

Original Article

p53 and MDM2 co-expression in tobacco and betel chewing-associated oral squamous cell carcinomas

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Oral cancers of tobacco and betel chewers represents a unique in-vivo model to understand the genotoxic effect of tobacco and betel carcinogens on oncogenes and tumor suppressor genes. Coordinated interactions of p53 and MDM2 play an important role in regulation of critical growth control gene following exposure to DNA damaging agents. The purpose of this study is to determine if the tumor suppressor function of p53 is inactivated by mutation or other alternative mechanisms in carcinogen-induced oral squamous cell carcinoma (SCC), and to investigate the clinicopathological significance of p53 and MDM2 expression. The p53 mutation in oral SCC of tobacco and betel chewers (n=40) was detected by polymerase chain reaction - single strand conformation polymorphism (PCR-SSCP) analysis and immunohistochemistry (IHC) was done to investigate p53 and MDM2 proteins overexpression. The incidence of p53 mutation was relatively low (17.5%), but there was a high prevalence of MDM2 overexpression (72.5%). In the total of 40 cases, IHC phenotype showed p53 positive immunostaining with MDM2 positive im-

munostaining (p53+/MDM2+) 62.5%, p53 negative immunostaining with MDM2 negative immunostaining (p53-/MDM2-) 15%, p53 positive immunostaining with MDM2 negative immunostaining (p53+/MDM2-) 12.5%, and p53 negative immunostaining with MDM2 positive immunostaining (p53-/MDM2+) 10%. A significant correlation was found between MDM2 and p53 overexpression ($p=0.0289$). Moreover, p53+/MDM2+ phenotype was significantly associated with poorly differentiated tumors ($p=0.0007$). These results conclude that other factors than p53 mutation is likely to be the targets of tobacco/betel carcinogens and MDM2 may play an important role in tobacco/betel chewing-related oral SCCs. Overexpression of MDM2 protein may constitute an alternative mechanism for p53 inactivation.

Key words: p53; MDM2; tobacco/betel chewing; oral squamous cell carcinoma

Introduction

Epidemiological evidence unequivocally confirms a causal association between the tobacco and betel chewing and the incidence of oral malignancies¹. Oral squamous cell carcinoma (SCC) is the most common

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malignant tumor in the oral cavity². Oral cancers of tobacco and betel chewers represent a unique in-vivo model to understand the genotoxic effect of tobacco and betel carcinogens on candidate oncogenes and tumor suppressor genes in the multi-step process of carcinogen-induced tumorigenesis.

Mutation of p53 gene, one of the tumor suppressor genes, is the most common inactivation mechanism of p53 tumor suppressor function. However, the incidence of p53 mutation in tobacco and betel-related oral cancer is relatively low^{3,4}. The other alternative mechanism of p53 inactivation includes interaction with viral oncoproteins (adenovirus E1B, HPV E6 and SV40) or other p53-binding cellular proteins like MDM2. It is interesting to find out whether tumor suppressor function of p53 is abrogated by other alternative mechanisms in carcinogen-induced oral SCC.

MDM2 (mouse double minute 2) was originally identified as a highly amplified gene present on double minutes in a spontaneously transformed tumorigenic derivative of a Balb/c cell line called 3T3DM⁵. It is a 90-kd cellular phosphoprotein (p90) capable of forming a complex with p53 protein, thus inhibiting its transcriptional activation function⁶ although MDM2 is a target protein of p53 and is up-regulated by transactivational function of p53. MDM2/p53 forms an auto-regulatory feedback loop that may be needed to maintain a fine balance between MDM2 and p53 proteins within the cell. The co-ordinated interactions of these genes have been proposed to play an important role in transcriptional regulation of a set of critical growth control genes and in mediating cell cycle progression following exposure to DNA damaging agents^{7,8}.

MDM2 acts as a ubiquitin E3 ligase which binds to the p53 transactivation domain and transports p53 to the cytoplasm where p53 is subsequently ubiquitinated and degraded in cytoplasmic proteasome⁹.

