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Original Paper

p53 Gene Mutations and p21 Protein Expression Induced Independently of *p53*, by TGF-β and γ-rays in Squamous Cell Carcinoma Cells

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***p53* gene mutation and the influence of TGF-β and γ-rays on p21 promoter activity, p21 mRNA and protein expression were investigated in nine cell lines (OSC-1 to -9) established from metastatic squamous cell carcinomas (SCC) of the cervical lymph nodes. The direct DNA sequence analysis of exons 2 to 11 of the *p53* gene revealed 16 point mutations in all cell lines, but neither deletions nor additions were observed. TGF-β upregulated *p21* promoter activity by approximately 2-fold of the control and concurrently increased p21 mRNA expression, except in OSC-8 and -9. However, γ-rays suppressed p21 promoter activity, although p21 mRNA expression in irradiated cells was increased except for OSC-8 and -9. In parallel with the messenger expression, p21 protein expression was strongly increased by TGF-β, but only weakly increased by γ-rays. These results indicate that point mutation of the *p53* gene is frequent in metastatic SCC cells and p21 mRNA and its protein expression is *p53*-independently induced by both TGF-β and γ-rays, although the mechanism of induction by TGF-β and γ-rays is different.** **© 1999 Elsevier Science Ltd. All rights reserved.**

Key words: *p53*, p21, TGF-β, γ-rays, squamous cell carcinoma, differentiation, apoptosis
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INTRODUCTION

THE *p53* GENE is one of the most commonly mutated genes in human tumours, and numerous studies have indicated that the inactivation or abnormality of *p53* is a critical step in neoplastic transformation. The *p53* gene product is a sequence-specific DNA-binding protein and a transcription factor [1, 2]. Nonmutated *p53* protein plays an important role not only in the prevention of malignancies in humans but also differentiation and apoptosis of tumour cells. Therefore, molecular analysis of the mutation of the *p53* gene is an exceptionally useful tool in the epidemiology of human cancer. Although the normal function of *p53* is still not completely defined, recent studies suggest that *p53* controls a cell cycle checkpoint [3–5]. In animals or in cell culture, DNA damage or abnormal proliferation of cells results in increased *p53* levels and enhanced transcriptional activity, which induces cell cycle arrest and apoptosis. The biochemical activity of *p53* that is responsible for the induction of cell cycle arrest and apoptosis depends on the expression of a cyclin-dependent kinase (Cdk) inhibitor, p21 [6, 7], and other growth inhibitory proteins such as GADD45 [8] and Bax [9]. *p53*-mediated apoptosis is induced not only by the transcriptional activation of these genes but also by direct binding to several cellular proteins. Among these proteins, a RNA polymerase II basal transcriptional factor, TF II H, has been shown to bind to the p53 protein and induce apoptosis [10].

The 20-kilobase human *p53* gene is composed of separate functional domains [3, 11, 12]. The N-terminal domain (amino acid 1 to 101) contains a large number of acidic residues and can act as a transcriptional activator. The core domain (amino acid 102 to 292) contains three highly hydrophobic regions which bind DNA. Two other domains, the tetramerisation domain and basic amino acid domain, are situated in the C-terminus (amino acid 293 to 393). Throughout vertebrate evolution, specific amino acid sequences are conserved in one region of the N-terminal domain and four regions of the core domain. The conserved regions of the core domain bind the *p53*-responsive element of the promoters. Mutations of these regions thus result in loss of normal *p53* function.

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The *p21* gene is induced by the p53 protein [13], transforming growth factor beta (TGF-β) [14, 15], and senescence [16]. p21 was found to be a component of cyclin-Cdk complexes and was able to modulate the activity of a number of Cdks. In addition, p21 inhibits DNA polymerase activity by complexing with PCNA through a motif different from that responsible for Cdk inhibition [17, 18]. Thus, p21 arrests tumour cells at the G1 phase in their cell cycle and induces differentiation and apoptosis of the cells.

