Induction of MDM2-P2 Transcripts Correlates with Stabilized Wild-Type p53 in Betel- and Tobacco-Related Human Oral Cancer

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MDM2, a critical element of cellular homeostasis mechanisms, is involved in complex interactions with important cell-cycle and stress-response regulators including p53. The *mdm2*-P2 promoter is a transcriptional target of p53. The aim of this study was to determine the association between *mdm2*-P2 transcripts and the status of the *p53* gene in betel- and tobacco-related oral squamous cell carcinomas (SCCs) to understand the mechanism of deregulation of MDM2 and p53 expression and their prognostic implications in oral tumorigenesis. Elevated levels of MDM2 proteins were observed in 11 of 25 (44%) oral hyperplastic lesions, nine of 15 (60%) dysplastic lesions, and 71 of 100 (71%) SCCs. The intriguing feature of the study was the identification and different subcellular localization of three isoforms of MDM2 (ie, 90 kd, 76 kd, and 57 kd) in oral SCCs and their correlation with p53 overexpression in each tumor. The hallmark of the study was the detection of *mdm2*-P2 transcripts in 12 of 20 oral SCCs overexpressing both MDM2 and p53 proteins while harboring wild-type *p53* alleles. Furthermore, *mdm2* amplification was an infrequent event in betel- and tobacco-associated oral tumorigenesis. The differential compartmentalization of the three isoforms of MDM2 suggests that each has a distinct function, potentially in the regulation of p53 and other gene products implicated in oral tumorigenesis. In conclusion, we report herein the first evidence suggesting that enhanced translation of *mdm2*-P2 transcripts (*S-mdm2*) may represent an important mechanism of overexpression and consequent stabilization and functional inactivation of wild-type p53 serving as an adverse prognosticator in betel- and tobacco-related oral cancer. The clinical significance of the functional inactivation of wild-type p53 by MDM2 is underscored by the significantly shorter median disease-free sur-

vival time (16 months) observed in p53/MDM2-positive cases as compared to those which did not show co-expression of these proteins (median time, 26 months; *P* = 0.02). (Am J Pathol 2000, 157:587-596)

Perturbations in the expression of the *mdm2* oncogene are implicated in the pathogenesis of human neoplastic diseases. The human *mdm2* gene mapped in chromosome 12q13-14 encodes a 90-kd cellular phosphoprotein (p90) capable of forming a complex with p53 protein, thus inhibiting its transcriptional activation function.^{1,2} Therefore it is an important regulator of the cell cycle. Amplification of the *mdm2* gene, with a corresponding increase in *mdm2* mRNA and protein(s), has been reported in a variety of human tumors, with a particularly high frequency in soft tissue sarcomas and gliomas.^{3,4} Overexpression of the *mdm2* gene has been shown to immortalize primary rat embryo fibroblasts and tumor cells thus acting as an oncogene.^{5,6} Two promoters were characterized in the *mdm2* gene; the first, P1, located upstream of the gene is expressed constitutively; whereas the second, P2, located in the first intron is regulated by the p53 protein,⁷⁻⁹ suggesting that the *mdm2*-P2 promoter is maintained in a nucleosome-free state which is primed for transcriptional activation by the p53 protein. Little is known about the constitutive P1 promoter.

MDM2 proteins of lower apparent molecular weight with deletions in the N- or C-terminal region are often present in addition to p90 in tumor cells.¹⁰ The full-length p90 form of the MDM2 protein has also been detected in human keratinocytes, even in the absence of the functional p53 protein.¹¹ A possible function of MDM2 protein in ribosome biosynthesis or in translational regulation is suggested by the specific interaction of the central acidic domain of the MDM2 protein with the L5 protein, a component of a large ribosomal subunit, which is itself asso-

Supported by the Indo-French Center For Promotion of Advanced Research. S. A. was supported by a Senior Research Fellowship from the University Grant Commission, India.

Accepted for publication April 24, 2000.

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ciated with 5s rRNA.^{12,13} A positive correlation was found between MDM2 binding to TAFII250 and activation of cyclin A, a regulator of the cell cycle.¹⁴ Thus, MDM2 protein may be involved in cellular proliferation and transformation, not only through its ability to modulate the transcriptional activity of p53, but also through additional functions which may be provided by both its N- and C-terminal domains.

Oral cancer, a major and growing global problem, exhibits considerable variation in incidence, etiology, and natural history in different population groups. Epidemiological evidence unequivocally confirms a causal association between the tobacco and betel chewing habits highly prevalent in India and the incidence of oral malignancies.¹⁵ Oral leukoplakia is a clinically distinct precancerous lesion with a high risk of transition to malignancy; however, little is known about its genetic basis. Intense efforts are directed toward identification of a molecular marker to predict the leukoplakic lesions at high risk of transition to malignancy. The development of betel- and tobacco-related oral cancer in the Indian population is a multistep process involving multiple genetic alterations and offers a unique model system to understand the mechanisms underlying tumorigenesis. The onset of oral malignancy causes severe morbidity. We have recently reported that alterations in MDM2 and p53 expression are early events in oral tumorigenesis.¹⁶ The aim of the present study was to 1) understand the mechanism of deregulation of MDM2 and p53 expression in oral tumorigenesis, and 2) determine the clinical relevance of MDM2/p53 aberrations in disease prognosis.

Materials and Methods

Materials

Surgical tissue specimens from 100 squamous cell carcinomas (SCCs) of the oral cavity, 40 leukoplakia lesions with histological evidence of epithelial hyperplasia (25 cases) or dysplasia (15 cases), and 40 normal oral tissues were obtained from the Department of Surgical Disciplines, All India Institute of Medical Sciences, New Delhi, India.