In the present study, we investigated the inactivation of p53 through two different mechanisms in tobacco and betel-related oral SCC from Myanmar as no molecular study on p53 inactivation has been conducted. The alterations of p53 gene in tobacco and betel-related oral SCC specimens were investigated by single strand conformation polymorphism (SSCP) analysis and secondly over-expression of both p53 and MDM2 proteins was analysed by immunohistochemistry (IHC) in these same specimens. Furthermore, we investigated the correlation between over-expression of p53 and MDM2 and clinicopathological features of these patients and found that over-expression of p53 and MDM2 was significantly correlated only with

pathological differentiation.

Materials & Methods

Patients & tumor samples

Forty specimens of oral SCCs were collected from patients at the Institute of Dental Medicine, Yangon, Myanmar. Patients with the chewing habit of tobacco and betel for more than 3 years were chosen for this study to observe the long-term effect of tobacco and betel carcinogens. None of these patients received chemotherapy or radiotherapy prior to tumor resection. These tumor tissues were processed into formalin-fixed paraffin-embedded specimens. The informed consent was taken from the patients involved in this study.

DNA preparation

DNA was isolated from formalin-fixed paraffin-embedded tumor tissue by proteinase K digestion, phenol/chloroform extraction and ethanol precipitation¹⁰.

PCR primers & amplification

We analysed p53 mutations in exons 5-8 as 87% of mutations were reported in these exons¹¹. Exons 5-8 of the p53 gene were amplified by PCR and radioactively labeled with ³²P-dCTP (ICN Biomedicals Inc., CA, USA, specific activity 3 TBq/mmol). The sequences of primers and the condition of PCR amplification were as described previously¹².

SSCP analysis

SSCP analysis of radioactively labeled PCR products was done by electrophoresis of denatured DNA in 6% non-denaturing polyacrylamide gel containing 5% glycerol, the running time was 3.5-4 h at 25°C with a constant power of 40 W. Gel was transferred to filter paper and bands were detected by exposing image plate and analysed by BAS 2000 (Fuji Film Co., Tokyo, Japan)^{13,14}.

Direct sequencing

Abnormal bands detected by SSCP were excised and DNA sequence of purified PCR product was investigated by using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA). The gel electrophoresis, data collection and analysis were performed with an Applied Biosystem model 373A automated sequencer.

Immunohistochemical staining

Immunohistochemical staining was performed according to the strept-avidin-biotin peroxidase complex (ABC) method¹⁵. Briefly, 4 μ m sections from paraffin-embedded specimens were deparaffinized with xylene and rehydrated with graded ethanol. Antigen retrieval was done by incubating the sections in 10 mM sodium citrate buffer (pH 6.0) for 10 min in a conventional microwave-oven. The endogenous peroxidase activity was blocked by immersing the sections in methanol with 0.3 % hydrogen peroxide. After blocking the non-specific reactivity, the sections were incubated with mouse monoclonal anti-p53 antibody (DO7, Dako, Glostrup, Denmark; dilution 1:100) and mouse monoclonal anti-MDM2 antibody (SMP 14, Santa Cruz, CA USA; dilution 1:100) overnight at 4°C, followed by incubation with a biotinylated anti-mouse secondary antibody and streptoavidin-biotin-peroxidase complex (Labeled Streptavidin Biotin kit, Dako, Glostrup, Denmark). The peroxidase reaction was developed using diaminobenzidine (DAB) as a chromogen. The sections were counter-stained with 1% methyl green. For negative controls, primary antibody was replaced with 0.05 M Tris-HCl buffer solution (pH 7.6).

The p53 and MDM2 positive cases were semi-quantitatively evaluated on a 4 point scale based on the percentage of cells staining p53 or MDM2: – = <10%; + = 10-30%; ++ = 30-50%; +++ = >50%.

Statistical Analysis

The correlation between the expression of p53/MDM2 and the clinicopathological features was analysed by using the Chi-square method and Fisher-exact test. A probability value of <0.05 was considered significant.

Results

p53 gene mutation

Seven of 40 specimens (17.5%) showed p53 gene mutation in exons 5-8. Three cases were missense mutations and 4 cases silent mutations through codons 141-279 (Table 1).

p53 protein overexpression

The p53-immunopositive cases mostly showed intense nuclear staining in tumor cells. The intensity of p53 immunoreactivity was shown in Table 2. In the total of 40 cases, 30 cases (75%) showed p53 immunos-

taining positive. Out of 30 positive cases, 25 cases (83.3%) co-expressed with MDM2 protein. All missense mutation cases showed positive immunostaining (Table 3).

