In this study, to better understand the mechanism of oral squamous cell carcinoma (SCC) carcinogenesis, alterations of the p53 gene and overexpression of MDM2 and p53 were analyzed in 38 oral SCC samples. Twelve of the 38 specimens revealed mutant-type p53. Moreover, coexpression of MDM2 and p53 was found most frequently in dysplastic lesions (P << 0.05). Expression of MDM2 and p53 was significantly increased in accordance with the histological progression of multistep carcinogenesis (P << 0.05). No significant correlation was found between the expression of MDM2 and the alteration of p53 protein or p53 gene status. MDM2 overexpression with mutant p53 was significantly associated with poorly differentiated SCCs (P << 0.05) and tumor stages III and IV of oral SCCs (P << 0.05). These results suggest that MDM2 overexpression is an early event in oral carcinogenesis through the functional inactivation of the wild-type p53, and corresponding alterations of MDM2 and p53 contribute to the oral carcinogenesis. We propose that it would be clinically more instructive to evaluate MDM2 overexpression combined with p53 gene status, compared to the evaluation of either MDM2 or p53 alteration alone.

Key words: MDM2 — p53 — Oral carcinogenesis

Oral squamous cell carcinoma (SCC) is the most common malignant tumor in the oral cavity. Nevertheless, the mechanism of oral carcinogenesis is still poorly understood. Previous studies have shown that inactivation of wild-type p53 function by mutation or interaction with a viral oncogene product plays an important role in carcinogenesis, including oral SCC. However, there are still oral SCCs which do not contain mutant p53 or viral protein. Therefore, it is reasonable to assume the presence of a p53-independent oral carcinogenesis pathway.

It was found that MDM2, a cellular oncogene product, can bind to the p53 N-terminal acidic domain to inactivate its transcription factor activity. Also, p53 can bind to the first intron of the MDM2 gene to activate MDM2 transcription, thus establishing a negative feedback loop to control the cell cycle.

MDM2 can inhibit wild-type p53-mediated G1 arrest and apoptosis. MDM2 also plays a role in promoting the S phase in the p53-independent pathway. It has been reported that overexpressions of both p53 and MDM2 are more frequent in high-grade bladder cancer. MDM2 overexpression is observed in various tumors with or without p53 mutation. However, the significance of the correlation between the overexpression of MDM2 and the p53 status in the multistep processes of oral SCC development has not yet been clearly elucidated.

In this study, to reach a better understanding of the mechanism of oral carcinogenesis, the alterations of the p53 gene in oral SCC specimens were investigated. Furthermore, using the same specimens, the overexpression of MDM2 and p53 was analyzed in normal mucosa adjacent to the tumor, and in premalignant and malignant lesions.

MATERIALS AND METHODS

Materials The 38 oral SCC specimens used in this study were obtained from the Department of Oral and Maxillofacial Surgery, Hamamatsu University School of Medicine from 1994 to 1999. Every specimen was divided into two sections. One section was immediately snap-frozen in liquid nitrogen, then stored at −80°C; the other was formalin-fixed and paraffin-embedded. A histological examination was carried out according to the WHO histological typing system, and 20 of the 38 cases were diagnosed as well-differentiated SCC, 14 as moderately differentiated SCC, and four as poorly differentiated SCC. Clinical staging of the 38 cases was also done based on the UICC TNM classification revised in 1997.

mRNA isolation and reverse transcriptase (RT) reaction mRNA was isolated from the frozen tissues using the “Micro-FastTrack” Kit (Invitrogen, San Diego, CA).
according to the manufacturer’s instructions. A reverse transcription master mix containing 5 mM MgCl₂, 50 mM KCl, 10 mM tri-HCl (pH 8.3), dNTPs (1 mM of each dNTP), RNase inhibitor (1 μg/μl), and the AMV reverse transcriptase (0.25 u/μl) (TaKaRa, Shiga) was prepared. Random 9-mers (2.5 μM) and mRNA (less than 1 μg) isolated from each specimen were added to the master mix. A 20 μl aliquot of the mixture was incubated at 30°C for 10 min, then at 42°C for 30 min, at 99°C for 5 min, 5°C for 5 min and finally cooled to 4°C.

