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Frequent Mutations of p53 and *MTS1/CDK4I* Tumor Suppressor Genes in Chinese Preneoplastic and Neoplastic Oral Tissues

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Aberrant expression and mutation in the p53 and *MTS1/CDK4I* genes were determined from 11 normal, 8 preneoplastic and 25 neoplastic oral tissues obtained from Beijing, China, using immunostaining, single strand conformational polymorphism analysis, and nucleotide sequencing. Normal tissue showed a negligible amount of p53 immunostaining, while 3 (38%) of 8 preneoplastic, and 16 (64%) of 25 cancer tissues demonstrated moderate to strong p53 immunostaining. Point mutations within exons 5 to 8 were not detected in normal tissue specimens, but were detected in 2 (25%) preneoplastic tissues and in 15 (60%) cancer specimens. Of the tissues with mutations, 2 (100%) preneoplastic and 14 (93%) cancer tissues contained a CGT to CAT mutation at codon 273 of p53 gene. One cancer tissue showed a silent mutation (CGC to CTC) at codon 283. Three cancer specimens containing a point mutation at codon 273 also showed additional silent mutations at codons 156, 157, or 275. These data indicate that p53 mutation is highly prevalent in tested preneoplastic and neoplastic oral tissues and that the codon 273 is the "hot-spot" for point mutations. The enhanced p53 immunostaining was, in general, closely associated with point mutations, but 1 (13%) preneoplastic sample and 5 (20%) neoplastic oral tissues not containing point mutations within exons 5 to 8 demonstrated enhanced immunostaining. Over 62% of preneoplastic and 80% of neoplastic oral tissues contained mutations in *MTS1/CDK4I* gene, but, unlike p53 mutations, the mutation pattern of *MTS1/CDK4I* gene was not specific. Two preneoplastic (25%) and 12 neoplastic (48%) tissues contained mutations in both p53 and *MTS1/CDK4I* genes, and 2 preneoplastic (25%) and 3 neoplastic (12%) tissues contained mutations neither in p53 nor in *MTS1/CDK4I* genes.

Key words : Oral cancer, p53, *MTS1/CDK4I*

Introduction

Although the incidence of oral cancer is relatively low in the Western countries, it is one of the most common malignant tumors in a number of Asian countries (Parkin *et al.*, 1988). Extensive consumption of tobacco is associated with cellular DNA damage and an increased incidence of oral cancer in humans (De Stefani *et al.*, 1990; Talamini *et al.*, 1990; Stich *et al.*, 1992). The constituents of tobacco responsible for cellular DNA damage are polycyclic aromatic hydrocarbons (PAH) and tobacco-specific nitrosamines, both of which show DNA alkylation and carcinogenicity in animals (Preston-Martin and Correa, 1988; Hecht *et al.*, 1988; Preston-Martin, 1991). Inasmuch as oral epithelial cells have direct contact with these chemicals in the oral cavity of tobacco users, the likelihood of cellular DNA damage is significantly higher in tobacco users compared to individuals who do not use tobacco.

Many studies have indicated that mutations in the p53 tumor suppressor gene are the most frequently found genetic disorders in most human cancers including oral cancer (Hollstein *et al.*, 1991; Ogden *et al.*, 1992; Sakai *et al.*, 1992; Levine, 1993). Mutations of the p53 gene through either rearrangement, deletion, or point mutation result in either no expression of wt p53 or overexpression of mutant p53 protein that, in turn, acts as an oncogene product (Hollstein *et al.*, 1991; Levine, 1993). The causes of p53 mutation are not known, but a significant body of evidence indicates

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that exposure to various mutagens may be responsible. For example, extensive consumption of tobacco, infections with hepatitis B and C viruses, and continuous exposure to aflatoxin B1 are closely associated with the point mutations of p53 gene (Ogden *et al.*, 1992; Aguilar *et al.*, 1994; Fujimoto *et al.*, 1994). A tumor suppressor gene, *MTS1/CDK 4I* (multiple tumor suppressor-1/cyclin-dependent kinase-4 inhibitor), was recently isolated and mapped at 9p21 of human genome (Kamb *et al.*, 1994; Nobori *et al.*, 1994). The *MTS1/CDK4I* gene encodes p16 that inhibits the function of active cyclin D-cdk4 complexes, and in doing so induces the arrest of cell cycle progression (Hunter and Pines, 1994). A recent study suggests a positive association between the loss of heterozygosity of 9p21 and the immortal phenotype of keratinocytes derived from head and neck carcinoma (Loughran *et al.*, 1994), indicating that loss of *MTS1/CDK4I* activity may, in part, be responsible for the immortalization of human keratinocytes. Extremely high mutation frequency of this gene has been reported in melanoma cell lines, lymphoblastoid cell lines derived from dysplastic nervous system, esophageal squamous cell carcinomas, primary non-small cell lung carcinomas, and pancreatic adenocarcinomas (Kamb *et al.*, 1994; Nobori *et al.*, 1994; Mori *et al.*, 1994; Hayashi *et al.*, 1994; Caldas *et al.*, 1994). However, there has been considerable controversy over the role of the *MTS1/CDK4I* gene product, p16, in neoplasia, because, unlike in the tumor cell lines, high frequency of *MTS1/CDK4I* mutations is not identified with some primary tumor tissues (Cairns *et al.*, 1994; Spruck *et al.*, 1994; Zhang *et al.*, 1994; Ohta *et al.*, 1994).

