Oncogenic mutation of the *p53* gene derived from head and neck cancer prevents cells from undergoing apoptosis after DNA damage

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Abstract. A p53 functional analysis system, which can identify the types of abnormality of p53, such as loss of function, dominant negative function, or gain of oncogenic function, is now required. In this study, we examined the functional diversity of several mutations of p53 derived from human head and neck cancer cells. The entire open reading frame of p53 cDNA was subcloned into a mammalian expression vector, pEGFP-C3, and genetic mutations were determined. Then, intracellular localization and transcriptional activity of the tumor-derived p53 proteins were examined in Saos-2 cells. A mutant-p53 (Glu17Lys, His193Leu) or a truncated p53 (Δ121) did not activate the reporters containing p53 responsive elements from *p21waf1, BAX, MDM2, p53AIP1*, and *PUMA* genes at all. However, a mutant-p53 (Asn30Ser) showed the transcriptional activity on all of the reporters as wild-type p53 did. On the other hand, a mutant-p53 (Asp281His) activated the *p21waf1* promoter strongly and the *MDM2* promoter faintly, but did not activate the *BAX* promoter. Interestingly, this mutant-p53 prevented Saos-2 cells from undergoing apoptosis after treatment with a DNA damaging agent, adriamycin. This mutant-p53 induced cell cycle arrest but not apoptosis. Furthermore, another mutant-p53 (Glu17Lys, His193Leu) also prevented the cells from undergoing apoptosis after DNA damage probably in a transcription-independent manner. These results suggest that some cancer cells may contain the oncogenic mutation of the *p53* gene, and the oncogenic p53 protein prevents cancer cells from undergoing apoptosis after DNA damage. Detailed information for mutated *p53* gene in cancer cells might provide useful suggestions for the therapeutic strategy.

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Abbreviations: ADR, adriamycin; DMEM, Dulbecco's modified Eagle's medium; D-PBS, calcium-free and magnesium-free Dulbecco's phosphate-buffered saline; FCS, fetal calf serum; GFP, green fluorescent protein; HPV, human papilloma virus; SCC, squamous cell carcinoma

Key words: p53, oncogenic mutation, apoptosis, head and neck cancer, p21waf1

Introduction

The annual incidence of head and neck cancer is 670,900 cases in the world, and it represents 6.7% of all human malignancies (1). The therapeutic strategy for head and neck cancer is determined according to the clinical stage of the disease and the general health condition of the patient (2). Most of the patients with head and neck cancer are treated by surgical resection of the tumor with or without adjuvant-chemotherapy and/or irradiation (2). The effect of chemotherapy and irradiation varies among patients. In some patients, tumors show a very good response to chemotherapy and/or irradiation, but in other patients, tumors are completely resistant to the therapy.

p53 is one of the most investigated tumor suppressor genes, and referred to as the 'guardian of the genome'. Early observations suggested that the *p53* gene might function as an oncogene, because of frequent overexpression of p53 in many kinds of human malignancies. However, the *p53* gene in most of the cancer cells was found to have mutation, and wild-type *p53* gene was shown to act as a tumor suppressor gene (3). The wild-type p53 protein is a transcription factor that binds to specific DNA sequences in its target genes and is implicated in cell cycle arrest, DNA repair and apoptosis (4,5). Thus, loss of function of *p53* is involved in malignant transformation of several types of cells.

In human cancers, missense mutation in the *p53* gene, often within the highly conserved DNA binding core domain of the protein, is the most frequent genetic alteration (6). Such a missense mutation of the *p53* gene is found in 40-75%

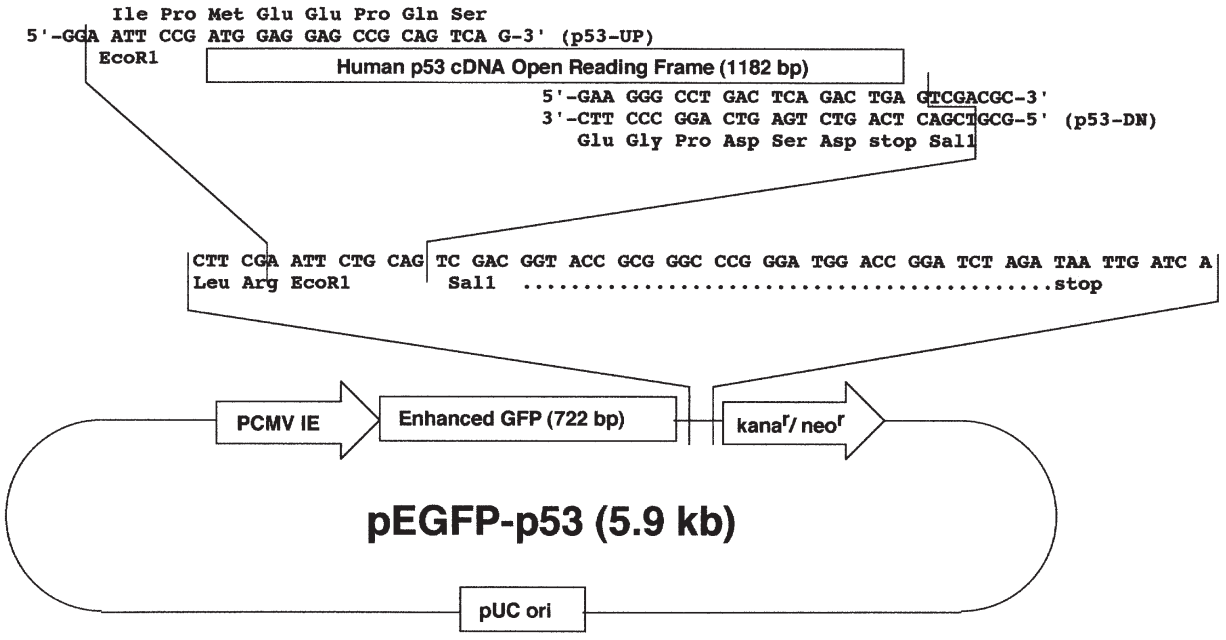

Figure 1. Schematic structure of the expression vector containing human *p53* cDNA derived from head and neck cancer cells. The plasmid contains human *p53* cDNA (1182 bp)-*GFP* fusion gene under the transcriptional control of the cytomegalovirus immediate early promoter (PCMV IE).

