Expression of metastasis-associated molecules in non-small cell lung cancer and their prognostic significance

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Abstract. Molecules associated with tumor invasion and metastasis have been actively investigated, but their prognostic significance has been variously reported by investigators. We evaluated the expression of matrix metalloproteinase (MMP)-1, MMP-2, MMP-3, MMP-9, MMP-10, tissue inhibitor of MMPs (TIMP)-1, TIMP-2, β-catenin, E-cadherin and cyclooxygenase-2 (COX-2) in 43 cases of non-small cell lung cancer (NSCLC). Immunohistochemistry of each marker was performed on tissue microarray paraffin blocks, and the results were determined by a semi-quantitative method using an intensity score (0-3) and percentage score (0-3). The expression scores of each marker were correlated with TNM stage and patient survival data. The expression of MMP-3 and COX-2 was significantly increased in higher stage tumors (P<0.001 and P=0.046, respectively), while a correlation with patient survival length was observed for MMP-1 and COX-2 (P=0.034 and 0.019, respectively). All stage I or II cases with increased MMP-1 expression succumbed to NSCLC within 34.1 months. Cases with low expression of both MMP-1 and COX-2 had a significantly longer survival time than cases with high expression of either of the two markers (P=0.002). These results suggest that MMP-1 and COX-2 are plausible candidate survival markers for NSCLC.

Introduction

Lung cancer remains a leading cause of death worldwide (1). Non-small cell lung carcinoma (NSCLC) comprises approximately 80% of all lung cancer cases (2). Many therapeutic options including adjuvant or post-operative chemotherapy and surgery have been applied to NSCLC, but with poor results (3,4). Therefore, newly emerging molecular prognostic markers are being meticulously studied by many investigators.

Zhu *et al* reviewed 462 papers on immunohistochemical prognostic markers for NSCLC published between 1987 and 2005, and summarized the prognostic significance of 50 markers (5). The markers were classified according to their function in tumor growth as molecules that: i) are involved in independent tumor growth [epidermal growth factor receptor (EGFR), HER-2, Ras, Ki-67]; ii) induce resistance to growth-inhibitory factors [transforming growth factor-β (TGF-β), p21, Rb]; iii) induce resistance to apoptotic pathways (p53, Bcl-2, Bax, Caspase 3); iv) induce sustained angiogenesis [vascular endothelial growth factor (VEGF) family members]; and v) are involved in invasion and metastasis [β-catenin, matrix metalloproteinase (MMP) family members, E-cadherin], among others. Seventeen of these molecules, including p53, Ki-67, EGFR, HER-2 and VEGF-A, have been frequently studied, but their reported prognostic significance as markers has varied (good, poor or none).

MMPs are proteolytic enzymes implicated in many physiological and pathological processes, including embryonic development and morphogenesis. Approximately 28 different MMPs have been identified. The activity of MMPs is controlled by tissue inhibitors of MMPs (TIMPs). To date, four TIMPs (TIMP-1 to TIMP-4) have been identified (6). In healthy lung tissue, fibroblasts express MMP-1, MMP-2 and TIMP-1, and bronchial epithelial cells release MMP-2, MMP-9 and TIMP-1. Type II pneumocytes produce TIMP-2 and MMP-1 (6,7). The expression of MMPs and TIMPs in NSCLC has been studied by several investigators, on the assumption that tumor metastasis is promoted by MMPs and suppressed by TIMPs. Among the MMPs, MMP-2 and MMP-9 (also called gelatinase A and gelatinase B, respectively) are most often reported to be associated with tumor metastasis.

Based on the report by Zhu *et al*, in addition to MMPs and TIMPs, we selected certain markers [cyclooxygenase-2 (COX-2), β-catenin and E-cadherin] that had either not been studied extensively or had produced controversial results, despite their known association with invasion and metastasis. COX-2 is a key enzyme of prostaglandin production and its expression has been observed in both adenocarcinomas and squamous cell carcinomas (8,9). This molecule contributes to tumor metastasis via several mechanisms, including the inhibition of apoptosis and promotion of angiogenesis and

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tumor invasion (10-12). Some authors have suggested that COX-2 has prognostic significance, particularly in early-stage NSCLC (13), but this finding remains controversial (5). E-cadherin exists ubiquitously on epithelial cells, and β-catenin binds to the intracellular domain of E-cadherin. The E-cadherin-catenin complex is a crucial component of intercellular junctions; thus, altered expression of the complex may promote tumor metastasis and confer a poor prognosis (14,15). However, this assumption has not been verified due to various studies reporting contradictory results regarding the prognostic significance of the E-cadherin-catenin complex (5).

