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Mutantional analysis of tumor suppressor gene p53 in human oral squamous carcinoma cell line YD-9

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Oral squamous carcinoma (OSC) is the most common malignant neoplasm of the oral mucosa. Although the etiology of OSC is not fully understood, accumulated evidences indicate that the activation of proto-oncogenes and the inactivation of tumor suppressor genes underlie the disease development. An OSC cell line, YD-9 was newly established and characterized. However, the mutational analysis of p53 gene was not performed. Thus, in this study, the presence of mutation in the p53 gene was examined by amplification of exon-4 to -8 and subsequent DNA sequencing. Two point mutations were found in exon-4 and -6: A to G, resulting in amino acid change Tyr to Cys in exon-4, and C to G, resulting in amino acid change Gly to Arg in exon-6, respectively. Any mutation was not found in the exon-5, -7 and -8. The presented results would contribute to basic research to understand the biological mechanism of OSC using YD-9 cells.

Key words: mutation of p53, oral squamous carcinoma

Introduction

Oral squamous carcinoma (OSC) is one of the most disfiguring types of cancer, since the surgical removal of the tumor may result in facial distortion. Treatment of OSC has primarily relied on classical modalities encompassing surgery, radiation and chemotherapy of a combination of these methods. At the late stage of malignancy, OSC cells are very resistant to cancer-therapy-mediated apoptosis. Understanding and overcoming resistance mechanism of OSC cells to chemotherapeutic agents would therefore facilitate identification of new therapeutic targets and development of new treatments.

Wilde-type p53, a nuclear phosphoprotein, is a negative regulation of cell proliferation and function as a tumor suppressor gene (Eliyahu *et al.*, 1989). Wide-type p53 protein inhibits the cotransforming activity of *ras* with either mutant p53, c-myc or E1A proteins in rodent cells (Mercer *et al.*, 1991). Alteration of the p53 gene through either rearrangement, deletion, or piont mutation result in either no expression of wild-type p53 or overexpression of mutant p53 protein that, in turn, act as oncogene products. Such products are often found in epithelial cells that have escaped from senescence, i.e. human squamous cell carcinoma cells (Lavigueur *et al.*, 1989; Hollstein *et al.*, 1990). Mutation in p53 may result not only in loss of the suppressor activity associated with the gene product, but may also induce expression of mutant p53 protein that may be intrinsically oncogenic. Transfection of primary cells with plasmids expressing mutant p53 yields immortalised cells that are tumorigenic in concert with activated ras (Jenkins *et al.*, 1985; Eliyahu *et al.*, 1984). Mutation of the p53 gene are present in most cancer subtypes, ie. Friend virus-induced mouse leukemic cells (Rovinski *et al.*, 1987), non-small cell lung carcinomas (Chiba *et al.*, 1990), colorectal carcinomas (Rodrigues et al, 1990), hepatocellular cellular carcinomas (Hsu *et al.*, 1991), cervical cancer cells (Scheffner *et al.*, 1991), and OSC cell lines (Kim *et al.*, 1993). The protein expressed from the mutant p53 gene has been implicated in growth deregulation and malignant progression of these cancers (Eliyahu *et al.*, 1989).

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Lee *et al.* (2005) newly established OSC cell lines derived from squamous cell carcinoma and investigated the characterization of the cell lines (YD-8, -9, -10B, -17 and -38). These YD cell lines will be expected to serve as useful tools in the study of the molecular pathogenesis and biological characteristics of OSC cells. However, mutaional analysis of p53 in YD-9 cell line is not performed and it is needed to understand the mechanism occurred in YD-9 cells. Thus, this study was undertaken to investigate whether p53 gene of YD-9 cells is mutated and which codons are mutated.

