Immunolocalisation of c-Myc an Oncoprotein of Canine Mammary Tumors

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ABSTRACT

The study was undertaken to detect the expression pattern of c-Myc, an important oncoprotein of canine mammary tumors by means of immunohistochemistry. A total 31 canine mammary tumors, 2 simple carcinomas, 14 complex carcinomas, 13 carcinosarcomas and 2 sarcomas were included in the study. Immunoreactivity of c-Myc was observed in 87% of cases. Benign lesions showed nuclear localization of *c-Myc* while it was found to be cytoplasmic in anaplastic malignant cancer cells. *c-Myc* expression was more prominent in benign and carcinoma cases while it was absent or almost undetectable in sarcomas. These findings suggest that c-Myc is highly expressed in canine mammary tumors and in carcinoma cases may indicate a poor prognosis. c-Myc appears to be involved in tumor progression and may serve as an important target for anti-cancer drugs.

Key Words: *c-Myc*, mammary tumor, carcinoma, carcinosarcoma, sarcoma, immunoreactivity, anti-cancer drug.

INTRODUCTION

MYC, the cellular homolog of *v-myc* is a well known proto-oncogene and the proteins it express c-Myc, L-myc and N-myc play different roles in cell cycle regulation (1). Among these different proteins the most important and extensively studied is c-Myc a basic helix-loop-helix leucine zipper transcription factor, which appears to be involved in embryogenesis, cell growth, cell differentiation, apoptosis and has transforming potential (2). All these activities are governed by myc–max heterodimer formation in cells and its interaction with DNA E box element.

Myc is an early response gene as its response is quickly attained following a variety of mitogenic stimuli (3). The *c-Myc* expression is undetectable or very low in the resting cell. Under the influence of growth factors or mitogenic stimuli there is rapid increase in the level of c-Myc which arouses the cell from resting phase to enter the G1 phase. This increased level lasts for a short period of time with a half life of 20-30 minutes. It is continuously produced during the cell cycle and then declines when cell the cycle is over and the growth factors are withdrawn (4). Normally the expression of *c-Myc* is low to absent and tightly regulated. The increase in the level of *c-Myc* induces apoptosis through p53-dependent and independent pathways which result in the release of cytochrome-c from mitochondria, the formation of the Apaf-1/caspase-9, the death effector complex and resultant cell death (5).

Various research studies have suggested that aberrant expression of altered or mutant c-Myc prevents the cell from exiting the cell cycle resulting in the accumulation of abnormal undifferentiated precursor cells which are transformed and which ultimately may lead to tumor progression (6). Thus c-Myc may be considered as a potential target for anti-cancer drugs to impede tumor growth.

The role of c-Myc has been studied in various tumors such as breast, cervical, head and neck, prostate, glioma and

skin cancer (7, 8, 9, 10). Many immunohistochemical studies have shown that about 50–100% of human breast cancer cases have increased levels of c-Myc proteins (11, 12, 13, 14, 15, 16). However there is a dearth of information available about the expression pattern of *c-Myc* in tumors of animals. This study was therefore carried out to study *c-Myc* expression in canine mammary tumors, a common tumor in dogs accounting for 25 and 50 percent of all tumors and second in incidence after skin tumors in dogs (17, 18, 19, 20). Furthermore, one third to half of surgically removed canine mammary tumors are malignant (18).

MATERIAL AND METHODS

The study was conducted on 31 cases of canine mammary gland tumors presented to the Department of Veterinary Pathology, College of Veterinary Science, GADVASU, Ludhiana, Punjab, India. After preparation of paraffin blocks and histological sections stained with Haematoxylin and Eosin (H&E), stained sections underwent initial screening. Lesions like alveolar hyperplasia, atypical hyperplasia, intraductal papillomas, ductal carcinoma *in situ* (DCIS), and lobular carcinoma *in situ* were considered as early lesions, whereas lesions such as invasive ductal carcinoma (IDC), invasive lobular carcinomas and epithelial–mesenchymal transitions (EMT) were considered as advanced lesions (21, 22, 23, 24, 25). EMT in canine mammary tumors were identified based on whether the epithelial structure was preserved or lost, polarity of epithelial cells, and on the basis of immunoreactivity of intermediate filaments like cytokeratin cocktail, vimentin and adhesion molecules like E-cadherin (BioGenex Laboratories Inc., San Ramon, California, USA). Epithelial cells undergoing EMT were positive for both cytokeratin and vimentin protein immunoreactivity, this indicating transition from epithelial to mesenchymal cells. Also cells undergoing EMT showed loss of immunoreactivity to intermediate protein E-cadherin which confirmed EMT (26, 27).

