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Short Communication

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Doxycyclin induces p53 expression in SaOs (osteosarcoma) cell line

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ABSTRACT

The p53 tumour suppressor gene plays an important role in preventing cancer development. This study determined if p53 can be induced in osteosarcoma cell line upon treatment with Doxycyline. It was demonstrated that induction of p53 resulted in the inhibition of the cyclin E/CDK2 complexes by the activation of p21. This mechanism could potentially therefore represent an important component of the p53 tumor suppressor pathway.

Keywords: Tumor suppressor, oncogene, mdm2, cyclinE, apoptosis

INTRODUCTION:

The p53 tumour suppressor gene plays an important role in preventing cancer development and loss of p53 function, or loss of the ability to activate a p53 response, appears to be a pre-requisite for malignant progression. In both mice and humans, germ line mutations in p53 result in a strong predisposition to cancer (Lozano and Zambetti, 2005). The mechanisms by which p53 functions to afford us this protection appear to be related to its ability to respond to stress and contribute to either the repair of stress-induced damage or the inhibition of further proliferation of stressed cells. In this way, disparate signals that could constitute oncogenic danger - such as oxidative stress, DNA damage, hypoxia, oncogene activation or loss of normal stromal support - all lead to the induction of a p53 response (Vousden, 2002). However, the ultimate response to p53 can be quite different, ranging from a reversible cell cycle arrest to the induction of a number of irreversible responses, such as cell death or senescence. This dramatic distinction in the outcome of p53 activation - death or survival - leads to obvious questions of how the choice of response is regulated and why p53 initiates these different responses. To some extent, the life or death of the cell is strongly influenced by the presence or absence of p53-independent death or survival signals that cooperate with the p53-activated responses. Probably the best understood activity of p53 is as a transcription factor that has sequence-specific DNA-binding activity and the potential to induce the expression of a large number of genes. Although bioinformatics studies suggested that there may be >4000 human genes that contain p53-binding sites (Lu, 2005), direct analysis using various

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chromatin-immunoprecipitation-based techniques have more recently placed this number between 500 and 1600 genes (Wei et al., 2006; Cawley et al., 2004) - still a daunting proposition. Key to the regulation of p53 ubiquitylation is MDM2, an ubiquitin ligase that is an essential negative regulator of p53 (Vousden, 2002). The amount of MDM2 available appears to be critical in determining the outcome: mono-ubiquitylation of p53 by low levels of MDM2 allows nuclear export, whereas higher levels of MDM2 result in poly-ubiquitylation and degradation of p53 by the proteasome (Li et al., 2003). The ability of different polymorphic forms of p53 to bind MDM2 and be exported has been linked to the efficiency of apoptotic activity (Dumont et al., 2003), which suggests that the regulation of export may help balance cell cycle arrest (which appears to be primarily due to transcriptional activity of p53) with apoptosis.

This work was undertaken to determine if p53 is induced in SaOs cell line upon treatment with doxycycline and also to determine if up-regulation of p53 results in the activation of p21 and subsequent down-regulation of cyclinE.

MATERIALS AND METHODS

Cell treatment

Cells were seeded in 100 mm dishes and grown for 24 hours to reach 20% or 30% confluence. Then, Doxycycline was added to a final concentration of 800µM. Cell lines were maintained in RPMI medium supplemented with 10% fetal bovine serum and grown to about 90% confluence for 48 hours, harvested and stored at -20.

RNA extraction and reverse transcription-PCR

RNA was extracted with RNeasy (Qiagen) with DNase treatment according to manufacturer's instructions. The integrity and quality of RNA was checked by agarose gel electrophoresis and absorbance reading at 260 nm. The purity was also determined according to standard procedures. RT-PCR was carried out with One Step (Qiagen) using gene-specific primers and programmes.

Preparation of Whole Cell Lysates

Lysis Buffer

(NP-40 Lysis): 150 mM $NaCl_2$, 50 mM Tris (pH 8.0), 1% NP-40 {containing Protease Inhibitor Cocktail (Table 1)}.