The purpose of this study was to evaluate the statistical significance of molecules that may affect tumor invasion and metastasis, and to identify the most significant prognostic markers among them. The expression of these molecules was evaluated by immunohistochemistry using tissue microarray blocks, and the degree of expression was compared with data on patient survival.

Table I. Summary of patient characteristics.

Variable	No. of patients
Age (years; range 41-77)	60.3±9.8
Sex (male/female)	37/6
Histologic type	
Squamous cell carcinoma	31
Adenocarcinoma	12
Stage	
I	11
II	8
IIIA	19
IIIB	3
IV	2

Materials and methods

Patient selection. Pulmonary adenocarcinoma and squamous cell carcinoma cases diagnosed following surgical lobectomy or pneumonectomy were selected from the surgical records of the Department of Pathology, Chungnam National University Hospital. Cases operated after 2003 were excluded to ensure a follow-up of more than 5-years. Cases without well-preserved paraffin embedded tissue or documented clinical records were also excluded after histologic examination and a review of the chart. A final total of 43 patients (37 male, 6 female) were enrolled in the study. The study was approved by the Institutional Review Board of Chungnam National University College of Medicine. Patient characteristics are summarized in Table I.

Tissue microarray construction. Slides of each case were reviewed and, during tissue preparation, a well-preserved tumor area without necrosis or artifacts was marked with an oil pen. Paraffin-embedded tissue was extracted from this area using a tissue microarray kit (3.0 mm, TM0006; Microm, Germany). Each extracted sample was delivered into one of the holes in recipient blocks (3.0 mm, 30 sp TM0011; Microm). Completely filled recipient blocks were placed in an embedding mold and incubated for 30 min at 60˚C. After becoming completely transparent, recipient blocks were solidified on a cold plate.

Immunohistochemistry. Each tissue microarray block was serially cut into 4-μm sections and stained using an avidin-biotin complex method. Briefly, sections were deparaffinized in xylene and rehydrated with graded alcohol. Antigen retrieval was performed using a microwave oven (2x5 min in citrate buffer pH 6.0). After treatment with 0.3% hydrogen peroxide, the sections were incubated with primary antibodies as follows: MMP-1, 1:25, overnight at room temperature (RT); MMP-2, 1:50, 30 min at RT; MMP-3, 1:50, 30 min at RT; MMP-9, 1:50, 30 min at RT; MMP-10, 1:50, overnight at RT; TIMP-1, 1:50, 30 min at RT; TIMP-2, 1:50, 30 min at RT; COX-2, 1:100, 30 min at RT; β-catenin, 1:50, 30 min at RT; E-cadherin, 1:60, 30 min at RT (all from Thermo Fisher Scientific, CA, USA). After washing with phosphate-buffered saline, biotin-labeled link antibodies and streptavidin-biotin peroxidase were applied using the LSAB kit (Dako). Bound peroxidase was detected by diaminobenzidine.

Analysis of immunohistochemical staining. Expression of each primary antibody was evaluated according to a previously described method (16) with modifications. Staining intensity was scored as follows: 0, no staining; 1, faint staining; 2, moderate staining; 3, strong staining. Moderate staining was determined when the staining intensity was the same as that of the internal control. The staining percentage was scored as follows: 0, no positive tumor cells; 1, <25% positive tumor cells; 2, 25-50% positive tumor cells; 3, >50% positive tumor cells. The intensity score and the percentage score were summed up and the final score was used to divide samples into low-expression (0-4) and high-expression (5-6) groups.

Statistical analysis. The correlation between the final scores of each marker and TNM stage was evaluated by Fisher's exact test. To perform this analysis, markers were re-grouped according to TNM stage into low and high groups (low T, T1-2; high T, T3-4; low N, N0-1; high N, N2-3; low stage, stages I and II; high stage, stages III and IV). Post-operative survival rates were evaluated by the Kaplan-Meier method and statistical significance was evaluated using the log-rank test. Multivariate analysis using the Cox proportional hazards model was performed to identify independent prognostic markers. A P-value <0.05 was considered significant.

