Original Article

MMP-2, TIMP-2 and CD44v6 Expression in Non-small-cell Lung Carcinomas†

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Abstract

Introduction: Factors that emerge as crucial participants in tumour invasion and metastases are matrix metalloproteinases (MMPs), tissue inhibitor of metalloproteinase (TIMP) inhibitors and cellular adhesion molecules (CD44 and similar molecules). They play important roles in tumour invasion and metastasis in non-small-cell lung carcinomas (NSCLCs). Materials and Methods: The study was performed using the data of 33 patients. MMP-2 from the metalloproteinase family, TIMP-2 from the metalloproteinase inhibitor family and the adhesion molecule CD44v6 expression were investigated immunohistochemically to search their role in the metastasis and the clinical outcome of the patients with NSCLCs. Results: Twenty-three tumours (70%) were squamous cell carcinoma (SCC), 9 (27%) were adenocarcinoma (AC), and 1 (3%) was large cell carcinoma (LCC). MMP-2 and TIMP-2 were expressed in high rates in NSCLC but CD44v6 expression was about 50%. Lymphatic invasion was less frequent in TIMP-2-positive patients and this difference was statistically significant (P = 0.005). There was a statistically significant difference between SCCs and ACs with respect to CD44v6 tumoral expression (P = 0.004). Also, there was a negative correlation between lymphatic invasion and the extent of CD44v6; lymphatic invasion was significantly less in CD44v6-positive cases (P = 0.013). Conclusion: We found that TIMP-2 and CD44v6 can decrease the lymphatic invasion in NSCLCs. Also there was observed histiotype-related pattern of CD44v6 variant expression in SCCs.

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Key words: Immunohistochemistry, Metastasis, Pulmonary neoplasms

Introduction

Studies have provided data that matrix metalloproteinases (MMPs) facilitate tumour invasion, the establishment of metastases. On the other hand, matrix metalloproteinase inhibitors have been shown to inhibit tumour growth and dissemination in non-small-cell lung carcinomas (NSCLCs).[1] The studies on metalloproteinases and their inhibitors in NSCLCs are limited and reports concluded that the results are heterogeneous and diverse positivity rates have been obtained.[1-4] CD44 which is a polymorphic family of cell surface glycoproteins with a variety of functions including participation in cell adhesion and migration as well as modulation of cell-matrix interactions. Expression of the standard form of CD44 and its variant isoforms has been shown in both normal and neoplastic tissue[5] and has been declared as a prognostic indicator in NSCLCs.[6,7] In lung carcinoma, CD44 expression has been reported to be a feature of NSCLC but not small cell lung carcinoma. A specific variant, CD44v6, was shown to be expressed only in a subset of NSCLC, namely the SCCs.[7]

Because CD44 is expressed in non-metastising tumours and even precancerous lesions, its role in the diagnosis of malignancy and the process of metastasis NSCLCs are controversial.[5-7] There is only one study that assessed CD44 concurrently with MMP and tissue inhibitor of metalloproteinase (TIMP) in NSCLCs: in that study, CD44 expression was found to be the result of complex set of mediators including prostaglandins and it was emphasised that there were possibly indirect relationships between tumoral MMP and TIMP positivity and CD44 expression in the tumour.[8] The current study examined the relationship between MMP, tissue inhibitors of MMP and CD44v6 and

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their role in the metastasis and the clinical outcome of the patients with NSCLCs.

Materials and Methods

Initially, 56 NSCLC patients who underwent surgery and received radiotherapy between 1986 and 1997 at the Cerrahpa Faculty of Medicine and had completed follow-up were considered for the study. The specimens and pathology reports of the patients who had been evaluated at the Department of Pathology of the same institution were retrieved from the archives and re-evaluated for the purposes of this study. In 10 patients, comparing the reports and reexamining the findings of the blocks revealed inadequate information. In 8 patients, the available blocks were inadequate for a re-evaluation. In 5 patients, immuno-histochemical staining failed in all 3 attempts and this was ascribed to inadequate fixation. Consequently, the study was performed on the data of the remaining 33 patients.

