# CHARACTERIZATION OF CANCER STEM CELLS FROM CANINE MAMMARY GLAND TUMORS AND ITS CORRELATION WITH METASTATIC MARKERS

## T H E S I S

Submitted

in partial fulfillment of the requirements for the Degree of

**MASTER OF VETERINARY SCIENCE**

**IN**

**VETERINARY PATHOLOGY**

**BY**

**GAONKAR PANKAJ PRAKASH**

**Enrollment No. V/11/006**

**NAGPUR VETERINARY COLLEGE, NAGPUR**

**MAHARASHTRA ANIMAL AND FISHERY SCIENCES UNIVERSITY, NAGPUR- 440 001**

**(INDIA)**

**2018**

## DECLARATION OF STUDENT

I hereby declare that the experimental research work and Interpretation of the thesis entitled "**CHARACTERIZATION OF CANCER STEM CELLS FROM CANINE MAMMARY GLAND TUMORS AND ITS CORRELATION WITH METASTATIC MARKERS.**" or part thereof has not been submitted for any other degree or diploma of any University, nor the data have been derived from any thesis/publication of any University or scientific organization. The sources of materials used and all assistance received during the course of investigation have been duly acknowledged.

Date : **Signature**

**(GAONKAR PANKAJ PRAKASH)**
**Enrolment No: V/11/006**

**Dr. N. V. KURKURE**
**Chairman**
**Advisory Committee**

## DECLARATION OF ADVISORY COMMITTEE

Mr. **GAONKAR PANKAJ PRAKASH** has satisfactorily prosecuted his course of research for a period of not less than one semester and that the thesis entitled, "**CHARACTERIZATION OF CANCER STEM CELLS FROM CANINE MAMMARY GLAND TUMORS AND ITS CORRELATION WITH METASTATIC MARKERS.**" submitted by him is the result of research work is sufficient to warrant its presentation to the examination in the subject of **Veterinary Pathology** for the award of **Master of Veterinary Science** degree by the Maharashtra Animal and Fishery Sciences University, Nagpur. We also certify that the thesis or part thereof has not been previously submitted by her for a degree of any other University.

Place: ……..
Date: .....

Signature
**N. V. KURKURE**
Associate Professor & Head
Dept. of Veterinary Pathology
Nagpur Veterinary College, Nagpur

**Advisory Committee**

| Name | Designation | Signature |
|---|---|---|
| **1. Dr. N. V. Kurkure (Chairman)** | Associate Professor & Head<br>Dept. of Veterinary Pathology<br>Nagpur Veterinary College, Nagpur | _____ |
| **2. Dr. P. M. Sonkusale (Member)** | Assistant Professor<br>Dept. of Veterinary Pathology<br>Nagpur Veterinary College, Nagpur | _____ |
| **3. Dr. S. V. Upadhye (Member)** | Associate Professor<br>Dept. of Veterinary Surgery & Radiology | _____ |
| **4. Dr. B. M. Gahlod** (Member) | Hospital Superintendent<br>TVCC, Nagpur | _____ |
| **5. Dr. M. R. Wani (Member)** | Scientist "G"<br>National Center for Cell Science, Pune | _____ |

## CERTIFICATE

This is to certify that the thesis entitled, "**CHARACTERIZATION OF CANCER STEM CELLS FROM CANINE MAMMARY GLAND TUMORS AND ITS CORRELATION WITH METASTATIC MARKERS.**" submitted by Mr. **GAONKAR PANKAJ PRAKASH** to the **Maharashtra Animal and Fishery Sciences University** in partial fulfillment of the requirement for the degree of **M.V.Sc** has been approved by the Student's Advisory Committee after examination in collaboration with the External Examiner.

| | | |
|---|---|---|
| Name & Signature of External Examiner | Signature with Seal Head of Department | **N. V. KURKURE**<br>Advisor/ Guide<br>Associate Professor & Head<br>Dept. of Veterinary Pathology<br>Nagpur Veterinary College, Nagpur |

**Advisory Committee**

| Name | Designation | Signature |
|---|---|---|
| **1. Dr. N. V. Kurkure (Chairman)** | Associate Professor & Head<br>Dept. of Veterinary Pathology<br>Nagpur Veterinary College, Nagpur | _____ |
| **2. Dr. P. M. Sonkusale (Member)** | Assistant Professor<br>Dept. of Veterinary Pathology<br>Nagpur Veterinary College, Nagpur | _____ |
| **3. Dr. S. V. Upadhye (Member)** | Associate Professor<br>Dept. of Veterinary Surgery & Radiology<br>Nagpur Veterinary College, Nagpur | _____ |
| **4. Dr. B. M. Gahlod (Member)** | Hospital Superintendent<br>TVCC, Nagpur | _____ |
| **5. Dr. M. R. Wani (Member)** | Scientist "G"<br>National Center for Cell Science, Pune | _____ |

**Associate Dean**
**Nagpur Veterinary College, Nagpur**

## *ACKNOWLEDGEMENT*

*For although my name may be on the cover, it is wrong to think that I could have ever done this alone. It will not be enough to express my gratitude in words to all those people who helped me. I would still like to give many many thanks to all these people.*

*I would like to express here the very thanks to my Guide,* ***Dr. N. V. Kurkure****, who provided me the opportunity to do such a meaningful research, ushered me in to the discipline of pathology, and instructed me the delicate pathological methodologies. His dynamism, vision, sincerity and motivation have deeply inspired me. He has taught me the methodology to carry out the research and to present the research works as clearly as possible. It was a great privilege and honor to work and study under his guidance. I am extremely grateful for what he has offered me.*

*Dr.* ***A. S. Bannalikar****, Associate Dean, Nagpur Veterinary College,Nagpur for providing necessary facilities for completion of research work.*

*I am highly indebted to* ***Dr. M. R. Wani*** *for providing me an opportunity to do research work in his lab at NCCS, Pune. His constructive suggestions and talented directions during my course and research work were highly valuable.*

*I express my whole hearted thanks to* ***Dr. P. M. Sonkusale****, for constant help and keen interest for completion of this research work.*

*I am highly grateful to the members of my advisory committee,* ***Dr. S. V. Upadhye*** *and* ***Dr. B. M. Gahlod*** *for their suggestions and encouragement throughout the span of my research work.*

*I thank and acknowledge the inspiration, encouragement and suggestions offered by my respected teachers* ***Dr. K. A. Sirothia , Dr. Megha Kaore*** *and* ***Dr. Madhuri Hedaoo.***

*I would like to express special thanks to* ***Dr. S. P. Awandkar*** *for providing excellent and invaluable guidance.*

*I wish to thank* ***Dr. Noopur Halmare*** *for her support and long term working rapport she has shared with me. I really appreciate our working term and your help throughout these two years of M.V.Sc.*

*Mere words are not enough to express the feelings of inspired association and evergreen cooperation of my seniors* ***Dr. Sneha Thorat, Dr. Siddhart Jamdade*** *and* ***Dr. Pratik Ingle.***

*I warmly thankful to my juniors* ***Dr. Akshay Chavan, Dr. Seema Shinde and Dr. Amit Kshirsagar*** *for their kind help during the research work.*

*I am extremely grateful to* ***Suhas Mhaske****, who has helped me selflessly in so many facets of my work and always had time to listen to my questions and problems.*

*I would also like to thank* ***Satish, Garima, Subhanath, Adrita, Juilee, Lekha, Soni*** *and* ***Mehrun*** *for their encouraging support and cooperation.*

*I would like to thank **Dr. Shambhavi, Dr. Chetan, Dr. Varun, Dr. Indrakshi, Dr. Niyati, Dr. Manish, Dr. Amit, Dr. A. R. Deshpande, Dr. Anand Neware** and **Neeraja** for their immense help in the collection of cancer samples.*

*I would also like to thank **Mrs. Dev** and **Mr. Dinesh** for their valuable contribution in processing of samples.*

*I am also thankful towards **Shri. V. N. Hiwase, Shri. G. M. Thakare, Mahendra Bhau,** for all the necessary help.*

*I would also like to thank my dear friends and seniors **Kaustubh, Pratik, Jayneel, Amit, Milind, Nitin, Abhishek, Ravi, Vinayak, Chetan, Nikita, Arnab, Kaushik, Prabhsimaran, Manish, Dr. Satyajit Kale, Dr. Aniruddha Jagtap and Dr. Shraddheya Shirankar** for their immense moral support.*

*Finally, to the ones I hold dearest to my heart- my family. I dedicate my achievements to my **Aai, Baba, Babaayi, Didi, Jiju and Prithviraj**. Nobody has been more important to me in the pursuit of this project than the members of my family. I would like to thank my parents, whose love and guidance are with me in whatever I pursue. They are the ultimate role models. Thank you for instilling the qualities in me that have made me the person that I am today.*

*Date:*

*Place:* *Gaonkar Pankaj Prakash*

## TABLE OF CONTENT

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF PLATES

## LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| % | : | Percentage |
| & | : | And |
| °C | : | Degree Celsius |
| ALDH | : | Aldehyde Dehydrogenase |
| ALP | : | Alkaline Phospatase |
| Ca | : | Calcium |
| CD | : | Cluster Of Differentiation |
| CMT | : | Canine Mammary Tumor |
| CSC | : | Cancer Stem Cells |
| DMEM | : | Dulbecco's Modified Eagel Medium |
| EDTA | : | Ethylenediaminetetraacetic Acid |
| EGF | : | Epidermal Growth Factor |
| EMT | : | Epithelial-Mesenchymal Transition |
| ER | : | Estrogen Receptor |
| FBS | : | Foetal Bovine Serum |
| FGF | : | Fibroblast Growth Factor |
| Gapdh | : | Glyceraldehyde 3-Phosphate Dehydrogenase |
| H & E | : | Haematoxylin And Eosin |
| Hb | : | Haemoglobin |
| IDC | : | Invasive Ductal Carcinoma |
| MCF7 | : | Michigan Cancer Foundation-7 |
| MDA-MB-231 | : | M.D. Anderson Metastasis Breast Cancer |
| MET | : | Mesenchymal To Epithelial Transition |
| mg | : | Milligram |
| Mg | : | Magnesium |
| ml | : | Mililiters |
| MP | : | Melting Point |
| MTT | : | 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide |
| ng | : | Nanogram |
| nm | : | Nanometer |
| NOD/SCID | : | Nonobese Diabetic/Severe Combined |

| | | |
|---|---|---|
| | | Immunodeficiency |
| Oct4 | : | Octamer-Binding Transcription Factor 4 |
| OD | : | Optical Density |
| P | : | Phosphorus |
| PCV | : | Packed Cell Volume |
| PR | : | Progesterone Receptor |
| RT-PCR | : | Reverse Transcriptase Polymerase Chain Reaction |
| SC | : | Stem Cell |
| Sca-1 | : | Stem Cells Antigen-1 |
| SNAI1 | : | Snail |
| SNAI2 | : | Slug |
| Sox2 | : | Sex Determining Region Y-Box 2 |
| STAT3 | : | Signal Transducer And Activator of Transcription 3 |
| TEC | : | Total Erythrocyte Count |
| TLC | : | Total Leukocyte Count |
| ug | : | Micro Gram |
| ul | : | Microlitre |
| VIM | : | Vimentin |
| WHO | : | World Health Organization |

## INTRODUCTION

Oldest description of cancer dates back to about 3000 BC which was discovered in Egypt and describes 8 cases of tumors or ulcers of the breast. The writing says about the disease, “There is no treatment.” Greek physician Hippocrates (460-370 BC) is credited with the origin of word cancer. The terms “carcinos” and “carcinoma” refer to crab suggesting finger-like spreading projections from a cancer to the shape of crab. Even after so many centuries there is still no cure for cancer. Understanding cancer remains a daunting task for everyone. Cancer continues to wreak havoc upon and remains as one of the leading cause of mortality.

Mammary tumors in canines are most frequent in intact bitches; they are extremely rare in male canines. The canine is so far the most frequently affected domestic species, with prevalence 3 times that in women; 50% of all tumors in the bitch are mammary tumors. In canines, mammary tumors are the second most common tumor (after skin tumors) over all and the most common tumor in female canines (Benjamin *et. al.,* 1999). Few studies show that significant similarities and differences exist between canine and human mammary tumors at the molecular level (Klopfleisch *et. al.,* 2010). Canine mammary tumors are specific tumors of females and are rare in males and are often associated with hormonal abnormalities (Moulton *et al.,* 1970). Gupta (2008) reported that the age of dogs affected with mammary tumors ranged from 2-16 years. The highest incidence was recorded in 10-12 years of age. There is maximum involvement of fifth pair of mammary gland followed by fourth pair, third, second and first pair. Maximum involvement of the caudal glands may be due to the fact that they have maximum glandular tissue and they maintain their secretary activity longer than other pairs (Fidler *et al.,* 1967). Bitches spayed before any estrous cycles has very less (approximately 0.5%) risk of the mammary cancer. Spaying after 1 estrous cycle, increased risk to 8%. Which further increased to 26% in case of 2 or more estrous cycles (Schneider *et al.,* 1969). Relative incidence of mammary gland lesions were 47.8% benign, 47.5% malignant and 4.7% diagnosed as non-neoplastic disorders (Salas *et al.*, 2015).

The high mortality rates affiliated with cancer are caused by the metastatic spread of tumor cells from the site of their origin. In fact, metastatic tumors are the cause of more than 90% of cancer related deaths due to the fact

that current therapies frequently fail to provide durable curative response if tumor is spread (Mehlen *et al.,* 2006). Mammary tumor frequently metastasizes to bone, liver, brain and lungs. Epithelial-mesenchymal transition (EMT) is a crucial step for metastasis. It is the stepping stone of metastatic cascade, generates cells with properties of stem cells (Mani *et al.,* 2008).

Most malignancies are composed of a subset of populations called cancer stem cells (CSC). CSC is a cell within the tumor that holds the capacity to self-renew and to generate the heterogeneous lineages of cancer cells that comprise the tumor (Clarke *et al.,* 2006). Up till now different theories have been proposed regarding origin of cancer stem cells. One theory suggests that CSC arise due to accumulation of special genetic mutation in normal stem cells over a period of time. Another theory suggests that accumulation of genetic and/or heterotypic alterations in normal somatic cells result into stem like characteristics along with malignant behaviour. Epithelial-mesenchymal transition is its classical examples. EMT is driven by transcription factors, including SNAI1/2, ZEB1/2, or TWIST1/2, which increase the invasiveness of epithelial cells (Yu *et al.,* 2012).

Conventional cancer treatments successfully target proliferating cells and induce tumor shrinkage, they frequently fail to prevent disease progression. CSCs are highly resistant to chemotherapeutics, they are believed to be a decisive factor responsible for tumor relapse and can be ultimate therapeutic target. Elimination of CSCs offers an exciting potential to obtain a durable clinical response with the prevention of tumor recurrence and metastasis (Agliano *et al.,* 2017).

CSC might serve as biomarker for early tumor detection, prognostication, and prediction of therapy response. However, different CSC targeted therapies are emerging as potentially curative anti-cancer treatment (Krause *et al.,* 2017).

The separation and identification of metastatic CSCs as a subpopulation could enhance the understanding of the initiation, progression, metastasis or relapse of cancer, as well as lead to the identification of novel molecules for the development of therapeutic agents targeting cancer. Expanding our knowledge on the metastatic mechanism of CSCs will help to discover the pertinent prognostic markers and develop novel therapeutic targets. So the present study is designed with following objectives:

1. To study haematological and biochemical alteration in serum of canines bearing tumors.
2. To study gross and histopathological changes in canine mammary tumors and their grading.
3. To isolate cancer stem cells from cancerous mammary glands in canines.
4. To characterize cancer stem cells using metastasis associated markers.

# REVIEW OF LITERATURE

The literature review on canine mammary gland tumor is discussed in relevance to following topics:

1. Epidemiology
2. Haemato-biochemical alterations
3. Histopathological examination and grading
4. Cancer stem cells and Metastasis associated markers

## 2.1 Epidemiology

Schneider *et al.,* (1969) studied canine mammary tumor (CMT) considering various factors like estrus, pseudopregnancy, parity, fecundity, and postsurgical survival as predisposing factors. They observed that neutered bitches had 12% of the mammary cancer risk as compared to intact animals. Bitches spayed before any estrous cycles had approximately 0.5% of the mammary cancer risk; those that had only 1 estrous cycle had 8%, and animals that had 2 or more estrous cycles before neutering, 26%. Within the group having 2 or more estrous cycles before being spayed, those neutered before $2^1/_2$ years of age exhibited a marked sparing effect on mammary cancer risk not shown for bitches neutered after $2^1/_2$ years of age. Pseudopregnancy, parity, and fecundity indicated no significant effects on mammary cancer risk. Survival data indicated that mortality was higher in the first year. Neutering after cancer diagnosis did not affect either survival or cause of death.

Moulton *et al.,* (1970) studied 1,366 cases of canine mammary tumors. The age for occurrence of mammary tumors varied between populations from the $9^{th}$ to $11^{th}$ years. At age of about 6 years there was increased risk of developing mammary tumors in dogs. Incidence of canine mammary tumor in relation to breeds didn't show any significance. Benign and malignant mammary tumors occur with increasing frequency from the most cephalad to the most caudal mammary glands. The abdominal and inguinal gland presented almost 60% of the mammary tumors.

Alenza *et al.,* (1998) correlated habitual diet and canine mammary tumor incidence. They found that intake of homemade meals (compared to that of

commercial foods) was significantly related to a higher incidence of tumors and dysplasia. High intake of red meat and low intake of chicken were significant risk factors associated with mammary tumors. Obesity at 1 year of age was significantly related to a higher prevalence of mammary tumors and dysplasia.

Polton (2009) inferred that even though in neutered bitches incidence of CMT is considered to be less, the chances of malignancy is more than 50%. CMT is generally observed as solitary mammary mass or frequently, as multiple lesions. It is mostly observed that dogs having multiple masses are been presented for veterinary intervention after a long delay only after the mammary tumor has become so big that it drags on floor and ulcerated along with its enormous size. Mammary tumors primarily undergo metastasis to the regional lymph nodes or to the lungs. Prognosis for patients with metastatic disease is usually poor.

Reddy *et al.*,(2009) breed-wise occurrence of mammary neoplasm's revealed highest number of tumors in German shepherd (35.0%) followed by Spitz (24.22%), non-descript (19.53%), Pomeranian (10.94%), Labrador (6.25%), Boxer (3.91%), Doberman (4.69%), Cocker Spaniel (3.13%), Bhutia (1.56%) and Great Dane (0.78%). The age group at which mammary tumors occurred most frequently was 8–10 years , followed by 6–8 years , 10–12 years , ≤ 6 years and >12 years .

Sleeckx *et al.,* (2011) in their review stated canine mammary tumor to be most common tumor in intact female dogs. Overall 40% of tumors in bitches are CMT. Almost fifty percent of CMTs are malignant. Caudal abdominal and inguinal mammary glands are most affected. Incidence of CMT is decreasing in some countries due to common practice of ovariohysterectomy at an early age.

