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Feline Mammary Tumors Show Altered *COX-2* and *BRCA1* Expression

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ABSTRACT

Cancer is the one of the main causes of death in cats and dogs, while it is the second most cause of deaths in humans. In third world countries, less attention is paid to the health of pets. The genes (*COX-2* and *BRCA1*) are selected to study the mutation and gene expressional profiling (qPCR, Real Time PCR) in mammary tumor of cats. Being mammals, cats are studied as a model as they may suffer from cancer in the same way as that of humans. Five (n=5) mammary tumors and twenty five (n=25) normal mammary tissues are collected from different cats respectively. All samples were collected from Lahore city, Punjab, Pakistan. High grade negative gene expression of *BRCA1* was observed in all mammary tumors. Here, we found that 40% of the mammary tumors showed up-regulation of *COX-2* gene with fold changes *i.e*. 1.76 and 1.84, respectively. While remaining mammary tumors showed positive behavior of *COX-2* gene expression, but it was down regulated as compared to normal samples included in the study with fold changes *i.e*. 0, 0.76 and 0.82. Sequencing of whole coding region of *COX-2* gene (1-10 exons) and the hot spot 5' (N terminal, *i.e*. 1, 2, 12 exons) and 3' region (C terminal, 18, 19, 20, 21, 22 exons) of *BRCA1* gene which comprised of 1812bp, 1128bp and 2024bp, respectively was also performed. There was not a single mutation observed in the selected regions. In conclusion, it was observed that the drastic reduction in *BRCA1* gene expression and over expression of *COX-2* may have a role in mammary tumor of cats. The fold change of *COX-2* gene in tumors has been associated with the extent of aggressive tumor's type. However, the mechanism behind the abnormal function of these genes is not clear yet and further studies are required.

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Authors' Contribution

HS conceived and designed the study, performed experimental work and analyzed the data. SM, MT, SF and TY analyzed the data. SM, AW and AKM collected samples. MW, AW, ZUM, AKM and ARA were involved in manuscript write up. MW supervised the study.

Key words

***COX-2, BRCA1*, feline, mammary tumor, gene expression**

INTRODUCTION

The term cancer is derived from the observations by Hippocrates in 400BC that the veins of breast are resembled with legs of a crab; hence it is termed as "Karkinoma" in Greek and "Cancer" in Latin (Diamandopoulos, 1996). Among all cancers, breast cancer is the most common cancer (Siegel *et al.,* 2011) and this cancer has a diverse pathogenesis. In the world, feline mammary tumors are the third in numbers after heamopoitic and skin cancers in cats and mostly they are malignant (Viste *et al.,* 2002). Feline mammary tumors are mostly adenocarcinomas (Hayes *et al.,* 1981) and the least common carcinomas are tumors with differentiation of squamous cells, lipid rich carcinoma and mucinous carcinoma (Takashima *et al.,* 1984). Feline mammary tumors are similar to human breast cancers in clinical behavior, histological features and pattern of metastasis, that's why many researchers have been suggested to study feline mammary tumors as a model for human breast cancers (Hahn *et al.,* 1999; Zappulli *et al.,* 2005). *BRCA1* is a tumor suppressor gene, located on chromosome E1 in felines, having 1871amino acids and 22 exons. In humans, *BRCA1* and *BRCA2* familial mutations have been reported in breast cancers and ovarian cancers and very common abnormality of these genes is the loss of heterozygosity, due to which these genes do not function normally (Miki *et al.,* 1994; Wooster *et al.,* 1995; Miller, 2003; Osorio *et al.,* 2003). In the majority of benign tumors and more especially of malignant tumors, the *BRCA1* gene expression has been observed and an increase in cytoplasmic expression, while a decrease in nuclear expression was observed (Fustier *et al.,* 2003). Rassi (2009) studied that the most common genes having mutations in breast cancers were *BRCA1* and BRCA2 as both genes had multiple exons. Genetic analyzer can detect even those mutations of *BRCA1* which were not detected by DGCG, HPLC and SSCP (Rassi, 2009), however, to collaborate these

