RESEARCH ARTICLE

Upregulated Myc Expression in N-Methyl Nitrosourea (MNU)-induced Rat Mammary Tumours

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Abstract

Background: The most common incident cancer and cause of cancer-related deaths in women is breast cancer. The Myc gene is upregulated in many cancer types including breast cancer, and it is considered as a potential anti-cancer drug target. The present study was conducted to evaluate the Myc (gene and protein) expression pattern in an experimental mammary tumour model in rats. Materials and Methods: Thirty six Sprague Dawley rats were divided into: Experimental group (26 animals), which received the chemical carcinogen N-methyl nitrosourea (MNU) and a control group (10 animals), which received vehicle only. c-Myc oncoprotein and its mRNA expression pattern were evaluated using immunohistochemistry (IHC) and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR), respectively, in normal rat mammary tissue and mammary tumours. The rat glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as internal control for semi-quantitative RT-PCR. Results: Histopathological examination of mammary tissues and tumours from MNU treated animals revealed the presence of premalignant lesions, benign tumours, *in situ* carcinomas and invasive carcinomas. Immunohistochemical evaluation of tumour tissues showed upregulation and heterogeneous cellular localization of c-Myc oncoprotein. The expression levels of c-Myc oncoprotein were significantly elevated (75-91%) in all the tumours. Semi-quantitative RT-PCR revealed increased expression of c-Myc mRNA in mammary tumours compared to normal mammary tissues. Conclusions: Further large-scale investigation study is needed to adopt this experimental rat mammary tumour model as an *in vivo* model to study anti-cancer strategies directed against *Myc* or its downstream partners at the transcriptional or post-transcriptional level.

Keywords: Myc - rat mammary tumour - overexpression - immunohistochemistry - semi - quantitative RT-PCR

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Introduction

Cancer is the leading cause of mortality in pets (dogs and cats), up to 41% in dogs (Bonnet et al., 2005) and second most in humans after cardiovascular diseases (Siegel et al., 2011). According to International Agency for Research on Cancer (IARC) estimates for 2008, there were 12.4 million incident cases of cancer, 7.6 million deaths from cancer and 28 million people alive with cancer worldwide (WHO, 2008), even after technologically advanced interdisciplinary approaches that have contributed significantly to the progress in cancer diagnosis and treatment. Globally, lung cancer is the commonest incident cancer and cause of cancer-related mortality in men; where as in women, the most common incident cancer and cause of cancer-related deaths is breast cancer, which originates in the inner lining epithelium of the alveolar ducts or lobules (WHO, 2008), comprising 26% of all female cancers (Jemal et al., 2008) with over 4.1 lakh deaths from breast cancer annually. The quest for newer cancer drug targets is ever expanding and most importantly highly challenging. The c-*Myc* oncogene (referred to hereafter as *Myc*) or c-Myc oncoprotein (referred to hereafter as Myc) identified themselves as an attractive target, but with the stigma of probable universal cellular catastrophe upon sudden down regulation. The *Myc* oncogene, which codes for a transcription factor Myc, is frequently altered in cancer cells by amplification, mutation, overexpression, or protein stabilization (Soucek et al., 2010). The basic helix-loop-helix zipper transcription factor Myc coordinates the expression of a vast and functionally varied repertoire of genes that are required for the orderly proliferation of somatic and germ cells within the body. These regulated genes control the cell cycle, cell metabolism and biosynthesis, cell architecture, and cell survival, as well as the multitude of processes that proliferating cells need to engage in their surrounding micro environment, such as angiogenesis, tissue remodelling, and recruitment of cells loaded with enzymes and growth factors needed to do this. Myc is