Pena *et al*., (2013) reported 21.53% of dogs had recurrences and/or metastases and 20.00% of the dogs died due to mammary cancer. They also compared the cancer associated deaths after two year of follow up reported by other studies which ranged from 23.33 to 44.44%.

Komazawa *et al*., (2016) analyzed 3,985 canine tumors and found out mammary tumors to be third most common tumors which accounted for 18%. Higher incidence was noted in smaller dog breeds as compared to medium and large dog breeds.

Vascellari *et al.,* (2016) reported increasing incidence of canine mammary tumor with more frequency of malignant tumor than benign tumor. CMT have second highest incidence among all tumors. Pure breed dogs were more likely to be affected with malignant neoplasm than mix breed dogs. Bitches between ages of 8 to13 years showed highest frequency of CMT. Lower frequency was recorded among younger bitches below 6 years of age. Mean age for diagnosis was higher for malignant CMT (9.74 years) than for benign neoplasm (8.95 years). Intact bitches were more susceptible to CMT. This study recorded 74% CMT in unspayed dogs.

Gabil *et al.,* (2017) mentioned overall prevalence of CMT was 19.53%. The average age of positively diagnosed animals was 9 ± 0.3 years old. A high rate was documented in Caniche (43.75%) as compared to Cross-breed (16.17%) and German shepherd (14.78%) bitches. Occurrence of tumor was more in the abdominal and thoracic glands (40.47% for each) than in inguinal ones (19.04%). Involvement of right mammary gland was more than the left ones (61.90% and 38.09% respectively).

**2.2 Hemato-biochemical parameters**

Losco (1986) upon examination of a mammary tumor affected dog found out white blood cell count was 15,700/ul with 11% eosinophils. Infiltration of eosinophils was evident in tumor sections. He suggested that peripheral eosinophilia is associated with disseminated disease and a resultant poor prognosis.

Fayolled *et al.,* (1987) found out that routine hematology and serum biochemistry were not useful in differentiating benign from malignant tumors. Post-surgical blood profiling was not useful in predicting tumor progression.

In case of benign tumors TLC was significantly increased. In benign and malignant tumor absolute neutrophil and lymphocyte count were increased respectively. Significant decrease was observed in serum calcium and serum inorganic phosphorus values in benign and malignant tumors (Chavan 2012).

Lallo *et al.,* (2016) investigated 246 bitches with mammary tumor for determining haematological observations. Prevalence of general hematologic abnormalities in dogs with breast cancer was 29%. Hematological abnormalities

were lower in bitches with benign tumors, whereas malignant mammary tumors showed high prevalence of hematologic abnormality. The main hematological abnormalities were thrombocytosis, hyperproteinemia and leucopenia with a predominance of neutropenia. Overall they observed high prevalence of thrombocytosis, leukopenia and hyperproteinemia (hypergammaglobulinemia) in bitches bearing mammary tumors.

Duda *et al.,* (2017) studied haematological, biochemical and hemostatic abnormalities in bitches affected with mammary tumors. Anemia, neutrophilic leukocytosis, monocytosis, eosinophilia, thrombocytosis, hypoalbuminemia, hypocalcemia, hypoglycemia, and low blood urea were evident. Hypoalbuminemia, hypocalcemia, low serum urea levels, and hypoglycemia were detected. Higher fibrinogen levels were observed which increased with tumor staging.

**2.3 Histopathology & Grading**

Misdorp and Hart (1976) studied CMT with histologic types sarcomas, simple carcinomas and complex carcinomas. A higher grade occurred more often in simple carcinoma than in complex carcinoma. Sarcomas, associated with a worse prognosis showing high grade of anaplasia than carcinomas. Complex carcinomas exhibited a lower grade of differentiation and anaplasia than simple carcinomas. Tumors with a higher nuclear grade of anaplasia or mitosis tended to infiltrate more than tumors with a lower grade. A higher histologic grade of malignancy was associated with a worse prognosis.

Pena *et al.,* (2003) histologically classified 20 canine inflammatory mammary carcinoma as simple carcinoma (n=15), adenocarcinoma (n=2), and lipid rich carcinoma (n=3). On immunohisochemical study the Ki-67 index was elevated, P53 was overexpressed, all cases were ER negative while 71.4% were PR positive. They suggested Canine primary inflammatory carcinoma to be aggressive form with distinct histopathological and immunohistochemical characteristics.

Karayannopoulou *et al.,* (2005) used Elston and Ellis grading method in dogs with mammary carcinoma to examine its relation to prognosis. According to the Elston and Ellis (1991) method, the grade for each case was derived from an assessment of tubule formation, nuclear pleomorphism, and mitotic counts. CMT

were histopathological characterized as simple carcinoma (64.7%), followed by carcinoma in benign tumor (21.2%), complex carcinoma (9.4%) and special type (spindle cell) carcinoma (4.7%).Of the 85 cases examined, 31.8% had well-differentiated (grade I), 32.9% had moderately differentiated (grade II) and 35.3% had poorly differentiated (grade III) carcinomas. survival was worse in dogs with grade III carcinomas than in those with grade II or grade I tumors.

Sontas *et al.,* (2009) classified 328 CMT of which 78.30% malignant Tumors ( Simple carcinoma 28.30% , Complex carcinoma 15.09%, Malignant mixed tumor 15.06% , Carcinosarcoma 13.85% , Osteosarcoma 5.66 % , Noninfiltrating (in situ) carcinoma 2.36% Other sarcomas 1.89% Fibrosarcoma 1.42% , Spindle cell carcinoma 0.47 % Squamous cell carcinoma 0.47%.), 12.27% Benign Tumors (Benign mixed tumor 4.72% , Adenoma 3.30% , Fibroadenoma 2.83% , Duct papilloma 1.42%). Unclassified tumors and hyperplasia/dysplasia accounted for 1.41% and 8.02% respectively and finally concluded that histopathological diagnosis of CMTs is crucial in prediction of tumor behaviour after surgical excision.

Reddy *et al.,* (2009) classified various CMTs based on WHO recommendations. The benign tumors were identified as fibroadenoma (41.66%), ductal papilloma (16.66%), benign mixed mammary tumor (29.16%), myoepithelioma (4.16%) and simple adenoma (8.33%). In malignant mammary tumors, epithelial tumors included papillary adenocarcinoma (25.96%), malignant mixed mammary tumor (25.96%), solid carcinomas (17.31%), infiltrative adenocarcinoma (11.54%), malignant myoepithelioma (7.69%), squamous cell carcinoma (2.88%), mucinous carcinoma (1.92%), intraductal carcinoma in situ (0.96%), whereas the connective tissue tumors were fibrosarcoma (2.88%), myxosarcoma (0.96%), carcinosarcoma (0.96%) and osteochondrosarcoma (0.96%).

Polton (2009) gave a simplified system incorporating elements of histological grade and clinical stage. In stage 1, lesions are non-infiltrative and resemble the tissue of origin and tubular structures are evident. In stage 2, there is loss of tubular lumen and/or invasion of the surrounding stroma but no evidence of vascular or lymphatic invasion. Stage 3, is determined by presence of lymphatic or vascular invasion. In stage 4, there is evidence of metastasis. Inflammatory carcinoma was dealt as separate entity as it didn't fit in grading

system. Histological evaluation of an incisional or excisional biopsy remains the gold standard recommendation for mammary tumor diagnosis.

Sleeckx *et al.,* (2011) in their review compared the classification methods for CMT proposed in the various literatures. World Health Organization (WHO) International Histological Classification of Mammary Tumors of the Dog of 1974 with the modification of 1999 and additionally discussed new histological subtypes that had been described since the publication of the WHO classification of 1999.

Kurilji *et al.,* (2011) investigated 146 CMT samples. Which revealed 110 (75.3%) malignant tumors (35 complex carcinomas, 28 tubulopapillary carcinomas, 17 solid carcinomas, 10 anaplastic carcinomas, 4 squamous cell carcinomas, 2 spindle cell carcinomas, 4 sarcomas, 2 carcinosarcomas, 8 carcinomas in benign tumors) and 36 (24.7%) benign tumors (14 complex adenomas, 8 simple adenomas, 9 benign mixed tumors, 1 fibroadenoma and 4 mammary hyperplasias).

Cerovšek *et al.,* (2013) made histopathological diagnosis of CMT that included 4 carcinomas in situ, 29 simple type carcinomas (17 tubulopapillary carcinomas, 10 solid carcinomas and 2 anaplastic carcinomas), 19 complex type carcinomas and 1 case of malignant mixed tumor, mucinus carcinoma, squamous cell carcinoma and malignant myoepithelioma. From the 50 carcinomas 22 (44 %) were grade I, 13 (26 %) were grade II and 15 (30 %) were grade III. Histological grade, histological type (simple/complex) and clinical stage were associated significantly with survival.

Tavasoly *et al.,* (2013) classified 37 CMT as carcinomas (86.5%) and sarcomas (13.5%). The carcinomas were classified as simple carcinoma 56.8%, complex carcinoma 13.5%, carcinoma arising from benign tumor 10.8% and special type of carcinoma 5.4%. Out of 32 carcinomas studied, 37.5% were grade I, 46.9% grade II and 15.6% grade III.

Kishor *et al.,* (2016) analyzed 40 spontaneous tumors of canine mammary glands of which 39 cases (97.5 per cent) were carcinomas and one case (2.5 per cent) was sarcoma. Tumors were classified as complex carcinoma (40.0%), simple carcinoma (22.5%), solid carcinoma (22.5%), mixed carcinoma (7.5%), anaplastic carcinoma (5.0%) and fibrosarcoma (2.5%). Tumor staging was done

on basis of neoplastic cells confined to mammary ducts and ductules without stromal invasion (tumor Stage 0), neoplastic cells found invading surrounding connective tissue stroma and capsule (Stage I), neoplastic cells found invading lymphatic or blood vessels (Stage II), neoplastic cells found metastasized to regional lymph nodes or to distant organs(Stage III). Out of which 21 cases (52.5%) were in stage I, 17 cases (42.5%) in stage II and two cases (5.0%) in stage III. Higher stage grade indicated guarded prognosis.

**2.4 Cancer stem cells and metastasis associated markers**

The history of research on cancer stem cells (CSCs) goes back to the 19$^{th}$ century and Virchow's embryonal-rest hypothesis. A series of analyses had led Virchow to speculate that cancer may arise from undifferentiated cells of connective tissue. He proposed that cancer is caused by the activation of dormant cells present in mature tissue that were remainders of embryonic cells. Similarities existing between these cells and cancer cells were the fundamental reason for proposing the modern SC theory of tumorogenesis (Blacking *et al.*, 2007; Gil *et al.*, 2008).

Paget *et al.,* (1889), more than 100 years ago, compared metastatic cancer cells to "seeds" that, once released from the plant (primary tumor), can spread widely but only when they fall on "congenial soil" (the metastatic microenvironment) they survive and proliferate.

Kang *et al.,* (2004) opined that cellular plasticity in stem cells may facilitate epithelial-mesenchymal transition, which has been postulated as a key event during the early phase of cancer metastasis.

Vega *et al.* (2004), Snail offers protection from both stress-induced cell death and that provoked by pro-apoptotic signals. This resistance to cell death is essential in the adult for malignant cells to disseminate and form metastasis.

Brabletz *et al.,* (2005) firstly established the hypothesis of migrating CSCs, which possess both an element of stemness and mobility. It is postulated that these cells undergo epithelial-mesenchymal transition at the invasive front of the primary cancer and migrate to colonize new tissues, where its acquired stemness to facilitate production of the cancer heterogeneity commonly observed in metastatic colonies.

Gimeno *et al.,* (2005) Snail induced epithelial-mesenchymal transition converts epithelial cells into mesenchymal cells with migratory properties that contribute to the formation of many tissues during embryonic development and to the acquisition of invasive properties in epithelial tumors. Loss of E-cadherin from tumors is associated with a poor prognosis. Snail genes being E-cadherin repressors are regarded as early markers of tumor malignancy.

Come *et al.,* (2006) suggested the involvement of Slug in maintenance of tubular structures in ductal carcinomas. They found that Slug and Snail were significantly over expressed in invasive ductal carcinoma (IDC) that metastasized to lymph nodes.

Eccles *et al.,* (2007) concluded that the metastasis, frequently a final and fatal step in the progression of solid malignancies, encompasses several fundamental biological processes: cancer initiation, epithelial–mesenchymal transition (epithelial-mesenchymal transition), breach of the basement membrane barrier, neighbour invasion, intravasation, mesenchymal–epithelial transition (MET), extravasation, colonization and outgrowth of micrometastases and secondary cancer.

Yang *et al.,* (2008) suggested that epithelial-mesenchymal transition represents a crucial step towards invasiveness and metastasis, and is strongly associated with poor clinical outcome in many tumor types.

Mani *et al.,* (2008) have also shown that epithelial-mesenchymal transition can induce differentiated cancer cells into a CSC-like state. These observations established a first functional link between cancer stemness and epithelial-mesenchymal transition and suggested that CSCs may underlie local and distant metastases by acquiring mesenchymal features which would greatly facilitate systemic dissemination from the primary mass.

Cocola *et al.,* (2009) isolated the first canine mammary CSC. They defined a canine cell suspension culture system that allows for the identification and propagation of dog cells with stem cell properties. Using their canine model system, they determined that spheres generated from normal and tumor samples could be serially regenerated for 3–5 passages in culture. They showed that cells from these spheres could also generate a mammary gland-like architecture with branching morphology in vitro in collagen and Matrigel gels. Cells from two tumor

samples that had sustained regeneration capacity up to passage 5 were used for further analysis. These cells were shown to have the capacity to generate tumors in NOD/SCID mice. The properties of sphere sustainability, tumor formation and multi-lineage differentiation potential suggest collectively that their protocol allows for the propagation and enrichment for canine tumor stem cells.

Lin *et al.,* (2009) showed that in invasive CSCs, an epithelial marker E-cadherin was down-regulated, mesenchymal markers were up-regulated, and Transgelin which regulates epithelial-mesenchymal transition associated genes were over-expressed , which indicated some link exit between epithelial-mesenchymal transition and CSCs in tumor invasion and metastasis.

Zeisberg *et al.,* (2009) documented that Snail family members of zinc finger proteins (Snail1, Snail2, and Snail3), the functionally equivalent Snail1 and Snail2 (which was formerly known as Slug) mediate epithelial-mesenchymal transition (EMT), which involves a loss of epithelial markers like E-cadherin and an increase in mesenchymal markers.

Yao *et al.,* (2011) recognized epithelial-mesenchymal transition and mesenchymal-epithelial transition as critical events for metastasis of carcinomas. The former are responsible for degrading the surrounding matrix to lead the way of invasion and intravasation and the latter is important for cancer cells then enter the blood stream and reestablish colonies in the secondary sites.

Simões *et al.,* (2011) the embryonic stem cell genes Nanog*,* Oct4*, and* Sox2 are expressed in normal breast stem cells and at higher levels in breast tumor cells and their expression decreases upon differentiation.

Pang *et al., (*2011) characterized tumorsphere using the embryonic stem cell markers, Nanog and Oct4. Canine tumorspheres expressed higher levels of the Oct4 and Nanog compared to parental cells, which suggests that tumorsphere have a primitive phenotype and are representative of a CSC population. They found that tumorspheres derived from a canine mammarycarcinoma cell line are more resistant to the chemotherapeutic drug doxorubicin and ionizing radiation, compared to parental cells.

Kim *et al.,* (2011) Injecting of Oct-$4^{high}$ sorted cells in mice mammary gland showed increased tumorogenic potential as compared to Oct-$4^{low}$ cell population.

Cancer stem cells expressing Oct-4$^{high}$ displayed tumorsphere forming ability in vitro and showed increased expression of stem cell markers Sca-1, CD133, CD34 and ALDH1.

Michishita *et al.,* (2011) found higher level of expression of CD44 and CD133 in spheres derived from canine mammary adenocarcinoma cell line, CHMp by flow cytometry and RT-PCR, which strongly supports the stem cell-like properties of the spheres in canine mammary tumors. Sox2, Oct4, Nanog and C-myc, which are associated with the maintenance of stem cells, were expressed in adherent cells as well as in sphere cells. They also reviewed the work done by others which claims spheres to be enriched with stem cells in normal breast tissues and in cancer in humans and dogs, and the sphere assay may be a valuable way to identify cells with the characteristics of cancer stem cells.

Ling *et al.,* (2012) suggested that there is higher Oct3/4A expression in the adenocarcinoma cells. Lower Oct3/4A expression in the ductal carcinoma cells since T-47D cells are derived from a ductal carcinoma. MCF7 and T-47D cells expressed significantly higher levels of Sox-2 and Nanog compared with MDA-MB-231 cells. Thus confirming expression of pluripotency associated markers in breast cancer cell lines.

Sampieri *et al.,* (2012) observed that cancer stem cells (CSCs) represent a subpopulation of tumor cells endowed with, just like normal Stem cells (SCs), self-renewal and multi-lineage differentiation capacity. Finely tuned homeostatic equilibrium is present in normal stem cell whereas its lost in CSCs. Whereas in the normal SC niche rates of self renewal, symmetric vs. asymmetric cell division, cell proliferation, migration, differentiation and apoptosis are coordinated so that for each newly produced cell another one undergoes programmed cell death, in the CSC niche subtle defects in any of the above cell functions alter the homeostatic equilibrium and result into unbalanced cell growth and the formation of the tumor mass.

Martin & Jiang *et al.,* (2014) demonstrated that, overall there was a reduction in expression of CD24, CD29, CD44 and CD133 with increasing Nottingham Prognostic Indicator (NPI), an increase in all markers with increasing grade and an increase in expression of CD29, CD34, CD44 and CD133 with increasing TNM status. Moreover, the majority of cancer stem markers were

reduced with metastatic disease and poor prognosis overall. There was a significant association between loss of expression and metastatic disease in patients with breast cancer and suggests that the current stem cell theory may not entirely hold true for all cancer types.

Wang *et al.,* (2014) indicated that Oct-4 and Nanog co-expression was associated with lymph node metastasis and the molecular type of breast cancer, as well as poor prognosis in breast cancer patients. Oct-4 and Nanog co-expression may be a valuable biomarker to predict the outcome of breast cancer patients.

Chung *et al.,* (2014) associated STAT 3 expression of breast tumors with tumorogenesis and drug resistant. When STAT3 gene was knocked down, the expression of the stem cell markers Oct4, Sox2 and CD44 were downregulated and tumorsphere formation was abolished. It suggests that STAT3 plays a clear role in inducing these expressions of stem cell markers.

Dominik *et al.,* (2016) demonstrated that CSC might serve as biomarker for early tumor detection, prognostication, and prediction of therapy response whereas different CSC targeted therapies are emerging as potentially curative anti-cancer treatment.

