Elevated Levels of Circulating Matrix Metalloproteinase-9 in Non-Small Cell Lung Cancer Patients

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SUMMARY

Elevated levels of matrix metalloproteinase have been implicated as playing important role in tumour progression in several types of cancers. Our aim was to determine whether these enzyme might be a useful tumour marker for lung cancer and also to evaluate the correlation of circulating levels of matrix metalloproteinase-9 (MMP-9) with tumour histology, staging, nodal status, metastasis and prognosis. Blood samples were collected from 35 nonsmall cell lung cancer patients who were diagnosed histologically, and 14 healthy controls. The MMP-9 levels were significantly higher in the cancer group (p< 0.001). However no significant correlation between several clinical features (such as histology of the tumour, staging, tumour status, or nodal status) and plasma MMP-9 levels have been observed. Though it does not show statistical significance, more patients with metastasis seemed to have higher MMP-9 levels. At the end of six month 11 patients were out of follow-up. Among the remaining 24 patients eight patients had lower MMP-9 levels, seven were survivors at the end of six months. Sixteen patients had MMP-9 levels above the threshold. Only 10 have survived to six months. In conclusion MMP-9 can serve as a marker for metastasis and can be valuable in the follow-up of lung cancer patients.

Key Words: *Nonsmall cell lung cancer, MMP-9, survival, prognosis.*

ÖZET

Küçük Hücreli Dışı Akciğer Kanserli Hastalarda Serum MMP-9 Düzeyi

Bazı kanser tiplerinde artmış matriks metalloproteinaz (MMP) seviyelerinin tümör progresyonunda önemli rol oynadığı bildirilmiştir. Amacımız; akciğer kanserinde MMP-9'un tümör belirleyici olarak yararını ve serum MMP-9'un tümörün tipi, evresi, nodal yayılımı, metastazları ve prognozla ilişkilerini değerlendirmektir. Histolojik olarak tanı konulan 35 küçük hücreli dışı akciğer kanserli (KHDAK) ve sağlıklı 14 kontrolün kan örneklerinde MMP-9 seviyeleri ölçüldü. Kanserli grubun MMP-9 seviyeleri anlamlı yüksek bulundu (p< 0.001). Kanser tipi, evresi ve nodal yayılımı gibi klinik bulgular ile serum

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MMP-9 arasında anlamlı ilişki saptanamadı. İstatistiksel anlamlılık bulunamamasına rağmen metastazlı hasta grubunun MMP-9 seviyeleri daha yüksek bulundu. Hastalardan 11'i altıncı ayın sonunda takibimizden çıktı. Kalan hastalardan MMP-9 düzeyleri düşük olan sekizinden yedisi altı ayın sonuna kadar hayattaydı. MMP-9 düzeyi yüksek olan 16 hastadan yalnız 10'u altı ayın sonuna kadar hayattaydı. Sonuç olarak; KHDAK'lı hastaların takibinde metastaz belirleyicisi olarak MMP-9 kullanılabilir.

Anahtar Kelimeler: *Küçük hücreli dışı akciğer kanseri, MMP-9, yaşam süresi, prognoz.*

Lung cancer is a major cause of death from malignant disease. Unfortunately its incidence and prevalance is increasing tremendously and today it is the most common cause of cancer death in Europe and United States of America (USA) (1). Nonsmall cell lung cancer is believed to account 80% of all the lung cancers (2). Therefore to find tumour markers which will be used for screening and diagnostic purposes is a crucial endpoint for cancer researchers however until now there is no tumour marker which has high sensitivity and specificity for nonsmall cell lung cancer. The possibility that matrix metalloproteinase-9 (MMP-9) has potential utility in this regard is very attractive and has been postulated by several authors. Besides knowledge of mechanisms responsible for tumour progression may result in new insights for therapeutic interventions.

Matrix metalloproteinase proteins (MMPs) are extracellular matrix degradative enzymes capable of degrading extracellular matrix proteins including basal membrane components (3). MMPs are expressed in many physiological conditions including embryogenesis and postinjurious tissue remodeling and also in different pathological conditions like arthritis, cancer and osteoporosis (4). MMPs are frequently upregulated in malignant disease where they facilitate tumour growth, angiogenesis and invasion. The elevation of MMP-9 in circulation has been demonstrated in several types of cancers however there are not so many reports revealing the clinical implications of circulating MMP-9 and the correlation of this enzyme by histology and staging of lung cancer (5-8).

Our aim was to determine whether these enzyme might be a useful tumour marker for lung cancer and also to evaluate the correlation of circulating levels of MMP-9 with tumour histology, staging, nodal status, metastasis and prognosis.

MATERIALS and METHODS

Study Population

Thirty-five nonsmall cell lung cancer patients who were diagnosed histologically, and 14 healthy controls were enrolled in the study. Staging of the patients are shown in Table 1. Twenty-two patients were diagnosed as squamous cell carcinoma and seven as adenocancer. Patients suffering from osteoporosis, asthma arthritis and acute infection were not included in the study, since this could contribute to high MMP-9 levels. Blood samples were drawn before therapy. Healthy subjects with no history of any infectious disease in the last three months had been included.