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an absolute requirement for the efficient proliferation of normal as well as cancer cells (Evan, 2012). Modern studies of experimentally induced chemical carcinogenesis began with the introduction of polynuclear hydrocarbons such as 3-methyl chloranthracene (MCA), 7, 12-dimethyl benzanthracene (DMBA) (Huggins et al., 1959) and MNU (Gullino et al., 1975) as chemical carcinogens. Tumours induced in rats by administration of chemical carcinogens such as 7,12-dimethylbenzanthracene(DMBA) (Russo and Russo, 2000) and N-methyl nitrosourea constitute useful tools for dissecting the multistep carcinogenesis, which involves initiation, promotion, and progression. In this study, an attempt was made to evaluate the expression pattern of *Myc*/Myc in chemically-induced rat mammary tumours which can serve as a model for *in vivo* anti-cancer studies targeted against *Myc*/Myc.

Materials and Methods

Experimental mammary tumour induction

Thirty six virgin female Sprague-Dawley rats (inbred strain) of matched age (28-30 days) and weight (25-30 g) were procured from Laboratory Animal Division of Central Drug Research Institute, Lucknow, India and acclimatized for a period of 12 days. Rats were divided into: experimental group (26 animals) and control group (10 animals). Experimental group received chemical carcinogen N-methyl nitrosourea (MNU) (Sigma-Aldrich, USA) dissolved in acidified saline (pH 4.0) at the dose rate of 50 mg/kg body weight (BW) intraperitoneally (i/p), two doses at 43 and 50 days of age. Control group received MNU vehicle (acidified saline, pH 4.0) only by the same route (Gullino et al., 1975; Thompson and Adlakha, 1991; Vegh and Salamanca, 2007; Mayilkumar, 2009). Following carcinogen or vehicle administration, all the animals were observed for a period of 28 weeks for the development of mammary tumours. The experimentation was performed as per the guidelines and approval of Institute Animal Ethics Committee (IAEC) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Observation and sample collection

The animals were palpated twice a week for any tumour growth in mammary glands and body weight was recorded at weekly intervals. Visible tumour growths were measured using dial caliper. Latency period was calculated from the time of last carcinogen administration to the appearance of the first tumour. Tumour incidence was determined as percentage representation of tumour-bearing animals in experimental group. At the end of observation period, animals were euthanized and mammary tissue, mammary tumours, lung, spleen, liver, heart, kidney, lymph nodes and long bones were collected for the assessment of the following: *i*) Histopathological examination and classification of mammary tumours (Russo and Russo, 2000), and screening of visceral organs and long bones for possible metastasis; *ii*) Immunohistochemical evaluation of Myc expression in normal and tumour tissues; and *iii*) Gene expressions by qualitative and semi-quantitative RT-PCR for *Myc* and GAPDH genes.

Histopathology

Formalin-fixed tissues were processed routinely through graded ethanol, xylene and paraffin embedding to obtain five μm thick sections and stained with haematoxylin and eosin (H&E) stain for histopathological examination. Good quality duplicate sections were also taken on 3-minopropyltriethoxysilane (APES) adhesive coated slides for immunohistochemical studies.

Immunohistochemistry (IHC)

Duplicate paraffin sections were deparaffinised and subjected to immunohistochemistry. IHC staining for Myc was performed employing mouse monoclonal anti-c-Myc antibody (clone 9E10, Sigma-Aldrich, USA). For antigen retrieval, the sections were microwaved in 10mM tri-sodium citrate buffer (pH 6.0) for 15 min (3 cycles of 5 min each). Endogenous peroxidase activity was quenched by incubating the sections with 3.0% hydrogen peroxide in distilled water for 15 min at room temperature (RT) in dark. For blocking non-specific antigen binding, sections were incubated with 5.0% normal goat serum (Invitrogen, USA) for 30 min at RT. Then, the sections were incubated with primary monoclonal anti-c-Myc antibody (1:100) for overnight at 4°C. Biotinylated goat anti-mouse IgG (Sigma-Aldrich, USA) was used as secondary antibody (1:15) and incubated for 45 min at RT, followed by ExtrAvidin peroxidase (1:15) (Sigma-Aldrich, USA) for 45 min at RT. Staining solution was prepared by dissolving 3, 3'-diaminobenzidine (DAB) and Urea-hydrogen peroxide tablets (Sigma-Aldrich, USA) in required volume of deionized distilled water and sections were stained for 3 min at RT. Counterstaining was done with Mayer's hematoxylin. All the steps were interceded by washing thrice (5 min each) in phosphate buffered saline (pH 7.4). Myc immunolabelling index was prepared by counting the number of positively stained (nuclear/cytoplasmic/both) cells among at least 1000 neoplastic cells (8-10 random high power field images) in the tumour tissue sections with the help of Adobe CS5 Photoshop count tool, and expressed in percentage.

