

Analysis of Expressed Genes in Normal and Tumoral Mammary Gland Tissue of the Terrier Dog

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Abstract

Mammary gland tumor is the most common type of tumor in female dogs. Data on genes that are involved in tumorigenesis and mechanism of tumor development are insufficient. Comparative studies have been conducted in order to see if tumorigenesis studies in the dog could be a model for human mammary gland tumors. In this study, we constructed two different cDNA libraries from mammary tissue, which were collected from a normal mammary tissue of a healthy Terrier dog and a tumoral mammary tissue of a sick dog. A total 2304 colonies which are randomly picked out from the two libraries were sequenced for developing a dog mammary gland ESTs collection. Raw EST data were analyzed with Phred/Phrap programs and readable EST sequences were assembled with the CAP3 program. All of EST sequences were grouped into 45 contig and 2203 singletons. Putative functions of all unique sequences were designated by NCBI BLAST based on gene homology and annotated by BLAST2GO. The results of this study are a very valuable resource for functional genome studies of the dogs.

Keywords: Terrier, Dog, Breast cancer, EST, cDNA library

Terrier Köpeklerinin Normal ve Tümörlü Meme Bezi Dokusunda Eksprese Edilen Genlerin Birincil Analizi

Öz

Meme bezi tümörü, dişi köpeklerde en sık rastlanan tümör türüdür. Tümör oluşumu ve tümör oluşum mekanizması ile ilgili genler hakkındaki veriler yetersizdir. Köpektaki tümörigenez çalışmalarının insan meme bezi tümörleri için bir model olabileceğini görmek için karşılaştırmalı çalışmalar yürütülmüştür. Bu çalışmada, sağlıklı bir Terrier köpeğinin normal meme dokusundan ve hasta bir köpekten tümörlü meme dokusundan toplanan iki farklı dokudan iki farklı cDNA kütüphanesi oluşturuldu. Bir köpek meme bezi EST koleksiyonu geliştirmek için, iki kütüphaneden rastgele seçilen toplam 2304 koloni dizilendi. Ham EST verileri Phred/Phrap programları ile analiz edildi ve okunabilir EST dizileri CAP3 programı ile bir araya getirildi. Tüm EST dizileri 45 kontig ve 2203 singlet grubuna ayrıldı. Tüm benzersiz sekansların varsayımsal fonksiyonları, NCBI BLAST tarafından gen homolojisine dayalı olarak belirlendi ve BLAST2GO ile açıklandı. Bu çalışmanın sonuçları, köpeklerin fonksiyonel genom çalışmaları için çok değerli bir kaynaktır.

Anahtar sözcükler: Terrier, Köpek, Meme Kanseri, EST, cDNA kütüphanesi

INTRODUCTION

Canis lupus familiaris is a useful model organism for medical studies because of its genetic and morphological diversity ^[1]. Dogs are pure animals as opposed to hybridized

rats, mice, and other laboratory animals. Many dog species are particularly susceptible to some of the same health conditions as humans, such as cancer, heart disease, rheumatoid arthritis, autoimmune disorders, deafness, and blindness ^[2,3]. In recent years, the sequencing of the



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dog genome and the similarity to the human genome has made the dog a suitable model for cancer studies. Dogs share a common environment with humans, are exposed to the same carcinogens and become an important model for breast cancer development in humans [3-7].

The mammary gland is an excellent system to study questions about organogenesis, cell differentiation and oncogenesis [8]. The most commonly diagnosed neoplasm in female dogs is mammary-gland tumor which is of an epithelial origin, and approximately 50% are malignant [9-11]. In dogs, breast cancer is complex and is formed by the interaction of genetic and environmental factors [1,11-14].

To further understand the molecular mechanism and differences between tumoral and normal mammary gland, it is necessary to obtain a collection of genes normal and tumoral stage specific gene expression profile. Expressed sequence tags (EST), single-pass and partial sequencing of randomly selected cDNA clones from cDNA libraries, has proved powerful for the discovery of novel genes, analysis of gene expression profile in a given tissue [15-18].

One thousand one hundred and fifty-two clones were sequenced from the normal mammary gland tissue cDNA library and 1152 clones were sequenced from the tumoral mammary gland tissue cDNA library. All of the ESTs were analyzed by using Phred and Contig Assembly Program 3 (CAP3) software. Annotation was performed by NCBI BLAST and BLAST2GO software.