Procedure

Cell pellets were incubated on ice with 250 µl of lysis buffer for 30mins, vortexing after every 5 mins. The solution was spun at 14,000 rpm for 10mins and the supernatant transferred to a fresh tube. The total protein concentration was determined by the Bradford assay.

Western Blotting

Cell lysates were resolved by 12% SDS-PAGE and blotted on a polyvinylidene difluoride membrane, blocked and incubated with appropriate antibodies, then washed with PBS-T and incubated with horseradish peroxidase-labelled secondary antibody. Specific immunoreactive bands were detected with enhanced chemoluminiscence (Pierce).

RESULTS

The results obtained by RT-PCR showed up-regulation of p53 upon treatment with 800 µM doxyxycline. No p53 expression was observed with the untreated control (Figure 1). There was no significant difference in the expression of β-actin which was used as loading control. Western blot analysis also revealed that the treated cells showed an up-regulation of p53 and a corresponding up-regulation of p21 and subsequent down-regulation of cyclinE (Figure 2).

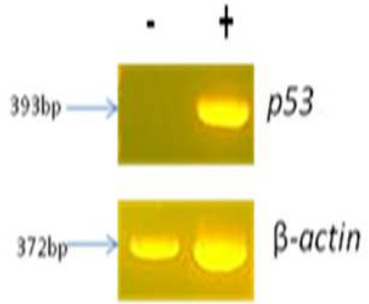

Figure 1: RT-PCR showing p53 expression in SaOs cell line.

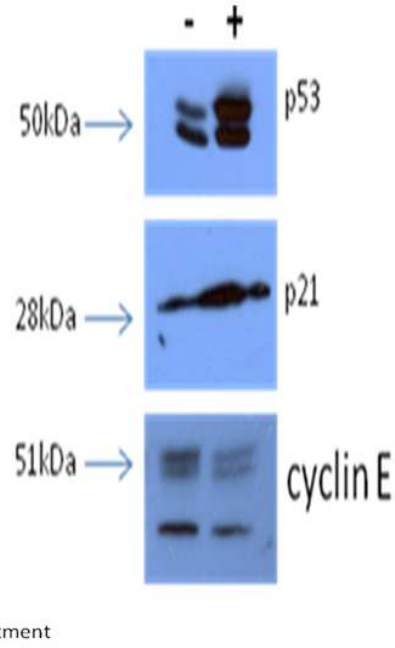

Figure 2: Western blot analysis.

Table 1: Protease Inhibitor Cocktail.

Protease Inhibitor	Stock Concentration		Final Concentration
Aprotinin	1 mg/ml	add 5 µl/ml	5 µg/ml
Leupeptin	1 mg/ml	5 µl/ml	5 µg/ml
PMSF	10 mg/ml	50 µl/ml	500 µg/ml
Na-ortho-vanadate	100 mM	2 µl/ml	0.2 M
NaF	1 M	10 µl/ml	0.01 M

DISCUSSION

In this report, it was demonstrated that induction of p53 can result in inhibition of the cyclin E/CDK2 complexes by activation of p21. Cyclins have previously been found to be an indirect target of p53. For example, the p53-inducible protein PC3 was shown to repress cyclin D transcription in NIH 3T3 cells (Guardavaccaro et al., 2000). Inhibition of cyclin E level is consistent with the tumor suppressor activity of p53 since both are proto-oncogenes. Cyclin E functions in the mid-G1 phase of the cell cycle where, in conjunction with its cyclin-dependent kinase partner CDK2, it acts to inhibit the retinoblastoma tumor suppressor (Harbour and Dean, 2000). Consistent with this proliferative function, cyclins are found to be over-expressed in most human breast cancers (Weinstat-Saslow et al., 1995). Our results indicate that Doxycycline treatment of SaOs cell line induces the expression of p53 leading to the activation of p21 and subsequent down-regulation of cyclinE. This mechanism could

potentially therefore represent an important component of the p53 tumor suppressor pathway.

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