Relative semi-quantitative analyses for Myc and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes

Total RNA was extracted from normal mammary tissue and mammary tumour tissue homogenates with TRI Reagent (Sigma-Aldrich, USA). The purity of isolated RNA was checked by measuring A260/A280 ratio; samples with ratio of 1.8 and above were selected for further processing. Total RNA was treated with Deoxyribonuclease-1 enzyme (AMPD-1 kit, Sigma-Aldrich, USA) to avoid any possible DNA contamination, as per instructions provided with the kit. cDNA was generated from DNase-1 treated RNA template with 0.2 µg of random hexamer primers and 200 units of RevertAid H-Minus M-MuLV reverse transcriptase enzyme (MBI Fermentas, USA). cDNA was synthesized at 25°C for 10 min; 42° C for 60 min, followed by 70°C for 10 min. Polymerase chain reaction was carried out to amplify rat *Myc* and *GAPDH* genes using published sequence specific primers for respective genes. The primer sequences used

were: *Myc*, 5'-CAACGTCTTGGAACGTCAGA-3' and 5'- CTCGCCGTTTCCTCAGTAAG-3' (Nakai et al., 2008); *GAPDH*, 5'- GTTACCAGG GCTGCCTTCTC-3' and 5'- GGGTTTCCCGTTGATGACC-3' (Shibata et al., 1999). The cycle number at which *GAPDH* amplification reaches plateau phase starting with a specific quantity of initial cDNA was calculated, and all the cDNA samples were diluted accordingly in such a way as to give uniform band intensity for *GAPDH* under UV transilluminator (Guénin et al., 2009). The same number of cycles was used for amplification of *Myc* and *GAPDH*, separately. The cycling conditions for both the genes were at 95°C for 5 min, and 35 cycles of 95°C for 15 s; 56°C for 1 min and 61°C for 1 min, followed by final extension at 61°C for 10 min. These steps were carried out in a thermal cycler (PTC-200, MJ Research, USA). Due to preferential amplification of one gene, a duplex PCR for both the genes was avoided though cycling conditions were same.

Exactly 6 μl of PCR product (for both *Myc* and *GAPDH*) was used for gel electrophoresis in 1.5% agarose gel stained with ethidium bromide (0.5μg/mL). Specific RT-PCR product bands were analyzed by densitometry using Quantity One 1D gel image analysis software (Bio-Rad, USA). Conditions for gel preparation, illumination, image acquisition and analysis were similar for both *Myc* and *GAPDH* products. The densitometric values of *Myc* and *GAPDH* products for each tumour (8 nos.), and control mammary glands (3 nos.) were compared and expressed as *Myc* to *GAPDH* ratio as previously described (Tao et al., 2002).

Results

Tumour development and histopathology

The administration of MNU at 50 mg/kg body weight twice at one week interval resulted in appearance of palpable tumorous nodules after 60 days post-injection (DPI) onwards in carcinogen treated animals. Most of the rats developed tumours at about 18 weeks post-carcinogen treatment. Out of 26 rats, 12 (46.15%) animals developed mammary tumours and 2 (7.69%) rats developed intraductal hyperplasia. A total of 18 tumours and 2 hyperplasias were diagnosed in 14 rats. Average latency period, determined by the time interval between last dose of carcinogen administration and appearance of the first tumour, was 144 days (ranged from 60 to 195 days). Grossly, most of the tumours were pink-reddish in colour, nodular, flat or globular in shape and measured between 0.2 cm and 3.1 cm in diameter. Number of pre-malignant lesions malignant detected and their histological types are given in Table 1. In control animals, no tumour growth was detected either grossly or microscopically.

