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ORIGINAL ARTICLE

Prognostic significance of overexpressed matrix metalloproteinase-2, mouse-double minute: 2 homolog and epidermal growth factor receptor in non-small cell lung cancer

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Summary

Purpose: *To evaluate the rate of overexpression of matrix metalloproteinase-2 (MMP2), mouse double minute 2 homolog (MDM2) and epidermal growth factor receptor (EGFR) in patients with non-small cell lung cancer (NSCLC), and evaluate their correlation with clinicopathological parameters and prognosis.*

Methods: *This was a prospective cohort study conducted from 2003 to 2008 among 184 NSCLC patients who underwent tumor resection. Each patient's clinical history and tumor characteristics were obtained from histopathology reports and medical records. EGFR, MDM2 and MMP2 expression were assessed by immunohistochemical (IHC) staining of the tissue specimens.*

Results: *MDM2 overexpression was observed in 70 (38%) of the patients studied, and was significantly higher in younger patients (p=0.01). Only 46 (25%) of patients had overexpression of MMP2. EGFR positive staining occurred in 105 (57%) of the evaluated tumor specimens and was more frequent in specimens with squamous cell carcinoma (p<0.001), the elderly (p<0.001), and in smokers (p<0.001). Independent risk factors for mortality were older age (adjusted odds ratio/aOR 1.3), being a smoker (aOR 10), having stage II disease (aOR 10.8) or stage III/IV disease (aOR 28.3), expression of EGFR (aOR 5.9) and MMP2 (aOR 4.1). However, the expression of MDM2 independently predicted a reduced risk of death (aOR 0.3).*

Conclusion: *Overexpression of MMP2 and EGFR were independent risk factors for mortality in NSCLC patients, while overexpression of MDM2 independently predicted a reduced risk of death.*

Key words: *biomarkers, EGFR, matrix metalloproteinase-2, MDM2, NSCLC, prognosis*

Introduction

Lung cancer is currently the most common human malignancy globally. It accounts for the highest mortality rate in patients with malignant tumors [1]. NSCLC represents over 85% of all lung cancers [2]. More than 60% of NSCLC patients are diagnosed with metastatic or advanced tumors, which precludes surgical resection. Thus, management of NSCLC requires alternative therapeutic approaches besides surgery [3,4]. A considerable therapeutic progress has been made in this field, however, the mechanism of lung carcinogenesis is not fully understood and prognosis of NSCLC patients is still unsatisfactory [5]. A better understanding of molecular biology associated with lung cancer will lead to more efficient patient management [6].

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Invasion and metastasis is the leading mechanism of spread in NSCLC patients. An important prognostic factor for the development of metastatic lung cancer is the involvement of the mediastinal lymph nodes. However, lymph node involvement has not been shown to be predictive of sensitivity to treatment, and does not provide understanding of tumor's complete biological features [3,4]. The use of biomarkers that can accurately envisage long term outcomes in NSCLC patients can overcome this limitation [7]. An ideal feature of these biomarkers should be that they can be detected both pre- and postoperatively, and they can also be used for developing targeted therapeutic interventions for NSCLC.

MMPs represent a broad family of zinc-dependent endopeptidases, capable of destruction of extracellular matrix and non-matrix proteins, playing a role at different steps of malignant tumor growth and in cancer cell survival. MMP2, also called gelatinase A, is able to cleave the helical domains of type IV collagen and its increased expression in malignant cell lines is related to angiogenesis, invasiveness and tumor metastasis. MMP2 overexpression has been reported in a variety of cancers, including lung cancers [8]. MDM2, a 90 kDa oncoprotein, inhibits p53 expression and activity. This protein was found to be abnormally amplified in human tumors and cell lines, and was associated with tumor growth and metastasis [9,10]. EGFR, a 170 kDa tyrosine kinase receptor, is expressed in various solid tumors, including NSCLC [11-13]. It influences angiogenesis, cell proliferation, invasion and metastasis by signaling JAK-STAT, Ras/Raf/mitogen-activated protein kinase and phosphatidylinositol 3-kinase (PI3K)/Akt pathways [14,15].

The objective of this study was to evaluate the rate of overexpression of MMP2, MDM2 and EGFR in patients with NSCLC and analyze their correlation with clinicopathological parameters and prognosis.

