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COX-2 AND BRCA1 HAVE ALTERED EXPRESSION PROFILE IN DIFFERENT CANINE TUMORS

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ABSTRACT

Cancer originates from the unnatural divisions of cells that have constant mutations in the genes which are involved in cell propagation and endurance. Dogs are beloved pet animals and they suffer from many cancers like humans. Mutated BRCA1 and COX-2 abnormal gene expression have been seen in a large number of cancers previously. In this research gene sequencing and gene expression of COX-2 and BRCA1 genes in 22 different canine tumors and 25 normal canine samples were performed. All samples were collected from Punjab Province, Pakistan. The whole coding region of COX-2, comprised of almost 1812bp with ten exons in dog and the hot spot N (1, 2 and 12 exons) and C- terminal (18, 19, 20, 21 and 22 exons) of BRCA1 gene which include 2024bp were included in mutational studies. There was no mutation in the coding region of COX-2 and 5'- and -3' region of BRCA1. The gene expression of COX-2 and BRCA1 in all aforementioned tumors was measured by real time qPCR. BRCA1 high grade negative expression and COX-2 positive gene expression were observed in this study. About 45% tumors showed up-regulation of COX-2 and four tumors showed highest gene expression, two CTVTs, one head tumor and one mammary tumor with fold changes 942.93, 122.79, 85.95 and 44.49 respectively. All granulomas showed down-regulation of COX-2 gene. It has been observed during this study that the loss of BRCA1 gene expression and up-regulation of the COX-2 gene may have a role in canine tumors. Altered expression of COX-2 gene in tumors may have link with the extent of aggressive tumor types. The mechanism behind the abnormal function of these genes is not clear yet, however, abnormal expression of COX-2 and BRCA1 in tumors may be associated with the most aggressive and poor prognosis of cancer types and further studies are required.

Key words: Tumor, Canine transmissible venereal tumors, gene expression, gene sequencing, fold change, mutation.

INTRODUCTION

Cancer is the first cause of death in cats and dogs while in humans it is the second most cause of death (Jemal *et al*., 2008). According to an assessment, cancer related deaths in the world are 13% and 70% of these aforementioned deaths are in poor countries (World Health Organization, 2012). Such natural cases of cancers in cats and dogs especially, in dogs offer an opportunity to use the dogs for comparative cancer studies and as an animal model for anticancer drug development (Pawaiya, 2008). In a series of more than 2000 autopsies, it was found that almost forty five percent dogs that lived for ten or more years expired because of cancer (Bronson, 1982). A German pathologist Johannes Muller first time demonstrated that cancer cells were originated from a bud called Blastema instead of normal cells (Kardinal and Yarbro, 1979). There are many exogenous and endogenous risk factors that are involved in caner. Tumor viruses (Bishop 1980), chemical carcinogens (Loeb *et al*., 2000), natural chemicals, (Ames *et al*., 1990), herbicides (Glickman *et al*., 2004), physical carcinogens like radiation (Upton, 1978) are exogenous factors while inherited genetic defects, immune system (Rosenthal, 1998) and hormonal factors (Rodney, 2001) are among endogenous risk factors. In order to induce cancer the mutations must affect a variety of genes that restrain somatic conflict (Frank and Nowak, 2004). These genes are known as cancer related genes.

BRCA1, a tumor suppressor gene is involved in repairing the DNA double strand breaks and in case of failure; it leads the cells towards apoptosis (Starita, 2003). In canine it is located on chromosome 9, it has 22 exons and it encodes a protein of 1882 amino acids. Many scientists from different research showed that women who have familial mutations in the *BRCA1* or BRCA2 (BRCA1/2) genes have increased risk of breast cancer (Struewing *et al*., 1997). Different mutations were found in BRCA1 of different ethnicities. Genetic analyzer can detect even those mutations of BRCA1, which were not detected by DGCG, HPLC and SSCP (Rassi, 2009). Another gene cyclooxygenase-2 (COX-2) synthesizes prostaglandins which participate in arachidonic acid

metabolism resulting in various mechanisms that leads to cancer development. COX-2 is usually absent in normal cells and it has been implicated in carcinogenesis of several neoplasms in companion animals. It was first isolated from prostate gland that's why it is called as prostaglandin. The extracellular inducible factors of COX-2 include growth factors, tumor promoters, cytokines, hypoxia, ionizing radiation and carcinogens (Singh, 2002; Howe, 2003; Wang, 2004). In canine genome COX-2 is present on chromosome 7; it has 604 amino acids and 10 exons. COX-2 was first reported in 1991 by a group of scientists and they described a viral oncogene or a tumor promoter had induced the COX-2 (Xie *et al*., 1991). Mutated p53 had also been detected in stronger expression of COX-2 gene in tumors as compared to tumors with non-mutated p53 (Leung *et al*., 2001). This correlation of COX-2 in cancer development suggests it as a new therapeutic target.

Seven types of tumors were included in this research work, including Mammary tumor, Canine transmissible venereal tumor (CTVT) (This tumor transmits sexually by genitalia of male and female dogs, Perianal adenoma (tumor of glands around the anus of dogs), Lymphoma (tumor of lymph nodes), Granuloma (tumor of granulocytes and macrophages) and Pelvic wart tumor (tumor of squamous cells of pelvic regions).

This research aimed to study the mutations in BRCA1 and COX-2 isolated from tumor samples as compared to normal samples, secondly to find out the gene expression of BRCA1 and COX-2 in the tissues of mammary tumor as compared to normal mammary tissues and thirdly to co-relate the type of mutations with mammary tumors as compared to normal samples.

MATERIALS AND METHODS

Sample Collection: In this research work total twenty two (n=22) tumors and twenty five (n=25) normal samples of dogs (*Canis familiaris*) were collected from Pet Centre UVAS, Asim Pet Clinic, Lahore and Outdoor Teaching Veterinary Hospital (Layyah Campus), UVAS (Table 1, 2). In dogs normal samples were obtained after autopsy (euthanasia). Samples were collected after proper diagnosis of tumor type and approval of ethical committee of the University of Veterinary and Animal Sciences, Lahore, Pakistan. Tumors of different breeds including German shepherds, Labrador, Rottweiler, English Springer, Sheepdog and non descriptive breeds were collected. These tissue samples were excisional biopsies obtained after surgery. In case of CTVT some flimsy tissues were collected. Tissues were also stored in RNAlater solution for long term storage according to manufacturer protocol (www.lifetechnologies.com).