Immunohistochemical analysis for Myc expression

Neoplastic cells with either nuclear and/or cytoplasmic immunolabelling were considered positive for immunostaining. The expression levels of Myc oncoprotein were significantly high (75-91%) in all the tumours. The cytoplasmic appearance of the Myc was more prominent in the malignant tumours of epithelial cells when compared to normal mammary epithelium and a fibroadenoma wherein mostly nuclear and moderate cytoplasmic staining pattern was observed. Different patterns of nuclear and cytoplasmic staining were observed between tumours as well as in different areas of the same tumour. The Myc immunostaining patterns included diffuse nuclear and cytoplasmic (Figure 1a), focal nuclear or perinuclear (Figure 1b), focal cytoplasmic, and pancytoplasmic (Figure 1c) immunostaining. Tumour cells evinced both nuclear and cytoplasmic or predominantly cytoplasmic with occasional nuclear immunostaining for

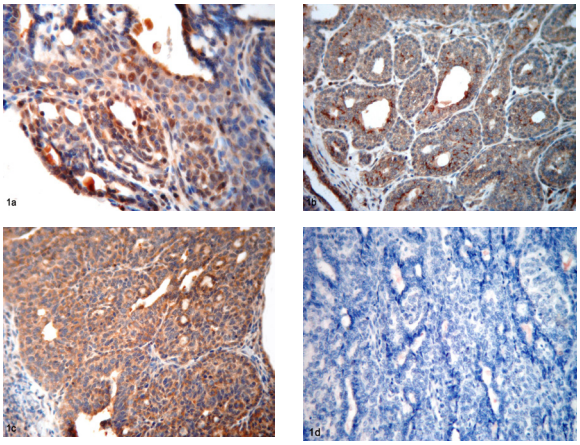

Figure 1. The Expression Pattern of Myc Protein in MNU-Induced Rat Mammary Tumours was High and Heterogeneous. a) In situ papillary carcinoma; diffuse nuclear and cytoplasmic immunostaining of Myc. DAB ×400; **b)** In situ solid and cribriform carcinoma; focal distinct perinuclear and mild diffuse cytoplasmic immunolabelling of Myc. DAB ×200; **c)** Invasive solid and cribriform carcinoma; strong pancytoplasmic immunostaining of Myc. DAB ×200; and **d)** Invasive solid and cribriform carcinoma; primary antibody control for Myc. DAB ×200