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findings further research is required (Antill *et al.,* 2010). Another gene cyclooxygenase (COX), also called as endo-peroxide synthase, is very important enzyme for the synthesis of prostaglandins from arachidonic acid. There are two isoforms of cyclooxygenase (Williams *et al.,* 1999), COX-1 is mainly involved in the production of prostaglandins and is constitutively produced (Williams *et al.,* 1999) while *COX-2* gene is only constitutively produced in kidneys, central nervous system and seminal vesicles, while in most of the cases it is inducible and being produced during ovulation, inflammation in cancers, anti-apoptotic activities, angiogenesis and immune suppression (Cha and DuBois, 2007). In feline genome *COX-2* is present at chromosome D4, having 633 amino acids and 10 exons. In 2008, three different mutations were reported for the first time in three different types of tumors *i.e*. squamous cell carcinomas, two large cell carcinomas and adenocarcinomas and these were never reported before in *COX-2* of any other tumors. These mutations were V102V (GTG>GTC), P593L (CCC>CTC) and H212H (CAT>CAC), respectively (Strazisar *et al.,* 2008). In many species the molecular structure of *COX-2* gene has been studied, however the molecular structure of *COX-2* in feline has not been reported (Kujubu *et al.,* 1991; Boerboom and Sirois,1998; Liu *et al.,* 2001b; Boutemmine *et al.,* 2002). In head and neck squamous cell carcinomas of feline and canine, p53 has been found mutated (Snyder, 2004). *COX-2* gene expression could be induced by a variety of growth factors and mitogens (Bakhle and Botting, 1996; Simon *et al.,* 2000) which might lead it to be involved in carcinogenesis of many tumors, while there are few reports of *COX-2* gene expression in feline cancers. The significant over expression of *COX-2* gene has been reported in transitional cell carcinomas (Beam *et al.,* 2003) and oral carcinomas, which recommended that feline could be good model to study anticancer behavior of *COX-2* inhibitors (Beam *et al.,* 2003; Hayes *et al.,* 2006; DiBernardi *et al.,* 2007). Feline mammary tumors had conflicting results in two different reports. One group described the absence of *COX-2* gene in all 9 mammary carcinomas while the other group found 96% *COX-2* gene expression in mammary tumors of cats (Millanta *et al.,* 2002; Beam *et al.,* 2003). Some tumors like, intestinal and pulmonary adenocarcinomas were found *COX-2* gene negative in cats (Beam *et al.,* 2003). In mouse animal models, 85% of the cases showed up-regulation of *COX-2* gene and it was suggested that up-regulation of *COX-2* gene alone was sufficient in breast tumors (Liu *et al.,* 2001a). In this research work 5 mammary tumors and 25 normal mammary tissues were included.

We aimed to study the mutations in tumor samples as a comparison 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 mutation with mammary tumors as compared to normal samples.

MATERIALS AND METHODS

Sample collection

In present research work five samples of feline mammary tumors and twenty five normal feline samples were collected from Pet Centre, University of Veterinary and Animal Sciences, Lahore (UVAS), Asim Pet Clinic, Lahore and Out-Door Teaching Veterinary Hospital (Layyah Campus), UVAS (Table I). These samples were collected since February 2012 to January 2014. In Pakistan, mammary tumors are the most prevalent tumors in cats. Samples were collected after proper diagnosis of tumor types and prior approval from ethical committee of UVAS, Lahore, Pakistan. Mammary tumors and normal samples belong to Siamese breed of cats. These tissue samples were the excisional biopsies obtained after surgery. Tissues were also stored in RNA later solution for long term storage.

Histopathological examination

Formalin fixed paraffin embedded tissues were examined histopathologically. Tissues were placed in 10% formalin solution. Core region of tumorous tissue was used for Heamatoxylin and Eosin staining (H&E). The grading and staging of tissues were determined (Lester, 2010).

