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An In-situ Mutational Analysis of BCL-2 Gene in Pets Cancer

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Abstract

To cure various cancers in veterinary medicine, the BCL-2 gene and its polymorphisms are very well known due to its anti-apoptotic and anti-cancerous activity. In the current study, different polymorphisms of the BCL-2 gene were studied to find a cure for lymphoma, TVT, and other cancers in dogs and cats. Various parameters are used in the present study, which aims to investigate coding regions of Bcl-2gene for analyzing cancer mutations. Different in-vivo and in-silico tools, including DNA extraction, PCR, spectrophotometry, NEB cutter online tool, and BLAST, were used to analyze the blood samples of 15 dogs and cats to find any association between BCL-2 and cancers. However, no significant result was reported, and couldn't find any link between polymorphisms of the BCL-2 gene with lymphoma, mammary gland tumor, oral tumor, peritoneal adenocarcinoma, and transmissible venereal tumor (TVT) in exons 1 and 2. In conclusion, further study with much larger blood and tissue samples is required to determine the polymorphisms and accurately identify the associated factors. There is a need to explore the other gene

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mutations causing cancers in the pet population, which will ultimately help develop genetic counselling strategies, gene therapies, and prenatal diagnostic procedures for the pet population.

Keywords: BCL-2 gene, Polymorphism, Cancer, Canine, Feline

1 Introduction

Veterinary and human health are both greatly affected by cancer diseases due to their significant impact (Chan & Rico, 2019; Ostrander et al., 2019). Aside from improving canine cancer therapy, research on cancer pathogenesis in pets such as dogs and cats is also translating into human oncology. Apoptosis disruption in cells that have undergone tumor transformation is a renowned process causing the progression of cancer (Carneiro & El-Deiry, 2020; Lang et al., 2019). The B-cell lymphoma 2 (BCL-2) family of proteins is one of the key players in this procedure, and an imbalance of pro- and antiapoptotic members contributes to cancer growth.

The Bcl-2 family, which shares over 99% of its amino acid sequence with human proteins, has been found to be crucial for human as well as canine cell function (Pawlak, & Henklewska, 2020; Wyżewski et al., 2020). The information from research on cats and dogs can be employed in investigating other species of organisms, such as humans. It has also been demonstrated that the aforementioned protein family plays a part in the emergence of cancer (Mohibi et al., 2019). There are various types of tumors associated with cats and dogs which are responsible for death of these pets. Metastasis to tissues like skin, nasal passage and oral cavity have also been reported in 5-6.9 percent of cases (Harmelin et al., 1995). To cure these fatal pathological diseases there is a great need to find out the cause of these diseases.

The BCL-2 is an interrelated family, comprises of pro- along with anti-apoptotic members. BCL-2 kin proteins carve up structural homology, because they all restrain as a minimum one BCL-2-homology domain, namely BH1, BH2, BH3 and/or BH4 (Zhai et al., 2020). The anti-apoptotic pro-survival protein BCL-2 is known to be associated with more aggressive behavior in humans, dogs, and cats with cancer (Correia et al., 2015; Edwards, & Johnson, 2021; Hardwick, 2021). The presence of BCL-2 has been linked to a better outlook for individuals with breast cancer in terms of disease-free life expectancy and overall survival (Avery, 2020; Dagher et al., 2019). The pro-apoptotic proteins of the BCL-2 family, given as BAX, BAD, BID as well as BCL-XS, smooth the progress of programmed cell death, whereas the anti-apoptotic members, given as BCL-2, BCL-XL along with Bcl-W put forth a contradictory deed, for that reason hindering commencement of apoptosis (Fendri et al., 2011; Pawlak & Henklewska, 2020). Among mammals' family of protein of Bcl-2 gene there are slightest 30 diverse interrelated proteins that consecrate by turnout of four relatively little succession model named as BCL-2 homology (BH) domains (Tzifi et al., 2012). The work on this gene has been done in developed countries and most of the research deciphered that mutations in this gene make it susceptible candidate gene for cancer. The objectives of this study were: (i) To study the reported and novel mutations in the BCL-2 gene isolated from the normal and diseased dog (Canis famiriaris) and cat (Feline catus) (ii) to Make an association between mutations (if found) in BCL-2 gene and different cancer types including lymphoma, MGT, TVT, PAC, and OT. In future, this work is aimed to be useful in designing anti-cancerous drugs according to genotypic makeup of the pets.