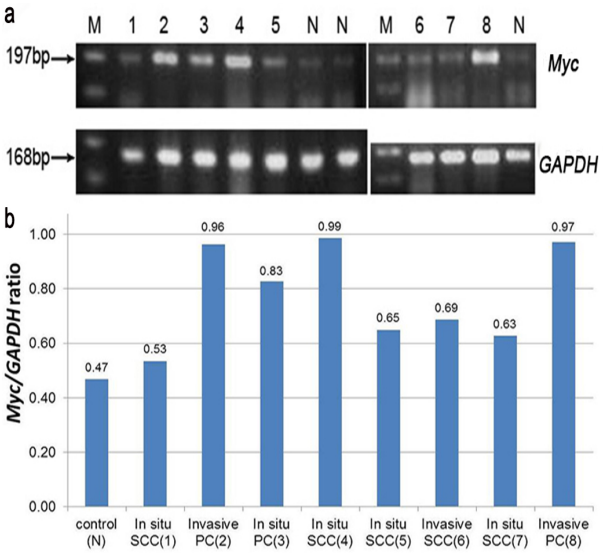

Figure 2. Relative Semi-Quantitative Analysis of Myc mRNA Expression Revealed Upregulation in Tumours. a) Myc and GAPDH PCR products in 1.5% agarose gel. Lane M: molecular marker, Lane 1-8: mammary tumour tissues, Lane N: normal control mammary gland; and **b)** Myc/GAPDH ratio (average of triplicates) in mammary tumour tissues (for Lane 1-8), and normal control mammary gland (average of Lanes N). SCC: solid and cribriform carcinoma, PC: papillary adenocarcinoma

Table 1. Myc Immunolabelling Index in Various MNU-Induced Tumour Types

Tumour type	No. (%)	Index (%±SE)
Normal mammary epithelium	-	56.42±2.7
Hyperplasia	2 (10)	64.08±27.71
Fibroadenoma	1 (5)	86.46±0
In situ solid cribriform carcinoma	7 (35)	86.18±3.67
In situ papillary carcinoma	2 (10)	77.46±2.37
Invasive cribriform carcinoma	4 (20)	89.90±1.01
Invasive papillary carcinoma	3 (15)	83.13±10.19
Invasive tubular adenocarcinoma	1 (5)	90.66±0

Myc. Focal nuclear and cytoplasmic staining was mainly observed in close proximity to the nuclear membrane, especially in cribriform carcinomas. Occasionally, stromal cells including fibroblasts, endothelial cells and infiltrated mononuclear cells also exhibited nuclear immunostaining for Myc. Myc immunolabelling index in various MNU-induced tumour types were presented in Table 1. Among tumour types, mean Myc immunolabelling index in descending order was: invasive tubular adenocarcinoma (90.66%), invasive cribriform carcinoma (89.90±1.01%), fibroadenoma (86.46%), in situ solid cribriform carcinoma (86.18±3.67%), invasive papillary carcinoma (83.13±10.19%), in situ papillary carcinoma (77.46±2.37%), hyperplasia (64.08±27.71%), and normal mammary epithelium (56.42±2.70%). The highest individual Myc index was noticed in an invasive cribriform carcinoma (90.97%). There was significantly difference ($p<0.01$; Student's t-test) between Myc immunolabelling indices of tumour and normal mammary gland tissues. No significant difference ($p>0.05$) was observed between invasive and in situ tumour types.

Semi-quantitative analysis for Myc and GAPDH mRNA expression

Specific amplicons for *Myc* (197 bp) and *GAPDH* (168 bp) genes were visualized in agarose gel. All 8 tumour tissues and 3 control mammary tissues processed for RT-PCR gave specific amplicons for *Myc* oncogene and *GAPDH* housekeeping gene. Densitometric values of RT-PCR product images for *Myc* and *GAPDH* for each sample (triplicates) were expressed as *Myc* to *GAPDH* ratio (Figure 2). In normal mammary gland the ratio of *Myc* to *GAPDH* ranged between 0.42 and 0.51, whereas in tumours it ranged between 0.53 and 0.99. Varied levels of *Myc* mRNA expression were observed in different (*in situ* and invasive) tumour types. Moderately strong positive correlation ($r^2=0.63$) observed between *Myc* mRNA expression and Myc IHC index in tumours.