In the present study, we investigated the frequency of aberrant expression and mutation of the p53 gene, mutation frequency of *MTS1/CDK4I* gene, and the association between a history of heavy tobacco consumption and the frequency of these mutations in normal, preneoplastic, and neoplastic (squamous cell carcinoma) oral tissues obtained from Beijing, China. In our study, 25% of preneoplastic and 64% of oral cancer tissues showed moderate to strong p53 immunostaining. Point mutations within exons 5 to 8 were detected in 25% of preneoplastic tissues and in 60% of cancer specimens. Of the tissues with mutations, 100% of the preneoplastic tissues and 93% of the cancer tissues contained a CGT to CAT mutation at codon 273 of the p53 gene. Thus p53 mutations are highly prevalent in preneoplastic and neoplastic oral tissues. Also codon 273 is the "hot-spot" for point mutations in the tested preneoplastic and neoplastic tissues. The p53 immunostaining data were, in general, in agreement with the point mutations: Enhanced staining was seen in all specimens containing point mutations. Sixty-two percent of preneoplastic and 80% of neoplastic oral tissues contained mutations of *MTS1/CDK4I* gene, but, unlike p53 mutations, the mutations of *MTS1/CDK4I* gene were nonspecific. Mutations of either p 53 or *MTS1/CDK4I* genes were found in 75% of preneoplastic tissues, indicating that those mutations may be an early event in the multistep carcinogenesis of human oral tissue. However, the mutations of both p53 and *MTS1/CDK4I* are not always positively linked to a history of heavy tobacco consumption (more than 20 pack-years), suggesting that risk factor(s) other than tobacco consumption or smoking history less than 20 pack-years may also be involved in the mutations of both p53 and *MTS1/CDK4I* genes in the tested tissues.

Materials and Methods

Patients, collection of samples, and histological examination

Eight biopsy-proven preneoplastic oral tissues (leukoplakia) and 25 oral squamous cell carcinoma specimens were obtained from patients undergoing biopsy and surgery, respectively, at the Beijing Medical University School of Stomatology, Beijing, China. Eleven normal oral tissues were also obtained from volunteers. All participants were native Chinese and have spent their whole lives in the metropolitan area. Histories of tobacco consumption were also obtained. Specimens were obtained from tongue, lip, buccal mucosa, palate, gingiva, or retromolar area. The tissues were fixed in neutral formalin, embedded in paraffin, and sectioned for histological and immunohistochemical analysis.

Immunohistochemistry

The avidin-biotin-horseradish peroxidase complex (ABC) procedure was used to stain p53 protein using the DAKO LSAB® Kit (DAKO Corp., Carpinteria, CA). Paraffin sections were deparaffinized and rehydrated using xylene, ethanol and water as recommended by the manufacturer. The sections were then treated with 3% hydrogen peroxide for

blocking the intrinsic peroxidase activity. After an initial blockage with nonimmune goat serum, the tissues were exposed to monoclonal antibody PAb 1801 (Ab-2; Oncogene Sciences, Manhasset, NY) that recognizes denaturation-resistant epitope in human p53 located between amino acid 32 and 79 (Banks *et al.*, 1986). The tissues were then treated with biotinylated antimouse IgG and peroxidase-conjugated streptavidin followed by incubation in peroxidase substrate solution (3-amino-9-ethylcarbazole) and counterstaining with Mayer's hematoxylin. The degree of nuclear staining in cells was arbitrarily graded none, moderate, and intense.

Polymerase Chain Reaction (PCR) amplification and single strand conformational polymorphism (SSCP) analysis

Unstained 20 μm paraffin-embedded sections were used to extract cellular DNA using standard procedures. The sections were incubated in xylene for 5 min in eppendorff tubes, and the supernatants were eliminated after centrifugation. This procedure was repeated with 10 min incubation in xylene. After washing with 95% and 75% ethanol for 10 sec each, the pellets were air-dried. The samples were then incubated overnight at 37°C in DNA extraction buffer (100 mM NaCl; 10 mM Tris HCl, pH 8.0; 25 mM EDTA; 0.5% SDS; 100 μg/ml proteinase K) and extracted twice with phenol and once with chloroform. DNA was precipitated with 10 M ammonium sulfate and 2.5 volumes of ethanol for 15 min at -70°C. DNA concentrations and purity were determined by optimal absorbance at 260 nm and 280 nm.

Amplification of p53 was carried out using a DNA PCR kit (Perkin-Elmer Cetus, Irvine, CA) for SSCP analysis. The oligonucleotide primers (Table 1) used for the amplification of p53 (exons 5, 6, 7, and 8) were custom-synthesized by Bio-Synthesis, Inc. (Louisville, TX). Each exon was amplified from 1 μg of DNA by addition of 10 μl of PCR mixture containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 5mM $MgCl_2$, 2.5 units of recombinant *Taq* DNA polymerase, 70 μM of dNTPs, 0.2 μM of p53 primers, and 1 μCi of [^{32}P]dCTP (7,000 Ci/mmol, ICN, Costa Mesa, CA). Each amplification cycle consisted of 1 min of denaturation at 94°C, followed by 2 min of annealing (60°C) and 3 min of extension (72°C). A total of 30 cycles were run with a final extension step at 72°C for 7 min. The 1.5 μl of PCR products were then mixed with 9 μl of loading buffer (0.05% bromophenol blue/0.05% xylene cyanol FF (Sigma, St. Louis, MO]/10 mM NaOH in formamide). One μl of the final product was loaded onto 0.5X MDE gel (AT Biochem., Malvern, PA) and electrophoresed at 6W for 10 hours at room temperature. The gel was dried onto a filter paper and exposed to X-ray film at -70°C for 12 hours with an intensifying screen.