A pretested, semistructured questionnaire was used to collect information on socio-economic factors and personal habits. Socio-economic factors were assessed on the basis of occupation, income, religion, marital status, education, and the locality of residence for the last 10 years. Personal habits considered were consumption of alcohol, tobacco in the form of chewing or smoking, or use of pan (an Indian masticatory consisting of areca nut, catechu, and tobacco rolled in a betel leaf coated with lime). Betel and areca nut use was defined as: moderate, 5 to 10 betels per day for 2 to 10 years; heavy, >10 betels per day for >10 years. Tobacco use was defined as: moderate, <20 bidis/cigarettes per day for 1 to 10 years or equivalent amount of chewable tobacco; heavy, >20 bidis/cigarettes per day for >10 years.

Punch biopsy specimens of normal oral tissues were obtained either from healthy volunteers working in the

institute or from patients with oral lesions from an area either contralateral or adjacent to the site of oral lesions. Of the 100 oral SCCs, 65 specimens were well-differentiated tumors and 35 specimens were poorly or moderately differentiated SCCs. The majority of the patients were habitual consumers of betel and/or tobacco. One piece of tissue was snap-frozen and stored at -80°C for immunohistochemical analysis, while another was preserved in formalin for histopathological examination.

Methods

Immunohistochemistry

Cryosections of oral tissue specimens (5 to 7 μm thickness) were fixed in acetone. For histopathological analysis, the representative sections were stained with hematoxylin and eosin, and immunostaining was carried out in serial sections as described previously.¹⁶ For immunohistochemical analysis, endogenous peroxidase activity was blocked with 0.3% (v/v) hydrogen peroxide in methanol for 20 minutes. Nonspecific binding sites were blocked by incubating the sections with 0.1% (w/v) bovine serum albumin in phosphate-buffered saline (PBS) for 1 hour. The sections were subsequently incubated with the primary antibody overnight at 4°C . Mouse monoclonal antibodies (1 $\mu\text{g/ml}$) SMP14, 2A10, and DO-1 (Santa Cruz Biotechnology, Santa Cruz, CA) were used for detecting MDM2 and p53 proteins, respectively. The primary antibody was detected using biotinylated secondary anti-mouse IgG antibody, by the avidin-biotin complex (ABC) method using 3,3'-diaminobenzidine as chromogen. Incubations were performed at room temperature in a moist chamber. Slides were washed several times in PBS after each step. In negative controls, the primary antibody was replaced by PBS or nonimmune mouse IgG of the same isotype to ensure specificity. Human esophageal SCC tissue sections known to overexpress MDM2 and p53 were used as the positive controls in each batch of sections analyzed (data not shown). The intensity of immunohistochemical staining was evaluated in five areas of the slide sections for correlation and confirmation of the tissue analysis. The MDM2 and p53-positive cases were evaluated semiquantitatively and graded on a 4-point scale based on the percentage of cells showing MDM2 or p53 staining: $-$, < 10%; +1, 10 to 30%; +2, 30 to 50%, and +3, >50%. The sections were counterstained with hematoxylin. The immunostained slides were graded on the above-mentioned 4-point scale by three of us independently (SA, RR, and MM). More than 10% positive staining in the nuclei was defined as the cut-off point chosen for both MDM2 and p53 protein expression.

Immunoblotting

Single-cell suspensions of normal, premalignant, and malignant tissues were lysed in buffer containing 2% Nonidet P-40, 0.2% (w/v) sodium dodecyl sulfate, 50 mmol/L NaCl, 25 mmol/L Tris-HCl (pH 7.5), and the protease inhibitors, phenylmethylsulfonyl fluoride (1 mmol/L)

and aprotinin (50 $\mu\text{g/ml}$) for 30 minutes on ice. Lysates were centrifuged at $10,000 \times g$ for 10 minutes and the supernatants were loaded onto 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and electrophoresed at constant voltage (150 V) for 3 hours. The gels were transferred to nitrocellulose membranes and probed with anti-MDM2 antibodies (SMP14 and 2A10 mAbs). Horseradish peroxidase-conjugated goat anti-mouse IgG was used as the secondary antibody and the signal was detected by the Enhanced chemiluminescence method (Life Sciences Inc., Arlington Heights, IL).

RNA and DNA Extraction

RNA was extracted by the acid-guanidium-thiocyanate-phenol-chloroform method.¹⁷ Briefly, tissues from normal, premalignant, and malignant lesions were homogenized in denaturing solution (4 mol/L guanidium thiocyanate, 25 mmol/L sodium citrate, pH 7, 0.5% sarcosyl, 0.1 mol/L 2-mercaptoethanol), and 0.2 mol/L sodium acetate (pH 4), an equal volume of phenol (water-saturated) and 0.1 volume of chloroform-isoamyl alcohol mixture (49:1, v/v) were added to the homogenate sequentially. The final suspension was shaken vigorously and cooled on ice for 15 minutes. After centrifugation the aqueous phase was recovered and RNA was precipitated with isopropanol. The RNA pellet was redissolved in denaturing solution, precipitated with isopropanol, dried, and dissolved in diethylpyrocarbonate-treated water.

For extraction of DNA, tissues from normal, premalignant, and malignant lesions were homogenized in lysis buffer (10 mmol/L Tris-Cl, pH 7.5, 1 mmol/L ethylenediaminetetraacetic acid, 5 mmol/L MgCl_2 , 0.5% Tween 20) and incubated overnight at 37°C in the presence of 10 mg/ml of proteinase K. Genomic DNA was purified by a standard extraction method using phenol-chloroform and chloroform-isoamyl alcohol.