Discussion

In the present investigation, the expression pattern of *Myc* mRNA and oncoprotein was assessed in normal rat mammary tissues and chemically induced mammary tumours employing semi-quantitative RT-PCR and immunohistochemistry, respectively. High percentage of neoplastic cells expressed Myc oncoprotein in mammary tumours. Different patterns of nuclear and cytoplasmic immunostaining were observed between the tumours and in different areas of the same tumour, with cytoplasmic predominance in some cases. Cytoplasmic Myc localization in addition to heterogenicity in Myc localization has also been observed by other workers (Pavelic et al., 1992; Blancato et al., 2004). Similar observations were recorded by earlier investigators (Royds, 1992; Pawaiya, 2003; 2012) on immunoelectron microscopy for Myc oncoprotein. In the present study, 70-90% of tumour cells and about 50% of normal mammary epithelial cells stained positive for Myc protein. Occasionally, tumour stromal cells also evinced c-Myc-positive nuclear immunostaining. Pietilainen et al. reported that expression of Myc in the stroma is related to the lack of estrogen receptors and to high S-phase fraction (Pietilainen et al., 1995). They also observed that >50% of normal heart cells were positive for Myc immunostaining, but with less intensity. Similarly in the present study, 56% of cells in normal mammary epithelium showed immunopositivity for Myc oncoprotein. McNeil et al. observed 30% of normal breast tissue cells stained positive for Myc with 2+ intensity or greater (McNeil et al., 2006). However, Imaoka et al. found only 30% of MNU-induced rat mammary tumour cells positive for Myc by IHC (Imaoka et al., 2005). Many studies on IHC showed that about 50-100% of breast cancer cases had increased levels of Myc oncoprotein expression (Pavelic et al., 1992; Pietilainen et al., 1995). In one of those reports, 95% of the cases showed positive staining of Myc in the cytoplasm, and only 12% of the cases revealed either nuclear or both nuclear and cytoplasmic staining (Pietilainen et al., 1995). In an excellent meta-analysis by Liao and Dickson, it has been observed that opinion and results on Myc localization, altered expression, and prognostic importance were equivocal (Liao and Dickson, 2000). Myc amplification and/or overexpression has been reported in many human and animal cancers including gastrointestinal (Sánchez-Pernaute et al., 2005), ovarian (Chen et al., 2005), testicular (Sikora et al., 1985), hepatocellular (Wang et al., 2002), rat skin (Pawaiya, 2012) and canine mammary cancers (Inoue and Shiramizu, 1999; Pawaiya and Ramkumar, 2009).

In the present study, all 8 cases (100%) of malignant tumours (invasive and non-invasive) subjected to semi-quantitative RT-PCR revealed varied levels of relative overexpression of *Myc* mRNA when compared to the housekeeping gene (*GAPDH*) in the same sample. Most, if not all, types of human malignancy including breast and other cancers have been reported to have amplification and/or overexpression of this gene, although the frequency of these alterations varied greatly among different reports (Mariani-Costantini et al., 1988; Nesbit et al., 1999; Blancato et al., 2004; McNeil et al., 2006). Even for a given type of malignancy, the frequencies of the alterations of *Myc* at the cytogenetic and expression levels varied greatly from one report to another (Liao and Dickson, 2000). Mariani-Constantini et al. (1988) opined that the overexpression might be related to gene amplification (a large scale mutation). *Myc* gene amplification occurs in approximately 15-20% of patients with breast cancers (Deming et al., 2000). However, mRNA and protein overexpression can occur with or