Sequencing of PCR product

Samples containing mutation of the p53 gene (as revealed by the SSCP analysis) were amplified using similar reaction cycle conditions to those described above, but without [^{32}P]dCTP in the reaction mixture. Exons 1 and 2 of *MTS1/CDK4I* gene were also amplified in a similar manner using custom-synthesized primers (Table 1). The amplified DNAs were ligated to pCRII vector using TA™ Cloning Kit (Invitrogen, San Diego, CA) under the conditions recommended by the manufacturer. The nucleotide sequences of the cloned p53 and *MTS1/CDK4I* DNA were determined by the primer extension method from at least 5 clones of each cDNA using Sequenase (USB Corp., Cleveland, OH) as described previously (Kim *et al.*, 1993).

Table 1. Sequences of primers used for the amplification of p53 (exons 5, 6, 7, and 8) and *MISl/CDK4I* (exons 1 and 2) genes

p53	Exon 5:	5'-TACTCCCCTGCCCTCAACAA-3' (sense)
		5'-CATCGCTATCTGAGCAGCGC-3' (antisense)
p53	Exon 6:	5'-GTCTGGCCCCTCCTCAGCA-3' (sense)
		5'-CTCAGGCGGCTCATAGGGCA-3' (antisense)
p53	Exon 7:	5'-TCTGACTGTACCACCATCCA-3' (sense)
		5'-CTGGAGTCTTCCAGTGTGAT-3' (antisense)
p53	Exon 8:	5'-TGGTAATCTACTGGGACGGA-3' (sense)
		5'-CGGAGATTCTCTTCCTCTGT-3' (antisense)
MIS1/CDK4I	Exon 1:	5'-GCGCTACCTGATTCCAATTC-3' (sense)
		5'-GAAGAAAGAGGAGGGGCTG-3' (antisense)
MIS1/CDK4I	Exon 2:	5'-TCTGACCTTTGGAAGCTCT-3' (sense)
		5'-GGAAATTGGAAACTGGAAGC-3' (antisense)

Results

Histological analysis and immunohistochemical staining of p53 protein

All preneoplastic tissues demonstrated epithelial dysplasia (2 with mild, 1 with moderate, and 4 with severe epithelial dysplasia). Of the 25 cancer specimens, 24 samples were histologically squamous cell carcinomas (1 well differentiated, 4 moderately differentiated and 20 poorly differentiated), and one specimen was osteosarcoma. To determine the level of p53 protein, the paraffin sections were immunostained using human p53 specific monoclonal antibody, PAb1801, after fixing as described in the Materials and Methods. There was no detectable p53 staining of any of the 10 normal tissues, but 3 (38%) of the 8 preneoplastic tissues demonstrated high p53 staining in the nuclei of the epithelial cells. Moreover, 19 (76%) of the 25 neoplastic specimens demonstrated moderate to intense nuclear p53 staining in the cancer cells (Table 2). No positive correlation between the degree of histological malignancy and the intensity of p53 protein staining was seen.

Table 2. Clinicopathological and immunohistochemical analysis of preneoplastic ane neoplastic human oral tissue

# of Specimens (Sex of patients)	Location	History of Heavy Tobacco Consumption	Histological Findings	p53 staining
Preneoplastic				
1 (M)	Tongue	Yes	Severe epithelial dysplasia	No (-)
2 (M)	Tongue	No	Mild epithelial dysplasia	No (-)
3 (M)	Lower lip	Yes	Severe epithelial dysplasia	No (-)
4 (M)	Gingiva	No	Moderate epithelial dysplasia	Yes (++)
5 (F)	Gingiva	No	Severe epithelial dysplasia	No (-)
6 (M)	Tongue	Unknown	Mild epithelial dysplasia	Yes (++)
7 (M)	Gingiva	No	Severe epithelial dysplasia	No (-)
8 (M)	Hard palate	No	Severe epithelial dysplasia	Yes (+)
Neoplastic Tissue				
1 (M)	Buccal mucosa	None	Mod. diff. squam. carc.	Yes (++)
2 (M)	Gingiva	Yes	Poor. diff. squam. carc.	Yes (+)
3 (M)	Gingiva	No	Poor. diff. squam. carc.	Yes (++)
4 (M)	Gingiva	No	Mod. diff. squam. carc.	No (-)
5 (M)	Gingiva	No	Well. diff. squam. carc.	Yes (+++)
6 (M)	Tongue	No	Poor. diff. squam. carc.	Yes (+)
7 (M)	Tongue	No	Poor. diff. squam. carc.	No (-)
8 (M)	Palate	No	Poor. diff. squam. carc.	Yes (++)
9 (M)	Lip	No	Poor. diff. squam. carc.	Yes (++)
10 (M)	Gingiva	No	Mod. diff. squam. carc.	Yes (+++)
11 (M)	Gingiva	Yes	Mod. diff. squam. carc.	No (-)
12 (M)	Palate	Yes	Poor. diff. squam. carc.	Yes (++)
13 (M)	Buccal mucosa	No	Poor. diff. squam. carc.	Yes (+++)
14 (M)	Tongue	Yes	Poor. diff. squam. carc.	Yes (+++)
15 (M)	Maxilla	No	Osteosarcoma	No (-)
16 (M)	Palate	No	Poor. diff. squam. carc.	Yes (+)
17 (M)	Tongue	No	Poor. diff. squam. carc.	Yes (++)
18 (M)	Gingiva	Yes	Poor. diff. squam. carc.	No (-)
19 (M)	Gingiva	No	Poor. diff. squam. carc.	No (-)
20 (M)	Tongue	Yes	Poor. diff. squam. carc.	Yes (+++)
21 (M)	Palate	Yes	Poor. diff. squam. carc.	Yes (+++)
22 (M)	Mandible	Yes	Poor. diff. squam. carc.**	Yes (+)
23 (M)	Tongue	Yes	Poor. diff. squam. carc.	Yes (+)
24 (M)	Unknown	Yes	Poor. diff. squam. carc.	Yes (+++)
25 (N/D)	Unknown	Yes	Poor. diff. squam. carc.	Yes (+++)