of human cancer (7). On the other hand, the frequency of nonsense or frame shift mutations (truncated protein-producing mutation) in the *p53* gene is relatively low, approximately 16% of all *p53* mutations (8). Thus, most of the cells with *p53* mutation express full-length proteins. This mutation spectrum in the *p53* gene is quite different from that in other tumor suppressor genes, such as *RB, APC*, and *PTEN* genes (9,10). Most of the full-length mutant-p53 is believed to inhibit the tumor suppressor function of wild-type p53 in a dominant negative fashion (11). However, it has been reported recently that at least certain types of full-length missense mutant-p53 can contribute actively to cancer progression through gain of new oncogenic function (7).

Pre-therapeutic evaluation of the *p53* gene is very important for treating patients with several malignancies including head and neck cancer, because the effect of DNA damaging therapy such as chemotherapy and irradiation is highly dependent on the status of the *p53* gene in the tumor cells (12). However, at present, analysis for the *p53* gene is performed usually by immunohistochemistry, PCR single-strand conformation polymorphism, and direct sequencing (13). A p53 functional assay system in yeast has been reported (14), however, regulation of the p53 transcriptional activity might be different in mammalian cells and yeast cells. Moreover, only one artificial p53 responsive promoter element is usually used in the yeast system. At present, a p53 functional analysis system in mammalian cells, which can identify the types of abnormality of p53, such as loss of function, dominant negative function, or gain of oncogenic function, is required.

In this study, we examined the functional diversity of several mutation of p53 derived from human head and neck cancer cells by a series of functional analysis in mammalian cells, and found some novel types of oncogenic mutation of the *p53* gene. This assay system can be utilized to identify the types of abnormality of mutated *p53* gene, and the detailed information of the mutated *p53* gene might provide useful suggestions for the therapeutic strategy.

Materials and methods

Cells and cell culture. TYS (15), HSG (16), AZA1 (17) and AZA3 (17) cells are human salivary gland cancer cell lines. BHY, HN, and HNt cells are all human oral squamous cell carcinoma (SCC) cell lines (18). Saos-2 is a human osteosarcoma cell line. All of the cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS; Sigma-Aldrich, St. Louis, MO), 100 μg/ml streptomycin, and 100 U/ml penicillin, 0.25 mg/ml Amphotericin B (Invitrogen Japan, Tokyo, Japan) in a humidified atmosphere of 95% air and 5% CO_2 at 37˚C.

RNA extraction and reverse transcription. Cancer cells grown in monolayers were harvested at early confluency. Cytoplasmic RNA was prepared by lysing cells in a hypotonic buffer containing Nonidet P-40 (Sigma-Aldrich), followed by removal of nuclei. RNA integrity was confirmed by visualizing intact 28S and 18S of ribosomal RNA on formaldehyde denaturing agarose gel. In order to produce cDNA, 5 μg of cytoplasmic RNA from cancer cells was reverse transcribed by Moloney murine leukemia virus reverse transcriptase (Invitrogen) at 42˚C for 60 min using random primers (5 μM, Invitrogen) in 20 μl of the reaction mixture.

Construction of mammalian expression vectors containing human p53 cDNA derived from head and neck cancer cells. The entire open reading frame of p53 cDNA (1182 bp) was amplified from several human head and neck cancer cell lines. One microliter of the cDNA products derived from the cancer cells was subjected to PCR amplification. The primers

used were 5'-GGA ATT ATG GAG GAG CCG CAG TCA G-3' (p53-UP) as an upstream primer and 5'-GAA GGG CCT GAC TCA GAC TGA GTC GAC GC-3' (p53-DN) as a downstream primer. PCR was performed as follows: the final concentration of dNTPs and primers in the reaction mixture were 200 μM and 1 μM, respectively. Taq DNA polymerase (Takara Biomedicals, Kusatsu, Japan) was added to the mixture at a final concentration of 0.05 U/μl, and the reaction was carried out in a Takara Thermal Cycler MP (Takara Biomedicals) under the following conditions: 94˚C for 5 min and then 94˚C for 1 min, 65˚C for 1 min, 72˚C for 1.5 min for 35 cycles, and extension at 72˚C for 7 min. PCR products were digested with *Eco*RI (Takara Biomedicals) and *Sal*I (Takara Biomedicals), and cloned into *Eco*RI/*Sal*I site of a mammalian expression vector pEGFP-C3 (Becton Dickinson Biosciences Clontech, Palo Alto, CA) (Fig. 1). The p53 cDNA sequence was determined by the dideoxy chain termination method by use of a BigDye™ terminator cycle sequencing kit (Applied Biosystems, Foster City, CA).

Transfection. Saos-2 cells ($5x10^3$ cells/dish) were seeded in 35-mm culture dishes (Becton Dickinson Biosciences Discovery Labware, San Jose, CA) in DMEM supplemented with 10% FCS. Twenty-four hours later, the cells were transfected with 1 μg of the mammalian expression vector containing p53 derived from cancer cells by use of Superfect reagent (Qiagen, Hilden, Germany). Before conducting the transfection assay, we determined the optimal condition for maximally introducing the plasmid into Saos-2 cells. The optimal concentration of the plasmid was 1 μg per 2 ml in a 35-mm culture dish and the optimal concentration of Superfect reagent was 4 μl per 2 ml in a 35-mm culture dish.

