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ARTICLE

Alterations in MDM2 Expression in Esophageal Squamous Cell Carcinoma: Relationship with p53 Status*

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In view of the significance of MDM2 as a regulator as well as critical target of wild type p53, this study was undertaken to determine the alteration in MDM2 expression in esophageal squamons cell carcinoma (ESCC) and its relationship to clinicopathological parameters as well as p53 gene and protein status. Immunohistochemical analysis of MDM2 and p53 proteins on paraffin embedded sections from 64 surgically resected ESCCs and matched histologically normal tissues showed overexpression of MDM2 protein in 23/64 (36%) ESCCs, while the histopathologically normal esophageal tissues did not show detectable level of MDM2 immunoreactivity. Interestingly, $MDM2^-/p53^+$ phenotype was observed in 37/64 (58%) cases. None of the cases with p53 missense mutations (12/30, 40%) showed detectable level of MDM2 protein. Missense p53 mutations were significantly associated with discordant $p53^+/MDM2^-$ immunophenotype (p= 0.004). The most intriguing feature of the study was accumulation of MDM2 in the absence of detectable p53 in 11% of and overexpression of MDM2 and p53 in 25% of ESCCs, suggesting a p53-independent role for MDM2 in a subset of tumors. These results underscore the involvement of MDM2 in p53-dependent and -independent pathways in the pathogenesis of esophageal cancer in the Indian population. (Pathology Oncology Research Vol 7, No 3, 203–208, 2001)

Keywords: MDM2, p53, mutation, oncogene, esophageal squamous cell carcinoma

Introduction

Esophageal squamous cell carcinoma (ESCC) is the eighth most common cancer worldwide, characterized by poor survival and wide geographical variation in incidence.[1-5] The molecular pathology underlying the development and progression of ESCC is poorly understood. Tobacco smoke and alcohol are major risk factors for cancer of the esophagus in Western countries. In addition, high consumption of sun dried and pickled vegetables, red chillies, spices and chewable tobacco have also been identified as important risk factors in India. Thus, chronic exposure to these dietary risk factors confounded by poor nutritional status results in insidious symptomatology and late clinical presentation of ESCC in the Indian population. Investigation of alterations in oncogenes and tumor suppressor genes implicated in esophageal tumorigenesis may lead to the identification of molecular markers for early diagnosis, therapeutic intervention and improved quality of life.

Perturbations in the expression of MDM2 oncogene are increasingly being implicated in the pathogenesis of human neoplastic diseases.[9,19] The MDM2 oncogene was first identified as an amplified gene in a spontaneously transformed mouse 3T3 DM cell line.[2] Overexpression of MDM2 in immortalized mouse cells was shown to confer tumorigenicity in nude mice.[5] The MDM2 oncoprotein is a potent inhibitor of p53. The transforming capacity of MDM2 has been attributed to its ability to abrogate transcription by binding to the transcriptional activation domain of p53 and blocking its ability to regulate target

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Abbreviations: ESCC: esophageal squamous cell carcinoma; MDM2: murine double minute 2; mAb: monoclonal antibody; SSCP: single strand conformation polymorphism

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genes and exert antiproliferative effects. The interaction of MDM2 with the tumor suppressor $p19^{ARF}/p14^{ARF}$ results in activation of p53. The p53 protein regulates the MDM2 gene at the level of transcription by an intronic promoter. This creates an auto-regulatory feed back loop. The interval between p53 activation and consequent MDM2 accumulation defines a time window during which p53 exerts its effects.[26] Sequential transfer of the wild-type p53 and E2F-1 genes induced apoptosis in human esophageal cancer cells by involvement of p53 accumulation via ARF-mediated MDM2 down-regulation.[11]

We showed that p53 alterations are frequent events in esophageal oncogenesis in the Indian population.[8,22] However, the alterations in expression of MDM2 protein in esophageal cancer in the Indian subcontinent remain to be determined. The present study was undertaken to analyze the expression of MDM2 protein in ESCCs and determine its relationship with clinicopathological parameters as well as p53 gene and protein status.

