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c-Myc has Altered Expression in Canine and Feline Tumors

Saba Manzoor[1,*], Ali Raza Awan[1], Abdul Wajid[1], Sehrish Firyal[1], Muhammad Tayyab[1], Muhammad Mansha[2], Asim Khalid Mahmood[3], Abu Saeed Hashmi[1] and Muhammad Wasim[1,*]

[1]*Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences, Outfall Road, Lahore 5400*
[2]*Deparment of Zoology, Division of Science and Technology, University of Education, Township, Lahore*
[3]*Pet Center, University of Veterinary and Animal Sciences, Outfall Road, Lahore 5400*

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Authors' Contribution
SM and MW conceived the project. SM, AW, SF performed experimental work and analysed data. ARA, ASH, MT and MM helped in manuscript writing and AKM helped in sampling. MW supervised the project.

Key words
c-Myc, Oncogene, Dog and cat cancer, qRT-PCR, Fold change.

ABSTRACT

Gene expression and mutation study in different cancer types of animal origin has its pivotal role in diagnosis, prognosis and comparative studies. *c-Myc* gene was selected to study the mutation and expression profiling of different canine and feline tumors due to its predetermined role in tumor pathogenesis. A total of 52 tumor and normal samples were examined, among these, 40 canine and 12 feline samples were analyzed using Sanger sequencing method and RT-qPCR for the mutation and expression profiling of *c-Myc* gene. The coding region of *c-Myc* gene (exon2 and 3) did not show any variation in both species. While expression of *c-Myc* gene was found to be up regulated in different canine (62.5% in mammary adenocarcinoma, 75% in oral squamous cell carcinoma, 66.6% in peri-anal sac adenocarcinoma, 80% in mast cell tumor and 100% in soft tissue sarcoma) and feline (60% in mammary adenocarcinoma and 100% in soft tissue sarcoma) tumors studied. These findings might support the established role of c-Myc in tumor pathogenesis but how this transcription factor affects the downstream targets to achieve the goal remains unanswered. Therefore, further research is desperately needed to understand the mechanism of tumor pathogenesis in animals in order to identify the molecular targets for proper cure of this ailment.

INTRODUCTION

Companion animals such as *Canis familiaris* (dogs) are considered the best preclinical model for cancer and many other complex human diseases (Rowell *et al*., 2011). Many features make the dog an attractive model for human cancer biology research. Firstly, there is high genetic homology between the human and dog genome, compared to other animals. Secondly, dogs live in the same environment and share the similar risk factors or disease characteristics with humans (Kirkness *et al*., 2003). The c-Myc proto oncogene, a transcription factor located on chromosome # 13 and F2 of dog and cat respectively, is involved in cell proliferation, cell cycle progression, differentiation and apoptosis (Meyer and Penn, 2008). c-Myc belongs to a family of related genes, N-Myc and L-Myc. Expression of N-Myc is tissue restricted and it can substitute for c-Myc in murine development (Malynn et al., 2000; Brodeur et al., 1984). The Myc (N-terminal domain) contains a conserved Myc boxes (boxes I, II followed by III, IV) and a nuclear targeting sequence (Cowling and Cole, 2007). The C-terminal domain of Myc contains bHLH-Zip which remain partially unstructured till its dimerization with MAX protein (Mustata *et al*., 2009). Myc-MAX heterodimer bends the DNA through E-box binding motifs (5'CACGTG3'). The N-terminal domain of Myc forms complexes with transcriptional factors including TRRAP, GCN5 and TBP (Liu *et al*., 2003). The expression of c-Myc mRNA and protein remains very low due to absence of positive regulatory signals in normal cells (Saccani *et al*., 1992; Hehir *et al*., 1993). Under the effect of mitogenic signals or growth factors, there is an increase in the level of c-Myc which promotes the cell from G_o to G_1 phase and this increased level lasts for approximately 30 min. c-Myc is continuously produced throughout the cell cycle until the growth factors are depleted (Mark and Sephen, 2000; Gavhane *et al*., 2013). The overexpression of *c-Myc* gene mostly occurs after post-translational modification, Thr58, Ser62 mutation of coding region of c-Myc is most prevalent in the case of Burkitt lymphoma (Salghetti *et al*., 1999). The role of c-Myc has been studied in different cancers such as breast cancer (Gavhane *et al*., 2013), glioma (Wang *et al*., 2008), colorectal cancer