In the present study, we aimed to gain gene expression profile of mammary gland based on the total 2304 ESTs as well as preliminary results of a comparison of normal and tumoral stages. These results facilitate the use of functional genomics approaches aiming at creating gene expression patterns of tumoral and normal mammary gland.

MATERIAL and METHODS

Tissue Samples

The mammary tissue samples were taken from the farm of the Faculty of Veterinary Science at the University of İstanbul. The normal mammary tissue of a healthy Terrier dog and neoplastic mammary tissue of a sick Terrier dog were taken by biopsy. These tissue samples were immediately frozen in liquid nitrogen and stored at -80°C.

cDNA Library Construction

Total RNA was separately extracted from 1.311 g normal and 1.056 g neoplastic mammary tissue with the RNeasy Kit (Qiagen) and mRNA was isolated from total RNA using the Oligotex Spin-Column Protocol (Oligotex mRNA Mini Kit, Qiagen, Valencia, CA). cDNA libraries were constructed with the CloneMiner cDNA Library Construction Kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Double stranded cDNA was cloned

into a pDONR222 vector and transformed into *Escherichia coli* strain DH5 (Invitrogen, Carlsbad, CA, USA). Each cDNA library was plated onto LB-kanamycin agar medium and individual grown colonies were picked into 384-well plates with SOB medium and inoculated overnight. After the addition of glycerol (10% v/v) the library stored at -80°C. Plasmid DNA was prepared by using alkaline lysis method. Isolated 96 DNAs were digested with Bsp1407I and analyzed by a 1% agarose gel electrophoresis for identifying insert size.

Generation of Expressed Sequence Tags

A total of 2304 clones from cDNA library were selected randomly and amplified by using M13 universal primers and DNA sequencing was performed at the Genome Sequencing Center, Washington University in St. Louis (WUSTL) using the ABI 3730 capillary sequencer (PE Applied Biosystems, Foster City, CA).

Sequencing Analysis

The cDNA sequences were analyzed with Phred/Phrap programs, vector and adaptor contamination were removed. Resulting sequences were separately assembled into contigs using the CAP3 program. The default values were used for all the parameters. Putative functions of all unique ESTs were designated by gene homology based on BLAST and were classified according to biological process, cellular component and molecular function based on Gene Ontology (GO) mapping provided by BLAST2GO which is a bioinformatics platform for the functional annotation and analysis of genomic data sets [19,20].

RESULTS

Characterization of cDNA Libraries

Two different cDNA libraries have constructed a pool of RNA extracted from normal and tumoral mammary gland tissue. As a result of the restriction enzyme digestion, the insert size distribution ranged from 380 to 1800 base pairs in the cDNA library constructed from normal mammary gland tissue which consisted of 6.2×10^4 clones and its average insert length was 0.845 kb. In the cDNA library for tumoral mammary gland tissue which consisted of 12.6×10^4 clones, its average insert length was 0.64 kb and the insert size distribution ranged from 250 to 1700 bps. After construction of cDNA libraries, 1152 clones were randomly sequenced from normal mammary gland tissue; 1152 clones were randomly sequenced from tumoral mammary gland tissue. Consequently, 2304 EST sequences were generated.

BLAST Analysis of ESTs

Raw EST data were processed and base-called by using Phred/Phrap computer programs. The EST sequences were trimmed to remove vector and adaptor sequences.