Materials and Methods

Tissue Specimens

Sixty four esophageal cancer cases enrolled in the Surgical Oncology Unit, Institute Rotary Cancer Hospital, All India Institute of Medical Sciences, India, were inducted into the study with prior consent. A structured questionnaire was used to collect information on various demographic, socio-economic, occupational, nutritional and life style variables. Surgical specimens from untreated primary esophageal tumors (64 cases), and matched histopathologically normal esophageal tissue specimens taken from a site distant from the cancerous lesion were formalin fixed, paraffin embedded. Histopathologically confirmed ESCCs and matched normal esophageal tissue sections with no contaminating tumor cells as determined by hematoxylin and eosin staining, were used for immunohistochemical analysis. Each patient's clinical status was classified according to the clinical tumor, node, metastasis (pTNM) classification system. The patients were grouped based on the tumor stage (pT_1/pT_2 cases; pT_3/pT_4 cases), Nodal metastasis (pN_0 cases; pN_1 cases), and distant organ metastasis (pM_0 cases; pM_1 cases).

Immunohistochemistry

Paraffin embedded sections (5 μ thickness) of human esophageal tissue specimens were collected on gelatin coated slides and fixed in acetone for 10 min. For histopathological analysis, the representative sections were stained with hematoxylin and eosin, whereas immunostaining was done on serial sections as described by us previously.[8] Briefly, the sections were deparaffinized and pretreated in a microwave oven (20 min, 120 W; 3x5min, 450 W) in citrate buffer [0.01M (pH6)]. The sections incubated sequentially in methanol containing hydrogen peroxide (0.3%, v/v) for 20 min to quench the endogenous peroxidase activity, non-specific binding was blocked with 1% (w/v) BSA in PBS for 1 hr and primary antibody (1 μg/ml) SMP-14 (anti-mdm-2, mAb recognizes MDM2 protein epitope 154-167 amino acids) for or DO-1 (anti-p53, mAb recognizes both wild type and mutant 1053 protein (Santa Cruz Biotechnology, CA) 16 hrs at 4°C and washed with PBS. The primary antibody was detected using biotinylated secondary antibody and avidin-biotin complex using the Vectastain Elite kit (Vector Laboratories) and diaminobenzidine as chromogen. Incubations were performed at room temperature in a moist chamber. Slides were washed several times with 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 oral squamous cell carcinoma tissue sections known to overexpress MDM2 were used as the positive control (data not shown). The sections were counterstained with hematoxylin. The immunostained slides were graded by three of us (SA, MM and RR) independently. The results were evaluated semiquantiatively and graded on a four point scale on the basis of percentage of cells showing p53 or MDM2 staining as follows: -ve, no detectable immunostaining was observed in cells; +1, <10%; +2; 10-30%; +3, 30-50%; or +4, >50% of tumors cells were stained. The intensity of staining was graded on the basis of color as intense, moderate, mild, or poor. The pattern of expression was described as focal, if localized wide clusters of positive cells were seen in some areas of the epithelium; diffused, if positive cells were found distributed throughout most areas of the lesions and scattered, if only a few isolated positive cells were identified in some regions of the epithelium.

Statistical Analysis

The association of MDM2 accumulation with p53 protein expression, p53 mutations and clinicopathological features was determined using MicroStat software by χ^2 and Fisher's Exact test. A p value less than 0.05 was considered significant. All the p values reported were two-tailed.

Results

Immunohistochemical analysis of MDM2 protein expression

The results of immunohistochemical analysis of MDM2 expression in 64 cases of untreated primary ESCCs, paired normal tissues and their correlation with clinicopathological parameters are summarized in *Table 1.* Overexpression of the MDM2 protein was observed in 23 of 64 (36%) ESCCs.

Immunoreactivity was predominantly localized to the nucleus with faint cytoplasmic staining (*Figure 1*). In few cases staining was observed in the cytoplasm and plasma membrane (*Figure 2*). The histopathologically normal tissue specimens obtained from sites distant from the site of the lesion did not show detectable MDM2 immunoreactivity. The pattern of MDM2 expression in ESCCs showed wide heterogeneity. Of the 23 cases expressing MDM2 protein, 3 showed intensely focal pattern, 6 showed intensely diffused pattern, 1 case showed moderately focal attern, 5 cases showed moderately diffused pattern, 5 cases showed mild scattered and 3 cases showed poorly scattered pattern of nuclear staining. However, a large number of cases (41/64, 64%) did not show detectable level of MDM2 protein. MDM2 expression was significantly associated with site of the tumor; with increased positivity in tumors in upper and middle one-third of the esophagus (p=0.005). No significant correlation was observed between MDM2 expression and age, gender, histopathological grade or TNM stage of tumors in this cohort.

