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Profile of p53 expression in bladder and oral tumours: effects of *in vitro* manipulations of p53 on the behaviour of established human tumour cell lines

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Abstract

In this investigation the profile of p53 and epidermal growth factor receptor (EGFR) expression in tumour tissue biopsies of transitional cell carcinoma of bladder (TCC) and of oral-pharyngeal carcinoma (OP) were compared using an immunocytochemical staining method. In addition, various techniques including sodium dodecyl sulphate-polyacrylamide gel elecrophoresis (SDS-PAGE), colorimetric assay and gene transfection were used to investigate the influence of p53 on the behaviour of human tumour cell lines *in vitro*. The results showed that: (a) p53 was detectable in more than 45% of cases in both tumour types, although the profile and intensity of expression differed. (b) Concomitant strong expression of EGFR and p53 for TCC and OP was 21% and 38% ($P > 0.05\%$), respectively. (c) Treatment of tumour cells by either gamma radiation or by cisplatin resulted in the induction of p53 independent of the origin of the tumour. (d) Susceptibility of two cell lines, one with and one without constitutive expression of p53 showed that the expressing cells were more sensitive to gamma radiation (the percentage inhibition at 250 cGy was 57% versus −15%, $P < 0.01$), and also cisplatin (the percentage inhibition at 1 μg/ml was 71.0±6.0 versus 2.6±7.0, $P < 0.001$). (e) Transfection of wild-type *TP53* gene into a bladder tumour cell line resulted in a rapid cell apoptosis (by as much as 90%) whereas cells receiving mutated *TP53* survived. A similar frequency of *TP53* mutation in TCCs and OPs was observed. In addition, the pattern of p53 expression within the squamous type of TCC was similar to that in OPs. If the data from the *in vitro* studies could be translated into an *in vivo* setting, one could envisage a situation where the introduction of wild-type *TP53* gene by gene transfection into tumour cells (independent of their *TP53* gene mutational status), would prove to be beneficial. If the cellular *TP53* gene is mutated, then an introduction of the normal *TP53* gene would induce cells to undergo apoptosis. Alternatively, if *TP53* is wild-type, then the increased levels of p53 expression would enable the cells to become more susceptible to DNA damaging treatments such as cisplatin or gamma radiation.

Keywords: p53; Gene transfection; Radiation; Bladder tumour; Head and neck tumour

1. Introduction

The pivotal roles of the tumour suppressor gene *TP53*, of *Bcl-2* and other related molecules, in determining the balance between cell proliferation and cell death (apoptosis) are well established [1]. The *TP53* gene is thought to be the most frequently mutated gene in human malignancies, and in some cases, the extent of mutation has been shown to correlate with poor prognosis and response to chemotherapy or radiotherapy [2–7]. The product of the *TP53* gene is not normally expressed or only expressed at extremely low levels. However, following exposure of cells to DNA-damaging stimuli, there is a rapid induction of p53, which induces a mitotic arrest of cells allowing them time to either repair the damaged DNA or to apoptose [8]. In addition, when in mutated form, the half-life of p53 is increased leading to its accumulation within cells.

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A previous report from this unit using the monoclonal antibody (MAb) 240, showed a strong expression of p53 in tissue biopsies in more than 80% of patients with testis cancer, especially in seminomas [9]. This taken together with the report that *TP53* is not mutated in these tumours led to the hypothesis that the overexpression of non-mutated p53 [10] may be a determining factor in their exquisite sensitivity to chemo- and radiotherapies.

Unlike testis tumours, a high frequency of *TP53* mutation has been reported within both bladder and oral tumours. In bladder, Watanabe and colleagues [11] demonstrated that the mutation status of *TP53* correlated with both poor prognosis and poor survival rate while Sidransky and colleagues [12] argued that this was a good predictor of the invasiveness of tumours. In separate studies, Esrig and colleagues [3] and Sarkis and associates [13] reported that patients whose tumour had poor prognosis overexpressed p53 and suggested that this could be used as a marker for the selection of patients for more aggressive chemotherapy regimens. This idea was extended by Dalbagni and associates [14] who argued that the response of TCC patients to immunotherapy was determined by their *TP53* status. Thus, patients showing no p53 before Bacillus Calmette-Guerin (BCG) and remaining negative after the treatment responded more favourably than those expressing p53 prior to BCG treatment and remaining positive thereafter. In three separate studies, mutation of *TP53* has also been shown to be a frequent event in oral-pharyngeal carcinomas (OPs) [5,15,16].

These observations have led many investigators to introduce the wild-type *TP53* gene into tumour cells with mutated *TP53* as a way of causing the cells to apoptose. Marki and colleagues [17] showed that the introduction of wild-type *TP53* gene into bladder tumour cell lines resulted in cell apoptosis. Similarly, Clayman and colleagues [16] and Liu and colleagues [18] showed the same effect using OP tumour cell lines. Fujiwara and colleagues [19] reported that such an introduction also increased tumour cell sensitivity to DNA-damaging agents such as cisplatin.

The aim of this investigation was to compare the pattern of p53 expression in TCC and OP tumours and correlate this with the expression of epidermal growth factor receptor (EGFR). In addition, *in vitro* experiments involving gene transfection, exposure to gamma irradiation and cisplatin were performed to study the effects of p53 modulation on tumour cell line behaviour.

2. Materials and methods

There were 34 cases of bladder cancers of which 21 were superficial, 10 invasive and 3 squamous cell carcinomas. Among the 21 superficial cases, 7 were new and the rest were recurrent tumours. The invasive cases were all recurrent tumours. All 24 cases of OP were of the invasive type.