Results

Expression pattern of immunohistochemical markers. Fig. 1 shows the intensity and extent of expression in various microarray slides. MMPs, TIMPs and COX-2 exhibited cytoplasmic immunoreactivity, and β-catenin and E-cadherin showed positive reactions along the cell membrane. Five of 43 cases (11.6%) showed nuclear positivity to β-catenin in addition to membrane staining.

Expression levels and TNM stage. The expression levels of each marker were compared with T stage, N stage and overall TNM stage grouping. Distant metastases (M1) were recorded

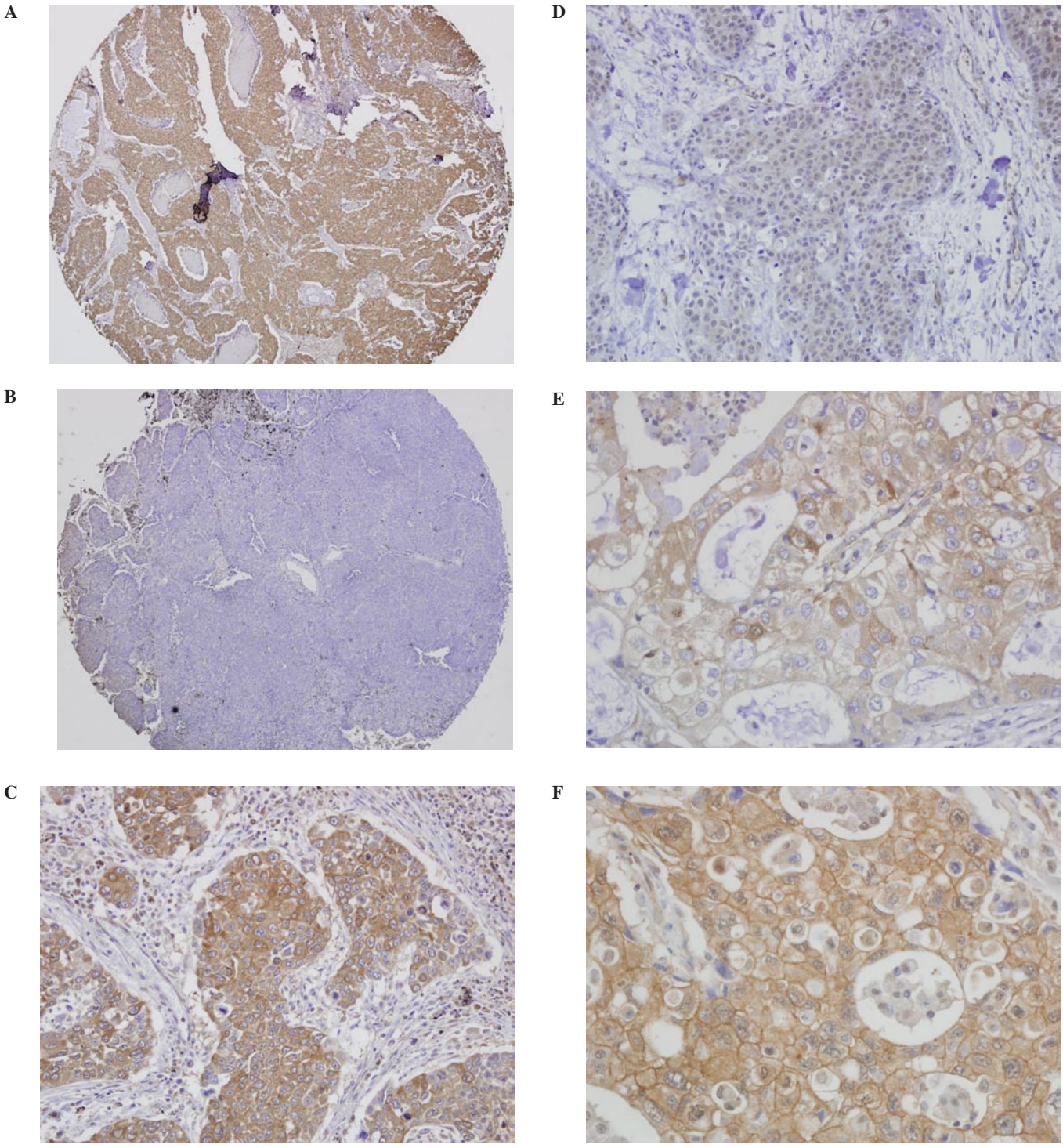

Figure 1. Immunohistochemical stainings of each marker were performed using tissue microarray paraffin blocks. Based on the results, stains were assigned an intensity score (0-3) and percentage score (0-3). A final score was calculated by summing up the intensity and percentage score, and was used to divide samples into low (0-4) and high (5-6) expression groups. (A and C) MMP-1 expression with intensity score 3, percentage score 3. (B) COX-2 expression with intensity score 0, percentage score 0. (D) MMP-1 expression with intensity score 1. (E and F) Membranous expression of E-cadherin (E) and β-catenin (F).

in only two cases, therefore comparison with M stage was not performed. None of the markers were correlated with T stage (tumor size). However, certain markers were correlated with N stage and overall stage grouping. The expression levels of MMP-3, MMP-9, TIMP-1 and COX-2 differed significantly between cases with low and high N stage ($P<0.001$, $P=0.025$, $P=0.011$ and $P=0.020$, respectively). Higher expression levels were correlated with higher stages. MMP-3 and COX-2 were significant markers associated with a high overall stage ($P<0.001$ and $P=0.046$, respectively). MMP-9 and TIMP-1 were also correlated with overall stage, though without statistical significance (Table II).