DNA isolation and PCR amplification and sequencing

DNA was isolated from tumors and normal tissues by using TIANGEN biotech genomic DNA tissue kit (Tiangen biotech Co., Mainland, and Beijing, China) from the tumorous tissues in accordance to manufacturer's guidelines and protocols. Total DNA concentrations were measured with NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Pittsburg, PA, USA). DNA of all samples was brought to same concentration *i.e*. 50ng/µl. Ten primers of all ten exons of *COX-2* gene (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 (Tables II, IV) and these PCR reactions were optimized by using different amounts of $MgCl_2$, dNTPs, buffer, primers and DNA (Tables III, V). The PCR of all primers were performed at touchdown PCR conditions having a range of 54-64°C for *COX-2* gene and 50-65°C for *BRCA1* gene. The following timings and ranges were used, 1sthold (1st) was 95°C for 5 minutes, then 94°C for 30 seconds, annealing at 64°C for *COX-2*

Table I.- Tissue samples collection, tumors and normal cat samples (age, sex, breed and collection site).

Animal	Breed	Gender	Age	Location	Type of tumor
Cat	Siamese	Female	12 years	Pet centre, UVAS, Lahore.	Mammary tumor
Cat	Siamese	Female	8 years	Pet centre, Lahore	Mammary tumor
Cat	Siamese	Female	8 years	Pet centre, Lahore.	Mammary tumor
Cat	Siamese	Female	5 years	Pet centre, Lahore.	Mammary tumor
Cat	Siamese	Female	7 years	Pet centre, Lahore	Mammary tumor
Cat	Siamese	Female	5 years	Surgery Department, UVAS, Lahore.	Mammary tissues (Normal)
Cat	Siamese	Female	3 years	Surgery Department, UVAS, Lahore	Blood (Normal)
Cat	Siamese	Female	2 years	Surgery Department, UVAS, Lahore	Mammary tissues (Normal)
Cat	Siamese	Female	4 years	Para Veterinary School, UVAS, Layyah Campus	Mammary tissues (Normal)
Cat	Siamese	Female	3.5 years	Para Veterinary School	Mammary tissues (Normal)
Cat	Siamese	Female	2.5 years	Para Veterinary School	Mammary tissues (Normal)
Cat	Siamese	Female	1.5 years	Para Veterinary School	Mammary tissues (Normal)
Cat	Siamese	Female	5 years	Para Veterinary School	Mammary tissues (Normal)
Cat	Siamese	Female	6 years	Asim Pet clinic, Lahore	Mammary tissues (Normal)
Cat	Siamese	Female	4 years	Asim Pet clinic, Lahore	Mammary tissues (Normal)
Cat	Siamese	Female	2 years	Asim Pet clinic, Lahore	Mammary tissues (Normal)
Cat	Siamese	Female	3 years	Asim Pet clinic, Lahore	Mammary tissues (Normal)
Cat	Siamese	Female	7 years	Asim Pet clinic, Lahore	Mammary tissues (Normal)
Cat	Siamese	Female	6 years	Asim Pet clinic, Lahore	Mammary tissues (Normal)
Cat	Siamese	Female	6 years	Pet centre, UVAS, Lahore	Mammary tissues (Normal)
Cat	Siamese	Female	7 years	Pet centre, UVAS, Lahore	Mammary tissues (Normal)
Cat	Siamese	Female	5 years	Pet centre UVAS, Lahore	Mammary tissues (Normal)
Cat	Siamese	Female	5 years	Pet centre UVAS, Lahore	Mammary tissues (Normal)
Cat	Siamese	Female	3 years	Pet centre UVAS, Lahore	Mammary tissues (Normal)
Cat	Siamese	Female	2 years	Pet centre UVAS, Lahore	Mammary tissues (Normal)
Cat	Siamese	Female	2 years	Pet centre UVAS, Lahore	Mammary tissues (Normal)
Cat	Siamese	Female	4 years	Pet centre UVAS, Lahore	Mammary tissues (Normal)
Cat	Siamese	Female	5 years	Pet centre UVAS, Lahore	Mammary tissues (Normal)
Cat	Siamese	Female	7 years	Pet centre UVAS, Lahore	Mammary tissues (Normal)
Cat	Siamese	Female	7 years	Pet centre UVAS, Lahore	Mammary tissues (Normal)

