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Suppression of tumourogenicity and overexpression of cell adhesion molecules in AK-5 cells transfected with wild-type p53 gene

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Abstract. We have previously shown overexpression of p53 and rearrangement of the p53 gene in AK-5 tumour. In order to study the role of p53 in AK-5 tumourogenicity, we introduced wild-type p53 in AK-5 cells. We have shown suppression of tumourogenicity in AK-5 cells after transfixion with wt p53. In one of the transfected clones 3B4, there was complete loss of tumour forming ability. Clone 3B4 also showed cellular aggregation which correlated well with the higher expression of cell adhesion molecules like fibronectin and hyaluronectin. These observations demonstrate tumour suppression in AK-5 after introduction of wt p53.

Keywords. wt p53; fibronectin; hyaluronectin; tumourogenicity.

1. Introduction

The p53 gene which was considered to be directly involved in the process of malignant transformation few years ago (Eliyahu *et al* 1984; Jenkins *et al* 1984) has now been shown to behave like a tumour suppressor gene (Finlay *et al* 1989; Chen *et al* 1990). Mutated form of p53 is overexpressed in murine as well as human cancers (Hollstein *et al* 1991) whereas, wild-type (wt) p53 protein has been shown to suppress transformation of cells in culture (Finlay *et al* 1989) and block the development of tumours in nude mice (Chen *et al* 1990). Wild-type p53 has been shown to bind specifically to DNA sequences and act as transcription factor (Farmer *et al* 1992), however this function is lost in mutated form of p53 (Farmer *et al* 1992; Bargonetti *et al* 1991). Wild-type p53 has also been shown to be involved in the repair mechanisms of damaged DNA in the cells (Maltzman and Czyzyk 1984) and induce the cells that cannot repair its damaged DNA into apoptotic pathway (Yonish-Rouach *et al* 1991), thus helping the system in the elimination process of abnormal cells.

Similarly, cell adhesion molecules have been shown to be directly involved in malignancy (Ruoslahti 1984). Tumour cells have been shown to present a number of adhesive abnormalities which contribute significantly to their ability to invade (Ruoslahti 1984; VanRoy and Mareel 1992). Gene transfer experiments demonstrated a direct link between the expression of integrins (Giancotti and Ruoslahti 1990), cadherins (Vleminckx *et al* 1991) and tumour invasion. Molecular characterization of adhesion molecules has established an important role of cell adhesion in tumour cell invasion and metastasis.

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AK-5, which is a rat macrophage tumour (Khar 1986) has been shown to overexpress mutated form of p53. We have also shown rearrangement of the p53 gene in AK-5 (Deshpande *et al* 1990). In order to understand the role of p53 in AK-5, we have introduced wild-type p53 gene into AK-5 cells and studied the tumourogenic potential of the transfected clones. In addition, we have also shown overexpression of cell adhesion molecules like fibronectin and hyaluronectin in the transfected clone, suggesting possible reversal of the phenotypic characteristics.

2. Materials and methods

2.1 *Maintenance of cells*

AK-5 tumour cells are maintained as ascites in the peritoneal cavity of Wistar rats (Khar 1986). However, to avoid cellular heterogeneity, we have used a single cell clone of AK-5 (BC-8 clone) for this study which was adapted to grow in culture (Khar and Mubarak Ali 1990). AK-5 cells were grown in DMEM containing 10% FCS and a mixture of penicillin and streptomycin.

2.2 *DNA transfections*

Cells (2×10^6) were co-transfected with 2·5 μg of pSV2 neo and 22·5 μg of linearized p53 plasmid p11-4, electroporation method (Stopper *et al* 1987). Cells were subjected to 6 pulses of pulse strength 150 volts with a 5 μs pulse duration using Biojet cell fusion/electroporation apparatus. After electroporation, the cells were incubated for 15 min at 37°C, transferred to resealing medium and incubated further for 20 min, Cells were washed, suspended in DMEM—FCS and plated in 96 well plates. 24 h later, Geneticin (G418) was added (400 μg/ml) and the cells were selected for 14 days. The surviving clones were expanded in 24 well plate and culture flasks.

