng for each sample. Efficiency of cDNA synthesis was tested by running GAPDH PCR, the primers for which were provided with the cDNA synthesis kit itself. PCR amplification of Bcl-2 gene using first set of primers resulted in a specific band of ~463 bp size (Fig. 1). The PCR product was gel purified successfully, evident by a single specific band of the eluted product in agarose gel electrophoresis. The transformation of ligated product resulted in development of mostly white colonies on the selective medium with few blue colonies indicating the optimum transformation efficiency. Plasmid extracted from the randomly selected six white colored colonies released a gene insert of ~463 bp on digestion with EcoRI (Fig. 2). Subsequently one clone having the confirmed plasmid was got sequenced. PCR amplification with the second set of primer pair resulted in amplification of ~207 bp product (Fig. 3). This purified PCR-product was cloned and sequenced in a similar manner as the other product. The initial sequence of ‘second set primer amplified product’ overlapped with the...
concluding sequence of ‘first set primer amplified product’. So, both the sequences were combined and a single nucleotide sequence was obtained. The resulting sequence was submitted to NCBI GenBank and an accession number (KC152952) was obtained.

**Sequence Analysis**

The nucleotide sequence and predicted amino acid sequence of canine Bcl-2 were aligned (Fig. 4 and 5) with the sequences of other species. In Fig. 4, the regions that are similar and conserved are shown by boxes and the regions with a number of substitutions have been shown by circles. The entire nucleotide sequence of canine Bcl-2 orf nucleotide sequence showed 98% homology with that of cat, 96% with cattle, 95% with pig, 94% with mouse and 93% with human. The amino acid sequence of 153 residues of dog Bcl-2 gene showed 86.3% similar to that of cattle, 93.1% to cat, 91.1% to mouse, 95.3% to pig and 94.4% to human sequence. Bcl-2 family of proteins can be defined by the presence of conserved sequence motifs20 that is evident from the sequence alignment results. The unraveling of the dog genome in 2005 set the foundation for comparative studies in many diseases, given the high homology between canine genome and its human counterpart.

**Phylogenetic Analysis**

The UPGMA tree did not produce a clear cut grouping. The neighbour joining and the minimum evolution methods were consistent in generating trees with similar topologies. The neighbor joining minimum evolution tree for the nucleotide sequences (Fig. 6) had relatively higher bootstrap values providing clear resolution of all the nodes. It was found that ruminants and monogastrics are derived from different clusters according to their closer evolutionary relationship. Among ruminants, cattle and sheep might have evolved from a common ancestor; pig positioned in between and diverged early from the bovid ancestors.

Thus, Bcl-2 might have evolved by positive selection among these species. Danio rerio and Salmo salar; fish sequences show dissimilarities suggesting different ancestry with an accelerated loss of trabecular bone, sites with a disproportionate.

**Biochemical Properties of Bcl-2 Protein Based on Deduced Amino Acid Sequence**

Predicted Bcl-2 protein (Fig. 7) of canine possesses molecular weight of 17230.78 daltons with 153 amino acids, out of which 11 are strongly basic (+) amino acids (K,R) and 15 strongly acidic (-) amino acids (D,E). Further, 63 amino acids are hydrophobic (A,I,L,F,W,V) and 32 (N,C,Q,S,T,Y) are polar in nature. Isoelectric point of Bcl-2 of canine is 6.060 and has got charge of -3.135 at pH 7.0. Bcl-2 family proteins are characterized by distinct domains. All members possess at least one of the four motifs known as Bcl-2 homology domains (BH1 to BH4).

Most pro-survival members which can inhibit apoptosis facing a wide variety of cytotoxic insults, contain at least BH1 and BH2 domains; those most similar to Bcl-2 have all four BH domains. All the pro-apoptosis family members possess BH3 domain which is the central domain22.
**Figure 1** PCR amplification of Bcl-2 gene fragment by KF/KR primer set (Lane 1 - PCR amplicon of Bcl-2 gene fragment from canine, M- TrackIt™ 100 bp DNA Ladder (Invitrogen))

**Figure 2** Restriction digestion of cloning vector by *EcoRI* carrying Bcl-2 gene insert (Lane 1, 2, 3, 4: *EcoR I* digested plasmids from clones containing Bcl-2 gene insert, M- TrackIt™ 100 bp DNA Ladder (Invitrogen))

**Figure 3** PCR amplification of Bcl-2 gene fragment by RF/RR primer set (Lane 1 - PCR amplicon of Bcl-2 gene fragment from canine, M- GeneRuler™ 100 bp plus DNA ladder (Fermentas, USA))
**Figure 4** Nucleotide multiple sequence alignment done with ClustalW method of MegAlign module of DNASTar Version 4.1 Inc. USA
Figure 5 Nucleotide multiple sequence alignment done with ClustalW method of MegAlign module of DNAsat Version 4.1 Inc. USA
Figure 6 Phylogenetic tree showing evolutionary relationship between Bcl-2 of canine and other species.
Figure 7 Physicochemical Properties of Bcl-2 orf

Figure 8 Canine Bcl-2 protein structure was predicted by SWISS-MODEL Workspace (http://swissmodel.expasy.org/workspace/) using PDB viewer software
Figure 9 Ramachandran plot of the predicted model of Canine Bcl-2 protein
Structural Model and Overall Architecture of Bcl-2

The ‘QMEANscore’ which is the global score of whole model reflecting the reliability of the predicted model from 0-1, was found to be 0.73 in predicted model of dog Bcl-2 (Fig. 8) which is a good score for a partial protein sequence. The assessment of the predicted model using the Ramachandran plot showed that the modelled structure has 93.4% residues in the most favorable regions, 6.1% residues occurring in the allowed regions and 0.5% of the residues in the disallowed regions. Such figures assigned by Ramachandran plot represent a good quality of the predicted model (Fig. 9). Ramachandran plot shows one labelled residues out of 218, whereas chi1-chi2 plots show one labelled residues out of 140. The main chain and side chain parameters for all of them were found to be concentrated/convoluted in the ‘better’ region. Two bad contacts were detected in the modelled structure.

To define a model reliable, the score for G-factor (a log odds score based on the observed distribution of stereochemical parameters such as main chain bond angles, bond length and phi-psi torsion angles) should be above -0.50. The observed G-factor score for the present model was 0.18 for dihedrals bonds, 0.36 for covalent bonds and 0.25 overall, assuring the reliability of the predicted model. The distribution of the main chain bond lengths and bond angles were 99.7% and 98.5% within the limits, respectively. The modelled structure of Canis familiaris Bcl-2 protein is comparable to the structurally resolved Homo sapiens Bcl-2 protein, wherein structural motifs have been identified to remain conserved.

The genetic and biochemical studies support Bcl-2 to be an interesting, potential drug target in a remarkably wide range of diseases. The present study reveals the biochemical properties of canine Bcl-2 along with its sequence analysis and structure prediction which may aid into new horizons for the drug designing or targeting this protein in various diseases or cancers.

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