Western blotting. Cell lysates were prepared from the transfectants. Cells were cultured to sub-confluence, and washed with calcium-free and magnesium-free Dulbecco's phosphate-buffered saline (D-PBS) three times and lysed with 50 mM HEPES (pH 7.5) containing 150 mM NaCl, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EDTA, 10 mM sodium pyrophosphate, 100 mM sodium orthovanadate, 100 mM NaF, 100 mM p-nitrophenyl phosphate, 5 U/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. The protein concentrations of the samples were determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA). Protein samples (10 μg) were electrophoresed on SDS-polyacrylamide gel. Proteins from gels were transferred to nitrocellulose (Bio-Rad), and the membrane was incubated with the anti-p53 antibody (NCL-p53-CM1; Novocastra, Benton Lane, UK). The specific bands were visualized by use of an Amersham ECL kit (Amersham Biosciences, Piscataway, NJ).

Luciferase assay. Saos-2 cells ($5x10^5$ cells/dish) were seeded on 35-mm culture dishes. Twenty-four hours later, the cells were co-transfected with 0.5 μg of p53 expression vector and luciferase reporter plasmid by use of Superfect reagent. Several luciferase reporter plasmids used in this experiment contained the p53 responsive element(s) of *p21waf1, BAX, MDM2, p53AIP1*, and *PUMA* (19). Twenty-four hours after transfection, cells were lysed with lysis reagent for luciferase assay (Promega, Madison, WI). Luciferase activities were measured with the use of a Promega luciferase assay kit (Promega) in a scintillation counter. The luciferase activities were normalized by the amount of protein. Each experiment was repeated at least three times.

Apoptosis inducing ability of the p53 protein derived from cancer cells in Saos-2 cells. Saos-2 cells ($5x10^5$ cells/dish) were seeded on the cover glass (Muto pure chemicals, Tokyo, Japan) in 35-mm culture dishes. Twenty-four hours later, the cells were transfected with 1 μg of *pEGFP-C3* (empty control vector), *pEGFP-wtp53* (wild-type *p53* cDNA), *pEGFP-TYS-p53* (*p53* cDNA derived from TYS), *pEGFP-HSG-p53* (*p53* cDNA derived from HSG), *pEGFP-HNt-p53* (*p53* cDNA derived from HNt), or *pEGFP-BHY-p53* (*p53* cDNA derived from BHY) by Superfect reagent. Twenty-four, 48 and 72 h after transfection, green fluorescent protein (GFP) was observed by fluorescent-microscopy (Nikon, Tokyo, Japan) excited by 490-nm blue light. The expression and the localization of p53 protein derived from head and neck cancer cells were visualized by GFP fluorescence in the Saos-2 transfectants. Apoptotic cells, which had condensed-chromatin fragments in GFP-positive cells, were counted, and a percentage of the apoptotic cells in the transfected cells was calculated.

Cell survival assay. Saos-2 cells ($5x10^5$ cells/dish) were seeded in 35-mm culture dishes in DMEM supplemented with 10% FCS. Twenty-four hours later, cells were transfected with 1 μg of *pEGFP-C3, pEGFP-wtp53, pEGFP-TYS-p53* or *pEGFP-HNt-p53* by use of Superfect reagent. After 24-h incubation, cells were cultured for 3 days in a medium containing both adriamycin (ADR; 5 μg/ml, Sigma-Aldrich) and Geneticin (770 μg/ml G418; Invitrogen). Then, the cells were cultured in a medium containing only Geneticin (770 μg/ml) for an additional 14 days. Surviving cells were stained by Giemsa solution (Merck, Darmstadt, Germany) and the number of the cells was counted under the microscope. Before conducting this experiment, we determined the optimal concentration of ADR for cell survival assay and we found that 5 μg/ml of ADR killed almost all of the cells with 3-day treatment.

Cell cycle analysis. Saos-2 cells ($1x10^7$ cells/dish) were seeded in 100-mm culture dishes in DMEM supplemented with 10% FCS. Twenty-four hours later, the cells were transfected with 5 μg of *pEGFP-C3* or *pEGFP-TYS-p53* by use of Superfect reagent. Forty-eight hours later, the cell suspensions were fixed with 70% ethanol, and washed in D-PBS. Cells were then resuspended in 100 μl of D-PBS containing 100 μg/ml of RNaseA (Sigma-Aldrich) and 40 μg/ml of Propidium Iodide (Sigma-Aldrich). After incubation at room temperature for 15 min, cell cycle of the GFP-positive cells was analyzed by flow cytometry (EPICS XL-MCL System II, Beckman Coulter, Fullerton, CA).

Results

Mutations of p53 genes of several head and neck cancer cells in the entire open reading frame. HSG, BHY, HN, HNt, and TYS had mutations of the *p53* gene (Table I). The mutations including point mutation (missense mutation) and nucleotide

Table I. Sequencing analysis of *p53* cDNA of head and neck cancer cell lines in entire open reading frame (1182 bp).