MDM2 protein overexpression

Immunoreactivity of MDM2 was seen predominantly nuclear with some degree of faint cytoplasmic staining. Twenty-five out of 29 MDM2 positive immunostaining cases (86.2%) showed p53 positive immunostaining with MDM2 positive immunostaining (p53+/MDM2+ phenotype) (Table 3). MDM2 overexpression was not found in p53 missense mutation cases suggesting that these mutations abrogated the transactivational function of p53.

Co-expression of p53 and MDM2 proteins

Twenty-five of 40 cases (62.5%) showed concordant overexpression of both MDM2 and p53 proteins. Localization of co-expressed p53 and MDM2 was shown in Table 4. Most of these 25 cases showed predominantly nuclear (Fig. 1) and a few are both nuclear and cytoplasmic.

Co-expression of p53 & MDM2 and clinicopathological parameters

In 19 cases of T₁-T₂, 10 cases (52.6%) showed p53+/MDM2+ phenotype whereas 15 cases (71.4%) were p53+/MDM2+ in the total 21 cases of T3-T4. In 18 cases with lymph node metastasis, 14 cases (77.8%) were p53+/MDM2+ phenotype and 4 cases (22.2%) showed lack of co-expression. In poorly differentiated cases, 19 cases (86.4%) were p53+/MDM2+ phenotype (Table 5).

Statistical analysis revealed that co-expression of p53 & MDM2 is not significantly associated with tumor size ($p=0.3685$) and lymphnode metastasis ($p=0.1396$), but this phenotype was significantly associated with poorly differentiated tumors ($p=0.0007$) (Table 5 & Fig. 2).

Discussion

Previous studies have reported that incidence of p53 mutation was 49-66% in oral SCCs from Europe¹⁶, USA¹⁷ and Japan¹². However, incidence of p53 mutation was 21% in tobacco/betel chewing-associated oral SCCs in two studies from India^{4,18} and one study from Taiwan¹⁹. Similarly p53 mutation was less frequent (17%) in betel quid chewers from Kerala, south India²⁰

Table 1. p53 gene mutations in 40 cases of tobacco & betel chewing-associated oral SCCs

No.	Exon	Codon No.	Mutated Codon	A/A Change	Base Change	Type
1	5	141	CCT → CCG	Pro → Pro	T → G	Silent
2	5	147	GAT → GAC	Asp → Asp	T → C	Silent
3	5	175	CGC → CAC	Arg → His	G → A	Missense
4	5	180	GAG → GAA	Glu → Glu	G → A	Silent
5	6	213	CGA → CGG	Arg → Arg	A → G	Silent
6	6	215	AGT → GGT	Ser → Gly	A → G	Missense
7	8	279	GGG → GAG	Gly → Glu	G → A	Missense

Table 2. Intensity of p53 & MDM2 immunoreactivity in tobacco & betel-related oral SCCs

Proteins	Degree of overexpression				Total
	-	+	++	+++	
MDM2	11 (27.5%)	10 (25%)	12 (30%)	7 (17.5%)	40
p53	10 (25%)	7 (17.5%)	11 (27.5%)	12 (30%)	40

Table 3. Phenotypes of p53/MDM2 protein overexpression and p53 gene status

Phenotype*	Wild-type p53	Mutant p53	Total (n=40)
	(n=37)**	(excluding silent mutation) (n=3)	
p53+ / MDM2+	25 (67.6%)	0	25 (62.5%)
p53+ / MDM2-	2 (5.4%)	3 (100%)	5 (12.5%)
p53- / MDM2+	4 (10.8%)	0	4 (10%)
p53- / MDM2-	6 (16.2%)	0	6 (15%)

+ = Positive immunostaining ; - = Negative immunostaining

* significant correlation between p53 & MDM2 overexpression was found ($p=0.0289$)

**4 cases of silent mutations were included

and the same frequency was reported in tobacco chewing-related oral cancers from the western part of India²¹. Less frequent mutation (10%) was also report-

ed from Papua-New Guinea³ and 5.4% in another study from Taiwan²².