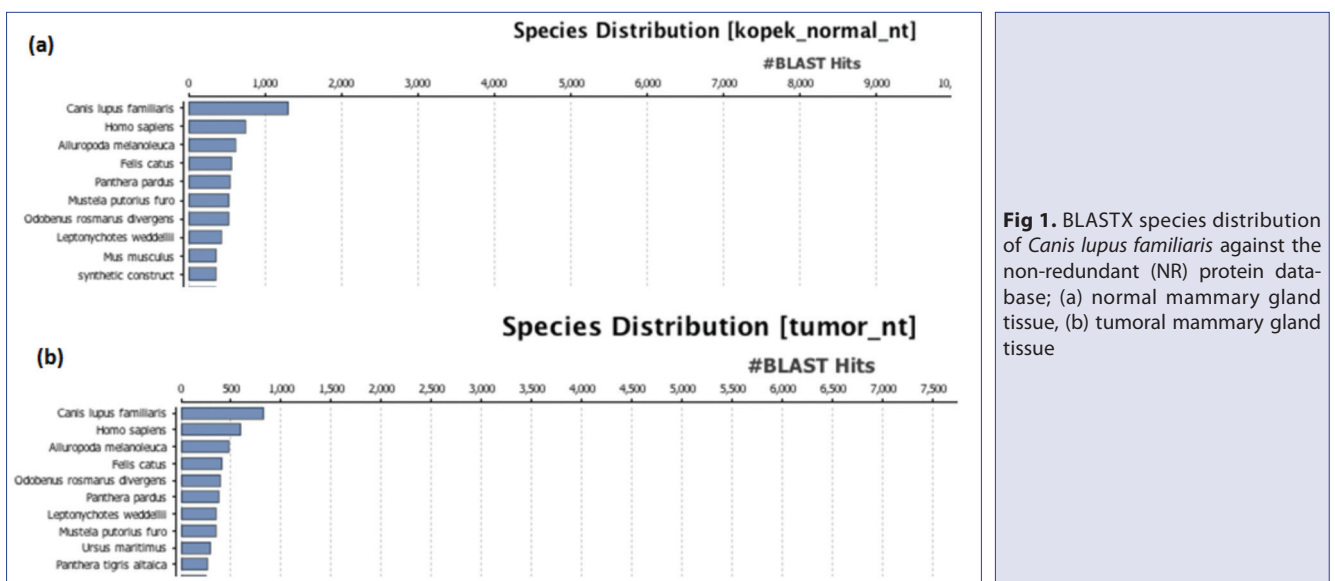


Fig 1. BLASTX species distribution of *Canis lupus familiaris* against the non-redundant (NR) protein database; (a) normal mammary gland tissue, (b) tumoral mammary gland tissue

After this process, for contig assembly, EST sequences were analyzed by using the CAP3 program. One thousand one hundred and eighteen normal ESTs were assembled into 21 contigs and length ranged from 510 to 1870. One thousand one hundred and fifty tumoral ESTs were assembled into 24 contigs and length ranged from 582 to 1527. All of the contigs were analyzed by BLASTN in NCBI. For putative functions, all the EST sequences were designated by gene homology based on BLAST. The database search algorithm BLASTX was done using Blast2GO software. Functional annotations were performed in 3 steps by using Blast2GO 4.1.7 tool. Firstly, EST sequences were used as a query in BLASTX searches against the NCBI non-redundant protein database to find homologous sequences with default parameters. Secondly, mapping was used to retrieve GO terms as associated with BLASTX hits. Finally, annotation was used to associate with queries reliable information.

The similarity results summarized in *Fig. 1* in the form of Species Distribution, showing the relationship to other sequenced species. The Species Distribution graph shows a list of the different species to which most sequences were aligned during the blast. According to all BLASTX results of 1152 normal tissue ESTs, the first five species in the species distribution graph were *Canis lupus familiaris*, *Homo sapiens*, *Ailuropoda melanoleuca*, *Felis catus* and *Panthera pardus*. The first five species in the species distribution graph of 1152 tumoral tissue ESTs were *Canis lupus familiaris*, *Homo sapiens*, *Ailuropoda melanoleuca*, *Felis catus* and *Odobenus rosmarus divergens*.