Tumour subtype, lymph node metastasis, lymphatic and vascular involvement were evaluated by light microscopy. Data on the results of initial staging and postoperative staging, postoperative survival and development of metastases were obtained from the patient files. After examination of the haematoxylin eosin (HE) sections, for each tumour, one of the paraffin blocks was chosen for immunohistochemical evaluation.

Immunohistochemical Staining Method

The procedure was started by taking 6-micrometre sections from paraffin blocks onto poly-L-lysine-coated slides. Deparaffinisation was accomplished by 3 incubations in xylene (5 minutes each) and 3 incubations in pure alcohol (5 minutes each). They were incubated 4 times (5 minutes each) at 750W to 850W with addition of 50 mL of water and 0.01 M sodium citrate. They were then kept at room temperature for 20 minutes, washed in distilled water and kept in phosphate-buffered saline (PBS) for 5 minutes. The antibodies (Neomarkers), their dilutions, incubation periods and temperatures were summarised in Table 1. They were then washed with PBS and stained with a universal kit (Antipolyvalent An HRP kit). After visualisation of the reaction with the AEC chromogen, counterstaining was performed with haematoxylin and the slides were covered with a glycerin gel. As positive control, a tonsil specimen was used for CD44v6, an ovarian carcinoma specimen for MMP-2, and a pancreatic carcinoma specimen for TIMP-2. For the stromal staining for MMP-2 ve TIMP-2, the endothelium and fibroblasts were used as internal controls.

Evaluation of the Immunohistochemical Staining

The extent and intensity of the staining were evaluated semiquantitatively. In the case of CD44, positive staining at the stromal margin was evaluated as well. However, statistical evaluation was made on the data from the tumour tissue. For CD44v6 staining, the intensity of membranous staining was graded as strongly positive (+++), moderately positive (++), weakly positive (+); punctate or negative staining was graded as (-). The extent of the staining was evaluated by calculating the ratio of the stained tumour cells to the total tumour area: absence of staining was graded as 0, <25% as 1, 25% to 50% as 2, 50% to 100% as 3. For the evaluation of MMP-2 and TIMP-2, only cytoplasmic staining was considered positive. Both tumoral and stromal elements were included in the assessment of intensity and extent of staining. Both tumoral and stromal elements were considered for the extent and intensity of the staining. Intensity was categorised as absent (-), weak (+), moderate (++) and strong (+++). Extent was graded as follows: absent “0”, <25% “1”, 20% to 50% ”2”, 50% to 100% “3”.

Statistical Analysis

Statistical analysis was carried out using the SPSS package with comparative tests followed by survival analyses. The chi-square test, Fisher’s exact test and Spearman correlation analysis were used to identify independent prognostic factors. The Kaplan-Meier method was used for univariate evaluation of survival.

Results

The median age of patients was 58 years (range, 33 to 83). Four patients (12%) were women and 29 were men (88%). Twenty-three tumours (70%) were squamous cell carcinoma (SCC), 9 (27%) were adenocarcinoma (AC) and 1 (3%) was large cell carcinoma (LCC). In the preoperative staging, 11 patients (33%) were in stage I, 16 (49%) were in stage II and 6 (18%) were in stage III. The results of postoperative staging were as follows: 2 (6%) in stage I, 4 (12%) in stage II, 20 (61%) in stage III, and 7 (21%) in stage IV. The final stage differed from the initial stage in 20 patients (61%) and remained the same in 13 (39%). The median survival was

Table 1. The Antibodies, their Dilutions, Incubation Periods and Temperatures

Antibody	Dilution	Incubation period	Temperature
MMP-2/72/(A-Gel vC2)	1/100	Overnight	Room temperature
TIMP-2/Ab-4(2TMP04)	1/60	Overnight	Room temperature
CD44v6 (Clone VFF-7)	1/40	2 hours	Room temperature

Table 2. MMP-2, TIMP-2 and CD44v6 Tumoral Expression Staining Intensity and Staining Extent

