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Original Paper

Transfection of Wild-type *TP53* Induces Differentiation in Human Gingival Carcinoma Cells

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We investigated the effects of transfection of wild-type *TP53* on the growth properties of a human gingival carcinoma cell line, KOSC-3, in which the *TP53* gene is mutated at codon 248 and overexpressed. The wild-type *TP53* expression plasmid, pCDM8-p53/neo and the control plasmid, pCDM8/neo, were each stably transfected into KOSC-3 cells by using the calcium phosphate method. The number of G418-resistant colonies from wild-type *TP53*-transfected cells was approximately half that from plasmid controls. Exogenous wild-type *TP53* transcripts were identified in four of the 20 G418-resistant clones analysed by reverse transcription PCR. Although the growth rates of the wild-type *TP53*+ clones did not drastically change during log phase, their saturation density was significantly reduced. The wild-type *TP53*+ cells were morphologically flat and enlarged when cultured *in vitro*, and were less able to form colonies in soft agar. In nude mice, the wild-type *TP53*+ clones formed subcutaneous tumours with conspicuous keratinisation and notable cell death that was not manifested in the parental and plasmid control cells. These findings indicate that the wild-type *TP53* gene, even when it coexists with a mutated form, may function as a growth suppressor and differentiation inducer under restricted conditions in gingival squamous cell carcinoma.

Key words: gingival cancer, *TP53* gene, transfection, squamous cell carcinoma
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INTRODUCTION

DOMINANT NEGATIVE effects and loss of function in mutations of the *TP53* tumour suppressor gene are frequently found in human malignancies [1, 2]. These mutations have also been seen in squamous cell carcinomas of the upper aerodigestive tract [3–6] but their significance in cellular function is not known. In oral carcinomas, three of 15 cell lines have *TP53* mutations at codon 248, which may be one of the hot spots according to a previous study [4]. Although many studies on *TP53* gene function have been reported, most were based on transient transfection experiments. Stable transfection with the *TP53* gene in human cancers has been achieved in only a few cases, including colorectal carcinoma [7], gastric carcinoma [8], osteosarcoma [9, 10] and oral cancer [11]. Transfection of wild-type *TP53* in oral squamous carcinoma cells was recently performed successfully by Brenner and associates [11] but their results were obtained *in vitro*. The mutations of parental cells at codon 142 or 176 are uncommon in oral squamous cell carcinoma [4]. To clarify the significance of the *TP53* mutation at codon 248 and to analyse the functional role of the *TP53* gene in oral carcinomas both *in vitro* and *in vivo*, we transfected a wild-type *TP53*-cDNA into a KOSC-3 cell line that we had previously established from a human gingival squamous cell carcinoma. The KOSC-3 cell line carries a point mutation at codon 248 of the *TP53* gene [12].

The purposes of our study were as follows: (1) to test whether wild-type *TP53* can stably coexist with mutant *TP53* after being transfected into the KOSC-3 cells; and (2) to further analyse the effects of wild-type *TP53* on cell growth *in vitro* and *in vivo* to ascertain whether the *TP53* gene plays an important role in oral squamous cell carcinoma.

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MATERIALS AND METHODS

Cell culture and stable transfection

A gingival carcinoma cell line, KOSC-3 [12], was cultured at 37°C in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS). Cells (5×10^5) were seeded into a 100-mm culture dish 24 h before transfection. Wild-type *TP53* expression plasmid, pCDM8-p53/neo, was previously constructed [8] by inserting a 1365 bp *TP53*-cDNA fragment containing the entire coding region of the *TP53* gene into a plasmid vector, pCDM8/neo (Invitrogen, San Diego, California, U.S.A.), which carries the neo^r marker (Figure 1). Either the wild-type *TP53* expression plasmid, pCDM8-p53/neo, or the control plasmid, pCDM8/neo was transfected according to the calcium phosphate method [13] using a Mammalian Transfection Kit (Stratagene, La Jolla, California, U.S.A.). After 24 h, the cells were shocked by treating them with 10% DMSO for 5–10 min. G418-resistant colonies were selected from the growth medium, which contained 400 μg/ml G418 (Gibco BRL, Grand Island, New York, U.S.A.) for 2–3 weeks, and the total number of colonies formed was counted using a haemocytometer. G418-resistant clones were maintained separately in G418-containing medium and propagated for further analysis.