As per Tume *et al.,* (2016), CD133 is recognized as an important biomarker to identify and isolate the specific cell subpopulation named ‘‘cancer stem cells’’ in many types of neoplasms including breast cancer. CD133+ cells have stemness properties such as drug-resistance, self-renewal, differentiation ability; high proliferation and they are able also to form tumors in xenografts. These cells with CD133+ are more resistant to radiation and standard chemotherapy than CD133 (−) cells.

Vazquez-Santillan *et al.,* (2016) derived Breast cancer stem cells from MCF7 and MDA-MB-231 cell line which expressed higher level of Oct4, Nanog, Aldh1a3, Aldh1a3 ,Aldh8a1 And Oct4, Nanog, Aldh1a3, Aldh1a3 and Aldh8a1 respectively.

Agliano *et al.,* (2017) in their review has stated that, amongst those cancer cells that escape the primary tumor, invade surrounding tissues, and enter

the bloodstream only a small fraction is able to initiate a secondary tumor; many disseminated cells remain viable but fail to form a clinical lesion.

Peitzsch *et al.,* (2017) has narrated landmark work done by Leroy Stevens and John Dick. Leroy Stevens was first to introduce the term “pluripotent” in relation to cancerous teratoma cells in 1953.The cancer stem cell concept of tumor development, which has been experimentally proven by John Dick and coworkers more than 20 years ago, suggests that cancer cells differ in their tumor initiating properties and population of tumor cells called cancer stem cells (CSC) resides on top of the tumor cell hierarchy and maintains tumor heterogeneity and tumorigenicity.

## MATERIALS AND METHOD

Thirty cases of mammary gland tumors in females of all possible breed types, age groups of canines in and around Nagpur and Pune region for the period of six months were studied. After obtaining the consent from owner the canine mammary tumor samples were collected after mastectomy or biopsy. Parts of the samples were subjected to histopathology and isolation of cancer stem cells. Further Real Time PCR was performed for characterization of cancer stem cells and to determine expression of metastatic marker. Blood and serum were subjected to haematological and biochemical analysis respectively.

### 3.1 Materials

#### 3.1.1 Chemicals and reagents

Chemicals and biological reagents used in this study were of molecular biology grade from Hi-Media (India), Gibco (USA) and Sigma-Aldrich (USA).

#### 3.1.2 Glass wares

The glasswares used in this study were obtained from Borosil (India). The glasswares were sterilized in hot air oven at 160°C for one hour prior to use.

#### 3.1.3 Plastic ware

The sterile plastic wares used in this study were obtained from Corning (USA), Axygen (USA) and Eppendorf (Germany).

### 3.2 Collection of materials

Mammary tumors were collected from canines presented to different clinics in and around Nagpur and Pune region after mastectomy. Samples were preserved in 10% formal saline solution for histopathological studies. Samples were collected in sterile Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% Foetal Bovine Serum (FBS), and antibiotics (penicillin 100U/ml, streptomycin 100µg/ml) for isolation of cancer stem cells. Part of the sample was also collected in TRIzol for further RNA studies.

Blood sample was collected on the day of mastectomy or biopsy in 2ml EDTA vials for haematology and without EDTA for estimation of serum biochemical parameters.

### 3.3 Haemato-biochemical Parameters

#### 3.3.1 Hematological Parameters

Determination of Hemoglobin (Hb), Packed cell volume (PCV), Total erythrocyte count (TEC) and Total leukocyte count (TLC) were done by using Mindray auto-haemoanalyzer.

#### 3.3.2 Serum biochemistry

The blood samples of ailing bitches were collected and serum was separated and stored at -20°C. They were subjected to biochemical analysis. The following estimations were carried out using clinical chemistry semi-auto analyzer machine with Coral clinical system kits (Goa, India).

1) Serum alkaline phosphatase (ALP)
2) Serum Calcium (Ca)
3) Serum Phosphorus (P)
4) Serum Magnesium (Mg)

### 3.4 Histopathological examination

The tumor tissues fixed in 10% neutral buffered formalin were embedded in paraffin wax (58-60°C M.P.) after dehydration in ascending grade of alcohol. Sections were cut at 4-5 m thickness and stained with standard haematoxylin and eosin (H & E) method (Luna, 1968). In present study histopathological classification of tumors was done as per WHO classification system followed earlier by Hampe and Misdorp (1974).

#### 3.4.1 Grading system

Grading of tumor was decided as per the Elston modification of the Bloom Richardson grading system and Nottingham method. Following parameters were observed for analysis of histological grading 1) tubule formation, 2) nuclear variation, and 3) mitotic activity (Elston and Ellis, 1991).

**a) Tubule formation**

Score 1: More than 75% of the area composed of definite tubules.
Score 2: Area between 10 to 75% occupied by tubule formation.
Score 3: Tubules occupy 10% or less of the tumor.

**b) Nuclear pleomorphism**

Score 1: Tumor nuclei are small, with little increase or variation in size compared to normal nuclei, have regular outlines and uniformity of nuclear chromatin.

Score 2: Nuclei are larger than normal, have more open vesicular nuclei with visible, usually single, nucleoli and there is moderate variation in size and shape.

Score 3: Marked variation in size and shape, very large and bizarre nuclei, nuclei are vesicular with prominent enlarged and often multiple nucleoli.

**c) Mitotic counts**

Mitotic activity was assessed by counting mitotic figures in 10 high power fields (40X).

Score 1: Upto 7 mitosis per 10 high fields.

Score 2: 8-16 mitosis per 10 high fields.

Score 3: More than 17 mitosis per 10 high fields.

**Elston Grade evaluation**

After employing above score criteria, the tumor scoring points in the range of 3 to 9 was considered as tumor and graded as per the following scores.

Grade I : Score between 3 to 5.

Grade II : Score between 6 to 7.

Grade III : Score between 8 to 9.

**3.5 Isolation of cancer stem cells**

Cancer stem cells isolation from mammary tumor sample was carried out by mammosphere assay as per Lombardo *et al*. (2015) with few modifications as mentioned below.

**a) Primary culture of Canine mammary tumor**

Tumor tissue obtained were washed in 70% ethanol and then thoroughly washed with PBS containing penicillin 100U/ml and streptomycin 100µg/ml. Tissue were minced into small pieces and enzymatically digested in 5ml DMEM containing collagenase (Gibco) and incubated at 37°C on rotary shaker for 30 minutes to 120 minutes (depending upon the hardness of tissue). Supernatant was collected in 15ml polypropylene tube containing complete media and centrifuged at 2000 rpm for 5-8 minutes. Supernatant was discarded and cell

pellet was resuspended in DMEM containing 10% FBS (complete media). Cells were seeded in 100 mm tissue Petri dish. Incubated at 37°C and 5% $CO_2$ till the cells were grown in monolayer. Cells were fed with complete media every 72 hrs. Before being used for mammosphere assay cells were amplified upto 5 passages.

**b) Mammosphere Culture**

Adherent cultured cell were detached with trypsin and collected in 15ml polypropylene tube containing DMEM/F12 and centrifuged at 1700 rpm for 5 minutes. Supernatant was discarded and cells were resuspended in DMEM/F12 supplemented with 100 U/ml penicillin, 100 U/ml streptomycin. Single cell suspension was obtained by 40 µm cell strainer. Low density of cells ($1.5 \times 10^4$ cells/ml) were resuspended in serum free conditional medium which contained DMEM/F12 supplemented with 20 ng/ml recombinant human epidermal growth factor (EGF; Sigma), 10 ng/ml recombinant human basic fibroblast growth factor (bFGF;Sigma) and 1x B27 supplement (Gibco). Then seeded in 6 well agar overlayed Plate. Incubation was done at 37°C and 5% $CO_2$ for 8 to 10 days. After every 72 hrs cultures were fed with above growth factor containing media. Precaution was taken to keep these plates undisturbed during initial growth phase. After 7-8 days representative views were photographed.

**3.6 MTT assay**

For the MTT assay previously described method was implemented with few modifications (Barhanpurkar-Naik *et al.,* 2017). Cells were cultured in 96-well plates ($3 \times 10^3$ cells/well) in four replicates. After 3hrs, 24hrs, 48hrs and 72hrs respective medium was replaced with 100 µl of MTT (0.5 mg/ml). Black crystals formed was dissolved with DMSO and absorbance measured at 570 nm.

**3.7 Population doubling (PD) time**

To determine PD time cells were cultured ($1 \times 10^4$ cells/well) in 24 well plate in growth medium. Cells were seeded in duplicate. After 3 days cells were harvested with TPVG and counted by trypan blue exclusion. The PD time was calculated as per method adopted by Roth (2006) mentioned as follows:

$$\text{PD time} = \frac{\text{duration} \times \log 2}{\log(\text{final concentration}) - \log(\text{initial concentration})}$$

**3.8 Wound healing assay**

For the wound healing assay previously described method was implemented (Barhanpurkar-Naik *et al.,*2017). Briefly, $10^4$ cells/well were plated in 24-well plates in triplicates and allowed to form a monolayer. The wounds were created by scratching monolayers with a 10 ul pipette tip. The cell layer was washed thoroughly with caution and incubated further for 18 hours. Images were captured at 0 and 18 hours using the phase-contrast microscope. Wound area was calculated using the "MRI Wound healing tool" in Image J software (open source, NIH, USA). Percent wound closure was calculated as described previously using the following formula:

$$\%\ \text{Wound closure} = \frac{\text{Wound area } (T_0) - \text{Wound area } (T_{18})}{\text{Wound area } (T_0)} \times 100$$

**3.9 Total RNA isolation:**

RNA was isolated from tissue, adherent cells and mammospheres using TRIzol reagent (Invitrogen) as per manufactures protocol with few modifications.

**a) Homegenization:**

The homogenization of the tissue was done by mortar and pestle in a liquid nitrogen bath. Homegenized tissue (100mg) was transferred to 1ml of TRIzol. Cancer cells ($10^6$) were stored in 1ml of TRIzol in -80°C and used for RNA isolation after thawing.

**b) Phase separation:**

For phase separation 0.2ml of chloroform per 1ml of TRIzol was used. Samples were vortexed vigorously for 15 s and incubated at room temperature for 5 min. Then centrifuged at 12000 g for 15 min at 4°C. Upon centrifugation two phases were formed. RNA is usually present in upper aqueous phase. Upper aqueous phase was cautiously transferred to fresh tube.

**c) RNA precipitation:**

RNA from upper aqueous phase was precipitated by mixing with 0.5ml isopropyl alcohol. Then incubated for 30 min on ice and centrifuged at 12000 g for 15 min at 4°C. RNA precipitate was seen as pellet on bottom of tube.

**d) Washing of RNA:**

Supernatant was discarded completely. RNA pellet was washed with 1ml of 75% ethanol by centrifuging at 7500 g for 5 min at 4°C. Leftover ethanol was decanted.

**e) Redissolving RNA:**

RNA pellet was air dried for 15-20 min. It was dissolved in 15ul of DPEC treated water. Further incubated for 5min at 65°C on thermostat and then on ice for 5 min.

**f) Quantification of RNA:**

Concentration and purity of isolated RNA was determined by spectrophotometric absorbance at 260 and 280 nm on NanoDrop 1000 spectrophotometer (Thermo scientific).

**3.10 Real Time PCR**

Total RNA (2ug) from each sample was be subjected to cDNA synthesis using QuantiTect® Reverse Transcription Kit as per manufacturer's instruction. The relative expression of the reported stem-cell markers (Oct4, Sox2, Nanog, and Stat3) and metastasis associated markers (Snail) were determined using the primers depicted in Table1 by real-time PCR performed on a StepOnePlus machine (Applied Biosystems). The PCR was carried out by 10 ul reaction mixture involving the ingredients given in the Table2**.** Cycling conditions used for amplification of cDNA template are mentioned in Table3. GAPDH expression was used as an endogenous control to normalize each sample.

The relative expression of individual gene was calculated by method adopted by Schmittgen & Livak, (2008) and Xu *et al.,* (2017) mentioned as follows:

$\Delta C_T$ = $C_T$ gene of interest - $C_T$ internal control

Relative expression = $2^{-\Delta CT}$

**Table 1. Primer sequences used for quantification of respective gene**

| Gene | Forward Primer (5'-3') | Reverse Primer (5'-3') | Base pair | Accession No. |
|---|---|---|---|---|
| Oct4 | ATGTGGTCCGAGTGTGGTTC | AGGGACTGAGGAGTAGAGCG | 200 | XM_538830.3 |
| Sox2 | CCAGCGCATGGACAGCTA | CCGTTCATGTAGGTCTGCGA | 195 | XM_005639752.3 |
| Nanog | CCAGACCTGGAACAGCCAAT | ACAGTTGTGGAGCGGATTGT | 106 | XM_022411387.1 |
| STAT3 | CCAAGCAACAGCTGAACAACA | CTGGGTCGGCTTCAGGATGT | 177 | XM_025439586.1 |
| Snail | CAGCTATGTCAGCGTCCTGT | TGGGAGACACATTGGTTGGG | 142 | XM_005635167.3 |
| Gapdh | CCATCTTCCAGGAGCGAGAT | TTCTCCATGGTGGTGAAGAC | 97 | XM_003435649.4 |

**Table 2. Ingredients for real time PCR**

| Ingredients | Volume (ul) |
|---|---|
| Power SYBR™ Green PCR Master Mix (Applied Biosystems) | 5 |
| Forward primer | 0.5 |
| Reverse primer | 0.5 |
| Nuclease free water | 3.5 |
| cDNA template | 0.5 |

**Table 3. Cycling condition for real time PCR**

| Stages | PCR condition | Temperature (°C) | Time | |
|---|---|---|---|---|
| Stage I | Pre incubation | 95°C | 10 min | |
| Stage II | Two step amplification | | | **40 cycles** |
| | Incubation | 95°C | 15 sec | |
| | Annealing | 60°C | 1 min | |
| Stage III | Holding | 37 | ∞ | |

## RESULTS & DISCUSSION

The aim of current study was to authenticate presence of Cancer stem cells (CSC) associated markers in Canine mammary tissue (CMT) and to further investigate whether expression of these markers correlate to expression of Snail (SNAI1) which is associated with metastasis, as well as their association with tumor grade. Overall all, 30 CMT samples were collected. The evaluation of 8 samples was done for cancer stem cells characterization and to determine expression of metastasis associated markers. All samples were subjected for histopathological studies for tumor diagnosis and grading. Results are summarized as follows:

4.1 Effect on haemato-biochemical parameters
4.2 Gross changes, histopathological diagnosis and grading of tumors
4.3 Migration, proliferation kinetics and population doubling time
4.4 Characterization of cancer stem cells in CMT
4.5 Cancer stem cells versus metastasis associated markers
4.6 Cancer stem cells versus tumor grade

### 4.1 Effect of haemato-biochemical parameters

### A) Haematological parameters

The observation of mean ± S.E. values of Haemoglobin (Hb), Packed cell volume (PCV), Total erythrocyte count (TEC) and Total leukocyte count (TLC) are presented in Table 4. In our study all the haematological parameters were well within the normal reference ranges. Earlier study had recorded increased TLC in bitches affected with benign mammary tumors (Chavan *et al.,* 2012). On par values of TLC in present study might be due to the fact that all tumors were malignant. Recently Mohapatra *et al*., (2016) also proclaimed that haematological parameters didn't show any deviation from normal reference ranges.

**Table 4. Haematological values of dog with canine mammary tumor**

| Parameters | Haematological Values |
|---|---|
| Haemoglobin (g/dl) | 12.58 ± 1.43 |
| PCV (%) | 44.57 ± 4.35 |
| TEC (x $10^{12}$/L) | 5.89 ± 0.61 |
| TLC (x $10^{9}$/L) | 14.06 ± 1.92 |

**B) Biochemical parameters**

The observation of mean ± S.E. values of serum ALP, Mg, Ca, P are presented in Table 5. In our study all the serum parameters were well within the normal reference ranges. Previous study have recorded decrease in serum P and Ca in benign and malignant canine mammary tumor (Chavan *et al.,* 2012). As per Fayolled *et al.,* (1987) routine hematology and serum biochemistry were not useful in differentiating benign from malignant tumors and post-surgical blood profiling was not useful in predicting tumor progression. So with our findings we infer that the haematological and biochemical values are not good indicators for the CMT development in bitches.

**Table 5. Serum biochemical value of dog with canine mammary tumor**

| Parameters | Serum Values |
|---|---|
| Alkaline Phosphatase(IU/L) | 129.15 ± 33.35 |
| Serum Magnesium (mg/dL) | 2.74 ± 0.99 |
| Serum Calcium (mg/dL) | 10.30 ± 0.21 |
| Serum Phosphorus (mg/dL) | 4.31 ± 0.15 |

**4.2 Gross changes, histopathological diagnosis and grading**

**4.2.1 Gross observation**

Variation in size of different tumors was observed in range of 2.5 cm x 1.5 cm X 1 cm to 28 cm X 15 cm X 16 cm. Poorly defined border, ulceration, multiple nodules were observed (Plate 1-3). Tumors shape observed were ovoid, pedunculated and bizarre structured affecting one or more glands. Consistency ranged from very hard to firm. Upon cutting the tumors the consistency was gritty. Cut surface revealed grey tan colour with fluid filled cystic pockets. Fluid was slimy amber coloured. Necrosis, inflammation and severe vascularisation were evident (Plate 4.). Observations were in accordance with Moulton (1999) and Dhaygude (2006).

**4.2.2 Histological classification:**

Tumor growths were classified histologically as per WHO recommendation and data is presented in Table 6.