In Situ Hybridization

The human *mdm2* cDNA, pCMV-Neo-Bam, plasmid was a kind gift from Bert Vogelstein (The Johns Hopkins University School of Medicine, Baltimore, MD).¹ This *mdm2* cDNA (1.9 kb) was cloned in pBluescript, pBSK⁺ phagemid (pBSK⁺ Hdm2). The pBSK⁺ Hdm2 was digested with *Xho*I for the T₃ polymerase reaction and with *Sac*I for the T₇ polymerase reaction.

The antisense and sense RNA probes were routinely synthesized using an *Xho*I-digested pBSK⁺ Hdm2 fragment and a *Sac*I-digested pBSK⁺ Hdm2 fragment in a standard T₇ or T₃ polymerase reaction (Stratagene enzymes and reagents; Stratagene, La Jolla, CA) using [³⁵S]-CTP followed by partial alkaline hydrolysis to reduce the average probe length to ~150 nucleotides. Cryosections, 5 to 7 μm thickness, were placed onto gelatin coated slides at 40°C, dipped in acetone for 5 minutes, and air-dried. The sections were then fixed in 4% buffered paraformaldehyde, acetylated and dried. After prehybridization the *in situ* hybridization was performed with [³⁵S]-labeled sense or antisense RNA probes transcribed from the above construct

Table 1. Immunohistochemical Analysis of MDM2 Protein in Oral Lesions*

Tissue type	Total cases	MDM2 positive	Expression negative
Squamous cell carcinoma	100	71 (71%)	29 (29%)
Histopathological grade [†]			
Well	65	42 (65%)	23 (35%)
Moderate/poor	35	29 (83%)	6 (17%)
Tumor stage [‡]			
T1/T2	31	17 (55%)	14 (45%)
T3/T4	69	54 (78%)	15 (22%)
Nodal status			
Nodes positive	51	33 (65%)	18 (35%)
Nodes negative	49	38 (78%)	11 (22%)
Leukoplakia			
Hyperplastic lesions	25	11 (44%)	14 (56%)
Dysplastic lesions	15	9 (60%)	6 (40%)
Normal mucosa	40	2 (5%)	38 (95%)

* χ^2 test.

[†]Well versus moderate/poor; $P = 0.017$.

[‡]T₁/T₂ versus T₃/T₄; $P = 0.017$.

with the probe concentration of 25,000 cpm/ μl in hybridization buffer (50% formamide, 0.3 mol/L NaCl, 10 mmol/L Tris-Cl, pH 8, 2 mmol/L ethylenediaminetetraacetic acid, 10% dextran sulfate, 10 mmol/L phosphate buffer, pH 8, 1 $\times$ Denhardt's solution, and 500 μg tRNA) after denaturation at 70°C for 2 minutes. The slides were incubated overnight at 52°C in a water bath. Thereafter, the slides were washed twice with 1 $\times$ standard saline citrate (SSC) and 50% formamide at 55°C for 1 hour. After rinsing in 2 $\times$ SSC, 2 $\times$ 5 minutes at room temperature, slides were incubated in 10 to 20 mg/ml RNase A and 1 unit/ μl RNase T1 at 37°C for 30 minutes. The slides were then washed sequentially in 2 $\times$ SSC-50% formamide, twice, for 1 hour, and 0.1 $\times$ SSC, 15 minutes at 55°C. The slides were rinsed in water, dehydrated, and exposed to Kodak NBT2 emulsion autoradiography (Eastman Kodak, Rochester, NY) for 2 to 6 weeks at 4°C and subsequently developed using Kodak D-19 developer. The tissue sections were stained with toluidine blue, dehydrated, mounted, and observed under the microscope.

Northern Blot Analysis

Total RNA (15 μg) was electrophoresed on a 1% agarose/formaldehyde gel in 10 mmol/L sodium phosphate buffer (pH 6.8) at a constant rate of 2 V/cm at room temperature. RNA was then transferred onto a nylon membrane in 1 $\times$ SSC overnight. The membrane was UV cross-linked and baked at 80°C for 2 hours. Prehybridization was carried out in a buffer containing 20% dextran sulfate, 40% formamide, 4 $\times$ SSC, 15 mmol/L Tris-HCl (pH 7.5), 0.8% Denhardt's solution, and 100 $\mu\text{g/ml}$ sonicated salmon sperm DNA at 42°C for 4 hours. The blot was subsequently hybridized overnight with [³²P]-dCTP random primer-labeled human *mdm2* cDNA probe. The *mdm2* cDNA ($\approx$ 1.9 kb) insert was excised from pCMV-Neo-Bam using *Bam*HI¹ at 42°C and then washed sequentially in a buffer containing 2 $\times$ SSC and 0.1% SDS at room temperature for 45 minutes, 0.2 $\times$ SSC 60°C and 0.1% SDS at room temperature for 45 minutes, followed

Figure 1. Immunohistochemical detection of MDM2 protein in oral lesions using mAb SMP14 and 2A10. **A:** Hyperplastic epithelium showing predominantly nuclear staining for MDM2 protein. **B:** Well-differentiated SCC showing MDM2 immunostaining exclusively in the nucleus. **C:** Well-differentiated SCC showing MDM2 immunostaining in the nucleus as well in the cytoplasm. **D:** Well-differentiated SCC showing MDM2 immunostaining only in the cytoplasm and the plasma membrane. All of the sections were counterstained with hematoxylin (original magnifications: **A**, $\times 100$; **B-D**, $\times 400$).

by incubation at 60°C for 5 to 20 minutes. The rRNAs (18S and 28S) were stained with ethidium bromide to ascertain uniform loading. β -actin was used as control.