2.1. Cell lines and monoclonal antibodies

The Fen and Wil bladder tumour cell lines were in-house established lines from patients with superficial bladder cancers [20]. The SKV14 (SV40 transformed foreskin epithelial cell line), 5637 (bladder), Scaber (bladder), MCF-7 (breast), Tera II (testis) and Ish (endometrial) were obtained from the American Type Culture Collection (ATCC). All the cell culture work was carried out in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal calf serum (FCS) and incubated in a 5% humidified CO_2 incubator at 37°C for 48 h. LP34 (anti-keratin) and EGFR.1 MAbs were obtained from the Imperial Cancer Research Fund (ICRF). DO1 (anti-p53 antibody) was a kind donation from D. Lane, Dundee, UK. Ki67-specific MAb was purchased from Dako, Denmark. All purified MAbs were used at a 1/50 dilution. Prior to screening all MAbs were titrated and in each experiment non-specific MAbs were used as negative controls.

2.2. Immunocytochemical staining technique

Surgically removed specimens from patients were snap frozen and placed in liquid nitrogen. Tissue sections were cut (6 mm), air-dried, acetone fixed and after hydration, were used for immunostaining as described in [21] using the peroxidase–anti-peroxidase staining technique.

2.3. SDS-PAGE

Cells were washed with sterile phosphate buffered saline (PBS), trypsinised and resuspended in 2 ml RPMI medium supplemented with 10% FCS. The cells were centrifuged at 1500 rpm (using MSE centrifuge, model 1.78) for 5 min. The pellet was washed twice in PBS and a total cell count determined using the trypan blue exclusion method. Once the total cell number had been determined, the cells were incubated in 0.5% Nonidet P40 (NP40) lysis (30 μl per 1×10^6 cells) buffer at 4°C for 30 min. The resulting sample was centrifuged at 15 000*g* for 15 min at 4°C, and the unsolubilised material removed. The total protein concentration of each resulting lysate was determined by the bicinchoninic acid protein assay [22]. The protein concentration was adjusted to 800 μg/ml by diluting with PBS/%azide. Eight micrograms of protein was then loaded onto 1 cm^2 squares of activated immobilon-P nitrocellulose paper. The standard method of the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (10% w/v) was used and protein at 40 μg/lane was loaded onto a gel and run at a constant current of 20 mA.

Table 1
Expression profile of various molecules in two human solid tumours

Molecule	TCC			OP		
	Strong *n* (%)	Other *n* (%)	Negative *n* (%)	Strong *n* (%)	Other *n* (%)	Negative *n* (%)
Keratins	34/34 (100)	0/34	0/34	24/24 (100)	0/24	0/24
EGFR	12/34 (35)	22/34 (65)	0/34	20/24 (83)	4/24 (17)	0/24
p53	8/34 (24)	12/34 (35)	14/34 (41)	6/24 (25)	5/24 (21)	13/24 (54)

Results of immunocytochemical staining are shown. Other denotes cases where the expression was either weak or only expressed in a proportion of the tumour cells. TCC, transitional cell carcinoma; OP, oral-pharyngeal carcinoma.

The transfer was carried out at a constant current of 200 mA for 2 h onto an immobilon-P membrane. After transfer, the membrane was washed with buffer and used for immunostaining.

2.4. Colorimetric (MTT) assay

The MTT (3-[4,5-dimethyziol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was carried out as described in [20] to determine cellular metabolic activity directly proportional to the number of live cells. Cells were cultured in microtitre plates after exposure to 125 and 250 cGy units of gamma irradiation. Three hours prior to termination of the experiment, 10 μl of MTT (5 μg/ml) was added to each well and the plate was incubated at 37°C in a 5% humidified CO_2 incubator. The medium from the plate was then discarded by flicking off and replacing it with 100 μl of acidified isopropanol. The plate was then left at room temperature for 30–45 min and read at 570 nm on a sandwich enzyme-linked immunosorbent assay (ELISA) reader. The mean optical density (OD) ± standard deviation (S.D.) for each group of replicates was calculated. The *P* values were obtained using the Student *t*-test.

2.5. Gene transfection

The gene transfection technique was carried out as previously described [23]. Briefly, 0.5×10^6 of exponentially growing Fen cells (in a 25 cm^2 flask) were washed with sterile PBS, and 5 ml of Optimem (BRL) was added; and the cells were incubated for 4 h at 37°C. Genomic DNA was diluted to a concentration of 2 μg/ml was diluted with 50 ml of PBS and 2.5 ml of Optimem in a bijou tube, and was mixed with the contents of a second bijou consisting of 150 ml Lipofectin (BRL) and 2.5 ml Optimem. This mixture was then added to the culture flask pretreated with Optimem. Cells were incubated overnight at 37°C before replacement of the medium with fresh RPMI containing 10% FCS for a further 10 h of incubation. The cells were then ready for investigation.

Table 2
Concomitant expression of EGFR and p53 in TCC and OP tissue biopsies

Intensity of EGFR/ p53 Expression	TCC *n* (%)	OP *n* (%)	*P* values
Strong/strong	7/34 (21)	9/24 (38)	>0.05
Strong/negative	5/34 (15)	11/24 (46)	$0.02>P>0.01$
Other	22/34 (65)	4/24 (17)	$0.02>P>0.01$

Numerical presentation of EGFR and p53 staining values in bladder and oral cancer tissue biopsies. Other includes cases where the intensity of EGFR/p53 expression appeared as negative/negative, strong/some positive (other), or some positive/some positive. *P* values were obtained using χ^2 test. TCC, transitional cell carcinoma; OP, oral-pharyngeal carcinoma.