Materials and Methods

Cell culture

YD-9 cells were cultured in a mixture of Dulbecco's modified Eagles medium (Gibco BRL, Grand island, NY) and Ham's nutrient mixture F12 (Gibco BRL, Grand island, NY), at 3 : 1 ratio, supplemented with 10% fetal bovine serum, 1% glutamine, 100 μg/m*l* penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO_2.

Genomic DNA purification in YD-9 cells

Cultured cells were trypsinized and harvested by centrifugation. Cells at 10^7 were resuspended with 150 μ*l* of buffer [50 mM Tris-HCl (pH 8.0), 25 mM EDTA and 400 mM NaCl]. The sample was mixed 200 μ*l* of lysis buffer [10% SDS, 20 μ*l* proteinase K (10 μg/m*l*)], The sample was homogenized and incubated at 65°C for 3 h. After incubation, proteins and cellular debris were precipitated by adding a 300 μ*l* 6 M NaCl, kept at 4°C for 15 min. Centrifugation was done at 25,000 g for 20 min. 500 μ*l* of the supernatant were transferred to a new tube, with 500 μ*l* 8 M guanidine hydrochloride (pH 8.0), and 0.49 M ammonium acetate solution, and kept in mild agitation for 90 min. DNA were precipitated by adding 800 μ*l* of cold 100 % isopropyl alchol, followed by centrifugation at 8,000 g for 5 min. Pellet was washed with 400 μ*l* of 70% isoprophyl alchol. After drying, pellet was resuspended in 100 μ*l* TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA and 50 μg/m*l* RNAse]. DNA sample was stored at 4°C

PCR and purification of product

The reaction mixture was contained DNA 50 ng, dNTP 250 uM, Tris-HCl (pH 9.0) 10 mM, KCl 40 mM, $MgCl_2$ 1.5 mM, forward primer 20 pmol, and reverse primer 20 pmol, Taq DNA polymerase 1 U. The sample was placed in a thermocycler (Corbett Research, Australia). The profile of thermocycling was one prereaction at 94°C for 5 minutes and 35 cycling reaction with 94°C 30 seconds denaturation, 50°C 30 seconds annealing, 72°C 1 minutes extension. After reaction, in order to recovery exciting band, after carrying out 1.0% low-melting-temperature agarose gel electrophoresis in 0.5 × TBE buffer, cutting out a slice of agarose containing the band with sharp razor blade and transfer it to clean 1.5 ml tube. To the gel slice, added 500 m*l* of 6 M sodium iodide solution, and melt the agarose by incubating for 5 minutes at 55°C. 10 m*l* of glass beads solution [0.1% silica in 3 M NaI] was added. The mixture was incubated for 5 minutes at room temperature with occasional shaking. The mixture was centrifuged at 10,000 xg for 5 minutes, and the supernatant was discarded. The pellet was washed three times with 500 m*l* of 70% ethanol, and resuspended in TE (pH 8.0). DNA and glass beads complex was incubated for 3 minutes at 45°C to elute the DNA from the bead. The sample was centrifuged at the 10,000 xg for 1 minute, and then transferred the DNA-containing supernatant to a fresh tube, and stored at 4°C.

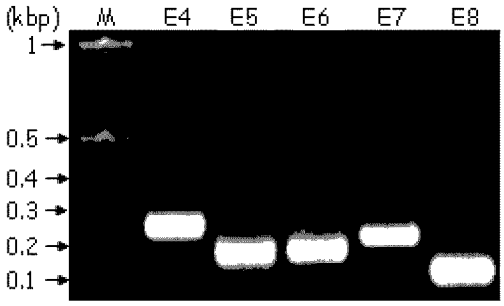

Fig. 1. Agarose gel electrophoresis of PCR products with each p53 exon oligonucleotides. (M: molecular DNA marker; E4: p53 exon4; E5: p53 exon5; E6: p53 exon6; E7: p53 exon7; E8: p53 exon8)