Southern Hybridization

DNA samples from normal, hyperplastic lesions, dysplastic lesions, and SCCs were completely digested with *EcoRI* and then resolved by electrophoresis in an 0.8% agarose gel followed by transfer to a nylon membrane. The membranes were hybridized with the biotinylated *mdm2* cDNA probe, pCMV-Neo-Bam.¹ The probe was labeled and detected as per the manufacturer's instructions (New England Biolabs, Beverly, MA). DNA extracted from normal oral tissues was used as the reference for the assessment of the gene copy number. Densitometric scanning of the *mdm2* gene was performed using Collage-2 software and a Fotodyne gel scanner (Hartland, WI). A relative increase in signal intensity of more than three times that of the normalized DNA signal was considered as a case with gene amplification.

RNase Protection Assay

The genesis of alternative 5'-UTRs of *mdm2* transcripts was described by Landers et al.¹⁸ The *mdm2* transcripts initiating from promoter region P1 contain sequences from exon 1 spliced directly to exon 3. 5'-UTR of the

L-*mdm2* transcripts initiating from the promoter P1 is derived from exon 1 of the human *mdm2* gene, whereas the 5'-UTR of the S-*mdm2* transcripts is derived from exon 2.¹⁸ Importantly, therefore, usage of the internal p53-responsive promoter region P2 generates *mdm2* transcript forms (S-*mdm2* transcribed from exon 2 and 3) with enhanced translational efficiency relative to those transcripts (L-*mdm2*) derived from the upstream P1 promoter. The probes were generated by amplifying JAR cell cDNA with primers corresponding to exons 2 to 3 of *mdm2* and GAPDH. A second round of polymerase chain reaction was used to introduce the T7 polymerase promoter. First round primers for *mdm2*: 829 5' CAGTGGC-GATTGGAGGGTAG 3', and 451 L 5'-GGCCGCGGGTGCTTTT-TGTGCACC-3'. Second round primers for *mdm2* and GAPDH: 829 and T7L.5'-GAGAATTCTAATACGACT-CACTATAG- GGCCGCGG-3'. First round primers for GAPDH: 151 5'-GTTGCCATCCATGGAATT-3'. Overlapping linker sequences are in smaller type and the T7 promoter is underlined. RNase protection assays were according to standard protocols, essentially as described by Landers et al.¹⁸ The expected lengths in bases of the products for RNA initiated at the P1 and P2 promoters are 140 and 208 and 89 for GAPDH.

The *mdm2* riboprobe was prepared using the Riboprobe Gemini System (Promega, Madison, WI). Approximately 10^6

Table 2. Analysis of MDM2 Protein Isoforms, mRNA, and Gene Status in Oral Lesions and Their Relationship with Clinicopathological Features

Case no.	Gender/age (years)	Site	Betel and areca nut	Tobacco	MDM2 IHC	MDM2 isoform (kDa)	Subcellular localization	Mdm2 mRNA
Hyperplastic lesions								
1	M/38	T	—	Moderate	+2	76	C,PM	—
2	F/55	BM	—	—	+2	57	N	—
3	F/36	BM	—	—	+3	90	N,C	—
4	M/33	T	Moderate	—	+1	90	N,C	—
5	M/53	L	—	Heavy	+2	76,90	N,C,PM	+
6	M/42	T	—	—	+1	76	C,PM	—
7	M/47	T	—	Moderate	+2	57,90	N,C	—
8	M/32	BM	Moderate	Moderate	+1	57	N	+
9	M/33	T	Moderate	—	+1	57	N	—
10	F/27	BM	—	—	+3	57	N	—
11	M/26	BM	—	Moderate	+3	90	N,C	+
Dysplastic lesions								
1	M/35	BM	—	Heavy	+2	57	N	—
2	M/20	T	—	—	+1	57	N	—
3	M/60	BM	—	Heavy	+1	57	N	—
4	M/45	T	—	—	+2	57,90	N,C	—
5	F/65	BM	—	Heavy	+2	76,90	N,C,PM	—
6	M/50	BM	Heavy	Heavy	+2	76	C,PM	—
7	M/24	T	Moderate	Moderate	+2	57	N	—
8	M/60	BM	—	Heavy	+1	57	N	—
9	M/45	T	—	—	+2	90	N,C	—

Abbreviations: BM, buccal mucosa; T, tongue; L, lip; M, male; F, female; HG, histopathological grading; H, hyperplasia; D, dysplasia; N, nucleus; C, cytoplasm; PM, plasma membrane; WT, wild type; NS, nonsense; MS, missense; S, silent.

Betel and areca nut use: moderate, 5 to 10 betel per day for 2 to 10 years; heavy, >10 betel per day >10 years.

Tobacco use: moderate, <20 bidis/cigarettes per day for 1 to 10 years or equivalent amount of chewable tobacco; heavy, over 20 bidis/cigarettes per day for more than 10 years.