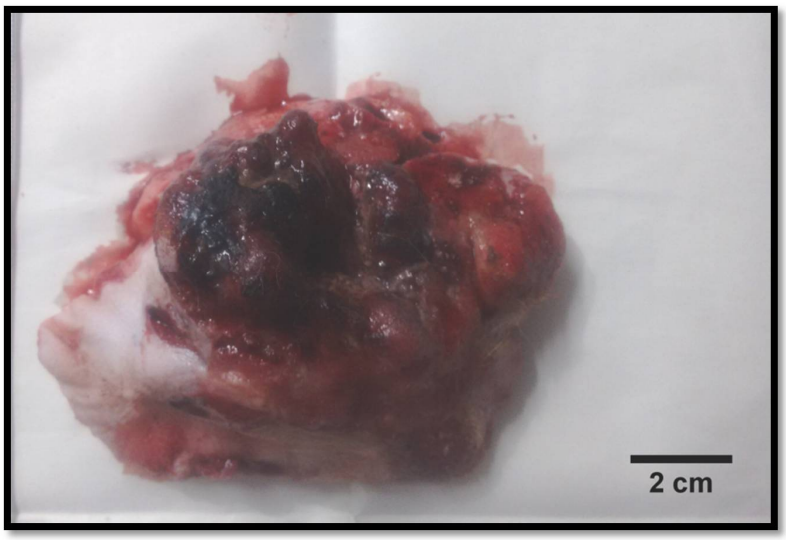


**Plate 1. Canine mammary tumor showed firm growth in inguinal region.**

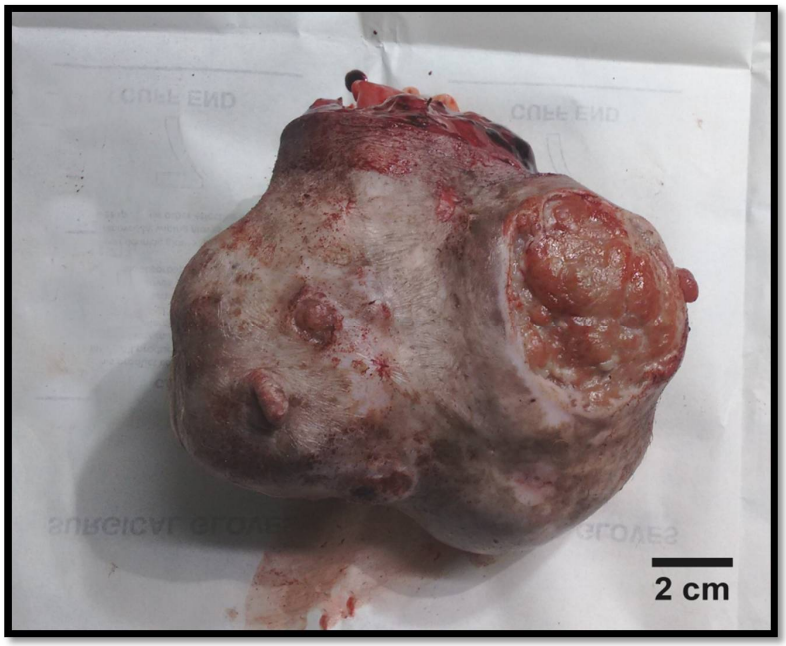


**Plate 2. Canine mammary tumor showing ulceration.**

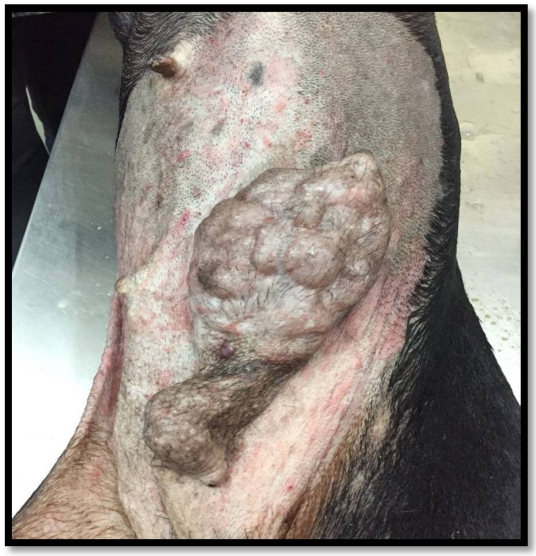

**Plate 3. Mammary tumor showing multiple nodular growth.**

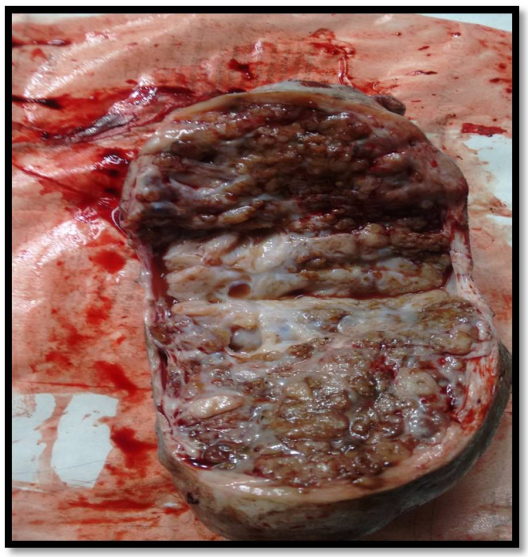

**Plate 4. Mammary tumor cut surface showing severe necrosis and vascularisation.**

**Table 6. Summary of histological classification of mammary tumor (n=30)**

| Histological types | No. of cases | Percentage |
|---|---|---|
| **Malignant Tumors** | | |
| Simple tubular adenocarcinoma | 7 | 23.33 |
| Complex tubular adenocarcinoma | 2 | 6.66 |
| Simple tubulopapillary adenocarcinoma | 3 | 10 |
| Complex tubulopapillary adenocarcinoma | 2 | 6.66 |
| Squamous cell carcinoma | 5 | 16.66 |
| Simple solid carcinoma | 1 | 3.33 |
| Fibrosarcoma | 3 | 10 |
| Chondrosarcoma | 2 | 6.66 |
| Malignant mixed | 5 | 16.66 |
| **Total** | **30** | |

Out of 30 spontaneous mammary tumors studied for histopathological classification, all cases (100%) were malignant mammary tumor. Higher incidence of malignant tumor have been mentioned in various reports (Simeonov & Stoikov, 2006, Tavasoly *et al*.,2013). It suggests that increased incidence of malignancy might play vital role in mammary tumor related deaths. Among them 30% were diagnosed as tubular carcinoma (n=9). Papillary carcinoma and squamous cell carcinoma each cases were recorded 16.66% (n=5). Solid carcinoma represented only 3.33% (n=1). Fibrosarcoma was recorded as 10% (n=3) and 2 cases of chondrosarcoma represented 6.66%. Malignant mixed tumor showed incidence of 16.66%. Findings were partly in agreement with previous researchers (Sontas *et al*.,2009, Reddy *et al*.,2009, Sassi *et al*.,2010, Cervosek *et al*.,2013). Overall epithelial origin tumors were highest (66.66%) as compared to mesenchymal tumors (33.33%). Our findings are in agreement with various reports which suggests CMT of epithelial origin to be more common than mesenchymal origin (Misdorp, 1999 and Sleeckx *et al*.,2011).

Histological findings of H & E stained sections of malignant mammary tumor are described as follows

**1. Tubular adenocarcinoma (Simple and complex)**

Tubules were observed with higher and lower grade. Tubular epithelial cells showing uniform architecture and lower mitotic figures indicated low grade. High grade tubules showed hyperchromasia, increased mitotic activity and severe nuclear pleomorphism. Simple carcinoma showed resemblance to only luminal epithelial cells. Complex carcinoma resembled luminal and myoepithelial cell type (Plate 5). Tubules were lined by double layer of epithelial cells (Plate 6). Proliferating and hyperchromic luminal epithelial cells were invading surrounding fibrous stroma. Epithelial cells were separated and compiled in the glandular lumen. Significant mitotic figures were observed. Haemosiderin laden macrophages indicated chronic haemorrhages (Plate 7). Accumulation cystic fluid was observed in various parts (Plate 8). Severe necrosis was observed in tubular adenocarcinoma indicating towards increased malignancy (Plate 9).

**2. Papillary carcinoma**

In simple papillary carcinoma proliferation of only epithelial cells was observed. In complex papillary carcinoma epithelial and myoepithelial cells were present. Both columnar and cuboidal cells within the neoplastic tubules were arranged in pedunculated papillary form (Plate 10). Papillary stroma was abundant. Extension of papilla beyond the fibrous tissue indicated infiltrative behaviour. Papillary epithelial cells showed hyperchromasia. Moderate nuclear pleomorphism and mitotic figures with few necrotic areas were observed.

**3. Tubulopapillary carcinoma**

Along with the features of tubular adenocarcinoma it has neoplastic cells arranged in papillary manner (Plate 11). The neoplastic features of cells on papillary projections are similar to papillary adenocarcinoma. Numerous mitotic figures were present.

**4. Squamous cell carcinoma**

Sheets of round to polyhedral cells were observed. Cells were pleomorphic and hyperchromic . Islands of keratin pearls were present within the sheet. Significant amount of mitotic figures were present. These keratin forming pearls were major feature for squamous cell carcinoma (Plate 12). Cords of

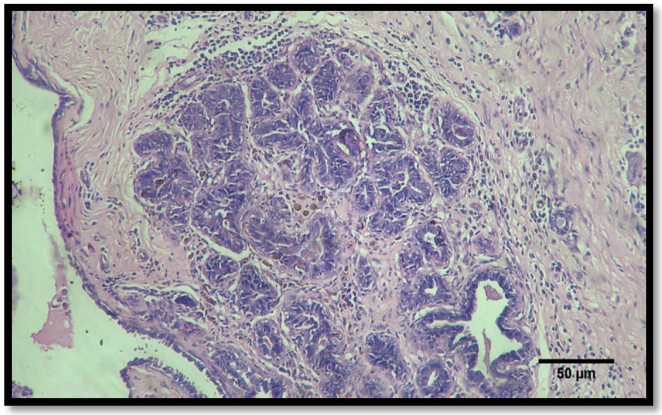


**Plate 5. Complex tubulo adenocarcinoma showing both myoepithelial cells and epithelial cells. (H & E 10X)**

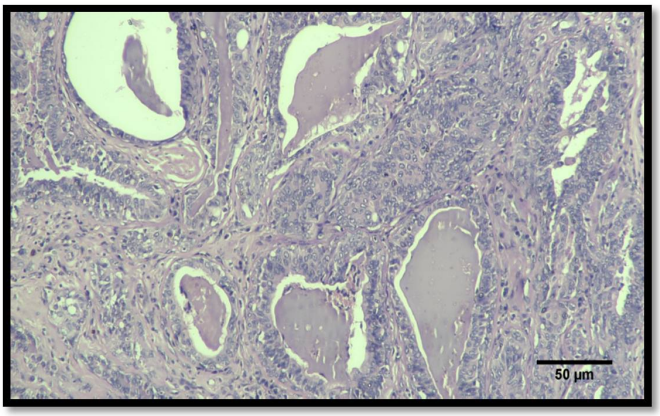


**Plate 6. Tubulo adenocarcinoma showing mitotic figures, double layered tubular epithelium and accumulation of epithelial cells in tubular lumen. (H & E 10X)**

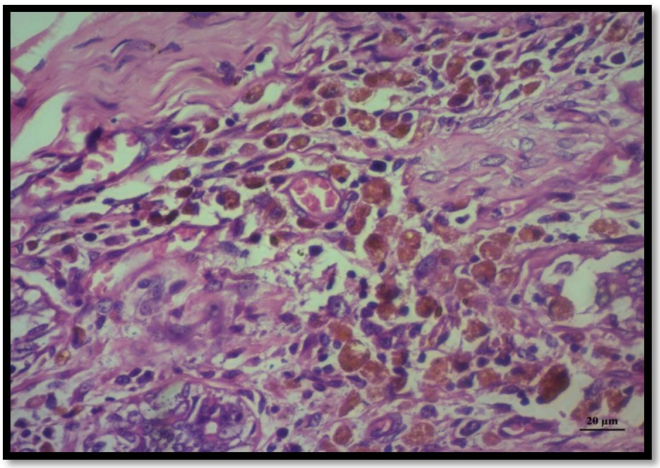


**Plate 7. Mammary tumor revealed presence of haemosiderin laden macrophages (H & E 40X)**

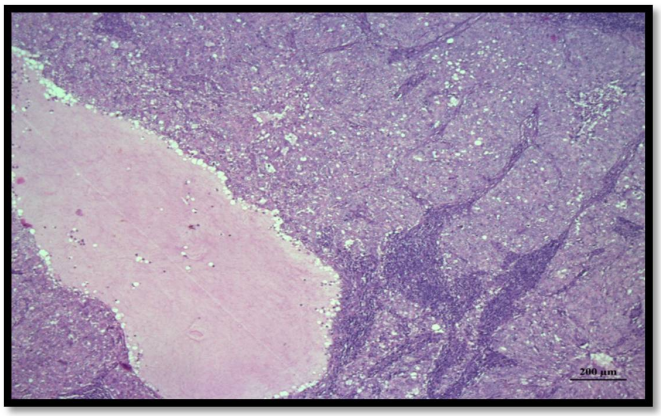

**Plate 8. Cystic fluid accumulation in tubular carcinoma (H & E 4X)**

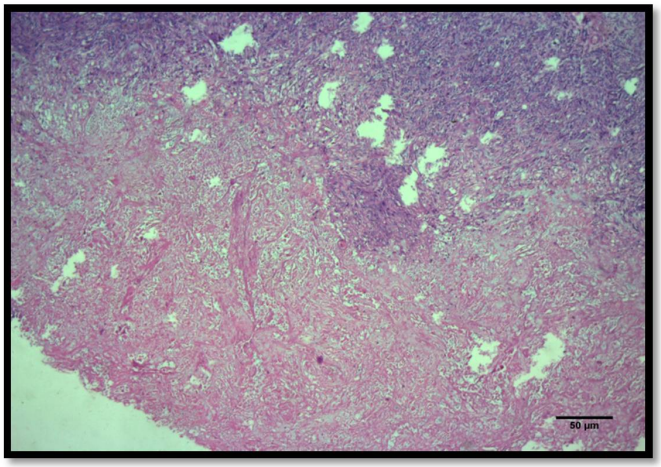

**Plate 9. Tubulo adenocarcinoma showing severe area of necrosis (H & E 4X)**

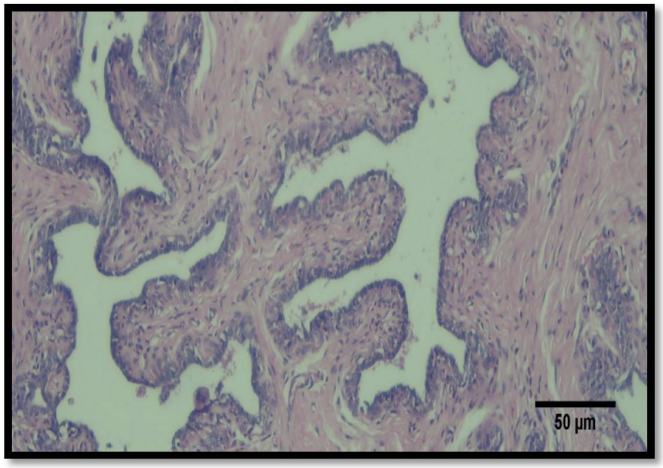


**Plate 10. Papillary carcinoma showing presence of both cuboidal and columnar epithelial cells. (H & E 10X)**

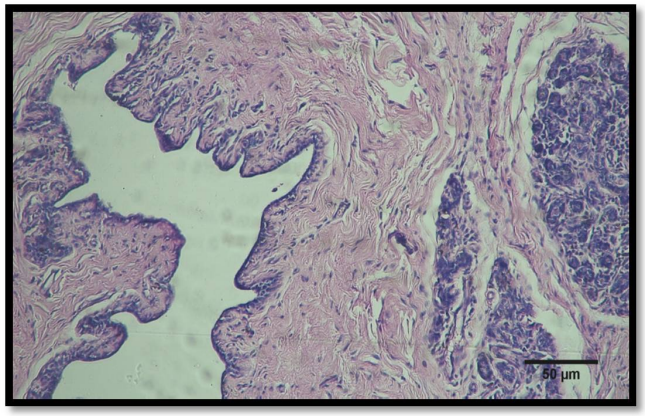


**Plate 11. Tubulopapillary carcinoma showing hyperchromatic lobular and papillary epithelium (H & E 10X)**

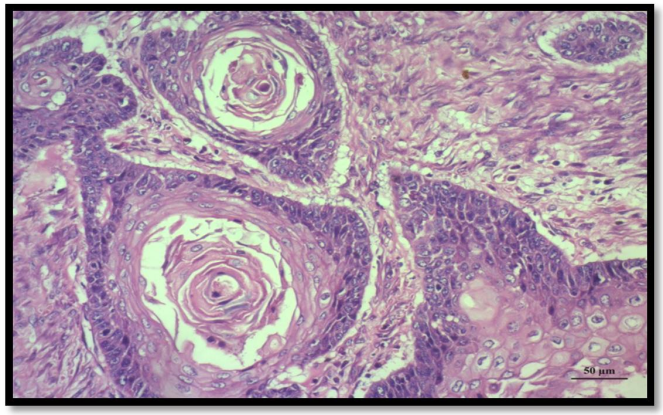


**Plate 12. Squamous cell carcinoma with stratified squamous epithelium leading to formation of keratinized pearl (H & E 20X)**

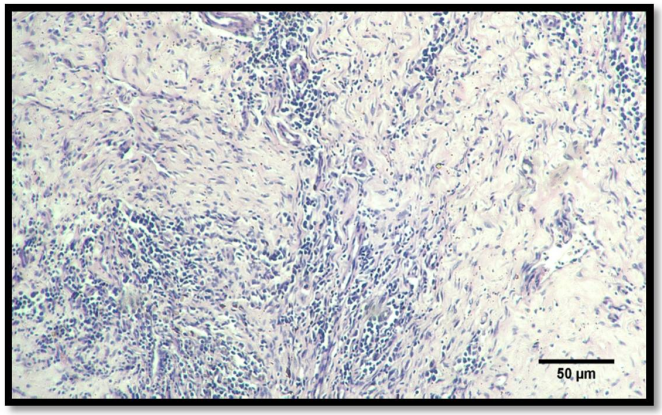


**Plate 13. Fibrosarcoma showing proliferation of spindle shaped cells with nuclear pleomorphism and increased microvasculation in tissue (H & E 10X)**

epithelial cell were seen and flat squamous like cells surrounded the keratin pearl.

**5. Fibrosarcoma**

Proliferation of spindle shaped cells with elongated nuclei were present (Plate 13). These proliferating cells architecture had fibroblast like morphology thus suggesting mesenchymal origin. Neoplastic fibroblasts were arranged haphazardly. There was stromal infiltration in tissue. Severe anisocytosis was observed. Intermittent mitotic figures were present.

**6. Chondrosarcoma**

Basophilic chondroid matrix is present in varied amount in which binucleated neoplastic chondroid cells are present (Plate 14). Chondroid cells are present in lacunae with atypical nuclei. Formation of cartilage in mammary gland indicates metaplasia of connective tissue. Prominent nucleoli were seen. Few mitotic figures were evident.

**7. Malignant Mixed tumor**

It had highly proliferating malignant epithelial and malignant mesenchymal cell types with hyperchromasia and nuclear pleomorphism (Plate 15). The third cell type we observed was of cartilage tissue along with malignant epithelial cells. Its appearance was in combination of tubular carcinoma, fibrosarcoma and chondrosarcoma. Severe intra tumoral micro vascularisation was evident.

**8. Solid carcinoma**

Neoplastic cells were densely packed ressembling solid sheet pattern without definite tubular formation (Plate 16). Severe nuclear pleomorphism, moderate cytoplasmic vacuolation and mitotic figures were evident.

### 4.2.3 Grading of mammary tumor

Grading of each tumor growth was executed in relation to histological classification as shown in Table 7.