2.6. Statistical methods

The Chi-squared test was used to compare the number of tumour biopsies showing strong, other and negative expression for both the TCC and OP tumours. A level of $P<0.05$ was taken as the level of significance. For the MTT data, comparisons between groups were made using the Student *t*-test.

3. Results

3.1. Expression of pan-keratin, EGFR and p53 in TCC and OP tumours

The intensities for various molecules were assigned as strong, other (where the expression was low or only a proportion of cells were positive) or negative. As shown in Table 1, in both TCC and OP tumour types, strong keratin expression (using LP34 MAb) was detected in all tumours, i.e. in 34/34 cases of TCC and in 24/24 of OP malignancies, although the expression was not always on all the tumour cells. In the case of EGFR, the proportion of cases showing strong expression in TCC was much lower, i.e. 12/34 (35%) compared with the OP tumours, i.e. 20/24 (83%, Chi^2 test; *P* value of <0.01).

For p53, the results showed a very similar staining profile for two tumour types. The percentage of cases strongly expressing p53 in TCC and in OP tumours was 8/34 (24%) and 6/24 (25%), respectively. However,

there was a distinct difference in the pattern of staining between the two tumour types. In OPs, the expression was mainly in the basal cells while in TCC tumours it depended on whether it was of the transitional or squamous type (Figs. 1 and 2). In the case of the transitional type, the expression was present in all tumour cells whereas in the squamous type the expression was like that of the OPs, i.e. confined mainly to the basal cells (Fig. 2a and b versus c and d). In 7 of 34 (21%) TCC cases (Table 2), there was a concomitant strong staining of EGFR and p53. The corresponding value for OP carcinomas was 9 of 24 (38%, $P > 0.05$) (Table 2). In cases where EGFR/p53 expression showed strong/negative pattern the values for TCC and OP were 5/34 (15%) and 11/24 (46%, $0.02 > P > 0.01$). It was interesting that the expression of p53 in OP carcinomas corresponded to the area where Ki67 (i.e. proliferation marker) expression was strongest (Fig. 1c and d). These data indicated that p53 expression in transitional and squamous bladder tumours was different. They also showed that p53 was mainly expressed in proliferating cells.

In a separate study, the expression of these molecules in tissue biopsies from a patient with oral mucosal warts was investigated. As can be seen from Fig. 3, the keratin expression as detected by LP34 MAb (Fig. 3a) was present on all the tumour cells while strong EGFR expression was mainly observed in the basal and some

