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# Candidate tumour suppressor genes associated with canine mammary tumour

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**Abstract**
Mammary neoplasia is one of the most frequently diagnosed tumours in bitches and a major cause of death. Certain breeds have higher incidence of canine mammary tumour than others, indicating a significant influence of genetic risk factor. Mainly three types of genes are responsible for carcinogenesis; oncogene, proto-oncogene and tumour suppressor gene. Among tumour suppressor genes the most repeatedly studied risk genes with respect to canine mammary tumour are *Tumour protein 53* (*p53*), *Breast Cancer1* gene (*BRCA1*), *Breast Cancer 2* gene (*BRCA2*), *Phosphatase and Tensin Homolog* gene (*PTEN*), *E-cadherin* gene and *Serine/Threonine kinase11* gene (*STK11*). Through different pathways they aid in tumour suppression. Various gene variants and alterations in genetic expression were identified in these genes with respect to canine mammary tumour. Despite the researches for the past few decades, scientists were not able to find out a promising molecular marker which help in early detection and better prognosis of the disease. So there exists a need for a deeper and closer research using most modern and sophisticated molecular technologies in canine mammary tumour. The better understanding of molecular mechanism of canine mammary tumour will help to choose better therapeutic strategies and thus bring an improvement in health and survival of canine population.

**Keywords:** Tumour suppressor genes, *p53*, *PTEN*, *BRCA1*, *BRCA2*, *E-cadherin*, *STK11*

## Introduction
The problem of mammary tumour is currently significant in human as well as veterinary medicine. Mammary tumour is one among the most frequently diagnosed and leading cause of death among bitches. It is observed that certain breeds are more predisposed to canine mammary tumour (CMT), indicating a crucial genetic involvement. The high risk breeds includes Boxer, English Springer Spaniel, Poodle, Cocker Spaniel, Dachshund, English Setter and Fox- Terrier [1, 2]. Like human breast cancer (HBC), CMT is also multifactorial. Besides genetic factors CMT is influenced by age, diet, hormonal status and environmental factors. Mammary tumours are usually reported in female dogs above seven years of age [1]. Obese dogs do have greater risk of CMT occurrence and certain hormones like oestrogen, prolactin etc. do have a significant role in canine mammary tumourogenesis [3]. Cancers are said to be polygenic; different genes contribute their part in the risk of tumour development. As far as cancer genes are considered, there include mainly three classes of genes: Oncogenes, which is considered as positive growth regulator and its overexpression results in abnormal growth and cell proliferation; second one is Proto-oncogenes, which normally functions as a cell growth regulator or as an apoptotic agent but under certain condition when it undergoes mutation it get converted into an oncogene and thus results in cancer; third type is Tumour suppressor genes(TSG), they are negative growth regulators and their down regulation can result in tumerogenesis.

Tumour suppressor genes or anti-oncogene represents opposite side of cell growth and involves in cell cycle arrest and apoptosis. The study of gene mutations is an assuring area in oncology, as it aids in understanding and identifying the initiating cause and process of cancer. Mutations within tumour suppressive genes are found to be recessive type, following knudson's two-hit hypothesis. As per the hypothesis individuals with one mutant TSG allele will not be having tumour currently but there exist every chance to develop tumour once they underwent with some forms of mutagents. Once both alleles of the gene are mutant it leads to tumour development. Deregulation of multiple TSGs is found to be a crucial episode that leads to malignancy of tumour. Several HBC susceptible TSG genes have been studied in association with CMT. Among them most repeatedly studied TSGs includes *Tumour protein p53* (*p53*), *Breast Cancer 1* gene (*BRCA1*), *Breast Cancer 2* gene (*BRCA2*), *Phosphatase and*

*Tensin Homolog* gene (*PTEN*), *E-cadherin* gene and *Serine/Threonine kinase11* gene (*STK11*). In this review we will look deep into these candidate genes and its involvement in CMT.