* Corresponding authors: saba_mmg@yahoo.com; muhammad.wasim@uvas.edu.pk
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(Smith *et al*., 1993) prostate cancer (Hawksworth *et al*., 2010) and cervical cancer (Sagawa *et al*., 2001).

The study aimed to find out the mutation and expression of *c-Myc* gene in different types of canine and feline tumors and assess the possibility; c-Myc is useful marker for these malignancies.

MATERIALS AND METHODS

Sample collection

In present study, thirty eight (n=38) tumors and (n=14) normal tissue samples of canine and feline species were collected from University Pet center and Asim Pet Clinic, Lahore, Pakistan, from 2012 to 2015, after the proper diagnosis of tumor type and approval of ethical committee of University of Veterinary and Animal Sciences, Lahore, Pakistan.

Histopathological examination

Formalin fixed tissues were embedded in paraffin and core region of tissues (5µm thickness) were mounted on the glass slides and stained with Hematoxline and Eosine. Histopathological examination of tumors were performed by veterinary pathologist according to the criteria of the WHO classification of canine and feline tumors.

DNA extraction

Genomic DNA was extracted from tumor and normal healthy control tissues by using DNeasy Blood and Tissue kit (Qaigen) following manufacturer's protocol. DNA concentration was measured with Nano Drop 2000 spcectrophotometer (Thermo fisher scientific, Pittsburg PA, USA) and visualized by 0.8% agarose gel electrophoresis. All DNA samples were normalized to 50ng/µL concentration.

PCR amplification and sequencing

Primers for exons (2, 3) were designed from intron exon boundaries of approximately 100bp sequence of *c-Myc* gene by primer 3 and Net primer software (Table I) and manufactured by Advance Bioscience International. Same primers were used for both species due to 98% sequence homology for exon 2 and 93% sequence homology for exon 3. Primers were optimized with wide range of annealing temperature (52-62°C) by gradient PCR and Touch down PCR along with variable concentration of $MgCl_2$ (2.5 to 3.0 mM), dNTPs, primers and templet DNA in thermo cycler (Applied Bio System). PCR reaction mixture contained templet DNA 1µL, Primer forward (10pmol) 0.75µL, primer reverse (10pmol) 0.75µL, $MgCl_2$ (2.5mM) 2.5µL, PCR buffer (2.0mM) 2.5µL, dNTPs (2.5mM) 2.5µL, Taq polymeras (5UµL) 0.5µL and DEPEC water 14.5µL to make total volume 25µL. Following conditions were used in thermo cycler for amplification of desired product; First hold, initial denaturation was 95°C for 5 min, 25 cycles (denaturation 94°C for 30 sec, annealing at 58°C for exon 2 and 60°C for exon 3 for 30 sec, extension at 72°C for 45 sec), final extension 72°C for 10 min and finally hold at 4°C. Amplified PCR products were analyzed on 1.5% agarose gel along with 1kb ladder (Fermentas, USA) the gel was stained with ethidium bromide and visualized under UV-trans illuminator (Bio Red). The PCR products were purified by ethanol precipitation and were sequenced at 3730 ABI genetic analyzer. The Bio Edit software version 7.0.5 was used to find out the novel SNP or mutation.

RNA extraction and cDNA synthesis

Total RNA was extracted and purified from tumors and normal tissues by using Gene Jet RNA purification kit (Thermo Scientific) and reverse transcribed using RevertAid first strand cDNA synthesis Kit (Themo Fisher Scientific, USA).