Methods

Patients and tissue collection

The study comprised 184 patients with pathologically confirmed NSCLC, consecutively recruited from 2003 to 2008 at a tertiary hospital in China. Each patient's clinical history and tumor characteristics were obtained from histopathology reports and medical records. The data obtained included patient age, gender, smoking status, clinical stage, histological grade, and regional lymph node involvement. The study was approved by the Institutional Review Board. All patients provided written informed consent prior to surgery. The tissue specimens were collected prior to any therapy. After resection, specimen were fixed in 10% phosphate-buffered formalin at room temperature for 24 hrs and then embedded in paraffin.

Tumor staging and pathological classification

Assessment of lung cancer stage was done according to the TNM classification [16]. Tumors were categorized into four major histological types – adenocarcinoma, squamous cell carcinoma, large cell carcinoma and adenosquamous carcinoma – by reviewing hemotoxylin and eosin stained slides of the tissue specimens.

Immunohistochemical staining

EGFR, MDM2 and MMP2 expression were assessed by immunohistochemical (IHC) staining. This was performed using avidin-biotin-peroxidase complex (ABC), using mouse monoclonal antibodies against MMP2, MDM2 phosphoprotein and external domain of EGFR. For immunostaining, 5 µm sections of embedded tumors were deposited on SuperFrost Plus Slides. The deposited slides were kept for overnight drying at 37°C and were later deparaffinized with toluene, rehydrated with ethanol and pretreated with EDTA buffer at 450 W for 20 min. Endogenous peroxidases were blocked by incubation at room temperature in 0.3% H_2O_2 in methanol for 30 min. This was followed by incubation with anti-MMP2, anti-MDM2 and anti-EGFR antibodies in immunostainers. Negative controls were created by omitting the respective primary antibody and also by substituting normal mouse monoclonal antibodies with their respective primary antibodies.

Interpretation of immunohistochemical staining results

Results of IHC staining were evaluated using tumor cells demonstrating positive immunoreactivity for the experimental protein. A semi-quantitative analysis of protein expression was made by adding up the scores calculated by multiplying the proportion of positive tumor cells with staining intensity. Tumors were classified into five grades, based on protein expression score (0-400). Tumors with expression score of 0 were classified as grade 0, score of 1-100 as grade 1, score of 101-200 as grade 2, score of 201-300 as grade 3 and score of 301-400 as grade 4. A tumor score exceeding grade 2 was considered as IHC-positive for statistical analysis (Figure 1). The evaluation was performed by investigators that were blinded to the tumor stage and grade.

Follow-up

All of the patients were followed-up for over 48 months with a median of 23 months. During the follow-up period, some of the patients developed relapse,

