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RESEARCH ARTICLE

Evaluation of Estrogen Receptor α and ß Genes Expression in Normal and Neoplastic Mammary Gland in Dogs by Real-time PCR

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Abstract: ***Background*:** Estrogen and progesterone play a key role in mammary tumorigenesis of female dogs. Malignant tumors are considered hormone-dependent neoplasms, which may reappear after surgical removal or might spread to other organs such as liver, lymph nodes, and lungs.

***Objective*:** The purpose of this study was to evaluate the expression of estrogen receptor α and ß genes in normal and neoplastic mammary glands of female dogs.

***Method*:** Ten neoplastic tumors (benign and malignant) and 10 normal samples were selected. After RNA extraction and cDNA synthesis, ERα and ERβ genes were amplified by Real-time PCR based on SYBR green related to β-actin reference gene.

***Result*:** The obtained C_t and ΔC_t values were analyzed by GLM procedure of SAS software v9.2. The results showed that the expression of ERα and ERβ genes in neoplastic cases was over- and down-expressed respectively as compared with normal samples ($p < 0.01$).

***Conclusion*:** The findings indicated that these genes could be used as reference genes for screening and also for early diagnosis of patients, and this method could be helpful for patient selection and monitoring of the anti-estrogen therapy.

Keywords: Breast cancer, ERα, ERβ, Relative real-time PCR, Canine, neoplastic mammary gland.

1. INTRODUCTION

Mammary gland tumors are known to be among the commonest of neoplasms in humans as well as dogs. About 52% of all tumors correspond to mammary neoplasia, which is repeatedly reported among dogs and is the most common malignant neoplasia among bitches [1, 2]. Malignant tumors can be found in roughly 50% of mammary tumors in canids [2-4]. Malignant tumors are hormone-dependent neoplasms and might reappear after surgical removal or spread to other organs such as liver, lymph nodes, and lungs [5, 6].

Epidemiological studies on animals disclose the link between 17-beta-estradiol (E2) and breast cancer. It is known that epoxidation can activate E2 reaction with the capability of inhibiting DNA synthesis by DNA-binding and adduct formation (*in vivo* and *in vitro*) which has an important role as a mammary carcinogenesis promoter [7].

The etiology of female breast cancer in humans is dependent on a number of factors including genetic predisposition, the timing between the onset of menarche, as well as first pregnancy. It also depends on the activity of hormone receptors in the mammary tissue [8, 9]. Progesterone and estrogen play a key role in controlling the proliferation of mammary glands and tumor formation [10, 11].

Estrogen has a direct effect on breast cancer development in females. Similarly, a 4-fold higher risk of tumor occurrence is seen in non-spayed female dogs <2 years old compared to spayed dogs at the same age [12].

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Long-term exposure of female dogs to progesterone acts as a stimulus for the proliferation of the mammary epithelium (due to treatment by exogenous progesterone in order to interrupt the ovarian cycle, or due to the endogenous progesterone that is secreted from the corpus luteum throughout the luteal phase of the menstrual cycle). Estrogen and progesterone are critical for the development of mammary glands; however, they elevate the likelihood of developing neoplasia in these glands. The physiological effects of such hormones are mostly moderated by receptors that are expressed in the mammary tissue [13, 14]. The mammary tumors of humans and dogs are regulated by hormones; therefore, the hormone dependency of mammary neoplasia among bitches is somewhat similar to breast cancer in humans [7, 15]. In this study, we established a real-time (RT)-quantitative PCR method in order to successfully quantify the expression of estrogen receptor α and ß genes in normal as well as neoplastic tissues of mammary glands among canids.

2. MATERIALS AND METHODS

2.1. Animals

The experiment protocol was approved by Animal Welfare Committee of Veterinary faculty, Ferdowsi University of Mashhad, Iran. In total, 10 adult bitches with mammary tumors were selected from different breeds. All individuals were brought to the veterinary teaching hospital of Ferdowsi University of Mashhad to be treated by surgical excision. To ignore differences in the endocrine status of bitches, we obtained mammary tumor tissue and normal tissue from the same individuals.