Cells	*p53* gene mutations	Alteration of p53 proteins
HSG	A 89 G (exon 3)	Asn 30 Ser
AZA-1	No mutation	
AZA-3	No mutation	
BHY	G 349 del (exon 4)	Frame shift (stop at 122)
	A 478 G (exon 5)	Met 160 Val
	T 1106 C (exon 11)	Leu 369 Pro
HN	A 578 T (exon 6)	His 193 Leu
	G 836 A (exon 8)	Gly 279 Glu
HNt	G 49 A (exon 2)	Glu 17 Lys
	A 578 T (exon 6)	His 193 Leu
TYS	G 841 C (exon 8)	Asp 281 His

deletion (frame-shift mutation) were located at the wide area of the open reading frame corresponding to exon 2 to exon 11 in the genomic DNA (Table I). No mutation was found both in AZA1 and AZA3, although AZA1 and AZA3 were subclones derived from HSG, which contained *p53* mutation at codon 30. AZA1 and AZA3 were isolated from HSG in 1985, a novel mutation might occur in the HSG clone during the long passage *in vitro*. BHY had one nucleotide deletion (G349del) and two point mutations (A478G, T1106C). The nucleotide deletion at exon 4 in BHY resulted in the frame shift and produced a stop codon at codon 122. HNt was a subclone derived from HN, these two cells had a corresponding mutation at codon 193. However, HNt had another mutation at codon 17 and HN also had another mutation at codon 279. These different mutations in each cell clone might also occur during the *in vitro* cultivation.

In order to invalidate the PCR-based artificial mutagenesis in the cloned plasmid, we sequenced at least 6 clones containing *p53* cDNA derived from each of the cancer cells. Furthermore, we confirmed the *p53* gene mutations in TYS cells by direct genomic sequencing.

Expression and localization of the p53 proteins derived from head and neck cancer cells in Saos-2. We observed the intracellular localization of the p53 protein derived from the cancer cells in Saos-2. Not only wild-type p53 but also HSG-p53 (Asn30Ser) and TYS-p53 (Asp281His) clearly localized to nucleus (Fig. 2). HNt-p53 (Glu17Lys, His193Leu) localized mainly to nucleus, but faintly to cytoplasm (Fig. 2). BHY-p53 (Δ122), which did not have a nuclear localization signal, diffusely existed at the entire area of the cells (Fig. 2), as the control GFP protein did (data not shown).

The artificially expressed p53-GFP fusion protein in Saos-2 cells clearly showed immuno-reactivity against an anti-p53 antibody (Fig. 3). All of the p53-GFP fusion proteins except that from BHY cells showed immuno-reactive bands at approximately 80 kDa on Western blotting (Fig. 3). The molecular weights of the immuno-reactive bands corresponded to the putative size of the fusion protein, p53 (53 kDa)-GFP (27 kDa). However, p53-GFP fusion protein derived from BHY cells showed one major band at 43 kDa and one minor band at 45 kDa. The molecular weight of the major band corresponded to the putative size of the truncated p53-GFP protein derived from the BHY-*p53* gene. The minor band at 45 kDa might be a post-translationally modified band.

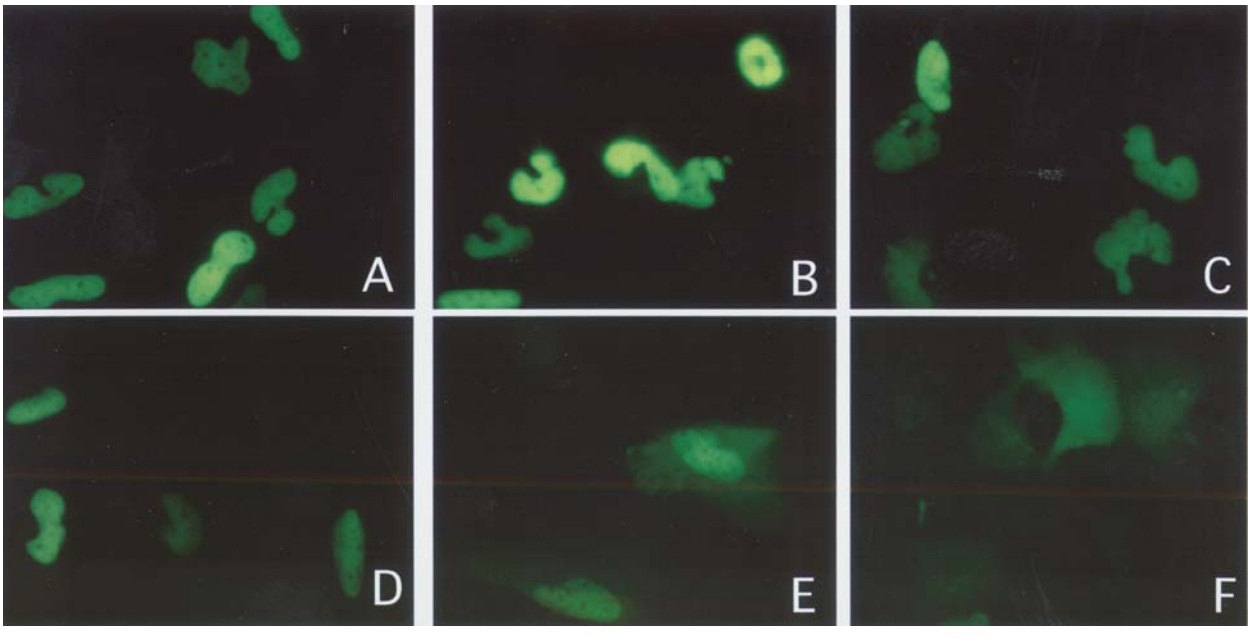

Figure 2. Expression and localization of the p53 protein derived from cancer cells in Saos-2. Saos-2 cells were transfected with 1 μg of the mammalian expression vector containing p53 derived from cancer cells by use of Superfect reagent. Twenty-four hours after transfection, GFP fluorescence was observed by fluorescent-microscopy excited by 490-nm blue light. A, wild-type p53-GFP; B, HSG-p53-GFP; C, AZA1-p53-GFP; D, TYS-p53-GFP; E, HNt-p53-GFP; F, BHY-p53-GFP.