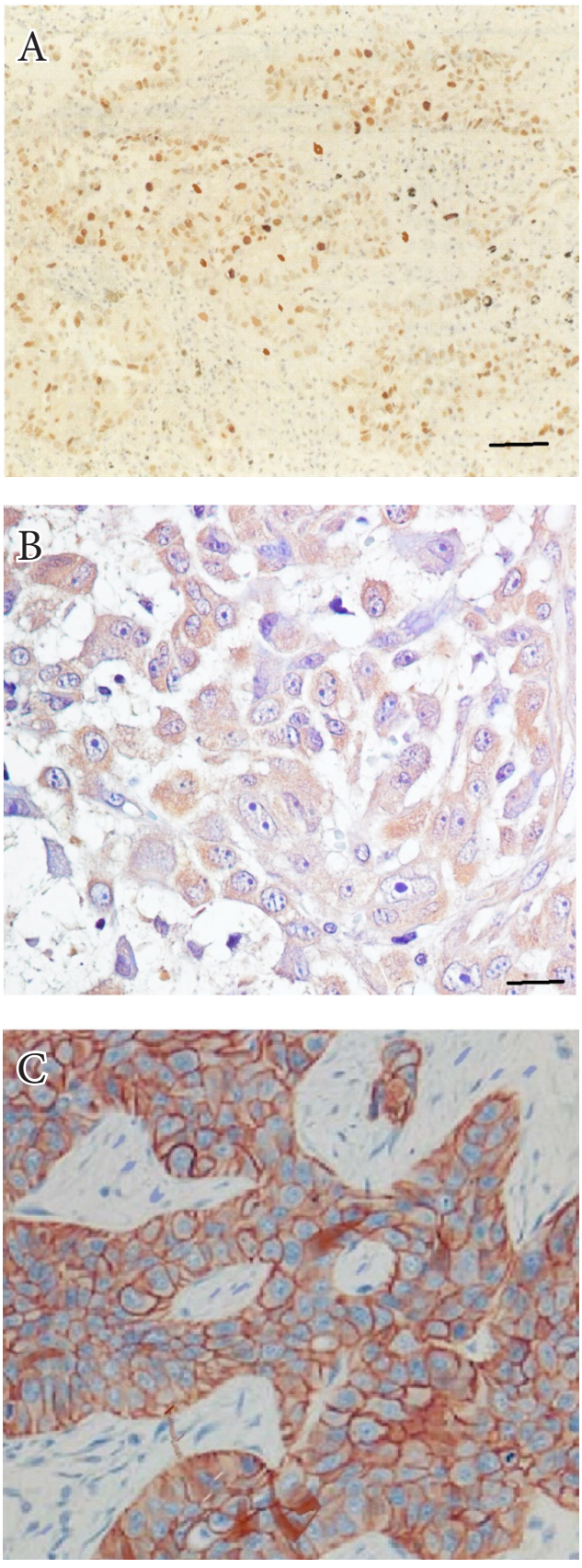

Figure 1. Immunostaining of MDM2, MMP2 and EGFR. (**A**) Positive immunoreactivity of MDM2 protein in adenocarcinoma, x40. (**B**) Strong MMP2 expression in the cytoplasm of adenocarcinoma, x40. (**C**) Membranous overexpression of EGFR in squamous cell lung carcinoma, x40.

Table 1. Demographic and clinical characteristics of the NSCLC patients (N=184)

Characteristics	*N (%)*
Age (years)	
<65	56 (30)
≥65	128 (70)
Gender	
Male	136 (74)
Female	48 (26)
Smoking status	
Smokers	106 (58)
Non-smokers (Ex-smokers+never)	78 (42)
Histologic type	
Squamous cell carcinoma	74 (40)
Adenocarcinoma	97 (53)
Large cell carcinoma	11 (6)
Adenosquamous carcinoma	2 (1)
Clinical stage	
I	91 (50)
II	40 (22)
III	34 (18)
IV	19 (10)

NSCLC: non-small cell lung cancer

had distant metastasis or both and survived /died.

Statistics

All statistical calculations were performed with SPSS 17.0 for Windows (SPSS Inc., Chicago, ILL, USA). Pearson's chi-square test was used to evaluate the association between clinicopathological variables and HIC-positivity for MDMs, MMP2 and EGFR. Multivariate logistic regression analysis was used to identify independent predictors of death during the follow-up period. A p value <0.05 was considered statistically significant.

Results

Patient characteristics

This study included 184 NSCLC patients. The characteristics of these patients are summarized in Table 1. All of the patients were in the age range of 40-83 years (median 66); overall, 70% of them were 65 years or older. Of the total patients enrolled, 136 (74%) were male and 48 (26%) female. Amongst them, 97 tumors (53%) were histologically diagnosed as adenocarcinoma, 74 (40%) as squamous cell carcinoma, 11 (6%) as large cell carcinoma, and 2 (1%) as adenosquamous carcinoma. A total of 91 tumors (50%) were in stage I,

Table 2. MDM2 expression and clinicopathological parameters in NSCLC patients

Parameters	*MDM2-positive N (%)*	*MDM2-negative N (%)*	*Chi-square*	*p value*
Total	70	114		
Age (years)			6.5	0.01
<65	29 (52)	27 (48)		
≥65	41 (32)	87 (68)		
Gender			0.61	0.43
Male	54 (40)	82 (60)		
Female	16 (33)	32 (77)		
Smoking status			3.04	0.08
Ever smoker	46 (43)	60 (57)		
Non-smoker	24 (31)	54 (69)		
Histological type			0.13	0.72
Squamous cell carcinoma	27 (36)	47 (64)		
Others	43 (39)	67 (61)		
Stage			0.63	0.42
I	32 (35)	59 (65)		
II	18 (45)	22 (55)		
III,IV	20 (38)	33 (62)		

NSCLC: non-small cell lung cancer, MDM2: mouse double minute 2 homolog

40 (22%) in stage II and 53 (28%) in stage III and IV. During a 4-year postoperative follow-up 105 (57%) of them died.

Protein expression by immunohistochemistry

MDM2

MDM2 overexpression was observed in 70 (38%) of the patients studied. MDM2 expression was significantly higher in younger patients (<65 years) compared with the elderly (52 vs 32%; p=0.01). Besides, MDM2 expression did not differ according to other clinicopathological characteristics of the patients (Table 2).

MMP2

Only 46 (25%) of the patients had overexpression of MMP2 while the majority (75%;138) of the 184 tumor specimens were MMP2-negative. Patients who had squamous cell carcinoma had significantly lower rates of MMP2 positivity compared with those who had other types of carcinoma (16 vs 31%; p=0.02). No significant difference was noticed between MMP2 staining status and clinicopathological parameters (Table 3).