Reverse transcription polymerase chain reaction and cDNA sequencing analysis

Total cytoplasmic RNA was isolated according to the guanidium thiocyanate method [14]. For sequencing analysis, cDNA was synthesised from 1 μg of total RNA by reverse transcription by using random hexamers as a primer. A region corresponding to exons 4 through 10 of the *TP53* gene was amplified from cDNA by using the polymerase chain reaction (PCR) with a set of primers (5′-CTTCTGTCCCTTCCCAGAAACC-3′ and 5′-CCTCATTCAGCTCTCGGAACATCTCG-3′). cDNA sequencing was performed by using an *fmol* cycle sequencing kit (Promega, Madison, Wisconsin, U.S.A.) The sequence ladder was detected by using a Sequencing High chemiluminescent detection kit (Toyobo, Tokyo, Japan) and a biotinylated sequencing primer that corresponds to the 3′ end of exon 7 (5′-XTAGTGTGGTGGTGCCCTATGAGCCG-3′, X: Biotin).

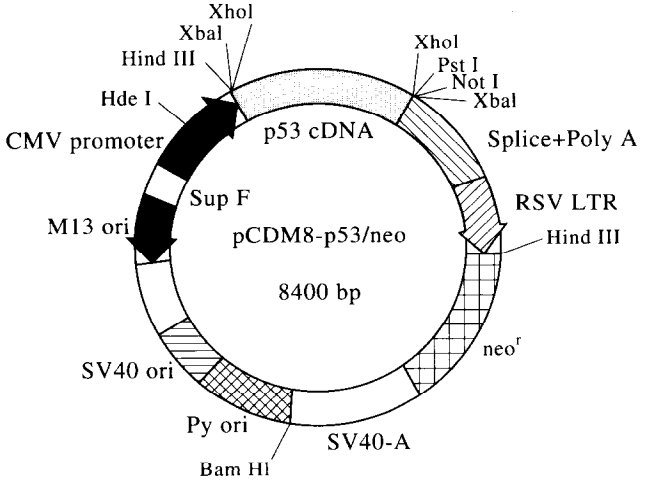

Figure 1. Schematic representation of *TP53* expression plasmid used for transfection.

Growth curve and colony formation in soft agar

Cells (1×10^5) were seeded in a 60-mm petri dish in medium supplemented with either 0.5 or 10% serum. Cells from replicate dishes were counted each day for 6 days using a haemocytometer. To form colonies in soft agar, 1×10^5 cells were seeded into each 35-mm dish, which contained 0.33% agarose with complete medium and 20% FBS. The medium was replenished every 7 days, and colonies (>60 μm in diameter, which is equivalent to approximately 100 cells) were counted using a haemocytometer after 3 weeks. All growth results were calculated as the average of colonies in five dishes. After being cultured in G418-free medium for a week, the cells were used for experiments.

Tumorigenicity test in nude mice

Cells (1×10^7) cells in phosphate-buffered saline (PBS) were injected subcutaneously into the flanks of nude mice (BALB/cA Jcl-nu, Clea, Tokyo, Japan). Tumour size was measured every 10 days for 30 days. Tumour volumes were estimated based on the Battelle Columbus Laboratories Protocol [15].

In situ *identification of DNA fragmentation*

DNA fragmentation in tumour cells undergoing apoptosis was identified by the TdT-mediated dUTP-biotin nick end-labelling (TUNEL) method [16,17]. The method is based on labelling of DNA strand breaks *in situ*. Briefly, the deparaffinisation and rehydration, tissue sections were incubated with 10 μg/ml proteinase K at room temperature for 10 min, washed with PBS and treated with 2% hydrogen peroxide at room temperature for 5 min. The sections were rinsed with water and then covered with TdT (0.3 units/μl) and biotinylated dUTP in TdT buffer, and incubated in a humidified chamber at 37°C for 1 h. The slides were washed with buffer containing 0.3 mM sodium chloride, 30 mM sodium citrate at room temperature for 15 min. The sections were immersed in PBS containing 2% BSA for 10 min, rinsed in PBS, covered with buffer containing Streptavidin Peroxidase (1:400), and incubated at 37°C for 30 min. The sections were washed, immersed in PBS, and treated with diaminobenzidine (DAB). For positive controls, sections were pretreated with DNAase I (1 ng/μl; Gibco BRL) in buffer containing 30 mM Tris/pH 7.2, 104 mM sodium cacodylate, 4 mM $MgCl_2$ and 0.1 mM diothiothreitol (DTT). The slides were then washed with water and processed for DNA nick end-labelling. For a negative control, TdT and biotinylated dUTP were omitted.