The frequency of p53 mutation was 17.5% in this

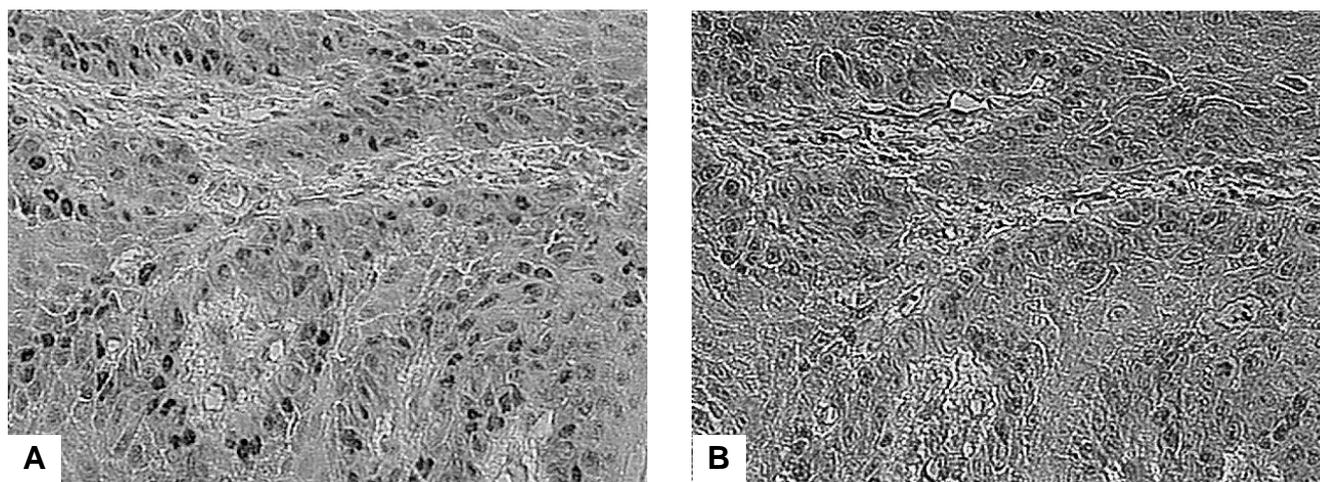


Fig. 1. The nuclear immunoreactivity of p53 (A) and MDM2 (B) in the same specimen.

Table 4. Subcellular localization of co-expressed p53 & MDM2 in p53+ / MDM+ phenotype

Proteins	subcellular localization			Total
	Nucleus	Cytoplasm	Nucleus+Cytoplasm	
p53	22 (88%)	1 (4%)	2 (8%)	25
MDM2	16 (64%)	3 (12%)	6 (24%)	25

Table 5. Relationship between co-expression of p53 & MDM2 and clinicopathological Parameters

Clinicopathological parameters	Co-expression of p53 & MDM2 (n=25)	Lack of co-expression (n=15)	Total
Tumor size ^(a)			
T ₁ -T ₂	10 (52.6%)	9 (47.4%)	19
T ₃ -T ₄	15 (71.4%)	6 (28.6%)	21
L/N metastasis ^(b)			
N (-)	11 (50%)	11(50%)	22
N (+)	14 (77.8%)	4 (22.2%)	18
Differentiation ^(c)			
well	2 (18.2%)	9 (81.8%)	11
moderate	4 (57.1%)	3 (42.9%)	7
poor	19 (86.4%)	3 (13.6%)	22

(a) $p = 0.3685$, (b) $p = 0.1396$, (c) $p = 0.0007$

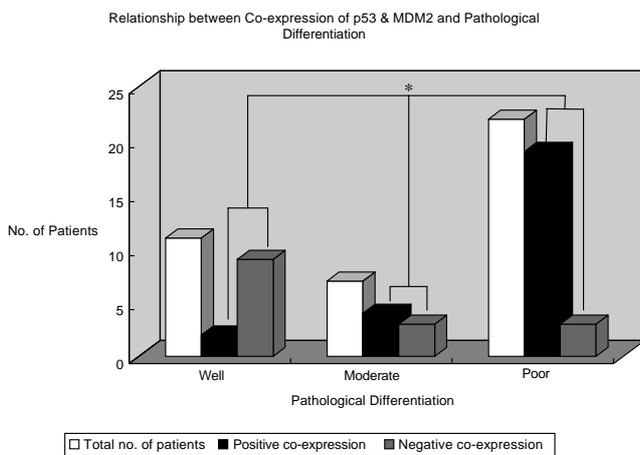


Fig. 2. Relationship between co-expression of p53 & MDM2 and pathological differentiation of tumor (* $p = 0.0007$).

study although about half of the mutations were not accompanied with amino acid substitution. This low incidence of p53 mutation in tobacco and betel chewing-associated oral SCCs is consistent with the previous findings from other countries. The different composition of tobacco/betel quid and the chewing style and pattern may be responsible for some variation in the incidence of p53 mutation. The low frequency of p53 mutation in tobacco and betel-related oral SCCs indicated that other factors than p53 mutation may be targets of tobacco/betel carcinogens.