Histopathological examination: Formalin fixed, paraffin embedded tissues were examined histopathologically. Tissues were placed in 10% formalin solution. The core region of tumortissue was used for Hematoxyline and Eosin staining. The grading and staging of tissues were determined (Lester, 2010) (Figure 1)

DNA isolation and PCR Amplification and Sequencing: DNA was isolated from tumors and normal tissues by using TIANGEN biotech genomic DNA tissue kit (Tiangin Biotech Co., Mainland, Beijing, China) from the tumor tissues according to manufacturer's guidelines and protocols. Total DNA concentrations were measured with NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Pittsburg, PA, USA). The DNA of all samples was brought to equal concentration (50ng/µL). Primers for COX-2 gene (10 exons, all the coding region) and primers for 5' (exon numbers 1, 2 and 12) and 3' region (exon numbers 18, 19, 20, 21 and 22) of BRCA1 gene were designed by primer 3 software. Primer details are given in Table 3 & Table 5. PCR for each primer pair was optimized by using different amounts of $MgCl_2$, dNTPs, buffer, primers and DNA (Table 4 & 6). All reactions were optimized at touchdown PCR conditions having a range of 54°C -64°C for COX-2 gene and 50 °C-65 °C for the BRCA1 gene. The following timings and ranges were used, 1st hold was 95°C for 5 minutes, then 94 °C for 30 seconds, annealing at 64°C for COX-2 and at 65°C for BRCA1 for 30 seconds and extension at 72°Cfor 45 seconds. The same was repeated for 10 times, with a decrease in 1°C with every one cycle (COX-2 gene) while for the BRCA1 primers, this cycle was repeated 15 times, the second hold (2nd) was at 95°C for 5 minutes, 94°C for 30 seconds, annealing at 54°C and extension at 72°C for 45 seconds, 2nd hold was repeated 30X times at 54°C for COX-2 and 50°C for BRCA1 and the final extension at 72°C was performed for ten minutes for COX-2 and for 25 minutes for BRCA1. To check the proper size of PCR products, all the PCR products were run on 1.5% agarose gel along with 1Kb Ladder (Fermentas, USA), the gel was stained with ethidium bromide and then it was visualized under ultraviolet radiations. Each PCR product was purified by QIAGEN PCR purification kit and PCR products were sequenced at ABI 3730 genetic analyzer (Sanger chain termination method).

Total RNA Isolation: RNA was extracted from tumors and normal tissues by thermo scientific Gene Jet RNA purification kit (Boom *et al*., 1990). RNA was also extracted manually by TRIzole method from those tissues which were in small amounts (Hummon *et al*., 2007). RNA integrity was determined by agarose gel electrophoresis and concentrations were measured by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Pittsburg, PA, USA) and were equalized to 200ng/µL.

Reverse transcriptase chain reaction for cDNA synthesis: Complementary DNA (cDNA) was prepared by Enzynomics cDNA synthesis kit (www.enzynomics.com). Oligo (dT)18 primers and random Hexamer primers were used simultaneously to produce first strand cDNA. Poly (A) tail of mRNA aneal with oligo (dT) 18 primers to synthesize cDNA while for the rest of RNA population, random primers initiate cDNA synthesis.

TaqMan primer-probe designing and RT-qPCRTaqMan detection chemistry: Pre-designed primers and probes for BRCA1 and COX-2 genes were used (Invitrogen) and for GAPDH (Housekeeping gene) was custom designed by using primer express software available with the Real Time PCR instrument (ABI). The following cat numbers of BRCA1 (cf02625922_m1), COX-2 (cf02625599_g1), GAPDH (cat #4331348 Custom) and reaction mixture (cat #4370048) were used (Table 7). GAPDH is a housekeeping gene and it was used for normalization in this qPCR. BRCA1 and COX-2 primers were FAM dye labeled at 5' end and TAMRA dye labeled at 3' end while GAPDH was labeled with a VIC dye at 5' end and TAMRA dye at 3' end.

RT qPCR Protocol: The qPCR was performed according to the protocol of the manufacturer (Applied Biosystem, USA). Twenty microlitre (20µL) reaction volume was used, which contained 10µL of 2X TaqMan gene expression master mixture, 1µL 20X TaqMan gene expression assay, 4µL of cDNA and 5µL of RNase free DEPC treated water was used in a single reaction.

RT-qPCR Experimental design: Real Time PCR was performed in triplicates according to ABI standard protocols (Both for target gene and control gene in tumors and normal samples as well). Singleplex 2 step qPCR was performed in triplicates according to ABI protocol (https://tools.lifetechnologies.com). Both targets (BRCA1, COX-2) and endogenous control/reference (GAPDH) were amplified in triplicates. Forty cycles of qPCR were performed for better amplification.

Rt qPCR data analysis by Livak Method (ΔΔCt method): Cycle threshold (Ct) values were obtained and gene expression was calculated in fold change. The gene expression was measured in fold changes by using the ΔΔCt method also called as comparative Ct method or relative quantification method (Livak and Schmittgen, 2001). Relative quantification is the most commonly used method of gene expression when target transcript of treatment group and control group is compared. Relative transcript abundance of the genes (BRCA1 and COX-2) was measured as ($\Delta Ct\ test = Ct^{target} - Ct^{reference}$) for tumors while normal samples as ($\Delta Ct\ calibrator = Ct^{target} - Ct^{reference}$). Relative changes in tumors and normal samples were measured as ΔΔCt ($\Delta\Delta Ct = \Delta Ct^{test} - \Delta Ct^{calibrator}$) and fold change values of tumor were measured by fold change= $2^{-\Delta\Delta Ct}$.