MDM2 and p53 immunostaining: —, <10%; +1 = 10–30%; +2 = 30–50%; +3 = >50% of the tumor cell positive.

mRNA expression: —: basal expression of mRNA; +: increased expression of mRNA.

counts of *mdm2* riboprobe were hybridized to equal amounts (10 μ g) of total cellular RNA, as determined by absorbance at 260 nm and by ethidium bromide staining in the presence of 50% formamide, 40 mmol/L PIPES Piperazine-N,N'-bis[2-ethane sulphonic acid] (pH 6.4), 0.4 mol/L NaCl, and 1 mmol/L ethylenediaminetetraacetic acid at 42°C overnight. ³²P]-labeled antisense probe complimentary to sequence in the shorter 5'-UTR as well as a small portion of the *mdm2* coding region was hybridized to RNA isolated from oral SCC tissues. After RNase A and RNaseT1 digestion, the reaction was stopped with the addition of SDS and proteinase K, followed by phenol-chloroform extraction. The protected fragments were precipitated, separated on 6% SDS-polyacrylamide gel electrophoresis gels, dried, and exposed to X-ray film.

PCR and Sequencing

Purified DNA samples were used as a template in the polymerase chain reaction. Exons 5 to 9 of the *p53* gene shown to have a high incidence of mutations were the target sequences. The following primers (oligonucleotide amplimers), complimentary to adjacent target sequences were used (BioServe Biotechnologies, Laurel, MD). Exon-5 Fwd: 5'-GTT TCT TTG CTG CCG TGT TC-3'; Rev: 5'-AGG CCT GGG GAC CCT GGG CA-3'; Exon-6 Fwd: 5'-TGG TTG CCC AGG GTC CCC AG-3'; Rev: 5'-GGA GGG CCA CTG ACA ACC A-3'; Exon-7 Fwd: 5'-ACC ATC CTG GCT ACCA GGT GA-3'; Rev: 5'-AGG GGT CAG CGG CAA GCA GA-3'; Exon-8 and 9 Fwd: 5'-TTG

GGA GTA GAT GGA GCC T-3'; Rev: 5'-AGT GTT AGA CTG GAA ACT TT-3'.

Samples were amplified using 40 pmol of each primer and 2.5 U of *Taq* DNA polymerase in 10× polymerase chain reaction buffer in a total volume of 50 μ l for one cycle at 94°C for 4 minutes and 35 cycles at 94°C for 1 minute, 60°C for 1 minute, and 78°C for 30 seconds. The polymerase chain reaction products were purified on low-melting agarose gels and sequenced using automated DNA sequencer (Applied BioSystems dye terminator cycle sequencing). The sequence data were analyzed using the p53 data base located at the National Center for Biotechnology Information web site: <http://www.ncbi.nlm.nih.gov>.

Statistical Analysis

The correlation between MDM2 and clinicopathological parameters of oral cancer patients was statistically evaluated using chi-squared test. Prognostic implication of MDM2/p53 co-expression in oral tumorigenesis was determined by Kaplan-Meier survival analysis.

Results

Immunohistochemical Analysis of MDM2 Protein Expression in Oral Tissues

The results of analysis of MDM2 expression in human oral normal tissues, hyperplastic and dysplastic lesions, as

Table 3. Analysis of MDM2 Protein Isoforms, mRNA and Gene Status in Oral SCCs and Their Relationship with Clinicopathological Features

Case no.	Gender/age (years)	Site	State	HG	Betel and areca nut	Tobacco	MDM2 IHC	MDM2 isoform (kDa)	Subcellular localization	Mdm2 mRNA	Mdm2 amplification (folds)
1	M/60	L	T ₄ N ₀ M ₀	W	Moderate	—	+1	76	C,M	—	1
2	M/40	BM	T ₂ N ₀ M ₀	W	Heavy	—	+3	90	N,C	—	1
3	M/54	BM	T ₄ N ₀ M ₀	W	Heavy	Heavy	+1	57	N	—	1
4	M/70	BM	T ₂ N ₀ M ₀	W	Heavy	—	+2	90	N,C	—	1
5	F/50	BM	T ₃ N ₁ M ₀	W	—	Moderate	+2	57	N	—	1
6	M/50	BM	T ₄ N ₂ M ₀	W	Moderate	—	+3	90	N,C	+	9
7	M/55	P	T ₄ N ₀ M ₀	W	Heavy	—	+2	57	N	—	1
8	F/45	T	T ₂ N ₀ M ₀	W	Heavy	—	+2	90	N,C	—	1
9	F/30	BM	T ₂ N ₀ M ₀	W	—	—	+3	76	C,PM	+	1
10	M/66	BM	T ₂ N ₀ M ₀	W	Heavy	Heavy	+2	57,90	N,C	—	1
11	M/44	BM	T ₂ N ₀ M ₀	W	Moderate	—	+1	57	N	—	1
12	M/42	BM	T ₂ N ₀ M ₀	W	—	Moderate	+1	90	N,C	—	1
13	F/49	BM	T ₄ N ₁ M ₀	M	—	Moderate	+1	76,90	N,C,PM	—	1
14	M/60	BM	T ₂ N ₀ M ₀	M	Heavy	Heavy	+2	90	N,C	+	1
15	M/82	P	T ₄ N ₀ M ₀	M	—	—	+1	76	C,PM	—	1
16	F/59	T	T ₄ N ₀ M ₀	M	Heavy	—	+3	57	N	—	1
17	F/52	BM	T ₄ N ₁ M ₀	M	—	Heavy	+2	76	C,PM	—	1
18	M/52	BM	T ₃ N ₁ M ₀	M	Heavy	—	+3	57	N	—	1
19	M/45	T	T ₂ N ₁ M ₀	P	Heavy	—	+2	57	N	—	1
20	F/22	BM	T ₃ N ₁ M ₀	P	Moderate	Moderate	+2	76,90	N,C,PM	—	1