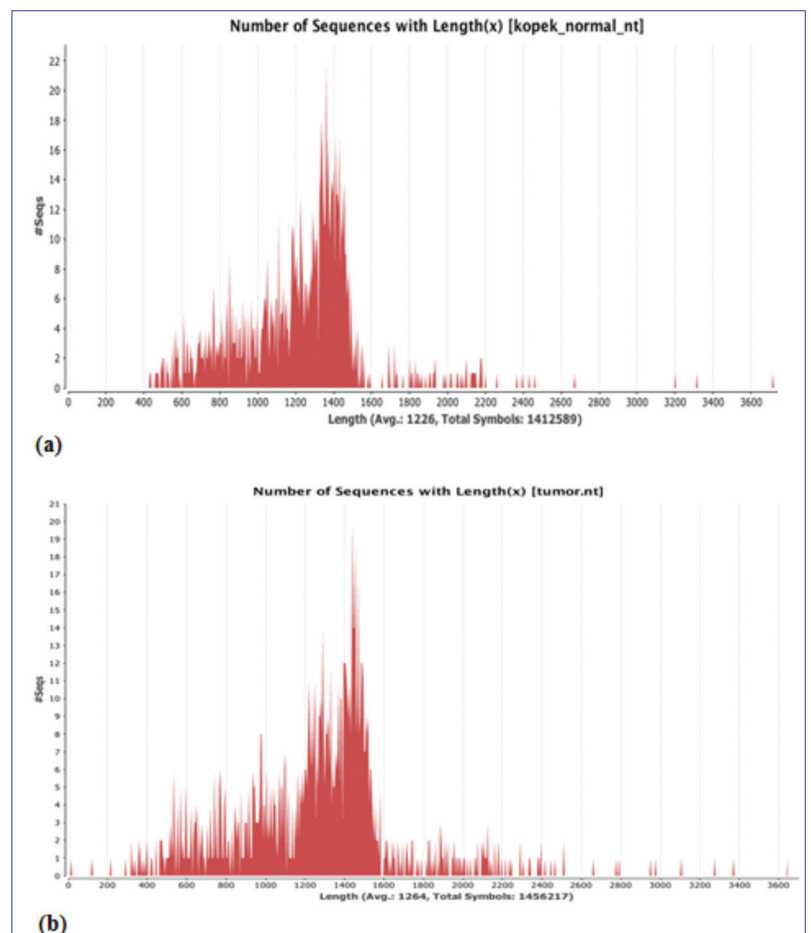


Fig 2. Number of sequences with length distribution of ESTs (a) normal mammary gland tissue, (b) tumoral mammary gland tissue

GO Analysis of ESTs

Blast2GO program performed BLASTX search against the NCBI non-redundant protein database, retrieved GO terms for the top 20 BLAST results and annotated the sequences

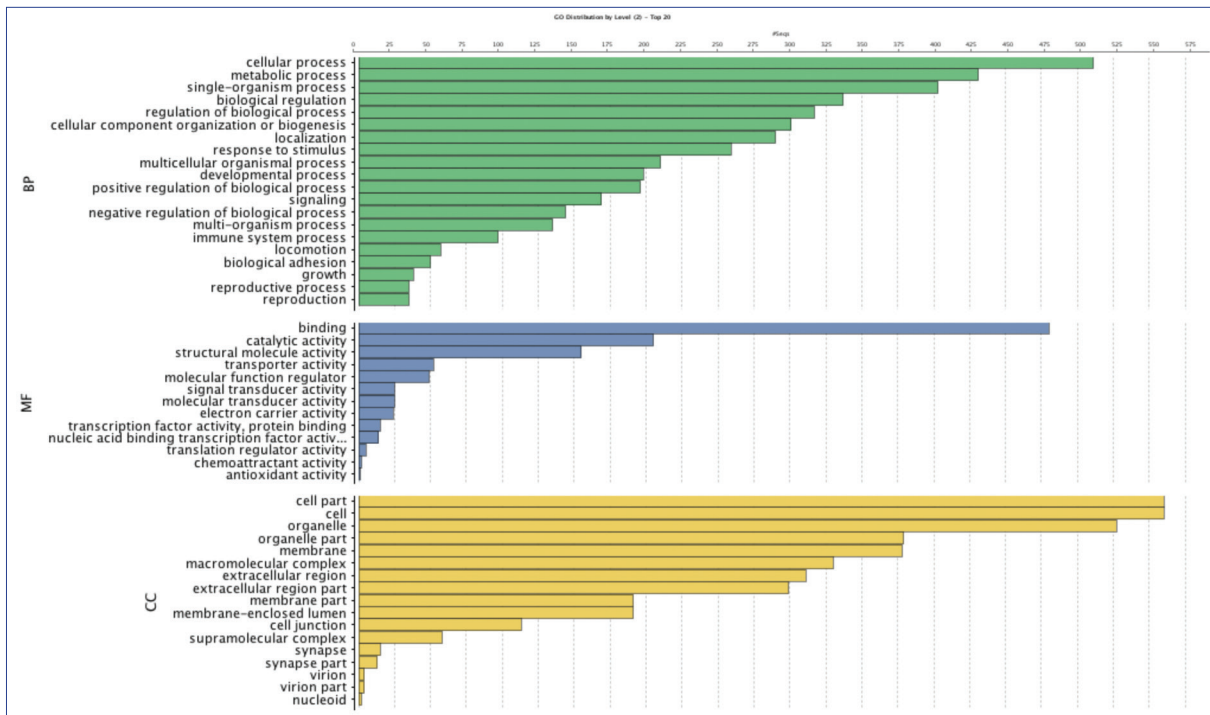


Fig 3. Distribution of normal ESTs by biological processes, molecular function, and cellular component GO categories at level 2