and at 65°C for *BRCA1* for 30 seconds and extension at 72°C for 45 seconds. The same was repeated for 10 times, with a decrease in 1^0C with every one cycle (*COX-2* gene) while for the *BRCA1* primers, this cycle was repeated 15 times, the second hold (2^{nd}) was at 95°C for 5 minutes, 94°C for 30 seconds, annealing at 54°C and extension at 72°C for 45 seconds, 2^{nd} 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*. PCR was performed for each individual primer. To check the proper size of PCR products, all the PCR products were run on 1.5% agarose gel along with Ladder, 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 (Thomas *et al*., 1990). Secondly, 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 NanoDrop2000 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 (http://www. enzynomics.com). Oligo (dT) 18 primer and random hexamer primers were used simultaneously to produce first strand cDNA. Poly (A) tail of mRNA react with oligo (dT) 18 primers to synthesize cDNA, while for the rest of RNA population, random primers initiate cDNA synthesis.

Table II.- Primers details of *COX-2* Gene.

Primers name	GC%	Sequence 5'-3'	Primer Length (I	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	41.47	CAGTTCACACCTTTATTTCTCCTG	24	59.24	416
COX2-5R	47.62	CAAGGGAGTGATTTGGTGTGT	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	GAAATATCAGGTTGTTCCATTG	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	AAAGATCACTTTGGTGGCAGA	21	59.73	399
COX2-9F	40	CCCAAGGAATGAATGCTTTT	20	59.02	381
COX2-9R	50	CAGCCATTTCCTTCTCTCCT	20	58.47	381
COX2-10F	35	TTGAAAGGAATTGAGCAAA	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 III.- PCR recipe of individual primers for *COX-2* gene mutation study.

Primers	DNA (50ng/µl) (µl)	PCR Buffer (2mM) (µl)	$MgCl_2$ (2mM) (µl)	Primer F (10pM) (µl)	Primer R (10pM) (µl)	dNTPs (25 mM) (µl)	Taq Polymerase (5U/µl) (µl)	Water (µl)	Total (µl)
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

The Table describes the amount of PCR buffer, $MgCl_2$, Primers, dNTPs, Taq polymerase in PCR reaction of each primer set.
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TaqMan primer-probe designing and RT-qPCR TaqMan detection chemistry

The primers and probes for *BRCA1* and *COX-2* were pre-designed by Invitrogen and GAPDH was custom designed by using Primer Express software available with the Real Time PCR instrument (ABI). The following cat # of *BRCA1*, COX-2, GAPDH and reaction mixture were used: cf02625922_m1, cf02625599_g1 cat #4331348 Custom and cat #4370048, respectively (Table V). GAPDH is a housekeeping gene and it was used for normalization in this qPCR. *BRCA1* and *COX-2* primers were FAM dye at 5' end and TAMRA dye 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). 20 µL

Table IV.- *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

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

Table V.- P C R Reaction Composition for *BRCA1* (Feline and Canine).

Primers	DNA (50ng/µl) (µl)	PCR Buffer (2mM) (µl)	$MgCl_2$ (2mM) (µl)	Primer F (10pM) (µl)	Primer R (10pM) (µl)	dNTPs (25 mM) (µl)	Taq Polymerase (5U/µl) (µl)	Water (µl)	Total (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

The Table describes the amount of PCR buffer, $MgCl_2$, Primers, dNTPs, Taq polymerase in PCR reaction of each primer set.

reaction volume was used, which contained 10µL of 2X TaqMan gene expression master mixture, 1uL 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 for 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 good amplification.

Table VI.- 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

The above Table describes the genes name, species, transcript ID, amplicon length, Kit ID and dye colors. Dog species was selected as it has 96% homology with cat gene sequences.