well as SCCs are summarized in Table 1. The expression of MDM2 protein was studied in 40 normal cases. Normal oral tissues obtained from cancer-free individuals (25 cases) and from a site contralateral to the hyperplastic lesions (5 cases) showed no detectable level of MDM2 protein. Two of the 10 tissues obtained from site adjacent to the dysplastic lesions showed faint MDM2 immunoreactivity in 12% and 15% of the cells localized in the nucleus. The histopathological examination of these tissues showed features of normal oral mucosal epithelium. The detection of MDM2 immunoreactivity in histologically normal oral epithelium adjacent to the dysplastic lesions may be because of the field cancerization effect in which the area surrounding the lesion may also show molecular alterations because of exposure to the carcinogens. Elevated levels of MDM2 were observed in 11 of 25 hyperplastic lesions and 9 of 15 dysplastic lesions. The representative tissue section of a hyperplastic oral lesion showing MDM2 staining is shown in Figure 1A. Of the malignant oral lesions examined, 71 of 100 (71%) oral SCCs were MDM2 positive (42 of 65 well-differentiated tumors, 29 of 35 moderately to poorly differentiated tumors). The positive cases showed intense nuclear staining in tumor cells in the majority of the cases (Figure 1B). However, the staining was observed predominantly in the nucleus and cytoplasm (Figure 1C) or cytoplasm and plasma membrane in a subset of cases (Figure 1D). Correlation of MDM2 protein overexpression with clinicopathological parameters of the patients showed significant association with tumor stage ($P = 0.017$) and loss of differentiation ($P = 0.017$) in oral SCCs. The overexpression of MDM2 protein in oral lesions may be because of gene amplification, enhanced transcription, or translation. Hence analyses of MDM2 protein isoforms, mRNA and gene status in MDM2 overexpressing hyperplastic lesions (11 cases), dysplastic lesions (9 cases), and SCCs (20

cases) were performed when inadequate tissue specimens were available to perform all of the assays.

Analysis of *mdm2* mRNA by *in Situ* Hybridization and Northern Blotting in Oral Lesions

The results of the *mdm2* mRNA status in MDM2 protein-overexpressing oral hyperplastic lesions, dysplastic lesions, as well as SCCs are summarized in Tables 2 and 3, respectively. Of the 11 hyperplastic lesions examined, increased *mdm2* mRNA transcripts were observed in three cases in the antisense probe-hybridized sections compared with those hybridized with the sense probe (Figure 2, A and B). Of the 20 oral SCCs investigated for *mdm2* mRNA, increased levels of *mdm2* mRNA transcripts were observed in three cases (Figure 2, C and D). The results of *in situ* hybridization were corroborated by Northern blotting. All of the oral hyperplastic lesions as well as SCCs showing increased *mdm2* transcripts by *in situ* hybridization also showed elevated RNA levels by Northern blotting. The representative results shown in Figure 3 illustrate increased RNA levels in hyperplastic lesions (lanes 1 and 2) and SCCs (lanes 6 to 8), basal levels in normal (lane N) and dysplastic lesions (lanes 3 and 4), respectively.

Genetic Analysis of *mdm2* by Southern Hybridization

mdm2 gene amplification was determined by Southern hybridization in MDM2-overexpressing oral hyperplastic lesions (11 cases), dysplastic lesions (9 cases), and SCCs (20 cases). Normal human placental DNA was

Figure 2. ISH analysis of *mdm2* mRNA in oral lesions: *mdm2* mRNA transcript in oral hyperplastic epithelium probed with sense cDNA (A), antisense cDNA (B), well-differentiated SCC sense cDNA (C), and antisense cDNA (D). The hybridization signals appear as black silver grains in bright-field illumination (original magnification, $\times 400$).

taken as control. One of 11 hyperplastic lesions showed sevenfold amplification of *mdm2* gene, whereas one of 20 SCCs showed ninefold amplification of *mdm2* gene (Figure 4, lanes 1 and 10, respectively).

Analysis of mdm2 P1 and P2 Promoter by RNase Protection Assay and Its Relationship with p53 Gene and Protein Status

Twenty oral SCCs were examined for assessing P1 and P2 promoter activity of the *mdm2* gene. Of the 20 oral SCCs examined, P2 promoter activity was observed in 12 cases. All of the 12 cases overexpressed both MDM2 and p53 proteins. Of the eight cases which did not show P2 promoter activity, only two cases co-expressed MDM2 and p53 proteins and in these two cases the *p53* gene was mutated (Table 3, Figure 5).

Analysis of MDM2 Protein Isoforms and Their Relationship with p53 Protein

MDM2 protein isoforms were assessed in oral lesions overexpressing MDM2 protein in 11 hyperplastic lesions, nine dysplastic lesions (Table 2), and 20 oral SCCs (Table 3, Figure 6) by immunoblotting as well as immunohistochemistry. In oral hyperplastic and dysplastic lesions, four cases showing the 90-kd MDM2 protein by immuno-

Figure 3. A: Northern blot of total RNA (10 μ g/well) extracted from oral normal tissue (lane N), leukoplakic lesions with dysplasia (lanes 3 and 4), leukoplakic lesions with hyperplasia (lanes 1, 2, and 8), and cancer tissues (lanes 5-7). B: Agarose gel stained with ethidium bromide (EtBr). The 28S RNA and 18S RNA bands were scanned to quantitate the amount of total RNA loaded in each lane.