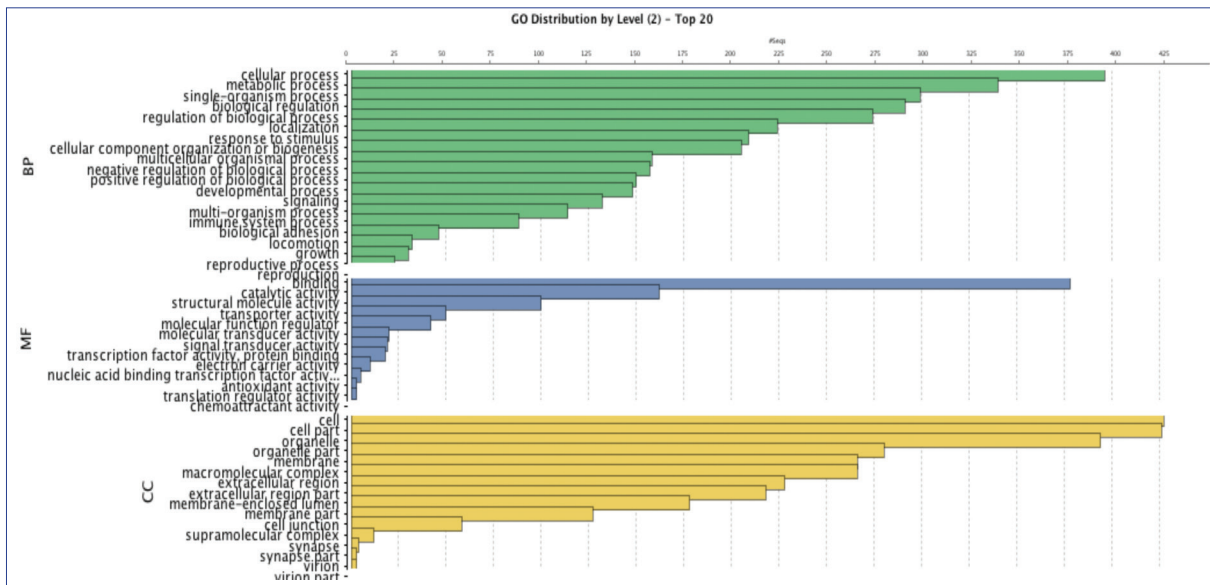


Fig 4. Distribution of tumoral ESTs by biological processes, molecular function, and cellular component GO categories at level 2

based on default parameters. The ESTs were classified according to GO terms such as molecular function, biological process, and cellular components.

As shown in Fig. 2, numbers of sequences with the length of the 1152 normal tissue ESTs were mostly between 1100 and 1500 base pairs, and the sequence length average was calculated as 1226 bp. Numbers of sequences with the length of the 1152 tumoral tissue ESTs were mostly between 1100 and 1500 base pairs, and the sequence

length average was calculated as 1264 bp.

Annotated genes were showed in Fig. 3 and Fig. 4 with biological process, molecular function, and cell component subclasses based on their annotation. Go Distribution by Level graph shows all GO terms for all 3 categories for GO level 2, taking into account the GO hierarchy. According to this graph of normal tissue ESTs, the terms in Biological Process were mostly distributed in the cellular process, metabolic process, single-organism process, biological