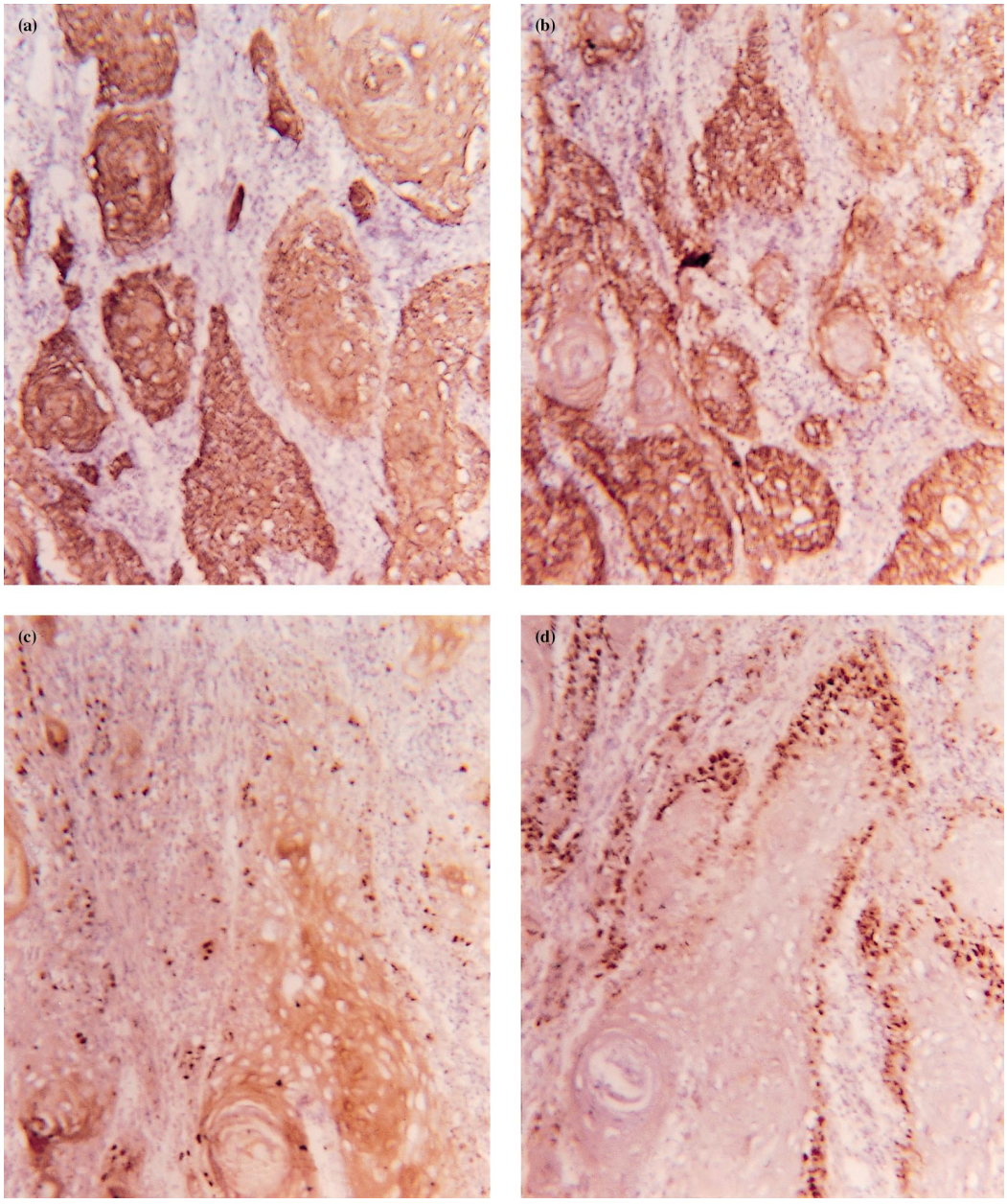

Fig. 1. Immunocytochemical staining of buccal warts as stained with LP34 (pan-keratin (a), EGFR (b), Ki67 (c) and p53 (d), respectively).

surrounding cells (Fig. 3b). The Ki67 staining was confined only to the basal cells (Fig. 3c) as was that of the p53 (Fig. 3d). These data demonstrated the similarities between the staining pattern of Ki67, EGFR and p53 in OPs and in oral mucosal warts. In addition, they showed that there was no direct correlation between the expression of EGFR and of Ki67.

3.2. *Effects of DNA-damaging agents on the induction of p53*

The bladder tumour cell line, Wil, was exposed to the optimal levels (based on earlier data) of cisplatin and gamma radiation (both known for their DNA-damaging potential) and p53 expression using MAb DO1 was assessed. As can be seen from Fig. 4, both treatments resulted in nuclear condensation and gamma radiation resulted in an extensive nuclear blebbing. In a separate study, a number of other tumour cell lines were exposed to these treatments and the cellular levels of p53 were detected using SDS-PAGE. As can be seen from Fig. 5, in all cases the treatments resulted in an induction of p53, with cisplatin being identified as a more efficient inducer of p53 than gamma radiation. These data demonstrated that DNA-damaging treatments induce p53, independent of the origin of the tumour cell lines.