BRCA1 Gene Expression in *Felis catus*

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 (Livak and Schmittgen, 2001). Relative transcript abundance of the genes (*BRCA1* and COX-2) were measured as (ΔCt test = Ct^{target} – $Ct^{reference}$) for tumors while normal samples as (ΔCt 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 slides of mammary tumors were analyzed. All tumor samples were benign, having no mitotic figure. In the cytoplasm, a large number of collagen fibers and fibroblasts were present.

Mutational studies

The gene sequencing of N (5') and C (3') terminal of *BRCA1* and whole coding region of *COX-2* was obtained 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 and BLAST softwares). All the selected regions of *BRCA1* and *COX-2* were conserved in mammary tumors of cats and results showed that there was no mutation in the exons of these genes studied in this research work.

Gene expression of COX-2 *and* BRCA1

Ct values of *COX-2* were obtained from RT-qPCR method and the fold change was determined by Livak method. Mean ΔCt (0.95) of normal tissues was calculated by subtracting mean Ct target (COX-2) from the mean Ct reference/endogenous (GAPDH) of 25 normal cat mammary tissues. Altered gene expression was observed in tumor samples as compared to normal mammary tissues. *COX-2* gene expression was positive in

Fig. 1. *BRCA1* gene expression in different tumors of *Felis catus* (cat) This indicates the loss of function in *BRCA1* leading to anti-apoptotic activity in cells which leads the cells towards tumorogenesis. FMT stands for feline mammary tumors.

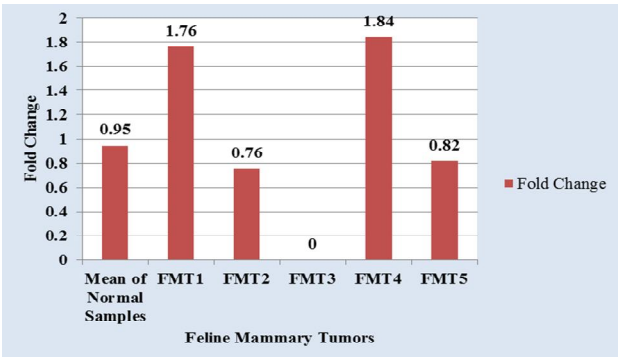

Fig. 2. *COX-2* gene expression in different feline mammary tumors (40% up-regulation) and others have low positive gene expression. FMT stands for feline mammary tumors.

all tumor samples and 2/5 (40%) tumors showed up-regulation with fold changes, *i.e.* FM1 1.76 and FMT4 1.84, respectively, while others showed down regulation of gene expression *i.e*. 0.76, 0 and 0.82 fold changes as compared to normal samples. The fold changes below 0.95, represent the positive gene expression, but less than normal gene expression in this study. Zero (n=0) fold change value also had lowest positive behavior almost one step near to undetectable Ct value (Fig. 2). In addition, these lower fold change values also showed a positive behavior of *COX-2* gene expression which could be co-related with the previous reported gene expression studies based on immunohistochemistry, indicating the positive gene expression as the abnormal function of *COX-2* gene. Gene expression of *BRCA1* was negative in all mammary tumors (Fig. 1), which indicated the abnormal behavior of *BRCA1* gene expression in mammary tumor of cats. 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 of cats.