2 Materials and Methods

The present study was conducted to find out the mutations in BCL-2 gene in cancer pets' patients. A total of 15 dog (Canis famiriaris) and cat (Feline catus) tumor patients identified from the pet center of University of Veterinary and Animal Sciences, Lahore and other private pet clinics in Lahore, and were enrolled in the present study. The work was carried out in Molecular Cytogenetic

and Genomics Laboratory in the Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences, Lahore. Both sexes were included, and all the patients were less than 14 years old by their age. The owners of the included patient pet agreed to take part in the present study and allowed to collect blood samples (3-5 mL) of affected patients. A consent form was signed by the owners and they were personally interviewed for information on clinical history, behavior of animals and their diet.

2.1 Data Collection

For this study, cats and dogs with diagnosed cancer were taken. History of all available patients were documented by asking relevant questions about pets from the pathologist, medical history or several other vital information interrelated to cancer, to diminish the existence of other idiosyncrasy and ecological grounds for the disarray. The printed clued-up consents were acquired as of all pretentious patients containing a variety of parameters like sexual category, age at inception, epoch at examination, conditions, number and brutality of malignancy and numerous others.

2.2 Blood Sampling, DNA Extraction and DNA Quantification

Blood samples were drawn from patients in a 5mL vacutainer tube containing ethylenediamine tetra-acetic acid (EDTA) (0.5M), as anticoagulant. Then the DNA was extracted from the samples and quantified by spectrophotometer.

3 Primer Designing

Primers were designed with the help of Primer3 software (http://frodo.wi.mit.edu/primer3/), via using the sequence information accessible at the NCBI website (www.ncbi.nlm.nih.gov) concerning the BCL-2 gene (Accession # NC_006583.3) found on chromosome 1 in dog and (Accession # NC_018734.1) found on chromosome D3 in cat. Mutation discovery was performed by the resequencing of amplicons from the affected individuals. These primers were blasted against the whole pet's genomes to check nonspecific binding at any other loci. Primer sequences were optimized using an online tool, OligoCalc (http://www.basic.northwestern.edu/biotools/oligocalc.html). Specificity of the primers was checked by insilico PCR online tool available at the University of California, Santa Cruz (UCSC) genome browser (http://genome.ucsc.edu/cgi-bin/hgPcr).

Table 1: Primer table (Bcl-2)

Sr. No.	Primer Name	Length (bp)	Primer Sequence (5'-3')	TM	Product Size (bp)
1	DBCL2F	20	GCAAAGCACATCCAATAAAA	56.4	445
	DBCL2R	18	GGACATCTCGGCGAAGTA	57.2	
2	DBCL2F	19	CACCTGTGGTCCACCTGAC	59.98	448
	DBCL2R	18	GCAACCCGACTGCTCTTC	59.50	
3	DBCL2F	20	TTTACTTTTCCCCACGTCTG	57.73	416
	DBCL2R	20	ATAGCTGATTCGACGCTGAG	58.23	

4 PCR Optimization and Amplification

After primer designing, PCR optimization of all primers (forward and reverse) was done. In the next step, both forward and reverse primers were used to amplify 15 DNA samples. Amplification was confirmed by agarose gel electrophoresis.

4.1 Precipitation of PCR Products for Sequencing

After the amplification of the desired portion of the DNA, PCR products were precipitated to see the concentration of DNA per µL.

4.2 Precipitation of Sequencing Samples

After the precipitation of PCR, products were sequenced on the principle of Sanger's chain termination method.

5 Analysis of Sequencing Samples

Analysis of the sequences was done with the help of appropriate Bioinformatics softwares and tools. Sequences were analyzed manually by using BioEdit software version (V.7.0). Nucleotide BLAST program available at NCBI website (http://www.ncbi.nlm.nih.gov/BLAST) was used for sequencing homology searches in public databases (Altschul et al., 1990). The sequences were also BLAST against the reported reference sequence. Polymorphism was detected from the sequences by multiple sequence alignment using ClustalW2 online tool available at EBI (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Any change in the DNA sequence was confirmed by sequencing both sense and antisense strands. Restriction analysis was also performed for mutated sequences using NEBcutter online tool available at BioLabs (http://tools.neb.com/NEBcutter2/).