After screening, diagnosis and classification of the mammary tumors, immunohistochemistry (IHC) was performed to observe c-Myc protein expression. Cells from tumor emboli were also observed for c-Myc protein expression.

Immunohistochemistry

For immunohistochemistry 4-5µ thick sections were taken on positively charged slides. Sections were dewaxed in xzylene and rehydrated in descending grades of alcohol. The antigen retrieval was carried out in citrate buffer (pH 6.0). Rabbit polyclonal antibody was used against c-Myc (A-14 Rabbit Polyclonal, SC-789, Santa Cruz Biotechnology Inc., Santa Cruz, California, USA).

Immunohistochemical staining was performed by using advanced SS™ One-Step Polymer–HRP IHC Detection System (BioGenex Laboratories Inc., San Ramon, California, USA) as per manufacturer's instructions. c-Myc expression was analyzed according to its distribution and positivity pattern. Immunoreactivity was expressed as the staining index on the basis of percentage (P) and intensity (I) in the cells showing positive reaction as per Balcanto *et al.* 2004 (28). Intensity scores were assessed as 0, 1, 2 and 3. Intensity score greater than 1 was considered as high. Depending upon the percentage of cells showing c-Myc immunoreactivity, percentage scores was assessed as 1 (1–25%), 2 (26–50%), 3 (51–75%) and 4 (76–100%). Percentage score more than 3 i.e. when the immunoreactive cells were detected in more than 50% of cells present in microscopic field, was considered as high.

RESULTS

A total 31 cases of canine mammary tumors were subjected to immunohistochemistry for the c-Myc protein. Among these 31 cases 2 were simple carcinomas, 14 were complex carcinomas, 13 were carcinosarcomas and 2 were sarcomas (Table 1). In our study the pattern of c-Myc immunolocalisation was both nuclear as well as cytoplasmic. 87% of cases showed the c-Myc expression (Figure 1, 2). Out of 31 cases 18 cases presented with more than 50% cells which were positive for c-Myc expression (i.e. percentage score ≥3). Among 27 tumors which were positive for c-Myc, expression of c-Myc was noticed in both the nucleus and the cytoplasm in 21 cases, whereas only cytoplasmic or nuclear staining was observed in 4 and 2 cases respectively.

The nuclear localization of c-Myc protein was predominant in early stage lesions. Invading cells, that is, the neoplastic cells spreading across the basement membrane into mammary tissue, showed nuclear exclusion of c-Myc protein. Thus advanced lesions showed c-Myc localization in cytoplasmic compartment. Some tumor emboli were found to be immunoreactive for c-Myc protein. Cells

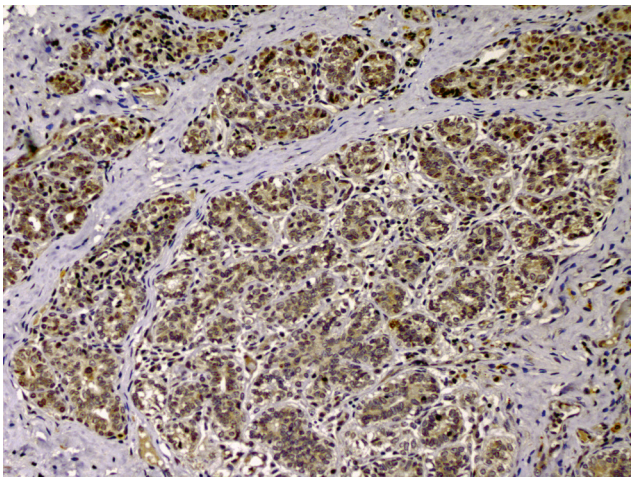

Figure 1: Section of canine mammary tumor showing nuclear immunoreactivity of c-Myc oncoprotein in a case of carcinoma. One Step Polymer HRP Detection System, counterstained with Gills hematoxylin. Original magnification -100X.