*Tissue specimens were originated from the patients from the Department of Oral & Maxillofacial Surgery, Beijing Medical University School of Stomatology, in Beijing, Peoples Republic of China.
M: Male; F: Female; N/D: not determined.
Heavy tobacco consumption indicates more than 20 pack-years in cigarette consumption.
No in heavy tobacco consumption indicate that the subjects may have history of smoking less than 20 pack-years.
Mod. diff. squam. carc.:Moderately differentiated squamous cell carcinoma.
Poor. diff. squam. carc.: Poorly differentiated squamous cell carcinoma.
Intensity of p53 staining: -, no staining; +, mild staining; ++, moderate staining; +++, strong staining.
**Metastatic carcinoma.

Inasmuch as point mutations in the p53 gene extend the half-life of p53 protein, resulting in an accumulation of p53 protein in tumor cells (Levine, 1993), the enhanced immunoreactivity may be associated with the point mutations of p53 gene.

Mutational analysis of p53 cDNA

To determine whether mutations in the p53 gene exist in normal, preneoplastic and neoplastic oral tissue, SSCP and sequence analyses of the conserved p53 region (exons 5, 6, 7, and 8) were conducted using DNA extracted from the tissue as described in Material and Methods (Table 3). SSCP analysis of exons 5 to 8 showed no abnormal migration of amplified DNA fragments from normal tissue, indicating no mutations of the p53 gene in the tested exons of p53. SSCP analysis of exon 7 from the preneoplastic and neoplastic oral tissue did not reveal an abnormal migration pattern. However, the migration pattern of amplified exon 8 was abnormal in 2 preneoplastic tissues and 15 cancer tissues. Abnormal migrations of amplified exons 5 and 8 were detected in two cancer specimens. In order to confirm and specify the location of point mutations in p53 gene, highly conserved regions of p53 gene (exons 5 to 8) were sequenced after amplification as described Materials and Methods. Point mutations within exons 5 to 8 were not detected in the normal tissue specimens, but were present in 2 (25%) of the 8 preneoplastic

Table 3. State of p53 mutation in preneoplastic and neoplastic Chinese oral tissue

# of Specimens	Codon number	Mutation	Amino acid change
Preneoplastic Tissue			
1	–	None	–
2	–	None	–
3	–	None	–
4	273	CGT→CAT	Arginine→Histidine
5	–	None	–
6	–	None	–
7	–	None	–
8	273	CGT→CAT	Arginine→Histidine
Neoplastic Tissue			
1	273	CGT→CAT	Arginine→Histidine
2	273	CGT→CAT	Arginine→Histidine
3	–	None	–
4	–	None	–
5	156	GCG→GCT	Silent mutation
	273	CGT→CAT	Arginine→Histidine
6	273	CGT→CAT	Arginine→Histidine
7	–	None	–
8	273	CGT→CAT	Arginine→Histidine
9	273	CGT→CAT	Arginine→Histidine
10	–	None	–
11	–	None	–
12	–	None	–
13	273	CGT→CAT	Arginine→Histidine
14	–	None	–
15	–	None	–
16	273	CGT→CAT	Arginine→Histidine
17	273	CGT→CAT	Arginine→Histidine
18	–	None	–
19	283	CGT→CAT	Silent mutation
20	273	CGT→CAT	Arginine→Histidine
21	–	None	–
22	275	TGT→TGC	Silent mutation
	273	CGT→CAT	Arginine→Histidine
23	157	GGT→GTT	Glycine→Valine
	273	CGT→CAT	Arginine→Histidine
24	273	CGT→CAT	Arginine→Histidine
25	273	CGT→CAT	Arginine→Histidine

The SSCP of the exones 5, 6, 7, and 8 of p53 gene was analyzed using PCR amplification and gel electrophoresis. Moreover, to confirm the SSCP results, all amplified exons were cloned and sequenced to determine the type of mutation as described in Material and Methods.

tissue and in 15 (60%) of the 25 cancer specimens. Of the tissues with mutations, 2 (100%) preneoplastic and 14 (93%) neoplastic tissues contained a CGT to CAT mutation at codon 273, consisting of an amino acid substitution of arginine to histidine. A silent point mutation at 283 codon (CGC to CTC) was noticed from one cancer specimen. Three cancer specimens contained silent point mutations at codons 156, 157 and 275, but they also showed point mutations at codon 273 (Table 3). The sequence analyses revealed that all samples with abnormal migration in SSCP contained point mutations. Sixty percent of cancer specimens derived from patients with heavy cigarette smoking (more than 20 cigarette smoking a day for over 30 years) contained point mutations of p53, while 66% of cancer samples from non-heavy smokers showed p53 mutations.