In treatment of head and neck squamous cell carcinomas (SCCs), inductive chemo- and radiotherapy are commonly used [19]. However, the strategy of tumour cell necrosis demands high doses of anticancer drugs and radiation, which is inevitably associated with impairment of the normal tissues. In this circumstance, differentiation- and apoptosis-inducing therapy has been recently tried [20, 21]. To obtain satisfactory therapeutic effects, the roles of *p53* and p21 in head and neck carcinomas should be understood. Here, we investigated *p53* gene mutation in SCC cell lines established from metastatic cervical lymph nodes, and examined p21 induction by TGF-β and γ-rays. The involvement of *p53* in differentiation as well as apoptosis was also investigated.

MATERIALS AND METHODS

Tumour cell lines

Cell lines used in the present study (OSC 1 to 9) were established from SCCs of the cervical lymph nodes metastasised from the oral cavity. These established cell lines are transplantable to nude mice and have colony forming ability in soft agar. Over 100 passages were used in all cell lines.

Mutation analysis of p53 *gene, exon* 2–*exon* 11

Genomic DNA was extracted from tumour cells of each cell line by phenol/chloroform extraction method. The extracted genomic DNA (250 ng) was used as the template for *p53* gene amplification (exon 2 to exon 11 in OSC-1 and -4 and exon 4 to 9 in the remaining cell lines) by polymerase chain reaction (PCR). PCR was catalysed by the thermostable Taq DNA polymerase (Perkin-Elmer, Foster, California, U.S.A.) using the recommended buffer and *p53* specific amplification primer sets (Bioserve Biotechnologies, Laurel, Maryland, U.S.A.). Thirty-five cycles of denaturation (94°C, 30 min), annealing (60°C, 1 min) and elongation (78°C, 30 sec) were performed. PCR products were directly subcloned to pGEM (Promega, Madison, Wisconsin, U.S.A.). Clones were individually isolated and sequenced using *p53* specific sequencing primers (Bioserve), a terminator cycle sequencing kit (Taq DyeDeoxy, Applied Biosystems, Foster, California, U.S.A.) and a DNA sequencer (type 373A, Applied Biosystems).

RT-PCR

Total RNA was extracted from cells of each cell line. Random hexamer primer was used to prime the extracted RNA (5 μg). The first-strand cDNA synthesis was catalysed by AMV reverse transcriptase (Takara Shuzo Co., Otsu, Japan). The synthesised first-strand cDNA was used as the template for PCR amplification as described above. Primers used for PCR were a *p53* specific exon 4-derived sense oligonucleotide (5′-CAGGGCAGCTACGGTTTCCGTCTG-3′), a *p53* specific exon 6-derived sense oligonucleotide (5′-CCTCAGCATCTTATCCGAGTGGAA-3′) and a *p53* specific exon 11-derived antisense oligonucleotide (5′-TCCTGGGGGAGGGAGGCTGTCAGT-3′). PCR products were electrophoresed on 3% agarose gel and stained with ethidium bromide.

Luciferase assay for p21 promoter

Genomic DNA extracted from peripheral blood mononuclear cells of a healthy volunteer was used as the template for PCR amplification of the 5′-flanking region of the *p21* gene containing two putative p53 protein binding sites. Primers used for PCR were a *p21* 5′-flanking region specific sense 20 mer oligonucleotide with additional 10 mer for restriction sites of Xho I and Sac I by 5′-end (5′-ATGAGCTCGAGAACATGTCCCAACATGTTG-3′) and a *p21* specific antisense 24 mer oligonucleotide located on the transcriptional start site augmented 8 mer to give the restriction site for Hind III (5′-GGAACAAGCTTCGGCAGCTGCTCACACCTCAG-3′). Thirty cycles of denaturation (98°C, 20 sec) and annealing with elongation (68°C, 20 min) were performed using LA-Taq polymerase (Takara Shuzo). In the last 16 cycles, 15 sec each were added to the time of annealing and elongation of the former cycle. After digestion with Xho I and Hind III, approximately 2.4 kbp PCR product was subcloned into the Xho I/Hind III site of luciferase reportor plasmid, pGL-3 enhance vector containing SV 40 enhancer. The vector was then transfected into tumour cells using Lipofectamin reagent (Life Technologies, Gaithersburg, Maryland, U.S.A.). pRL-SV40 vector was co-transfected as an internal control for transfection efficiency. The activity of the p21 promoter was regarded as the luciferase chemiluminescent activity, which was counted by a luminometer, Lumat LB 9501 (EG & G Berthold, Bad Wildbad, Germany).