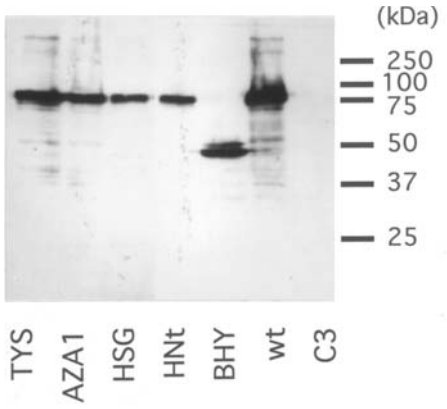

Figure 3. Western blot analysis for the p53 protein derived from cancer cells expressed in Saos-2 cells. Cell lysates were prepared from the transfectants, and 10 μg of protein samples were electrophoresed on SDS-polyacrylamide gel. Proteins from gels were transferred to nitrocellulose, and the membrane was incubated with the anti-p53 antibody. The specific bands were visualized by use of an Amersham ECL kit. Positions of molecular-weight markers (kDa) are indicated.

Transcriptional activity of the p53 proteins derived from head and neck cancer cells on several p53-target genes in Saos-2 cells. Wild-type p53 and HSG-p53 (Asn30Ser) showed transcriptional activity on all of the reporter plasmids (*p21waf1, MDM2, BAX, p53AIP1* and *PUMA*) tested in this experiment (Table II). BHY-p53 (Δ122) and HNt-p53 (Glu17Lys, His193Leu) did not activate the reporters at all (Table II). Interestingly, TYS-p53 (Asp281His) showed higher transcriptional activity on the *p21waf1* promoter than wild-type p53 did. TYS-p53 also showed transcriptional activity on the *PUMA* promoter, but did not activate *MDM2, BAX*, and *p53AIP1* promoter (Table II).

Apoptosis inducing ability of the p53 proteins derived from head and neck cancer cells in Saos-2 cells. Wild-type p53 induced apoptosis in almost all of the transfected Saos-2 cells at 48 h after transfection (Figs. 4 and 5C and D). HSG-p53 (Asn30Ser) induced apoptosis in half of the transfected Saos-2 cells at 48 h, and almost all of the transfectants at 72 h after

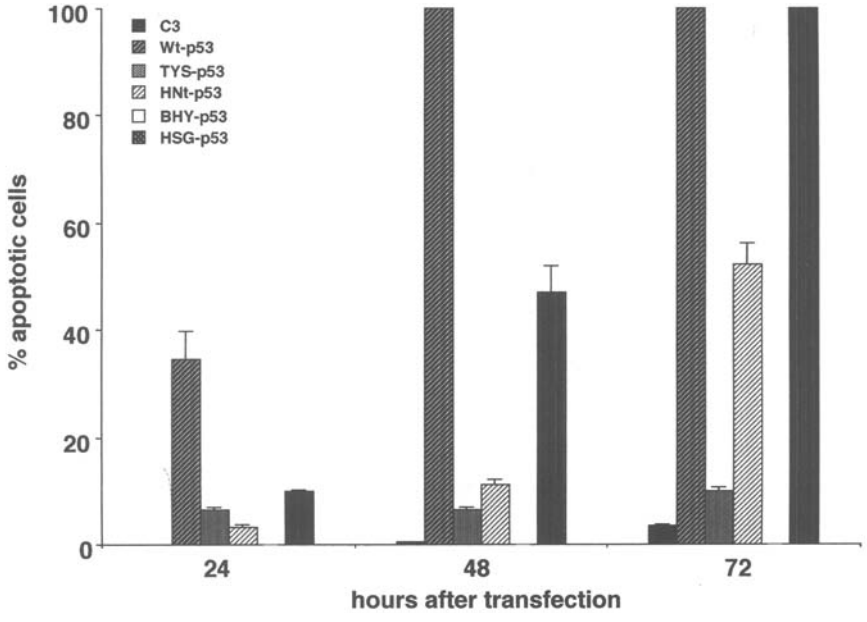

Figure 4. Apoptosis inducing ability of the p53 proteins derived from cancer cells in Saos-2 cells. Saos-2 cells were transfected with 1 μg of expression vectors containing human *p53* cDNA derived from cancer cells. The expression of the p53 protein was visualized by GFP fluorescence, and apoptotic cells, which had condensed-chromatin fragments in GFP-positive cells, were counted. The percentage of apoptotic cells in transfected cells was calculated.

Table II. Transcriptional activity of cancer-derived p53 on *p21waf1, MDM2, BAX, PUMA*, and *p53AIP1* genes in Saos-2 cells.

	Target genes				
Type of p53	*p21waf1*	*MDM2*	*BAX*	*PUMA*	*p53AIP1*
Wild-type p53	++	++	++	++	++
HSG-p53 (Asn 30 Ser)	++	++	++	++	++
AZA-1-p53 (no mutation)	++	++	++	++	++
AZA-3-p53 (no mutation)	++	++	++	++	++
BHY-p53 (truncated: stop at 122)	-	-	-	-	-
HN-p53 (His 193 Leu, Gly 279 Glu)	-	-	-	-	-
HNt-p53 (Glu 17 Lys, His 193 Leu)	-	-	-	-	-
TYS-p53 (Asp 281 His)	++++	+	-	++	-