2.2. Collection of Samples

A surgery was performed on each individual to collect contralateral normal tissue and tumors from mammary glands of the same bitch. Each tissue sample was divided into two parts. One part was maintained in liquid nitrogen for real-time PCR. The other half was fixed in 10 percent neutral buffered formalin, then dehydrated and submerged in paraffin. Afterward, sections with 5-µm thickness were separated and then stained using eosin and hematoxylin, and finally used for histopathology, as stated in the histopathological classification reported by Goldschmidt [16].

2.3. RNA Extraction and cDNAs Synthesis

Total RNA was extracted from mammary gland specimens by Trizol kit (Invitrogen, USA). Quality and quantity of RNAs were measured by nanodrop ND-2000 spectrophotometer (Thermo, Wilmington, USA). RNAs were reverse transcribed and cDNAs were synthesized using RevertAid™ H minus Reverse Transcriptase kit (Fermentas, Burlington, USA). Measurement of the cDNAs quality and quantity were carried out by nanoDrop ND-2000 spectrophotometer. In order to accomplish cDNA normalization, all synthesized cDNAs were diluted to 30 ng/µl concentration for uniformity by diethyl pyrocarbonate (DEPC) water.

2.4. Primer Design

Primers for β-actin as the reference gene, and for ERα and ERβ as the target genes were designed by the Primer premier software, version 5. To validate total gene specificity of primers' nucleotide sequences, we performed BLASTN searches and Primer Database such as RTPrimerDB. The primers were synthesized in Bioneer Co. South Korea.

2.5. Real-time Quantification PCR Assay

Real-time polymerase chain reaction (PCR) was conducted for amplification and quantification of ERα, β-actin and ERβ genes using SYBR® Green PCR Master Mix (Applied Biosystems, USA). Real-time PCR was carried out in 25 µl volume (7300 Real-Time PCR System, Applied Biosystems, USA). The optimum concentration of primers was set by a series of preliminary experiments. β-actin was used as the reference gene. Thermal cycling contained an initial denaturation at 95°C in 1 cycle of 10 minutes, then 45 cycles of 30 seconds at 95°C, followed by 30 seconds at 60°C, as well as 30 seconds at 72°C and for melting curves in 1 cycle of 15 seconds at 95°C, one minute at 60°C, and finally 15 seconds at 95°C and 15 seconds at 60°C. Reactions without reverse transcriptase or without template served as negative controls for ERα, ERβ and β-actin genomic DNA contamination. To confirm the specificity of the amplified products, the real-time PCR products were randomly analyzed by electrophoresis on 1.5% agarose gel.

2.6. PCR Efficiency and Statistical Analysis

PCR efficiency and data analysis were performed using the Pfaffl, 2001 method [17]. A standard curve was constructed from a set of known concentrations of complementary DNA generated from universal canine reference RNA to determine PCR efficiency of ERα, ERβ and β-actin genes. The dilution series consisted of concentrations of 300, 30, 3, 0.3, 0.03, and 0.003 ng/µL. The ΔC_t for ERα, ERβ and β-actin genes was determined for each concentration, and the slope of the line of best fit was calculated from a plot with ΔC_t on the y-axis and the log of total complementary DNA on the x-axis. For analysis, the ratio was calculated by normalizing the copy of ERα and ERβ in each sample by dividing obtained ERα and ERβ C_t by the C_t obtained for β-actin ($normalization = Ct\ Er\alpha\ and\ ER\ \beta$ [$sample$]/$Ct\ \beta - actin$ [$sample$]). Normalized values for each triplicate sample were averaged to give the final data used. The fold change in the target gene for the