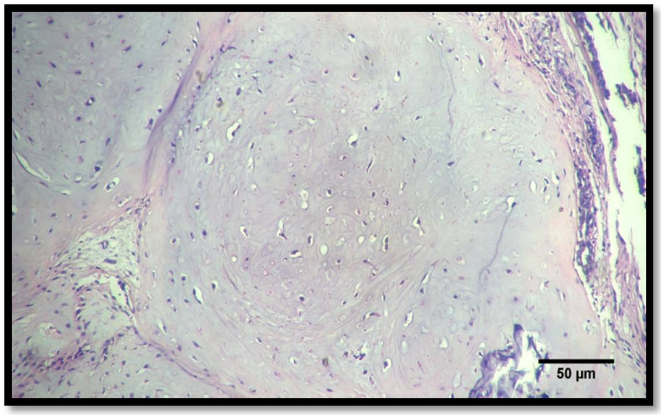


**Plate 14. Chondrosarcoma revealed presence pf binucleated neoplastic cells in basophilic matrix (H & E 10X)**

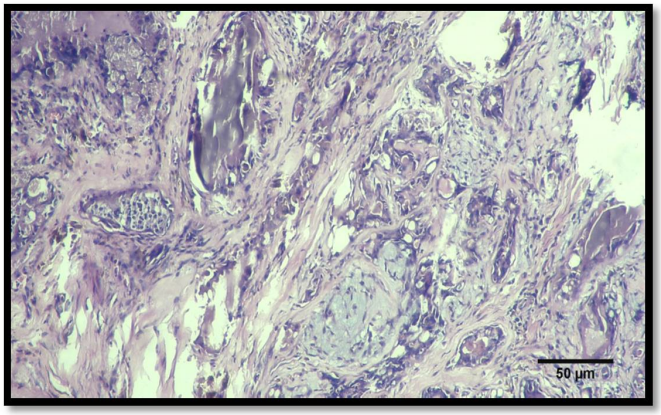


**Plate 15. Malignant mixed mammary tumor showing neoplastic cells of epithelial and mesenchymal origin with distorted tissue morphology (H & E 20X)**

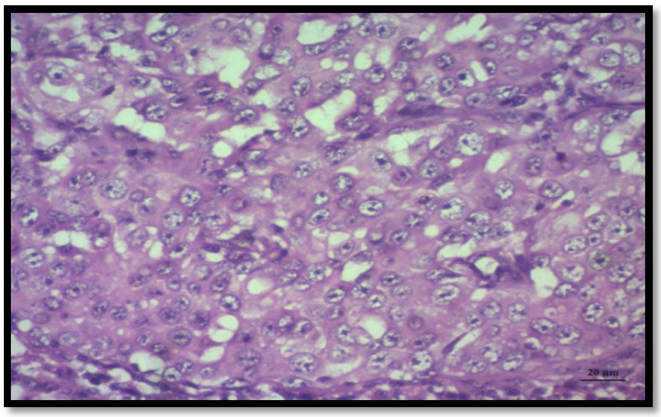

**Plate 16. Solid carcinoma showing densely packed neoplastic cells (H & E 40X)**

**Table 7. Summary of grading of mammary tumor (n=30)**

| Histological Grading | Histological type | No. of cases | Percentage |
|---|---|---|---|
| Stage I | Simple tubular adenocarcinoma | 5 | 16.66 |
| | Complex tubular adenocarcinoma | 1 | 3.33 |
| | Simple tubulopapillary adenocarcinoma | 2 | 6.66 |
| | Malignant mixed | 1 | 3.33 |
| | **Total** | **9** | **30** |
| Stage II | Simple tubular adenocarcinoma | 2 | 6.66 |
| | Complex tubular adenocarcinoma | 1 | 3.33 |
| | Simple tubulopapillary adenocarcinoma | 1 | 3.33 |
| | Complex tubulopapillary adenocarcinoma | 2 | 6.66 |
| | Squamous cell carcinoma | 5 | 16.66 |
| | Fibrosarcoma | 2 | 6.66 |
| | Malignant mixed | 1 | 3.33 |
| | **Total** | **14** | **46.66** |
| Stage III | Simple solid carcinoma | 1 | 3.33 |
| | Fibrosarcoma | 1 | 3.33 |
| | Chondrosarcoma | 2 | 6.66 |
| | Malignant mixed | 3 | 10 |
| | **Total** | **7** | **23.33** |

Regarding grades, out of 30 malignant mammary tumors 30% (n=9) tumors were of grade I, 46.66% (n=14) were grade II and 23.33% (n=7) were grade III. Previous workers have reported variable incidences of tumor grade (Martin de las Mulas *et al*. 2005, Rezaie *et al*. 2009 and Sassi *et. al*. 2010).

Prognosis is poor in tumors of higher grade (Elston and Ellis, 1991). Pin pointing prognostic factor has always been a challenge for oncologist. Identifying tumor type and histological grading is excellent prognostic tool (Pena *et al*.,2003, Tavasoly *et al*.,2013). Tumor grades determined the survival of dogs. Grade III carcinoma had worst survival whereas grade II and III cases had poor prognosis and dogs with higher grade were at increased risk of mortality (Karayannopoulou *et al*.,2005).

**4.3 Migration, proliferation kinetics and population doubling time**

From 8 cases of CMT the primary cell culture was prepapred and propagated for 5 passages. Attempt was made to determine migration, proliferation kinetics and population doubling time of canine mammary tumor cells and data with mean value is presented in Table 8. and Fig. 1,2,3.

**Table 8. Values of migration, proliferation kinetics and population doubling time of canine mammary tumor cells (n=8).**

| Sample No. | Migration (% wound closure) | Proliferation (OD at 570nm) | Population Doubling Time (hrs) |
|---|---|---|---|
| 2T | 44.09 | 0.45 | 14.29 |
| 3T | 84.59 | 0.42 | 13.65 |
| 4T | 49.75 | 0.22 | 14.61 |
| 5T | 64.23 | 0.49 | 14.45 |
| 6T | 69.2 | 0.25 | 14.11 |
| 7T | 89.10 | 1.09 | 12.34 |
| 8T | 91.5 | 0.97 | 12.44 |
| 9T | 53.75 | 0.66 | 13.49 |

Population doubling (PD) time negatively correlated with the proliferation kinetics of adherent cancer cells ($r = -0.9245$, $p=0.001$).Thus lower PD time will have increased proliferation of cancer cells and vice versa. Proliferation is the major force for cancer progression. Increased proliferation is related to poor

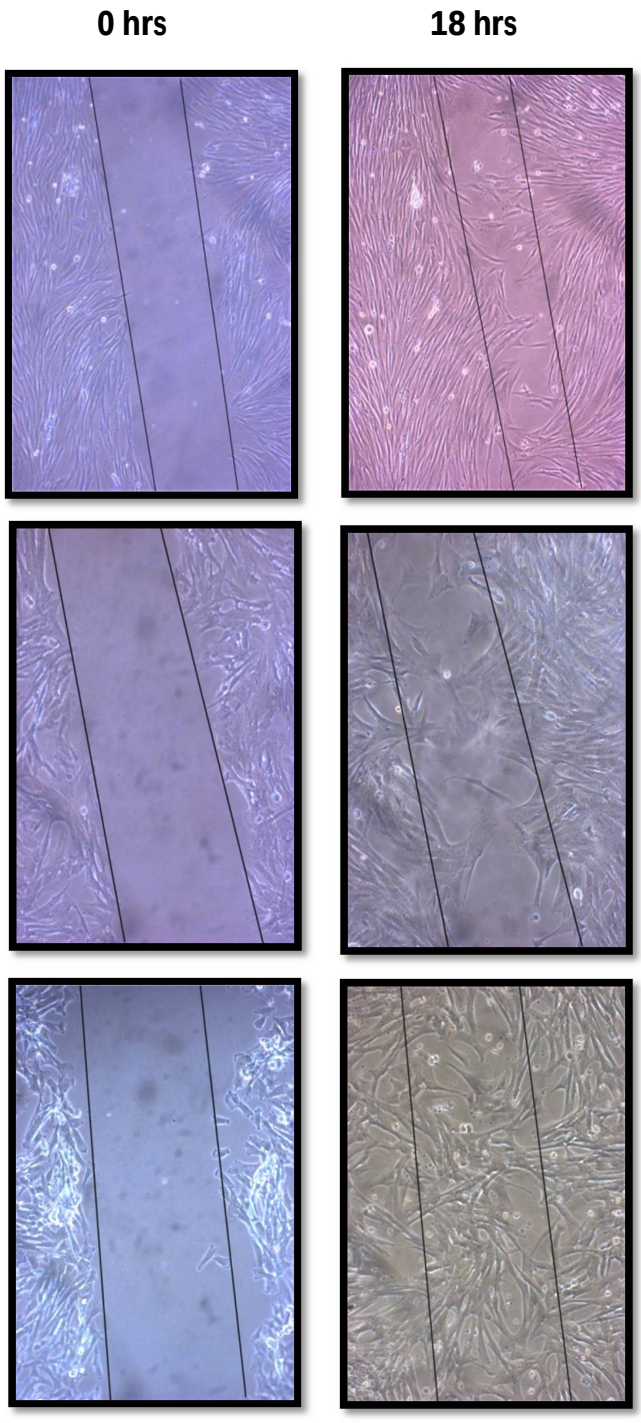


**Plate 17. Wound healing assay (0hrs and 18hrs observation at 10x)**

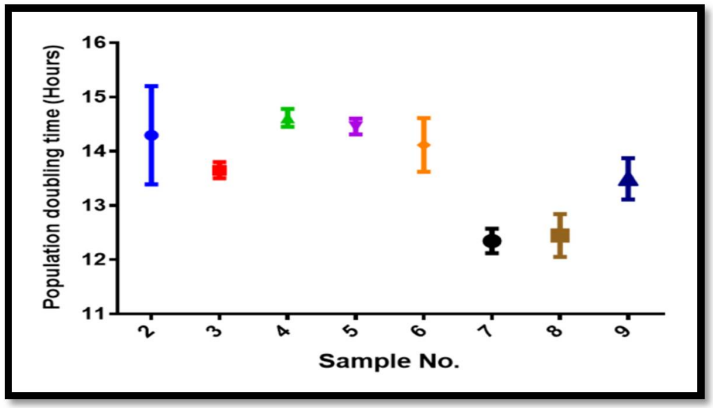


**Fig 1. Population doubling time of mammary tumor cells(error bar depicts Standard Error Mean{SEM})**

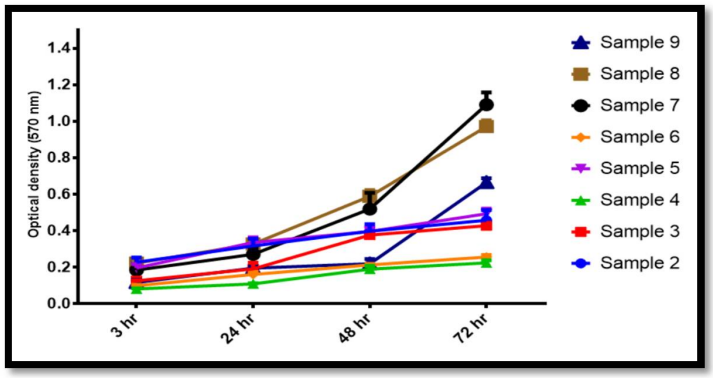


**Fig 2. Proliferation kinetics of mammary tumor cells (error bar depicts SEM)**

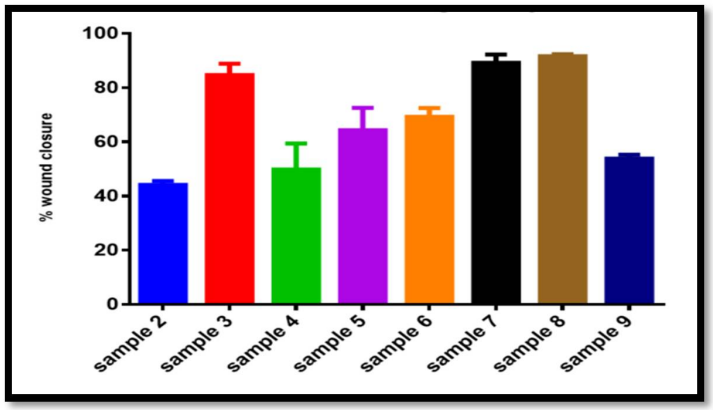


**Fig 3. Wound healing assay of mammary tumor cells (error bar depicts SEM)**

prognosis (Dai *et al*., 2005). Oct4, Nanog, and myc is responsible for reprogramming of differentiated cells at epigenetic level which results into long lasting proliferation (Vicente-Dueñas *et al*., 2013). If cancer stem cells play role in proliferation then targeting them might increase population doubling time which would provide better prognosis and increased survivability. Progression in cell cycle depends upon balance between proliferative and antiproliferative signals. Cell cycle arrest takes place in G1 phase. When arrest is ignored by the cell, resultant increase in replication occurs which results into induced mutation and telomere degeneration (Feitelson *et al*., 2015). Tumor proliferation is regulated by several molecular pathways (β catenin signalling, Notch signalling, insulin-like growth factor signalling, and NF-B signalling). Previous works have reported their regulatory role in proliferation (Fietelson *et al*., 2015). Various tumor proliferation rate among the mammary tumor cells in current study might owe to these signalling pathways. Uncontrolled proliferation is due to imbalance between cell proliferation and apoptosis (Lopez *et al*., 2015).

Migration of cancer cells was assessed by wound healing assay (Plate 17). Our study positively correlated migration with Snail expression in adherent cancer cells ($r = 0.5035$, $p = 0.0468$). For any cancer cell to invade the surrounding, it first needs to detach from tumor mass and then migrate. Cell migration by epithelial to mesenchymal transition (EMT) is due to downregulation of e-cadherin as a result of activation of transcription factors such as Snail and Slug. Such cells loose epithelial polarity and acquire fibroblast like shape. Mesenchymal phenotype leads to migration of cancer cell (Krakhmal *et al*.,2015). *In vitro* experiments have demonstrated that fibroblasts induce cell migration (Gaggioli *et al*., 2007). With wound healing assay determining long term migration (>24hrs) was not possible in current study because it cannot distinguish cell proliferation after longer periods (Kramer *et al*., 2013). Table 9 depicts the aggressiveness of tumor with increasing tumor grade leading to increased migration and proliferation of cancer cells.

**Table 9. Tumor grade versus migration, proliferation kinetics and population doubling time of canine mammary tumor cells**

| Tumor Grade | Migration (% wound closure) | Proliferation (OD at 570nm) | Population Doubling Time (hrs) |
|---|---|---|---|
| Grade I | 58.045 | 0.43 | 14.01 |
| Grade II | 66.715 | 0.37 | 14.28 |
| Grade II | 89.1 | 1.09 | 12.34 |

In the present study migration, cell proliferation and population doubling time varied in every canine mammary tumor **(**Fig.1, 2, 3). This suggests that every mammary tumor cells behave differently in spite of their similar classification and cell origin.

**4.4 Characterization of cancer stem cells in CMT:**

During the course of study samples evaluated included 8 CMT tissues, for which 8 adherent cancer cells were prepared and from them mammospheres were isolated from 4 cases (Plate 20-22). Time taken to attain confluency varied among the cultures from 5-10 days. Adhernet cells established from mammary tumor tissues revealed combination of fibroblast like and epithelial like morphology (Plate 18) or only fibroblast like cell population (Plate 19). As per Cancer stem cell hypothesis, tumor contains subset of cancer cells having properties of stem cells like self renewal and pluripotency. There is hierarchical organization with a rare population of cells that enable tumor formation and progression. Tumorogenic mammary cancer cells manifest properties of CSC (AlHajj and Clarke, 2004). To characterize primary adherent cells and tumorsphere as a primitive sub-population, the expression of embryonic stem cells markers Oct4, Sox2, and Nanog was analysed. Genes Oct4 and Sox2 were significantly expressed at higher level in mammospheres compared to parental adherent cells (Fig.7). So the cells that isolated from naturally occurring CMT contain subpopulation of undifferentiated cells that can flourish in absence of attachment and show the expression of embryonic stem cell markers. Differentiated cells cannot survive in non adherent conditions. Observations of

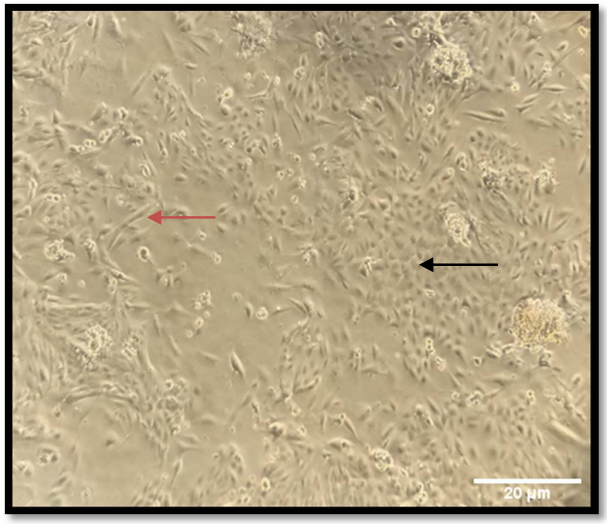


**Plate 18. Cells isolated from mammary tumor showing mix population of epithelial like (black arrow) and fibroblast like (red arrow) cells (10x)**