Table I.- Primer sequence of *c-Myc* gene.

Exons	Primer sequences (5' - 3')	Product size (bp)
Exon-2Fa	CCGTAACTCAAGATCGCCCC	476
Exon-2Ra	CGCTCCACATGCAGTCCT	
Exon- 2Fb	GGACGACGAGACCTTCATCA	499
Exon- 2Rb	GAAAAGGCTGGGGTCAACTG	
Exon- 3Fa	TGGCTTGAAGGACACTGTTG	585
Exon- 3Ra	TGTTTCAACTGTTCTCGCCG	
Exon- 3Fb	TACATCCTGTCCGTCCAAGC	813
Exon- 3Rb	GGCCCCAGACCCATTTTAAC	

Table II.- RT-qPCR primers for *c-Myc* and housekeeping gene.

Gene	Sp.	Kit ID	Amplicon length	Assay location/exon boundaries	Dye
c-Myc	Dog	cf-02628821_ m1	126bp	Exon 2- 3	FAM
GAPDH	Dog	cat#4331348 custom designed	59bp	655bp	VIC

Quantitative real time PCR

Pre designed primers and probes for *c-Myc* gene were selected and purchased from Applied Bio System and for

data normalization, GAPDH housekeeping gene primer was custom designed using primer express software (ABI) (Table II). The qRT-PCR reactions were carried out in 96 well plate that contained triplicates of each cDNA sample for target and housekeeping gene according to the AB1 standard protocol. The 20µL reaction mixture contained 4µL cDNA, 1µL 20X TaqMan gene expression assay, 10µL of gene expression master mix and 5µL of RNase free DEPC water. Cycling conditions were 10 min at 95°C followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C.

Table III.- Outline of canine and feline patients' cohort.

***Canis familiaris* (n=30)**	
Age	
= >5 Year	17(56.7%)
=< 5 Year	13(43.3%)
Gender	
Male	13(43.3%)
Female	17(56.7%)
Breed	
Germen Shepherd	17(56.7%)
Rottweiler	4(13.3%)
Golden Retriever	3(10.0%)
Labrador	6(20.0%)
Tumors	
Mammary adenocarcinoma	8(26.7%)
Oral squamous cell carcinoma	4(13.3%)
Peri-anal sac adenocarcinoma	3(10.0%)
Mast cell tumor	5(16.7%)
Soft tissue sarcoma	10(33.3%)
***Felis catus* (n=08)**	
Age	
= > 5 years	4(50%)
= <5 years	4(50%)
Gender	
Female	8(100%)
Breed	
Siamese	8(100%)
Tumors	
Mammary adenocarcinoma	5(62.5%)
Soft tissue sarcoma	3(37.5%)

Quantification of target gene expression

Relative expression of the target gene (c-Myc) was calculated using ΔΔCt method (Livak and Schmittgen, 2002). Data was presented as fold change in gene expression level in the target sample normalized to housekeeping gene and relative to the control sample. The fold change based on the delta Ct value was calculated using Microsoft Excel 2010. Cut off values for the fold change was set at >1.0 was higher and folds change value <1.0 was accepted lower gene expression.

RESULTS AND DISCUSSION

In the present study, incident rate of canine soft tissue sarcoma and mammary adenocarcinoma was found higher as compared to other tumors. The study tumor was most prevalent in female Germen Shepherd and Siamese breed of dogs and cats, respectively (Table III). Priester and Mantel (1971) reported that particular breed of pedigree dogs show higher incidence of particular type of tumor. The Siamese cat breed has twice the risk of developing cancer when compared to other breed of cats (Weijer *et al*., 1972). The same predisposition was observed in our data, since all cat cases belonged to Siamese breed.