EGFR

Immunohistochemical staining results for EGFR are described in Table 4. EGFR positive staining was observed in 105 (57%) of the evaluated tumor specimens; the expression was more frequent in specimens with squamous cell carcinoma (p<0.001), among the elderly (p<0.001), and in smokers (p<0.001). No statistically significant difference in EGFR expression was observed relative to patients' gender or the clinical stage of disease.

Postoperative risk of mortality

Overall survival was defined as the interval from diagnosis to death; the outcome of this variable was either death or alive at the end of study. The median overall survival was 23 months (range 12–48). During this period, 105 (57%) of the patients died. Independent risk factors for mortality determined using multivariate logistic regression analysis are shown in Table 5: older age (≥65 years) aOR 1.3; 95% confidence interval (95% CI) 1.1–1.7), being a smoker (aOR 10; 95% CI, 5.5–19.4), having stage II disease (aOR 10.8; 95% CI, 3.7–19.6) or stage III/IV disease (aOR 28.3; 95% CI, 7.9–38.2), expression of EGFR (aOR 5.9; 95% CI, 3.4–11.8) and MMP2 (aOR 4.1; 95% CI, 1.7–7.9) . However, the expression of MDM2 was protective – independently predicting a reduced risk of death (aOR 0.3; 95% CI, 0.1–0.6).

Table 3. MMP2 expression and clinicopathological parameters in NSCLC patients

Parameters	*MMP2-positive N (%)*	*MMP2-negative N (%)*	*Chi-square*	*p value*
Total	46	138		
Age (years)			0.14	0.71
<65	15 (27)	41 (73)		
≥65	31 (24)	97 (76)		
Gender			0.15	0.70
Male	33 (24)	103 (76)		
Female	13 (27)	35 (73)		
Smoking status			0.27	0.61
Ever smoker	28 (26)	78 (84)		
Non-smoker	18 (23)	60 (77)		
Histological type			5.1	0.02
Squamous cell carcinoma	12 (16)	62 (84)		
Others	34 (31)	76 (69)		
Stage			3.4	0.05
I	30 (33)	61 (67)		
II	10 (25)	30 (75)		
III,IV	6 (11)	47 (89)		

NSCLC: non-small cell lung cancer, MMP2: matrix metalloproteinase-2

Table 4. EGFR expression and clinicopathological parameters in NSCLC patients

Parameters	*EGFR-positive N (%)*	*EGFR-negative N (%)*	*Chi-square*	*p value*
Total	105	79		
Age (years)			12.6	<0.001
<65	21 (38)	35 (62)		
≥65	84 (66)	44 (46)		
Gender			0.02	0.89
Male	78 (57)	58 (43)		
Female	27 (56)	21 (44)		
Smoking status			14.2	<0.001
Ever smoker	73 (69)	33 (31)		
Non-smoker	32 (41)	46 (59)		
Histological type			29.1	<0.001
Squamous cell carcinoma	60 (81)	14 (19)		
Others	45 (41)	65 (59)		
Stage			2.2	0.14
I	47 (52)	44 (48)		
II	31 (78)	9 (22)		
III,IV	27 (51)	26 (49)		

NSCLC: non-small cell lung cancer, EGFR: epidermal growth factor receptor

Discussion

This study was designed to assess the role of EGFR, MDM2 and MMP2 overexpression in NSCLC pathogenesis and prognosis for NSCLC patients. MDM2 protein overexpression was found in 38% of NSCLC patients studied. The rate of expression of MDM2 in other lung cancer series ranged from 6-78% [10,17-20]. One reason for this variation may be due to differences in the type of

Table 5. Multivariate logistic regression analysis of postoperative predictors of mortality in NSCLC patients

Variables	*Total N*	*Death N (%)*	*Crude OR (95% CI)*	*Adjusted OR (95% CI)*	*Adjusted p value*
Total	184	105			
Age (years)					
<65	56	28 (50)	1.2 (0.9 – 1.6)	1.3 (1.1 – 1.7)	
≥65	128	77 (60)	1	1	0.04
Gender					
Male	136	79 (58)	1.2 (0.6 – 2.4)	1.2 (0.7 – 2.4)	0.64
Female	48	26 (54)	1	1	
Smoking status					
Ever smoker	106	86 (81)	13.4 (6.2 – 29.0)	10 (5.5 – 19.4)	<0.001
Non-smoker	78	19 (24)	1	1	
Histological type					
Squamous cell carcinoma	74	48 (65)	1.7 (0.9 – 1.6)	1.6 (0.9 – 1.7)	0.08
Others	110	57 (52)	1	1	
Stage					
I	91	24 (26)	1		
II	40	32 (80)	11.1 (4.2 – 21.8)	10.8 (3.7 – 19.6)	<0.001
III, IV	53	49 (92)	34.2 (10.3 – 77.6)	28.3 (7.9 – 38.2)	<0.001
MDM-2 status					
positive	70	12 (17)	0.25 (0.1 – 0.5)	0.3 (0.1 – 0.6)	<0.001
negative	114	93 (82)	1	1	
MMP2 status					
positive	46	36 (78)	3.6 (1.6 – 8.5)	4.1 (1.7 – 7.9)	<0.001
negative	138	69 (50)	1	1	
EGFR status					
positive	105	79 (75)	6.2 (3.1 -12.5)	5.9 (3.4 -11.8)	<0.001
negative	79	26 (33)	1	1	

