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Roles of *p53* Mutation in Cell Line Establishment and Identification of the Minimum Transactivation and Transform Suppression Domains

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The mutation of the *p53* tumour suppressor gene is the most frequently recognised genetic alteration in human cancer. We recently showed that the frequency of *p53* gene mutations in oral squamous cell carcinomas (SCCs) from which cell lines were established (group A) did not significantly differ from that in SCCs from which cell lines could not be established (group B), suggesting that the presence of a *p53* mutation by itself is not sufficient. To assess the relevance of *p53* mutations to cell line establishment, we determined sequences of the mutated genes, constructed the expression plasmids, and compared biological and biochemical activities. Both groups contained typical mutant type mutations at a similar frequency. However, two mutations in group A had strong transforming activity. One of the mutants, codon 306 Stop mutant with C-terminal truncation, was found to have the transactivation and transform suppression activities similar to wild type. The minimum transactivation and transform suppression domains of p53 were thus determined based on analysis of various C-terminal deletions. Activity disappeared between codons 300 and 282, an interval which contains the C-terminal end of the sequence-specific DNA binding domain, which suggests that the DNA binding domain is essential for the above activities.

Keywords: p53, cell line establishment, transactivation, transform suppression

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INTRODUCTION

THE *p53* TUMOUR suppressor gene is altered frequently in many human malignancies [1]. Biologically, overexpression of wild type p53 reduces oncogene-mediated focus formation [2] and when introduced in colorectal carcinoma cells not expressing any p53, *p53* suppresses the neoplastic phenotype [3]. In contrast to wild type, mutant *p53*s immortalise rat embryo fibroblasts (REF), and transform REF in collaboration assay with activated *ras* oncogenes [4, 5]. Mutant *p53*s fail to suppress or slightly enhance oncogene-mediated transformation [2].

Biochemically, wild type p53 has the features of a transcriptional factor with a potent transactivational domain in its acidic N-terminal portion. The basic C-terminal domain is involved in oligomerisation [6] and the central portion in specific DNA binding [7]. Transactivation by wild type *p53* can be seen only when a promoter has a p53 binding site, whereas mutant *p53*s lack this activity [8, 9]. Recently, a gene induced by wild type *p53* was identified [10], which encodes an inhibitor of cyclin-dependent kinases, p21, a protein playing a key role in the G1 block of the cell cycle.

The frequency of mutations in cell lines has been shown to exceed that in primary tumour samples in lymphoid malignancy [11]. It is thus suggested that tumour cells carrying *p53* mutations may be more suitable for *in vitro* establishment. However, *p53* mutations were detected in five (group A) of six tissue samples from which cell lines were established and in four (group B) of five specimens from which no cell line could be established [12, 13]. Thus, possibly *p53* mutations by themselves are not capable of establishing cell lines [12, 13]. In this study, since the properties of *p53* were variable, depending on mutation positions [14], comparison was made of the biological and biochemical activities of *p53* mutations of both groups to determine whether *p53* mutation features are essential to cell line establishment. The two groups were found to be essentially the same. Interestingly, one of the mutants, the oligomerisation deficient *p53* 306 Stop mutation, suppressed transformation but supported p53-binding-site-dependent transactivation. The minimum domains of p53 involved in transactivation and transform suppression were identified using various C-terminal truncated *p53* genes.

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Drastic reduction in activity was observed when *p53* was truncated from codon 300 to codon 282.

MATERIALS AND METHODS

DNA preparation

DNA was extracted from tumour tissues as described previously [13].

PCR-SSCP analysis

PCR (polymerase chain reaction) primers, thermal cycling conditions and SSCP (single strand conformation polymorphism) analysis were described by Sakai *et al.* [13].