**Tumour Protein 53**

The *p53* is one of the most extensively studied gene in cancer research. It is known as "guardian of the genome" or "cellular gatekeeper". The gene was first identified in 1979 by Arnold Levine, David Lane and William Old. In dog the gene is located at fifth chromosome with 10 exons. The gene codes for the protein with 53 kilodalton molecular mass and hence the gene got the name. The *p53* being a transcriptional factor involves in regulation of several target genes responsible for tumour suppression. The *p53* gets activated in response to cellular stress, hypoxia, DNA damage and oncogenic activation. The p53 protein comprises of mainly three parts; N terminal domain, core domain or DNA binding domain and C terminal domain. The level of p53 protein is maintained by its negative regulator Mouse Double Minute2 homolog (Mdm2) protein. Under normal condition *p53* itself activates the transcription of *Mdm2* gene. The Mdm2 protein binds with p53 and cause ubiquitin ligase mediated degradation of p53. But during cellular stress condition p53 undergoes certain post- translational modifications like phosphorylation that prevent p53-Mdm2 binding and thus the p53 level increases. The p53 functions as cell-cycle check point; by arresting cell cycle. It also involves in cell senescence, autophagy, apoptosis, DNA repair mechanism and cell metabolism.

The *p53* variations were identified in several canine cancers including mammary tumour. Investigations in *p53* mutations and its association with CMT had begun from last century. On expression study conducted on frozen mammary tumour tissue samples, a mutation G to A within intron five splice acceptor site that affecting splicing of p53 transcript were reported [4]. The first germline mutation reported within canine *p53* were indels within exon two, four and five in a comparative study within CMT predisposed dog breeds [5]. The exons five, seven and eight is most altered region within canine *p53*. Studies showed a mutation frequency of 23.4 percent, 31.6 per cent and 23.4 percent in exon five, seven and eight respectively [6]. This region of the gene codes for the DNA binding domain of p53 protein and hence interfere regulative action of *p53* on the target genes. This region is considered to be mutation hotspots of canine *p53*. So itself several researches had been carried out to study the variants within these mutation hotspots. On analysis of alterations within exon five to eight in 20 CMT cases, four missense and one non-sense mutations in 10 malignant cases and two missense and one non-sense mutation in benign mammary tumour were observed [7]. Another study analysed 63 CMT cases of both benign and malignant to evaluate the mutations within the hot spot region. They observed four missense mutations in 38 benign tumour, one non-sense and five missense mutations in 25 malignant carcinoma [8]. Several variants are identified in canine *p53* but every alterations need not result in CMT, in a study two novel single nucleotide polymorphism (SNPs) at exon three and intron seven were reported, but upon statistical analysis they didn't found these SNPs significant in CMT development [9]. During expression studies on *p53* certain conflicting results had been obtained, that one may be expecting reduced expression, since *p53* is a TSG. But studies showed an over expression of altered *p53*. On an expression study of *p53* in CMT tissue using monoclonal antibodies or anti-p53 antibodies, which specifically identifies epitome of p53 protein. They observed an over-expression of p53 in those mammary tumour samples [10]. Similar findings were also obtained later in another study where they investigated on p53 expression in CMT of different histological type in comparison to healthy mammary tissue and they found out an over-expression of p53 in tumour condition; 66.6 percent in CMT compared to 33.33 percent in normal conditions [11]. So there exists a significant correlation between p53 mutation and its over-expression in CMT and extent of over-expression indicates poor prognosis [12]. The alteration in *p53* is considered to be an early event in CMT development.