RESULTS

Transfection and identification of exogenous TP53 *gene expression*

The wild-type *TP53* expression plasmid was transfected into KOSC-3 cells, and the number of G418-resistant colonies from quadricate dishes was counted 3 weeks later. On average, G418-resistant clones transfected with pCDM8-*TP53*/neo or pCDM8/neo formed 32.0 or 68.5 colonies, respectively, per dish. Since endogenous mutant *TP53* expression interferes with wild-type *TP53* for analysis at the protein level, transcripts from G418-resistant colonies were sequenced after reverse transcription polymerase chain reaction (RT-PCR) to identify the exogenous wild-type *TP53* allele. The presence of the normal sequence that corresponds to codon 248 as well as the mutated sequence proved that the wild-type *TP53* was integrated and expressed in four G418-resistant clones, K3/p53-1, K3/p53-2, K3/p53-3 and K3/p53-4. Represent-

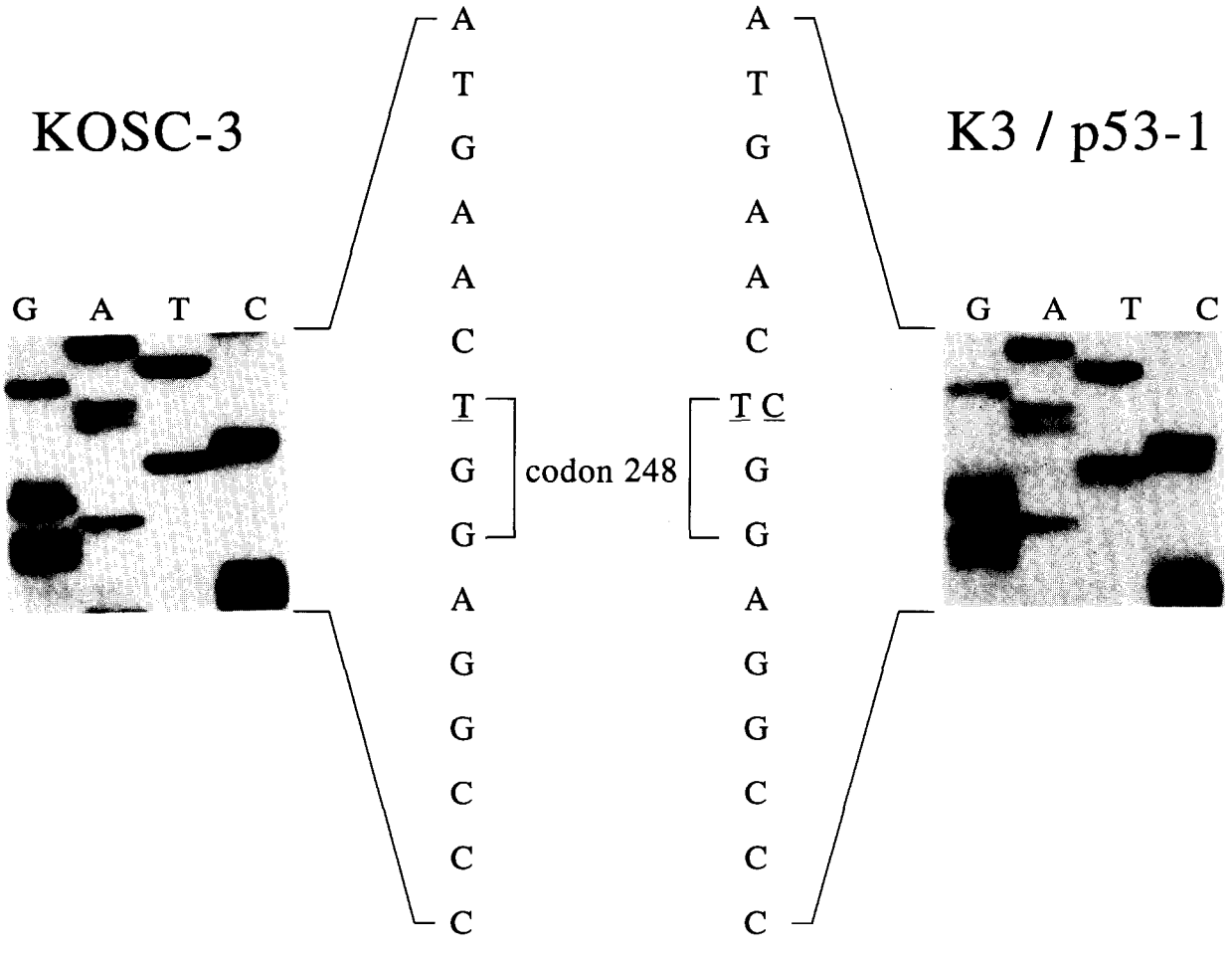

Figure 2. Sequence analysis of the PCR-cDNA at codon 248 of the *TP53* gene in parental and K3/p53-1 cells.

ative results of RT-PCR sequencing from the parental KOSC-3 and wild-type *TP53*+ K3/p53-1 clone are shown in Figure 2.

Phenotypic changes of wild-type TP53+ cells in culture