Northern blot analysis

Total RNA was extracted from cells of each cell line using a standard method and 5 μg/lane RNA was electrophoresed on a 1.2% agarose gel containing 2.2 M formaldehyde. Then the electrophoresed RNA was transferred on to HyBond N^+ nylon membrane (Amersham International plc, Amersham, U.K.), which was prehybridised at 42°C for 24 h in a prehybridisation solution [50% (v/v) de-ionised formamide, 1×Denhardt's solution (Sigma, St Louis, Missouri, U.S.A.), 1% SDS, 1 mM NaCl, 5 mM Tris-HCl pH 7.4 and 10% dextran sulphate]. The membrane was then hybridised with ^{32}P-dCTP (Amersham)-labelled p21 specific probe provided by Dr Kawamata (Tokushima University, School of Dentistry, Tokushima, Japan). After washing in 2×standard saline citrate (SSC)/0.1% SDS at 55°C for 1 h and further washing in 0.16×SSC/0.1% SDS at 55°C for 30 min, the membrane was exposed to Kodak X-ray film (Eastman Kodak, Rochester, New York, U.S.A.) at −70°C for 24 to 72 h. Densitometric analysis of the signals was performed by the BAS-2000 image analysing system (Fuji Photo Film Co., Tokyo, Japan).

Western blot analysis

Cells harvested were lysed in a lysis buffer solution [50 mM Tris–HCl (pH 7.8), 1% Nonidet P-40, 20 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin] for 30 min on ice. By centrifugation at 14 000 rpm for 10 min at 4°C, cell lysates were obtained and the protein content in the lysate was determined using a

protein assay kit (Bio-Rad Laboratories, Richmond, California, U.S.A.). 50 μg of protein was heated in 3×SDS sample buffer solution [195 mM Tris–HCl (pH 6.8), 30% glycerol, 9% SDS, 15% 2-mercaptoethanol] for 5 min at 95°C and subjected to SDS-PAGE. The protein was transferred to an immobilon-P filter (Nippon Millipore Ltd, Tokyo, Japan). After incubation for 60 min in 3% powdered-skim milk at room temperature, the filter was incubated with anti-p53 MAb (DO-7, DAKO Japan Co., Tokyo, Japan), anti-p21 MAb (UBI, Lake Placid, New York, U.S.A.) or anti-Rb protein MAb (Pharmingen, San Diego, California, U.S.A.). These antibodies were detected with peroxidase-conjugated rabbit anti-mouse IgG (Cappel, West Chester, Philadelphia, U.S.A.) and peroxidase-positive bands were detected by an enhanced chemiluminescence system (Amersham).

RESULTS

Identification of alteration of p53 *exon* 2*–exon* 11

The sequence analysis in exons 2–11 of the *p53* gene revealed point mutations in all cell lines, although neither deletions nor additions were observed (Table 1). The point mutations were observed in exons 4 ($n=3$), 5 ($n=4$), 6 ($n=2$), 7 ($n=1$), 8 ($n=3$), 9 ($n=1$) and 10 ($n=1$). The cell line OSC-4 revealed one silent mutation (174 AGG→AGA) and OSC-2 and OSC-7 possessed one missense mutation (OSC-2: 280 AGA→ACA, Arg→Thr, OSC-7: 54 TTC→TAC, Phe→Tyr). In OSC-8 and OSC-9, two missense mutations (OSC-8: 193 CAT→TAT, Hys→Tyr, 256 ACA→ATA, Thr→Ile, OSC-9: 273 CGT→CAT, Arg→His, 309 CCC→ACC, Pro→Thr) were detected. The *p53* gene in OSC-3 and OSC-5 was mutated revealing one silent mutation and one missense mutation (OSC-3: 116 TCT→TCC, 176 TGC→TTC, Cys→Phe, OSC-5: 150 ACA→GCA, Thr→Ala, 190 CCT→CCC). Two silent mutations and one missense mutation were observed in OSC-1 (283 CGC→CGG, 299 CTG→CTA, 346 GAG→AAG, Glu→Lys). In addition, OSC-6 revealed a missense mutation at codon 95 (TCT→CCT, Ser→Pro) and one nonsense mutation at codon 126 (TAC→TAG, Tyr→termination).