**Phosphatase and Tensin Homolog (Pten) Gene**

The *PTEN* is also known as *Mutated in Multiple Advance Cancer 1* (*MMAC1*) or *TGF-β regulated and epithelial cell-enriched Phosphatase 1* (*TEP1*). In dogs the gene is located in chromosome 26 with eight exons. It codes for 403 amino acid long protein which has got both lipid and protein phosphatase activity. The PTEN protein comprises of PIP2 binding domain, phosphatase domain, $C_2$ domain, C-terminal region and PD2-binding domain. The gene do have both cytoplasmic and nuclear functions. In cytoplasm *PTEN* acts as negative regulator of Phosphatidylyinositol 3 kinase (PI3K) pathway/ Protein kinase pathway/ Akt pathway. The PTEN will hydrolyse phosphatidylinositol 3, 4, 5 trisphosphate (PIP3) into phosphatidyl 4, 5 bisphosphate (PIP2) and thus makes PIP3 second messenger unavailable for Akt pathway. Akt regulates a plethero of downstream activities which causes cell survival and apoptosis inhibition. Hence by negative regulation of this Akt pathway *PTEN* induces apoptosis and cell survival inhibition. In nucleus, *PTEN* promotes the transcriptional activity of *p53*. The gene also phosphorylate Mitogen Activated Protein (MAP) kinase pathway which lead to inhibition of cyclin D at nuclear level and prevent cell proliferation. It helps in maintaining centromere stability by binding with Centromere Specific Binding Protein C (CENP-C). Interaction of *PTEN* with Rad51 helps in DNA repair. It interacts with Anaphase Promoting Complex (APC) and result in proteolysis of mitotic cyclin. It cause inactivation of Focal Adhesion Kinase (FAK) pathways and thus bring about inhibition on cell proliferation, migration, tumour cell invasion and tumour directed angiogenesis. Thus both nuclear and cytoplasmic function of PTEN enables it to act as a tumour suppressor.

The *PTEN* was expressed almost similar in normal mammary tissue and benign mammary tumour, but there was a loss of expression in malignant tumour [13]. There was 100 per cent expression of *PTEN* in normal tissue and benign mammary tumour tissue whereas it was only 67 per cent in malignant mammary tumour tissue [14]. On evaluating PTEN expression and its prognostic implication in canine and feline mammary tumour and a significant correlation ($p< 0.05$) between loss of PTEN protein expression in carcinoma histotype, lymphatic vessel invasion, distant organ metastasis, tumour differentiation and short overall survival in both feline and canine mammary tumour were obtained [15]. In a study conducted to investigate on copy number aberrations which results in development of CMT, a homogenous deletion in chromosome at PTEN region were observed in several malignant mammary tumour [16]. Expression of *PTEN* was not related to age of animal and tumour size but it closely correlates with lymphnode metastasis and differentiation of

mammary gland tumour.

**Breast Cancer 1 Gene (Brca1)**

The BRCA1 is a nuclear phosophoprotein that participates in the regulation of cell cycle. In dog the gene is located within chromosome nine with 27 exons. It codes for a protein with 190 kilodalton molecular weight. The BRCA1 protein consist of a ring domain, exon 11-13 domain and 2 BRCT domain. The gene has got several synonyms *IRIS*, *OSCP*, *BRCC1*, *FANCS*, *PNCA*, *RNF53*, *BROVCA1* and *PPPIR53*. The *BRCA1* undertakes the activities like negative regulation of cell-cycle in mammary epithelial cells [17], arresting cell cycle, increased apoptosis and maintaining genetic stability. It involves in number of signalling pathways and act on several genes to execute its tumour suppressive activity. The ring domain of BRCA1 forms hetero-dimer with BARD1 protein and enhances ubiquitin ligase activity of BRCA1 which is essential for tumour suppression function. The exon 11-13 domain, forms the largest part constituting more than 65 percent of the BRCA1 protein. This domain has got binding site for different genes. With Retinoblastoma (Rb) gene it involves in cell cycle suppression, along with Rad 50/51 and PALB2 it aids in DNA repair mechanism. The gene activates ATM/ATR kinase pathway involves in DNA repairing. The two BRCT domain align in a head to tail manner so as to make a cleft in between these two proteins which will result in the formation of consensus sequence pSer-X-X-pThr. This consensus sequence help in recognizing the target proteins involved in DNA repair mechanism.