We, thus, examined the overexpression of MDM2 as well as p53 combined with p53 gene mutation. Twenty-nine of 40 cases (72.5%) were positive for MDM2 staining. This result is comparable with those obtained from India²³ in which MDM2 overexpression was detected in 78% (51/65) and those from Taiwan²⁴ in which it was 69% (26/38). High prevalence of MDM2 overexpression indicates that MDM2 may play an important role in carcinogenesis of tobacco and betel chewing-related oral SCCs.

In this study, high frequency of MDM2 overexpression was frequently associated with p53 protein overexpression and low p53 mutation rate. Co-expression of wild-type p53 and MDM2 proteins has been reported in melanoma²⁵, bladder cancer²⁶ and bronchiogenic carcinoma²⁷. We found that p53 was co-expressed with MDM2 in 25 of 40 cases (62.5%). A statistically significant correlation between p53 and MDM2 overexpression was found in tobacco/betel chewing-related oral SCCs ($p=0.0289$). In contrast, there was no significant correlation between expression of p53 and MDM2 in oral SCCs which were not associated with

tobacco/betel chewing and have relatively high p53 mutation rate²⁸.

We found that immunoreactivity of both p53 and MDM2 was within nucleus in the majority of cases with p53/MDM2 co-expression. A well-documented model for MDM2-mediated regulation of p53 is that MDM2 binds to the p53 transactivation domain and p53 is transported to cytoplasm, ubiquitinated and degraded in cytoplasmic proteasomes. However, it is also known that other regulating-proteins like $p14^{ARF}$ prevents MDM2 nucleocytoplasmic shuttling and thus inhibits MDM2-directed export of p53 to the cytoplasm. Subsequently $p14^{ARF}$ stabilizes p53 by attenuating MDM2-mediated degradation. The underlying mechanism may be lack of $p14^{ARF}$ epigenetic silencing, that is, absence of $p14^{ARF}$ promoter hypermethylation is associated with tumors showing exclusive nuclear MDM2 staining. However, it has been reported that aberrant methylation of $p14^{ARF}$ promoter was a relatively common epigenetic event in colorectal, gastric, renal, esophageal, and endometrial neoplasms and gliomas²⁹. It is, thus, interesting to find out the role of $p14^{ARF}$ in the interaction with p53/MDM2 in tobacco/betel chewing-associated oral SCCs. The functional role of p53 which escaped from MDM2-mediated degradation and its fate also remain a puzzling question. High frequency of MDM2 overexpression with low p53 mutation rate corroborate the previous reports that MDM2 amplification and p53 genomic mutations may be mutually exclusive^{30,31,32}.

The relationship between p53/MDM2 co-expression and clinico-pathological parameters was evaluated for clinical implication. Among these parameters such as tumor size, lymphnode metastasis and pathological differentiation, p53/MDM2 co-expression was significantly related only to poorly differentiated phenotype of tumors ($p=0.0007$). Although molecular basis for this relationship is not so clear, p53/MDM2 co-expression may represent an indicator for more aggressive behaviour of tumor indirectly resulting in poor prognosis.

In conclusion, p53 tumor suppressor gene was more profoundly affected by its negative regulator, MDM2, rather than mutation itself in the carcinogenesis of tobacco/betel chewing-associated oral SCCs. The present study also proved that MDM2 protein overexpression was significantly associated with p53 overexpression, as a contradiction to no significant association in non-tobacco/betel-related oral SCCs. All these findings suggest that overexpression of MDM2 protein may constitute an alternative mechanism for p53 pro-

tein dysfunction and other regulating protein(s) are likely to be involved in the p53/MDM2 interaction in tobacco/betel chewing-associated oral SCCs.

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