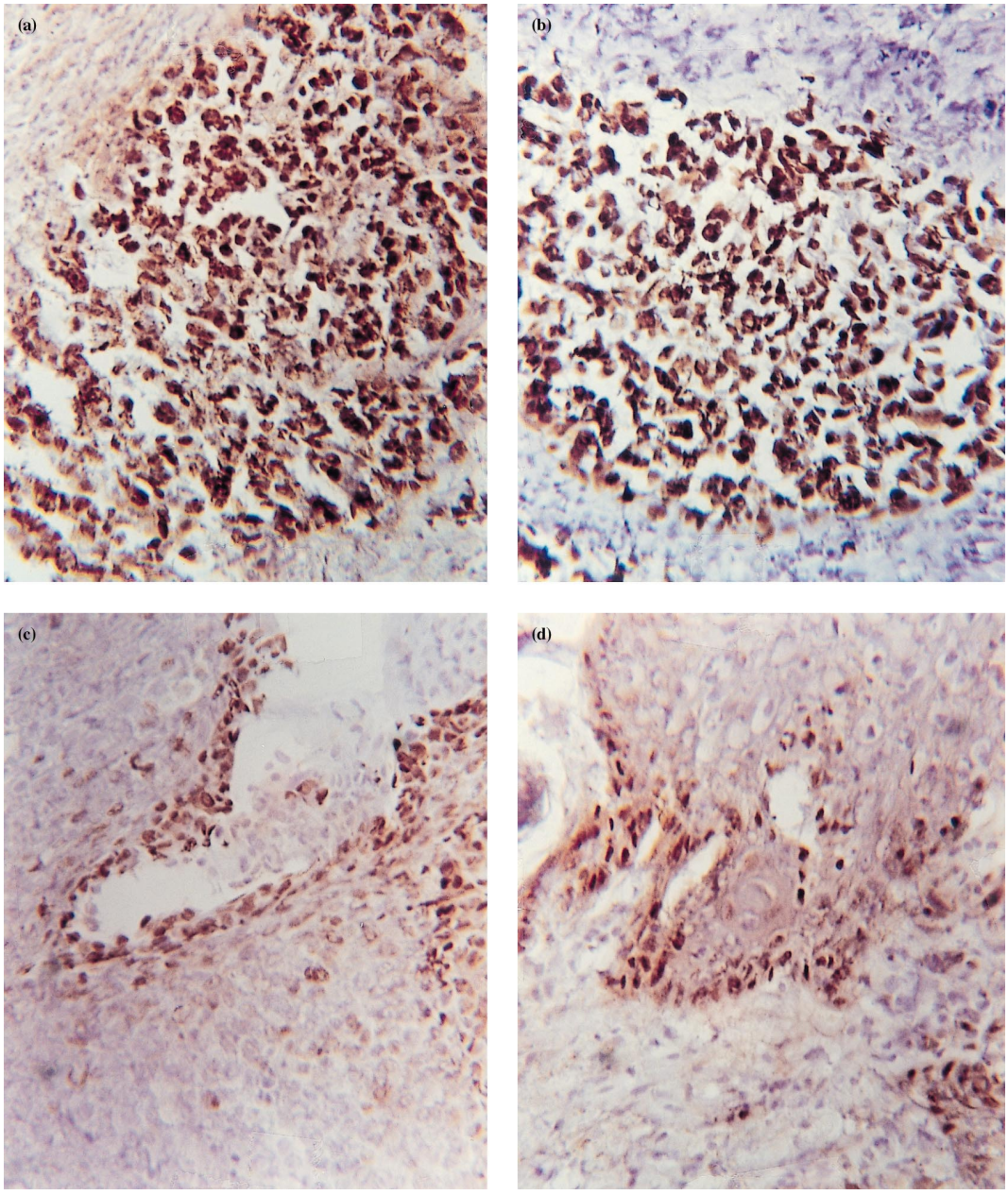

Fig. 2. Expression of p53 as detected by DO1 on four tissue sections from four areas of bladder cancers. (a) and (b) are transitional and (c) and (d) are squamous cell carcinomas.

3.3. Influence of constitutive expression of p53 on tumour cell susceptibility to cisplatin and gamma radiation

Two tumour cell lines, one our established bladder line Fen and one SV40 transformed foreskin epithelial line SKV14 (known to express p53) were exposed to varying amounts of gamma radiation and concentrations of cisplatin and cell killing was assessed by the MTT colorimetric assay. As can be seen from Table 3 and Fig. 6, in the case of SKV14 line, there was a direct relationship between the degree of radiation and tumour cell survival. Thus, the percentage decrease in mean optical densities at 125 and 250 cGy were 14% and 57%, respectively (in both cases the P values were below 0.01). In contrast, Fen cells showed a slight, but significant increase in the optical densities at both

Table 3
Effects of gamma radiation on two established human tumour cell lines

Dose (cGys)	Cells/well ($\times 10^3$)	Mean optical density (OD)±S.D.	
		SKV14 line (%)	Fen line (%)
NT	10	0.28±0.01	0.27±0.01
	5	0.19±0.01	0.18±0.01
125	10	0.24±0.01 (14)*	0.30±0.01 (−11)
	5	0.19±0.01	0.20±0.01 (−11)
250	10	0.12±0.01 (57)*	0.31±0.03 (−15)†
	5	0.11±0.01 (42)*	0.21±0.02 (−17)†

Means are from at least three replicates of cells cultured in microtitre plates for 48 h after exposure to gamma radiation. Per cent inhibitions were obtained by comparing treated with untreated cells of the same group. *$P < 0.01$. †$P < 0.05$. NT, no treatment.