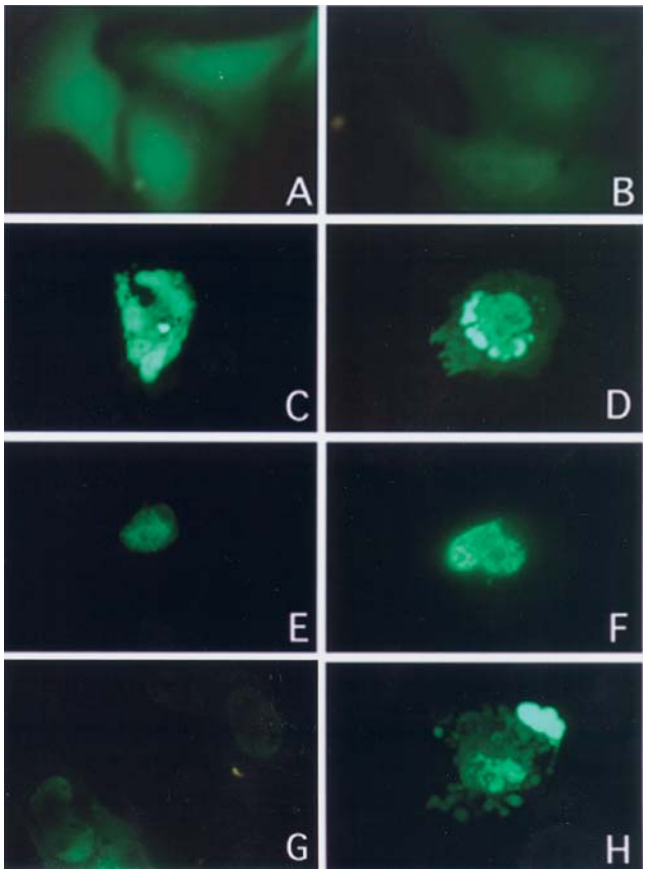

Figure 5. Induction of apoptosis by the p53 protein derived from cancer cells in Saos-2 cells. Morphology of the cells at 48 h (A, C, E, G) and 72 h after transfection (B, D, F, H). Apoptotic cells had condensed-chromatin fragments in GFP-positive cells (C, D, H). A and B, GFP; C and D, wild-type p53-GFP; E and F, TYS-p53-GFP; G and H, HNt-p53-GFP.

transfection (Fig. 4). Thus, the effect of HSG-p53 was similar to that of wild-type p53, but the action of HSG-p53 was slightly delayed when compared to that of wild-type p53. BHY-p53 (Δ122) did not induce apoptosis in Saos-2 cells at all (Fig. 4). TYS-p53 (Asp281His) induced apoptosis in <10% of the transfected Saos-2 cells at 24, 48 and 72 h after transfection (Figs. 4 and 5E and F). HNt-p53 (Glu17Lys, His193Leu) induced apoptosis in <10% of the transfected Saos-2 cells at 24 and 48 h after transfection (Figs. 4 and 5G). However HNt-p53 induced apoptosis in approximately half of the cells at 72 h after transfection (Figs. 4 and 5H).

Effect of the p53 proteins derived from head and neck cancer cells on the survival of Saos-2 cells after treatment with ADR, a DNA damaging agent. ADR (5 μg/ml) killed almost all of Saos-2 cells after 3-day treatment (Fig. 6A). Wild-type p53 acted synergistically with ADR to kill the Saos-2 cells in this experimental condition (Figs. 6B and 7). However, surprisingly TYS-p53 (Asp281His) and HNt-p53 (Glu17Lys, His193Leu) clearly increased the number of the surviving colony after cytocidal DNA damage by ADR treatment (Figs. 6C and D and 7). These mutant-p53 proteins also protected the cells from the cytocidal effect of 5-fluorouracil and cisplatin, which are usually used to treat patients with head and neck cancer (data not shown). The effect of HSG-p53 (Asn30Ser) on the cell survival was the same as that of wild-type p53 (data not shown), and BHY-p53 (Δ122) did not affect the cell survival in this assay (data not shown).

Cell cycle analysis. Saos-2 cells transfected with TYS-p53 (Asp281His) showed a marked increase of G1 (70.3%) and subG1 (10.1%) populations, and decrease of S (8.7%) and