Mutational analysis of MTS1/CDK4I

Normal tissues did not demonstrate any mutations in the *MTS1/CDK4I* gene. Three of 8 preneoplastic tissues contained no mutations, but the remaining 5 specimens showed point mu-

Table 4. State of *MTS1/CDK4I* gene mutation in preneoplastic and neoplastic Chinese oral tissue

# of Specimens	Codon number	Mutation	Amino acid change
Preneoplastic Tissue			
1	12	GCG→GCT	Silent mutation
	13	GCC→CC (Deletion)	Frame shift
2	95	CGG→CAG	Arginine→Glycine
	106	CCC→CC	Frame shift
3	–	None	None
4	31	AAC→GAC	Asparagine→Asparatic acid
5	–	None	None
6	–	None	None
7	111	GAG→GTG	Glutamic acid→Valine
8	72	CGA→TGA	Arginine→Amber
Neoplastic Tissue			
1	118	GTC→GAC	Valine→Asparagine
	124	GCC→GTG	Alanine→Valine
2	Deletion	Deletion	No amplification
3	91	CGG→CTG	Arginine→Valine
4	40	CCG→TCG	Proline→Serine
5	74	GTG→GCG	Valine→Alanine
6	25	GAG→AAG	Glutamic acid→Lysine
	29	CTG→CTT	Silent mutation
7	–	None	None
8	40	CCG→TCG	Proline→Serine
9	34	AAT→AGT	Asparagine→Serine
	53	AGT→GGT	Serine→Glycine
	91	CGG→CTG	Arginine→Leucine
10	–	None	None
11	40	CCG→ACG	Proline→Threonine
12	–	None	None
13	Deletion	Deletion	No amplification
14	34	AAT→AGT	Asparagine→Serine
15	Exon 2	Deletion (16 bp)	Frame shift
16	131	GGC→GAC	Glycine→Asparagine
17	Exon 1	Deletion	No amplification
18	Exon 1	Deletion	No amplification
	130	AGA→AAA	Arginine→Lysine
19	103	GGC→GAC	Glycine→Asparagine
	111	GAG→GAA	Silent mutation
20	–	None	None
21	Exon 2	Deletion	No amplification
22	143	CCC→CTC	Proline→Leucine
23	–	None	None
24	46	ATG→ATT	Methionine→Isoleucine
25	–	None	None

Both exons 1 and 2 of the *MTS1/CDK4I* gene were amplified, cloned in pCRII vector, and sequenced as described in Material and Methods.

tations: 4 missense mutations and 1 silent mutation. The preneoplastic tissue showing a silent point mutation also contained a deletion of one nucleotide at codon 13, resulting in a frame shift (Table 4). Of the 25 tested neoplastic tissue samples, 20 specimens showed either point mutations or deletion of the gene: (1) 4 tissues with multiple point mutations, (2) 1 specimen containing both deletion (no amplification of exon 1) and point mutation of codon 130, (3) no amplification of the gene (either exon 1 or 2, or both) in 5 samples probably due to deletion of the gene or deletion at the primer binding sites, (4) 9 specimens with single point mutations, and (5) 1 sample with frame shift of the gene because of the deletion of 16 nucleotides (Table 4). However, unlike the p53 mutations which showed specific point mutations in codon 273, we were not able to detect any specific pattern of mutations or mutation "hot-spots" in the *MTS1/CDK4I* gene from the tested preneoplastic or neoplastic tissues.

Discussion

The presented data demonstrate that a high percentage of preneoplastic and neoplastic oral tissues from Beijing, China show mutations in p53 gene. Moreover, a high percentage of preneoplastic and neoplastic tissues also contained a high level of p53 protein. Inasmuch as the point mutation of unstable wt p53 protein results in the prolongation of the half-life of this protein (Kemp *et al.*, 1993), the high level of mutant p53 protein in the preneoplastic and neoplastic tissue containing the point mutations was expected. Positive correlation of p53 point mutations with an accumulation of p53 protein indicates that immunostaining of p53 protein is a reliable analysis to predict p53 point mutations in oral cancer specimens. However, we also observed a high level of p53 in certain preneoplastic and neoplastic tissues that did not contain point mutations at the tested exons. Though the mode of enhanced p53 protein levels in these tissues remains unknown, the increase may result from either the mutations at untested exons (e.g. exons 1, 2, 3, 4, and 9) or due to the overexpression of wt p53 gene. The SSCP analysis demonstrated an extremely high reliability in screening for the mutations of p53 gene: All amplified exons with abnormal migration showed point mutations.

The frequencies of p53 point mutations in the tested preneoplastic and neoplastic oral tissue were similar to other reports studied in Caucasians and other races (Sakai *et al.*, 1992; Caamano *et al.*, 1993; Brachman *et al.*, 1992; Burns *et al.*, 1993). The most striking observation from this study is the frequency of mutation in codon 273 of p53 gene: Over 90% of specimens containing point mutations showed point mutations in the codon 273. We have repeated the paraffin sectioning, amplification, cloning, and sequencing from each tissue sample for at least 3 times to rule out the possibility of cross contamination, and have found the point mutation at codon 273 to be consistent. Although codon 273 of p53 gene is one of the "hot-spots" for point mutations in all types of human tumors (Hollstein *et al.*, 1991), the unusually high frequency in point mutation in this codon seen in preneoplastic and neoplastic oral tissue specimens from China deserves further investigation to establish risk factor(s) involved in oral carcinogenesis. Frequent mutations in codon 273 in both preneoplastic and malignant oral tissue specimens also indicates that the mutation may occur early in oral carcinogenesis. Although the mutation of p53 is relatively rare in the early stages of colorectal and bladder cancers (Magrisso *et al.*, 1993; Sidransky *et al.*, 1991), a significant body of evidence suggests that altered function of p53 gene (overexpression or mutation) is an early event in most other human cancers (Donehower and Bradley, 1993; Levine *et al.*, 1994). Moreover, many studies have also shown either the overexpression or mutation of p53 in preneoplastic oral (head and neck) lesions, supporting the concept that p53 dysfunction is an early event in oral squamous cell carcinogenesis (Wang *et al.*, 1993; Shin *et al.*, 1994; Sauter *et al.*, 1994). Similar to these reports, our data also show that aberrant expression or p53 mutation is common in a high percentage of preneoplastic oral tissues, indicating that the dysfunction of p53 appears to be an early event in oral carcinogenesis.