Marker	Intensity Extent	Absent (-) Absent (0)	Weak (+) <25% (1)	Moderate(++) 20-50% (2)	Strong (+++) 50-100% (3)
		Cases n (%)	Cases n (%)	Cases n (%)	Cases n (%)
MMP-2	Intensity	7 (21%)	14 (42%)	7 (21%)	5 (15%)
	Extent	7 (21%)	13 (39%)	9 (27%)	4 (12%)
TIMP-2	Intensity	7 (21%)	15 (46%)	8 (24%)	3 (9%)
	Extent	7 (21%)	13 (39%)	10 (30%)	3 (9%)
CDv6	Intensity	15 (46%)	5 (15%)	8 (24%)	5 (15%)
	Extent	15 (46%)	8 (24%)	3 (9%)	7 (21%)

21 months (range, 4 to130). The patients in whom the stage changed postoperatively and those in whom it remained the same, did not differ with respect to immune marker expression (P >0.05). On the other hand, the frequency of MMP-2 expression was 17/20 (85%) in the first group and 9/13 (69%) in the second group. Distant metastases developed in 7 patients (21%) during follow- up.

MMP-2, TIMP-2 and CD44v6 tumoral staining intensity and staining extent results are shown in Table 2. All tumours expressed MMP in the stroma (Fig. 1). Eight (24%) were <25%, 19 (58%) were 25% to 50% and 6 (18%) were 50% to100%. The extent of TIMP-2 in the stroma was absent in 6 (18%), <%25 in 17 (51%), 25% to 50% in 3 (9%), and 50% to 100% in 7 (21%). Positive staining with TIMP-2 was weak in most of the patients (Fig.2). In some patients, the CD44v6 positivity in the tumour cells was particularly strong in cells near the stroma (Fig. 3). This feature was observed in all areas in 6 patients (18%), in most areas in 4 patients (12%), and was focal in 6 patients (18%) and absent in 17 patients (52%).

Comparisons of the MMP-2 tumoral expression immunohistochemistry results with clinicopathologic parameters are presented in Table 3. MMP-2 positive staining was observed in the single LCC. As for the relationship between MMP-2 and tumour stage, only 1 of the stage I patients was positive whereas 3 of the stage II patients (75%), 14 of the stage III patients (70%) and all of the 7 stage IV patients (100%) were positive. There was no significant association between MMP-2 and the investigated parameters; histological subtype, gender, stage, nodal involvement, vascular invasion, lymphatic invasion, and development of distant metastases. However, there was a strong correlation between the extent and intensity of tumoral MMP-2 staining (Spearman rho = 0.88). There was no correlation between the extent of MMP-2 in the tumour and the desmoplastic stroma (Spearman rho <0.30).

Table 3. Comparisons of the MMP-2 Tumoral Expression Results with Clinicopathologic Parameters

Clinicopathologic parameters		**MMP-2 (-)** **Cases n (%)**	**MMP-2 (+)** **Cases n (%)**	***P***
Gender	Female	2 (6%)	2 (6%)	0.19
	Male	5 (15%)	24 (72%)	
Histological subtype	Squamous cell carcinoma	5 (15%)	18 (54%)	0.56
	Adenocarcinoma	2 (6%)	7 (21%)	
Stage	Stage I-II	2(6%)	4 (12%)	>0.05
	Stage III-IV	5 (15%)	22 (66%)	
Nodal involvement	N0-N1	3 (9%)	12 (36%)	0.99
	N2-N3	4 (12%)	14 (42%)	
Vascular invasion	+	5 (15%)	17 (51%)	0.57
	-	2 (6%)	9 (27%)	
Lymphatic invasion	+	5 (15%)	16 (48%)	0.49
	-	2 (6%)	10 (30%)	
Distant metastases	+	3 (9%)	4 (12%)	0.19
	-	4 (12%)	22 (66%)	

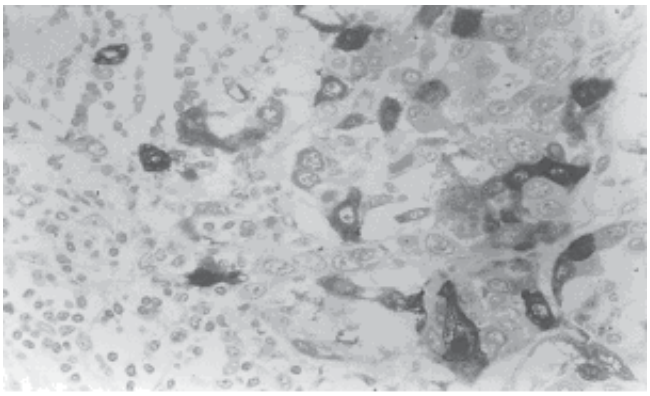

Fig. 1. Adenocarcinoma. Strong, diffuse cytoplasmic staining for MMP-2 in the tumour (MMP-2 x400).