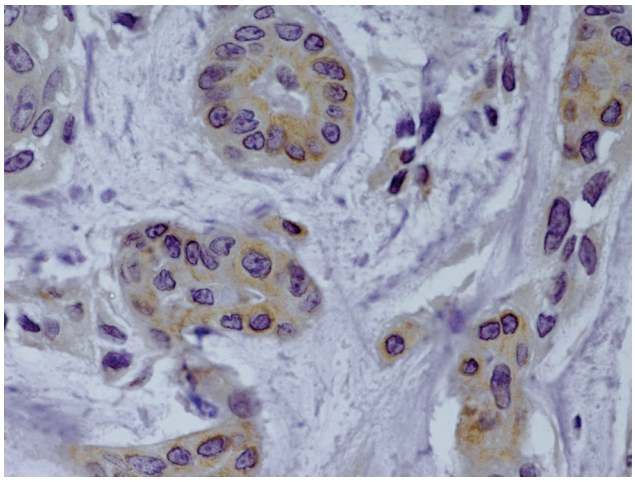

Figure 2: Section of canine mammary tumor showing predominantly cytoplasmic immunoreactivity of c-Myc oncoprotein in a case of carcinosarcoma. One Step Polymer HRP Detection System, counterstained with Gills hematoxylin. Original magnification -400X.

from epithelium to mesenchymal transitions (EMT) also showed immunoreactivity to c-Myc protein. Other cells from parenchyma like connective tissue and inflammatory cells were negative for c-Myc immunoreactivity.

DISCUSSION

In the present study increasing nuclear frequency of c-Myc protein was seen in lesions like simple carcinoma particularly ductal carcinoma *in situ* and lobular carcinoma *in situ* and complex carcinoma while it was more cytoplasmic in carcinosarcomas and it was found to be down regulated as tumors progressed to the pure sarcomatous type. The results suggests that the c-Myc protein expression is initially nuclear and then later it becomes progressively more cytoplasmic. Balcanto *et al.* 2004 (28) also reported the loss of nuclear expression from normal to invasive cells with predominant cytoplasmic expression in invasive cells. As the c-Myc immunoreactivity was more pronounced in simple and complex carcinomas, it suggests that c-Myc expression is predominant in early lesions. Many immunohistochemical studies have shown that about 50–100% of human breast cancer cases have increased levels of c-Myc proteins (11, 12, 13, 14, 15, 16). In a few studies nuclear localization and the predominant cytoplasmic localization of c-Myc proteins have also been observed (29). Mazzini *et al.* 2009 (30) found more c-Myc expression in invasive, malignant cells where benign lesion showed less c-Myc expression. Overall results suggested that the c-Myc expression is greater in neoplastic cell populations than in simple or complex carcinomas. These findings along with the findings of other researchers indicate that c-Myc may be important in progression of neoplasms from benign to invasive (31).

Interestingly the c-Myc was also expressed in the cells of epithelial to mesenchymal transition (EMT). The association of c-Myc with EMT indicates that c-Myc may play an important role in cell switch from one cell type to another type, which are reminiscent of the properties of stem cells and may be indicative that c-Myc may be an important cancer stem cell regulator (9, 32). Localization of c-Myc in tumor emboli in few cases indicates that c-Myc may also play a role in tumor metastasis but this requires further study for verification.

Table 1: Immunostaining of c-Myc oncoprotein in canine mammary tumor (N=31)

Type of tumor	Total no. of cases	No. of cases showing c-Myc expression	Immunoreactivity
Simple Carcinoma	2	2	More Nuclear
Complex Carcinoma	14	11	More Nuclear
Carcinosarcoma	13	12	More cytoplasmic
Sarcoma	2	2	Cytoplasmic (very low expression)

Overall findings of this study indicated by the immunoreactivity to c-Myc protein suggests that there might be a relation between nuclear expression with early tumor cell populations and cytoplasmic expression with the advanced and invasive tumor cell population as well as EMTs. These findings are based of limited number of cases and therefore it is suggested that further studies be carried out to confirm the results presented here. Predominant cytoplasmic expression and nuclear exclusion suggests a poor prognosis as these tumor cells might have acquired invasive and metastatic potential however this requires confirmation using other metastatic markers. The frequent over expression or altered expression of c-Myc in tumors may be interrelated to the initiation and progression of tumors (33, 34). These findings need to be evaluated in larger number of mammary neoplastic cases.

The authors propose that these finding may denote a role for c-Myc as a potential target for anti-cancer drugs.