In early 1990s, researchers discovered *BRCA1* as a breast and ovarian cancer susceptibility gene [18]. Studies showed *BRCA1* germline mutations accounts for five to ten percent of human breast cancer [19]. Similarly the germline mutation of the gene is also observed among dogs and those mutations has an increased risk in CMT development [20]. Two SNPs in intron eight and exon nine of BRCA1 were identified in association with CMT, and the SNP A/G in exon nine of *BRCA1* (SS748770617) was found to be significantly associated with CMT development [21]. Yet another study found out three novel SNP within *BRCA1* among which a SNP (-1173C>T) at promoter region was found to be associated with development of CMT (with odds ratio 2.57 and 95 percentage confidence interval) [22]. The genetic variations in coding regions of *BRCA1* were studied and found out two mutations (4702C>T) and (4765G>T) in benign and two mutations in malignant tumour (3619A>G) and (4006G>A). Further the physical effect of these mutations were analysed and found that all these variants interfere the protein formation and thus affect the tumour suppressive activity [23]. In a study it was evaluated, the mutation and methylation status of *BRCA1* in both normal and mammary tumour affected bitches. They identified a novel SNP (C>T transition) at 5'UTR region. Further their study suggested that methylation is not the main *BRCA1* inactivation mechanism, as they didn't get any proof of hypermethylation in the samples analysed [24]. Regarding the expression of *BRCA1*, it is less expressed in tumour condition especially in malignant mammary tumour [20] more specifically exon 11 region of the gene even though there are reports of reduced expression from exon 10-14 of canine *BRCA1* [25]. Even in immune-histochemical analysis done to find out the expression and distribution of BRCA1, a reduced nuclear expression and increased cytoplasmic expression in both benign and malignant tumour were observed. On further evaluation it was inferred that reduction in *BRCA1* expression was significantly associated with malignancy of tumour [17]. Certain breed specific difference in expression of *BRCA1* is also reported and the study showed that among different dog breeds considered Shitzu dog breed has higher percentage of altered strong over expression of *BRCA1* which related with triple negative phenotype [26]. On an experiment conducted to determine canine *BRCA1* promoter sequence and its mutation status in CMT. It was observed that the mRNA expression of *BRCA1* was significantly reduced in benign and malignant tumour ($P< 0.05$) and protein expression of BRCA1 was significantly reduced in malignant mammary tumour ($p< 0.05$) [27]. So far all the studies within canine *BRCA1*, either mutation study or expression profile had revealed that just like HBC, *BRCA1* can also indicted in CMT.

**Breast Cancer 2 Gene (Brca2)**

The *BRCA2* is a very huge gene located within chromosome 25 of dogs with 28 exons and codes for 3418 amino acid long protein. The *BRCA2* gene is strongly implicated in familial HBC. The gene has got metonyms like *BRC2*, *BROVCA2*, *FACD*, *FAD*, *FAD1*, *FANCD*, *FANCD1*, *GLM3*, *PNCA2* and *XRCC11*. The BRCA2 protein is made up of BRC region and C-terminal region. The BRC region comprises of eight BRC repeats which is functionally most active region of the protein and it interacts with Rad51 protein and inturn results in repairing of double strand DNA (dsDNA) breaks through homologous recombination. The C-terminal region comprised of Nuclear Localisation Sequence (NLS) region and three oligosaccharide binding domain (OB1, OB2 and OB3). Considering tumour suppressive activity, *BRCA2* mainly involves in DNA repair mechanisms.

The first specifically reported polymorphism in canine *BRCA2* in association with CMT is an *indel* named 10204 *indel* AAA within exon 27 [28]. Exon 27 codes for the NLS region of BRCA2 protein. Such a polymorphism will affect the translocation of BRCA2 protein towards site of dsDNA breaks. The same group did further investigation within the same exon and identified four novel SNPs in addition to 10204 *indel* AAA, all of them disturbing NLS region of protein. Significant CMT associated SNPs were also reported within exon 24 of *BRCA2* (SS748770619) [20]. Exon 11 of *BRCA2* codes for the BRC repeats of the protein, which is functionally most active region and along with Rad51 involves in DNA repairing mechanism. So itself this region draws the attention of many scientists paving way for several studies. On evaluation of sequence variation of BRC1-BRC8 and C-terminus region of the protein which corresponds to exon 11 and exon 27 of *BRCA2* respectively, it was observed that there exist multiple SNPs in exon 11 and indels in exon 27 among CMT affected dogs [29]. An elaborative study regarding the variants within exon 11 had identified seven novel SNPs, out of which three were found to be deleterious. It was observed that 97.9 percent of bitches in the study had atleast one of these SNPs. Hence there exist every possibility for a strong correlation between canine *BRCA2* exon 11 alterations and CMT development [30]. Recently a group of scientists evaluated genetic variation of several HBC susceptible gene with CMT and on statistical analysis they found that two variants C386W and M3332K in *BRCA2* were significantly associated with CMT [31]. Even mutations which was similar to that reported in human HBC were also identified in canine patients, two mutations in canine *BRCA2*, T1425P and K1435R were almost similar to human *BRCA2* mutation K1440R and K1440E [32, 33]. The variations in

*BRCA2* can be exploited further for early diagnosis and better treatment of mammary tumour in canine patients.