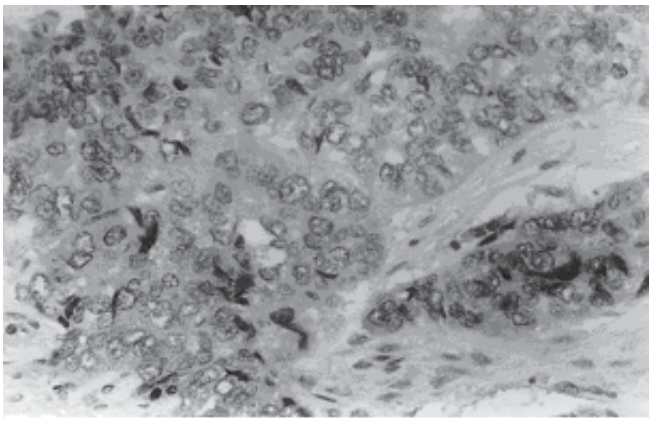

Fig. 2. Squamous cell carcinoma. Weak, diffuse cytoplasmic staining for TIMP-2 in the tumour (TIMP-2 x400).

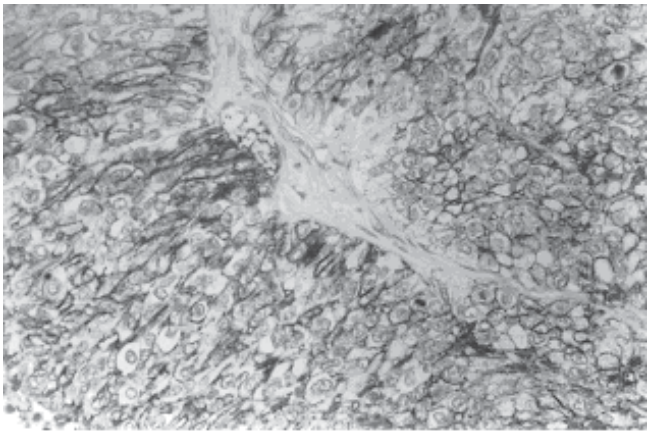

Fig. 3. Squamous cell carcinoma. Strong, diffuse membranous staining for CD44v6 in the tumour near the stroma (CD44v6 x400).

TIMP-2 tumoral expression immunohistochemistry results and clinicopathologic parameters are summarised in Table 4. The only LCC was positive with TIMP-2. As for the relationship between the extent of TIMP-2 and tumour stage, 2 of the stage I patients (100%), 3 of the stage II patients (75%), 14 of the stage III patients (70%) and 7 of the stage IV patients (100%) exhibited positive staining. There was a moderately strong correlation between the extent and intensity of TIMP-2 staining in the tumour tissue (Spearman rho = 0.70). There was no correlation between the extent of TIMP-2 in the tumour and the desmoplastic stroma (Spearman rho <0.30). Lymphatic invasion was less frequent in TIMP-2-positive patients and this difference was statistically significant ($P = 0.005$). But there was no correlation between TIMP-2 and the investigated

Table 4. Comparisons of the TIMP-2 Tumoral Expression Results with Clinicopathologic Parameters

Clinicopathologic parameters		**TIMP-2 (-) Cases n (%)**	**TIMP-2 (+) Cases n (%)**	***P***
Gender	Female	1(3%)	3 (9%)	0.63
	Male	6 (18%)	23 (69%)	
Histological subtype	Squamous cell carcinoma	5 (15%)	18 (54%)	0.56
	Adenocarcinoma	2 (6%)	7 (21%)	
Stage	Stage I-II	1 (3%)	5 (15%)	>0.05
	Stage III-IV	6 (18%)	21 (63%)	
Nodal involvement	N0-N1	2 (6%)	13 (39%)	0.41
	N2-N3	5 (15%)	13 (39%)	
Vascular invasion	+	2 (6%)	9(27%)	0.57
	-	5 (15%)	17 (51%)	
Lymphatic invasion	+	6 (18%)	6 (18%)	0.005
	-	1 (3%)	20 (60%)	
Distant metastases	+	3 (9%)	4 (12%)	0.27
	-	4 (12%)	22 (66%)	