Figure 4. Southern blot of *EcoR*-I-digested DNA (10 μ g/well) obtained from oral normal tissue (lane N), leukoplakic lesions with hyperplasia (lanes 1–3), leukoplakic lesions with dysplasia (lanes 4 and 5), and cancer tissues (lanes 6–10). DNA amplification was observed in lane 1 (sevenfold) and lane 10 (ninefold).

blotting showed staining in the cytoplasm as well as in the nucleus by immunohistochemistry. Three cases showed the 76-kd protein with localization of the protein in the cytoplasm and plasma membrane and 10 cases showed the 57-kd protein with staining exclusively in the nucleus. We have found the simultaneous expression of 57- and 90-kd MDM2 proteins in case nos. 7 and 15 and simultaneous expression of 76- and 90-kd MDM2 proteins in case nos. 5 and 16. No mutations were detected in exons 5 to 9 of the *p53* gene in cases overexpressing 57-kd or 90-kd MDM2 proteins (Table 2) suggesting alternate mechanisms for overexpression of wild-type *p53* in these cases. In oral SCCs, a 90-kd MDM2 isoform localized in the nucleus as well as cytoplasm (Figure 1c) was observed in six cases, a 76-kd protein showing staining in the cytoplasm and plasma membrane (Figure 1d) was observed in four cases, and a 57-kd protein showing staining exclusively in the nucleus (Figure 1b) was observed in seven cases. In case no. 10, simultaneous expression of 57- and 90-kd MDM2 proteins and in case nos. 13 and 20 simultaneous expression of 76- and 90-kd MDM2 proteins were observed. The accumulation of

Figure 5. RNase protection assay of oral SCCs. RNase protection analysis illustrates differential expression of the two *mdm2* transcript classes. 32 P-labeled antisense probe was hybridized to RNA isolated from oral SCCs (lanes 1–10). In lane 11, only the antisense probe of 208 nucleotides, those *mdm2* transcripts containing the longer (301 bp) 5'-UTR will protect a fragment of 140 nucleotides. GAPDH was used as control for RNA loading.

Figure 6. Western blot analysis of MDM2 protein isoforms in oral lesions. The immunoblot shows MDM2 protein isoforms (57 kd, 76 kd, and 90 kd) in oral leukoplakic lesions with hyperplasia (lanes 1 and 2), leukoplakic lesions with dysplasia (lane 3), and SCCs (lanes 4–6). Lane 1 shows the simultaneous expression of the 76- and 90-kd MDM2 proteins.

wild-type *p53* was observed in cases overexpressing 57- or 90-kd MDM2 proteins (Table 3).

Correlation of *p53*/MDM2 Alterations with Clinical Outcome of Patients with Oral Premalignant and Malignant Lesions

Of the 100 oral SCC cases analyzed in this study, 46 patients could be followed for up to 180 months. Among these 46 cases, 31 cases were *p53* positive and 15 cases were *p53* negative. The Kaplan-Meier survival analysis (Figure 7) showed that median disease-free survival time

Figure 7. Kaplan-Meier estimation of cumulative proportion of tumor recurrence/metastasis in relation to co-expression of *p53*/MDM2 proteins. Forty-six oral SCC patients were followed up to 180 months. The median time for disease-free survival in the *p53*/MDM2-positive cases (group A) was 16 months; in cases not co-expressing *p53*/MDM2 (group B), it was >26 months ($P = 0.02$).

in p53-positive cases was significantly shorter (16 months) than in p53-negative cases (41 months) ($P = 0.008$). Of the 31 oral tumors expressing p53 protein, 24 cases (77%) showed recurrence within 5 to 36 months, whereas eight of the 15 (53%) p53-negative cases showed recurrence within a period of 12 to 180 months.

Among the 46 oral cancer patients followed in regular follow-up clinics for 15 years from the time of diagnosis, 24 cases co-expressed MDM2 and p53 proteins, whereas 22 cases did not show concomitant expression of these two proteins. The Kaplan-Meier survival analysis showed the median disease free survival time (no recurrence/metastasis) to be significantly shorter in p53/MDM2-positive cases (median time, 16 months) than in cases which did not show co-expression of p53/MDM2 proteins (median time, 26 months) ($P = 0.02$) illustrated in Figure 7.

Discussion

The results presented herein demonstrate overexpression of MDM2 protein in oral hyperplastic lesions (44%), dysplastic lesions (60%), and a majority of betel- and tobacco-related oral SCCs (71%) in the Indian population. The overexpression of MDM2 protein may be because of gene amplification, enhanced transcription, or translation. Nevertheless, the extremely low frequency of *mdm2* amplification observed in our study in oral hyperplastic lesions (one out of 11 cases) and SCCs (one out of 20 cases) suggests that *mdm2* amplification is an infrequent event in oral tumorigenesis. *In situ* hybridization analysis showed increased *mdm2* mRNA transcripts in three out of 11 hyperplastic lesions and three out of 20 SCCs suggesting that enhanced transcription may account for MDM2 overexpression in subset of oral lesions.