**E Cadherin Gene**

*E-cadherin* is one among the several gene which is expressed in early embryonic stage and it play a major role in maintaining homeostasis and overall survival of organism through effective intercellular communications. *E-Cadherin* is among the most important molecules that regulate cell- cell adhesion in epithelial tissues. *E-cadherin* is located in regions of cell-cell contact known as adherens junctions, and it belongs to the family of genes encoding for the so-called calcium-dependant CAMs or the cadherin gene family. It is also known as *Cadherin 1* (*CDH1*) or *Uvomorulin*. In dog the gene is located in chromosome five with 16 exons. The gene codes for 120 kilodalton glycoprotein which embodies a large extracellular N-terminal domain, a trans-membrane segment and a short cytoplasmic C-terminal domain. The cytoplasmic domain interacts with actino-cytoskeleton using α-β-γ catenins, and aids in cell-adhesion [34]. The N-terminal region with the help of $Ca^{2+}$ion, interacts with adjacent E-cadherin molecule resulting in formation of adherent junction. The adherent junction formation play a major in homophilic contact inhibition of cell proliferation by physically blocking movement of cells [35]. So a loss of function of *E-cadherin* can lead to metastasis. The gene involved in several signalling pathways like Wnt, PI3K, MAPK and Hippo pathway. As far as tumour suppressive activity is considered, regulation of Wnt pathway is most important. The β-catenin dependant Wnt pathway or the canonical pathway act on several genes that involves in DNA repairing, stabilises cancer stem cells and regulates epithelial to mesenchymal transition of cell [36].

The vast majority of studies of cell adhesion in tumerogenesis and invasion have focused on *E-cadherin*, as this molecule is the major cadherin expressed in epithelial cells [37]. Most of the mammary tumour in bitches are of epithelial origin hence a down-regulation of the gene can results in tumour development, tumour aggressiveness, metastasis and short survival [34]. Reduced membrane expression of *E-cadherin* was significantly associated with tumour histological type. Solid type carcinoma showed frequent loss of *E-cadherin* expression in contrast to tubulopapillary carcinoma type [38]. Reduced *E-cadherin* expression was associated with invasion and several classic prognostic features, namely proliferation and lymphnode metastasis, suggesting this molecule as a strong prognostic marker [39]. The analysis of *E-cadherin* expression in lymph node metastasis was rarely reported and several pattern of expression have been described, namely downregulation, upregulation or similar expression levels with regard to primary mammary tumour lesion [40]. E-cadherin expression is preserved in 80 per cent of benign tumour where as a reduction in expression in 20 per cent malignant tumour [41]. In an immune-histochemical analysis of 54 canine mammary lesion (15 hyperplasia, 7 adenoma and 32 carcinoma) for evaluation of *PTEN* and *E-cadherin* co-expression. All hyperplasia samples stained positive for both markers, 100 percentage adenoma were positive for *PTEN* and 86 percentage for *E-cadherin* and 69 per cent and 34 per cent of carcinoma were positive for *PTEN* and *E-cadherin* respectively. It was observed that those female dogs having both markers positive had larger survival and absence of lymphatic invasion and this confirmed tumour suppression effect of both genes [42]. The gene do have a membranous expression in normal mammary tissue and non-invasive type of mammary tumour, whereas cytoplasmic expression in case of invasive malignant tumour [43]. In a study regarding the co-expression of E-cadherin, β-catenin and APC, a loss of expression of E-cadherin along with altered β-catenin co-expression in malignant tumour was observed [44]. On evaluating the epithelial-mesenchymal transition of the gene in CMT using canine mammary cancer cell-lines, strong correlation between reduced *CDH1* expression and malignancy of tumour was obtained [45]. Reduced expression of *E-cadherin* was significantly associated with progression low infiltrating to high infiltrating tumour but not with cell proliferation and survival [46]. Certain non-deleterious polymorphisms and *indels* were also reported within both coding and non-coding regions of this gene in association with CMT [47]. Certain variants within the gene that had a protective role also; in an assessment of CDH1 variants in different gradients of mammary tumour samples, it was observed that three *CDH1* variants rs8508055755, rs852280880 and rs852639930 caused small less invasive type of tumour which implies that these variants of *CDH1* possess a protective role in CMT [48]. Certain post-translational modifications like N-glycosylation of the gene can be an altered regulatory mechanism in E-cadherin in CMT cases [49]. The diagnostic and prognostic role of CDH1 seems to be promising but it requires further studies for better confirmation.