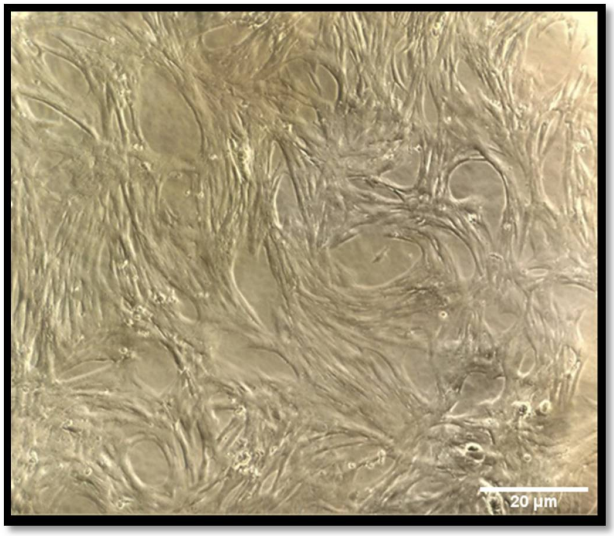


**Plate 19. Cells isolated from mammary tumor showing confluent growth of fibroblast like cells (10x)**

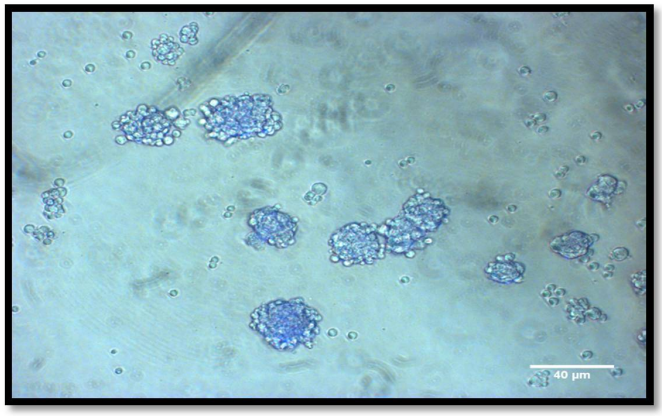


**Plate 20. Mammospheres isolated from adherent mammary tumor cells (10X)**

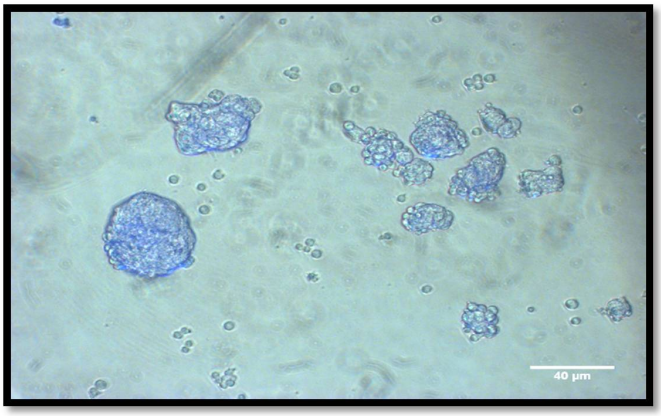


**Plate 21. Mammospheres isolated from adherent mammary tumor cells (10X)**

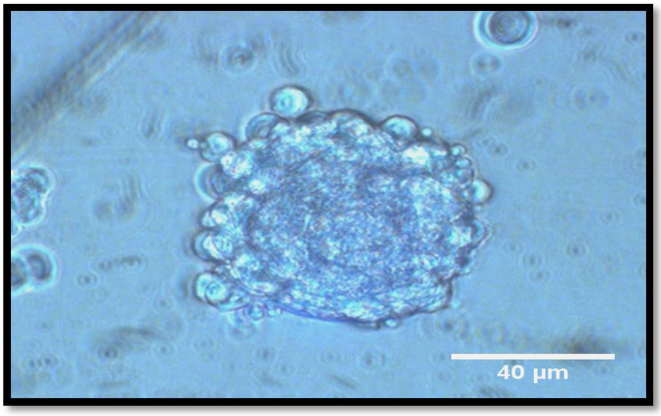


**Plate 22. Mammospheres isolated from adherent mammary tumor cells (20X)**

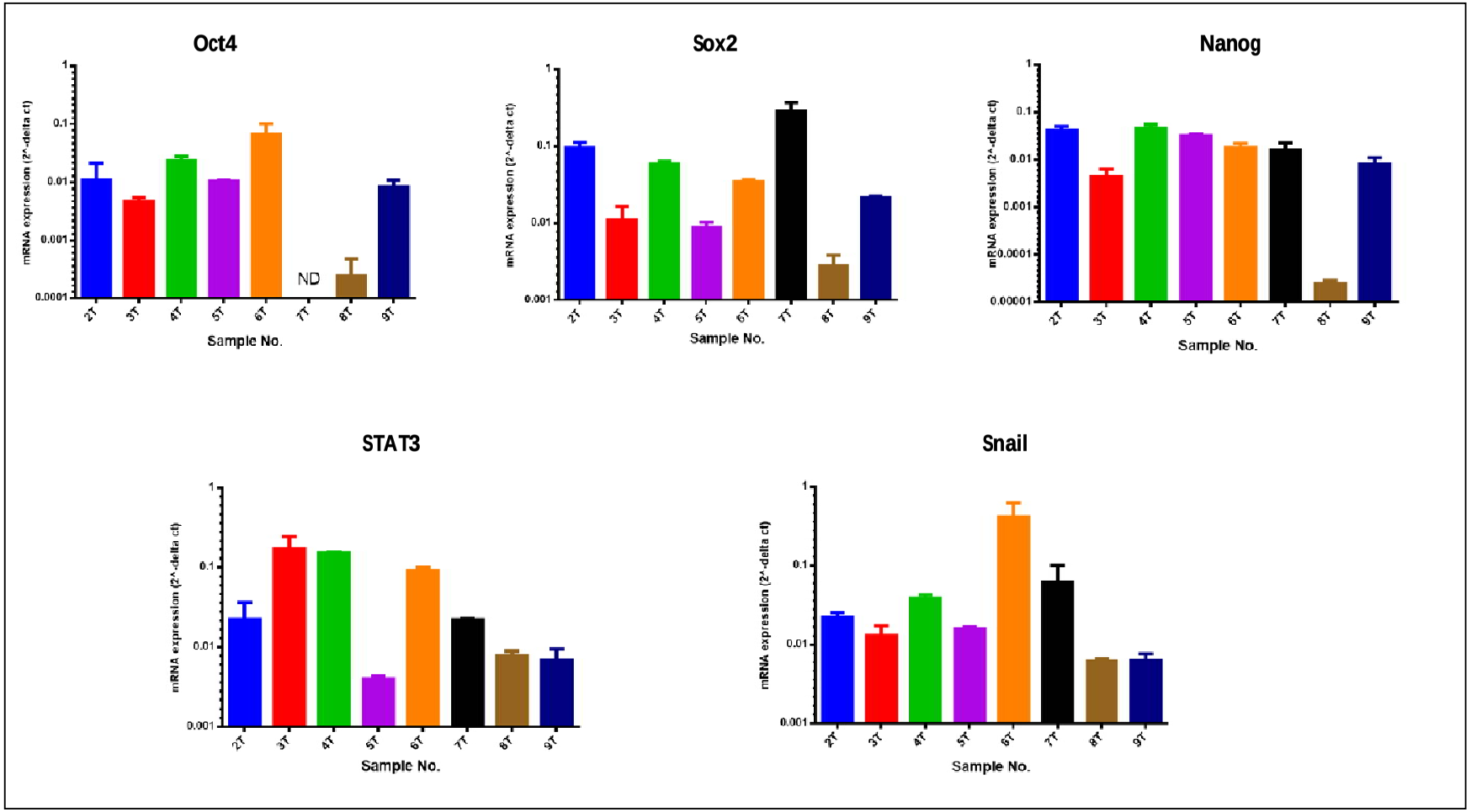


**Fig 4. mRNA expression in tumor tissues (error bar depicts SEM)**

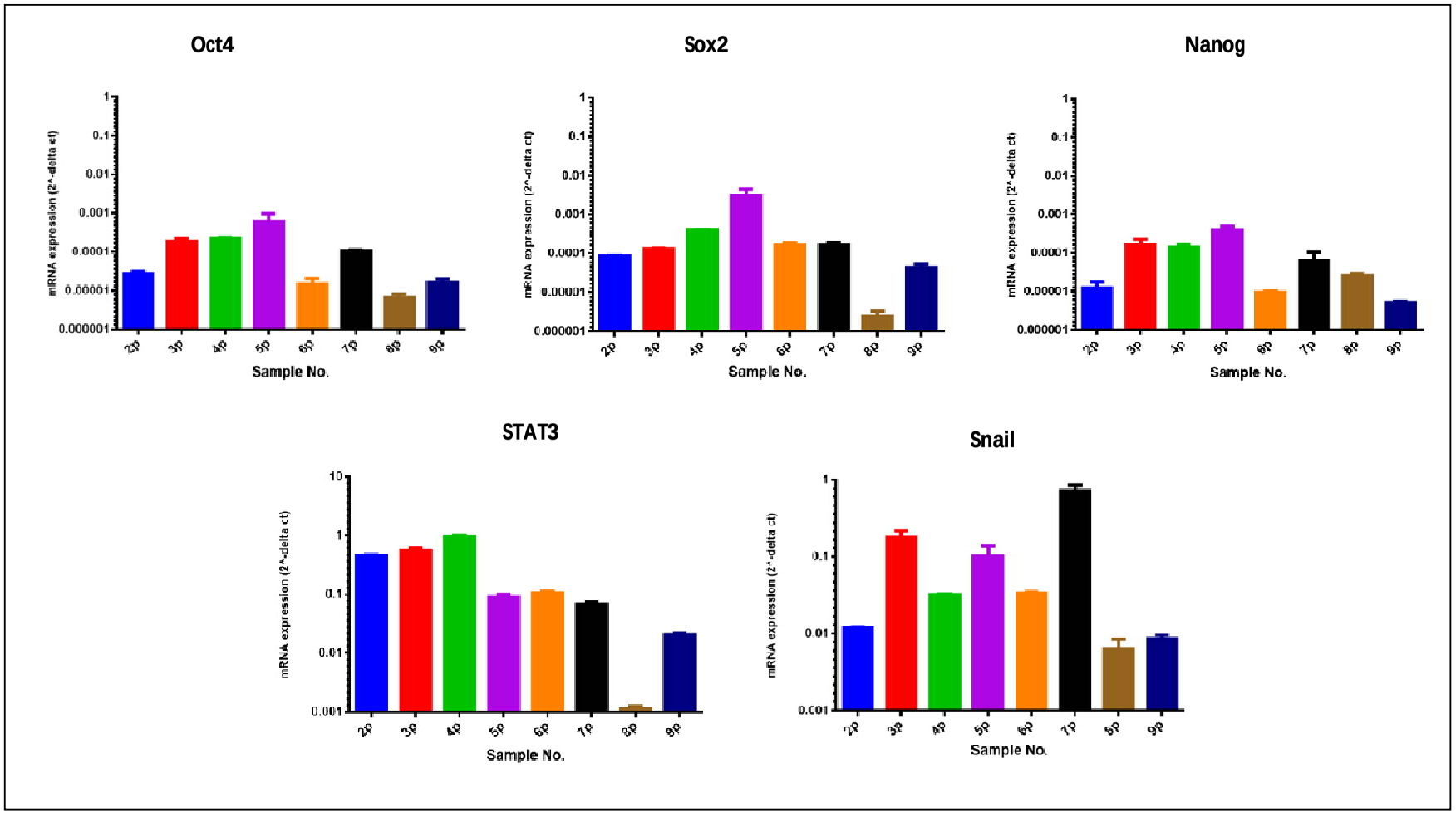


**Fig 5. mRNA expression in adherent cancer cells (error bar depicts SEM)**

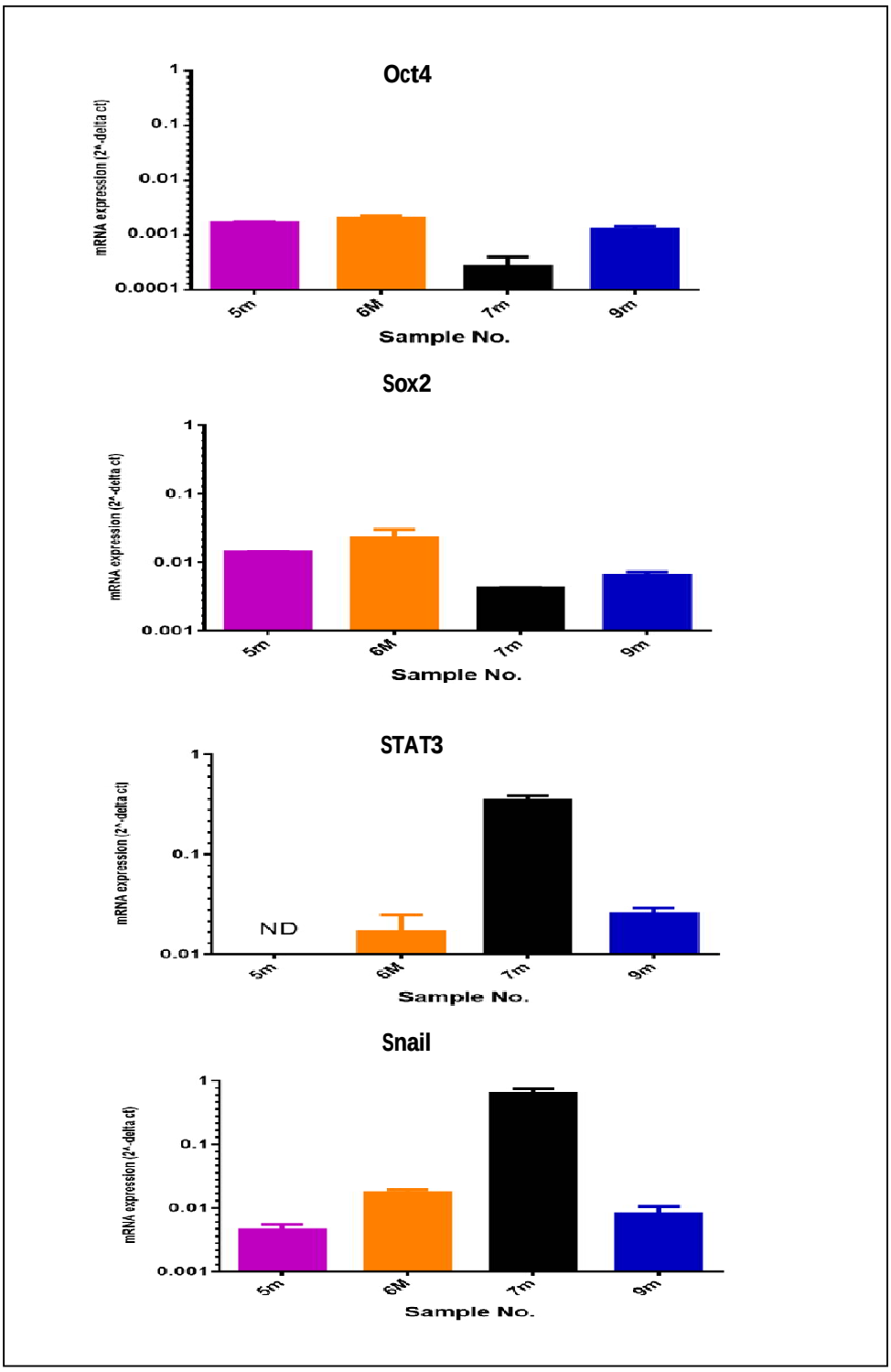


**Fig 6. mRNA expression in mammosphere (error bar depicts SEM)**

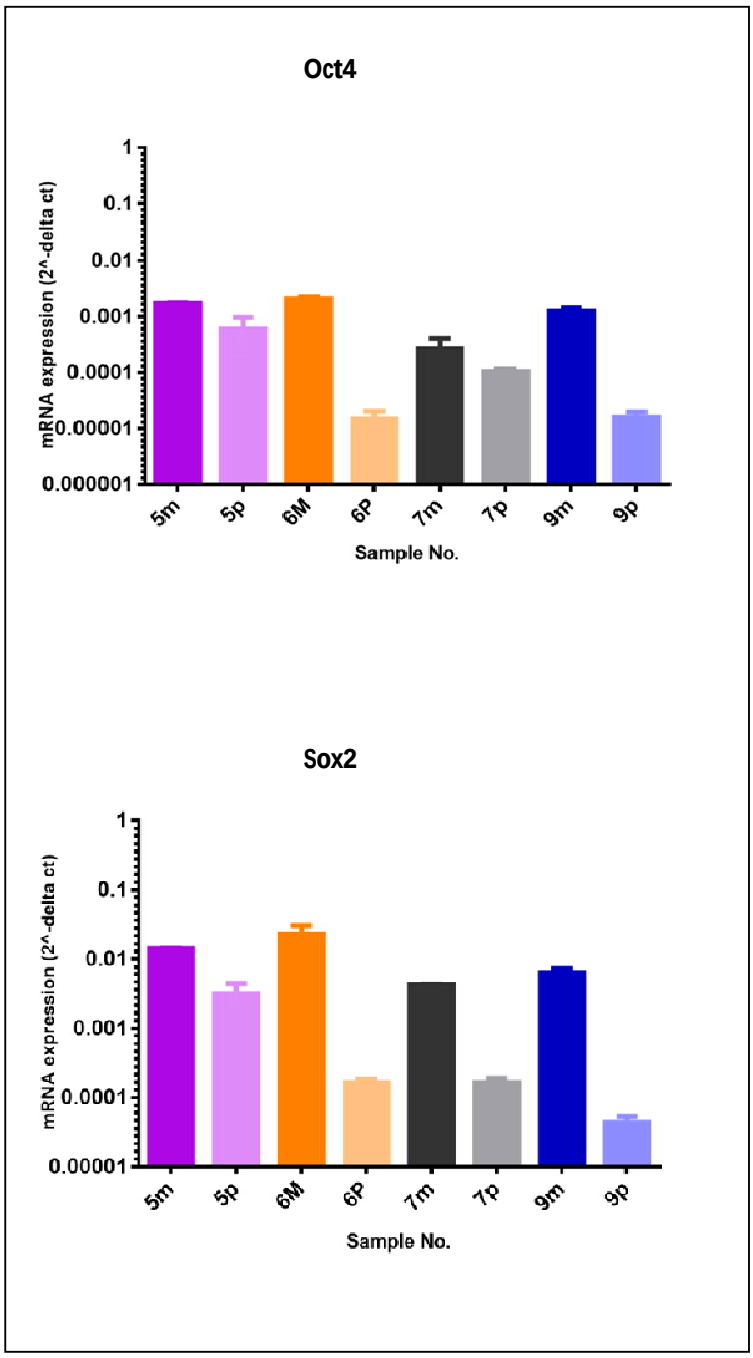


**Fig 7. mRNA expression of mammosphere (m) versus adherent cancer cells (p) (error bar depicts SEM)**

present study were in accordance with Pang *et al*., (2011) and Klevebring *et al*., (2014).

All the tumor tissue and adherent cancer cells expressed evident Oct4, Sox2 and Nanog at various expression levels (Fig.4 and Fig.5). Oct4, Sox2 and Nanog are pluripotency associated markers which play vital role in progression of breast cancer (Ling *et al*., 2012). A previous study has demonstrated the expression of Oct4 and Sox2 in adherent cells as well as in sphere cells in canine mammary gland adenocarcinoma cell lines (Michishita *et al*., 2011). Wang et al., ( 2014) linked Oct-4 and Nanog co-expression with EMT, lymph node metastasis, tumor size, histological grade and poor prognosis. Role of Sox2 has also been reported for increasing cell proliferation in breast cancer and drug resistance (Kaufhold *et al*., 2016).

In present study tumor tissue, adherent cancer cells and mammosphere showed relative expression of Signal transducer and activator of transcription 3 (STAT3) (Fig.4 - 6). Breast cancer cell growth and survival is regulated by STAT3 activation (Burke *et al*., 2001). STAT3 is frequently activated during tumorogenesis and responsible for modulating cell growth, differentiation, and apoptosis (Sherry *et al*., 2009). STAT3 is associated with tumorogenesis, drug resistant and expression of Oct4 and Sox2 (Chung *et al*., 2014).