Analysis of the p53 *transcripts*

No truncated fragments were observed by amplification of the *p53* transcripts spanning exons 4–11 and exons 6–11 by RT-PCR and expected length fragments were developed except in one cell line, OSC-7 (Figure 1). In this cell line, the RT-PCR product was not obtained, although the genomic DNA from exon 4 to exon 9 revealed no deletion or nonsense mutation.

Expression of p53 and p21 protein

p53 protein was constitutively expressed in all cell lines except OSC-7 in which p53-mRNA was not observed (Figure 2). Compared with OSC-4 with the wild type *p53* gene, mutant type *p53* gene possessing cell lines, including OSC-6 in which the stop codon was observed, strongly expressed constitutive p53 protein. When carcinoma cells were cultured in the presence of 3 ng/ml TGF-β for 24 h, the expression of p53 protein was increased in OSC-1 and -2, but was not increased or was negligibly increased in OSC-3, -4, -5, -6, -8 and -9. The influence of γ-rays (10 Gy) on p53 protein expression was similar to that of TGF-β.

Table 1. *Mutation of the* p53 *gene in cell lines established from oral carcinomas*

	Cell line								
Exon	OSC-1	OSC-2	OSC-3	OSC-4	OSC-5	OSC-6	OSC-7	OSC-8	OSC-9
Exon 2	–	ND	ND	–	ND	ND	ND	ND	ND
Exon 3	–	ND	ND	–	ND	ND	ND	ND	ND
Exon 4	–	–	116 TCT→TCC Silent	–	–	95 TCT→CCT Ser→Pro	54 TTC→TAC Phe→Tyr	–	–
Exon 5	–	–	176 TGC→TTC Cys→Phe	174 AGG→AGA Silent	150 ACA→GCA Thr→Ala	126 TAC→TAG Tyr→Stop	–	–	–
Exon 6	–	–	–	–	190 CCT→CCC Silent	–	–	193 CAT→TAT His→Tyr	–
Exon 7	–	–	–	–	–	–	–	256 ACA→ATA Thr→Ile	–
Exon 8	283 CGC→CGG Silent 299 CTG→CTA Silent	280 AGA→ACA Arg→Thr	–	–	–	–	–	–	273 CGT→CAT Arg→His
Exon 9	–	–	–	–	–	–	–	–	309 CCC→ACC Pro→Thr
Exon 10	346 GAG→AAG Glu→Lys	ND	ND	–	ND	ND	ND	ND	ND
Exon 11	–	ND	ND	–	ND	ND	ND	ND	ND

ND, not done; –, no mutation.

The constitutive expression of p21 protein was observed in OSC-2, -3, -5, -6, and -7 (Figure 2). Protein expression was increased by TGF-β although the increase was very weak in OSC-4, -7, and -8. γ-rays increased protein expression in OSC-5, but p21 protein was not increased by γ-rays in the other cell lines. There was no relationship between the expression of p21 protein and *p53* gene mutation or p53 protein.

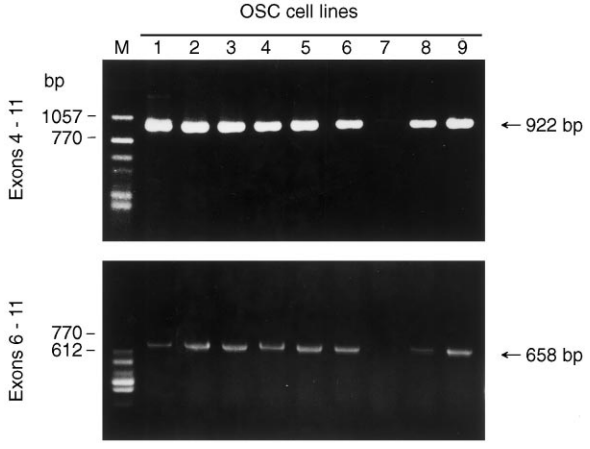

Figure 1. Amplification of the *p53* transcripts spanning exons 4-11 and 6-11 by RT-PCR. Cytoplasmic RNA was prepared from tumour cells in each OSC cell line and RT-PCR products were electrophoresed and visualised as described in Materials and Methods.

p21 promoter activity and p21-mRNA expression

TGF-β increased p21 promoter activity in all OSC lines except OSC-7 and -8, whilst radiation did not increase, but rather decreased luciferase activity (Figure 3). The promoter activity was upregulated by approximately 2-fold in OSC-1 and OSC-6.