2.3 *Tumourogenicity assay*

Tumourogenic potential of AK-5 cells and the p53 gene transfected clones was assessed by injecting 1×10^6 cells intraperitoneally in 4-6 week old Wistar rats from the inbred colony of this laboratory. Appearance of the peritoneal bulge and the presence of viable AK-5 tumour cells in the peritoneal lavages followed by the death of the host denoted positive tumour growth. However, absence of all the above parameters denoted suppression of tumour growth.

2.4 *Southern hybridization*

DNA from control cells and the transfected clones was isolated by phenol: chloroform extraction procedure. DNA samples (10μg) were digested with *EcoRI* for 12 h and fractionated on 1 % agarose gel. The digested DNA was transferred onto nylon membrane and the blot was hybridized with the insert (1·7 kb) obtained from wild-type p53 cDNA containing plasmid p11-4 (Kelekar and Cole 1986). The blots were washed with 0·2 × SSPE/0·1% SDS at 65°C and exposed to X-ray film at — 70°C.

2.5 *Isolation of RNA and Northern hybridization*

Total cellular RNA was isolated by guanidinium thiocyanate—phenol—chloroform method (Chomozynski and Sacchi 1987). RNA preparations (20 μg/lane) were subjected to electrophoresis on 1% agarose gels containing 2% HCHO. The fractionated RNAs were transferred to nylon membrane in 50 mM NaOH. The blots were hybridized with the p53 cDNA insert. The blots were washed to a final stringency of 0·2 × SSPE/0·1% SDS at 65°C and exposed to X-ray film at — 70°C.

2.6 *Immunoprecipitation*

Cells were washed and suspended in phosphate-free DMEM containing 2% dialysed FCS. Carrier-free [^{32}P] orthophosphoric acid (150 μCi/ml) was added to the cultures and incubated for 6-8 h. Cells were washed thoroughly, extracted with extraction buffer and immunoprecipitated (equal number of TCA precipitable counts) with monoclonals 421 and 246 specific for p53. Immunoprecipitates were separated by adding protein A-Sepharose and the precipitated proteins were fractionated by SDS-PAGE and exposed to X-ray film at — 70°C.

2.7 *Western blotting*

Cells (1×10^6) were washed thoroughly with PBS and the pellet was boiled in 200 μl of electrophoresis sample buffer. Samples (50 μ1) were fractionated by SDS-PAGE and the proteins were transferred to nitrocellulose membrane using Novoblot apparatus (LK B). The blot was blocked with 3% BSA and probed with rabbit anti-hyaluronectin (l:1000) for 1 h. The blot was washed and treated with AP-conjugated anti-rabbit Ig (1:5000) for 30 min and developed with BCIP—NBT substrate.

2.8 *Flow cytometer*

Washed cells ($1\text{-}2 \times 10^6$) were incubated with FITC-conjugated anti-fibronectin (1:200, Serotec, UK) for 1 h at 4°C. The cells were washed and analysed on a FAC Star Plus flow cytometer.

3. Results

3.1 *Transfection of AK-5 with p53 gene*

The p53 gene organization in AK-5 and BC-8 clone was similar as checked by Southern hybridization (results not shown). BC-8 clone of AK-5 tumour was transfected with wild-type p53 gene by electroporation. After selection with Geneticin, the clones were subjected to Southern hybridization and 3 clones which showed additional bands (figure 1) were expanded. Expression of p53 message in these clones was tested by Northern hybridization. Clones 3B4 and 2C4 showed higher expression of the p53 message when compared to the parent clone BC-8 (figure 2).

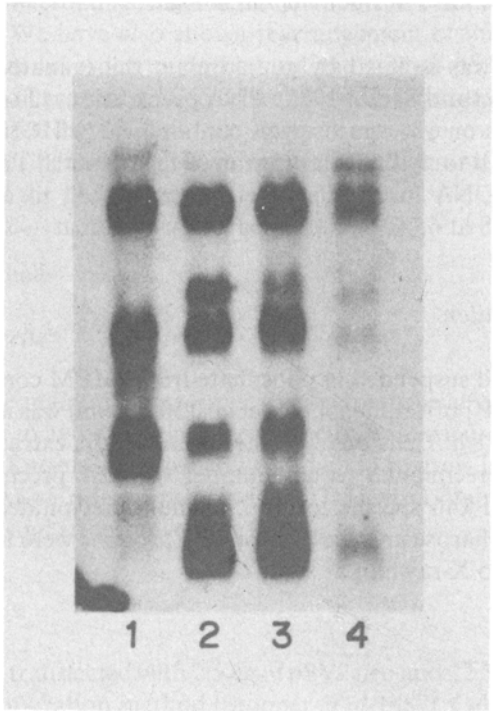

Figure 1. Southern blot analysis of BC-8 and wt p53 transfected clones. Lanes: 1, BC-8 control; 2, 2C4; 3, 3CI; 4, 3B4. Transfected clones show additional bands.