Determination of p53 *mutations in tumour tissues*

p53 mutations in four cases of tumour tissues from which cell lines could not be established were determined by the method of Suzuki *et al.* [15]. Briefly, the tumour samples often contained very small amounts of mutated DNA fragments in PCR-SSCP analysis, and thus mutated fragments were isolated from dried SSCP gel by cutting out corresponding portions. DNAs of the mutated alleles were eluted from the gel pieces in 50 μl water by heating at 80°C for 30 min. The eluted DNA fragments were subjected to sequencing using DNA fragments amplified by asymmetric PCR as templates according to Sakai *et al.* [13]. By this method, missense mutations were determined in three cases: codons 135 (TGC to TTC), 173 (GTG to ATG), 179 (CAT to TAT), respectively, while a nonsense mutation of codon 306 (CGA to TGA) was detected in the 4th case (data not shown).

CAT reporter plasmids

Reporter plasmids p53CONTK-CAT and RGCTK-CAT were constructed by inserting the two p53 binding sequences, GGACATGCCCGGGCATGTCC (CON) [8] and a sequence in the human ribosomal gene cluster, CCAGGCAAGTCCACTGCAGG (RGC) [16], respectively, upstream of the Herpes virus thymidine kinase promoter in the CAT reporter plasmid (pBLCAT2Δ). pBLCAT2Δ was constructed by removing the 60 bp BamHI-RsrII fragment from pBLCAT2 [17] after changing the RsrII site to a BamHI site.

p53 *expression plasmids*

LTRp53^{W} containing human wild type *p53* cDNA under the control of the Molony strain of a murine sarcoma virus long terminal repeat (Molony LTR), was constructed by inserting the 1.85 kb XbaI fragment of php53c.1 [18], after converting the XbaI sites to BamHI sites by blunt-ending with a Klenow fragment, followed by a BamHI linker ligation, into the unique BamHI site of pLTR-SA [19].

CMVp53^{W} was made by inserting the 1.85 kb BamHI fragment of LTRp53^{W} into a CMV (human cytomegalovirus major immediate-early promoter) vector. This vector was constructed on pUC18 by inserting (a) at the BamHI site the 0.85 kb BamHI-BglII fragment of pLTR-SA, and (b) between the salI and HindIII sites the 3.3 kb HindIII-XhoI fragment of BCMGSneo (neomycin resistant gene, the human CMV major immediate-early promoter, and the second intron of the rabbit β-globin gene) [20].

For the expression of mutant *p53* genes from Group A, the 468 bp Aor51HI fragment isolated from the 699 bp fragment amplified from mutant *p53* cDNA [12] was replaced with the corresponding wild type fragment of LTRp53^{W} or CMVp53^{W}. To express mutant genes within exon 5, a 613 bp DrdI-PflMI fragment was replaced with the corresponding wild type fragment.

p53 mutant plasmids of group B were constructed by site-directed mutagenesis by the gapped duplex method with a Mutan®-G kit (Takara Shuzo, Kyoto, Japan). Site-directed mutagenesis of the *p53* cDNA was performed in M13mp18 using the following synthetic nucleotides: 135 Phe, 5′-AAGATGTTTTTCCAACTGGC-3′; 173 Met, 5′-GACGGAGGTTATGAGGCGCTG-3′; 179 Cys, 5′-CTGCCCCCACTATGAGCGCT-3′; 306Stop, 5′-GAGCACTAAGTGAGCACTGC-3′. The mutant 1.85 kb BamHI

Table 1. *Transformed foci of REF by activated* ras *plus* p53 *mutants*

	Plasmid	No. of foci				Average no. of foci
		Exp1	Exp2	Exp3	Exp4	
Controls						
	LTR-SA + *ras*	3	0	0	0	0.8
	LTRp53^{W} + *ras*	1	0	0	0	0.3
Group A						
	LTRp53^{285Lys} + *ras*	ND*	ND	7	7	7.0
	LTRp53^{126Stop} + *ras*	ND	ND	3	5	4.0
	LTRp53^{205Cys} + *ras*	ND	ND	15	7	11.0
	LTRp53^{281Glu} + *ras*	ND	ND	2	2	2.0
	LTRp53$^{Exon6s.d.†}$ + *ras*	ND	ND	2	2	2.0
Group B						
	LTRp53^{135Phe} + *ras*	5	8	8	6	6.8
	LTRp53^{173Met} + *ras*	12	12	18	17	14.8
	LTRp53^{179Tyr} + *ras*	13	26	16	20	18.8
	LTRp53^{306Stop} + *ras*	2	0	0	0	0.5