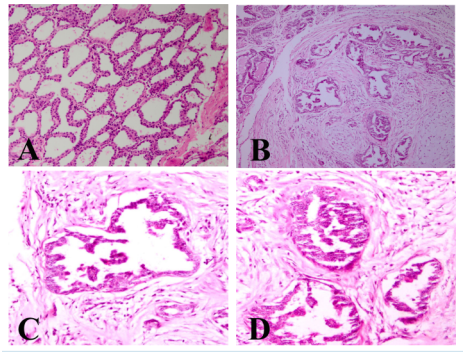

Fig. (1). The cancerous and normal breast samples pathology results. A- Normal mammary gland: There are alveoli with cuboidal epithelium. **B-** Complex-type carcinoma: There are malignant epithelial component, spindle-shaped cells and chondromucinous compartments. **C & D-** ERα protein expression in female dog breast cancer tissues: A strong dark blue staining on the nucleus of breast ductal epithelium demonstrates the high protein expression of ERα.

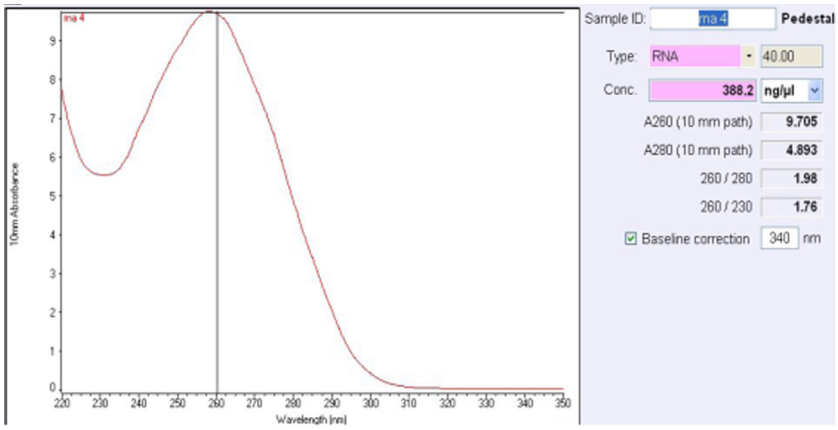

Fig. (2). The nanodrop spectrophotometry result. The presence of sharp peak demonstrated that the RNA concentration and purity.

results of quantitative amplification was also calculated for each sample using $2^{-\Delta\Delta Ct}$ method [17], where $\Delta\Delta C_t$ = (C_t*Era and ERb* - C_t *B – actin*) *cancer* – (C_t*ERa and ERb* –C_t) normal. Ratios were expressed as inverse ratios (1/ratio) to reorient changes in ratio to reflect actual behavior of ERα and ERβ (*increased ration = increase in Era and ERb copy number*). For categorical variables, we used Fisher's exact test. Also, student's t-test procedure was performed in SAS v9.2 software and Microsoft Excel determine statistical significance. A significance level of the tests was taken at $p < 0.01$.

3. RESULTS

3.1. Histopathology and RNA Extraction

The histopathology results indicated that the ten neoplastic tumors comprised 3 benign and 7 malignant mammary tumors (Fig. **1**). The remaining seven malignant mammary gland tumors included 1 tubulopapillary carcinoma, 2 simple carcinomas, 1 cystic papillary carcinoma and 3 complex-type carcinomas (Table **1**). The spectrophotometry results showed high purity of total RNAs and cDNAs without any phenol or EDTA contamination (Fig. **2**).

Table 1. Histopathological classification of tumor.

Histological Diagnosis	No.
Benign tumour	
Benign mixed tumor	2
Fibroadenoma	1
Malignant tumor	
Tubulopapillary carcinoma	1
Simple carcinoma	2
Cystic papillary carcinoma	1
Complex-type carcinoma	3
Total	10

Table 2. Properties of primers.