Polymerase chain reaction (PCR) amplification of p53 gene

First PCR: The pair of oligonucleotide primers used to amplify the cDNA of the p53 gene (codon 113 to codon 343) was 5'-TCTTGTACATCTGAGACAG-3' (p5, sense) and 5'-TCTCGGAACATCTCAGAG-3' (p6, antisense). One hundred microliters of the reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl₂, 0.2 μM of each dNTP, 0.2 μl of each first set primer, 0.5 units of Taq DNA polymerase (TaKaRa) and 20 μl of reverse transcription product was prepared. The PCR amplification was performed for 30 cycles, each of 94°C for 30 s, annealing at 52°C for 1 min, and extension at 72°C for 2 min, with pre-denaturation at 94°C for 10 min and post-cycling extension at 72°C for 10 min.

Second PCR: Using the 1st PCR products of the p53 gene as a template, three fragments including A (113 to 195 codon, the end of exon 4 to beginning of exon 6), B (181 to 261 codon, the end of exon 6 to the end of exon 7) and C (238 to 343 codon, part of exon 7 to part of exon 10) were re-amplified with the following primers: p5 and 5'-GATAAGATCTGGAGG-3' (for fragment A), 5'-GCGTGCTCAGATGCCAT-3' and 5'-CTGGAGTCTTCAGTGG-3' (for fragment B), 5'-TGTAACAGTTCCTGACG-3' and p6 (for fragment C). For each specimen, a master mix of 50 μl was prepared as previously described, containing 1 μl of the 1st PCR product, and the PCR mixture was subjected to 35 cycles with the specific pair of primers under the same conditions as used for the 1st PCR.

Single-strand conformation polymorphism (SSCP)

‘Cold SSCP’ was performed according to a previously described method. Briefly, 5 μl of the 2nd PCR products of the p53 gene was diluted 4-fold with a solution containing 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol. The PCR products were denatured at 95°C for 15 min and electrophoresed on 6–10% nondenaturing polyacrylamide gel with or without 5% glycerol. The electrophoresis was performed at 90–120 volts for 12–17 h at room temperature. Separated single-stranded DNAs were detected using a silver stain II kit (Wako, Osaka).

DNA sequencing and restriction fragment length polymorphism (RFLP)

The 2nd PCR products of the p53 gene with an abnormal migration pattern in SSCP were purified using the Qiaquick PCR purification kit (QiAgen, Chatsworth, CA) or Microcon/Micropure kit (Millipore Co., Bedford, MA). Direct sequencing was then performed using the specific primers and a cycle sequencing core kit (Perkin-Elmer, Chiba) with an automated DNA sequencer (ABI Model 373A, Applied Biosystems, Foster City, CA). The sequencing procedure was performed at least twice on both the sense and anti-sense strands. To ensure consistency and reproducibility and to eliminate PCR artifacts, all assays were performed a minimum of 2 times on separate occasions. To exclude false positive results, some point mutations were confirmed by RFLP analysis. PCR products (5 μl) were directly used for digestion with 15 u restriction enzyme (HhaI orMspI, TaKaRa) and corresponding buffer, followed by electrophoresis on 3.5% gel (including 2.5% NuSieve Agarose and 1.0% regular agarose) and staining with ethidium bromide.