Table 1. **Relationship of MDM2 protein expression with clinicopathological parameters, p53 protein expression and MDM2 mRNA expression in ESCCs**

Group	*Total cases*	*MDM2 expression*		*p value*[a]
		Positive (%) n	*Negative (%) n*	
Normal	64	6 (9)	58 (91)	(0.0007)
ESCCs	64	23 (36)	41 (64)	
Age (years)				
<40	8	4 (50)	4 (50)	NS
>40	56	19 (34)	37 (66)	
Gender				
Male	37	12 (32)	25 (68)	NS
Female	27	11 (40)	16 (60)	
Histopathological grade				
Well	31	11 (35)	20 (65)	Well+Moderate
Moderate	23	7 (31)	16 (69)	vs. Poor
Poor	10	5 (50)	5 (50)	NS
Tumor stage (n=61)[b]				
T_1/T_2	15	5 (33)	10 (67)	NS
T_3/T_4	46	16 (35)	30 (65)	
Nodal metastasis				
N_0	35	9 (26)	26 (74)	NS
N_1	26	12 (46)	14 (54)	
Distant organ metastasis				
M_0	50	17 (34)	33 (66)	
M_1	11	4 (36)	7 (64)	NS
Site				
U 1/3 +M 1/3	27	15 (56)	12 (44)	
L 1/3	37	8 (22)	29 (78)	(0.005)
p53 expression status				
Positive	53	16 (30)	37 (70)	(0.04)
Negative	11	7 (64)	4 (36)	
MDM2 mRNA expression (n=19)				
Basal expression	16	3 (19)	13 (81)	(0.02)
Overexpression	3	3	0	

* p value by Chi Square test b TNM stage could not be ascertained for 3 tumors.

MDM2 mRNA analysis was carried out by *in situ* hybridization in a subset of ESCCs and normal tissues (in which the tissues were available). Of the 19 ESCCs examined, increased level of MDM2 mRNA transcripts was observed in 3 cases which also showed mdm-2 protein accumulation, while basal level of MDM2 mRNA transcripts was observed in 16 ESCCs, as well as in all the 19 histologically normal esophageal tissues examined (data not shown). There was a significant association between MDM2 protein and mRNA expression (p=0.02) (*Table 1*). These results suggest that enhanced transcription may account for overexpression of MDM2 protein in a subset of ESCCs. Analysis of MDM2 gene status in these 19 ESCCs (wherein adequate tissue specimens were available for Southern hybridization) did not show MDM2 gene amplification in this cohort of tumors (data not shown).

Relationship between MDM2 protein expression, p53 protein level and p53 gene alterations

Correlation of MDM2 and p53 protein expression revealed four groups (A-D) as follows: Discordant MDM2 positive/p53 negative expression (A; $MDM2^+/p53^-$) was observed in 7 of 64 (11%) ESCCs. Concordant nuclear