Annealing Temperature	Product Size	Primer Sequence	Genes
60	136	CGGAAAACTGCTCCTGTAAATGC	ERα-F
		CGTCAAAGAGGTCACAGAGGGTA	ERα-R
59	100	CATCCATTGCCAGTCGTCAC	ERβ-F
		GAACTTGGACTAGTAATGGGGCT	ERβ-R
60	103	CAAATGTGGATCAGCAAGCAG	β-Actin-F
		GAAAGGGTGTAACGCAACTAAAG	β-Actin-R

3.2. Primer Design and Real-time PCR Assay

The nucleotide sequences and properties of the designed primers of the current study are shown in Table **2**. Qualitative PCR assay of cDNA was implemented to be confident that the size of amplified segments of ERα, ERβ and β-actin genes were in expected range. These results demonstrated that all primers were designed correctly and PCR reaction was done accurately (Fig. **3**). Based on the outputs of the Primer premier software, the optimum annealing temperature was 60°C for ERα and β-actin genes and 59°C for ERβ gene. The results of amplification of ERα, β-actin and ERβ genes by real-time PCR assay indicated that amplification was done without any noise. SYBR green florescence dye does not act specifically in real-time PCR reaction. To verify amplification specificity, a melting curve was run and plotted after PCR to be sure of specific segments production, the absence of nonspecific band, secondary structures such as hairpin loops, self-dimer and cross-dimer primer (Fig. **4**). The results of the melting plot indicated that all primers acted specifically and there were no nonspecific segments and secondary structures.

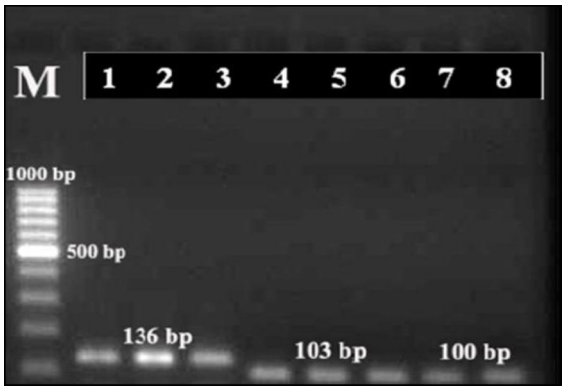

Fig. (3). The results of qualitative PCR assay was performed on 1.5% agarose gel. M: 100 bp Size Marker (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 bp), 1-3: PCR product of ERα gene. 4-6: PCR product of ERβ gene, 7-8: PCR product of β-Actin gene.

3.3. PCR Efficiency and Statistical Analysis

The standard curve design could be reliably used as a substitute for real-time PCR efficiency evaluation. We used the slope of the standard curve to measure the efficiency of PCR amplification in the real-time PCR, in which a slope of –3.32 shows that the PCR reaction was 100% efficient. Negative slopes higher than –3.32 demonstrate efficiency of less than 100%, which might be an indication of problems with manual pipetting or the quality of samples. The ERα, ERβ standard curve by real-time PCR was plotted as serial dilution of cDNA versus C_t values. The slope of this curve was 3.1, which was in the expected range. The obtained results demonstrated that PCR efficacy was 95% for both ERα and ERβ genes.

A ratio of higher or lower than the average ratio of noncancerous samples was considered as gain or loss of ERα and ERβ copy number/ amplification, respectively. The obtained results indicated that there was a significant difference between neoplastic and normal tissues for ERα and ERβ expression amount. Fold change was calculated about 12.65 and 0.06 ($p < 0.01$), which indicated that ERα and ERβ expression in cases with breast neoplasm was over- and low-expressed, respectively (Fig. **5**). Over-expression of ERα in breast tumors led to an increase in the ratio of $ER\alpha / ER\beta$ in adenocarcinoma tissue compared to normal tissue.