Deregulation of *c-Myc* gene expression in tumors and tumor cell lines have been identified by gene amplification, translocation, mutation and higher level of c-Myc RNA. The genomic DNA of tumor and healthy tissues were amplified using coding exon 2 and 3 of *c-Myc* gene. The amplified product was subjected to DNA sequencing analysis by dideoxy chain termination method, which showed no mutation in coding exons of both species. Similarly, no rearrangement of *c-Myc* gene was found in cutaneous B cell lymphoma (Garatti *et al*., 1995). *c-Myc* gene amplification mostly occurs late during tumor formation and is consistently observed in tumor aggressiveness, correlating with poor prognosis and distant metastasis (Singhi *et al*., 2012). Gain of *c-Myc* gene was reported in (16.7%) hyperplasia, (9.1%) benign and (30.4%) malignant canine mammary tumors by means of microarray (Borge *et al*., 2015). Similarly, Prayitno *et al*. (2013) reported that 9.1% *c-Myc* gene mutation happen in oral sequmaous cell carcinoma with HPV positive patients.

c-Myc *gene expression in canine tumors*

All the study tumors, showed abnormal *c-Myc* gene expression with different fold change values. In the present study, *c-Myc* gene was up regulated in 5/8 (62.5%) canine mammary adenocarcinomas (specifically in C-MAM-2, C-MAM-4 and C-MAM-8 samples) with the highest fold change of 21.9, 18.2 and 14.9, respectively compared to normal mammary tissues (Fig. 1). The worth mentioning point of discussion about the *c-Myc* gene over expression in studied tumor samples is that, all of these dogs belong to German Shepherd breed. Gavhane *et al*. (2013) reported that c-Myc protein expression was increased 87 % in benign as well as carcinoma tumors.

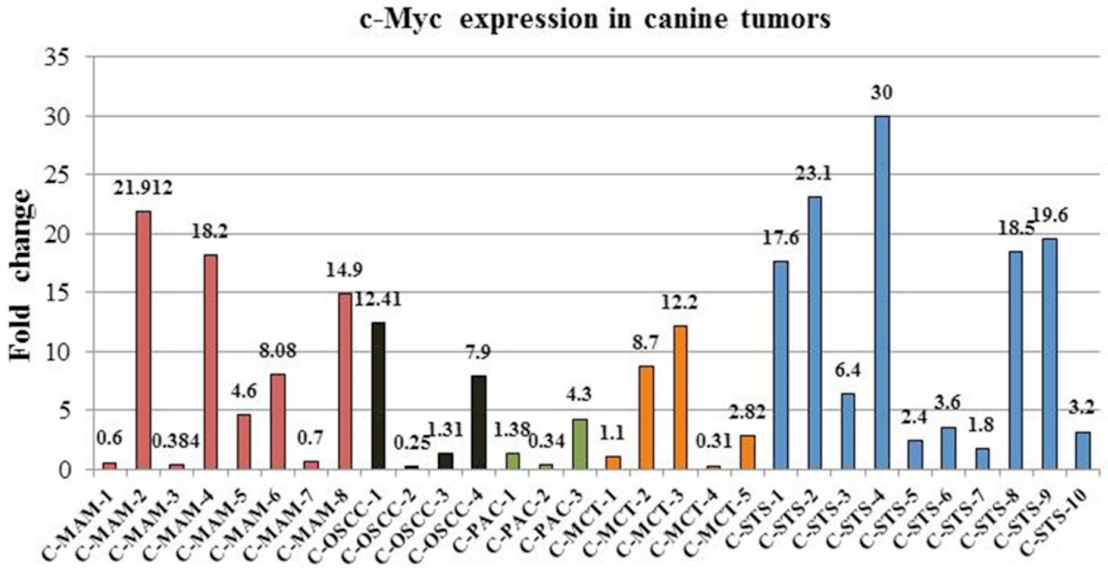

Fig. 1. Expression level of *c-Myc* gene in canine mammary adenocarcinoma (C-MAM), oral squamous cell carcinoma (C-OSCC), peri anal sac adenocarcinoma (C-PAC), mast cell tumors(C-MCT) and soft tissue sarcoma (C-STS). Values are represented as fold change relative to normal sample (Livak method). Values >1.0 were considered up regulation of gene.