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REFERENCES:

1. Adhikary, S. and Eilers, M.: Transcriptional regulation and transformation by Myc proteins. Nat. Rev. Mol. Cell Biol. 6:635-645, 2005.
2. Prochownik, E.V. and Li, Y.: The ever expanding role for c-Myc in promoting genomic instability. Cell Cycle. 6:1024-1029, 2007.
3. Zhang, S. K.: Immediate-Early Response Genes as Targets for Breast Cancer Treatment. Final Report, the Burnham Institute, La Jolla, California. pp: 4-7, 2002.
4. Mark, A. G. and Stephen, R. H.: c-Myc Proteolysis by the Ubiquitin-Proteasome Pathway: Stabilization of c-Myc in Burkitt's Lymphoma Cells. Mol. Cell Biol. 20: 2423–2435, 2000.
5. David, N. B., Ying, Q., Zhaoliang, L., and Stephen, R.H.: Egr1 mediates p53-independent c-Myc–induced apoptosis via a noncanonical ARF-dependent transcriptional mechanism. PNAS. 108: 632-637, 2011.
6. Taira, N., Mimoto, R., Kurata, M., Yamaguchi, T., Kitagawa, M., Miki, Y. and Yoshida, K.: DYRK2 priming phosphorylation of c-Jun and c-Myc modulates cell cycle progression in human cancer cells. J. Clin. Invest. 122:859-872, 2012.
7. Smith, D. R., Myint, T. and Goh, H. S.: Over-expression of the c-Myc proto-oncogene in colorectal carcinoma. Br. J. Cancer. 68: 407-413, 1993.
8. Sagawa, Y., Nishi, H., Isaka, K., Fujito, A. and Takayama, M.: The correlation of TERT expression with c-Myc expression in cervical cancer. Clin. Oncol. 168: 45-50, 2001.
9. Wang,J., Wang, H., Li, Z., Wu, Q., Lathia, J. D., McLendon, R. E., Hjelmeland, A. B., and Rich, J. N.: c-Myc Is Required for Maintenance of Glioma Cancer Stem Cells. PLoS ONE. 3: e3769. 2008.
10. Hawksworth, D., Ravindranath, L., Chen, Y., Furusato, B., Sesterhenn, I. A., Mcleod, D. G., Srivastava, S. and Petrovics, G.: Overexpression of c-Myc oncogene in prostate cancer predicts biochemical recurrence. Prostate Cancer Prostatic Dis. 13: 311-315, 2010.
11. Agnantis, N. J., Mahera, H., Maounis, N. and Spandidos, D. A.: Immunohistochemical study of ras and myc oncoproteins in apocrine breast lesions with and without papillomatosis. Euro. J. Gynaecol. Oncol. 13: 309–315, 1992.
12. Pavelic. Z.P., Pavelic. K., Carter. C.P. and Pavelic, L.: Heterogeneity of c-Myc expression in histologically similar infiltrating ductal carcinomas of the breast. J. Cancer Res. Clin. Oncol. 118: 16–22, 1992.
13. Saccani, J.G., Fontanesi, M., Bombardieri, E., Gabrielli, M., Veronesi. P., Bianchi, M., Becchi, G., Bogni, A. and Tardini, A.: Preliminary study on oncogene product immunohistochemistry (c-erbB–2,c-Myc, ras p21, EGFR) in breast pathology. Int. J. Biol. Markers. 7: 35–42, 1992.
14. Hehir, D. J., McGreal, G., Kirwan, W. O., Kealy, W. and Brady, M. P.: c-Myc oncogene expression: A marker for females at risk of breast carcinoma. J. Surg. Oncol. 51: 207-210, 1993.
15. Spaventi, R., Kamenjicki, E., Pecina, N., Grazio, S., Pavelic, J., Kusic, B., Cvrtila, D., Danilovic, Z. and Spaventi, S.: Immunohistochemical detection ofTGF-alpha, EGF-R, c-erbB-2, c-H-ras, c-Myc, estrogen and progesterone in benign and malignant human breast lesions: a concomitant expression. In Vivo 8: 183–89, 1994.
16. Pietilainen, T., Lipponen, P., Aaltomaa, S., Eskelinen, M., Kosma, V.M. and Syrjanen, K.: Expression of retinoblastoma gene protein (Rb) in breast cancer as related to established prognostic factors and survival. Europ. J. Cancer 31: 329-333, 1995.
17. Moulton, J. E.: Tumours of mammary gland. In: Moulton J E (ed) Tumours in Domestic Animals (3rd Eds.): University of California Press, Berkeley and Los Angeles, pp. 518-520, 1990.
18. Misdorp, W.: Tumours of the mammary gland. Tumors in Domestic Animals, DJ Meuten (4th ed.), Iowa State Press, Blackwell Publishing Company, U.K. pp. 575-606, 2002.
19. Sorenmo, K.: An update on canine mammary gland tumors. ACVIM Forum Proceedings. 16:387-388, 2003.
20. Pang, L Y., Cervantes-Arias, A., Else, R. W. and Argyle, D.: Canine Mammary Cancer Stem Cells are Radio- and Chemo- Resistant and Exhibit an Epithelial-Mesenchymal Transition Phenotype. Cancers. 3: 1744-1762, 2011.
21. Schmitt, J. F., Keogh, M. C., Dennehy, U., Chen, D., Lupu, F., Weston, K., Taylor, F., Kakkar, V. and Lemoine, N. R.: Tissue-selective expression of dominant-negative proteins for the regulation of vascular smooth muscle cell proliferation. Gene Therapy. 6: 1184-1191, 1999.
22. Ely, K. A., Carter, B. A., Jensen, R. A., Simpson, J. F. and Page, D. L.: Core biopsy of the breast with atypical ductal hyperplasia: a probabilistic approach to reporting. Am. J. Surg. Path.. 25:1017–1021. 2001.