4. DISCUSSION

Molecular markers could be utilized to have an accurate prognosis and predict the response, resistance, or toxicity to therapy. To profile a tumor completely, the diversity of genomic alterations involved in malignancy needs a diversity of assays. Gene expression is the main factor in some new molecular classifications of tumors which requires a paradigm shift in specimen processing to keep the integrity of RNA for analysis. More stable markers, such as DNA and protein, could be readily processed in the clinical laboratory. Quantitative real-time PCR is able to analyze gene

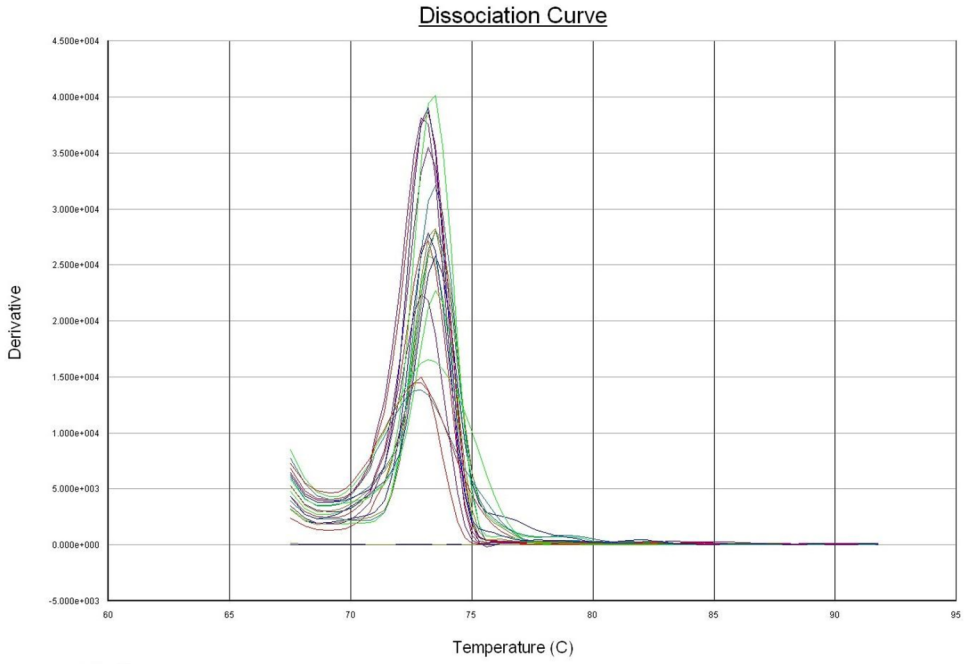

Fig. (4). Confirmation of qRT-PCR amplification specificities of ERα gene. Melting peaks were examined for the ERα with the samples and NTC.

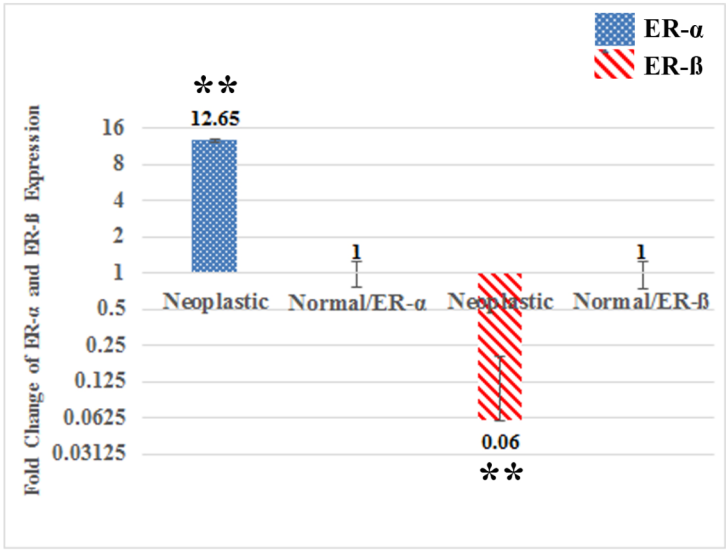

Fig. (5). Plot of logarithmic Fold change expression of ER-α and ER-β in cancerous and normal samples. The ER-α and ER-β expression rate were distinguished using a qRT-PCR. The ER-α and ER-β expression was significantly upregulated and downregulated in cancerous breast tumors in compared to normal breast tissues.

duplications or deletions. In addition, the melting curve analysis shortly after PCR can determine the small mutations, down to single base changes. Such methods are becoming faster, more accessible, and possible to be multiplexed. Real-time PCR methods are a proper alternative for the analysis of cancer markers [18].