Similar finding was reported by Pavelic *et al*. (1992), Hehir *et al*. (1993) and Spaventi *et al*. (1994) that showed the increased level of c-Myc protein in 50-100 % of human breast cancer patients. In canine oral squamous cell carcinoma, the up regulation of *c-Myc* gene was observed in 3/4 (75%) of tumor samples with the highest fold change of 12.4 and 7.9 in C-OSSC-1 and C-OSSC-4 samples, respectively (Fig. 1). Up regulation of *c-Myc* gene was observed in 2/3 (66.6%) peri-anal sac adenocarcinomas with a fold change of 4.3 in the F-PAC-3 samples (Fig. 1). Several studies reported the c-Myc amplification and expression in anus squamous cell carcinoma (Crook *et al*., 1991; Ogunbiyi *et al*., 1993). *c-Myc* gene was up regulated in 4/5 (80%) canine mast cell tumor samples with highest fold change 8.7, 12.2 in C-MCT-2 and C-MCT-3 sample, respectively (Fig. 1). Similar finding was reported by Rodrigo *et al*. (2010) that showed 77.7 % occurrence and 80% expression of c-Myc mRNA in dog eyelid tumor. In another study, 93.3 % expression of *c-Myc* gene was reported at mRNA and protein level in human cutaneous melanoma (Utikal *et al*., 2002). All canine soft tissue sarcoma samples showed, up regulation of *c-Myc* gene with the highest fold change of 23.1, 30.0 in C-STS-2 and C-STS-4 sample, respectively (Fig. 1). A significant amplification of c-Myc was observed in canine osteosarcoma samples (Kochevar *et al*., 1990).

c-Myc *gene expression in feline tumors*

Feline mammary adenocarcinoma is third in number after hematopoietic and skin carcinoma. Sadia *et al*. (2016) expressed profiling of COX-2 and BRCA-1 gene in feline mammary tumors. *c-Myc* gene was up regulated in 3/5 (60%) of feline mammary adenocarcinoma with the highest fold change of 3.1 and 3.0 in the F-MAM-3 and F-MAM-4 samples, respectively and 100% up regulation in soft tissue sarcoma sample with highest fold change 21.8 (Fig. 2). Shon *et al*. (2014) reported the overexpression of *c-Myc* gene in cutaneous angiosarcoma.

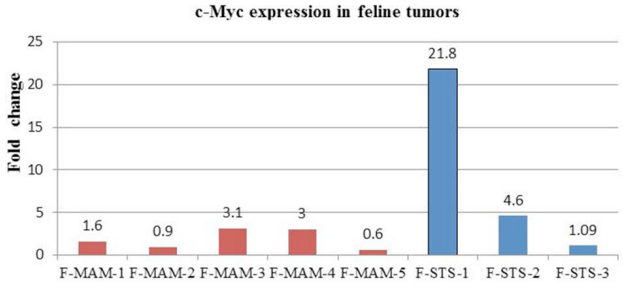

Fig. 2. Expression level of *c-Myc* gene in feline mammary adenocarcinoma (F-MAM), soft tissue sarcoma (F-STS). Values are represented as fold change relative to normal sample (Livak method). Values >1.0 were considered up regulation of gene.

CONCLUSION

This is first report from this part of world in which we have observed over expression of c-Myc in most of analyzed

canine and feline tumor samples but no polymorphism was detected in them. Enhanced expression of this transcription factor might regulate the downstream players implicated in tumor pathogenesis. These findings might support the established role of c-Myc in tumor pathogenesis but how this transcription factor affects the downstream targets to achieve the goal remains unanswered. Therefore, further research is desperately needed to understand the mechanism of tumor pathogenesis in animals in order to identify the molecular targets for proper cure of this ailment.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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