It has been proposed that the molecular basis of enhanced translation of MDM2 mRNA is the increased activity of the internal (wild-type, p53-responsive) P2 promoter of the gene that generates a *mdm2* transcript with a distinct 5'-UTR (*S-mdm2*), which is translated approximately eightfold more efficiently than transcripts (*L-mdm2*) produced by the constitutive *mdm2*-P1 promoter.¹⁸ To test the possibility that the increased production of *S-mdm2* transcript because of *mdm2*-P2 promoter activation by wild type p53 is the major mechanism of MDM2 overexpression in this cohort of betel and tobacco related oral SCCs, we performed RNase protection assay using probes that distinguish between *S-mdm2* and *L-mdm2* transcripts. The most intriguing feature of our study was the presence of *S-mdm2* transcripts in the majority of oral SCCs (12 out of 20 cases) overexpressing MDM2 protein in the absence of gene amplification or enhanced transcription. Our results suggest that *mdm2*-P2 promoter activation may account for overexpression of the MDM2 protein in a subset of oral tumors. To our knowledge this is the first study showing the activation of *mdm2*-P2 promoter and production of *S-mdm2* transcripts in oral SCCs.

The *mdm2*-P2 transcripts were correlated with the p53 gene status in the same subset of oral SCCs. Interest-

ingly, we observed a low frequency of p53 gene mutations in these cases. It was reported earlier by us and other workers that p53 gene mutations are indeed infrequent events in betel- and tobacco-related oral cancers in the Indian population.^{19,20} However, the stabilization of wild-type p53 protein despite the low frequency of p53 mutations and its role in oral tumorigenesis remained a puzzling question. The significant correlation between low frequency of p53 mutations and MDM2 overexpression in a large subset of cases in this cohort suggests that MDM2 may be implicated in the accumulation of p53 in oral tumorigenesis. Inactivation of wild-type p53 by MDM2 has also been reported in Burkitts lymphoma.²¹ Interestingly, the increased susceptibility for the early onset of a variety of malignancies in individuals in breast cancer-prone families has also been ascribed to the inactivation of wild-type p53 protein by MDM2.²²

The salient feature of the study was the identification and characterization of three different isoforms of MDM2 protein (ie, p90, p76, and p57) in betel- and tobacco-related oral hyperplastic lesions, dysplastic lesions, and SCCs and their relationships with clinicopathological parameters. The p90/85 isoforms contain all MDM2 epitopes, p76 protein is devoid of N-terminal epitope that maps at residues 19 to 50, the region required for binding to p53, and p57 protein has deletion of the C-terminal epitopes. Our data demonstrated that 9 out of 11 hyperplastic lesions and 7 out of 9 dysplastic lesions as well as 14 out of 20 SCCs showed the presence of MDM2 protein isoforms p90 and p57 that can bind to the p53 protein and thus may account for the accumulation of wild-type p53 in oral tumorigenesis.

Several alternatively spliced *mdm2* transcripts were detected in transformed cell lines/tumors, some of which give rise to proteins that have lost the ability to bind to p53 protein.^{23,24} Some of the variant forms of MDM2 lack the p53 binding domain and hence are not able to regulate p53 transactivation function. The existence of multiple forms of MDM2 proteins with differential capacities to bind to p53 raises the question of different functional roles for these proteins in cell-cycle regulation, although MDM2 acts as an oncogene mainly by virtue of its ability to inactivate p53, it remains possible that other MDM2 isoforms (such as p76) contribute directly to its oncogenic potential by alternative pathways. The p53-independent oncogenic role of MDM2 has been demonstrated by its ability to activate E2F1/DP1 transcription factors²⁵ and interact with Rb protein.²⁶ To our knowledge this is the first report showing the presence of different MDM2 isoforms (p90, p76, and p57) in betel- and tobacco-related oral tumorigenesis.

Furthermore, we have investigated the subcellular localization of these protein isoforms. Our studies by immunohistochemistry and immunoblotting in a subset of cases revealed the presence of MDM2 isoforms (p57 and p90) which bind to p53 protein in both the nucleus or nucleus and cytoplasm, respectively, whereas p76 (does not bind p53) was localized in the cytoplasm and plasma membrane. The differential subcellular compartmentalization of these protein isoforms further supports the possibility that each may have a distinct function in the reg-

ulation of p53 and other gene products. These protein isoforms may arise from alternatively spliced mRNAs and it is possible that MDM2 protein isoforms and p53 protein may function in different, but synergistic transformation pathways. Selective compartmentalization of different MDM2 proteins derived from differential splicing of the 5.5-kb mRNA with distinct functional roles have also been observed in non-small-cell lung carcinoma²⁷ and bronchogenic carcinoma.²⁸

The central point of clinical significance of any molecular event involved in tumorigenesis is its prognostic value as an indicator of disease outcome. The follow-up data presented herein showed that the median disease-free survival time was significantly shorter in patients with oral SCCs showing co-expression of MDM2 and p53 proteins compared with tumors that did not show concomitant overexpression of these proteins ($P = 0.02$). These findings underscore MDM2/p53 overexpression as an adverse prognosticator in oral tumorigenesis.

Furthermore, a significant association of MDM2 expression was observed with tumor stage ($P = 0.017$) and dedifferentiation ($P = 0.017$) in oral SCCs, features indicative of aggressive tumor behavior.

In conclusion, the present study reports the identification of various MDM2 isoforms, their different subcellular localization, and their relationship with p53 in oral hyperplastic and dysplastic lesions as well as SCCs. The enhanced translation of *mdm2*-P2 transcripts (*S-mdm2*) correlated with stabilized wild-type p53 and may represent an important mechanism for accumulation of p53 in oral tumorigenesis, particularly with regard to identification of oral SCCs with poor prognosis.

Acknowledgments

The authors wish to thank the IGBMC core and oligonucleotide synthesis and DNA sequencing facilities for tremendous help and support, the members of Boh Wasyluk's lab for help and constructive discussions, and the members of Centre Franco-Indien Pour La Promotion De La Recherche Avancée for their scientific support.

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