Progesterone and estrogen receptors can be naturally expressed and found in normal as well as neoplastic tissues [19]. To estimate whether hormonal treatment could be considered more beneficial compared to cytotoxic therapy, one can use the receptor expression status of mammary tumors in dogs [20]. Ninety-five percent of normal mammary tissues in canids have progesterone and/or estrogen receptors [21]. Most recent study results indicated that 65 to 70% of human breast tumors and more than 50% of mammary tumors in dogs [1, 2, 22] expressed estrogen and progesterone receptors. The current study was conducted to assess

gene expression of the estrogen receptor α and ß in normal and neoplastic mammary glands in dogs. Analysis of the results indicated that the expression of ERα was significantly associated with malignancy which is similar to the results of previous reports [2, 23, 24]. Moreover, our obtained results demonstrated that more than 75% of neoplastic mammary gland tissues express the estrogen receptors and those receptors stimulate breast cancer increase and progression. PCR efficiency evaluation includes some theoretical and practical problems that are not present in the standard curve method, particularly due to its simplified calculations. We derived a standard curve out of serial dilutions, which was then displayed in the form of a semi-log regression line graph representing C_t values versus logarithm of input nucleic acid. To produce the standard curve with sufficient quality, we had an adequate number and range of standard dilutions. Basically, percentage of tumors with estrogen receptors varies considerably depending on the study method. Hence, in different studies percentage of ER expression in malignant cancerous tissues varied, and was reported 12.96-92.3 [2, 24-27]. On the other hand, similar results were reported for percentage of ER expression in benign cancerous tissues, which varied from 49 to 100 [2, 23, 24, 26, 28]. Surprisingly, Kim *et al.*, 2015 have shown the ER and PR are decreased in malignant canine mammary tumors [3]. Some investigations indicated that the expression of progesterone and estrogen receptors is associated with tumor size and malignancy grade.

There are some factors that trigger growth of neoplastic as well as normal mammary tissues, for instance growth factors that bind to the surface of receptors of the target cells, or steroid and peptide hormones. Many studies have confirmed the presence of a number of receptors for progesterone (PR), estrogen (ER), epidermal growth factors (EGF-R), and prolactin (PRL-R) in normal mammary glands of healthy as well as tumorous dogs [29]. Such studies also revealed that about 40-60 percent of malignant and benign mammary tumors were positive for estrogen, progesterone, and prolactin [16], as well as epidermal growth factors [7] and androgen receptors [30]. Moreover, the concurrent presence of a number of receptors was also observed in several tumors. As a result of admixture of epithelial cells with high levels of estrogen and progesterone, false positivity of receptor values might be observed in malignant mammary tumors. As a matter of fact, false-positive values of receptors were more common, and receptor levels were considerably greater in admixture of carcinomas and normal tissues compared to pure or non-mixed carcinomas [31]. Malignant mammary tumors were mostly observed in the process of treatment either with high doses of progesterone or with combinations of progestin-estrogen, while low doses of progesterone appeared to provide some levels of protection [16, 32]. In mammary tumors, there was a significant association between receptor-rich carcinomas and long-term survival after the surgery [33].

CONCLUSION

Quantitative analysis of real-time PCR data as C_t value showed that ERα and ERβ genes expression in malignant cases was over- and down-expressed, respectively ($p < 0/01$). The significant difference between gene expressions in malignant cases compared with the benign samples showed that dogs with malignant tumors were significantly more likely to have a higher ERα expression, whereas ERβ expression had decreased. Results of the present study indicated that these genes could be used as marker genes for screening and also an early diagnosis of patients, even at the very beginning stages of breast cancer. Furthermore, this method could be helpful for patient selection and monitoring the anti-estrogen therapy.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

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