The ability of all wild-type *TP53*+ clones except K3/p53-3 clone to form colonies in soft agar was markedly suppressed, whereas parental and plasmid control cells produced large colonies with high plating efficiency (Table 1). The wild-type *TP53*+ clones showed flatter and enlarged morphology *in vitro* (Figure 3d,e,f) compared to those of the parental and plasmid control clones (Figure 3a,b,c). The effects of wild-type *TP53* expression on cell proliferation were analysed at serum concentrations of 0.5 and 10%. The growth rate of K3/p53-1, -2, -3 and -4 cells was not notably reduced at day 5, but showed a drastic decline at day 7, unlike those of parental KOSC-3 and plasmid control K3/neo1 and K2/neo2 cells

Table 1. Changes in growth properties of wild-type TP53-transfected KOSC-3 cells

	Saturation density ($\times 10^6$)*		100 × CFE† (%)	No. of tumours /mice‡	Tumour weight (mg) average§
	10% FBS	0.5% FBS			
KOSC-3	4.78	2.16	5.54	4/4	86.2
K3/neo1	5.41	2.41	7.51	4/4	27.2
K3/neo2	3.82	1.56	4.90	4/4	32.4
K3/p53-1	2.55	1.05	0.28	4/4	26.6
K3/p53-2	3.43	1.15	0.06	4/4	14.7
K3/p53-3	3.71	1.30	2.49	4/4	13.9
K3/p53-4	4.08	1.10	0.07	4/4	14.9

*1×10^5 viable cells per 60-mm dish were seeded and counted on the seventh day after plating. Values represent the average of triplicates. †CFE, colony-forming efficiency in soft agar; ‡Tumour size at 1 month was given for 1×10^7 injected cells each in four mice; §Tumour weight (mg) was given based on the Battelle Memorial Institute protocol.