Numerous studies have indicated a positive correlation between p53 mutations and the history of tobacco consumption in head and neck cancer (Brennan *et al.*, 1995). Exposure of cells to benzo (a)pyrene, the main chemical carcinogen found in smoked tobacco tar has been shown to cause mutations of p53 (Ruggeri *et al.*, 1994). Our data also shows such a positive correlation between tobacco

consumption and p53 mutations: 60% of cancer specimens derived from patients with a history of heavy tobacco consumption showed p53 mutations. However, a similarly high percentage of cancer samples obtained from subjects with history of non-heavy tobacco consumption also demonstrated p53 mutations. A high percentage of p 53 mutation rate in cancer specimens from non-heavy smokers could be due to (1) the small number of samples analyzed and/or (2) other environmental and genetic factors playing a major role in the mutation of p53. Precise determinants of correlation will require further studies.

Our data also indicates that tested preneoplastic and neoplastic specimens contain extremely high frequency of mutations in *MTS1/CDK4I* gene. The high frequency of mutations of *MTS1/CDK4I* gene in tested preneoplastic and neoplastic oral tissues is similar to those in melanoma cell lines, lymphoblastoid cell lines derived from dysplastic nervous system, oesophageal squamous cell carcinomas, primary non-small cell lung carcinomas, and pancreatic adenocarcinomas (Kamb *et al.*, 1994; Nobori *et al.*, 1994; Mori *et al.*, 1994; Hayashi *et al.*, 1994; Caldas *et al.*, 1994). Though the mutation frequency of *MTS1/CDK4I* is similar to that of p53 in Chinese oral cancer specimens, the type of mutations was different from that of p53 gene including: Different types of anomalies such as (1) point mutations, (2) no PCR amplification of the gene probably because of complete deletion of the gene or deletion at the primer binding sites, and (3) frame shift because of deletion of one or more nucleotides were observed from the tissue specimens and may account for the difference. These data indicate that, unlike the p53 gene, site of point mutations of *MTS1/CDK4I* gene may not be specific in the tested preneoplastic and neoplastic tissues. Presence of *MTS1/CDK4I* mutations in a high percentage of preneoplastic oral tissues deserves further attention, and the presented data indicates that the mutation of this gene may, in part, be responsible for a conversion of normal cells to preneoplastic cells in the human oral cavity.

The role of mutations or aberrant expression of p53 and *MTS1/CDK4I* in oral carcinogenesis remains unknown. However, inasmuch as both p53 and *MTS1/CDK4I* gene products play an important role as the check point determinants of cell cycle progression, mutation of either p53 or *MTS1/CDK4I* gene may perturb the control of cell cycle progression of cells. In fact, wt p53 plays a critical role in establishing G_1 cell cycle arrest when cells are challenged by DNA damaging agents (Kastan *et al.*, 1991; Kuerbitz *et al.*, 1992; Gujuluva *et al.*, 1994). The transient arrest of cell cycle progression may permit the repair of damaged DNA prior to DNA replication and thus prevent the propagation of heritable genetic errors caused by DNA damaging agents (Weinert *et al.*, 1988). Moreover, wt p53 is involved in DNA repair processes via enhancing the expression of *gadd*45 gene (Smith *et al.*, 1994). Though detailed functions of the *MTS1/CDK4I* gene product, p16, remain to be investigated, the p16 protein inhibits the function of cdk4 and cdk6 by binding to them in competition with cyclin D (Serrano *et al.*, 1993; Hannon and Beach, 1994). The loss of functional p16 activity could result in the loss of G_1/S phase cell cycle control and promote growth advantage of cells. A closely related gene $p15^{INK4B}$ has been mapped next to *MTS1/CDK4I* gene in human chromosome (Hannon and Beach, 1994). Thus mutation of either p53 or *MTS1/CDK4I* gene would cause cells to be genetically unstable especially when challenged by genotoxic agents, resulting in the generation of tumor cells. Inasmuch as human oral epithelial cells are continuously challenged by innumerable genotoxic agents from the oral cavity, preneoplastic cells having mutations in either p53 or *MTS1/CDK4I* gene could easily convert to tumor cells. Our data show that mutations of both p53 and *MTS1/CDK4I* genes are frequently found in preneoplastic oral tissue, indicating that these mutations may occur at an early stage of oral carcinogenesis and may play a major role in further conversion of these preneoplastic cells to cancer cells.

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References

Aguilar, F., Harris, C.C., Sun, T., Hollstein, M. and Cerutti, P.: Geographic variation of p53 mutational

profile in nonmalignant human liver. Science 264: 1317-1319, 1994.

Banks, L., Matlashewski, G. and Crawford, L.: Isolation of human-p53-specific monoclonal antibodies and their use in the studies of human p53 expression. Eur. J. Biochem. 159: 529-534, 1986.

Brachman, D.G., Graves, D., Vokes, E., Beckett, M., Haraf, D., Montag, A., Dunphy, E., Mick, R., Yandell, D. and Weichelbaum, R.R.: Occurrence of p53 gene deletions and human papillomavirus infection in human head and neck cancer. Cancer Res. 52: 4832- 4836, 1992.