23. Antuofermo, E., Miller, M. A., Pirino, S., Xie, J., Badve, S. and Mohammed, S. L.: Spontaneous mammary intraepithelial lesions in dogs: A model of breast cancer. Cancer Epidemiol. 16: 2247-2256, 2007.
24. Mediana, D.: Premalignant and malignant mammary lesions induced by MMTV and chemical carcinogens. J. Mammary Gland Biol. Neoplasia. 13:271–27, 2008.
25. Kretschmer, C., Sterner-Kock, A., Siedentopf, F., Schoenegg, W., Schlag, P. M., and Kemmner, W.: Identification of early molecular markers for breast cancer. Mol. Cancer. 10: 15, 2011.
26. Chaw, S. Y., Majeed, A. A., Dalley, A. J., Chan, A., Stein, S. and Farah, C. S.: Epithelial to mesenchymal transition (EMT) biomarkers--E-cadherin, beta-catenin, APC and Vimentin--in oral squamous cell carcinogenesis and transformation. Oral Oncol. 48:997-1006, 2012.
27. Kallergi, G., Papadaki, M. A., Dimitris, E. P., Georgoulias, M. V. and Agelaki, S.: Epithelial to mesenchymal transition markers expressed in circulating tumour cells of early and metastatic breast cancer patients. Breast Cancer Res. 13:R59, 2011.
28. Blancato, J., Singh, B., Liu, A., Liao, D. J. and Dickson, R B.: Correlation of amplification and over expression of the *c-Myc* oncogene in high-grade breast cancer: FISH, in situ hybridization and immunohistochemicalb analyses. Brit. J. Cancer 90:1612-1619, 2004.
29. Mizukami, Y., Nonomura. A., Noguchi, M., Taniya, T., Koyasaki, N., Saito, Y., Hashimoto, T., Matsubara, F. and Yanaihara, N.: Immunohistochemical study of oncogene product ras p21, c-Myc and growth factor EGF in breast carcinomas. Anticancer Res. 11: 1485–1494, 1991.
30. Mazzini, R. C., Elias, S., Nazário, A. C., Kemp, C. and Logullo, A. F.: Prevalence of c-Myc expression in breast lesions associated with microcalcifications detected by routine mammography. Sao Paulo Med. J. 127: 66-70, 2009.
31. Corzo, C., Corominas, J. M., Tusquets, I., Salido, M., Bellet, M., Fabregat, X., Serrano, S. and Solé, F.: The MYC oncogene in breast cancer progression: from benign epithelium to invasive carcinoma. Cancer Genet. Cytogenet. 165:151-156, 2006.
32. Kim, J., Woo, A. J., Chu, J., Snow, J. W., Fujiwara, Y., Kim, C. G., Cantor, A. B. and Orkin, S. H.: A Myc network accounts for similarities between embryonic stem and cancer cell transcription programs. Cell. 143:313-324, 2010.
33. Dang, C. V.: c-Myc Target Genes Involved in Cell Growth, Apoptosis, and Metabolism. Mol. Cell Biol. 19: 1–11, 1999.
34. Cappellen, D., Schlange, T., Bauer, M., Maurer, F. and Hynes, N. E.: Novel c-Myc target genes mediate differential effects on cell proliferation and migration. EMBO Rep. 8: 70–76, 2007.