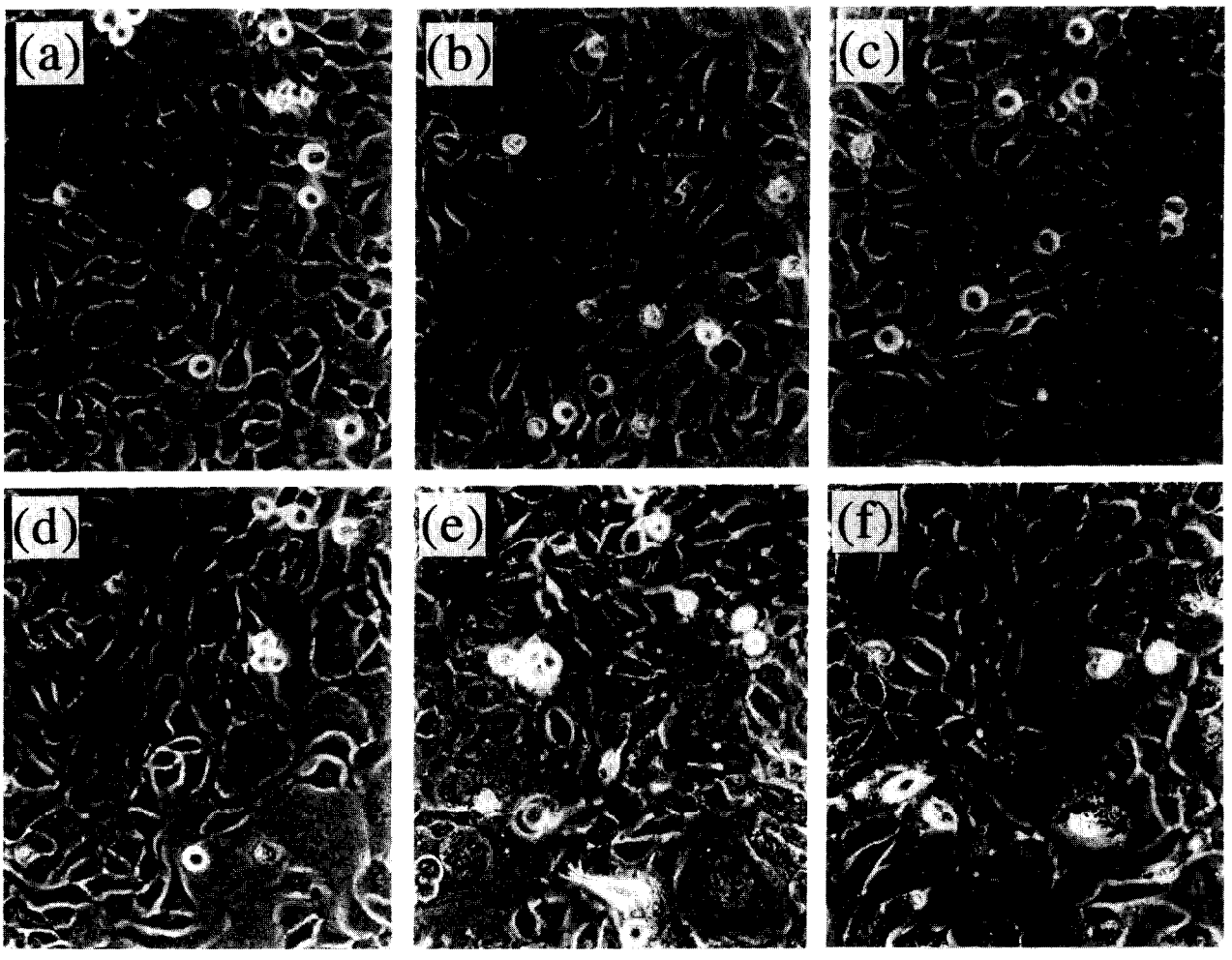

Figure 3. Morphological changes of parental and transfected cells *in vitro*. Cells were cultured in 10% FBS, (a) parental KOSC-3 cells; (b) and (c) plasmid control, K3/neo1 and K3/neo2, respectively; (d), (e) and (f) wild-type *TP53* transfectant, K3/p53-1, K3/p53-2, K3/p53-3, respectively. Magnification: ×200.

grown in 10% FBS containing medium (Figure 4). When the cells were grown in 0.5% FBS-containing medium, the growth rates of wild-type *TP53*+ clones were consistently lower, but did not show any decline in the growth curve. Accordingly, the saturation density of wild-type *TP53*+ clones was lower than that of parental KOSC-3 and plasmid control cells (Table 1).