NSCLC: non-small cell lung cancer, MMP2: matrix metalloproteinase-2, MDM2: mouse double minute 2 homolog, EGFR: epidermal growth factor receptor

antibody used and the detection system [10,17,18]. In the regression analysis, MDM2 expression was a predictor of survival. The precise role of MDM2 in NSCLC progression, pathogenesis and prognosis remains unclear [21,22]. A previous study reported that MDM2 gene amplification was associated with poor prognosis [23]; however, another study documented that MDM2 mRNA expression to be a favorable prognostic marker in patients with NSCLC [24]. Furthermore, studies that evaluated the prognostic value of MDM2 IHC protein overexpression were also inconclusive [10,16-20,23,24]. While four studies did not find MDM2 expression to be prognostic, two studies [23,24] have reported MDM2 staining to be a favorable prognostic marker in patients with NSCLC; this agrees with the findings of the present study.

Data from previous studies on EGFR overexpression and its role in prognosis are conflicting, as different antibodies and evaluation criteria were used for immunohistochemical analysis of EGFR [22,25]. Most of the tissues in our study demonstrated EGFR overexpression, being more frequent in squamous cell carcinoma, compared with other histologic types. Consistent with the findings of this study, a systematic review of 16 studies – of which 14 were based on IHC – found that the overall EGFR expression rate in NSCLC was 51%. Expression was less frequent in adenocarcinoma and other histologic types (46.2%) than in squamous cell carcinoma (82.6%) [25]. In this study, patients with EGFR expression were 6 times more likely to have died during follow-up compared with patients without EGFR expression. A quantitative meta-analysis of 8 evaluable IHC studies showed that EGFR IHC expression was

marginally significant as a negative (poor) prognostic marker [22]. However, findings from recent studies have remained conflicting, with some suggesting EGFR IHC expression as a poor prognostic marker while others not making this observation [26-28]. Recent studies suggest that EGFR IHC expression plays an important role in patient selection and as a predictive marker for response and survival benefit in patients with advanced NSCLC who may benefit from EGFR inhibitors (gefitinib and erlotinib) [22,29].

An increase in MMP2 expression has been found to be associated with increased metastatic potential and advanced stage in a number of tumors including NSCLC [30-32]. We observed a higher death rate among NSCLC patients with overexpression of MMP2 protein in this study, and overexpression of MMP2 protein was an independent predictor of mortality. Results from several studies have suggested that high expressions of MMP2 and MMP9 are poor prognostic markers for NSCLC, especially the expression of MMP-2 [22,33-38]. Passlick et al. demonstrated a significant association of MMP2 expression with shortened overall survival in NSCLC patients [34]. The role of MMP IHC overexpression in the prognosis of NSCLC should be studied further, but this needs to be accompanied by a very vigorous validation of the specificity of the antibodies used. And, further studies on the impact of MMP2 overexpression in identifying patients with advanced NSCLC who may benefit from treatment with biologic inhibitors of matrix metalloproteinases should be carried out [39].

A potential limitation of this study is the relatively small sample size which precluded a combined analysis of the impact of MDM2, MMP2 and EGFR overexpression on mortality. A prospective multicentre study which investigates the expression of multiple biomarkers, their combinations and their impact on mortality in NSCLC patients will improve these limitations.

In conclusion, this study demonstrated that the overexpression of EGFR, MDM2 and MMP2 correlated with some clinicopathological parameters of NSCLC patients. Furthermore, this study found that overexpression of MMP2 and EGFR was independent risk factor for mortality in NSCLC patients, while overexpression of MDM2 independently predicted a reduced risk of death. Our findings have implications in the selection of NSCLC patients with advanced disease who may benefit from targeted biologic therapies and this needs to be explored further.

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