without gene amplification (Blancato et al., 2004). Normal breast tissue is dominated by fat tissue; it differs greatly from tumour tissue in its epithelial cellularity, and thus it is not a rigorously normal counterpart for comparisons involving mRNA extraction (Liao and Dickson, 2000; Blancato et al., 2004). Overexpression of *Myc* mRNA in tumour tissues might account for the *Myc* gene expression not only in tumour cells, but also in stromal cells including lymphocytes, macrophages and fibroblasts (Masramon et al., 1988; Blancato et al., 2004). In the present study too stromal cells in few cases showed diffuse nuclear and cytoplasmic staining on IHC for Myc. Thus, heterogeneous population and contamination by normal cells might mask the real degree of amplification. For, these reasons fold difference in *Myc mRNA* expression in tumours compared to normal mammary gland was not calculated. Moreover, Myc has several functions in a cell; the role(s) which it plays in a normal cell might be entirely different from what it does in a neoplastic cell. So, direct comparison of the *Myc*/*Myc* activity or expression in normal mammary gland and tumours needs rethinking. Estrogen receptor (ER) deregulation plays a critical role in breast cancer development and progress, and targeting ER with selective ER modulators (SERMs) has achieved significant reduction of breast cancer incidence in women at high risk for breast cancer. However, not all breast cancers are amenable to SERMs, because 30-40% of the tumours are ER- negative (Shen and Brown, 2003) and 20-30% of ER-positive and all ER-negative breast cancers failed to be prevented by SERMs (Fisher et al., 1988). Myc is one of the targets of ER-α and is the key effector gene for estrogen action (Kininis and Kraus, 2008). Myc overexpression is known to occur in both ER-positive as well as negative breast cancers. Hence, an effective drug target against Myc or its downstream target might be useful in both ER-positive and ER-negative breast cancers, among other cancer types with Myc overexpression. It is believed that tumours remain dependant or addicted to the activities of oncogenic pathways that drive tumorigenesis (Luo et al., 2009). However, many oncogenes such as *Ras* and *Myc* have proven difficult to be inhibited pharmacologically, highlighting the need for complementary approaches (Kessler et al., 2012). An unusual property of *Myc* gene is that the antisense strand of the gene also yields transcripts (Spicer and Sonenshein, 1992), which needs to be considered while designing RNAi strategies against *Myc*. Nevertheless, *Myc* inactivation or blocking strategies to combat various cancers have shown encouraging results *in vitro* (Wang et al., 2005; Hongxing et al., 2008; Zhang et al., 2009; Kessler et al., 2012), and *in vivo* models (Jain et al., 2002; Shachaf et al., 2004; Wang et al., 2005; Zhang et al., 2009). Soucek and Evan (2010) concluded in their review of *Myc* biology that systemic *Myc* inhibition is possible without affecting homeostasis in normal resting and proliferating tissues, and *Myc* is a supreme choice of cancer therapeutic target. Transformation by Myc depends on several additional protein-protein interactions, besides the interaction with Max protein (Oster et al., 2002). Kessler et al. provided insight into how Myc's oncogenic activity might be suppressed by suppressing non-oncogenic proteins whose functions help Myc to transform cells (Kessler et al., 2012). So far, a considerable attention has been paid to the human breast cancer cell lines and mouse mammary tumour models for modelling human breast cancer, despite the fact that cell lines do not represent true tissue environment and, the majority of the mouse lesions are alveolar, while in humans and rats they are predominantly ductal (Thompson et al., 1985). The tumour incidence (46.15%) and frequency (1.5/rat) observed in this study is relatively low (Thompson and Adlakha, 1991) owing to seasonal influence. The study was conducted during the period of winter months. Seasonal influence is known to affect pineal melatonin production and immune function in rodents, thus modulating carcinogenesis (Loscher et al., 1997; De Jonage-Canonico et al., 2003; Sumova et al., 2004). A substantial decrease in tumour frequency in winter (2.6/rat) as compared to the observation in summer (4.56/rat) was previously reported (Kubatka et al., 2002).

In conclusions, Myc is an oncogenic nuclear transcription factor that is frequently deregulated in many human and animal cancers. The results of the present study showed that MNU-induced rat mammary tumours overexpress *Myc*/Myc. Preliminary data in this study showed upregulation of *Myc*/Myc in MNU-induced rat mammary tumours. A large scale study is needed to reassure Myc expression pattern in MNU-induced mammary tumours. If found suitable, this experimental mammary tumour model can be effectively used as an *in vivo* model to study the anti-cancer strategies directed against Myc or its downstream targets at the transcriptional or post-transcriptional level. The same model can also be used to study any plausible off-target effects in normal resting or proliferating cells in adult tissues caused by global *switching off* of *Myc* activity. Further studies to elucidate the mechanism of overexpression of *Myc*/Myc in chemical-induced rat mammary tumour models are warranted before attempting therapeutic studies.

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