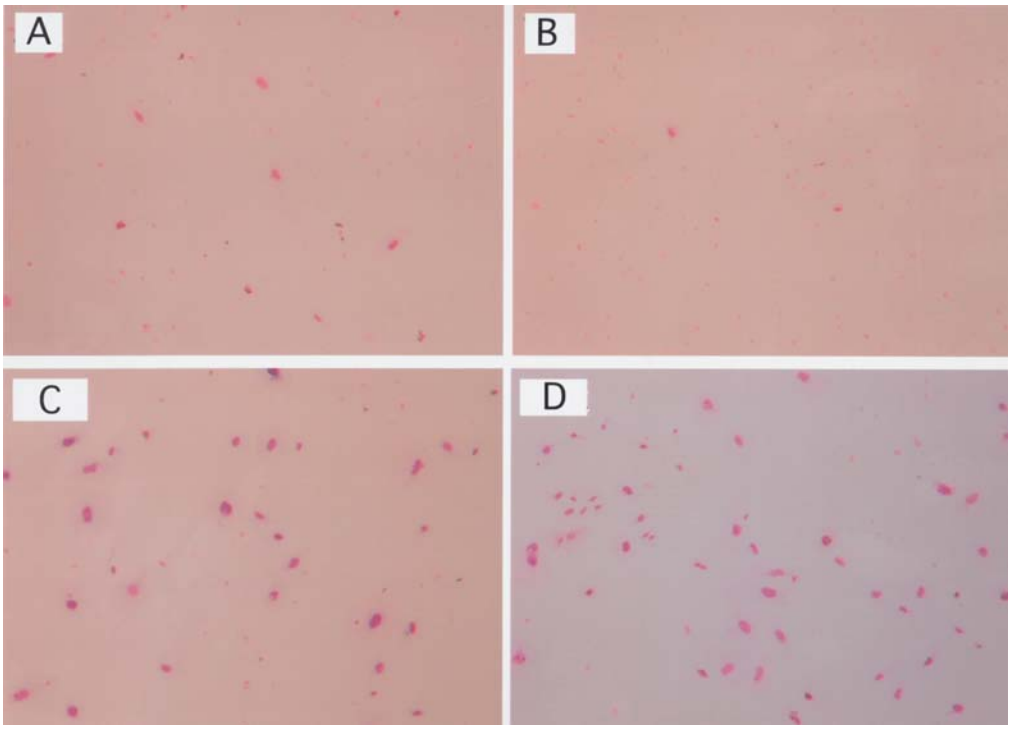

Figure 6. Effect of the p53 protein derived from cancer cells on the survival of Saos-2 cells after treatment with ADR. Saos-2 cells were transfected with 1 μg of *pEGFP-C3* (A), *pEGFP-wtp53* (B), *pEGFP-TYS-p53* (C) or *pEGFP-HNt-p53* (D). Cells were cultured for 3 days in a medium containing both ADR and Geneticin. Then the cells were cultured in a medium containing only Geneticin for an additional 14 days. Surviving cells were stained by Giemsa solution.

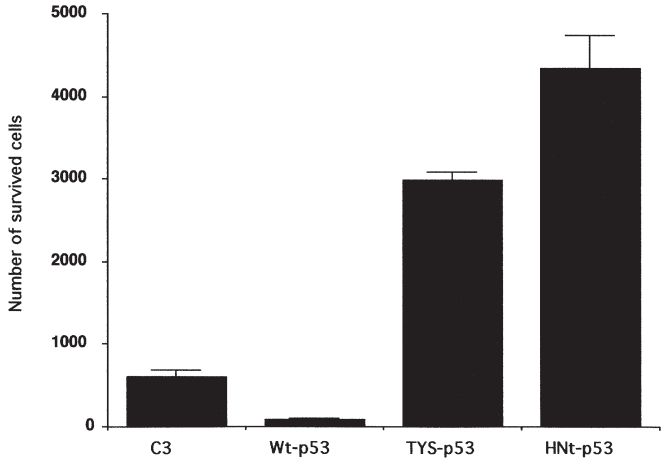

Figure 7. Number of surviving Saos-2 cells after treatment with ADR, which expressed the p53 protein derived from cancer cells. Saos-2 cells were transfected with 1 μg of *pEGFP-C3, pEGFP-wtp53, pEGFP-TYS-p53* or *pEGFP-HNt-p53*. Cells were cultured for 3 days in a medium containing both ADR and Geneticin. Then the cells were cultured in a medium containing only Geneticin for an additional 14 days. After staining with the Giemsa solution, the number of surviving cells was counted under a microscope. The values shown are the mean of three determinations. The error bars indicate the standard deviation.

G2/M (10.0%) populations when compared to the control cells (G1, 60.3%; subG1, 0.5%; S, 16.5%; and G2/M, 24.9%) (Fig. 8). Thus, TYS-p53 (Asp281His) induced G1 arrest, but rarely induced apoptosis in Saos-2 cells.

Discussion

It has been reported that mutations of the *p53* gene occur in approximately 60% of invasive head and neck cancers (20). Therefore, the clinico-pathological characteristics of head and neck cancers, such as responsiveness to chemotherapy and irradiation, local invasiveness, metastatic potential, and prognosis have been compared between cancers bearing mutated *p53* and those with wild-type *p53*. Some studies have noted that head and neck cancers with mutated *p53* show poor prognosis (20), high invasiveness and metastatic potential (21), and resistance to chemotherapy and irradiation (12,13) in comparison with those bearing wild-type *p53*. However, some other studies have found no clear relationship between *p53* mutational status and the clinico-pathological characteristics of the tumor (22).

In immunohistochemical analysis, cells with homozygous deletion of the *p53* gene or cells with a *p53* gene mutation producing a truncated protein may show negative results (23). According to the IARC TP53 Mutation Database (R11. September 2006), the incidence of *p53* gene mutation producing a truncated protein is approximately 16% of all *p53* mutations. On the other hand, PCR single-strand conformation polymorphism and direct sequencing has usually been performed in exons 5 to 8 of the *p53* gene, and again, according to the database, the incidence of the mutation outside these exons is approximately 15% of all *p53* mutations. As most mutational analysis of *p53* over the last decade has been performed for exons 5 to 8, most of the information on *p53* mutations available on the database is for these exons. Thus, approximately 30% of tumors with *p53* mutation may have given false negative results in previous analyses, and these tumors may have been considered to harbor the wild-type *p53* gene.

Balz *et al* reported that 95% (117/123 cases) of head and neck cancers showed mutation in the entire open reading frame of the *p53* gene, or expression of human papilloma virus (HPV) E6 oncoprotein even if the cancer cells had the wild-type *p53* gene (24). HPV E6 oncoprotein is well known to inactivate wild-type p53 protein. We previously investigated 26 cases of oral SCCs, and showed that 13 (50%) of them were IHC-positive for p53 (25). However, among the remaining 13 IHC-negative cases, 10 showed marked down-regulation of p53-regulatory factors such as *p33ING1* and *p19ARF*. Thus,