accumulation of MDM2 and p53 (B; MDM2$^+$/p53$^+$ phenotype) was observed in 16 of 64 (25%) cases. Patients with no detectable MDM2 expression and p53 overexpression (C; MDM2 /p53$^+$ phenotype) emerged to be the major group 37/64 (58%) cases. A significant association was observed between MDM2 negative phenotype and p53 overexpression (p=0.04), thus emphasizing a reciprocal relationship between these proteins in ESCCs. Among these 37 ESCCs showing MDM2$^-$/p53^1 phenotype, there were 17 well differentiated tumors, 15 moderately differentiated and 5 poorly differentiated tumors. Significant association was observed between MDM2$^-$/p53^1 immunophenotype and differentiation status of these tumors (p=0.05). Four of 64 cases (6%) did not show detectable levels of either of the proteins (D; MDM2$^-$/p53$^-$). We have earlier reported the data on p53 protein overexpression and gene mutational analysis by PCR-SSCP[8]. Specific mutations were observed in exons 5-9 of p53 gene by direct DNA sequence analysis.[22] The relationship between MDM2 expression and p53 gene and protein status was determined in 30 ESCCs, wherein the resected tissue specimens were in adequate amount to carry out analysis of all these parameters. Nine of 30 cases showed elevated levels of MDM2, 4 of these harboured frameshift mutations (2 each in exon 6 and 9) in p53 gene (*Table 2*). Twenty one ESCCs did not show detectable MDM2 immunoreactivity, 18 of which harboured p53 mutations within exons 5-9. Twelve of the 18 single base substitutions were missense mutations and all these 12 cases did not show detectable level of MDM2 protein. In this group (dissociate phenotype) the frequency of p53 missense mutations was significantly higher than in the other groups (p=0.004). All the twelve cases harbouring p53 missense mutations exhibited MDM2 /p53$^+$ phenotype.

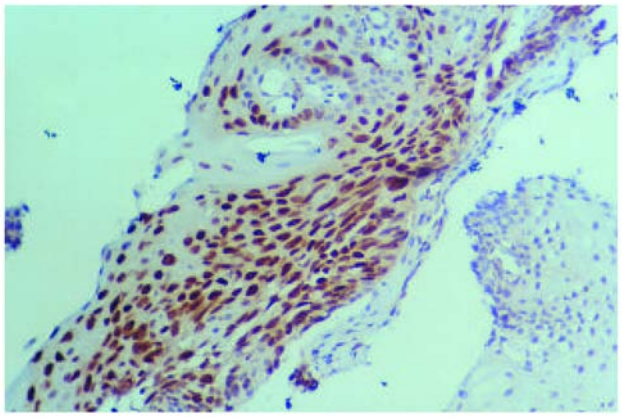

Figure 1. *Moderately differentiated ESCC showing MDM2 positive nuclear staining (x200)*

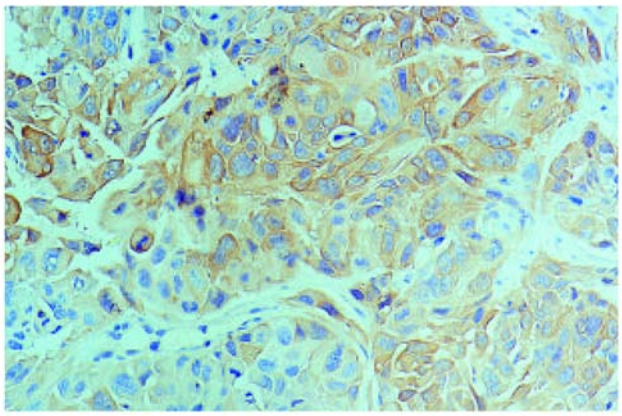

Figure 2. *Moderately differentiated ESCC showing cytoplasmic and membrane staining for MDM2 (x200)*

Table 2. **Correlation of MDM2 expression with p53 gene and protein status**

MDM2 expression (n=30)	*ESCCs*	*p53 mutation** (+)	(–)	*p53 expression* (+)
Positive	9	4**	3	9
Negative	21	18	3	21

*Ralhan et al: Int J Cancer 85: 791-795, 2000.
**Frameshift mutations

Discussion

Herein, we present the first account of MDM2 expression in ESCC and its reciprocal relationship to p53 status in the Indian population, with wide variation in ethnic and etiological risk factors in comparison with the western population.

Overexpression of MDM2 in 7 of the 64 cases (11%) harbouring wt p53 suggests MDM2 transactivation by p53 and a p53-independent oncogenic potential of MDM2 protein in the development of esophageal carcinoma. Overexpression driven by the entire MDM2 gene predisposes to spontaneous tumor formation, revealing a p53-independent role for MDM2 in tumorigenesis.[12] It is well known that overexpression of MDM2 oncogene contributes to tumorigenesis, at least in part by interferring with p53 function. Initial analyses have shown MDM2 amplification in tumors that rarely harboured detectable alterations in p53,[16,20] suggesting that MDM2 overexpression may have a comparable effect as mutation in p53 gene in the cell transformation process. However, very little is known about the physiological functions of MDM2 and it is possible that its oncogenic potential is effected in other mechanistic pathways as well. Interestingly, exogenous MDM2 has recently been shown to increase papilloma formation induced by chemical carcinogenesis and predispose to the appearance of premalignant lesions and squamous cell carcinoma, suggesting that the oncogenic potential of MDM2