Table 5. Comparisons of the CD44v6 Tumoral Expression Results with Clinicopathologic Parameters

Clinicopathologic parameters		**CD44 v6 (-) Cases n (%)**	**CD44v6 (+) Cases n (%)**	***P***
Gender	Female	3 (9%)	1 (3%)	0.56
	Male	12 (36%)	17 (51%)	
Histological subtype	Squamous cell carcinoma	7 (21%)	16 (48%)	0.004
	Adenocarcinoma	8 (24%)	1 (3%)	
Stage	Stage I-II	3 (9%)	3 (9%)	>0.05
	Stage III-IV	12 (36%)	15 (45%)	
Nodal involvement	N0-N1	7 (21%)	8 (24%)	0.89
	N2-N3	8 (24%)	10 (30%)	
Vascular invasion	+	3 (9%)	8 (24%)	0.14
	-	12 (36%)	10 (30%)	
Lymphatic invasion	+	9 (27%)	3 (9%)	0.013
	-	6 (18%)	15 (45%)	
Distant metastases	+	4 (12%)	3 (9%)	0.13
	-	11 (33%)	15 (45%)	

parameters; histological subtype, gender, stage, nodal involvement, vascular invasion and development of distant metastases.

Immunohistochemistry results and clinicopathologic parameters for CD44 v6 tumoral expression are presented in Table 5. The only LCC showed positive staining with CD44v6 staining. As for the relationship between clinical stage and CD44v6 tumoral staining, 2 of the stage I patients (100%), 3 of the stage II patients (75%), 11 of the stage III patients (55%) and 4 of the stage IV patients (57%) showed positive staining. There was a statistically significant difference between histological subtypes of SCCs and ACs with respect to CD44v6 (P = 0.004). Also, there was a negative correlation between lymphatic invasion and the extent of CD44v6; lymphatic invasion was significantly less in CD44v6-positive cases (P = 0.013). On the other hand, there was no significant association between CD44v6 and the investigated parameters; gender, stage, nodal involvement, vascular invasion and development of distant metastases. The extent and intensity of the tumoral CD44v6 positivity showed a very strong correlation (Spearman rho = 0.89). Also, increased tumour CD44v6 expression was associated with more marked positivity in the tumour-stroma margin and these parameters showed a strong correlation (Spearman rho = 0.83).

The Spearman correlation test used to investigate the relationship between MMP-2, TIMP-2 and CD44v6 yielded no statistically significant correlation between immunohistochemical staining positivity and intensity (Spearman rho <0.30). Although the expression of MMP-2 and TIMP-2 expressions in the tumour stroma was not statistically significant, MMP-2 was observed in all cases and usually at moderate intensity; whereas TIMP-2 was expressed at a lower frequency (79%) and generally at weak intensity. When the positive results with each marker was investigated, MMP-2-positive tumours showed high frequencies of TIMP-2 (85%) and CD44v6 (58%). TIMP-2-positive tumours showed high frequencies of MMP-2 (85%) and CD44v6 (54%). CD44v6-positive tumours showed high frequencies of MMP-2 (83%) and TIMP-2 (78%). When the expressions of the 3 markers were compared using the chi-square test and Fisher's exact test, no significant results were obtained: MMP-2 and CD44v6 (P = 0.67), CD44v6 and TIMP-2 (P = 0.99), MMP-2 and TIMP-2 (P = 0.14). When the initial and postoperative stages were compared, stage was altered in 20 patients (61%) and remained the same in 13 (39%). The chi-square test and Fisher's exact test yielded no significant differences in the extent of tumoral expression. The probability values were 0.39 for MMP-2, and 0.98 for TIMP-2 and 0.51 for CD44v6. The extent of MMP-2 expression was 85% in the subgroup in which the stage changed postoperatively and 69% in the subgroup in which the stage was not altered. The corresponding values were 76.9% and 80% for TIMP-2, 50% and 62% for CD44v6.