*ND, not determined; †s.d., splicing donor site mutation. Early passaged REF was cotransfected with pEJ6.6 and one LTR-promoted *p53* expression plasmid. Morphologically distinct foci were counted 7–10 days following transfection.

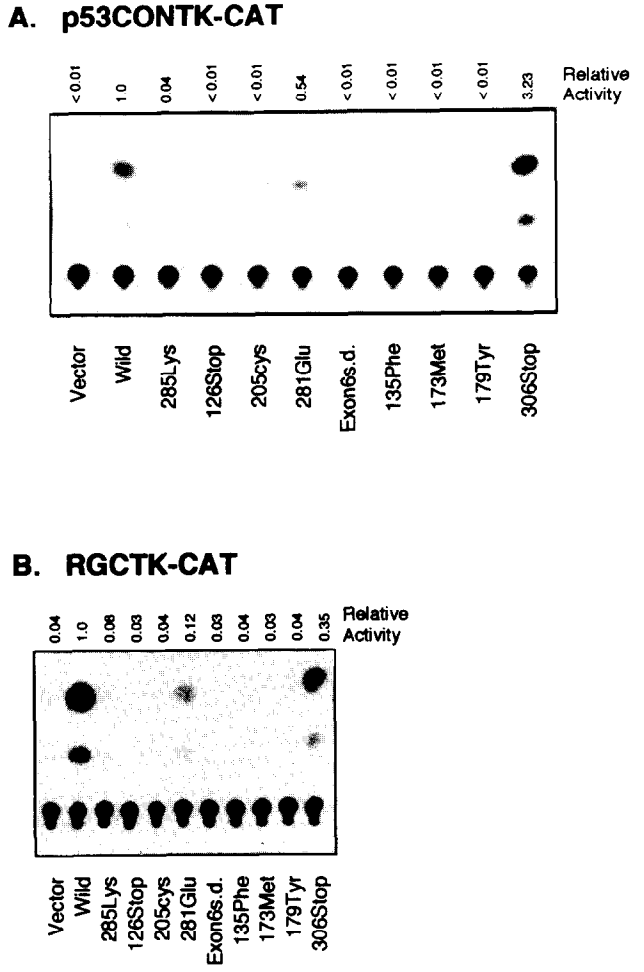

Fig. 1. Transactivation of CAT reporter genes containing p53 binding sequences by group A and group B *p53* mutants. Saos2 cells were transfected with the CAT reporter gene containing a p53 binding sequence and each CMV-promoted p53 expression plasmid. p53CONTK-CAT (A) and RGCTK-CAT (B) constructs contain p53 binding sequences of GGACATGCCCGGGCATGTCC [8] and CCAGGCAAGTCCAGGCAGG [16], respectively.

fragments thus constructed were inserted at the BamHI site of LTR-SA and CMV expression vectors. These *p53* mutant plasmids were designated, for example, as LTRp53^{285Lys} or CMVp53^{285Lys} with CMV or LTR expression plasmids containing the 285Lys mutation in the *p53* gene.