Expression levels and patient survival. MMPs, TIMPs and COX-2 showed higher survival rates in low expression cases. By contrast, β-catenin and E-cadherin showed a tendency toward a

Table II. Relationship between the expression of each marker and tumor stage.

	T		N		Stage	
	Low	High	Low	High	Low	High
MMP-1						
Low	28	7	16	4	14	20
High	4	1	19	1	4	1
P-value	1.000		0.342		0.162	
MMP-2						
Low	16	4	12	8	11	8
High	18	5	9	14	8	15
P-value	1.000		0.227		0.213	
MMP-3						
Low	14	2	14	2	13	2
High	19	6	7	18	6	19
P-value	0.448		<0.001		<0.001	
MMP-9						
Low	21	5	17	9	15	10
High	12	3	4	11	4	11
P-value	1.000		0.025		0.055	
MMP-10						
Low	26	5	18	13	16	14
High	6	3	2	7	2	7
P-value	0.348		0.127		0.139	
TIMP-1						
Low	14	3	13	4	11	5
High	19	5	8	16	8	16
P-value	1.000		0.011		0.051	
TIMP-2						
Low	28	5	17	16	15	17
High	6	3	4	5	4	5
P-value	0.336		1.000		1.000	
COX-2						
Low	23	5	18	10	16	11
High	10	3	3	10	3	10
P-value	0.692		0.020		0.046	
β-catenin						
Low	25	9	17	17	15	18
High	9	0	4	5	4	5
P-value	0.166		1.000		1.000	
E-cadherin						
Low	29	8	19	18	17	19
High	5	1	2	4	2	4
P-value	1.000		0.664		0.673	

P-values were calculated by Fisher's exact test. Low T, T1-2; high T, T3-4; low N, N0-1; high N, N2-3; low stage, stages I and II; high stage, stages III and IV.

higher survival rate in high-expression cases. However, statistical significance was obtained only with MMP-1 (P=0.034; Fig. 2A) and COX-2 (P=0.019; Fig. 2B). Based on multivariate analysis with MMP-1, COX-2 and MMP-9, COX-2 was the most important prognostic factor (P=0.040; Table III).