RESULTS

Histopathological (Hematoxyline and Eosin stained) slides of mammary tumors were analyzed (Figure-1a). All tumor samples were benign, having no cell at mitosis stageIn the cytoplasm, a large number of collagen fibers and fibroblasts were present. Samples were properly diagnosed by qualified veterinarians and histopathologists. In perianal adenoma, there was no cell in mitotic stage, so it was looking an adenoma not the malignant tumor (Figure-1a) while in one granuloma, multiple granulomas were present, which were surrounded by fibrous connective tissues and were applying pressure on surrounding tissues (Figure-1b). Another granuloma had mononuclear inflammatory cells in the dermis and there was necrosis of epidermal layers and inflammation (Figure–1c). CTVT histochemistry revealed that there were round oval nucleoli, coarsely aggregated chromatin, usually a prominent nucleus and abundant cytoplasm and cells at mitotic stage (Figure – 1d).

Mutational studies: The sequencing of N (5') and C (3') terminal of BRCA1 and whole coding region of COX-2 was done by the Sanger sequencing method. The sequences of reference genes of BRCA1 gene (Ensemble Accession, ENSCAFG00000014600) and COX-2 gene (Ensemble Accession, ENSCAFG00000013762) were used as a query and the sequences of the BRCA1 gene and COX-2 gene obtained by sequencing were aligned together. The complete analysis of sequences was performed manually and by using computational methods (Bio edit & BLAST softwares). All the selected regions of BRCA1 and COX-2 were conserved in all tumors of dogs. The results showed that there was no mutation in the studied regions of these genes.

Gene expression of COX-2 and BRCA1: Cycle threshold (Ct) values of COX-2 were obtained by RT-qPCR method and the fold change was determined by Livak method. Mean ΔCt (0.75-0.9) of normal tissues was calculated by subtracting mean Ct target (COX-2) from the mean Ct reference/endogenous (GAPDH) of respective normal tissues. Altered gene expression was observed in tumor samples as compared to normal mammary tissues. COX-2 gene expression was positive in all tumor samples (Figure 3). The highest fold changes were observed in four samples, two CTVTs, one head tumor and one mammary tumor of the dog. They have 942.93, 122.79, 85.95 and 44.49 fold change, respectively (Figure 4). These tumor samples were aggressive tumors, having larger sizes as compared to other tumors. There may be co-relation of up-regulation of COX-2

withaggressiveness of tumors. In addition, these lower fold

Table 1. Tissue sample collection, tumors of dogs (age, sex, breed and collection site).

Sr#	Animal	Breed	Gender	Age	Location	Type of tumor
01	Dog	German Shepherd	M	7years	Pet Centre, Lahore.	Lymphoma
02	Dog	German Shepherd	F	9 years	Pet Centre, Lahore.	Mammary tumor
03	Dog	English Springer	F	6 years	Pet Centre, Lahore.	CTVT
04	Dog	German Shepherd	F	8 years	Pet Centre, Lahore.	Mammary tumor
05	Dog	German Shepherd	M	8 years	Pet Centre, Lahore.	CTVT
06	Dog	German Shepherd	M	13 years	Asim Pet Clinic, Lahore.	Perianal adenoma
07	Dog	German Shepherd	M	2 years	Asim Pet Clinic, Lahore.	Oral tumor
08	Dog	German Shepherd	F	2 years	Asim Pet Clinic, Lahore.	CTVT
09	Dog	German Shepherd	F	10 years	Asim Pet Clinic, Lahore.	Mammary tumor
10	Dog	German Shepherd	F	11 years	Pet Centre, Lahore.	Mammary tumor
11	Dog	German Shepherd	M	10.5 years	Pet Centre, Lahore.	Peri-anal adenoma
12	Dog	German Shepherd	F	3.5 years	Pet Centre, Lahore.	Peri-anal adenoma
13	Dog	Non descriptive	M	5 years	Pet Centre, Lahore.	Oral tumors
14	Dog	Non descriptive	M	12 years	Pet Centre, Lahore.	Lymphoma
15	Dog	Non descriptive	F	11 years	Pet Centre, Lahore.	Mammary tumor
16	Dog	German Shepherd	F	2.5 years	Pet Centre, Lahore.	CTVT
17	Dog	Rottweiler	F	2 years	Pet Centre, Lahore.	Granuloma
18	Dog	German Shepherd	M	3 years	Pet Centre, Lahore.	Granuloma
19	Dog	German Shepherd	F	2 years	Pet Centre, Lahore.	CTVT
20	Dog	German Shepherd	F	2.5 years	Pet Centre, Lahore.	CTVT
21	Dog	German Shepherd	M	2.5 years	Asim Pet Clinic, Lahore.	Head tumor
22	Dog	German Shepherd	M	2.5 years	Pet Centre, Lahore.	Pelvic wart tumor

M is abbreviated for male and F for female. MT stands for mammary tumors, CTVT for canine transmissible venereal tumors, PA for perianal adenoma, HT for head tumor, PWT for pelvic warts tumor.

Table 2. Tissues sample collection, normal dog samples (age, sex, and breed and collection site).

Sr#	Animal	Breed	Gender	Age	Location	Type of tissues
01	Dog	Non descriptive	F	3 years	Surgery Department, (SD), UVAS, Lahore.	Mammary tissues
02	Dog	English Springer	F	2years	SD, UVAS, Lahore.	Mammary tissues
03	Dog	Non descriptive	F	1.5years	SD, UVAS, Lahore.	Mammary tissues
04	Dog	German Shepherd	F	4 years	SD, UVAS, Lahore.	Mammary tissues
05	Dog	German Shepherd	F	4 years	SD, UVAS, Lahore.	Mammary tissues
06	Dog	German Shepherd	M	5 years	SD, UVAS, Lahore.	Liver tissues
07	Dog	German Shepherd	F	3 years	SD, UVAS, Lahore.	Vaginal tissues
08	Dog	German Shepherd	F	3 years	SD, UVAS, Lahore.	Vaginal tissues
09	Dog	German Shepherd	F	3 years	SD, UVAS, Lahore.	Vaginal tissues
10	Dog	German Shepherd	M	3 years	SD, UVAS, Lahore.	Penis tissues
11	Dog	German Shepherd	M	3 years	SD, UVAS, Lahore.	Prepuce tissues
12	Dog	German Shepherd	M	3 years	SD, UVAS, Lahore.	Penis tissues
13	Dog	German Shepherd	M	5 years	SD, UVAS, Lahore.	Pelvic tissues
14	Dog	German Shepherd	M	3.5 years	SD, UVAS, Lahore.	Pelvic tissues
15	Dog	German Shepherd	M	3 years	SD, UVAS, Lahore.	Pelvic tissues
16	Dog	English Springer	M	4years	SD, UVAS, Lahore.	Oral tissues
17	Dog		M	4.5 years	SD, UVAS, Lahore.	Oral tissues (Normal)
18	Dog	German Shepherd	F	3 years	Out-Door Teaching Veterinary Hospital (TVH) Layyah, Campus, UVAS.	Oral tissues (Normal)
19	Dog	Rottweiler	M	3.5 years	TVH, Layyah Campus	Lymph nodes (Normal)
20	Dog	Rottweiler	F	2 years	TVH, Layyah Campus	Lymph nodes