C-terminal deletion plasmids

Various deletion mutants were generated as follows. First, LTRp53^{W} linearised with StuI and deletion was introduced by digestion with ExoIII and S1 nuclease to various lengths. The resultant DNAs were recircularised in the presence of the "stop" linker containing the BamHI site capable of generating a stop codon in any one of the three reading frames (5'TGACTGACTGAGGATCCTCAGTCAG TCA3'). To construct the corresponding CMV deletion plasmids, BamHI fragments from LTR plasmids were inserted into the CMV vector. The deletion mutants were designated LTRp53^{345}, CMVp53^{345}, LTRp53^{300}, CMVp53^{300}, LTRp53^{282}, CMVp53^{282}, LTRp53^{269}, CMVp53^{269}, LTRp53^{254}, CMVp53^{254}, LTRp53^{242}, CMVp53^{242}. The deletion points of the resultant plasmids were determined by sequencing with an Applied Biosystems DNA sequencer, using a Prism® Ready Reaction DyeDeoxy® Terminator Cycle Sequencing kit.

CAT assay

The human osteosarcoma-derived cell line, Saos2, devoid of any p53 expression [21], was plated at a density of 3.5×10^5 per 60 mm dish and incubated at 37°C in a humidified 5% CO_2 atmosphere for 18–24 h. The cells were refed with 4 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (fetal bovine serum) 4 h prior to transfection. The cells were then transfected, by the calcium phosphate coprecipitation method [22], with 1 μg of CAT (chlolamphenicol acetyletransferase) reporter gene and 1 μg of each CMV-promoted *p53* expression plasmid. The transfected cells were cultured at 37°C in a 3% CO_2 atmosphere for 18 h, washed with PBS (phosphate buffered saline) (−) containing 0.1% EDTA, (ethylenediaminetetraacetic acid) and refed with fresh medium. Following additional incubation for 24 h, the cells were harvested, and equal quantities of protein were assayed for CAT activity as described previously [23].

Transformation and transform suppression assays

Early passaged rat embryo fibroblasts (REF) prepared as shown previously [24] were seeded at a density of 3.5×10^5 cells per 60 mm dish. One day later, the cells were cotransfected by the calcium phosphate method with 1 μg of pEJ6.6 [25] and 1 μg of each LTR-promoted *p53* expression plasmid. In the transform suppression assay, in addition to these plasmids, 1 μg of pMomyc [19] was also cotransfected. One day following transfection, the cells in a 60 mm dish were rinsed with PBS(−) containing 0.1% EDTA and plated in 100 mm dishes. The cells were refed with DMEM supplemented with 10% FBS at 3- to 4-day intervals. Morphologically distinct foci were counted at 7–10 days after transfection.

RESULTS

Transforming activity

A previous comparison of the frequencies of *p53* mutations in two groups of oral SCC tissues (one from which cell lines were established and the other from which no cell line could be established), indicated no significant difference in the two groups [13]. Determination was thus made of the biological activity of these groups. To assess the transforming activity of *p53* mutants of the two groups, we constructed the expression vectors containing *p53* mutant cDNA under the direction of Molony LTR. Constructs were cotransfected into REF with the activated *ras* gene. pLTR-SA without the *p53* insert served as a background control. Five independent transfections are summarised in Table 1. Two in group A (285Lys, 205Cys) and three in group B (135Phe, 173Met, 179Cys) showed relatively high transforming activity, while nonsense mutations (126Stop, 306Stop) and exon 6 splicing donor site mutation generating the stop codon in exon 7 [12] showed relatively low numbers of foci.

Transactivational activity

Wild type *p53* activates promoters containing a p53 binding sequence by direct interaction, while mutant *p53* genes are inactive [8, 9]. The transactivational activity of various *p53* mutants detected in both groups was studied in Saos2 cells