Immunohistochemical staining

Immunohistochemistry was performed using the strepto-avidin-biotin-peroxidase complex (ABC) method. Briefly, a series of 6 μm sections from paraffin-embedded blocks was deparaffinized with xylene and rehydrated with graded ethanol. Antigen retrieval was performed by incubating the sections in 10 mM sodium citrate buffer (pH 6.0) for 10 min in a microwave oven. After blocking of endogenous peroxidase and nonspecific reactivity, the sections were incubated with mouse monoclonal anti-p53 antibody (DO7, Dako A/S, Glostrup, Denmark. Dilution 1:100) and mouse monoclonal anti-MDM2 antibody (SMP14, Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Dilution 1:100) overnight at 4°C, followed by incubation with a biotinylated goat anti-mouse secondary antibody and streptavidin-biotin-peroxidase complex (Universal kit, ICN Biomedicals, Inc., Aurora, OH) according to the manufacturer’s recommendations. The peroxidase reaction was developed using diaminobenzidine as a chromogen (DAB kit, ICN Biomedicals, Inc.). The sections were counterstained with 3% methyl green. Negative controls were included from each sample, in which the primary antibody was omitted. The extent of the nuclear reactivity was classified into four categories: (−), no nuclear reactivity; (+), a few focally positive cells <10% tumor cells; (++), nuclear reactivity in 10 to 50% tumor cells; (+++), nuclear reactivity >50% tumor cells. The sections were observed and evaluated by a pathologist.

Statistical analysis

The correlation between the expression of MDM2 and the alteration of p53 was analyzed by using the χ² method or Fisher’s exact test. The levels (mean±SD, %) of MDM2 or p53 expression during multistep oral SCC were compared using the t test. A probability value of <0.05 was considered significant.
RESULTS

*p53* gene status Twelve of 38 specimens evaluated by RT-PCR-SSCP were found to have mobility shifts. Out of 12 specimens identified by direct DNA sequencing, 8 showed missense mutations, 1 had a large deletion and 1 had a silent mutation; 2 (cases 6 and 8) could not be confirmed by direct sequencing. The enzyme *HhaI* was used

| Case | RT-PCR-SSCP | DNA sequencing                   | RFLP(b) |
|------|-------------|---------------------------------|---------|
|      | Fragment(a) | Exon, Codon | Nucleotide change | Amino acid change |         |
| 2    | A +         | 5 151 | CCC→TCC | Pro→Ser | ND(c) |
| 5    | B +         | 6 220 | TAT→TGT | Tyr→Cys | ND     |
| 6    | A +         | 7 249 | CGG→CGG | Silence mutation | ND     |
| 8    | C +         | 8 282 | CGG→TGG | Arg→Trp | ND     |
| 13   | B +         | 7 248 | CGG→CTC | Ala→Leu | +/HhaI |
| 15   | A +         | 5 175 | CGC→CAC | Arg→His | +/HhaI |
| 17   | B +         | 7 248 | CGG→TGG | Arg→Trp | +/MspI |
| 28   | A +         | 5 175 | CGC→CTC | Ala→Leu | +/HhaI |
| 29   | A +         | 5 120–163 | 130 bp deletion | ND     |
| 31   | B +         | 5 234 | TAC→CAC | Tyr→His | ND     |
| 34   | C +         | 8 273 | CGT→TGT | Arg→Cys | ND     |
| 38   | B +         | 7 234 | TAC→CAC | Tyr→His | ND     |

(a) Described in “Materials and Methods.”
(b) RFLP to confirm the point mutations, with *HhaI* or *MspI*.
(c) Not done.

Fig. 1. Immunostaining of p53 and MDM2 in oral carcinogenesis. p53 expression in adjacent normal mucosa (A), dysplasia (B) and tumor lesion (C). MDM2 expression in adjacent normal mucosa (D), dysplasia (E) and tumor (F) lesion. Bar=10 µm.
for cases 15 and 18 to confirm the point mutations
CGC→CAC/CGC→CTC of codon 175, and MspI was
used for case 17 to confirm the mutation CGG→TGG of
codon 248 (Table I).

**p53 and MDM2 protein expression in premalignant
and malignant lesions** In the adjacent normal mucosa,
the expression of p53 or MDM2 was weakly displayed
only in the basal layer (Fig. 1, A and D). Expression of
both extended from the basal layers to the stratum spinosum
layers in dysplastic lesions (Fig. 1, B and E) and spread
throughout the tumor lesions (Fig. 1, C and F). The level
(mean±SD, %) of MDM2 or p53 protein expression
was significantly increased with the histological progres-
sion of multistep carcinogenesis (Fig. 2). In the SCC
lesions, 29 of 38 (76%) and 19 of 38 (50%) specimens
showed overexpression of p53 and MDM2, but no correla-
tion was found between the expression of p53 and MDM2
(r=−0.25, P>0.05). The coexpression of p53 and MDM2
in oral SCC was observed most frequently in dysplastic
lesions (Table II).