21	Dog	Rottweiler	M	2.5 years	TVH, Layyah Campus	Lymph nodes
22	Dog	German Shepherd	M	5 years	TVH, Layyah Campus	Lung tissues
23	Dog	Rottweiler	M	2 years	TVH, Layyah Campus	Perianal tissues
24	Dog	Rottweiler	M	3 years	TVH, Layyah Campus	Perianal tissues
25	Dog	German Shepherd	M	4 years	TVH, Layyah Campus	Perianal tissues

M is abbreviated for male and F for female

Table 3. Primers details of COX-2 Gene (Canine).

Primers name	GC%	Sequence 5'-3'	Primer Length (	Tm	Product length (bp
COX2-1F	55	AGGAAGGTTCCGTCCGTTAG	20	60.49	370
COX2-1R	50	AAACGGTCCAAGCCCTTTAC	20	60.39	370
COX2 2F	50	TCCCTGGTTGAACGTTGT	18	60.01	412
COX2-2R	45	ATTTGGAGTGGGTTTCAGGT	20	58.35	412
COX2-3F	57.89	CACGTAAGTGTGCCCTTGG	19	60.16	382
COX2 3R	55	CCCCACTCAGGTTCATTCTC	20	59.15	382
COX2-4F	47.62	TCGGTCTTTAGTGCCACTTTG	21	60.29	376
COX2-4R	33.33	TTCACAGATATCCTCAAGCAAAAA	24	60.13	376
COX2 5F	41.17	CAGTTCACACCTTTATTTCTCCTG	24	59.21	416
COX2-5R	47.62	CAAGCGAGTCATTTCGTCTCT	21	59.88	416
COX2-6F	43.48	TTAGTGGTTGTGAGAGAAACGTG	23	59.34	374
COX2-6R	50	CAAACTGCAGGTGTTCAGGA	20	59.87	374
COX2-7F	34.78	CAAATATCACGTTGTTCCATTC	23	57.56	395
COX2-7R	45	GGGGAGAGGGTTTTATTGAA	20	57.97	395
COX2-8F	38.1	GATTGCATTTCAGTTGCTTGA	21	58.91	399
COX2-8R	42.86	AAACATCACTTTCGTCGCCACA	21	59.73	399
COX2-9F	40	CCCAAGGAATGAATGCTTTT	20	59.02	381
COX2-9R	50	CAGCCATTTCCTTCTCTCCT	20	58.47	381
COX2-10F	35	TTCAAAGCAATTCAGCAAA	19	58.33	555
COX2-10R	29.15	AATTAAGTTAAAAGGAATCGTCCA	24	57.5	555

The above table represents the primer names, GC%, sequences of forward and reverse primers, primers length; Tm and all PCR expected product sizes.

Table 4. PCR recipe of individual primers for COX-2 gene (Canine).

Primers	*Reagents*								Total
	DNA (50ng/ul)	PCR Buffer (2mM)	MgCl (2mM)	Primer F (10pM)	Primer R(10pM)	dNTPs (25mM)	Taq Polymerase (5U/µl)	Water	
	ul	ul	ul	ul	ul	ul	ul	ul	ul
COX-2 1	2	2.5	2	1	1	2.5	0.5	13.5	25
COX-2 2	1	2.5	2.5	1	1	2.5	0.5	14	25
COX-2 3	1	2.5	2.5	0.75	0.75	2.5	0.5	14.5	25
COX-2 4	3	2.5	2.5	0.75	0.75	2	0.5	13	25
COX-2 5	3	2.5	2.5	0.75	0.75	2.5	0.5	12.5	25
COX-2 6	3	2.5	2.5	1	1	2	0.5	12.5	25
COX-2 7	2	2	2	1	1	2	1	14	25
COX-2 8	2	2.5	2.5	1	1	2.5	1	12.5	25
COX-2 9	2	2.5	2	1	1	2.5	0.5		25
COX-2 10	1	2.5	2.5	1	1	2.5	0.5	14	25

change values also showed a positive behavior of COX-2 gene expression which could be co-related with the previously reported gene expression studies based on immunohistochemistry, indicating the positive gene expression as the abnormal function of COX-2 gene. There was up-regulation of 3/6 (50%) CTVTs, 2/5 (40%) mammary tumors of dogs, 1/2 (50%) oral tumors, 2/2 (100%) lymphoma, 1/3 (33%) perianal adenoma, while single cases of head tumor and pelvic wart tumor were studied. Pelvic wart tumor was down regulated with 0.22 fold change and all granulomas 0/2 (0%) were down regulated with fold changes i.e. 0.11 and 0.05, respectively (Figure 3). Gene expression of BRCA1 was negative in all tumors of dogs (Figure 2), which indicated the abnormal behavior of BRCA1 gene expression in all tumors. The BRCA1 gene expression has been found to be down regulated or having no expression in breast cancers in humans, the same pattern was observed in mammary tumors in dogs.

Table 5.BRCA1 selected portion for Sequencing and primers' details.