Results of the Survival Analysis

The data on the histological subtype, gender, T status, stage, nodal involvement and development of metastases during follow-up, expression of MMP-2, TIMP-2 and

CD44v6 and survival were investigated. None of the parameters were found to be significantly associated with survival.

Discussion

Researches have provided evidence that MMPs, a family of zinc-containing proteolytic enzymes, facilitate tumour invasion, the establishment of metastases and the promotion of tumour-related angiogenesis. Matrix metalloproteinase inhibitors have been shown to inhibit tumour growth and dissemination in preclinical models.[1] Not all lung cancers express the MMPs believed to be most important in promoting the neoplastic process, and there are different reports about the prognostic importance of MMPs and TIMPs in lung cancer.[1-4]

A review of the studies on metalloproteinases in lung carcinomas concluded that the results are heterogeneous and diverse positivity rates, ranging from 20% to 90%, have been obtained.[1] Passlick et al[2] found a MMP-2 positivity rate of 34% in NSCLCs. Yamamura et al[3] reported MMP-2 expression in 26% of lung carcinomas. Cox et al[9] found immunoreactivity frequencies of 24% in the tumour and 62% in the stroma. Whether tumoral MMP expression is determined by the induction of different stromal factors is controversial. Herbst et al[10] used in situ hybridisation in NSCLCs and reported that the source of the MMP is the fibroblasts and endothelial cells in the tumour stroma. Other investigators reported that in MMP-2-positive tumours, expression is most marked in the tumour-stroma margin.[11]The extent of MMP-2 positivity was 78% in both the SCCs and ACs. Similarly, Passlick et al[2] detected no difference in MMP expression in different histological subtypes of NSCLCs. Studies measuring serum MMP-2 levels also found no differences between subtypes of the NSCLCs.[10,12] In contrast, other studies showed high levels of MMP-2 expression in SCCs[3,9] and ACs.[13] Cox et al[11] reported high levels of MMP-9 in SCCs and LCCs. In the present study, there was no statistically significant association between tumour stage and the extent of tumoral MMP-2 expression. In the literature, there are reports that found no relationship between stage and MMP-2 in NSCLCs.[9,13-15] as well as others that detected a significant association between stage and MMP-2 expression demonstrated by immunohistochemistry and other methods.[2,3,11,12] In the present study, there was no association between nodal status and MMP-2 positivity. Studies investigating the relationship between nodal involvement and MMP-2 used various methods such as immunohistochemistry, and in situ hybridisation, Northern blot, polymerase chain reaction (PCR) and serum level measurements[2,3,9,11,13,14]; however, in accordance with the present study, no significant association was identified. In contrast, in NSCLCs, Albelda et al[4] and Delebecq et al[14] reported that MMP-11 and nodal involvement show significant association with MMP-2. Fujise et al[15] reported that in pulmonary ACs, there is no significant association between tumoral MMP-9 expression and vascular invasion. In the present study, there was no significant association between the development of distant metastases and MMP-2 positivity. This may be ascribed to the small number of patients with distant metastases (n = 7) and the site of the evaluation the primary tumour rather than the metastatic site. In the literature on NSCLCs, there are studies reporting no association between distant metastases and MMP-2 expression demonstrated by immunohistochemistry[2,14] and MMP-9 expression demonstrated by immunohistochemistry and serum level measurement.[15] However, contrasting results have also been obtained for MMP-2 using immunohistochemistry and in situ hybridisation,[3,10] and MMP-11 using Northern blot and immunohistochemistry.[4] In the present study, no association between gender and tumoral MMP-2 level was found. This is in accordance with previous reports.[2,10] In NSCLCs, there was moderately strong correlation between the extent and intensity of TIMP-2 expression (Spearman rho = 0.70); as was the case with MMP-2, it was considered sufficient to use one of these parameters in future studies. The TIMP-2 positivity was 79% in the tumour and 82% in the desmoplastic tumour stroma. Bonomi[1] reported TIMP-2 positivity rates of between 45% and 87% in lung tumours. Iizasa et al[13] reported a TIMP-2 expression frequency of 67% in the tumour cells and 79% in the desmoplastic stroma. Some patients have investigated TIMP-2 expression in NSCLCs in the tumour cells and the surrounding lung tissue and detected higher levels of TIMP-1 and TIMP-2 in the tumour cells in comparison with the surrounding tissue.[4,16] In the present study, there was no statistically significant association between histological type and TIMP-2 extent in the tumour. Similar results have been reported in the literature.[13,16] Albelda et al[4] argued that TIMP-2 is important in the transformation of the metaplastic epithelium to SCC. Similarly, we found no statistically significant association between clinical stage and tumoral TIMP-2 expression. One study using the immunohistochemical method found similar results[13] whereas another found an association between increased serum TIMP-1 level and advanced tumour stage.[16]