6 Results

The principal goal of this study was molecular characterization of BCL-2 gene in different canine and feline tumors in order to identify SNPs as it is considered to have association with certain types of mutations that can cause different types of tumors. In total, 3 pairs of primers were designed and synthesized to amplify the exons 1 and 2 of BCL-2 gene against 15 DNA samples taken from dogs and cats.

After the amplification of the desired regions of the DNA, PCR products were precipitated and loaded on 1.2% gel to see the concentration of DNA. The purpose of precipitation was to eliminate the nonspecific products from the amplified sample. These precipitated products were than sequenced for mutational analysis. The sequences of amplified BCL-2 gene fragments were aligned with the help of software Blast2 in the NCBI database (http://www.ncbi.nlm.nih.gov). All three amplifying and sequencing primer sets were used for the analysis of mutation in exon 1 and 2.

6.1 Results of exon 1

All 15 samples of both cats and dogs were read carefully with the help of BLAST to find mutations in Exon 1 of BCL-2 gene. None of them showed any significant results with no mutation in exonic region as well as in flanking region. These samples were tested against different kinds of cancers including lymphoma, MGT, TVT, PAC, and OT. The details of all mutations and their relationship with tumor type are given in Table 2.

Table 2: Mutation Details of exon 1

Sample no.	Exon no.	Pet Nama	Tumor type	Age (years)	Sex	Exonic mutation	Flanking region mutation
DP1	1	(*Canis famiriaris*)	Lymphoma	7	Male	NO	NO
DP2	1	*Canis famiriaris*	MGT	9	Female	NO	NO
DP3	1	*Canis famiriaris*	TVT	6	Female	NO	NO
DP4	1	*Canis famiriaris*	MGT	6	Female	NO	NO
DP5	1	*Canis famiriaris*	MGT	8	Female	NO	NO
DP6	1	*Canis famiriaris*	TVT	8	Male	NO	NO

DP7	1	*Feline catus*	MGT	12	Female	NO	NO
DP8	1	*Canis famiriaris*	PAC	13	Male	NO	NO
DP9	1	*Canis famiriaris*	OT	2	Male	NO	NO
DP10	1	*Canis famiriaris*	MGT	10	Female	NO	NO
DP11	1	*Canis famiriaris*	MGT	11	Female	NO	NO
DP12	1	*Feline catus*	MGT	8	Female	NO	NO
DP13	1	*Canis famiriaris*	PAC	10.5	Male	NO	NO
DP14	1	*Canis famiriaris*	PAC	3.5	Female	NO	NO
DP15	1	*Feline catus*	MGT	3.5	Female	NO	NO

(1) Mammary Gland Tumor (MGT)
(2) Oral tumor (OT)
(3) Transmissible venereal tumor (TVT)
(4) Perinealadinocarcinoma (PAC)

6.2 Results of Exon 2

After analyzing the Exon 2 of BCL-2 gene of dog and cat, it was concluded that no exonic or flanking region mutations were reported in this region like exon 1. No significant relationship between BCL-2 gene and the cancer was found in both dogs and cats. The details of the all 15 samples of exon 2 are given below (Table 3):

Table 3: Mutation Details of exon 2

Sample no.	Exon no.	Pet Nama	Tumor type	Age (years)	Sex	Exonic mutation	Flanking region mutation
DP1	2	(*Canis famiriaris*)	Lymphoma	7	Male	NO	NO
DP2	2	*Canis famiriaris*	MGT	9	Female	NO	NO
DP3	2	*Canis famiriaris*	TVT	6	Female	NO	NO
DP4	2	*Canis famiriaris*	MGT	6	Female	NO	NO
DP5	2	*Canis famiriaris*	MGT	8	Female	NO	NO
DP6	2	*Canis famiriaris*	TVT	8	Male	NO	NO
DP7	2	*Feline catus*	MGT	12	Female	NO	NO
DP8	2	*Canis famiriaris*	PAC	13	Male	NO	NO
DP9	2	*Canis famiriaris*	OT	2	Male	NO	NO
DP10	2	*Canis*	MGT	10	Female	NO	NO

		famiriaris					
DP11	2	*Canis famiriaris*	MGT	11	Female	NO	NO
DP12	2	*Feline catus*	MGT	8	Female	NO	NO
DP13	2	*Canis famiriaris*	PAC	10.5	Male	NO	NO
DP14	2	*Canis famiriaris*	PAC	3.5	Female	NO	NO
DP15	2	*Feline catus*	MGT	3.5	Female	NO	NO