Table 1. Primers used to amplify each exon of p53

Exon number	Primer sequence	Length (bp)
4	5'-TTCCTCTTCCTGCAGTACTC-3' 5'-ACCTGGGCAACCAGCCCTGT-3'	245
5	5'-ACAGGGCTGGTTGCCCAGGGT-3' 5'-AGTTGCAAACCAGACCTCAG-3'	175
6	5'-GTGTTGTCTCCTAGGTTGGC-3' 5'-GTCAGAGGCAAGCAGAGGCT-3'	190
7	5'-TATCCTGAGTAGTGGTAATC-3' 5'-AAGTGAATCTGAGGCATAAC-3'	212
8	5'-GCAGTTATGCCTCAGATTCAC-3' 5'-AAGACTTAGTACCTGAAGGGT-3'	138

Subcloning in pGEM®-T vector

Eluated PCR products were subcloned pGEM®-T vector system (Promega, Madison, WI). As host *E. coli* strain, XL1-Blue (F'::Tn10 proA$^+$B$^+$ lacI^q Δ(lacZ) M15/recA1 endA1 gyrA96 (Nal^r) thi hsdR17 (r_k^- m_k^+) supE44 relA1 lac) was transformed with the ligated PCR product with vector DNA. The *E. coli* colony inserted DNA was selected as white colony in LB media plate containing X-gal, isopropylthio-β-D-galactosidase (IPTG) and ampicillin (100 μg/ml). The cell was inoculated in LB broth including ampicillin, inserted plasmid DNA was purified and confirmed by enzyme digestion reaction.

Plasmid DNA purification and sequence analysis

Plasmid DNA was purified using Wizard® plus SV minipreps DNA purification system (Promega, Madison, WI). Bacterial cells were harvested by centrifugation at 10,000 xg for 1 minute. Cell resuspension solution was added and the cell pellet was completely resuspended by vortexing, and then 250 μl of cell lysis solution was mixed by inverting the tube 4 times. The mixture was added 350 μl neutralization solution and centrifuged at 10,000 xg for 10 minutes. The supernatant was filtrated in DNA binding column and washed two times. Plasmid DNA was purified 50 μl of TE buffer. To analyse of cloned DNA sequences, we requested Genotech DNA sequencing service company.

Results

The presence of mutation of the p53 gene was examined by performing PCR and by subsequent DNA sequencing of exons-4 ~ -8 of the p53 gene in the YD–9 cells. In order to analysis p53 gene, origin p53 DNA sequence was obtained from NCBI GenBank (NM 000546).

For cloning of human p53 gene, the sequences of p53 gene was analyzed with blast program of National Center for Biotechnology Information (NCBI). In order to perform PCR,

Exon 4 (245 bp)

TTCCTCTTCCTGCAGTACTCCCCTGCCCTCAACAAGATGTTTTGCCAACTGGCCAAGACCTGCC
CTGTGCAGCTGTGGGTTGATTCCACACCCCCGCCCGGCACCCGCGTCCGCGCCATGGCCATCTGC
AAGCAGTCACAGCACATGACGGAGGTTGTGAGGCGCTGCCCCCACCATGAGCGCTGCTCAGAT
AGCGATGGTGAGCAGCTGGGGCTGGAGAGACGACAGGGCTGGTTGCCCAGGT

>gi|56157802|gb|AY838896.1| Homo sapiens tumor protein p53 (Li-Fraumeni syndrome) (TP53) gene, complete cds, Length=23164, Score = 466 bits (235), Expect = 2e-128, Identities = 241/243 (99%), Gaps = 0/243 (0%), Strand=Plus/Plus

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Query  1      TTCCTCTTCCTGCAGTACTCCCCTGCCCTCAACAAGATGTTTTGCCAACTGGCCAAGACC  60
              ||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  14329  TTCCTCTTCCTACAGTACTCCCCTGCCCTCAACAAGATGTTTTGCCAACTGGCCAAGACC  14388