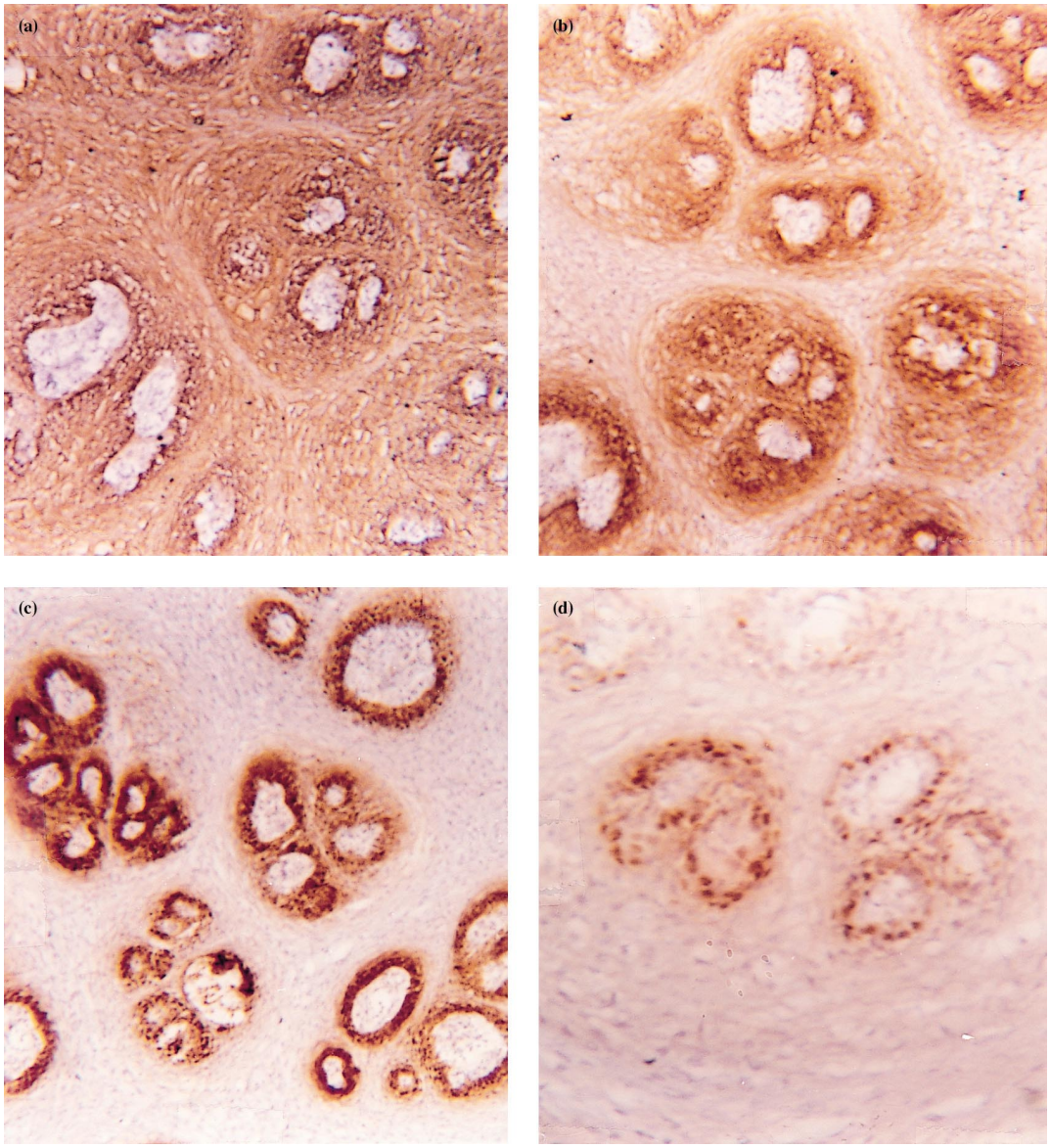

Fig. 3. Immunocytochemical staining of tissue sections from a patient with OP. (a), (b), (c), (d) represent LP34 (pan-keratin), EGRF, Ki67 and p53, respectively.

radiation levels. The figures in each case show p53 nuclear staining in the cell lines. The exposure of the two cell lines to cisplatin again showed a greater sensitivity for SKV14 compared with Fen cell line. Thus, the percentage inhibition for SKV14 and Fen cells at cisplatin concentrations of 0.5 and 1 μg/ml were 52±8, 71±6.0 and 1.0±3.1 ($P<0.001$), 2.6±7.0 ($P<0.001$), respectively. These results showed a greater sensitivity of SKV14 compared with Fen cells to DNA-damaging stimuli.

3.4. *Effect of insertion of wild-type and mutated* TP53 *genes on tumour cell survival in culture*

Fen cells were split into equal cell densities in two culture flasks (0.5×10^6/flask). After 24 h in culture, the cells in one flask were transfected with wild-type and the other with mutated *TP53* gene. Over a 3-day period, the cells were trypsinised and counted, and the number of cells recorded. Thus, the cell number at time 0 and 3 days for Fen cells with wild-type or mutated type were 2, 2 (for time 0) and 0.2, 2.5×10^5 (for 3 days), respectively. The changes in cell number are shown graphically in Fig. 7. These data demonstrated that the insertion of the wild-type but not mutated *TP53* gene into the Fen cell line resulted in significant tumour cell apoptosis.

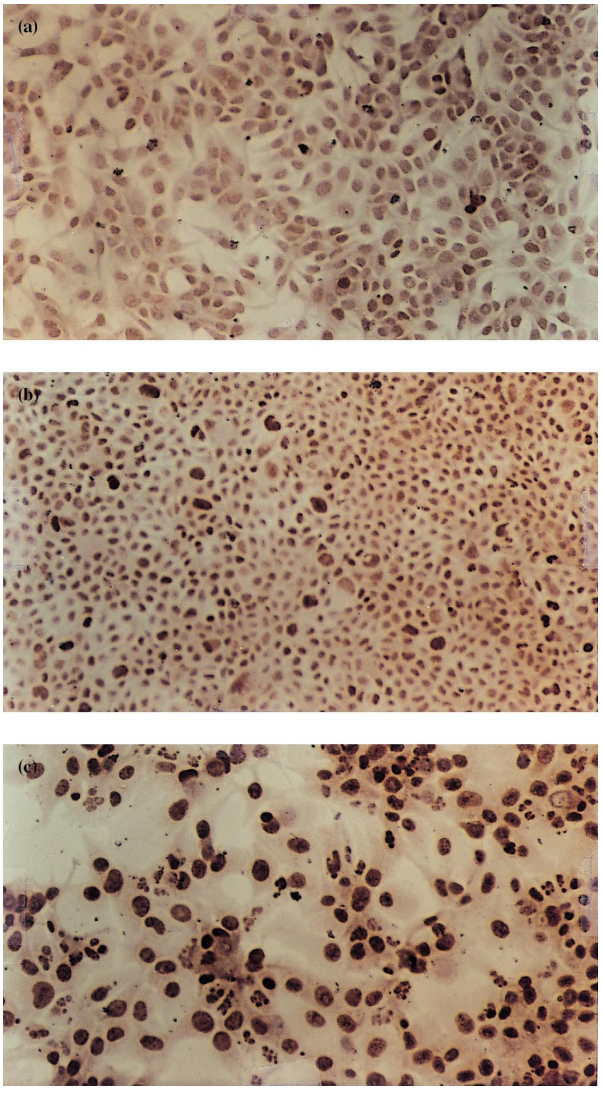

Fig. 4. Expression of p53 (DO1) on a bladder cell line, untreated (A), treated with cisplatin (1 μg/ml) (B), and gamma irradiation (500 cyc) (C), respectively.

4. Discussion

The findings of this investigation can be summarised as follows.

p53 could be detected in more than 45% of TCC and OP malignancies (combination of cases with strong and not very strong expressing cases) although the pattern of expression differed within the two tumour types. In 7/34 (21%) of bladder carcinomas and 9/24 (38%) of oral carcinomas there was a concomitant strong expression of EGFR and p53. In addition, there were a greater number of cases expressing EGFR among the OP carcinomas. Both gamma radiation and cisplatin treatments of the tumour cell lines (independent of their origin) resulted in the induction of p53. The tumour cell line SKV14 with a constitutive expression of p53 showed an exquisite sensitivity to gamma radiation compared with the bladder tumour line Fen with no p53. Insertion of wild-type and not mutated *TP53* gene into a bladder tumour line resulted in tumour cell apoptosis. These data confirmed the mutational status of *TP53* in a major proportion of bladder and oral carcinomas. They also indicate that the alteration of p53 expression determined the way tumour cells responded to different treatments.