Primers Names	GC%	Primer Sequence 5'-3'	Base pairs (bp	Tm	Product length (bp)
BRCA1-1F5t	40	GACATCTAATGAAACTAGGCTGTTC	25	57.66	358
BRCA1-1R5t	40.91	CCAAAGCTCCTGAGTTAAGAAA	22	57.83	358
BRCA1-2F5t	45	CGCAGCTTAAAGTTGTGCTT	20	58.4	297
BRCA1-2R5t	50	TGGCTTGCTAAGTACTCTGAGG	22	58.81	297
BRCA1-12F5t	47.62	TGATTGTCACAGGTTGCTCCT	21	60.71	473
BRCA1-12R5t	50	CCTGACCTTCAAAAGGGACA	20	60.08	473
BRCA1-18F3t	52.38	CAGCAGCTGAGATACTGGTCA	21	59.2	481
BRCA1-18R3t	45	TTGGGCTTGGTCTCTCAAAT	20	59.67	481
BRCA1-19F3t	50	TCTCTGGGAAGGAGCAGAAA	20	60.07	400
BRCA1-19R3t	55.56	GGGCACAGGGCTGTTTTT	18	61.05	400
BRCA1-20F3t	45	TGTGTTTTGGAGCAAAGACG	20	59.88	374
BRCA1-20R3t	55	ATCCTCCACAGAGGGGAGTT	20	59.93	374
BRCA1-21F3t	55	CTATCCCTCCGACCCTTCAT	20	60.29	382
BRCA1-21R3t	52.38	CCCATCTCTCACAGGCACAT	20	59.93	382
BRCA1-22F3t	55	TTGCACCTACCTGAGGAACC	20	60.11	387
BRCA1-22F3t	42.86	TTCAAAGGGAGACTTGAAGCA	21	59.98	387

Table 6. PCR Reaction compositions for BRCA1 for PCR (Canine)

Primers	*Reagents*								Total
	DNA (50ng/µl) µl	PCR Buffer (2mM) µl	$MgCl_2$ (2mM) µl	Primer F (10pM) µl	Primer R(10pM) µl	dNTPs (25mM) µl	Taq Polymerase (5U/µl) µl	Water µl	µl
BRCA1-1	1	2.5	2.5	0.75	0.75	2.5	0.5	14.5	25
BRCA1-2	1	2.5	2.5	1	1	2.5	0.5	14	25
BRCA1-12	2	2.5	2	1	1	2.5	0.5	13.5	25
BRCA1-18	3	2.5	2.5	0.75	0.75	2	0.5	13	25
BRCA1-19	3	2.5	2.5	1	1	2	0.5	12.5	25
BRCA1-20	3	2.5	2.5	0.75	0.75	2.5	0.5	12.5	25
BRCA1-21	2	2.5	2.5	1	1	2.5	1	12.5	25
BRCA1-22	2	2	2	1	1	2	1	14	25

Table 7: Primers and probes selected kits 'details of BRCA1, COX-2 and GAPDH genes

Gene	Species	Transcript	Amplicon length	Exon boundary/Assay location	Kit ID	Dye
BRCA1	Dog	NM_001013416	65	14-15, 5001	cf02625922_m1	FAM-MGB
COX-2 (PTGS2)	Dog	NM_001003354.1	105	3-4, 341	cf02625599_g1	FAM-MGB
GAPDH	Dog	N2M_00100314	59	655	cat # 4331348 Custom	VIC-MGB

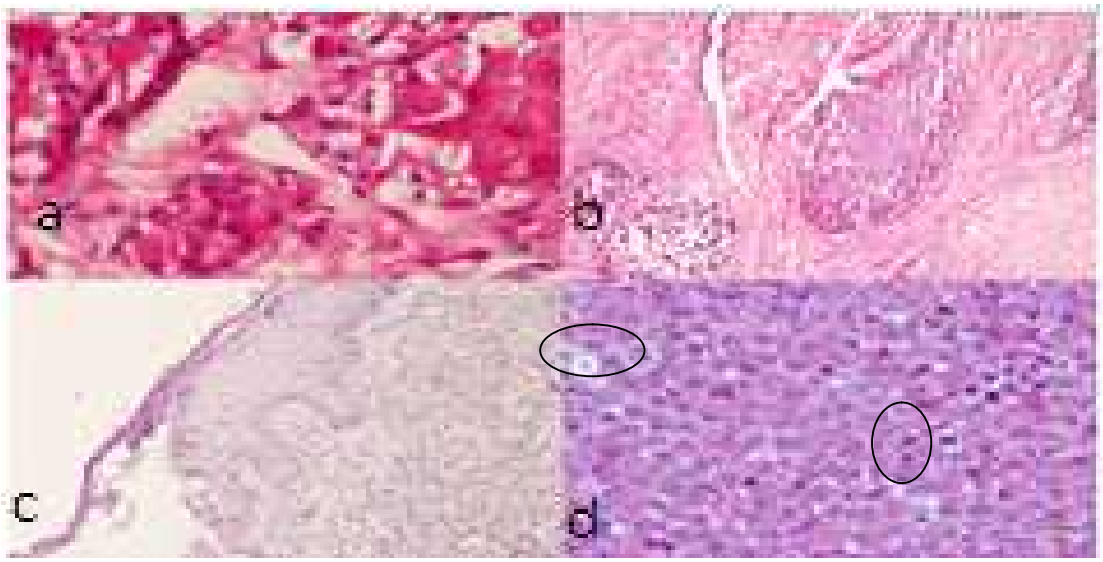

Figure 1: Perianal adenoma, 40*10X (a),Granuloma, 4*10X (b), Granuloma, 4*10X (c), CTVT, 4*10X (d).