Table 2. p53 *mediated suppression of REF transformation by* ras *and* myc

	Plasmid	No. of foci				Average no. of foci	Ratio to number of LTR-SA
		Exp1	Exp2	Exp3	Exp4		
Controls							
	LTR-SA + *ras* + *myc*	68	34	41	40	45.8	1.0
	LTRp53^{W} + *ras* + *myc*	12	6	9	15	10.5	0.2
Group A							
	LTRp53^{285Lys} + *ras* + *myc*	ND*	ND	51	80	65.5	1.4
	LTRp53^{126Stop} + *ras* + *myc*	ND	ND	28	33	30.5	0.7
	LTRp53^{205Cys} + *ras* + *myc*	ND	ND	59	79	69.0	1.5
	LTRp53^{281Glu} + *ras* + *myc*	ND	ND	42	37	39.5	0.9
	LTRp53$^{Exon6s.d.}$† + *ras* + *myc*	ND	ND	37	42	39.5	0.9
Group B							
	LTRp53^{135Phe} + *ras* + *myc*	53	63	30	30	44.0	1.0
	LTRp53^{173Met} + *ras* + *myc*	70	72	32	28	50.5	1.1
	LTRp53^{179Tyr} + *ras* + *myc*	90	56	24	39	52.3	1.1
	LTRp53^{306Stop} + *ras* + *myc*	10	8	9	6	8.3	0.2

*ND, not determined; †s.d., splicing donor site mutation. Triple transfection of REF with pEJ6.6 and pMomyc and *p53* mutants was performed. Morphologically distinct foci were counted 7–10 days after transfection.

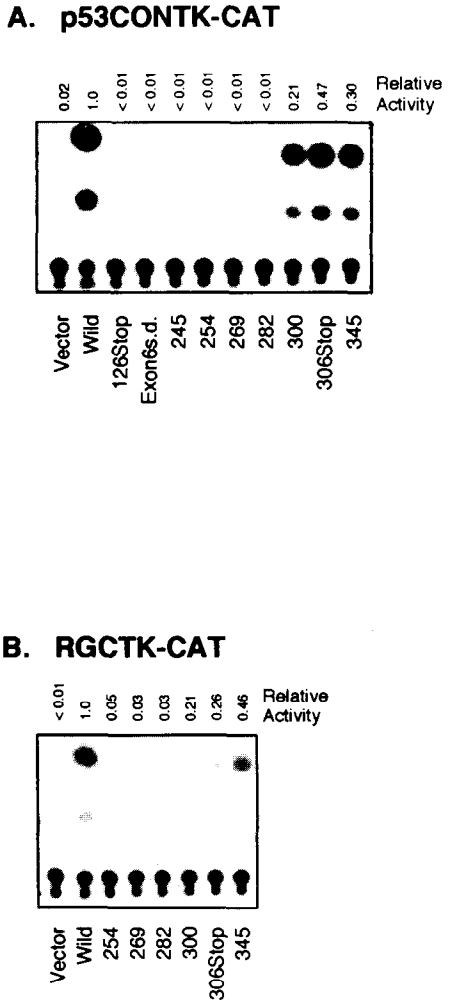

Fig. 2. 3′ side boundary for transactivation. Using p53CONTK-CAT (A) or RGCTK-CAT (B) reporters, the transactivational activity of *p53* truncated at the C-terminal region was determined as shown in Fig. 1. Relative conversion ratios of several samples to that of CMVp53^{W} are indicated at the top of the figure.

using p53CONTK-CAT and RGCTK-CAT as reporter genes. As with the wild type *p53* gene which stimulated the transcription of both reporter constructs, one of each group expressed a certain degree of transactivational activity (Fig. 1). CMVp53^{306Stop} from group B with the p53CONTK-CAT reporter showed high CAT expression comparable to that of wild type *p53* (Fig. 1A, lanes 11 and 2). With the RGCTK-CAT reporter construct, the activity was clearly less than that of the wild type. This was also seen for the *p53* mutant derived from the HSC3 cell line with a 4 bp insertion containing a stop codon between codons 305 and 306 (data not shown). CMVp53^{281Glu} from group A with p53CONTK-CAT or RGCTK-CAT resulted in low CAT expression (Fig. 1A, B) but in the other plasmids, no transactivation of the two reporter genes could be seen. Also, no significant difference between groups A and B could be seen in this study.