Northern blot analysis revealed that treatment with TGF-β and γ-rays increased p21-mRNA by approximately 2-fold the original level in OSC-1, -3, and -4 (Figure 4). However, p21-mRNA was almost constant in OSC-6 and OSC-9 and even decreased in OSC-8. In OSC-2, -5 and -7, the mRNA level was slightly increased. These results were not correlated with the p21 promoter activity or 21 protein expression.

DISCUSSION

All cell lines examined revealed point mutation of the *p53* gene and, in total, 16 point mutations were detected in exon 4 to exon 10 although insertion or deletion was not observed. It is well known that genomic mutation is frequent in metastatic tumours. In addition, cell lines are more easily established from tumour cells possessing *p53* mutations than those without the mutation. Therefore, the frequent mutation

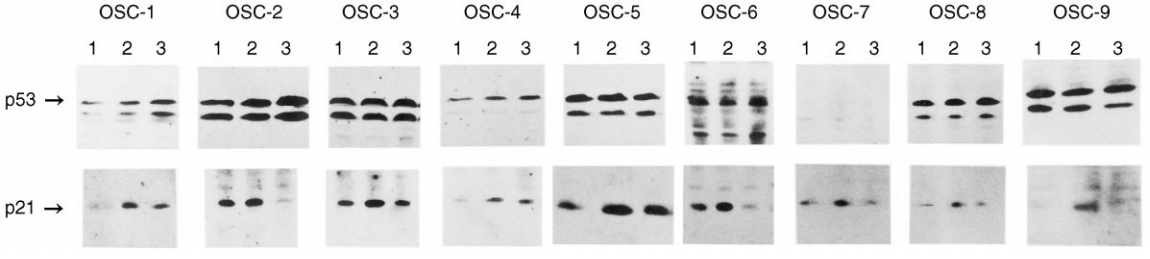

Figure 2. Effect of TGF-β and γ-rays on p21- and p53-protein expression. Total cellular proteins were prepared from tumour cells, which were incubated in the absence (lane 1) or presence (lane 2) of 3 ng/ml TGF-β for 24 h and from tumour cells irradiated with ^{137}Cs (10 Gy) and cultured in the absence of TGF-β for 24 h (lane 3). These proteins were blotted for the detection of p21 and p53 expression. The results are representative of three separate experiments.