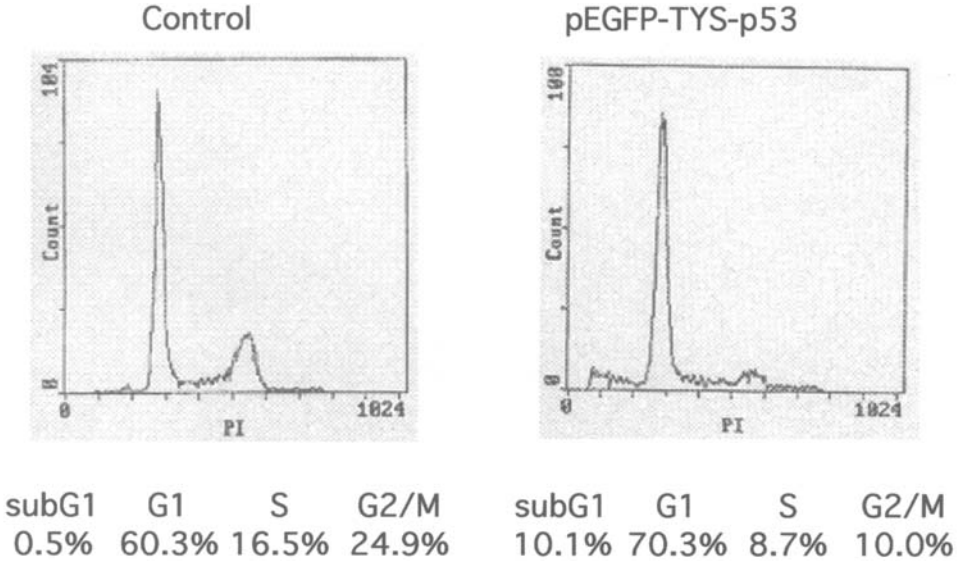

Figure 8. Cell cycle analysis of the Saos-2 cells transfected with TYS-p53. Saos-2 cells were transfected with 5 μg of *pEGFP-C3* or *pEGFP-TYS-p53*, and the cells were fixed with 70% ethanol. Cells were then resuspended in 100 μl of D-PBS containing RNaseA and Propidium Iodide, and cell cycle of the GFP-positive cells was analyzed by flow cytometry.

almost all oral SCCs show either abnormality of p53 itself, loss of expression of p53-regulatory factors, or expression of HPV E6 oncoprotein.

If these false negative cases can be discriminated from cases with a normal p53-signaling pathway, is such information clinically useful? The answer is no, because previous data have shown that most head and neck cancers have some abnormality in the p53-signaling pathway. Therefore, it will be necessary to analyze the functional diversity of the altered *p53* gene, such as complete loss of function, dominant negative function, or gain of oncogenic function.

Several mechanisms for the chemoresistance of cancer cells with oncogenic p53 have been proposed. Pugacheva *et al* reported that oncogenic p53 (Arg175His, Thr284Trp) enhanced the transcription of dUTPase, and provided the cells with 5-FU resistance (26). Furthermore, several types of mutant-p53 (Val143Ala, Arg175His, Arg248Trp, Arg273His, Asp281Gly) have been reported to enhance the expression of MDR-1. It has also been noted that the transcriptional regulatory domain (codons 22 and 23) (27) and tetramerization domain (codon 344) (28) are important for transcriptional activity of the oncogenic p53 protein on the *MDR-1* gene. Blandino *et al* have reported that a mutant-p53 (Arg175His) protects against etoposide-induced cell death, whereas another mutant-p53 (Arg273His) does not (29). However, both mutant-p53 proteins enhance the protective effect against cisplatin (29). Hence, a particular mutant form of *p53* may confer a selective survival advantage upon tumor cells during chemotherapy.

In the present study, we found one interesting mutation of the *p53* gene in TYS cells: a point mutation (G to C transversion) at the first nucleotide of codon 281 (exon 8). This mutation resulted in an aspartic acid to histidine amino acid change, and apparent oncogenic activity. TYS-p53 (Asp281His) clearly protected Saos-2 cells from the cytocidal effect of ADR. Because Saos-2 cells lack both alleles of the p53 locus, TYS-p53 might act as an oncogenic protein but not as a dominant negative protein. TYS-p53 showed higher transcriptional activity on the *p21waf1* promoter than wild-type p53. We confirmed up-regulation of the p21waf1 protein in Saos-2 cells transfected with the *TYS-p53* gene (data not shown). Moreover, in our previous experiment (19), the *p21waf1* promoter in TYS cells was highly activated by DNA-damaging stress, whereas the *BAX, p53AIP1* and *PUMA* promoters were not activated. Thus, TYS-p53 did not induce apoptosis, but clearly induced G1 arrest in the transfected Saos-2 cells. Therefore, under DNA-damaging stress, TYS-p53 induced p21waf1 and arrested the cell cycle, but did not induce apoptosis. Hence, TYS-p53 prevented cells from undergoing apoptosis after DNA damage, and might have accelerated the cumulative genetic alterations and malignant progression of the cells resulting from DNA-damaging therapy.

In this experiment, we also found another oncogenic mutation of the *p53* gene, HNt-p53 (Glu17Lys, His193Leu). HNt-p53 also protected Saos-2 cells from the cytocidal effect of ADR. HNt-p53 completely lost its transcriptional activity on the original target genes for wild-type p53. However, overexpression of HNt-p53 protein in Saos-2 cells resulted in the induction of apoptosis in 50% of the transfectants. Although the precise mechanism was unclear at this time, HNt-p53 might protect against cell death due to DNA damage probably in a transcription-independent manner, such as an interaction with the mitochondria proteins (30), or other p53 family proteins, p63 and p73 (31).

As mentioned previously, most head and neck cancers have some abnormality in the p53-signaling pathway. Therefore, it is necessary to establish a high-throughput functional analysis system for identifying the various types of *p53* gene abnormality. We are currently attempting to introduce the target gene promoter fused to *GFP* using an adenoviral vector to primary-cultured cancer cells from biopsy materials. After treating the cancer cells with a chemotherapeutic drug scheduled for possible clinical use, transactivation of the p53 target gene will be assessed in terms of their expression of GFP protein within the cells. Kinetic analysis of mutated *p53* may help to determine the optimal therapeutic strategy for individual patients with head and neck cancer.

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