Table 1. The BLASTN results of normal contigs

Contig Number	Description	Query Coverage	Max. Ident.	Length (bp)	Number of ESTs
Contig 1	PREDICTED: <i>Mustela putorius furo</i> gelsolin (GSN), transcript variant X4, mRNA	73%	89%	1870	2
Contig 2	<i>Canis lupus familiaris</i> isolate HXHdog mitochondrion, complete genome	56%	99%	1359	4
Contig 3	PREDICTED: <i>Canis lupus familiaris</i> troponin T type 3 (skeletal, fast) (TNNT3), transcript variant X2, mRNA	60%	99%	1585	2
Contig 4	<i>Canis lupus familiaris</i> ribosomal protein S27a (RPS27A), mRNA	58%	100%	910	2
Contig 5	<i>Canis lupus familiaris</i> ribosomal protein SA pseudogene (LOC610641) on chromosome 20	69%	99%	1489	4
Contig 6	PREDICTED: <i>Homo sapiens</i> zinc finger protein 484 (ZNF484), transcript variant X2, misc_RNA	83%	74%	728	3
Contig 7	<i>Canis lupus familiaris</i> ribosomal protein L19 (RPL19), mRNA	87%	100%	783	2
Contig 8	PREDICTED: <i>Canis lupus familiaris</i> thymosin beta-4 (LOC100683370), mRNA	59%	100%	1059	2
Contig 9	<i>Canis lupus familiaris</i> ribosomal protein L7 (RPL7), mRNA	83%	100%	971	2
Contig 10	<i>Canis lupus familiaris</i> isolate HXHdog mitochondrion, complete genome	87%	100%	920	2
Contig 11	<i>Canis lupus familiaris</i> ribosomal protein L10a pseudogene (LOC474656) on chromosome 11	66%	99%	1095	2
Contig 12	PREDICTED: <i>Odobenus rosmarus divergens</i> annexin A2 (LOC101364324), mRNA	29%	94%	1409	2
Contig 13	<i>Canis lupus familiaris</i> selenoprotein M (SELENOM), mRNA	61%	98%	1114	2
Contig 14	<i>Canis familiaris</i> chromosome 12, clone XX-511E22, complete sequence	13%	85%	1262	4
Contig 15	<i>Canis lupus familiaris</i> ribosomal protein S15a (RPS15A), mRNA	88%	99%	510	2
Contig 16	<i>Canis lupus familiaris</i> metallothionein 1E (MT1E), mRNA	48%	99%	899	2
Contig 17	<i>Canis lupus familiaris</i> ribosomal protein S6 (RPS6), mRNA	71%	99%	1153	2
Contig 18	<i>Canis lupus familiaris</i> vimentin (VIM), mRNA	68%	99%	1379	2
Contig 19	PREDICTED: <i>Neomonachus schauinslandi</i> eukaryotic translation initiation factor 4A2 (EIF4A2), transcript variant X6, mRNA	70%	99%	998	2
Contig 20	PREDICTED: <i>Canis lupus familiaris</i> S100 calcium binding protein A11 (S100A11), mRNA	63%	100%	884	2
Contig 21	PREDICTED: <i>Canis lupus familiaris</i> ribosomal protein L28 (RPL28), mRNA	49%	100%	934	3

regulation and regulation of the biological process. The terms in Molecular Function were mostly distributed as binding, catalytic activity, structural molecule activity, transporter activity and molecular function regulator. The terms in the Cellular Component were mostly distributed cell part, cell, organelle, organelle part, and membrane.

The terms of tumoral tissue ESTs, Biological Process were mostly distributed in the cellular process, metabolic process, single-organism process, biological regulation and regulation of the biological process. The terms in Molecular Function were mostly distributed as binding, catalytic activity, structural molecule activity, transporter activity and molecular function regulator. The terms in the Cellular Component were mostly distributed cell, cell part, organelle, organelle part, and membrane.

Analyzing of Contigs

According to BLASTN results in [Table 1](#), 8 of 21 normal tissue contigs are similar to "ribosomal protein". Ribosomal protein forms subunits of ribosomes and is involved in protein synthesis. The results of the other normal tissue contigs are as follows: "Gelsolin" is an actin-binding protein, "troponin T" is responsible for muscle contraction, "zinc finger protein" functions in protein transcription,

"thymosin beta-4" plays a role in regulation of actin polymerization, "annexin" is a cellular protein linked to phospholipid membranes by calcium, "selenoprotein" contains selenium, "metallothionein 1E" binds heavy metals, "vimentin" anchors the position of the organelles in the cytosol, "translation initiation factor" stabilize the formation of the functional ribosome around the start codon, and "calcium-binding protein" participates in calcium cell signalling pathways.