Suppression of oncogene-mediated transformation

Wild type *p53* suppresses the focus formation of REF by transforming and immortalising oncogenes in combination [1, 2]. *p53* mutants not only fail to inhibit oncogene-mediated focus formation but even on occasion, slightly stimulate it [2]. The effects of *p53* mutants from groups A and B on the transformation of REF by *ras* plus *myc* were examined by triple transfection with *ras* plus *myc* plus one *p53* mutant (Table 2). 285Lys and the 205Cys mutants (group A) slightly stimulated focus formation. This is consistent with the result of a focus formation assay in which relatively high transforming activity was noted. Only the 306 Stop mutation, from which a protein truncated with the C-terminal region (306–393) is predicted to be made, reduced the number of foci to the same extent as wild type. No other mutations did so significantly. Since protein made from the 306 Stop mutation does not contain the entire oligomerisation domain, this domain is suggested to be dispensable for *p53* mediated suppression, a view consistent with recent findings [26, 27].

Minimum transactivation and transform suppression domains of p53

In contrast to the 306 Stop mutant, 126Stop and exon6 s.d. mutants, possessing stop codons upstream of codon 306, were

Table 3. Suppression of oncogene mediated transformation by truncated p53

Transfected plasmids	Foci per dish			Average	Ratio
	Exp1	Exp2	Exp3		
LTR-SA + *ras* + *myc*	22, 15	24	13, 10, 15	16.5	1.0
LTRp53^{W} + *ras* + *myc*	9, 11	1, 6, 3, 3	4, 3	5.0	0.30
LTRp53^{345} + *ras* + *myc*	7, 2	4, 6	4, 4	4.5	0.27
LTRp53^{306Stop} + *ras* + *myc*	1, 1	11, 10	8, 5	6.0	0.36
LTRp53^{300} + *ras* + *myc*	5, 1	4, 6, 8	21, 24	10.1	0.61
LTRp53^{282} + *ras* + *myc*	2, 6	18, 16	29, 27, 9	15.3	0.92
LTRp53^{269} + *ras* + *myc*	10, 3	15, 17	13, 23, 13	13.4	0.81
LTRp53^{254} + *ras* + *myc*	5, 10	17, 29	ND	15.3	0.92
LTRp53^{242} + *ras* + *myc*	8, 2	34, 21	ND	16.3	0.99
LTRp53$^{s.d.}$ + *ras* + *myc*	3, 36	25, 19	ND	20.8	1.26
LTRp53^{126stop} + *ras* + *myc*	19, 2	26, 26	ND	18.3	1.11

Triple transfection of REF with *ras*, *myc* and C-terminal truncated *p53* were performed. Experimental details as in Table 2. For each sample, the ratio of average foci number to that of LTR-SA is indicated in the right column of the table. ND, not determined.

Table 4. Summary of transforming activity, transformation suppression activity and transactivational activity in the mutants of groups A and B

	No.	Mutated codon	Cell line establishment	Cell line	Transforming activity	Transformation suppression activity	Transactivational activity	
							CONTK-CAT	RGCTK-CAT
		Wild type p53	/	/	−	++	++	++
A*	1	285 GAG to AAG (Glu to Lys)	+	HOC313	++	−‡	−	−
	2	126 TAC to TAG (Tyr to Stop)	+	HOC605	+	−		
	3	205 TAT to TGT (Tyr to Cys)	+	HOC815	++	−‡	−	−
	4	281 GAC to GAG (Asp to Glu)	+	HOC719	−	−	+	+
	5	Exon 6 s.d.† AG/GT to AAGT	+	NU	−	−	−	−
B	6	135 TGC to TTC (Cys to Phe)	−	/	+	−	−	−
	7	173 GTG to ATG (Val to Met)	−	/	++	−	−	−
	8	179 CAT to TAT (His to Tyr)	−	/	++	−	−	−
	9	306 CGA to TGA (Arg to Stop)	−	/	−	++	++	+

*Data from Sakai and Tsuchida, 1992; †s.d., splicing donor site mutation generating a stop codon in exon 7; ‡number of transformed foci was enhanced.