Tumour formation in nude mice

We investigated the effects of stable *TP53* expression on the tumorigenicity of KOSC-3 cells in nude mice. All wild-type *TP53*+ clones, parental KOSC-3 and three plasmid control clones, K3/neo1 and K3/neo2 formed progressively growing subcutaneous tumours in nude mice (Table 1). Although there was no significant difference in tumour size, on histological examination all tumours from the wild-type *TP53*+ clones exhibited marked keratinisation as compared to those of the parental and plasmid control cells. Representative histopathology of parental KOSC-3 and wild-type *TP53*+ K3/p53-1 cells in nude mice is shown in Figure 5. Apoptotic cell death was also prominent in the keratinising regions of the tumours from the wild-type *TP53*+ clones demonstrated by the *in situ* nick end-labelling method (Figure 6).

DISCUSSION

In this study, we transfected a wild-type *TP53* expression plasmid into *TP53*-mutated gingival carcinoma cells and obtained four stable transfectants, K3/p53-1, K3/p53-2, K3/p53-3 and K3/p53-4. The chances of obtaining a wild-type *TP53*-transfected clone are extremely low according to previous studies using various cell types. We also had smaller numbers of G418-resistant colonies in wild-type *TP53*-transfected dishes which may have reflected such growth-inhibitory effects of *TP53*.

The growth-inhibitory effects of wild-type *TP53* over endogenous mutant *TP53* on KOSC-3 cells were more prominent in colony formation assays in selective medium and soft agar than in mass culture analysis. When wild-type *TP53* expression was introduced, colonies were less able to grow in soft agar, but no significant alteration of growth rates in culture was seen, which is similar to those of Chen and associates [18]. Of particular interest in our results, is that marked cancer pearl formation was induced when the wild-type *TP53*+ clones were grown subcutaneously in nude mice, although tumour size was not greatly affected. These findings *in vivo* may be correlated to the flat morphology and low saturation density of these cells *in vitro*. The difference in

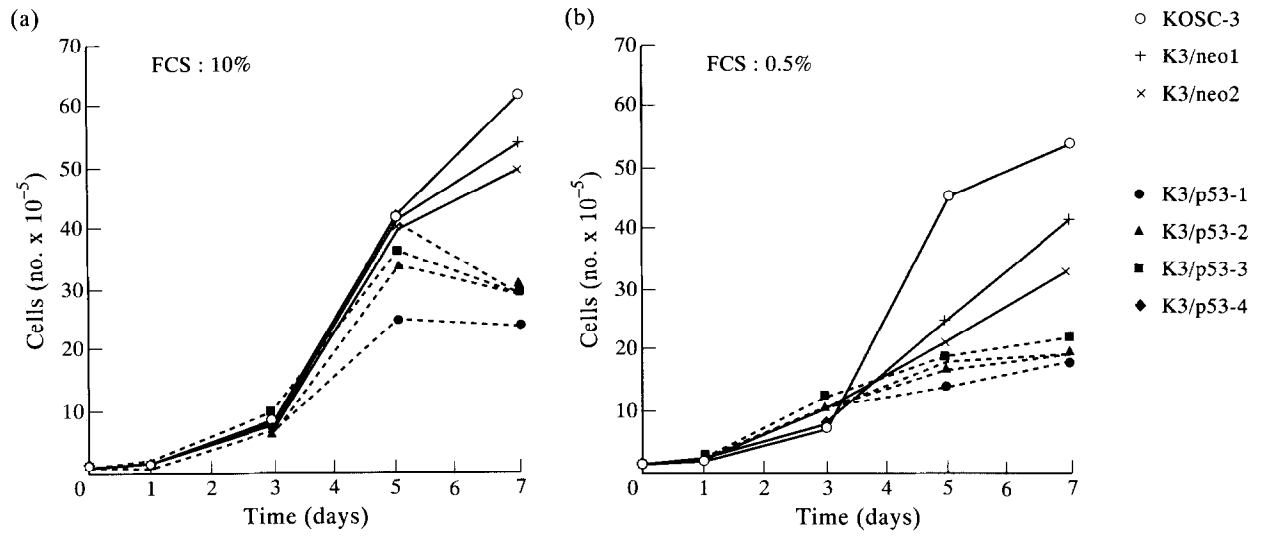

Figure 4. Effects of wild-type *TP53* expression on growth rates of KOSC-3 cells *in vitro*. Cells were seeded at a concentration of 1×10^5 cells per 60-mm dish on day 0. Two different concentrations of FBS were used, 10% (a) and 0.5% (b).