Query  61     TGCCCTGTGCAGCTGTGGGTTGATTCCACACCCCCGCCCGGCACCCGCGTCCGCGCCATG  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  14389  TGCCCTGTGCAGCTGTGGGTTGATTCCACACCCCCGCCCGGCACCCGCGTCCGCGCCATG  14448

Query  121    GCCATCTGCAAGCAGTCACAGCACATGACGGAGGTTGTGAGGCGCTGCCCCCACCATGAG  180
              ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  14449  GCCATCTACAAGCAGTCACAGCACATGACGGAGGTTGTGAGGCGCTGCCCCCACCATGAG  14508

Query  181    CGCTGCTCAGATAGCGATGGTGAGCAGCTGGGGCTGGAGAGACGACAGGGCTGGTTGCCC  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  14509  CGCTGCTCAGATAGCGATGGTGAGCAGCTGGGGCTGGAGAGACGACAGGGCTGGTTGCCC  14568

Query  241    AGG  243
              |||
Sbjct  14569  AGG  14571
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Fig. 2. Comparison with wild-type p53 exon 4 and YD-9 cell p53 exon 4.

specific forward primer and reverse primer were designed (Table 1). After specific primer was designed to obtain five p53 exon genes, each exon gene was amplified by PCR with genomic DNA. We obtained 245 bp (exon-4), 175 bp (exon-5), 190 bp (exon-6), 212 bp (exon-7) and 138 bp (exon-8) p53 gene of YD-9 cells (Fig. 2).

The presence of mutant p53 gene was examined by executing PCR and subsequent DNA sequencing of exon-4 ~-8. We found the point mutation in Exon-4 (Fig. 2) and -6 (Fig. 3). The TAC sequence was changed to TGC, which resulted in an amino acid mutation of Tyr to Cys in exon-4 (Fig. 2 and Table 2). The CGG sequence was changed to GGG, which resulted in an amino acid mutation of Gly to Arg in exon-6 (Fig. 3 and Table 2). Other mutations were not found in the exon-5, -7 and -8.

Table 2. Mutational analysis of p53 in YD-9 cells.

Exon number	Base change	Amino acid change
4	TAC → TGC	Tyr → Cys
5	none	none
6	CGG → GGG	Gly → Arg
7	none	none
8	none	none

Discussion

Many studies on the establishment of cancer cell lines contribute to various basic research on cancer, as well as to diagnostic and therapeutic development. However, the establishment of an OSC cell line is especially difficult from the primary site, and only a few successful attempts have been reported (Easty *et al.*, 1981; Hu *et al.*, 1984; Ji *et al.*, 2001). Since Lee *et al.* (2005) have had a interest in establishing cell lines from Korean OSC, they established the five new OSC cell lines were established from untreated OSC.