**Serine/ Threonine Kinase 11 Gene (Stk11)**

*Serine/threonine kinase 11* is a part of Ca2+/ calmodulin kinase group of kinases. It also associates with four pseudokinase and help in maintaining basal structures. The gene otherwise called as *LKB1* or *PJS*. In dog the gene is located in chromosome 20 with 11 exons. The protein structure is made up of N-terminal domain which holds the NLS region, kinase domain and C-terminal domain with prenylation motif. The gene aids in maintaining energy homeostasis, chromatin remodelling, cell-cycle arrest, ras-induced cell transformation, p53 mediated apoptosis and activation of tumour suppressive Wnt signalling.

When compared to human *STK11* and its association studies with HBC, the role of *STK11* in CMT risk is yet to be explored a lot. A group of scientists evaluated ten HBC susceptible gene and its variants in association with CMT and they identified a SNP rs229288114 within STK11, but its physical effect was not studied in detail [50]. Later another group did a detailed study by evaluating several oncogene, proto-oncogene and TSG variants in association with CMT. They found out that two SNPs in *Rad51* and one SNP in *STK11* were significant in CMT development. The identified SNP rs22928814 (C>T) was same as previously reported and it was analysed statistically. On analysis it was observed that those bitches with T allele were more prone to CMT, the T allele was present in 40.3 percent of bitches with CMT whereas 26.7 percent of animals in control group [51]. Recently an investigation in CMT by whole genome sequencing revealed that three variants in *STK11* C109T, A286G and T293C play major role in CMT risk [31]. Inspite of these researches further breakthroughs are required in this gene by conducting expression profiling, comparative study and association studies for better understanding the gene involvement in CMT.

**Conclusion**

Over last few decades there has been an explosion about the

molecular biology of cancer. Cancer is a leading cause of death in both human and canine population. Mammary tumour is the second most diagnosed tumour among dogs after skin cancer. The whole genome sequencing of dog revealed that there exists a great similarity between human and canine genome. As dogs have a long history of inbreeding with low levels of genetic variation, it has been suggested CMT development in single breed should have a more defined homogeneous origin compared with that of human breast cancer, which occur within a diverse population. The major goals in human and veterinary medicine is to identify diagnostic and prognostic factors that help in early detection and prognosis of disease, which will aid in better therapeutic strategies. In humans so many molecular markers are identified with respect to HBC and to some extent used in routine clinical practices. But such kind of markers are absent in case of canine patients. This brings the importance of a rigorous molecular study with regard to CMT. Accumulating the similarity in genome between human and dogs along with the common environment they share; advancement in CMT research can equally be applied in biochemical characterisation of HBC. The currently published studies could able to find out certain significantly associate genetic variants but no study found to be confirmatory. This situation drags the need of deeper and closer study in this field so that a confirmatory risk factor could be proposed; which can help in better prediction of possible chance of tumour occurrence at an early stage of animal. If so breeders could be advised to spay the animal at early age, to avoid breeding of that animal, to control animal's diet to prevent them from being obese and to go for frequent check-ups for hormone status of the animal; such that the chances of CMT occurrence can be avoided. An extensive research in this field will enable better understanding of molecular mechanism of tumerogenesis for effective screening of 'at risk' population and improve the health of canine patient.

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