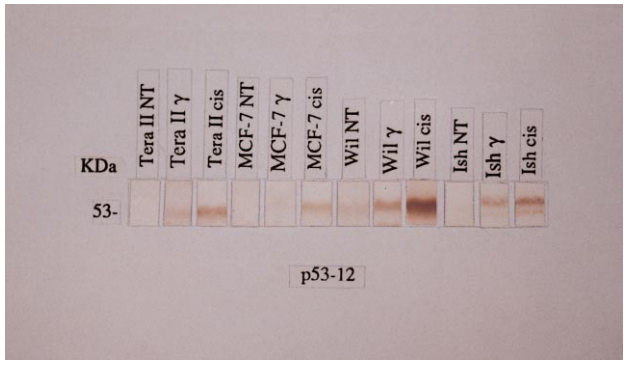

Fig. 5. Effects of γ irradiation and cisplatin treatment on p53 expression. Immunoblot of lysates prepared from four human tumour cell lines, namely Tera II, MCF-7, Wil and Ish from testis, breast, bladder and endometrium, respectively. The cells were treated with cisplatin (cis 1 μg/ml) or γ-irradiated (500 cGy) and cultured for 48 h before lysis. NT, no treatment.

The high frequency of *TP53* mutation in TCC and in OP cancers is consistent with the reports of others [3,7,11–17]. The significantly higher degree of EGFR expression in OP compared with the TCC cancer is suggested to reflect the more invasive nature of the former [24]. This is particularly relevant as 62% of the bladder cases investigated were of superficial type, the relevance of which was clearly demonstrated by Watanabe and colleagues [11]. It is tempting to argue that expression of p53 in combination with high intensity of EGFR staining in these tumours could be taken as a poor clinical indicator and may prove to be relevant to their clinical management, an issue currently under investigation.

Another point of interest was the pattern of expression of p53 in transitional versus squamous carcinoma of bladder cancers. In the case of squamous carcinomas the expression tended to be mainly on the basal cells, i.e. proliferative cells whereas in the transitional types the expression was similar throughout the tumour. Concomitant staining of tissues with Ki67 and p53 confirmed that p53 expression appeared mainly on Ki67-positive cells, a finding also reported by Warnakulasuriya and colleagues [25]. Experiments are in progress to see whether tumours with only basal cell positivity have different patterns of *TP53* gene mutation compared with those showing overall p53 positivity.

A previous report from this unit [9] and reports from others showed the presence of p53 expression in human testis tumours, particularly in seminomas [26,27]. The gene sequencing has confirmed the non-mutated status of the *TP53* gene in these tumours [10]. As seminoma is known to be the most chemo- and radiosensitive tumour, this led us to argue that the high expression of non-mutated p53 may be related to their exquisite sensitivity to treatment [28]. We argued that such a hypothesis could be tested experimentally. To do this, two cell lines were chosen, i.e. SKV14, with a high constitutive expression of non-mutated p53 (as it was originally established by transformation of normal