The molecular mechanisms underlying the development

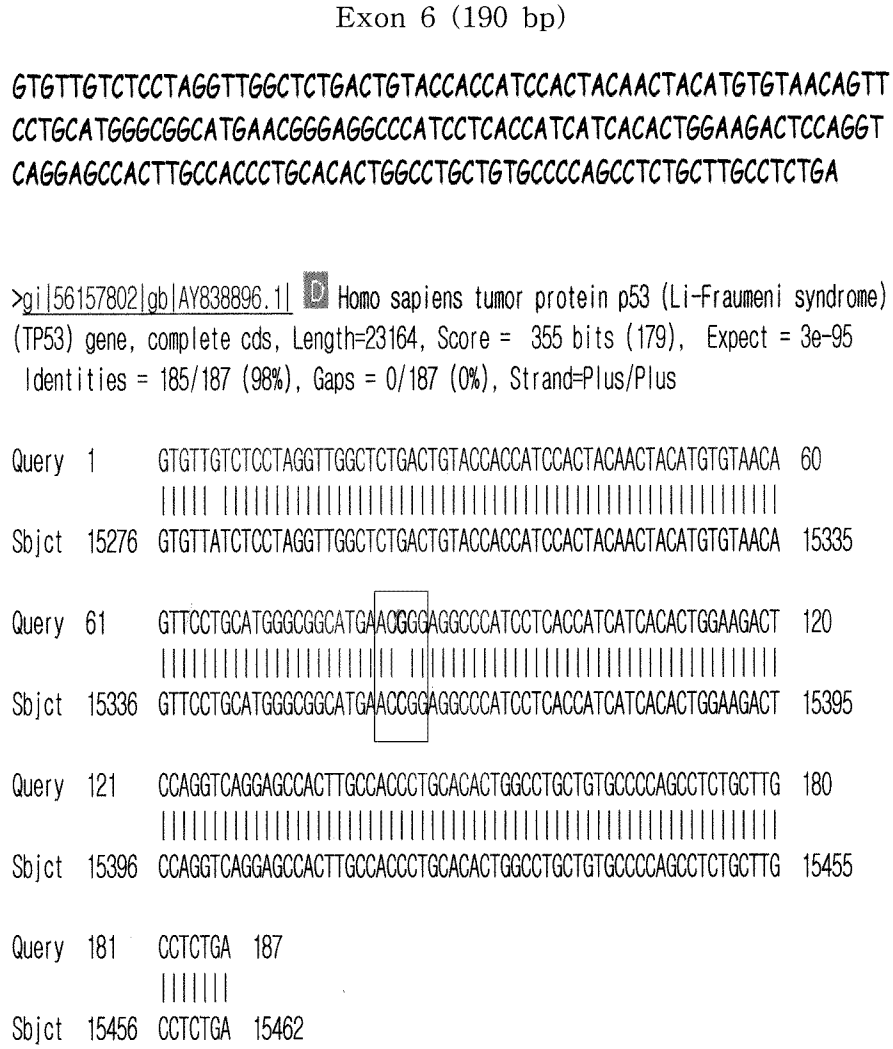

Fig. 3. Comparison with wild-type p53 exon 6 and YD-9 cell p53 exon 6.

of OSC remain largely unknown. Several studies indicate the overexpression and/or amplification of *c-erb*B-1/epidermal growth factor receptors (EGFR), *c-myc, c-bcl* and c-Ha-*ras* are closely associated with the development of oral cancer in humans (Matlashewski, 1989; Romano *et al.*, 1989; Saki *et al.*, 1990). Inactivation of tumor suppressors through gene mutations and high-risk HPV infections are also linked to oral carcinogenesis. For example, high levels of p53 protein resulting from point mutation of the p53 gene are frequently found in head and neck cancer tissue (Husain *et al.*, 1989; Wong and Biswas, 1987). Therefore, it appears that the inactivation of tumor suppressor genes, along with the dominant activation of proto-oncogenes is involved in oral carcinogenesis.

In this study, the p53 gene was point mutated in YD-9 cells and all of these mutations occurred in highly conserved regions. The mutation in exon 4 occurred in the central conserved part of the protein or 'core domain' (residues 102-209), which was responsible for sequence specific DNA binding. In general, this mutation is the missense type and leads to loss of DNA binding, which is believed to be critical for the biological activity of p53. Since the half-life of wild type p53 protein is several minutes, p53 protein level in normal cells are relatively very low and generally undetectable by immunoblotting. However, abnormal p53 protein can be easily detected by immunoblotting because of the prolongation of its half-life. The overexpression of p53 protein was found in p53-mutated YD-9 cells. The results of DNA sequencing demonstrated p53 gene mutations associated with abnormal accumulation of p53 protein in YD-9 cells. Since p53 is closely related to various proteins regulating the cell cycle, abnormal p53 function may cause accelerated cell proliferation leading to multicentric tumorgenesis and malignant tumor. The presented result of p53 gene in YD-9 cells will provide new understanding the biological mechanism in OSC cells.

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