According to BLASTN results in [Table 2](#), 9 of 24 tumoral tissue contigs are similar to "ribosomal protein". The results of the other tumoral tissue contigs are as follows: "Vacuole membrane protein 1" is a cancer-relevant cell cycle modulator, "protein kinase" modifies other proteins by chemically adding phosphate, "casein beta" is found in mammalian milk, "lipopolysaccharide-binding protein" modulates the LPS-induced immune response, "guanine nucleotide binding protein-like 3" regulates the cell cycle and affects cell differentiation, "biorientation of chromosomes in cell division 1-like 1" protects stalled/damaged replication forks from uncontrolled DNA2-dependent resection, "lysyl oxidase" catalyses collagen cross-link formation, "high mobility group nucleosomal binding domain 2" maintains an open chromatin

Table 2. The BLASTN results of tumoral contigs

Contig Number	Description	Query Coverage	Max. Ident.	Length (bp)	Number of ESTs
Contig 1	PREDICTED: <i>Canis lupus familiaris</i> ribosomal protein L10 (RPL10), transcript variant X1, mRNA	59%	100%	1217	2
Contig 2	<i>Canis lupus familiaris</i> ribosomal protein S11 (RPS11), mRNA	39%	99%	1446	2
Contig 3	<i>Canis lupus familiaris</i> ribosomal protein L32 (RPL32), mRNA	50%	100%	962	3
Contig 4	<i>Canis lupus familiaris</i> ribosomal protein S27a (RPS27A), mRNA	51%	100%	1072	2
Contig 5	<i>Canis lupus familiaris</i> ribosomal protein S15a (RPS15A), mRNA	50%	99%	883	2
Contig 6	PREDICTED: <i>Mustela putorius furo</i> vacuole membrane protein 1 (VMP1), transcript variant X2, mRNA	81%	86%	1091	4
Contig 7	PREDICTED: <i>Ailuropoda melanoleuca</i> protein kinase AMP-activated non-catalytic subunit gamma 2 (PRKAG2), mRNA	81%	91%	1067	2
Contig 8	<i>Canis lupus familiaris</i> isolate HXHdog mitochondrion, complete genome	57%	100%	1152	2
Contig 9	PREDICTED: <i>Canis lupus familiaris</i> ribosomal protein S9 (RPS9), mRNA	87%	100%	795	2
Contig 10	<i>Canis lupus familiaris</i> casein beta (CSN2), mRNA	93%	99%	1298	2
Contig 11	PREDICTED: <i>Canis lupus familiaris</i> lipopolysaccharide binding protein (LBP), mRNA	94%	95%	1232	2
Contig 12	PREDICTED: <i>Canis lupus familiaris</i> guanine nucleotide binding protein-like 3 (nucleolar) (GNL3), transcript variant X1, mRNA	97%	99%	982	2
Contig 13	PREDICTED: <i>Canis lupus familiaris</i> ribosomal protein S20 (RPS20), mRNA	75%	100%	655	2
Contig 14	PREDICTED: <i>Canis lupus familiaris</i> biorientation of chromosomes in cell division 1-like 1 (BOD1L1), mRNA	40%	98%	1103	2
Contig 15	<i>Canis familiaris</i> chromosome 31, clone XX-443F3, complete sequence	51%	96%	945	2
Contig 16	PREDICTED: <i>Canis lupus familiaris</i> lysyl oxidase (LOX), transcript variant X1, mRNA	57%	99%	944	2
Contig 17	<i>Canis lupus familiaris</i> high mobility group nucleosomal binding domain 2 (HMGN2), mRNA	77%	95%	1419	2
Contig 18	PREDICTED: <i>Canis lupus familiaris</i> ribosomal protein S2 (RPS2), transcript variant X1, mRNA	61%	99%	1522	2
Contig 19	<i>Equus caballus</i> MHC class II region on chromosome ECA20	15%	78%	1190	2
Contig 20	PREDICTED: <i>Canis lupus familiaris</i> guanosine monophosphate reductase 2 (GMPR2), transcript variant X1, mRNA	98%	97%	1192	2
Contig 21	<i>Canis lupus familiaris</i> ribosomal protein L23 (RPL23), mRNA	40%	100%	1182	2
Contig 22	PREDICTED: <i>Canis lupus familiaris</i> adipogenesis regulatory factor (ADIRF), mRNA	60%	99%	582	2
Contig 23	<i>Canis lupus familiaris</i> secretory leukocyte peptidase inhibitor (SLPI), mRNA	39%	99%	1527	2
Contig 24	<i>Canis lupus familiaris</i> aldehyde dehydrogenase 1 family member A1 (ALDH1A1), mRNA	57%	99%	1118	2

configuration around transcribable genes, “guanosine monophosphate reductase 2” catalyzes the irreversible and NADPH-dependent reductive deamination of GMP to IMP, “adipogenesis regulatory factor” plays a role in fat cell development, “secretory leukocyte peptidase inhibitor” protects epithelial tissues from serine proteases, and “aldehyde dehydrogenase 1 family member A1” is in the major pathway of alcohol metabolism.