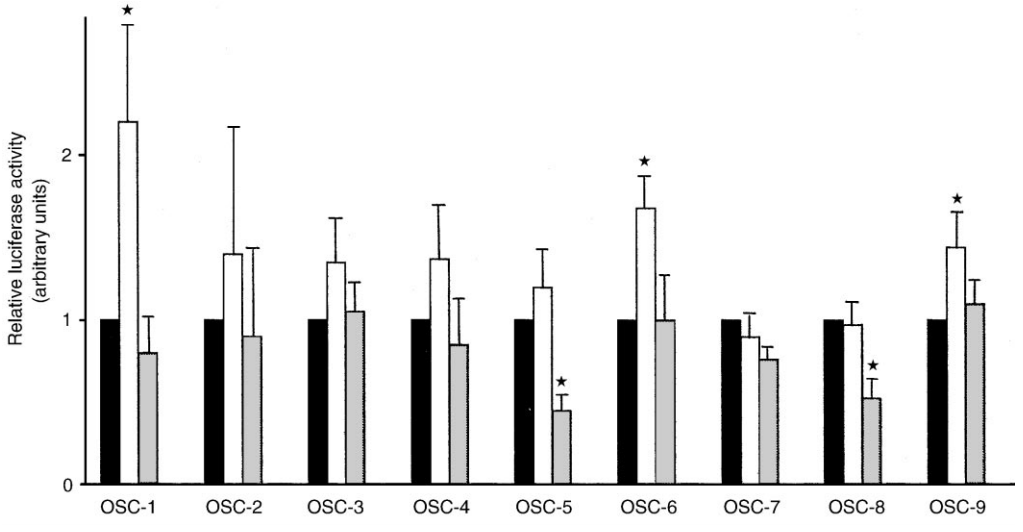

Figure 3. Effect of TGF-β and γ-rays on p21-promoter activity. OSC cells were transiently transfected with the p21 promoter luciferase reporter construct and the cells were incubated in the presence (□) or absence (■) of 3 ng/ml TGF-β for 48 h, or irradiated with ^{137}Cs (10 Gy; ■) and cultured for 48 h. Each luciferase activity was counted by a luminometer and each promoter activity was normalised to the internal control. Bars indicate the mean ±1 S.D. of three independent experiments. *P<0.05 versus medium control (Mann–Whitney U test).

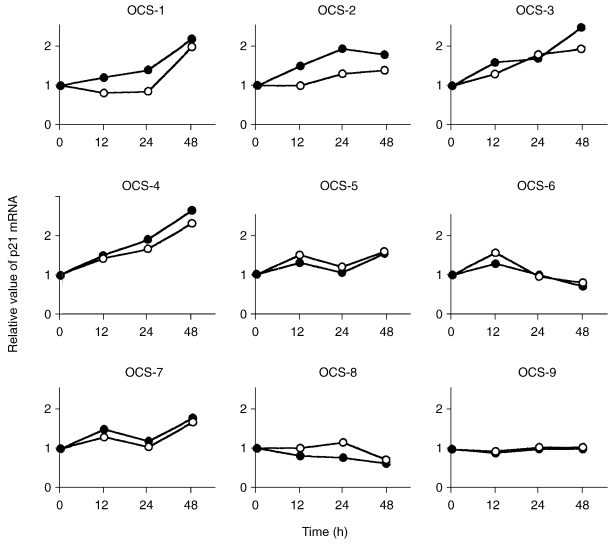

Figure 4. Effect of TGF-β and γ-rays on p21 mRNA expression. Cytoplasmic RNA was prepared from tumour cells, which were incubated with 3 ng/ml TGF-β (closed circles) for the indicated times or incubated without TGF-β for the indicated times after 10 Gy irradiation with ^{137}Cs (open circles) and 5 μg/lane of total RNA was blotted as described in Materials and Methods. The results are representative of two separate experiments.

of the *p53* gene appears to depend, at least partially, on the establishment of cultures from metastatic cells.

OSC-4 revealed only one silent mutation of the *p53* gene without amino acid change. Therefore, both transcriptional and DNA binding activities of the gene product appear to be intact. In OSC-1 and OSC-7, missense mutations were detected in codon 346 and codon 54, respectively, which are not involved in the transcriptional activation domain or core domain. However, codon 346 is situated at the tetramerisation domain and this codon mutation appears to inhibit specific DNA binding [3]. Apart from the mutation at codon 54, p53 protein in OSC-7 could not be detected in the Western blot analysis because p53-mRNA was not expressed. In the remaining cell lines, missense and nonsense mutations were observed between codon 102 and codon 292, which is the functional area for DNA binding, but hot spot mutation was observed only in OSC-9 (273 CGT→CAT). The genomic mutations observed were random in the site, which suggests that oral squamous cells are transformed by a variety of carcinogenic agents and that this variety causes the multiplicity of the *p53* gene mutation.

When p53 protein recognises the consensus binding site and binds to the target gene, the four conserved regions of the core domain, in which the 'hot spot' is included, play an important role. However, the transcriptional activity is not regulated by these regions alone. For instance, mutation in the tetramer-forming region of the C-terminal domain changes the conformation of p53 protein and causes loss of p53 function [22]. In the cell lines examined, four (OSC-1, OSC-4, OSC-5 and OSC-7) had no mutation in the conserved regions. However, p21-promoter activity was not increased by γ-rays in any cell lines by the transfection of the expression vector for luciferase including the p21-promoter region. Therefore, it is possible that the function of the *p53* gene is regulated not only by the four conserved domains, but also by other regions.