**4.5 Cancer stem cells markers versus metastasis associated marker**

Present study revealed expression of Snail in tumor tissue, adherent cancer cells and mammosphere (Fig.4 - 6). Attempt was made to correlate the expressions of Oct4, Sox2, Nanog and STAT3 with the Snail (Table 10). We found that only Oct4 expression from tissue was positively and significantly correlated with Snail expression ($r = 0.9611$, $p = 0.0001$). While other markers didn't show any significant correlation. Previous studies have mentioned that expression of Snail is closely related to the metastasis because of its regulatory role in EMT and downregulation of e-cadherin, as it plays vital role in metastatic cascade (Zeisberg *et al*.,2009, Wang *et al*.,2013,). Snail induces the expression of Oct4 and confers cancer stem cell like features (Wang *et al*., 2013). Ability of Snail to evade apoptosis, repress e-cadherin and endow cells with migratory properties makes it good marker determining malignancy (Vega *et al*., 2004, Gimeno *et al*., 2005, Come *et al*.,2006).

Mladinich *et al*., (2016) reviewed a previous report which suggests variation in expression of cancer stem cells markers and Snail. They opinioned that Oct4 upregulates Snail and facilitates EMT. But also narrated the contrary report that Oct4 and Sox2 were down regulating Snail thus suppressing EMT. In present study we had significant positive Oct4 correlation with Snail thus there is possibility that Oct4 expression in mammary tumors of present study might be responsible for upregulation of Snail. Although in tumor tissue Sox2 was negatively correlated with Snail (r =0.028) it didn't reach statistical significance. Variation among correlation as mentioned in above reports and diverse correlation of different cancer stem cell markers with Snail (Table 10) in mammary tumors of present study suggests the complexity among their dependence on each other for subsequent expression and further studies are needed to establish a concrete relation among cancer stem cells and Snail in canine mammary tumor.

**Table 10. Summary of correlation of Snail with Cancer stem cell markers**

| **Gene** | **Mammary tumor tissue (Snail)** | | **Mammary cancer cells (Snail)** | |
|---|---|---|---|---|
| | r | Significance | r | Significance |
| Oct4 | 0.9611 | P < 0.0001 | 0.07114 | NS |
| Sox2 | -0.02852 | NS | -0.02070 | NS |
| Nanog | -0.06403 | NS | 0.02774 | NS |
| STAT3 | 0.1867 | NS | -0.1797 | NS |

**4.6 Cancer stem cells versus tumor grade.**

In present study mammospheres were successfully isolated from all types of tumor grade. Thus cancer stem cells are expressed in mammary tumor irrespective of tumor grade**.** Expression of Oct4, Sox2 and Nanog in tumor tissue was increased in high grade (III) as compared to low grade tumor(I and II) (Fig 8). Grade I was well differentiated, grade II was moderately differentiated, grade III was poorly differentiated during histopathological observation. Various researchers have reported different finding with some positively correlating CSC expression with tumor grade and some didn't find any significant correlation.

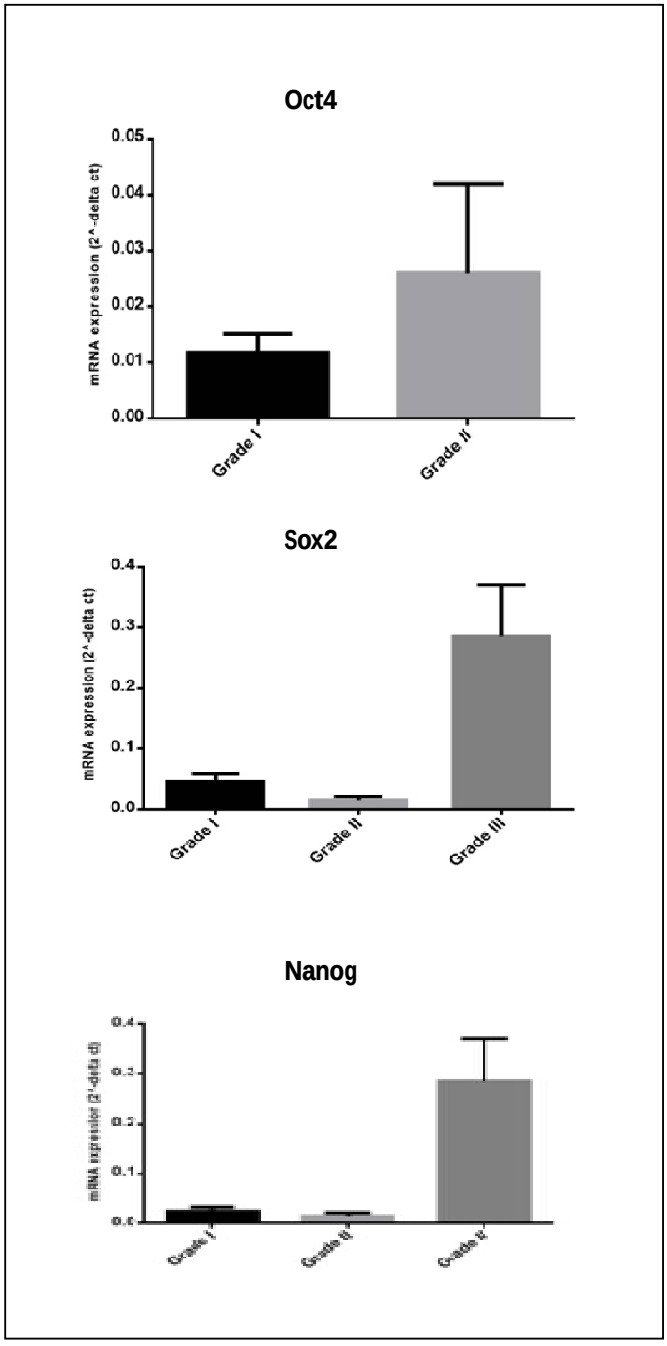


**Fig 8. Cancer stem cell marker expression (tumor tissue) versus Tumor Grade. (error bar depicts SEM)**

Elevated Sox2 is expressed in early breast carcinoma and results into increased metastasis but Sox2 expression didn't correlate with tumor grading (Lengerke *et al*.,2011). Increased expression of Oct4 and Sox2 were affiliated to high histologic grade and Nanog expression was associated with low histologic grade breast cancer (Gwak *et al*., 2017). In the present study increased expression of Oct4, Sox2 and Nanog are evident in high grade mammary tumors. Histologically poorly differentiated tumors are molecularly similar to embryonic stem cells hence such tumors cells are characteristically related to undifferentiated stem cells of tumor, embryonic stem like signature is seen in poorly differentiated tumors (Ben-porath *et al*.,2008). It might be the reason for increased expression of pluripotency associated markers (Oct4, Sox2, Nanog) in high grade tumor tissue in current study.

After analysis of all the above reports and finding of current study inference can be drawn as expression of cancer stem cell markers (associated with pluripotency) varies among different tumor grade. As per present study "Consistent expression of CSC marker (pluripotency marker) within different tumor grade is not the case yet and further studies are needed". Our statement is supported by successful isolation of mammospheres (characterized as CSC in the present study) from high and low grade canine mammary tumors.

## SUMMARY AND CONCLUSION

In unspayed bitches mammary tumors are frequently noted. Among the domestic species canids are most frequently affected. Prevalence of mammary tumors in canine is 3 times more than the woman. By far metastasis is responsible for cancer related deaths. Mammary tumors consist of subpopulation of cells called as cancer stem cells (CSC) which possess ability of self renewal and originates heterogeneous lineages of cancer cells. Current therapies have targeted proliferating cells and lead to shrinkage of tumor size but have failed to prevent recurrence. Recurrence might owe to subpopulation of cancer stem cells. Present study was designed to characterize cancer stem cells population in canine mammary tumor in addition to its correlation with metastasis associated marker and histologic tumor grade.

Haematological and serum biochemical profiling of bitches affected with mammary tumors didn't show any deviation from normal reference ranges. Grossly, mammary tumors of various size showed nodular, pedunculated and bizarre structure along with ulceration, necrosis and cyst filled pockets. Tumor growths were classified histologically as per WHO recommendation. Various tumors were diagnosed as tubular adenocarcinoma, papillary adenocarcinoma, squamous cell adenocarcinoma, solid adenocarcinoma, fibrosarcoma, chondrosarcoma and malignant mixed. Overall epithelial origin tumors were highest as compared to mesenchymal tumors. Thus suggesting CMT of epithelial origin are more common than mesenchymal origin. All the tumors studied were malignant. Histologically tumors were graded by Elston-Ellis system. In present study grade II tumors were frequently observed followed by grade I and grade III.

Aggressive cancer cells behaviour with increasing tumor grade was observed *in vitro* in terms of increased migration and proliferation of cancer cells along with decreased population doubling time in high grade tumors. Decreased population doubling time leads to increased proliferation activity of cancer cells. For any cancer cell to invade the surrounding, it first needs to detach from tumor mass and then migrate. In present study Snail which is an e-cadherin repressor was positively correlated with wound healing assay, which points towards role of Snail in increased migratory activity of cancer cells.

During course of study naturally occurring canine mammary tumors were subjected to primary cell culture. Cells isolated from 3rd to 5th passages revealed population ranging from mixture of fibroblast like cells and epithelial like cells to only fibroblast like cells. From them mammospheres were isolated in serum free and non adherent condition. Mammospheres of 8-10 day old were used for further studies. Characterization of mammary tumor tissue, adherent cells and mammosphere for cancer stem cells was achieved in this study by determining expression of Oct4, Sox2, Nanog and STAT3 at mRNA level.

Metastasis associated marker (Snail) was positively and significantly correlated with the Oct4 expression in tumor tissue. Upon investigation of expression of Oct4, Sox2 and Nanog in tumor tissue revealed increased in higher grade (III) as compared to low grade tumor (I and II). In present study cancer stem cells were isolated from all three grades of tumor. Present study reports that, “cancer stem cells are expressed in canine mammary tumor irrespective of tumor grade”**.**

From present study following conclusions are drawn:

1. Canine mammary tumor contains subpopulation of cells known as cancer stem cells.
2. CSC expressed similar embryonic stem cells markers to human CSC as reported in other studies. Dog can serve as model for studying mammary CSCs.
3. Cancer stem cells can serve as biomarker for mammary tumor detection and holds promising future in tumor prognostication.
4. Snail, a transcription factor, results into increased migration potential of cancer cells.
5. Every mammary tumor cells behave differently in spite of their similar classification and cell origin.
6. Expression of Oct4 in canine mammary tumor tissue is positively related to expression of Snail. Though further studies are required to establish concrete link between them.

7. Cancer stem cells are expressed in canine mammary tumor irrespective of tumor grade. Their expression is elevated in higher grade than lower grade. However increased sample size is required to have the definite conclusion.
8. Understanding cancer stem cell behaviour is need of an hour, in order to develop therapeutics to target CSCs.

## BIBLIOGRAPHY

Agliano, A., A. Calvo, C. Box (2017) The challenge of targeting cancer stem cells to halt metastasis. In *Seminars in Cancer Biology*. Academic Press.

Alenza, D. P.,G. R. Rutteman, L. Peña, A. C. Beynen, P. Cuesta (1998) Relation between habitual diet and canine mammary tumors in a case-control study. *Journal of veterinary internal medicine*, *12*(3):132-139.

Al-Hajj, M., and M. F. Clarke (2004) Self-renewal and solid tumor stem cells. *Oncogene*, *23*(43):7274.

Barhanpurkar-Naik, A., S. Mhaske, S. Pote, K. Singh, and M. Wani (2017). Interleukin-3 enhances the migration of human mesenchymal stem cells by regulating expression of CXCR4. *Stem cell research and therapy*, *8*(1), 168.

Benjamin S, Lee A, Saunders W (1999). "Classification and behavior of canine mammary epithelial neoplasms based on life-span observations in beagles". *Vet Pathol*. 36 (5): 423–36.

Ben-Porath, I., M. W. Thomson, V. J. Carey, R. Ge, G. W. Bell, A. Regev and R. A. Weinberg (2008) An embryonic stem cell–like gene expression signature in poorly differentiated aggressive human tumors. *Nature genetics*, *40*(5), 499.

Blacking, T. M., H. Wilson, D. J. Argyle (2007) Is cancer a stem cell disease? Theory, evidence and implications. *Veterinary and comparative oncology*, *5*(2):76-89.

Brabletz, T., A. Jung, S. Spaderna, F. Hlubek, T. Kirchner (2005) Migrating cancer stem cells-an integrated concept of malignant tumour progression. *Nature Reviews Cancer*, *5*(9):744-749.

Burke, W. M., X. Jin, H. J. Lin, M. Huang, R. Liu, R. K. Reynolds, J. Lin, (2001) Inhibition of constitutively active Stat3 suppresses growth of human ovarian and breast cancer cells. *Oncogene*, *20*(55): 7925.

Cerovšek, M., T. Plavec, P. Zrimšek, M., ogačnik, and J. Zabavnik (2013) Clinicopathological survey of 56 canine malignant mammary tumours in Slovenia—Prognostic value of clinical stage and histological grade. *Slov Vet Res*, *50*: 93-102.

Chavan, C. A. (2012) *Immunohistochemical Characterization of Mammary Gland Tumors In Dog* (Doctoral dissertation, MAFSU).

Chung, S. S., N. Giehl, Y. Wu, J. V. Vadgama (2014) STAT3 activation in HER2-overexpressing breast cancer promotes epithelial-mesenchymal transition and cancer stem cell traits. *International journal of oncology*, *44*(2): 403-411.

Clarke, M. F. and A. T. Hass (2006). Cancer stem cells. *Reviews in Cell Biology and Molecular Medicine*.

Cocola, C., P. Anastasi, S. Astigiano, E. Piscitelli, P. Pelucchi, , L. Vilardo, O. Barbieri (2009) Isolation of Canine Mammary Cells With Stem Cell Properties and Tumour-Initiating Potential. *Reproduction in Domestic animals*, *44*(s2): 214-217.

Côme, C., F, Magnino, F. Bibeau, P. De Santa Barbara, K. F. Becker, C. Theillet, P. Savagner (2006) Snail and slug play distinct roles during breast carcinoma progression. *Clinical cancer research*, *12*(18): 5395-5402.

Dai, H., L. van't Veer, J. Lamb, Y. D. He, M. Mao, B. M. Fine and R. Stoughton (2005) A cell proliferation signature is a marker of extremely poor outcome in a subpopulation of breast cancer patients. *Cancer research*, *65*(10), 4059-4066.

Dhaygude, V. S. (2006) The study of canine mammary tumors with special reference to mutations in p53 tumor suppressor gene by PCR-SSCP. M.V.Sc Thesis, College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand.

Dominik Kuhlmann, J., L. Hein, I. Kurth, P. Wimberger, A. Dubrovska (2016) Targeting cancer stem cells: promises and challenges. *Anti-Cancer*

*Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents)*, *16*(1):38-58.

Duda, N. C., S. D. F. Valle, J. P. Matheus, N. C. Angeli, L.C. Vieira, L. O. Oliveira, F. H. González (2017) Paraneoplastic hematological, biochemical, and hemostatic abnormalities in female dogs with mammary neoplasms. *Pesquisa Veterinária Brasileira, 37*(5): 479-484.

Eccles, S. A., D. R. Welch (2007) Metastasis: recent discoveries and novel treatment strategies. Lancet 2007; 369:1742–57.

Elston, C. W. and I. O. Ellis (1991) Pathological prognostic factors in breast cancer. I. The value of histopathological grade in breast cancer: Experience from a large study with long term follow up. Histopathology. 19: 403-410

Fayolled, P., V. Broulet, J. P. Braun, A. G. Rico, J. P. Magnol, J. P. Thouvenot (1987) Routine hematology and clinical chemistry in female dogs with benign and malignant mammary tumors. *Veterinary clinical pathology*, *16*(3):73-75.

Feitelson, M. A., A. Arzumanyan, R. J. Kulathinal, S. W. Blain, R. F. Holcombe, J. Mahajna, and D. Sharma (2015) Sustained proliferation in cancer: Mechanisms and novel therapeutic targets. In *Seminars in cancer biology*(Vol. 35, pp. S25-S54). Academic Press.

Fidler, I. J., and R. S. Brodey, R. S. (1967). The biological behavior of canine mammary neoplasms. *Journal of the American Veterinary Medical Association*, *151*(10) : 1311-1318.

Gabil Z., L. Beddar, Z. Djerrou and E. Gomez-Reyes (2017) Prevalence and Histopathologic Analyses of Mammary Tumors in Female Dogs in the Northeast of Algeria. Online Journal of Biological Sciences. 17. 166-177. 10.3844/ojbsci.2017.166.177.

Gaggioli, C., S. Hooper, C. Hidalgo-Carcedo, R. Grosse, J. F. Marshall, K. Harrington, and E. Sahai (2007) Fibroblast-led collective invasion of

carcinoma cells with differing roles for RhoGTPases in leading and following cells. *Nature cell biology*, *9*(12), 1392.

Gil, J, A. Stembalska, K. A. Pesz, M. M. Sąsiadek (2008) “Cancer stem cells: the theory and perspectives in cancer therapy”. J Appl Genet 49:193–199.Gujarat

Gimeno, A., and M. A. Nieto (2005) The Snail genes as inducers of cell movement and survival: implications in development and cancer. *Development*, *132*(14):3151-3161.

Gupta, P. 2008 ‘Diagnosis of canine mammary neoplasms and their management with adjuvant chemotherapy using vincristicine, methotrexate and cyclophosphamide’. M.V.Sc. Thesis, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana.

Gwak, J. M., M. Kim, H. J. Kim, M. H. Jang, and S. Y. Park (2017) Expression of embryonal stem cell transcription factors in breast cancer: Oct4 as an indicator for poor clinical outcome and tamoxifen resistance. *Oncotarget*, *8*(22), 36305.

Hampe, J. F., and W. Misdorp (1974) Tumours and dysplasias of the mammary gland. *Bulletin of the World Health Organization*, *50*(1-2), 111.

Kang, Y., and J. Massagué, J. (2004) Epithelial-mesenchymal transitions: twist in development and metastasis. *Cell*, *118*(3): 277-279.

Karayannopoulou, M., E. Kaldrymidou, T. C. Constantinidis, A. Dessiris (2005) Histological grading and prognosis in dogs with mammary carcinomas: application of a human grading method. *Journal of comparative pathology*, *133*(4): 246-252.

Kaufhold, S., H. Garbán, and B. Bonavida (2016). Yin Yang 1 is associated with cancer stem cell transcription factors (SOX2, OCT4, BMI1) and clinical implication. *Journal of Experimental and Clinical Cancer Research*, *35*(1), 84.

Kim, R.J. and J. S. Nam (2011) OCT4 expression enhances features of cancer stem cells in a mouse model of breast cancer. *Laboratory animal research*, *27*(2): pp.147-152.

Kishor, T. K., S. Rao, M. L. Satyanarayana, H. D. Narayanaswamy, S. byregowda, B. N. Nagaraja, N. Kavya (2016) Classification and staging of canine mammary gland tumours. *Journal of Cell and Tissue Research Vol*, *16*(3): 5787-5792.

Klevebring, D., G. Rosin, R. Ma, J. Lindberg, K. Czene, J. Kere, and J. Hartman (2014) Sequencing of breast cancer stem cell populations indicates a dynamic conversion between differentiation states in vivo. *Breast cancer research*, *16*(4), R72.