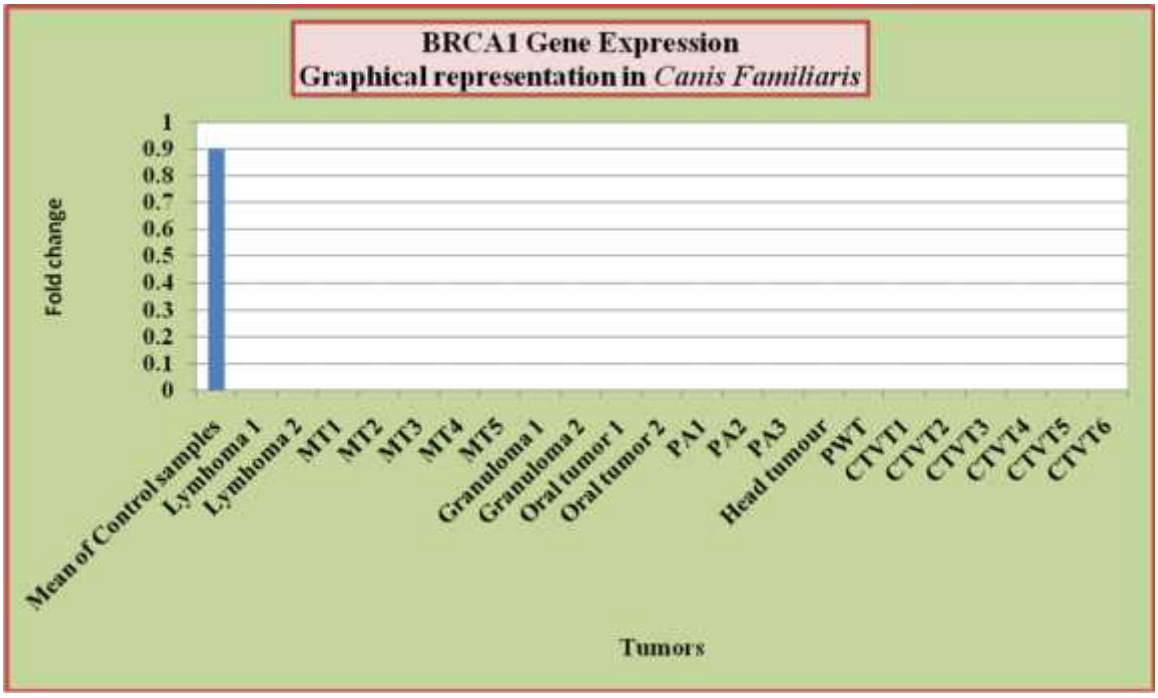

Figure 2: Gene expression of BRCA1 in all tumors. BRCA1 gene expression was highly negative in all tumors. MT stands for mammary tumors, CTVT (canine transmissible venereal tumors) PA for perianal adenoma, PWT for pelvic warts tumor

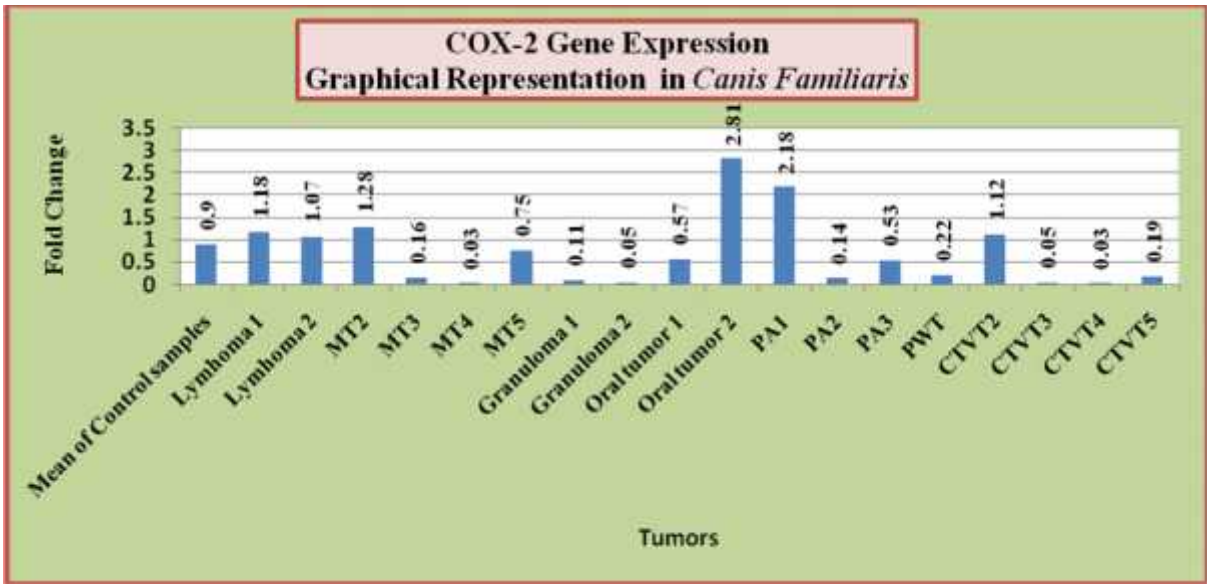

Figure 3: Gene expression of COX-2 in all tumors. Positive gene expression was observed in all tumors. Almost 45% tumors showed up-regulation. MT stands for mammary tumors, CTVT (canine transmissible venereal tumors) PA for perianal adenoma.