It has been reported that mutative p53-mRNA may be generated by aberrant splicing due to abnormal introns, even though no exons are mutated [23]. To ascertain the splicing, the mRNA was examined by RT-PCR, but no abnormality was detected. However, unexpectedly no PCR-product of the *p53* gene was observed in OSC-7. The regularity, specific for the transcriptional start site of each gene, has been reported and normal transcription does not start if abnormality exists in the start site. In addition, transcription of the *p53* gene is inhibited in cells which possess abnormalities in the promoter region of the *p53* gene, which is involved in the regulation of transcriptional activity. Therefore, the lack of a PCR-product in OSC-7 suggests that, despite the existence of a *p53* gene in exons 4 to 9, p53-mRNA could not be transcribed because of an abnormality in the promoter region or the transcriptional start site. Correspondingly, p53 protein was not detected by Western blot in this line. However, the p53 protein was detected in OSC-6, which revealed a nonsense mutation of the *p53* gene. The p53 protein appears to be derived from the nonmutated opposite allele, because it is known that only one allele is involved in *p53* gene mutations.

Expression of constitutive p53 protein was observed in all cell lines except for OSC-7 in which p53 mRNA was not detected. The constitutive expression of p53 protein appears to be one of the characteristics of malignant cells, but the role of constitutive expression is not satisfactorily explained. Interestingly, p53 expression in cells with the mutant *p53* gene was stronger than that in cells with the wild type *p53* gene. The degradation of the mutant type p53 protein takes longer than the wild type and thus results in the strong expression of the constitutive p53 protein in cells with missense mutations of the *p53* gene [24].

It is well known that the *p21* gene is one of the targets of p53. The *p21* gene possesses p53 protein-binding sites at 1.3 and 2.3 kb upstream of the transcription starting point in the promoter region and transcription of the *p21* gene is enhanced by binding of wild type p53 at the sites [13]. However, the *p21* promoter activity in OSC-4 was not increased by γ-rays, although wild type p53 protein expression was increased. The cause of this result is difficult to explain. Possibly, p53-inhibiting proteins were induced by γ-rays, such as Mdm-2 protein. Mdm-2 binds p53 protein directly and inhibits its transcription-activating activity [25–27]. Therefore, induction of Mdm-2 by γ-rays should be examined in OSC-4 in future studies.

Although the promoter activity was not increased by γ-rays, increases in p21 mRNA expression were observed. The degree of mRNA expression depends not only on the transcriptional activity, but also the stability of the synthesised RNA. Generally, degradation of mRNA is regulated by AU-rich instability determinant (ARE) motif in the 3′ untranslated region (3′UTR) [28]. However, degradation of p21 mRNA is strongly regulated by an endonuclease which specifically recognises other motifs in 3′UTR [29]. Therefore, the suppression of endonuclease activity by radiation appears to be one of the mechanisms by which X-rays increase p21 mRNA expression. p21 protein expression was not increased, but the p21 mRNA level was upregulated by γ-rays. This result suggests that translation and protein synthesis was inhibited by γ-rays.

TGF-β-reaction sites exist in the *p21* promoter region and TGF-β induces *p21* in a *p53*-independent manner [14, 15]. In all cell lines except for OSC-7 and OSC-8, TGF-β upregu-

lated *p21* promoter activity and concomitantly the expression of its protein. However, increased p21 mRNA expression was not always concurrent with increased protein expression. In OSC-8 and OSC-9, p21 protein expression was increased even though its mRNA was almost constant. Therefore, it is likely that TGF-β upregulates the translation efficacy of p21 mRNA.

In conclusion, the cell lines revealed a variety of *p53* gene mutations and they also expressed p21 mRNA and protein in a p53-independent manner. The induction of p53 and p21 proteins by γ-rays and TGF-β was remarkable in some cell lines, but not obvious in others. This heterogeneity suggests a reason for the difficulty in treatment of squamous cell carcinomas. Detail analysis of the expression of cell cycle regulating proteins, which is advantageous for establishment of the SCC treatment strategy, depends on future studies.

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