Brennan, J.A., Boyle, J.O., Koch, W.M., Goodman, S. N., Hruban, R.H., Eby, Y.J., Couch, M.J., Forastiere, A.A. and Sidransky, D.: Association between cigarette smoking and mutation of the p53 gene in squamous-cell carcinoma of the head and neck. New Engl. J. Med. 332: 712-1717, 1995.

Burns, J.E., Baird, M.C., Clark, L.J., Burns, P.A., Edington, K., Chapman, C., Mitchell, R., Robertson, G., Soutar, D. and Parkinson, E.K.: Gene mutations and increased levels of p53 protein in human squamous cell carcinoma. Br. J. Cancer 67: 1274-1284, 1993.

Caamano, J., Zhang, S.Y., Rosvold, E.A., Bauer, B. and Klein-Szanto, A.J.: p53 alterations in human squamous cell carcinomas and carcinoma cell lines. Am. J .Pathol. 142: 1131-1139, 1993.

Cairns, P., Mao, L., Merlo, A., Lee, D.J., Schwab, D., Eby, Y., Tokino, K., Van Der Riet, P., Blaugrund, J. E. and Sidransky, D.: Rates of p16 (MTS1) mutations in primary tumors with 9p loss. Science 265: 415-416, 1994.

Caldas, C., Hahn, S.A., Da Costa, L.T., Redston, M.S., Schutte, M., Seymour, A.B., Weinstein, C.L., Hruban, R.H., Yeo, C.J. and Kern, S.E.: Frequent somatic mutations and homozygous deletions of the p16 (MTS1) gene in pancreatic adenocarcinoma. Nature genetics 8: 27-32, 1994.

Donehower, L.A. and Bradley, A.: The tumor suppressor p53. Biochim. Biophys. Acta. 1155: 181-205, 1993.

Fujimoto, Y., Hampton, L.L., Wirth, P.J., Wang, N.J., Xie, J.P. and Thorgeisson, S.S.L.: Alterations of tumor suppressor genes and allelic losses in human hepatocellular carcinomas in China. Cancer Res. 54: 281-285, 1994.

Gujuluva, C.N., Baek, J.-H., Shin, K.-H., Cherrick, H.M. and Park, N.-H.: Effect of UV irradiation on cell cycle, viability and the expression of p53, gadd153, and gadd45 in normal and HPV-immortalized human oral keratinocytes. Oncogene 9: 1819-1827, 1994.

Hannon, G.J. and Beach, D.: p15INK4B is a potential effector of TGF-b induced cell cycle arrest. Nature 371: 257-261, 1994.

Hayashi, N., Sugimoto, Y., Tsuchiya, E., Ogawa, M. and Nakamura, Y.: Somatic mutations of the MTS (Multiple tumor suppressor) 1/CDK4 (Cyclin-dependent Kianse-4 Suppressor) gene in human primary non-small cell lung carcinomas. Biochem. Biophys. Res. Comm. 202: 1426-1430, 1994.

Hecht, S.S., Spratt, T.E. and Trushin, N.: Evidence for 4-(3-pyridyl)-4-oxobutylation of DNA in F344 rats treated with the tobacco-specific nitrosamines 4-(methylnitrosamino)-1-(3- pyridyl)-1-butanone and N'-nitrosonornicotine. Carcinogenesis 9: 161-165, 1988.

Hollstein, M., Sidransky, D., Vogelstein, B. and Harris, C.C.: p53 Mutations in human cancers. Science 253: 49-53, 1991.

Hunter, T. and Pines, J.: Cyclins and cancer II: Cyclin D and CDK inhibitors come of age. Cell 79: 573-582, 1994.

Kamb, A., Gruis, N.A., Weaver-Feldhaus, J., Liu, Q., Harshman, K., Tavtigian, S.V., Stockert, E., Day, R. S. Iii, Johnson, B.E. and Skolnick, M.H.: A cell cycle regulator potentially involved in genesis of many tumor types. Science 264: 436-440, 1994.

Kastan, M.B., Onyekwere, O., Sidransky, D., Vogelstein, B. and Craig, R.W.: Participation of p53 protein in the cellular response to DNA damage. Cancer Res. 51: 6304-6311, 1991.

Kemp, C.J., Donehower, L.A., Bradley, A. and Balmain, A.: Reduction of p53 gene dosage does not increase initiation or promotion but enhances malignant progression of chemically induced skin tumours. Cell 74: 813-822, 1993.

Kessis ,T.D., Slebos, R.J., Nelson, W.G., Kastan, M.B., Plunkett, B.S., Han, S.M., Lorincz, A.T., Hedrick, L. and Cho, K.R.: Human papillomavirus 16 E6 expression disrupts the p53-mediated cellular response to DNA damage. Proc. Natl. Acad. Sci. USA 90: 3988- 3992, 1993.

Kim, M.S., Li, S.-L., Bertolami, C.N., Cherrick, H.M. and Park, N.-H.: State of p53, Rb and DCC tumor suppressor genes in human oral cancer cell lines. Anticancer Res. 13: 1405- 1413, 1993.

Kuerbitz, S.J., Plunkett, B.S., Walsh, W.V. and Kastan, M.B.: Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proc. Natl. Acad. Sci. USA 89: 7491-7495, 1992.

Levine, A.J.: The tumor suppressor genes. Ann. Rev. Biochem. 62: 623-651, 1993.

Levine, A.J., Perry, M.E., Chang, A., Silver, A., Dittmer, D., Wu, M. and Welsh, D.: The 1993 Walter Hubert Lecture: the role of the p53 tumour-suppressor gene in tumorigenesis. Br. J. Cancer 69: 409-416, 1994.