3.2 *Production of p5.3 by transfected clones*

Clone 3B4 was analysed for the production of p53 protein by immunoprecipitation reaction using p53 specific monoclonals 421 and 246. Clone 3B4 expressed higher levels of p53 compared to the parent cell line BC-8 (figure 3B). Clone 3B4 makes wild-type p53 which is recognized by mAb246. Clones 2C4 and 3C1 did not produce large amounts of p53 as determined by immunoprecipitation (data not shown).

3.3 *Suppression of tumourogenicity*

In order to study the effect of wild-type p53 on the tumourogenicity of AK-5 cells, *in vivo* studies were carried out. Syngeneic breed of Wistar rats were injected intraperitoneally with 1×10^6 cells from different clones (table 1). Animals which had received the control cells i.e., BC-8 died by day 12, however, the p53 transfected clones were resistant to tumour growth. Clones 3B4 and 2C4 showed 100% and 93% survival respectively, whereas clone 3C1 showed only 60% survival. These observations suggest suppression of tumourogenic potential of AK-5 cells especially in clone 3B4, after introduction of wild-type p53 gene.

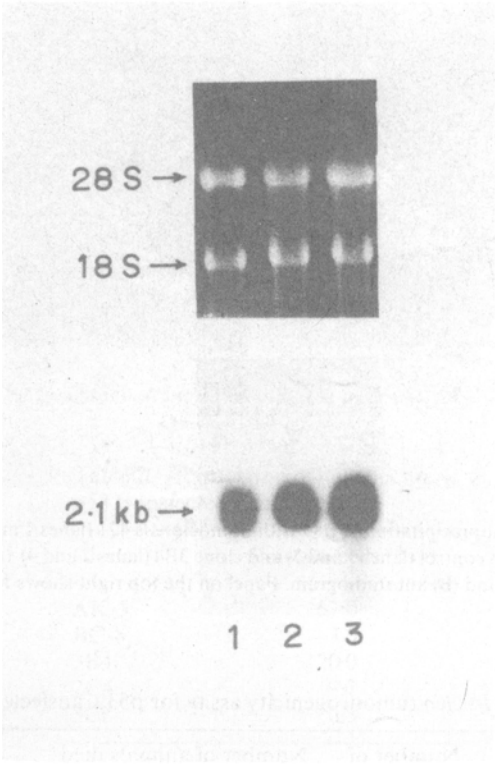

Figure 2. Expression of p53 message in the transfected clones. Lanes: 1, BC-8 control; 2, 2C4; 3, 3 B4. Top panel shows the loading and integrity of RNA samples.

3.4 *Expression of cell adhesion molecules by p53 transfected clone*

During the expansion of clones after selection with Geneticin, clone 3B4 was observed to form cell aggregates (figure 4B) which were not seen for other clones and the BC-8 cells (figure 4A). The transfected clones were analysed for the presence of fibronectin, hyaluronic acid binding protein, hyaluronectin. Clone 3B4 had the highest percentage of fibronectin positive cells, whereas 3C1 had the lowest number of cells positive for fibronectin when compared with the parent clone BC-8 (table 2). AK-5 which is a heterogeneous population (Pande and Khar 1988) showed 67% fibronectin positive cells. Expression of hyaluronectin was analysed by Western blotting after loading of equal amounts of protein in the gel, clone 3B4 showed the highest levels of hyaluronectin (figure 5). These observations suggest the possible role of fibronectin and hyaluronectin in inducing cell adhesion in clone 3B4.