**MDM2 protein expression and p53 gene status** The p53
protein expression was significantly associated with p53
gene mutation (Table III). Cases (14/26, 54%) with wild-
type p53 and cases (5/12, 42%) with mutant p53 showed
MDM2 overexpression. The MDM2 protein expression
was not associated with the p53 gene status (Table IV). To
investigate a possible relationship between p53 over-
expression and the accumulation of the MDM2 oncoprotein,
all p53-positive and p53-negative cases were anal-
alyzed for MDM2 expression, and the expression was com-
pared with the p53 gene status (Table V). Among the cases
with wild-type p53, 6 showed a high level (+++++) of
MDM2 expression with a low level (−−−) of p53 expres-
sion; nuclear immunoreactivity of p53 is not associated
with MDM2. In 5 cases with p53 mutation, simultaneous
p53 and MDM2 overexpressions were found (Table V and
Fig. 3).

**MDM2 expression and clinicopathological parameters**
In the wild-type p53 cases, the expression of MDM2 pro-
tein was not significantly associated with differentiation
(P=0.418), tumor stage (P=0.774) or lymph node status
(P=0.107). In contrast, the MDM2 protein expression in

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**Fig. 2.** The levels of MDM2 and p53 protein expression were
significantly increased in carcinogenesis (mean±SD, %). * P<0.05, ** P<0.01 (t test).

**Table II. p53- and MDM2-positive Phenotype in Oral Carcinogenesis**

| Histology | p53+/MDM2+ (%) |
|-----------|---------------|
| Normal    | 5/21 (24)     |
| Dysplasia | 12/26 (46)*   |
| SCC       | 13/38 (37)*   |

a) Dysplasia vs. Normal, $P=0.0046$, $\chi^2$ test.
b) SCC vs. Normal, $P=0.06$, $\chi^2$ test.

**Table III. Expression of p53 Protein and p53 Gene Status**

| p53 gene | p53 expression | p53 expression | p* |
|-----------|----------------|----------------|-----|
| (n=38)    | −              | +              | ++  | +++ | <0.05 |
| Wild-type p53  | 9              | 5              | 6   | 6   |       |
| Mutant p53   | 0              | 0              | 3   | 9   |       |

* $P=0.0007$, $\chi^2$ test.

**Table IV. Expression of MDM2 Protein and p53 Gene Status**

| p53 gene | MDM2 expression | p53 expression | p* |
|-----------|-----------------|----------------|-----|
| (n=38)    | −               | +              | ++  | +++ | >0.05 |
| Wild-type p53  | 13             | 3              | 5   | 5   |       |
| Mutant p53   | 7              | 0              | 4   | 1   |       |

* $P=0.42$, $\chi^2$ test.

**Table V. Phenotypes of p53 and MDM2 Protein Expression in SCC**

| Patterns | Wild-type p53 (n=26) | Mutant p53 (n=12) |
|----------|----------------------|--------------------|
| p53+/MDM2+ | 8                    | 5                  |
| p53+/MDM2− | 9                    | 7                  |
| p53−/MDM2+ | 6                    | 0                  |
| p53−/MDM2− | 3                    | 0                  |
mutant p53 cases was significantly associated with low differentiation and late tumor stage (III–IV), but no correlation was found with the lymph node status (Table VI).

In addition, p53 gene status was not significantly associated with these clinicopathological parameters (data not shown).

**DISCUSSION**

In this study, increment in the expression levels of p53 and MDM2 protein was found to be related to histological progression of oral carcinogenesis. The highest rate of p53 and MDM2 coexpression was found in dysplastic lesions.

MDM2 expression by reactive nontumor cells has been reported in lymphoid tissue. DNA damage induces nuclear accumulation of p53 and a transient inhibition of DNA synthesis via both G1 and G2 arrests. Meanwhile, overexpression of wild-type p53 can induce MDM2 accumulation. Therefore, the coexpression of p53 and MDM2 in normal cells of the oral mucosa might be a consequence of an early cellular response to mutagen exposure and DNA damage. The coexpression of MDM2 and p53 protein without p53 mutation suggests that functional inactivation of p53 by MDM2 is an important pathway in oral carcinogenesis.