not able to suppress transformation or transactivation. To determine the boundaries of both transform suppression and transactivation upstream of codon 306, we made *p53* expression plasmids which produce protein truncated at various lengths from the C-terminal (see Materials and Methods), and transactivational activity was determined using the above two CAT reporter genes and Saos2 cells. In both reporter genes, CAT activity was reduced dramatically when the gene was deleted at a position between codons 300 and 282 (Fig. 2), although the RGCTK-CAT, activity was somewhat less than with p53CONTK-CAT. To examine transform suppression activity, triple transfection of REF with *ras*, *myc* and one truncated *p53* gene was carried out to determine the boundary of transform suppression activity. LTRp53^{345} and LTRp53^{306Stop} showed significant capacity for transform suppression. LTRp53^{300} also expressed suppression activity although weakly (Table 3). The other truncated *p53* caused no significant suppression. Thus, suppression activity and transactivational activity are lost when *p53* is truncated to codon 282.

DISCUSSION

In this study, we compared *p53* mutation features to investigate precisely the relevance of *p53* mutation to cell line establishment, and found that the frequencies of mutations were essentially the same for the two groups A and B. Data for transformation, transactivation, and transform suppression assays indicated that the ratios of the number of strong mutant type mutations are basically the same for the two groups (Table 4). It is thus suggested that the features of *p53* mutations alone have no significant effect on the cell line establishment from oral SCC tumours. However, a careful examination of the features of individual mutations indicated that 285Lys and 205Cys mutations in group A possibly promote cell line establishment since these mutants increased the number of foci in triple transfection with *ras* and *myc*. The activity of the 306 Stop mutation in group B was similar to the wild type, indicating that it possibly inhibits cell line establishment (Table 4). However, essentially the same mutation was found in the HSC3 cell line which had a 4 bp insertion containing a stop codon between codons 305 and 306 [13]. Thus, this *p53* mutation may not be so important for cell line establishment.

Inactivation of the *p53* gene was suggested to play a role in immortalisation of rodent cells as well as human cells. Spontaneously immortalised REF cultures often contain mutated or missing *p53* alleles [28]. The transfection of mutant *p53* alone into primary rat cells induces cell immortalisation

[4]. Diploid human fibroblasts from Li–Fraumeni patients possessing a single germline mutant *p53* allele were shown to undergo spontaneous immortalisation in seven of eight cases [29]. These observations seem to contradict the present observations. However, these results are based on those obtained with primary normal human or rat cells. Other observations suggest that the *p53* mutation is not by itself sufficient for the immortalisation of rodent and human cells [30, 31]. Taken together, present result suggests that the *p53* gene inactivation is not particularly important for cell line establishment of human oral tissues but rather that other factor such as the abrogation of genes which are necessary for mortality stages 1 and 2 [32] and the inactivation of genes involved in complementation for indefinite division [33], may be importantly involved in the cell line establishment from human oral SCC tissues.

While comparing nine mutants identified in oral SCCs, we found that the 306Stop mutants presumed to generate a truncated protein still retain transactivation and transform suppression activity, which is consistent with recent reports [26, 27]. The C-terminal boundary of these two activities was determined and both were found to greatly decrease when the deletion extended from codons 300 to 282. Tarunina *et al.* noted transactivational activity disappeared when the deletion extended from codons 335 to 295 [27]. Taking this together with the present study, the boundary of transactivation may thus be present around codons 300 and 295. A transform suppression assay with truncated *p53* genes demonstrated that the region of amino acids 1–300 retain some suppression activity while amino acids 1–282 do not. It should be noted that the C-terminal boundary of activity corresponded to that of the sequence-specific DNA binding domain [7], thus indicating a rough correlation between transactivation and transform suppression. This basically agrees with the observation of Pietenpol *et al.* who showed sequence-specific transcriptional activation to be necessary for the growth suppression of tumour cells [34]. However, in their study, the boundary of sequence-specific transactivational activity was downstream of codon 333, possibly as a result of using different reporters and cells [35].

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