Although no association between TIMP-2 and vascular invasion was found in the present study, there was a negative correlation with lymphatic invasion ($P = 0.005$). Studies on vascular and lymphatic invasion in lung carcinomas are limited; no other similar study could be found. That high TIMP-2 expression was negatively associated with lymphatic invasion is an expected finding. In principle, high levels of the “inhibitors” that may participate in both activation and inhibition processes of

MMP-2 will decrease tumoral MMP release, which will lead to decreased degradation of the extracellular matrix and consequently impaired capacity for lymphatic and vascular invasion. Surprisingly, there was no association found between lymph node metastasis and TIMP-2 positivity. In studies investigating the association between nodal involvement and TIMP-2 extent in lung SCCs,[4] high TIMP-2 expression was related to nodal involvement. In the present study on NSCLCs, CD44v6 positivity was found in 52% of the cases. Hirata et al[17] found a lower value of 29%. In the literature on NSCLCs, frequencies of 50% and above were also reported.[18-20] There was a statistically significant association between histological subtype and CD44v6 expression, which was different from MMP-2 and TIMP-2 expression (P = 0.004); Seventy per cent of the SCCs and only 11% of the ACs showed CD44v6 expression. Accordingly, Fasano et al[7] found positivity in 97% of the SCCs but only 10% of the ACs. There are also studies showing large differences in SCCs[19,20] but weak associations.[21,22] In one study, the highest CD44v6 was detected in bronchioloalveolar carcinoma.[18] It appears that CD44v6 may be considered a "specific" marker of SCCs. It has been argued that in pre-neoplastic lung lesions, CD44v6 is very important in tumorigenesis. It is expressed at high levels in squamous metaplasia and dysplasia and at the same time participates in carcinogenesis and neoplastic differentiation.[5,7] In the present study and published literature, there was no association found between tumour stage and the expression of CD44v6, as determined by immunohistochemistry[7,18,21,22] and serum CD44v6 levels.[19] On the other hand, although there was as a significant association between tumoral CD44v6 expression and lymphatic invasion (P = 0.013), there was no association with vascular invasion. Hirata et al[17] found no correlation of CD44v6 with vascular and lymphatic invasion. In contrast, Fasano et al[7] reported that there was an association between lymphatic invasion and increased CD44v6 expression. High levels of CD44v6 expression cause strong adhesion between tumour cells. In the present study, there was no association between distant metastasis and CD44v6 expression. The results of the studies analysing the relationship between CD44v6 and distant metastasis are controversial. Some authors reported that decreased CD44v6 expression was associated with metastasis in NSCLCs.[17,22] In accordance with previous studies, we found no association between CD44v6 expression and gender.[19,21,22]

Studies on metalloproteinases and their inhibitors in NSCLCs are limited. There is only one study that assessed CD44 concurrently with MMP and TIMP in NSCLCs: in that study, CD44 expression was found to be the result of complex set of mediators including prostaglandins and it was emphasised that there were possibly indirect relationships between tumoral MMP and TIMP positivity and CD44 expression in the tumour.[8] In the present study, although the expressions of MMP-2 and TIMP-2 in the tumour and the desmoplastic tumour stroma showed no statistically significant correlation with tumoral CD44v6 expression, most of the tumours expressing MMP-2 also expressed TIMP-2 (85%) and more than half of the MMP-2-negative patients (57%) were also CD44v6-negative. In view of the metastatic process, these are expected findings. The co-expression of MMP-2 and TIMP-2 can be accounted for, because, in addition to its inhibitory role, TIMP also participates as a co-factor in the activation of MMP.[23,24]