4. Discussion

Recent studies have shown that cancer cells do not express a normal p53 protein, because of both alleles being inactivated by a combination of single allele deletion and

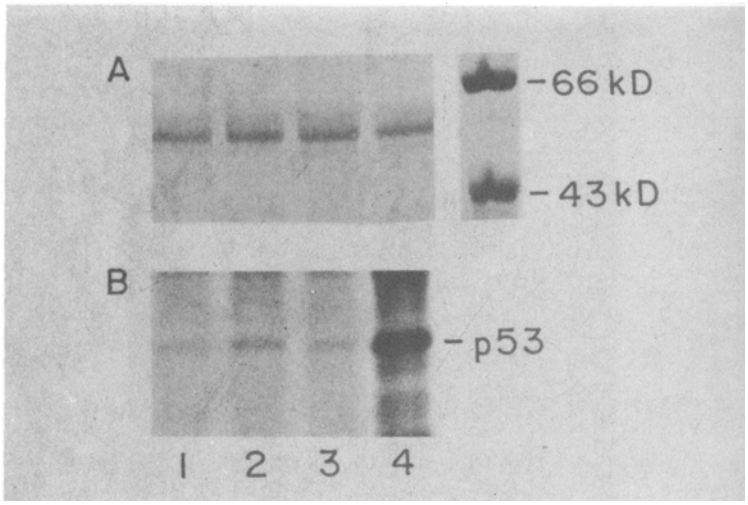

Figure 3. Immunoprecipitation of p53 with monoclonals 421 (lanes 1 and 2) and 246(lanes 3 and 4) from BC-8 control (lanes 1 and 3) and clone 3B4 (lanes 2 and 4). (**A**) Silver stained gel after SDS-PAGE and (**B**) autoradiogram. Panel on the top right shows MW markers.

Table 1. *In vivo* tumourogenicity assay for p53 transferred clones[a].

Clone	Number of rats rejected	Number of animals died/ survived	Survival(%)
AK-5[b]	15	15/0	0
BC-8[b]	15	15/0	0
3B4	15	0/15	100
2C4	15	1/15	93
3C1	15	6/15	60

[a]1×10^6 cells were injected i.p. and the animals were monitored up to 60 days after transplantation.
[b]Control animals died by day 12.

point mutation of the remaining allele (Nigro *et al* 1989). We have also shown rearrangement in one of the alleles of p53 gene in AK-5 tumour cells (Deshpande *et al* 1990). The present study was aimed at studying the role of p53 (mutated) in maintaining the tumourogenic potential in AK-5 cells. It is not clear as to how the mutated form of p53 protein contributes to tumourogenesis.

Investigators have found it difficult to express wild-type p53 in transformed cells because the continuous presence of this protein in these cells was selected against. This problem was overcome by using the regulatable p53 expression vectors (Michalovitz *et* al 1990). In our system, we have been successful in obtaining stably transfected clones which express high quantities of p53 that could be immunoprecipitated with mAb246, which is shown to recognize wild-type p53 in mice (Finlay *et al* 1988). In one of the clones, 3B4, the overexpression of p53 was concomitant to total suppression of tumourogenicity in these cells (table 1).

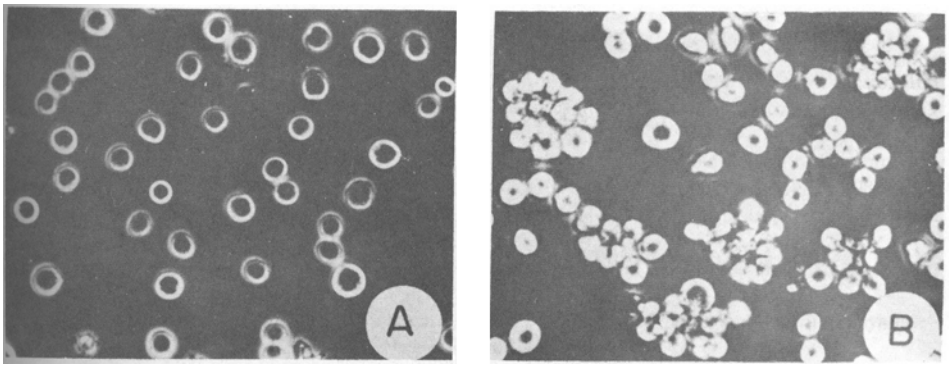

Figure 4. Aggregation of clone 3B4 cells. (**A**) BC-8 control, **(B)** 3B4. (X 150).

Table 2. Expression of fibronectin by p53 transfected clones".

Clone	Positive cells (%)
AK-5	67·0
BC-8	3·5
3B4	20·0
2C4	9·7
3Cl	0·3

[a]2×10^4 cells were analysed by flow cytometry.