Klopfleisch R, Klose P, Weise C, Bondzio A, Multhaup G, Einspanier R, Gruber AD (2010). "Proteome of metastatic canine mammary carcinomas: similarities to and differences from human breast cancer". *J Proteome Res*. 9 (12): 6380–91.

Komazawa, S., H. Sakai, Y. Itoh, M. Kawabe, M. Murakami, T. Mori, K. Maruo (2016) Canine tumor development and crude incidence of tumors by breed based on domestic dogs in Gifu prefecture. *Journal of Veterinary Medical Science*, *78*(8): 1269-1275.

Krakhmal, N. V., Zavyalova, M. V., Denisov, E. V., Vtorushin, S. V., and Perelmuter, V. M. (2015) Cancer invasion: patterns and mechanisms. *Acta Naturae)*, *7*(2 (25)).

Kramer, N., A. Walzl, C. Unger,M. Rosner, G. Krupitza, M. Hengstschläger, and H. Dolznig, (2013) In vitro cell migration and invasion assays. *Mutation Research/Reviews in Mutation Research*, *752*(1), 10-24.

Krause, M., Dubrovska, A., Linge, A., and Baumann, M. (2017). Cancer stem cells: Radioresistance, prediction of radiotherapy outcome and specific targets for combined treatments. *Advanced drug delivery reviews*, *109*, 63-73..

Kurilj, A. G., M. Hohsteter, B. Artuković, K. Severin, I. C. Sostarié-Zuckermann and A. Beck (2011) Histopathological evaluation and immunohistoch

emical study of estrogen receptor α, HER-2 and Ki-67 in canine neoplastic mammary lesions. *Veterinarski arhiv*, *81*(6): 709-722.

Lallo, M. A., T. M. Ferrarias, A. Stravino, J. F. Rodriguez, R. L. Zucare (2016) Hematologic abnormalities in dogs bearing mammary tumors. *Revista Brasileira de Ciencia Veterinaria*, *23*(1-2): 3-8.

Lengerke, C., T. Fehm, R. Kurth, H. Neubauer, V. Scheble, F. Müller, and F. Fend (2011). Expression of the embryonic stem cell marker SOX2 in early-stage breast carcinoma. *BMC cancer*, *11*(1), 42.

Lin, Y., P. J. Buckhaults, J. R. Lee, H. Xiong, C. Farrell, R. H. Podolsky, W. S. Dynan (2009) Association of the actin-binding protein transgelin with lymph node metastasis in human colorectal cancer. *Neoplasia*, *11*(9): 864IN2-873IN5.

Ling, G. Q., D. B. Chen, B. Q. Wang, and L. S. Zhang (2012) Expression of the pluripotency markers Oct3/4, Nanog and Sox2 in human breast cancer cell lines. *Oncology letters*, *4*(6): 1264-1268.

Lombardo, Y., A. de Giorgio, C. R., Coombes, J. Stebbing, and .L. Castellano (2015). Mammosphere formation assay from human breast cancer tissues and cell lines. *Journal of visualized experiments: JoVE*, (97).

Lopez, J., and S. W. G. Tait (2015) Mitochondrial apoptosis: killing cancer using the enemy within. *British journal of cancer*, *112*(6), 957.

Losco, P. E. (1986) Local and peripheral eosinophilia in a dog with anaplastic mammary carcinoma. *Veterinary pathology*, *23*(4): 536-538.

Luna, L. G. (1968) Manual of histological staining methods of armed forces institute of pathology. 3rd Ed. McGraw Hill, New York.

Mani, S. A., W. Guo, M. J. Liao, E. N. Eaton, A. Ayyanan, A. Y. Zhou, L. L. Campbell (2008) The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell*, *133*(4): 704-715.

Martin de las Mulas, J., Y. Millan and R. Dios (2005) A prospective analysis of immunohistochemically determined estrogen receptor and progesterone

receptor expression and host and tumor factors as predictors of disease free period in mammary tumors of the dog. Vet. Pathol. 42: 200-212.

Martin, T. A. and W. G. Jiang (2014) Evaluation of the expression of stem cell markers in human breast cancer reveals a correlation with clinical progression and metastatic disease in ductal carcinoma. *Oncology reports*, *31*(1): 262-272.

Mehlen, P. and A. Puisieux (2006) Metastasis: a question of life or death. *Nature reviews cancer*, *6*(6): 449.

Michishita, M., R. Akiyoshi, H. Yoshimura, T. Katsumoto, H. Ichikawa, K. Ohkusu-Tsukada, T. Nakagawa, N. Sasaki, and K. Takahashi, (2011) Characterization of spheres derived from canine mammary gland adenocarcinoma cell lines. *Research in veterinary science*, *91*(2): pp.254-260.

Misdorp, W., and A. A. M. Hart (1976) Prognostic factors in canine mammary cancer. *Journal of the National Cancer Institute*, *56*(4): 779-786.

Misdorp, W., R. W. Else, E. Hellmen, and T. P. Lipscomb (1999) WHO International Histological Classification Tumors of Domestic Animals. *Histological Classification of Mammary Tumors of the Dog and the Cat. 2nd series*, *7*.

Mladinich, M., D. Ruan, and C. H. Chan (2016) Tackling cancer stem cells via inhibition of EMT transcription factors. *Stem cells international*, *2016*.

Mohapatra, A. K., D. Das, S. K. Panda, B. Jena, and J. Singh (2016) Spontaneous canine mammary neoplasia: a clinicopathological study. *Journal of Cell and Tissue Research*, *16*(2).

Moulton, J. E. (1999) Tumors in Domestic animals. University of California Press, Berkley.3: 518-543.

Moulton, J. E., D. O. N. Taylor, C. R. Dorn, and A. C. Andersen (1970) Canine mammary tumors. *Pathologia veterinaria*, *7*(4): 289-320.

Paget, S. (1889) The distribution of secondary growths in cancer of the breast. *The Lancet*, *133*(3421): 571-573.

Pang, L. Y., A. Cervantes-Arias, R. W. Else, and D. J. Argyle (2011) Canine mammary cancer stem cells are radio-and chemo-resistant and exhibit an epithelial-mesenchymal transition phenotype. *Cancers*, *3*(2): 1744-1762.

Peitzsch, C., A. Tyutyunnykova, K. Pantel, and A. Dubrovska (2017) Cancer stem cells: the root of tumor recurrence and metastasis. In *Seminars in Cancer Biology*. Academic Press.

Peña, L., M. D. Perez-Alenza, A. Rodriguez-Bertos, and A. Nieto (2003) Canine inflammatory mammary carcinoma: histopathology, immunohistochemistry and clinical implications of 21 cases. *Breast cancer research and treatment*, *78*(2): 141-148.

Peña, L., P. D. Andrés, M. Clemente, P. Cuesta, and M. D. Perez-Alenza (2013) Prognostic value of histological grading in noninflammatory canine mammary carcinomas in a prospective study with two-year follow-up: relationship with clinical and histological characteristics. *Veterinary Pathology*, *50*(1): 94-105.

Polton, G. (2009) Mammary tumours in dogs. *Irish Vet J*, *62*(1): 50-56.

Reddy, G. M., P. Kumar, R. Kumar, V. S. Pawaiya, and R. Ravindran (2009) Histopathological classification and incidence of canine mammary tumours. *Indian J. Vet. Pathol*, *33*(2): 152-155.

Rezaie, A., A. Tavasoli, A. Bahonar and M. Mehrazma (2009) Grading in canine mammary gland carcinoma. Journal of Biological Sciences. 9(4): 333-338.

Roth V. (2006) Doubling Time Computing, Available from: http://www.doubling-time.com/compute.php

Salas, Y., A. Márquez, D. Diaz, and L. Romero (2015) Epidemiological study of mammary tumors in female dogs diagnosed during the period 2002-2012: A growing animal health problem. *PloS one*, *10*(5): e0127381.

Sampieri, K., and R. Fodde (2012) Cancer stem cells and metastasis. In *Seminars in cancer biology* 22(3): 187-193. Academic Press.

Sassi, F., C. Benazzi, G. Castellani and G. Sarli (2010) Molecular based tumor subtypes of canine mammary carcinomas assessed by immunohistochemistry. BMC Veterinary Research. 6: 5-13.

Schmittgen, T. D., and K. J. Livak (2008) Analyzing real-time PCR data by the comparative C T method. *Nature protocols*, *3*(6): 1101.

Schneider, R., C. R. Dorn, and D. O. N. Taylor (1969) Factors influencing canine mammary cancer development and postsurgical survival. *Journal of the National Cancer Institute*, *43*(6): 1249-1261.

Sherry, M. M., A. Reeves, J. K. Wu, and B. H. Cochran (2009) STAT3 is required for proliferation and maintenance of multipotency in glioblastoma stem cells. *Stem cells*, *27*(10): 2383-2392.

Simeonov, R., and D. Stoikov (2006) Study on the correlation between the cytological and histological tests in the diagnostics of canine spontaneous mammary neop-lams. *Bulg J Vet Med*, *9*(3): 211-219.

Simões, B. M., M. Piva, O. Iriondo, V. Comaills, J. A. López-Ruiz, I. Zabalza and M. Vivanco (2011) Effects of estrogen on the proportion of stem cells in the breast. *Breast cancer research and treatment*, *129*(1): 23-35.

Sleeckx, N., H. De Rooster, E. J. B. Veldhuis Kroeze, C. Van Ginneken, and L. Van Brantegem (2011) Canine mammary tumours, an overview. *Reproduction in Domestic Animals*, *46*(6): 1112-1131.

Sontas, B. H., A. H. Ozyogurtcu, A. Gurel, and H. Ekici, (2009) Evaluation of clinical and pathological characteristics of 155 canines with mammary tumours: a retrospective study. *Archivos de Medicina Veterinaria*, *41*(1).

Tavasoly, A., H. Golshahi, A. Rezaie and M. Farhadi, (2013) Classification and grading of canine malignant mammary tumors. In *Veterinary research forum: an international quarterly journal,* 4(1): 25.

Tume, L., K. Paco, R. Ubidia-Incio, and J. Moya (2016) CD133 in breast cancer cells and in breast cancer stem cells as another target for immunotherapy. *Gaceta Mexicana de Oncología*, *15*(1): 22-30.

Vascellari, M., K. Capello, A. Carminato, C. Zanardello, E. Baioni, and F. Mutinelli, (2016) Incidence of mammary tumors in the canine population living in the Veneto region (Northeastern Italy): Risk factors and similarities to human breast cancer. *Preventive veterinary medicine*, *126*: 183-189.

Vazquez-Santillan, K., J. Melendez-Zajgla, L. E. Jimenez-Hernandez, J. Gaytan-Cervantes, L. Muñoz-Galindo, P. Piña-Sanchez, G. Martinez-Ruiz, J. Torres, P. Garcia-Lopez, C. Gonzalez-Torres and V. Ruiz (2016) NF-kappaB-inducing kinase regulates stem cell phenotype in breast cancer. *Scientific Reports*, *6*, p.37340.

Vega, S., A. V. Morales, O. H. Ocaña, F. Valdés, I. Abregat, and M. A. Nieto (2004) Snail blocks the cell cycle and confers resistance to cell death. *Genes and development*, *18*(10): 1131-1143.

Vicente-Dueñas, C., I. Romero-Camarero, C. Cobaleda, and I. Sánchez-García (2013) Function of oncogenes in cancer development: a changing paradigm. *The EMBO journal*, *32*(11): 1502-1513.

Wang, D., P. Lu, H. Zhang, M. Luo, X. Zhang, X. Wei, and C. Liu (2014) Oct-4 and Nanog promote the epithelial-mesenchymal transition of breast cancer stem cells and are associated with poor prognosis in breast cancer patients. *Oncotarget*, *5*(21): 10803.

Wang, Y., J. Shi, K. Chai, X. Ying, and B. P Zhou (2013) The role of Snail in EMT and tumorigenesis. *Current cancer drug targets*, *13*(9): 963-972.

Xu, B., C. Cheval, A. Laohavisit, B. Hocking, D. Chiasson, T. S. Olsson, and M. Gilliham, (2017) A calmodulin-like protein regulates plasmodesmal closure during bacterial immune responses. *New Phytologist*, *215*(1): 77-84.

Yang, J., and R. A. Weinberg, (2008) Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. *Developmental cell*, *14*(6): 818-829.

Yao, D., C. Dai, and S. Peng (2011) Mechanism of the mesenchymal–epithelial transition and its relationship with metastatic tumor formation. *Molecular cancer research*, *9*(12): 1608-1620.

Yu, Z., T. G. Pestell, M. P. Lisanti, and R. G. Pestell (2012) Cancer stem cells. *The international journal of biochemistry and cell biology*, *44*(12): 2144-2151.

Zeisberg, M., and E. G. Neilson (2009) Biomarkers for epithelial-mesenchymal transitions. *The Journal of clinical investigation*, *119*(6): 1429-1437.

## VITA

The author Dr. Gaonkar Pankaj Prakash was born on 12th September, 1993 in Mumbai of Maharashtra state. He has completed his Secondary School Certificate examination in the year 2009 from Raigad Military School, Raigad and Higher Secondary School Certificate examination in the year 2011 from A. G. High school and Junior College of Science, Dapoli. The author completed B.V.Sc. and A.H. degree course in the year 2016 in 1st division from KNP College of Veterinary Science, Shirwal. In the year 2016 he joined M.V.Sc. (Veterinary Pathology) degree course at Nagpur Veterinary College, Nagpur due to his keen interest in veterinary pathology.

During graduation the author was an active member of National Service Scheme and successfully completed N.C.C.

## THESIS ABSTRACT

a) Title of the thesis : "**CHARACTERIZATION OF CANCER STEM CELLS FROM CANINE MAMMARY GLAND TUMORS AND ITS CORRELATION WITH METASTATIC MARKERS.**"

b) Full name of student: **GAONKAR PANKAJ PRAKASH**

c) Name and address of advisor : **Dr. N. V. Kurkure**
Associate Professor and Head,
Department of Veterinary Pathology, N.V.C.,
Nagpur.

d) Degree to be awarded : Master of Veterinary Pathology

e) Year of award of degree : 2018

f) Major Subject : **Veterinary Pathology**

g) Total no. of pages in thesis : 38

h) Number of words in abstract : 209

i) Signature of student:

j) Signature, Name and Address of forwarding authority :

(**Dr. A. S. Bannalikar)**
Associate Dean,
Nagpur Veterinary College,
Nagpur.

## Abstract

Mammary tumor is most frequently reported neoplasm in bitches. Mammary tumours consist of subpopulation of cells called as cancer stem cells (CSC) which is responsible for tumor recurrence as current therapies have failed to clear them. Present study was undertaken to characterize CSC in canine mammary tumor and determine expression of metastasis associated marker.

Present study revealed that haematological and serum biochemical profiling of bitches affected with mammary tumours didn't show any deviation from normal reference ranges. Overall epithelial origin tumours were highest as compared to mesenchymal tumours. *In vitro* studies showed aggressive cancer cells behaviour with increasing tumor grade. Positive correlation between Snail expression and migration of cancer cell was achieved. Oct4 in canine mammary tumor tissue was positively related to expression of Snail. Results of present study show that cells isolated from primary mammary tumours have ability to form mammospheres. CSCs predominantly express pluripotency markers than the adherent cancer cells. Our findings indicate that CSCs are present in canine mammary tumor irrespective of tumor grade. Expression of Oct4, Sox2 and nanog in tumor tissue were increased in high grade (III) as compared to low grade tumor (I and II). Cancer stem cells can serve as biomarker for mammary tumor detection and holds promising future in tumor prognostication.

ic/k lkjk×k

| | | | |
|---|---|---|---|
| v- | ic/kkp f'k"kd | % | **d=ke/; LrukP;k ddjkxkP;k LVe i'khp fu:i.k vkf.k R;kp eVkLVhfll ekdj lkcr lgic/k** |
| c- | fo|kF;kp i.k uko | % | **Xkkodj idt idk'k** |
| d- | ekxn'kdkp uko o iRrk | % | Mk- ,u-0gh- djdj<br>lg;kxh ik/;kid o foHkkxie[k<br>fodrh 'kkL= foHkkx<br>ukxij i'ko|d egkfo|ky;]ukxij |
| M- | inku dj.;kr ;.kkjh inoh | % | Lukrdkrj inoh ¼e-0gh-,l-lh-½ |
| b- | inoh inku dj.;kp o"k | % | 2018 |
| Q- | e[; fo"k; | % | fodrh 'kkL= |
| x- | ic/kkrhy ,d.k i"B | % | 38 |
| g- | lkjk'kkrhy ,d.k 'kCn | % | 165 |
| b- | fo|kF;kph lgh | % | |
| t- | vxf"kr dj.kkÚ;k vf/kdkÚ;kph lgh] uko vkf.k iRrk | % | ¼Mk- ,-,l- cUUkkGhdj½<br>lg;kxh vf/k"Bkrk<br>ukxij i'ko|d egkfo|ky;<br>ukxij |

**lkjk'k**

Lrui'kh vcn lkeU;rk% d«;ke/; fnlrkr -Lrukpk ddjkxk e/; cgrd oGkk LVe i'kh vk<Grkr d=jkxkfo"k;d vcn e/; LVe

i'kh x.k/ke lknj dj.;klkBh vkf.k eVkLVfll lcf/kr ekdjph vfHk;Drh fu/kkfjr dj.;klkBh l/;kp vH;kl dy xy-

l/;kP;k vH;klkru vl fnlu vky dh Lrukpk djkx iklu jDrkp vkf.k lhjep x.k/ke lkEkkU; lnHk J.khiklu dk.krkgh cny n'kfoyyk ukgh- eludk;Eytu; xkBhP;k ryur ,fiFkyh;ytu; xkBh vf/kd gkR;k- l/;kP;k vH;klkr 100 VDd no'k;Dr LrukPkk ddjkx >kY;kp uknoy xy ok<R;k vcn xMlg ddjkx i'khp vkded >ky - ,l,u,vk; 1 vfHk;fDr vkf.k ddjkxkP;k i'kh LFkykrj.k njE;ku ldkjkRed lgdk; ikIr >ky- d«;kpk Lru vcn e/; vkDV 4 ldkjkRed ,l,u,vk; 1 vfHk;Drh lcf/kr gkr- l/;kP;k vH;klkP;k fu"d"kko:u vl fnlu vky-dh ikFkfed LRkukPkk ddjkx e/khy i'khe/; eekfLQvl r;kj dj.;kph {kerk vlr- vkEP;lk fu"d"kko:u vl lfpr gkr dh vcn xMpk fopkj u djrk Lru vcn LVe i'kh vfLrRokr vkgr- mPPk xM vcn e/; vkDV 4 lkDl 2 vkf.k uukx ddjkx fV';e/; ok< >kyh ddjkxkp LVe i'kh Lrukpk ddjkx 'kk/kklkBh ck;kekdj Eg.ku dke d: 'kdr vkf.k Hkfo";k r gk.kkj vk'olu nr-