In addition, MMP-1 and COX-2 were combined, and were defined as dual marker negative when both the markers showed low expression and as dual marker positive when more than one of these markers showed high expression. When survival analysis using this dual marker was performed, there

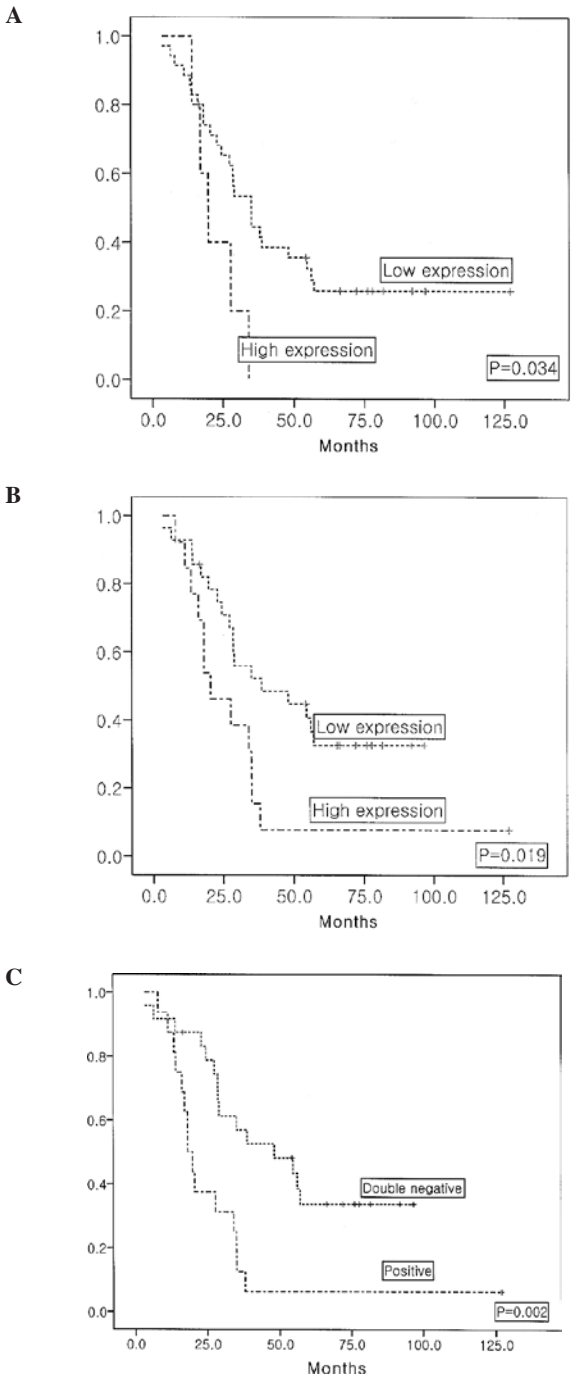

Figure 2. The prognostic significance of each marker was analyzed using the Kaplan-Meier method. Significant correlations were observed with MMP-1 (P=0.034; A) and COX-2 (P=0.019; B). When these two markers were combined, the prognostic significance was intensified (P=0.002; C).

was a significant difference between the dual marker negative and positive group, revealing this dual marker to be the most significant prognostic marker (P=0.002; Fig. 2C).

Discussion

The markers included in this study are molecules which mainly contribute to tumor invasion and metastases (one exception is COX-2; as mentioned in the Introduction, various mechanisms have been suggested for this molecule). These molecules are under active investigation, but to date the reported results have been controversial.

Table III. Univariate and multivariate analysis of the expression of each marker and patient survival.

Markers	Univariate analysis	Multivariate analysis
MMP-1	0.034	0.138
MMP-2	0.714	
MMP-3	0.360	
MMP-9	0.124	0.320
MMP-10	0.289	
TIMP-1	0.625	
TIMP-2	0.976	
COX-2	0.019	0.040
β-catenin	0.634	
E-cadherin	0.819	
Dual marker (MMP-1 + COX-2)	0.002	

MMPs were among the first markers reported to play a possible role in tumor invasion and the spread of NSCLC, and consequently their inhibitor, TIMP, also received attention. In this study, we evaluated the expression of five MMPs (MMP-1, -2, -3, -9 and -10) and two TIMPs (TIMP-1 and -2) by immunohistochemistry.

When compared with the TNM staging system, none of the molecules were associated with primary tumor size. However, increased expression of MMP-3 was associated with nodal spread and increased overall stage, and expression of MMP-9 and TIMP-1 showed an association with nodal spread and a possible association with increased overall stage. In spite of these correlations with stage, survival analyses revealed only MMP-1 to have statistical significance.