Using p53 transfected clones, a number of p53-mediated activities are being identified. A variety of tumour cells have been shown to be growth arrested when transfected with p53 tumour suppressor gene (Casey *et al* 1991) and these cells are blocked in G1-S transition phase (Mercer *et al* 1982). These effects are seen only when the transformed cells possess mutated p53. No effect on *in vitro* growth was observed if the cancer cell possessed endogenous wild-type p53 (Casey *et al* 1991). Cancer cells have also been shown to undergo apoptosis when they are exposed to high levels of wild-type p53 (Yonish–Rouach *et al* 1991). Similar observations have been made by us where p53 transfected clones show higher levels of apoptosis than the parent cell line (data not shown).

In addition to the suppression of tumourogenicity by p53, we have also shown overexpression of cell adhesion molecules which induce cellular aggregation. Clone 3B4 shows high levels of fibronectin on the cell surface and also overexpression of hyaluronectin which has been shown to be involved in cell adhesion. AK-5 has previously been shown to possess both fibronectin (Pande and Khar 1988) and hyaluronectin (Gupta and Dutta 1991), the levels of which probably are not sufficiently high to induce cell aggregation. However, the fact that clone 3B4 which has lost its tumourogenic potential shows significantly higher levels of both fibronectin and hyaluronectin indicates a possible inverse relationship between loss of tumourogenicity and cellular adhesion in this clone. The role of wt p53 in the induction of

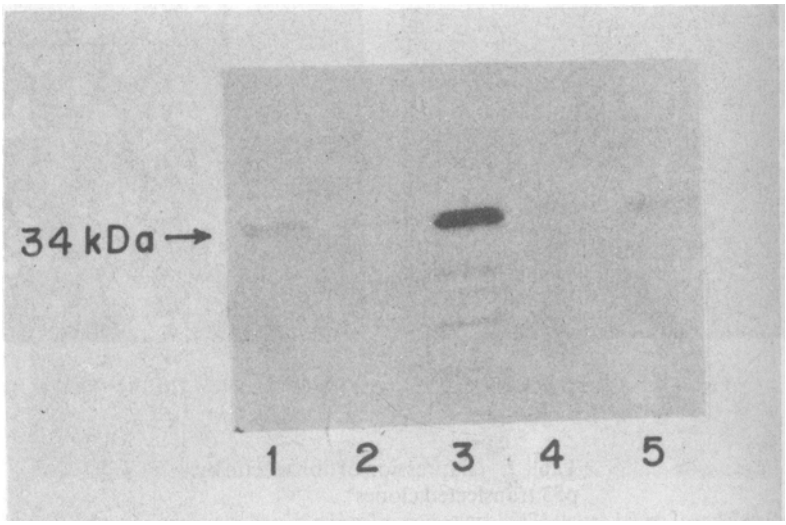

Figure 5. Expression of hyaluronectin by p53 transfecied clones. Western blot analysis of 1, BC-8 control; 2, lymphocytes; 3, 3B4; 4, 3C1 and 5,2C4 proteins.

hyaluronectin is not clear at this stage. The wt p53 may have a direct effect on the expression of hyaluronectin or alternatively the integration of p53 vector in the clone 3B4 somehow activates the expression of hyaluronectin. The overexpression of hyaluronectin/ fibronectin could also be consequence of the transfection-associated genome rearrangement in 3B4. Loss of cell surface fibronectin (Hynes 1973) and other extracellular matrix constituents like lamin, collagens and proteoglycans (Martin and Timpl 1987) on oncogenic transformation have been previously reported. The loss of fibronectin is closely related to the reduced cellular adhesion and loss of cytoskeletal organization typical of tumour and transformed cells (Hynes 1990). However, restoration of cell adhesion and contact inhibition was demonstrated in a variety of transformed cells by addition of cell surface protein from normal cells (Yamada *et al* 1976; Ali *et al* 1977). Thus our observations suggest a possible role for fibronectin and hyaluronectin in the aggregation of clone 3B4 cells. However, the link between the expression of wild-type p53 and overexpression of cell adhesion molecules in AK-5 is not clear at present.

Our present observations suggest a significant role for p53 in the maintenance of tumourogenicity in AK-5 cells and assign an important function to wild-type p53 in suppressing the malignant phenotype of AK-5 cells.

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