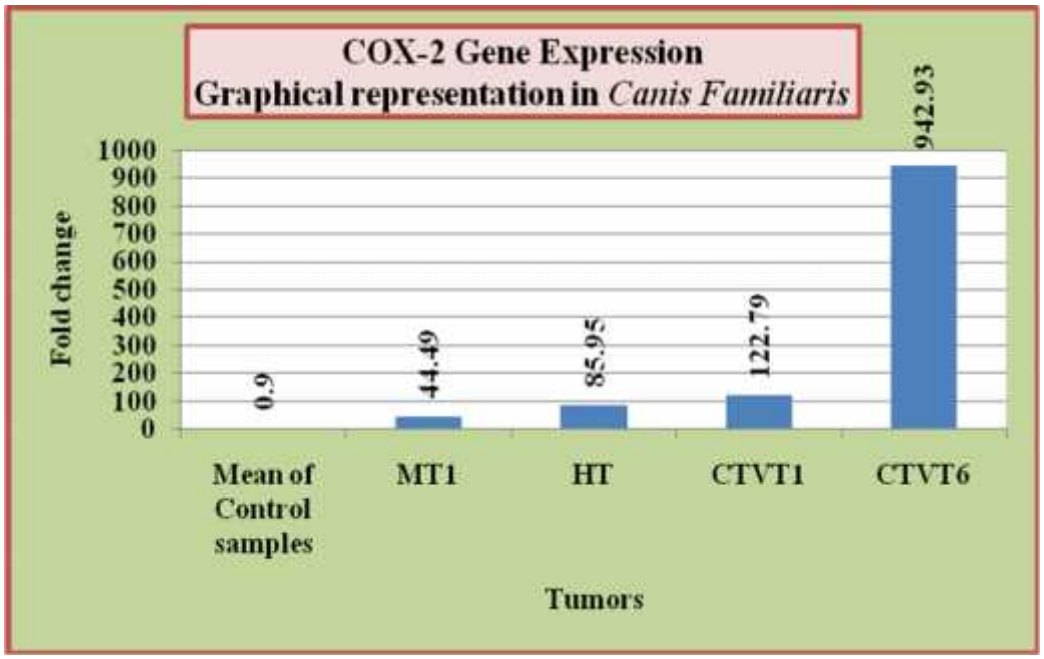

Figure 4: Gene Expression of COX-2 in tumors having the highest up-regulation. MT stands for mammary tumors, CTVT (canine transmissible venereal tumors) HT head tumor.

DISCUSSION

In this study the role of BRCA1 and COX-2 genes in different canine tumors was studied. Expression and mutational studies were performed to find out any abnormality in these genes in tumor samples as compared to normal samples. The regulation of cell proliferation, stability of the genome and programmed cell death are important for systemic homeostasis. Two types of genes are very important in cell cycle, tumor suppressor genes and proto-oncogenes. The proto oncogenes that enhance DNA synthesis, cell division and its growth become cancerous when they are mutated (Adamson, 1987). One important enzyme cyclooxygenase-2 (COX-2) synthesizes prostaglandins which participate in arachidonic acid metabolism resulting in various mechanisms that lead to cancer development.

BRCA1 is a tumor suppressor gene and C (3') and N (5') terminals of BRCA1 gene are considered as hot spot for BRCA1 mutations and mutated or abnormal C and N terminals lead to disturbing many cell signaling processes of cell division. As the role of BRCA1 and COX-2 genes have been studied in human tumorogenesis and oncogenesis. This study was designed to see the role of these genes in canine tumors. COX-2 is not constitutively produced in the cells while it is induced in inflammation and cancer. The extracellular stimuli that induce COX-2 include growth factors, cytokines, tumor promoters, hypoxia, ionizing radiation and carcinogens (Singh, 2002; Howe, 2003; Wang, 2004). Gene sequencing and expression of the 22 different canine tumors and 25 different normal tissues for COX-2 and BRCA1 genes were performed. Our focus was to study the C (3') and N (5') terminus of BRCA1 and whole coding region of COX-2 for mutational analysis in different tumors of dogs and also the gene expression study of BRCA1 in these tumors as well. The 5' and 3' terminal of BRCA1 gene was sequenced to see variants in it and its comparison to the reference sequence. Exon 1, 2, 12, 18, 19, 20, 21, and 22 of BRCA1gene were selected for mutational analysis but no mutation was found in these exons. Although in humans, the BRCA1 mutations have been strongly related to hereditary breast cancers, however, the types of mutations differ in distribution according to geography and ethnicity. For Ashkenazi Jewish the "hot spot" mutations are present at 5382insC and 185delAG (Abeliovich *et al*., 1997), whereas in Swedish people 3171ins5 is considered as the highest risk familial mutation (Einbeigi *et al*., 2001). The prevalence of BRCA1mutations also varies in diverse populations. For example, the BRCA1mutation frequency in Sweden is 7% (Zelada-Hedman *et al*., 1997) while Finnish breast cancer patients have 0.4% (Syrjakoski *et al*., 2000). But in case of dogs, we did not find any mutation in sporadic mammary tumor. There was no variation in the entire coding region of COX-2 as well. There is limited literature about knowledge of mutation of COX-2 in human and in canine tumors. Role of COX-2 and IL-10 SNPs was studied in 290 squamous cell carcinoma of head and neck tumor (SCCHN) of Korean samples and in COX-2, -1329A>G,+1266C>T and +6365 T>C SNPS were found but there was not a significant association between (SCCHN) and the SNPs (Jeong *et al*., 2010). A group of researchers examined the association between mutation in*p53* and COX-2 expression in gastric cancer (Leung *et al*., 2001). Similarly, different groups showed the mutation of any other gene, which in turn induced the COX-2 gene expression.

Gene Expression of BRCA1 and COX-2 was also measured in canine tumors under this study. In the human BRCA1 role has been studied in different cancers, especially in, ovarian (Matsushima *et al*.,1995), head and neck (Buchholz *et al*., 2002), renal form of tumors (Kawakami *et al*., 2003), lung (Taron *et al*., 2004), pancreatic (Cybluski, 2007) and skin tumors (Monnerat *et al*., 2007) but its extensive role was studied in majority of ovarian and breast cancers (both sporadic and hereditary) (Easton *et al*., 995). As the dogs live in the same environment as that of humans, so the etiology and pathogenesis of dog tumors is considered as the most similar to human tumors (MacEwen, 1990, Vail, 2000; Khanna 2006; Pinho, 2012; Marconato, 2013). In all studied tumor samples we found BRCA1 negative-regulation as compared to normal samples. These results are in line with the findings of Navaraj and colleagues (2009). They found that the loss of BRCA1 function in knockdown mice showed the angiogenic potential and tumorogenesis (Navaraj *et al*., 2009). BRCA1 deficiency and haploinsufficiency accelerated tumorogenesis was also determined by karyotyping analysis in knockdown mouse model having deletion of BRCA1 gene (Trilett *et al*., 2008). So, the loss of BRCA1 gene expression in this research work showed a loss of function of this gene in our tumor samples as well. BRCA1 putative role was also studied in Japanese population. The whole coding region of BRCA1 had no mutation in somatic ovarian cancers however, 4 SNPs, two frame shifts, one nonsense mutation and one intronic substitution of near to exon 22 was observed (Matsushima *et al*., 1995). Although BRCA1 is found responsible in the majority of breast cancers in humans with familial history, however, its involvement in sporadic breast cancer (especially of Pakistani population) is an area that requires further research. One group from Pakistan (Malik *et al*., 2008) studied the N and C terminal of the BRCA1 gene and they found 5 silent and one splice site mutation in the BRCA1 gene. However, most of the BRCA1 region analyzed by this group remained conserved at the genomic level. BRCA1 promoter methylation was reported to be involved in tumor progression. BRCA1 expression is often reduced in sporadic breast cancers, and it has been reported even in the absence of its genetic modifications, but the molecular basis for this is not clear (Gustavo, 2003; Penney, 2010).