in vivo is revealed when it is targeted to cells in which p53 suppresses tumorigenesis.[7] MDM2 overexpression was not observed in 37/64 (58%) of the ESCC cases showing accumulation of p53 protein, suggesting a reciprocal relationship between p53 and MDM2 proteins (p=0.04) in this cohort of population.

Concomitant accumulation of MDM2 and p53 proteins was observed in 16/64 (25%) ESCCs. Intriguing, MDM2/p53 co-expression has been observed in various human tumors including oral cancer[23] and clear cell renal carcinoma[10] and correlated with aggressive tumor behaviour and poor prognosis. It has been suggested that mutation in p53 gene and overexpression of MDM2 ($p53^+$/$MDM2^+$) in the same tumor are biologically redundant, in that both alterations are articulated in the same pathway leading to the same effect, that is the loss of p53 function.[6] However, this argument can be completely valid only if both the proteins are devoid of any off shoot functional pathways affecting the cell cycle regulation independently of the principal pathway.[18] MDM2 mediates cell cycle regulation in more than one way and interacts with various cellular macromolecules in a p53-independent manner. Therefore, this immunophenotype which represents concomitant alteration in two major cell cycle regulatory proteins may be clinically important. The interaction of MDM2 with the tumor suppressor p19ARF/p14 ARF results in activation of p53 by two independent mechanisms.[21] p53 independent overexpression mechanisms of MDM2 have been implicated which include gene amplification and enhanced translation. Therefore, we sought to investigate the possibility of gene amplification playing a causal role in the MDM2 overexpression in a subset of cases where p53 gene was mutated. However, we could not find any evidence of MDM2 gene amplification in ESCCs in this cohort of patients. Many human tumors show MDM2 overexpression without MDM2 gene amplification.[3,17,21] Interestingly, we have recently reported that induction of MDM2-P2 transcripts correlates with stabilized wild type p53 suggesting that enhanced translation of MDM2-P2 transcripts may represent an important mechanism of overexpression and consequent stabilization and functional inactivation of wild type p53 in betel and tobacco related human oral cancer.[21] However, induction of MDM2-P2 transcripts could not be demonstrated in this cohort of ESCCs due to paucity of tissues specimens available for analysis.

Analysis of MDM2 overexpression in this subset of patients in relation to p53 gene status revealed significant association between p53 missense mutations and lack of detectable MDM2 protein expression. Similar association has been previously reported in non-Hodgkin's lymphomas.[25] The absence of detectable MDM2 protein expression in these missense p53 mutant cases can be explained by the fact that missense mutations in the core domain of p53 gene give rise to mutant protein that fails to bind to MDM2 promoter elements and upregulate its transcription.[13,14] Several studies in experimental systems have shown that after DNA damage , mutant p53 lacks transcriptional activity and is unable to activate the expression of proteins such as p21, and MDM2.[1,4] The MDM2 negative phenotype found in this series of ESCCs with p53 mutated gene is indicative of the functional inactivation of p53 protein. This observation is further supported by the pronounced accumulation of MDM2 observable at both protein and mRNA levels in 3 of the 5 cases harbouring wt p53 gene. These results suggest that expression of p53 and p53 regulated protein MDM2 is related to p53 gene status in a subset of patients. Furthermore, existence of only a small number cases (n=4) not showing detectable levels of either p53 or MDM2 protein strengthens the implication of alterations in these genes in esophageal tumorigenesis.

In conclusion, the findings presented suggest p53-independent and -dependent implication of MDM2 in esophageal tumorigenesis. The overexpression of MDM2 may play a role in the progression of tumors by a yet unknown mechanism, in addition to the one indicated by inactivation of p53 protein. However, these different patterns of expression of p53 and MDM2 need to be explored further in terms of clinical behavior of ESCCs to evaluate their diagnostic and prognostic relevance, and are currently under investigation in our laboratory.

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