MMP-3 expression and its prognostic significance have yet to be intensively studied. Thomas *et al* reported that MMP-3 overexpression evaluated by immunohistochemistry was observed only in stage I NSCLC (17). In contrast, in our study 19 (44.2%) stage III or IV cases exhibited high expression. This difference can be explained by the different counting methods used; we used the combined score of intensity and percentage, while Thomas *et al* used only the intensity score to evaluate MMP-3 overexpression. Based on the fact that MMP-3 expression did not affect patient survival despite its correlation with nodal spread and overall stage, it can be reasoned that MMP-3 does not directly promote tumor aggressiveness in NSCLC, but rather is a molecule whose expression increases in the late stages of the tumor. MMP-3 was observed to have a protective role in mouse squamous cell carcinoma (18), partially supporting this hypothesis.

It is possible to apply similar interpretations to the expression of MMP-9 and TIMP-1. MMP-9 expression in tumor cells did not affect patient survival in our study, though it was roughly related to tumor progression. However, despite some disagreement (19), the poor prognostic influence of MMP-9 expression has been relatively uniformly reported (20-22).

Based on the observed survival curve pattern and relatively low P-value (0.124), the possibility of reaching statistical significance is likely to increase with a larger sample number. There are few reports regarding the prognostic significance of TIMP-1 in NSCLC. Gouyer *et al* reported TIMP-1 expression to be an indicator of worse prognosis, but this was not related to TNM stage (23). In contrast, Simi *et al* reported that TIMP-1 expression was related not to patients survival, but rather to nodal spread and metastasis (24). Our results were in accord with those of Simi *et al*. The fact that TIMP-2 was not related to prognosis and disease progression was also in agreement with previous reports (23,25). As its name implies, the basic function of TIMP-1 is inhibiting matrix degradation by MMPs, therefore it is somewhat ironic that TIMP-1 is related with poor prognosis or at least advanced stage disease. This phenomenon can be explained by certain additional functions of TIMP-1 (23), including the promotion of cellular growth (26,27) and the inhibition of apoptosis (28,29).

In the present study, MMP-1 was the only molecule associated with survival among the MMPs and TIMPs. MMP-1 overexpression and its prognostic significance have been more frequently studied in other malignancies (30-32); there are relatively few reports regarding its role in NSCLC. Various authors have reported that lung cancer is associated with a polymorphism in the promoter of MMP-1 (33-35); Sauter *et al* reported a single nucleotide pleomorphism of MMP-1 to be associated with early-onset lung cancer (36). High expression of MMP-1 was observed in only six cases in our study population; therefore, it is difficult to confirm the prognostic significance of this marker. However, it is notable that all of the six patients died within 14.1-34.1 (median 23.8) months of surgery, though five patients had only stage I or II disease. In other words, MMP-1 overexpression in early-stage NSCLC may be a significant indicator of shorter survival time. This hypothesis should be verified in a larger study population composed of early-stage cases.

The prognostic significance of COX-2 expression in NSCLC has been the subject of heated debate. Certain authors have reported that COX-2 confers a poor prognosis (37-39), while others did not find it to have prognostic significance (40,41) and yet others found it to have a good prognostic influence (21). The present study demonstrated a strong relationship between COX-2 overexpression and shorter patient survival, in addition to the correlation between COX-2 expression and nodal spread and overall stage. The significance of this marker was confirmed in univariate and multivariate analysis. As Yamaguchi *et al* discussed, these contradictory results might be due to differences in histologic type (adenocarcinoma vs. squamous cell carcinoma) and the degree of differentiation of the tumors included in each study (21). A more controlled study should be conducted in order to settle the controversy.

The predictive value of COX-2 expression became more intensive when combined with MMP-1 expression. Cases with low expression of both these markers showed significantly extended survival time compared to cases with high expression in at least one of the markers. Based on these results, we propose the possibility of a new prognostic marker discriminating cases with a good prognosis in NSCLC.

This study had several limitations in its design. Firstly, cases should have been stratified according to their TNM stage and cell type for a more precise analysis. The failure to obtain statistical significance for some markers, in particular MMP-9, β-catenin and E-cadherin, was possibly influenced by the small number of cases included. Additionally, It was not possible to evaluate stromal expression of the markers, as each microarray section contained relatively few stroma in comparison with the tumor cells. Despite these weaknesses, the results of the study raise the possibility of new candidate prognostic markers, which should be validated in further investigations.

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