COX-2 gene expression was positive in all tumors. All canine tumors showed abnormal expression of COX-2 varying in different fold change values from lowest to highest fold changes. The highest fold change values were observed in 2/6 CTVT tumors that were 122.79 and 942.93 respectively. The second and third one highest fold change values were observed in head tumor and in 1/5 canine mammary tumor i.e. 85.95& 44.65 fold change, respectively. Similar results were obtained by other scientists and one group observed that COX-2 was not expressed in normal tissues, but showed increased expression in benign (24%) and malignant (56%) tumors, indicating a possible role in tumorogenesis (Dore *et al*., 2003). All tumor samples showed positive gene

expression of COX-2, however, the 45% of all tumors studied showed up-regulation as compared to the normal samples studied in this research work. Molecular studies have revealed that COX-2 is inducible only and either negative or very low expression of COX-2 gene in normal samples has been seen. The up regulation of COX-2 in many types of neoplasms and regular use of nonsteroidal anti-inflammatory drugs reduce the risk of colon cancer (43%), breast cancer (25%), lung cancer (28%) and prostate cancer (27%) (Harris, 2009). Five (n=5) mammary tumors of dogs were included in this study. Out of five, two tumors showed up regulation, MT1 and MT2 with 44.49 and 1.28 fold change values while other three MT3, MT4 and MT5 showed 0.16, 0.3 and 0.75 fold changes, respectively. About 20% canine mammary tumor samples had highest up-regulation and the same percentage was for intermediate up-regulation while remaining 60% showed low positive expression of COX-2 gene. These results co-relate with previously reported gene expression studies based on immunohistochemistry, indicating the positive gene expression as the abnormal function of COX-2 gene.

All six CTVT samples were found to have positive expression for COX-2. Two samples CTVT1 and CTVT6 showed very high expression with 942.93 and 122.29 fold up-regulation respectively. One CTVT showed intermediated up-regulation of COX-2 with fold change 1.12 while other three samples of CTVT i.e 3, 4, 5 showed positive expression for COX-2 with 0.05, 0.03 and 0.19 fold change which was down- regulated as compared to normal samples. Two canine lymphomas were studied in this research work. Both showed intermediate up-regulation and 100% positive COX-2 gene expression with 1.18 and 1.07 fold changes. Scientists investigated that stromal cells of follicular lymphoma produced large amount of prostaglandins and COX-2 inhibitors like celecoxib were used successfully against this tumor (Gallouet *et al*., 2014). In another study, the researchers found the correlation of COX-2 with primary effusion lymphoma (PEL) and they identified that nimesulide was acting as an inhibitor of COX-2 in treating primary effusion lymphoma (Paul *et al*., 2011).

Three perianal adenomas of dogs were examined for COX-2 gene expression. One tumor PA1 had 2.18 fold change in gene expression while PA2 and PA3 had 0.14 and 0.53 fold changes, respectively. So, 33.33% perianal tumors had up-regulation while others 66.66% were positive but did not show up-regulation as compared to normal samples. There are also reports about the role of COX-2 in companion animals. In one study, seventy-six percent (76%) anal sac adenocarcinoma had 50% positive COX-2 immunostaining, so this study also suggested using the cyclooxygenase-2 inhibitors for anal sac adenomas and adenocarcinomas (Knudsen *et al*., 2013). Two granulomas studied in this research work showed down regulation of COX-2, although both tumors were positive in COX-2 gene expression (0.11 and 0.05 fold change). COX-2 and IL-10 higher expression has been seen in oral pyogenic granuloma (Isaza-Guzman *et al*., 2012).

The pelvic wart tumor was purposeful for this study as it was a different type of tumor, which was present in the form of boils in the pelvic region of dog. It had very low positive COX-2 expression which was also lower as compared to normal samples. The COX-2 over expression in actinic keratosis, squamous cell carcinoma and inflammatory dermatoses of canine and feline was determined (Bardagi *et al*., 2012) and COX-2 inhibitors have been used for these tumors (Pestili de Almeida *et al*., 2001). One head tumor was also considered in this study. This tumor was present on the head and was very aggressive (large sized tumor). This tumor had 85.95 fold-change which was very high fold change as compared to normal samples. The anti-metastatic effect of COX-2 inhibitors has been seen in head and neck tumor of human (Fuiji *et al*., 2014; Kao *et al*., 2009). Similarly, two oral tumors were studied. Both tumors showed positive gene expression with 0.57 and 2.81 fold change values. The latter had moderate up-regulation of COX-2 while the first one was within normal range. Scientists studied the COX-2 gene expression (immunohistochemistry) in oral squamous cell carcinoma; oral lichen planus and normal oral mucosa. The highest gene expression was in oral squamous cell carcinoma, i.e. 68.4%, then oral lichen planus (24.2%) and there was no expression in normal oral mucosa (Li *et al*., 2013).

Conclusion: These findings support a role of BRCA1 and COX-2 genes in the pathogenesis of these tumors. This is the first study in Pakistan to investigate the role of aforementioned genes in different tumors of dogs. The mechanism behind the loss of function of BRCA1 and up-regulation of COX-2 still needs more research. We did not find any tumor associated mutations, however, our results co-relate with the previous studies of BRCA1 and COX-2 involvement in inflammation and tumorogenesis. There is a dire need to study the other coding as well as non-coding regions of BRCA1 to find out the reasons of abnormal function of BRCA1 and also it is extremity to find out other proto oncogenes which are directly linked in up-regulation of COX-2 gene expression.

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