DISCUSSION

Cancer is a noxious disease of the 21st century and multiple factors are involved in its progression. Mammary tumors in cats are third in number after hematopoietic and skin cancers in cats and the role of different genes has been studied in these cancers. The most abundantly studied gene in breast cancer is *BRCA1* gene and higher expression of *COX-2* has been also observed in breast cancers. *BRCA1* showed decrease gene expression in breast cancers (Sugiura *et al.*, 2007). In the human *BRCA1* role has been studied in different cancers, especially in ovarian (Matsushima *et al.*, 1995), head and neck (Buchholz *et al*., 2001), a renal form of tumor (Kawakami *et al*., 2003), lung (Taron *et al*., 2004), pancreatic (Ferrone *et al*., 2009) and skin tumor (Monnerat *et al.*, 2007) but its extensive role was studied in majority of ovarian and breast cancers (both sporadic and hereditary) (Easton *et al*., 1995). Similarly, the role of *COX-2* has been suggested that *COX-2* and VEGF are involved to increase angiogenesis in malignant tumors (Queiroga *et al.*, 2011). The Immuno histochemical study showed that strong expression of *COX-2* was seen in tumors with 67% chances of reoccurrence of tumor (Strazisar *et al.*, 2008). 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 mammary tumors of cats and also the gene expressional 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 numbers 1, 2, 12, 18, 19, 20, 21, and 22 of *BRCA1*gene were selected for mutational analysis but no mutation found in the exons which were studied. Although in humans, the *BRCA1* mutations are strongly related to hereditary breast cancers. However, the types of mutations differ in distribution according to geography and ethnicity. *BRCA1* is involved in supervising the cell cycle, however, mutations in *BRCA1* have been found strongly related to familial breast cancers, which are different, related to ethnicity and geographic location. For Ashkenezai Jewish the "hot spot" mutations are present at 5382insC and 185delAG (Abeliovich *et al*., 1997), whereas in Swedish people 3171ins5 is considered as the high risk familial mutation (Einbeigi *et al*., 2001). The prevalence of *BRCA1*mutations also varies in diverse populations. For example, the *BRCA1* mutation 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 cats, we did not find any mutation in sporadic mammary tumor. There was no variation in the entire coding region of *COX-2* as well. A group of researchers examined the association between *p53* mutation 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* genes was measured in all mammary tumors and normal mammary tissues. There was very good amplification of housekeeping gene (GAPDH) in all the tumors. However, *BRCA1* in mammary tumors showed negative gene expression. 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 (Triplett *et al.*, 2008). So, the loss of *BRCA1* gene expression in this research work showed the loss of function of this gene in our tumor samples as well while the *COX-2* gene expression was positive in all tumors. Out of five (n=5) feline mammary tumors of this research work, two tumors (40%) showed medium range of *COX-2* up-regulation, FMT1 having 1.76 and FMT4 having 1.84 fold changes while others showed low positive gene expression *i.e.* 0.76, 0 and 0.82 fold changes as compared to normal samples. The same results have been studied by Haleema Sadia and her co-workers in different canine tumors (Sadia *et al*., 2016). The fold change below 1 represented the positive gene expression but less than normal gene expression in this study. 0 fold change also had lowest positive behavior almost one step near to undetectable Ct value. Most of the previous studies were

based on immunohistochemistry and were described in a manner of positive and negative gene expression and positive gene expression represented the abnormal behavior of *COX-2* gene. This study was organized to perform the qPCR having gene expression results in fold change values, to compare the results of our research with previous studies, positive and negative behavior was also described here. Researchers described the positivity of *COX-2* gene in 83% canine and 81% of feline mammary carcinomas (Millanta *et al.,* 2014). Another report also showed the positive gene expression of *COX-2* at different levels in 87% tumors in which 50% cancers showed low, 32% showed intermediate while 5% cancers demonstrated high gene expression. These results showed that *COX-2* was expressed in majority of feline mammary carcinoma (Sayasith *et al.,* 2009). *COX-2* role was also estimated as a troubleshooter of angiogenesis in canine and feline invasive mammary carcinomas and among them 100% canine and 96% feline showed positivity of *COX-2* (Millanta *et al.,* 2006). Different trials investigated the benefits of *COX-2* inhibitors along with existing treatments in the management of cervical, ovarian and breast cancers (Munkarah *et al.,* 2002, 2003). *COX-2* inhibitors "Clecoxib" were found very effective in down regulation of *COX-2* protein expression in canine mammary tumor (Saito *et al.,* 2014).

CONCLUSION

These findings support a role of *BRCA1* and *COX-2* genes in the pathogenesis of these tumors. Those tumors that do not have up-regulation of *COX-2* may have role of other genes in tumorigenesis. This is the first study in Pakistan to investigate the role of aforementioned genes in mammary tumors of cats. The same type of results have been obtained by other scientists all over the world while working on different tumors of animals and humans. 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*. *COX-2* inhibitors may be used in the treatment of those tumors having upregulation of *COX-2* gene expression.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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