Loughran, O., Edington, K.G., Clark, L.J. and Parkinson, E.K.: Loss of heterozygosity of chromosome 9p21 is associated with the immortal phenotype of

neoplastic human head and neck keratinocytes. Cancer Res. 54: 5045-5049, 1994.

Magrisso, I.J., Richmond, R.E/, Carter, J.H., Pross, C.B., Gilfillen, R.A. and Carter, H.W.: Immunohistochemical detection of RAS, JUN, FOS, and p53 oncoprotein expression in human colorectal adenomas and carcinomas. Lab. Invest. 69: 674-681, 1993.

Mori, T., Miura, K., Aoki, T., Nishihira, T., Mori, S. and Nakamura, Y.: Frequent somatic mutation of the MTS1/CDK4I (Multiple Tumor Suppressor/Cyclin-dependent Kinase 4 Inhibitor) gene in esophageal squamous cell carcinoma. Cancer Res. 54: 3396-3397, 1994.

Nobori, T., Miura, K., Wu, D.J., Lols, A., Takabayashi, K. and Carson, D.A.: Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. Nature 368: 753-756, 1994.

Ogden, G.R., Kiddie, R.A., Lunny, D.P. and Lane, D.P.: Assessment of p53 protein expression in normal, benign, and malignant oral mucosa. J. Pathol. 166: 389-394, 1992.

Ohta, M., Nagai, H., Schimizu, M., Rasio, D., Berd, D., Mastrangelo, M., Singh, A.D., Shields, J.A., Shields, C.L., Croce, C.M. and Huebner, K.: Rarity of somatic and germline mutations of the cyclin-dependent kinase 4 inhibitor gene, CDK4I, in melanoma. Cancer Res. 54: 5269-5272, 1994.

Parkin, D.M., Laara,E. and Muir, C.S.: Estimates of the worldwide frequency of sixteen major cancers in 1980. Int. J. Cancer 41: 184-197, 1988.

Preston-Martin, S.: Evaluation of the evidence that tobacco-specific nitrosamines (TSNA) cause cancer in humans. Crit .Rev. Toxicol. 21: 295-298, 1991.

Ruggeri B.A., Bauer, B., Zhang, S.-Y. and Klein-Szanto, A.J.: Murine squamous cell carcinoma cell lines produced by a complete carcinogenesis protocol with benzo[a]pyrene exhibit characteristic p53 mutations and the absence of H-ras and cyl 1/cyclin D1 abnormalities. Carcinogenesis 15: 1613-1619, 1994.

Sakai, E. and Tsuchida, N.: Most human squamous cell carcinomas in the oral cavity contain mutated p 53 tumor-suppressor genes. Oncogene 7: 927-933, 1992.

Sauter, E.R., Cleveland, D., Trock, B., Ridge, J.A. and Klein-Szanto, J.P.: p53 is overexpressed in fifty percent of pre-invasive lesions of head and enck epithelium. Carcinogenesis 15: 2269-2274, 1994.

Serrano, M., Hannon, G.T. and Beach, D.: A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/cdk4. Nature 366: 704-707, 1993.

Shin, D.M., Kim, J., Ro J.Y., Hittelman, J., Roth, J.A., Hong, W.K. and Hittelman, W.N.: Activation of p53 gene expression in premalignant lesions during head and neck tumorigenesis. Cancer Res. 54: 321-326, 1994.

Sidransky, D., Von Eschenbach, A., Tsai, Y.C., Jones, P., Summerhayes, I., Marshall, F., Paul, M., Green, P., Hamilton, S.R. and Frost, P.: Identification of p53 gene mutations in bladder cancers and urine samples. Science 252: 706-709, 1991.

Smith, M.L., Chen, I.-T., Zhan, Q., Bae, I., Chen, C.-Y., Gilmer, T.M., Kastan, M.B., O'conner, P.M. and Fornace, A.J. Jr.: Interaction of the p53-regulated protein gadd45 with proliferating cell nuclear antigen. Science 266: 1376-1380 , 1994.

Spruck, C., Gonzalez-Zuleta, M., Shibata, A., Simoneau, A., Lin, M., Gonzales, F., Tsai, Y. and Jones, P.: p16 gene in uncultured tumors. Nature 370: 183-184 , 1994.

Stich, H.F., Parida ,B.B. and Brunnemann, K.D.: Localized formation of micronuclei in the oral mucosa and tobacco-specific nitrosamines in the saliva of "reverse" smokers, Khaini- tobacco chewers and gudakhu users. Int. J. Cancer 50: 172-176, 1992.

Talamini, R., Francheschi, S., Barra, S. and La Vecchica, C.: The role of alcohol in oral and pharyngeal cancer in non-smokers, and of tobacco in non-drinkers. Int. J. Cancer 46: 391- 393, 1990.

Wang, L.-D., Hong, J.-Y., Qiu, S.-L., Gao, H. and Yang, C.S.: Accumulation of p53 protein in human esophageal precancerous lesions: a possible early biomarker for carcinogenesis. Cancer Res. 53: 1783-1787, 1993.

Weinert, T.A. and Hartwell, L.H.: The RAD 9 gene controls the cell cycle response to DNA damage in Saccharomyces cerevisiae. Science 241: 317-322, 1988.

Zhang, S.Y., Klein-Szanto, A.J.P., Sauter, E.R., Shafarenko, M., Mitsunaga, S., Norobi, T., Carson, D.A., Ridge, J.A. and Goodrow, T.L.: Higher frequency of alterations in the p16/CDKN2 gene in squamous cell carcinoma cell lines than in primary tumors of the head and neck. Cancer Res. 54: 5050-5053, 1994.