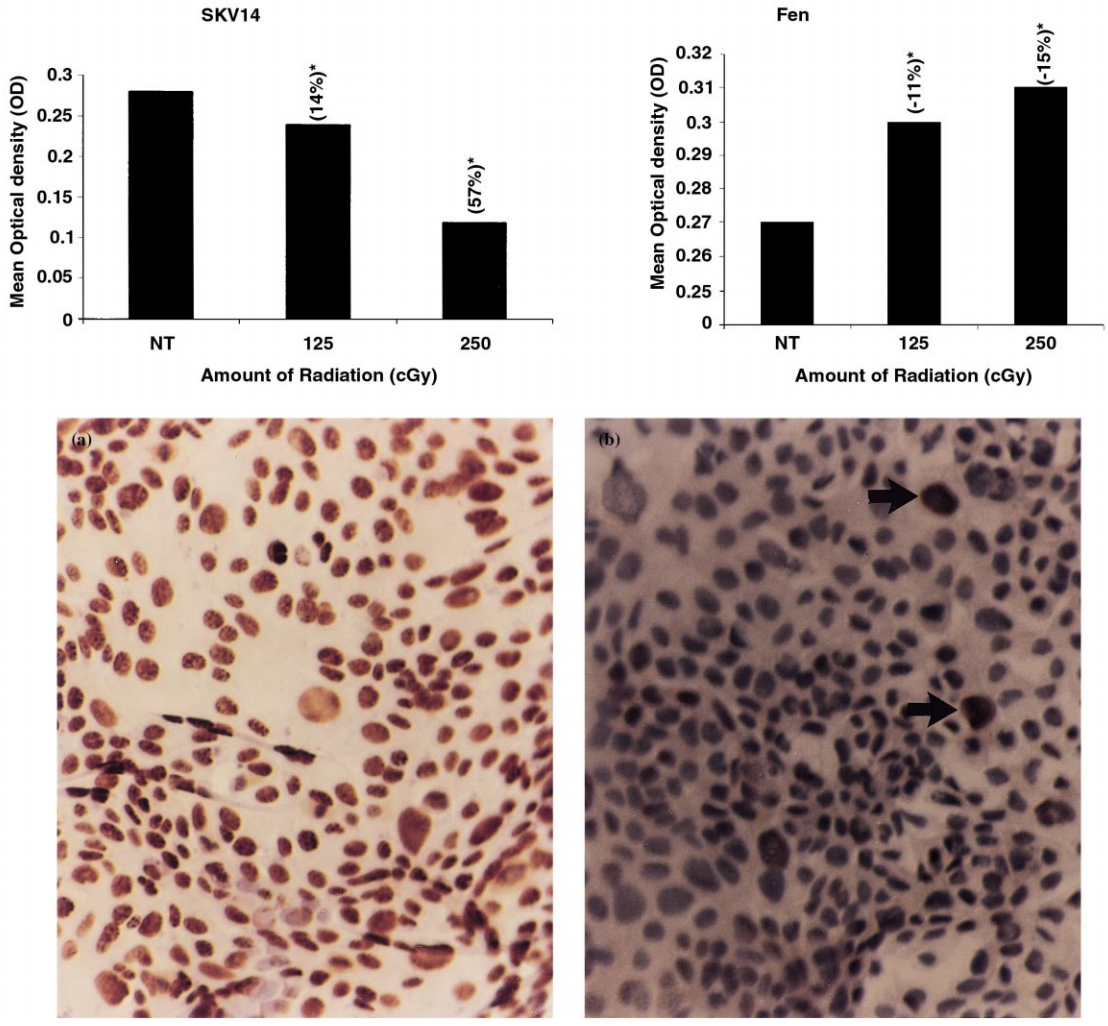

Fig. 6. Effect of γ radiation on two tumour cell lines. Results are expressed as mean± S.D. (standard deviation) of optical density in a MTT assay. **P* value of <0.01 compared with NT (non-treated) cells. Immunocytochemical staining of (a) SKV14 and (b) Fen for expression of p53 with DO1 MAb are presented. Arrows indicate DO1 positive cells.

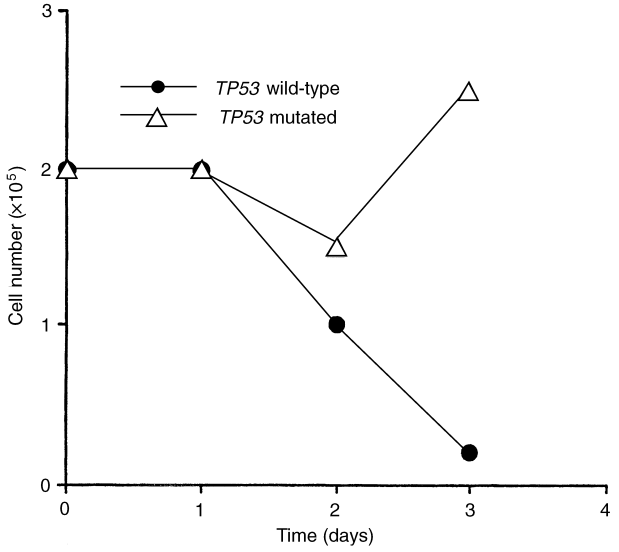

Fig. 7. Cell survival curve for a bladder tumour cell line (Fen) transfected with wild-type and mutated *TP53* genes.

foreskin epithelial cells by SV40) and one in-house established bladder tumour line Fen with mutated p53 (see below). The cells were exposed to gamma radiation and it was found that the SKV14 cells were more sensitive than the Fen cell line, a finding also observed when the cells were exposed to cisplatin. The mechanism(s) of the slight but significant increase in the Fen cell response to radiation remains to be investigated. These data confirmed that the presence of non-mutated p53 increased tumour cell susceptibility to DNA-damaging treatments. This is in keeping with the data reported by Fujiwara and associates [19] and Perego and colleagues [28].

In their normal status, cells express little or no (at least as assessed by the immunocytochemical staining technique) p53, however, following exposure to DNA damaging stimuli there is a rapid p53 induction. In this study, a bladder tumour cell line Wil when exposed to cisplatin or gamma radiation showed nuclear condensation and severe nuclear blebbing. These treatments also induced p53 in other tumour cell lines independent of their origin. The inducibility of p53 in response to DNA-damaging stimuli is consistent with the report of Kastan and colleagues [29] and Fritsche and coworkers [30] and many others.

The normal function of p53, as indicated earlier, is critical for the normal working of the apoptotic processes [8]. This was the basis for the next set of experiments in which a bladder tumour cell line Fen was transfected with either wild-type or mutated *TP53* gene. The data showed that the cells with transfected wild-type *TP53* apoptosed, whereas those with mutated *TP53* survived. This implied that the *TP53* gene in Fen cells was mutated and the introduction of the wild-type *TP53* gene led to the apoptosis of these cells. These data are consistent with those reported by Liu and colleagues [18], Shaw and co-workers [31] and Kayo and colleagues [32]. They are also in accordance with the insensitivity of the Fen cells to radiation and cisplatin reported in this study.

Taken together, these data have confirmed the high frequency of *TP53* mutation in two human solid tumours. They also indicate that the constitutive expression of non-mutated *TP53* in cells resulted in their increased sensitivity to DNA-damaging treatments. In addition, the findings show that introduction of wild-type *TP53* gene by gene transfection into tumour cells led to their apoptosis. If these could be translated into a clinic, it could prove to be a powerful way of debulking tumour mass in patients with localised accessible tumour expressing mutated *TP53* without a need for major surgery. This, of course, would necessitate the use of an efficient gene delivery system.

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