If the MMPs, which participate in the surface activation of CD44v6, are not expressed, cell surface-associated CD44v6 will not be activated and it will be difficult to detect the inactive CD44.[8] Although we expected to find a significant association between MMP-2 and TIMP-2 (the number of doubly positive patients was 22), statistical significance was approached but not attained; this may be due to the small number of patients. More extensive and more intense MMP-2 expression in comparison with TIMP are expected in the neoplastic process. As also stated above, the equilibria between MMPs and TIMPs in different organs are diverse. In previously published work, the relationship between MMP and TIMP was usually investigated in the tumour cells and there are studies that did not even include the stromal reaction.[23,24]

In the present study, MMP-2, TIMP-2 and CD44v6, which are thought to affect metastatic potency, did not show any association with distant metastases or nodal involvement. This may be accounted for by the fact that the number of patients with distant metastases was small and measuring only primary tumour may be inadequate for analysis. One study showed that increased simultaneous expression of TIMP-2 and MMP-11 as demonstrated by immunohistochemistry and Northern blot analysis showed positive correlation with metastases, particularly in SCCs.[4] Current studies focus on the relationships between MMPs and the hyaluronate receptor CD44. MMP-9 and MMP-2 activate CD44, increase TGF-β synthesis, and thus participate in cell growth and neoangiogenesis.[25] It has been hypothesised that MMP-2, MMP-9, TIMP and CD44 form a complex; after the necessary matrix degradation is realised by MMP, the tumour cells adhere to the matrix elements via CD44 and in stepwise fashion, achieve migration and metastasis.[20] We have shown that in tumoral CD44v6- and TIMP-2-positive patients, lymphatic invasion was less and each association was statistically significant (P = 0.005, P = 0,013). The patients in whom the stage changed postoperatively and those in whom it remained the same did not differ with respect to immune marker expression (P >0.05). On the other hand, the frequency of MMP-2 expression was 17/20 (85%) in the first group and

9/13 (69%) in the second group. Although the difference did not reach statistical significance, the frequency of the expression was slightly higher in the subgroup whose stage changed postoperatively. To the best of our knowledge, this is the first such study on NSCLCs.

In the univariable analysis on 33 patients, none of the clinicopathological factors – gender, histological subtype, T status, nodal involvement, stage, and expressions of MMP-2, TIMP-2 and CD44v6 – significantly affected survival. In the study by Passlik et al[2] on NSCLCs, increased MMP-2 expression as demonstrated by immunohistochemistry was associated with decreased survival. Herbst et al,[10] using in situ hybridisation, found increased MMP-2/E-cadherin to be associated with poor prognosis. Studies on the effects of TIMPs on the survival of patients with lung carcinomas are limited. High serum TIMP-1 levels were associated with poor prognosis.[1,16] Previous studies on the relationship between CD44v6 and survival of patients with NSCLCs, as well as our study, found no effect on survival; Fukuse et al[22] and Tran et al[20] used immunohistochemistry and Takigawa et al[19] measured serum levels by ELISA.[9,11,14] In contrast, Nguyen et al[21] reported that low CD44v6 level is associated with long survival.

In conclusion, we detected negative correlation between lymphatic invasion and TIMP-2 and CD44v6 tumoral expression when these markers were investigated separately in NSCLCs. On the other hand, no significant correlation was observed in the expression of these markers in common. This findings may suggest the independent effects of these markers in preventing the lymphatic invasion. There are ongoing researches on the MMP inhibitors, but the results from different studies are difficult to be interpreted instantly. Further studies on the the CD44 receptor family, with emphasis on regulatory mechanisms, organ and tissue specific synthesis, interactions with MMP and TIMP will better define the roles of these molecules in tumorigenesis, invasion and metastasis in the NSCLCs. Also our data point to a clear histiotype-related pattern of CD44v6 expression in SCCs.

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