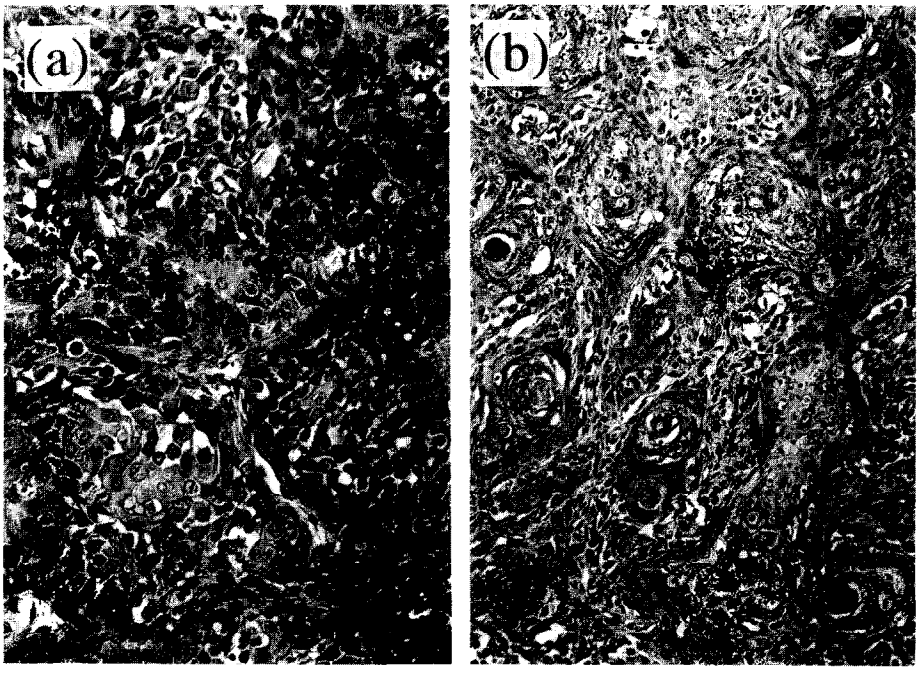

Figure 5. Histopathology of tumours formed in nude mice. (a) Parental KOSC-3 cells; (b) K3/p53-1 cells. Magnification: ×125.

growth properties between wild-type *TP53*+ and wild-type *TP53* clones in culture became apparent when the cells were more confluent, resulting in a marked reduction of the saturation density in *TP53*+ clones. Other reports [9–11,19–21] have also produced evidence that cell differentiation could be induced by transfecting the *TP53* gene in many types of cancer cells. Our results demonstrated induction of differentiation *in vivo* in squamous carcinoma cells after re-expression of the wild-type *TP53* gene.

In addition to morphological changes of the *TP53*+ cells both *in vitro* and *in vivo*, molecular markers for squamous cell differentiation, such as involucrin and transglutaminase (TGase), may support our findings [22–25]. Expression of involucrin of wild-type *TP53*+ clones in culture was extremely low, and no significant changes were found according to Western blot analysis (data not shown). In contrast, immunohistochemical staining of xenograft tumours using anti-involucrin antibody showed extremely high expression not only in the wild-type *TP53*+ clones but also in parental and plasmid control clones (data not shown). These molecular markers may be too sensitive to detect morphological changes.

Mutant *TP53* may have either dominant positive or dominant negative effects on the transcriptional activities of wild-type *TP53*, as suggested by Zhang and associates [26], depending on the type of mutation. Both wild and mutant types of *TP53* also interact with each other by means of