DISCUSSION

In recent years, genome studies have progressed rapidly. These genomic studies are aimed to determine when and how genes are active. In genomic studies, one of the shortest and fastest way to reach information about genes is EST studies. Expressed Sequence Tags provide the discovery of new genes, genomic mapping, identification of coding regions in genomic sequences, data on gene expression and regulation [17]. For the reliability of the study results, the ESTs must be analyzed correctly [16].

In this study, we have constructed two different cDNA libraries from a normal mammary tissue of healthy Terrier dog and a tumoral mammary tissue of another sick Terrier dog with breast cancer. From these two libraries EST clones were obtained and sequence analysis was performed. As a result, 2304 ESTs were generated. With Phrad program, vector and adapter sequences in ESTs are removed to prevent vector and adapter sequence contamination. After the vector removal process, the mapping of the EST fragments with the CAP3 program was performed. With this program, overlapping parts of ESTs were determined and contigs were created. In addition, the CAP3 program creates singleton sequences that do not generate contig. The number of contigs for normal mammary tissue was 21 while the number of contigs for tumoral mammary tissue was 24. The number of singlets for normal mammary tissue was 1102 while the number of singlets for tumoral mammary tissue was 1101. The contig sequences obtained in order to determine the similarities of the sequences in the NCBI database and

to estimate their possible functions were examined with the BLASTN analysis by BLAST program. Putative functions of all unique ESTs were designated by gene homology based on BLAST and were classified according to biological process, cellular component and molecular function based on Gene Ontology (GO) mapping provided by Blast2GO. B2G is comprehensive bioinformatics tool for functional annotation and analysis of gene or protein sequences^[19,20]. With this program, BLASTX analysis, mapping and annotation steps of ESTs were made, and the obtained graphics and results were evaluated and compared separately.

Based on the information given by the analysis results, the BLAST results of the normal mammary tissue showed similarities with the genes in the structural parts of the cells, which play a role in the regulatory, chemical reactions, and the growth and differentiation of the cell.

Genes which often predispose to cancer have been proven to play a role in the regulation of cell growth and differentiation, DNA repair and genomic integrity^[21]. The results of BLAST analysis of tumoral mammary tissue support this situation. Compared to normal mammary tissue, genes that play a role in cell growth and differentiation, DNA repair, etc. are more common in tumoral mammary tissue. Genes such as "vacuole membrane protein 1", "lipopolysaccharide-binding protein", "guanine nucleotide binding protein-like 3", "biorientation of chromosomes in cell division 1-like 1", and "adipogenesis regulatory factor" which is active in cell cycle, signal pathways, cell growth control, proliferation and differentiation, showed homology in the tumoral mammary tissue. "Ribosomal protein", which plays a role in protein synthesis in both mammary tissues and constitutes the subunits of the ribosomes, is the most common gene in our ESTs. In this study, ESTs of normal and tumoral mammary tissues of Terrier dogs are similar to *Homo sapiens* after *Canis lupus familiaris* according to the results obtained by BLASTX analysis.

The ESTs will be a valuable source for many researchers working with tumor tissue^[8]. These ESTs, especially specific for canine mammary gland tissue, provide significant information for canine mammary cancer studies. High-throughput genomic studies in recent times allow understanding the molecular complexity of breast cancer. The fact that canine mammary tumors are very similar to human breast tumors makes them important model organisms in terms of breast cancer studies in humans. Research with molecular biology techniques, the identification of genes that cause malignant characters in healthy cells and cause tumor formation is getting more and more important every day. It is thought that this study will help to identify the unknown genes in the breast tissue of Terrier dog, which is used as a model organism in cancer research, will be an important source for future studies and will help to understand the molecular biology of cancer.

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