On the other hand, a high level of MDM2 expression with a low level of p53 expression was found in 6 wild-type p53 cases. This could be due to other mechanisms, such as chromosomal translocation or mutation, which could increase the level of the MDM2 protein. An in vitro study has recently shown that MDM2 can also be induced via a p53-independent pathway. No association was found between the expressions of p53 and MDM2 in colorectal and bronchial neoplasms. In this study, it was found that the MDM2 overexpression was not significantly associated with either p53 expression or the absence of p53 gene mutation in oral SCC. These findings suggest that p53-independent mechanisms may also be important in oral SCC, and that MDM2 expression may not be specifically linked to the functional status of p53.

To explain the absence of MDM2 expression in our study, the following possibility might be considered. First, p53 interacts with some viral antigen that might alter the rate of the p53 protein degradation or p53 mutation affects the binding sites, rendering MDM2 products inactive. Second, multiple forms of MDM2 proteins may express different combinations of MDM2 epitopes such that the monoclonal antibody used here might fail to detect the MDM2 protein.

The coexpression of p53 and MDM2 protein was also found in the mutant p53 cases. This is consistent with the report that not only do some mutant p53 products retain several functions, but they also acquire or ‘gain’ specific properties, such as a selective growth advantage and trans-activation of certain genes that are not affected by wild-type p53.

![Image](image-url)

**Table VI. Expression of MDM2 Protein and p53 Gene Status Associated with Clinicopathological Parameters**

| Clinicopathological parameters | MDM2 expression | Wild-type p53 (%) | Mutant p53 (%) |
|-------------------------------|-----------------|-------------------|---------------|
|                               | (n=26)          | (n=12)            |               |
| Differentiation               |                 |                   |               |
| Well                          | 6/14 (43)       | 2/6 (33)          |               |
| Moderate                      | 7/10 (70)       | 0/4 (0)           |               |
| Poorly                        | 1/2 (50)        | 2/2 (100)<sup>a</sup> |               |
| Stage                         |                 |                   |               |
| I–II                         | 5/9 (56)        | 0/5 (0)           |               |
| III–IV                       | 9/17 (47)       | 5/7 (71)<sup>b</sup> |               |
| LN status                     |                 |                   |               |
| N(−)                         | 9/12 (75)       | 0/5 (0)           |               |
| N(+)                         | 5/14 (36)       | 3/7 (43)          |               |

<sup>a</sup> Well vs. Moderate vs. Poorly, P=0.0497, Fisher’s exact test.

<sup>b</sup> I–II vs. III–IV, P=0.026, χ² test.

Fig. 3. The nuclear immunoreactivity of p53 (A) and MDM2 (B) in a case with mutant p53. Bar=5 µm.
In this study, the tumors that contained both mutant p53 and MDM2 overexpression tended to show low differentiation and to be in a late stage (III or IV). These findings indicate that both events could promote the progression of oral SCC.

In general, functional inactivation of the p53 gene by MDM2 overexpression may be an early event of carcinogenesis, and corresponding alterations of MDM2 and p53 could contribute to oral carcinogenesis. Therefore, evaluation of MDM2 overexpression in combination with the p53 gene status should have more clinical value than the evaluation of either MDM2 or p53 alteration alone.

ACKNOWLEDGMENTS

We thank Associate Professor Oda Toshiaki, Associate Professor Uezato Tadayoshi, Research Associate Obayashi Masaharu, Research Associate Yamaguchi Kazue, and Wei Li for their technical assistance. We also thank Research Associate Shou-zhi Gu for the statistical analysis. This work was supported in part by Grants-in-Aid for Scientific Research (No. 08771853 and No. 09771751) to Takashi Koyama from the Ministry of Education, Science, Sports and Culture, Japan.

(Received October 8, 1999/Revised November 26, 1999/2nd Revised February 4, 2000/Accepted February 19, 2000)

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