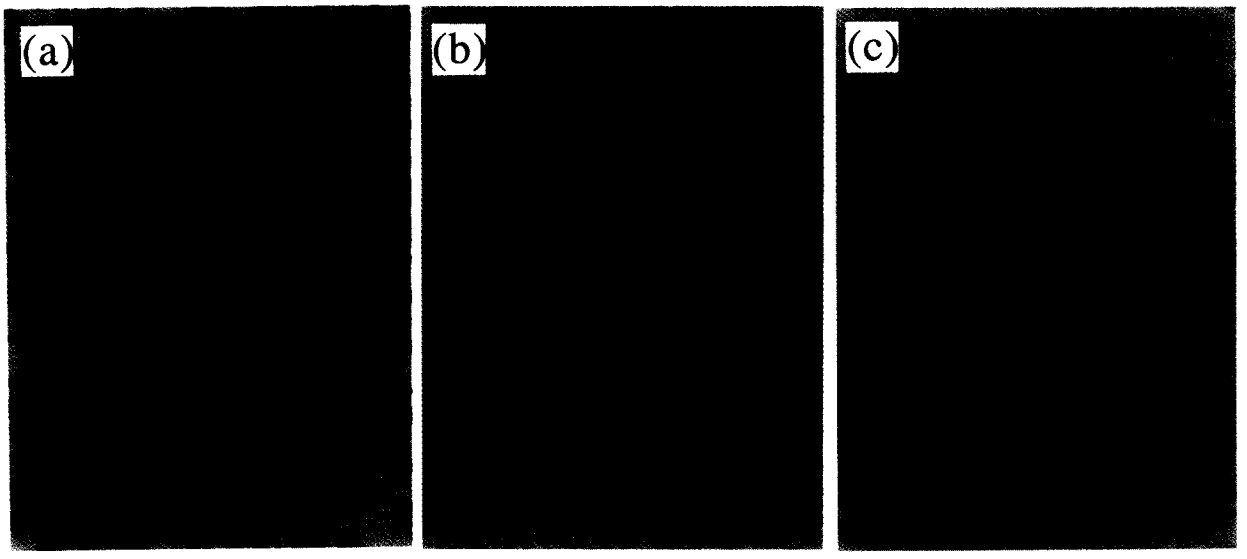

Figure 6. Detection of DNA fragmentation *in situ* in wild-type *TP53*+ cells in xenograft tumours. (a) Parental KOSC-3 cells pretreated with DNase as a control; (b) parental KOSC-3 cells without DNase treatment; (c) K3/p53-1 cells without DNase treatment.

oligomerisation. Therefore, the ratio of wild-type *TP53* to mutant *TP53* at the expression level should be an important indicator for their effects on transcription. These effects of wild-type *TP53* on KOSC-3 cells carrying the *TP53* mutation at codon 248, which is thought to be a recessive mutation [27, 28], are particularly interesting. Apparently, wild-type *TP53* has some overriding effects on mutant *TP53*, as evidenced by reduced colony formation, low saturation density, and other phenotypic changes, including flat morphology in culture and increased keratinisation in xenograft tumours. If the parental KOSC-3 cells had other types of *TP53* mutation, the effects of wild-type *TP53* would not be the same. Thus, our data could be dependent on the types of pre-existing mutation in parental cells.

Since the parental KOSC-3 cells overexpressed *C-MYC* and had a *TP53* mutation, we speculated that *C-MYC* expression is transcriptionally upregulated by the overexpression of mutant *TP53*. This hypothesis is relevant to a previous report [29] that wild-type *TP53* represses transcription from the *C-MYC* promoter in a transient transfection experiment. According to our preliminary data, only a slight reduction of *C-MYC* expression after wild-type *TP53* transfection was seen. It is, therefore, conceivable that the *C-MYC* gene is modulated by many other factors in *TP53* transfectants, but could be more down regulated by wild-type *TP53* in a transient expression experiment, including one that involves an inducible promoter.

Recently, Oren and coworkers [30] reported that wild-type *TP53* induces apoptosis in myeloid leukaemia cells. The rarity of such observation suggests that cell type and the state of differentiation before transformation may be important factors determining whether or not wild-type *TP53* expression causes apoptosis. Of particular interest in our results is that the effect of wild-type *TP53* on KOSC-3 cells was manifested by squamous differentiation through apoptotic cell death demonstrated by the TUNEL method. Our model may provide a clue to development of a new molecular cancer therapy using apoptosis-inducing agents.

In conclusion, the wild-type *TP53* gene apparently not only functions as a potent growth suppressor gene in human gingival carcinoma while carrying multiple genetic alterations, but also has additional effects on other phenotypes, including the differentiation of squamous cell carcinoma.

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