(1) Mammary Gland Tumor (MGT)
(2) Oral tumor (OT)
(3) Transmissible venereal tumor (TVT)
(4) Perineal adinocarcinoma (PAC)

7 Discussion

Research on the polymorphisms in genetic code has a great scope in the medicine field and more specifically it is getting attention in veterinary medicine to cure various types of cancers in pets (Campion, & Dowell, 2019; Martinez et al., 2008). Many examples of finding therapeutic ways to cure cancers in canines and felines can be found in recent research studies. The present study was aimed to investigate the relationship of one of the renowned polymorphism of BCL-2 gene i.e., (-938C > A) with the different kinds of cancer in dogs and cats however, no significant result has been reported regarding this gene yet (Al-Zubaidy et al., 2022).

Polymorphism functions as a modifier of breast cancer development; in this study, the distribution of genotype regularity, as well as the association of genotype with clinic-pathological properties were analyzed (Hamdi et al., 2018; Shadrina et al., 2016). In addition, it was also examined that the upshot of SNP on BCL-2 expression in-vitro. The BCL-2 (-938C > A) was genotyped in 114 patients and 107 controls, and analyzed the estrogen receptor (ER), progestogen receptor (PR), C-erbB2 and Ki67 status with immune-histochemistry (IHC). Different BCL-2 protein levels in breast cancer cell lines were determined using western blot analysis (Du et al., 2018). Logistic regression model was applied in statistical analysis. It was found that homozygous AA genotype was associated with an increased risk (AA vs AC+CC) by 2.37- fold for breast cancer development and significant association was observed between nodal status and different genotypes of BCL-2 (-938C > A) ($p = 0.014$). AA genotype was more likely to develop into lobular breast cancer ($p = 0.036$). The result of western blot analysis indicated that allele A was associated with the lower level of BCL-2 expression in breast cancer cell lines. AA genotype of BCL-2 (-938C > A) is associated with susceptibility of breast cancer, and this genotype is only associated with the nodal status and pathological diagnosis of breast cancer. The polymorphism has an effect on Bcl-2 expression but needs further investigation (Paul et al., 2023).

In literature, description of no important connection was practically caught between BCL-2 expression status as well as BCL-2 (-938C > A) polymorphism ($p = 0.485$). Consequently, the connection of BCL-2 (-938C > A) polymorphism by way of BCL-2 expression into prime cancer was supposed to be considered into a bigger specimen size as well as the concerned molecular methods necessitate additional exploration (Al-Zubaidy et al., 2022; da Silva Lawisch et al., 2022). Along with it except lymphoma all of the tumors are tissue related so, it also requires tissue samples to find out the polymorphism.

As per results, no significant result was seen as this was a descriptive study to find out the relation of BCL-2 gene, its polymorphisms and their relationship with multiple types of cancers. However, the variability found in this study may be due to some other factors like it requires tissue sample instead of blood, heterogeneity present at genomic level and/or some clinical variables might also be involved that were not readily identifiable this time because the sample number of heterogeneous patients were very small. Hence a much larger sample number will be required to accurately identify associated factors. Some electrophysiological experiments are also needed to revise the effects of this polymorphism and to analyze whether after this mutation in Bcl-2 gene would express its normal function properly.

8 Conclusion and Future Perspectives

In the last few decades, several mutations in many genes have been identified that were associated with lymphoma, mammary gland tumor, oral tumor, perineal adenocarcinoma and TVT. As per results, no significant result was seen as this was a descriptive study to find out the relation of BCL-2 gene, its polymorphisms and their relationship with multiple types of cancers. This study has opened a new avenue in medical sciences in Pakistan, which will help the scientist to work on genetic disease following the methodologies used in this study. The outcome of this study can be further used to confirm the hypotheses through human and other animal modeling and proteomics. This study has few limitations including it has not revealed any genetic factors involved in these diseases in blood sample because these were tissue related diseases except lymphoma. Moreover, no genetic factor involvement in lymphoma was shown.

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