Histological type, pathological stage and tumour-node metastasis classification were classified according to the criteria of the American Joint Committee on cancer (9).

At the 6th month after the therapy the patients were evaluated.

Method of The Assay

After the sample was taken it was centrifuged. The sera was separated and refrigerated until assayed. Samples were diluted by adding 10 µL of serum into 500 µL of wash buffer. Microtitration strips were transferred to a strip frame. 100 µL of monoclonal anti-MMP-9 antibody were pipetted into wells. The plate was incubated for 60 minutes at room temperature with gentle shaking at 900 rpm. The wells were washed three times with 300 µL of diluted Wash buffer.

100 µL of standards and diluted samples in duplicates were pipetted into the wells. The plate was incubated for 60 minutes at room temperature with gentle shaking. The wells were washed for three times with 300 µL of diluted wash buffer. 100 µL of polyclonal anti-MMP-9 antibody

Table 1. Mean and median MMP-9 levels according to staging and TNM status.

	# of patients	Mean ± SD	Median	Minimum-maximum
Stage				
IB	4	165.4 ± 150.73	94.35	81.6-391.3
IIA	1	86.7		
IIIA	5	88.68 ± 35.63	76.5	61.2-150.15
IIIB	9	258 ± 336.33	91.8	56.1-1066.3
IV	16	183.29 ± 199.58	129.03	58.65-900.5
T				
1	2	80.33 ± 16.23	80.33	68.85-91.8
2	9	137.6 ± 104.33	86.7	66.3-391.3
3	10	113.53 ± 51.14	96.9	61.2-232.52
4	14	279.43 ± 322.53	143.93	56.1-1066.3
N				
0	7	151.93 ± 112.88	96.9	81.6-391.3
1	2	563.75 ± 710.71	563.75	61.2-1066.3
2	26	163.67 ± 178.14	91.8	56.1-900.5
M				
0	20	184.87 ± 238.83	89.25	56.1-1066.3
1	15	183.27 ± 206.59	117.3	58.65-900.5

were pipetted into all wells and then the plate was incubated for another 60 minutes. The wells were washed three times with 300 µL of diluted Wash buffer. 100 µL of HRP-Anti-Chicken IgG was added into wells. The plate was again incubated for another 60 minutes. And the wells were washed three times with 300 µL diluted Wash buffer. 100 µL of just prepared substrate solution was pipetted into all wells and incubated 20 minutes in dark.

25 µL of Stop Reagant was pipetted into all wells. The contents of the wells were mixed thoroughly and the absorbance for each standard and sample were read at 490 nm wavelength. The mean absorbance were plotted. The values for each unknown sample were read from the standard curve in µg/L. The values were multiplied by the dilution factor.

Statistical Analysis

Comparison of MMP-9 levels between the healthy subjects and cancer patients was evaluated by Mann-Whitney U test. Correlations between patients' cancer histology, nodal or metastasis status with plasma concentrations of MMP-9 were evaluated with χ^2 test. $p < 0.05$ was considered to be statistically significant.

RESULTS

The plasma MMP-9 concentration was 71.06 ± 31.13 (minimum-maximum: 40.80-132.60) in the healthy controls and 184.19 ± 222.37 (minimum-maximum: 56.10-1066.30) in the lung cancer patients. This difference was statistically significant ($p = 0.001$). The mean ± SD and median levels with the minimum and maximum levels in each group are shown in Table 1. Stage IIIB and IV patients had higher MMP-9 levels. Also patients with higher tumour status had higher MMP-9 levels as shown in Table 1.

We wanted to evaluate whether there was any difference according to tumour histology, tumour status, nodal status, metastatic status. We determined the normal range of plasma MMP-9 concentration as 53.09-89.04 µg/L (95% confidence interval). The upper limit is taken as a threshold. A total of 21 (60%) patients demonstrated MMP-9 levels above the upper limit of the normal range. No significant correlation between several clinical features (such as histology of the tumour, staging, tumour status, or nodal sta-

tus) and plasma MMP-9 levels have been observed. However though it does not show statistical significance, more patients with metastasis seemed to have higher MMP-9 levels as shown in Table 2.

Six month follow-up: At the end of six month 11 patients were out of follow-up. Among the remaining 24 patients eight patients had lower MMP-9 levels, seven were survivors at the end of six months. Sixteen patients had MMP-9 levels above threshold. Only 10 have survived to six months.

DISCUSSION

After neoplastic transformation tumour-host interactions promote coordinated molecular and cellular processes. Cancer researchers has directed considerable interest toward outlining the cascade of events. MMPs are associated with degradation of extracellular membrane. When it was first described it was believed that MMPs were responsible for tumour invasion, entry and exit of tumour cells from the circulation and local migration at metastatic sites via the damage of the basement membrane. It is now recognised that MMPs are not solely responsible for the changes mentioned above but also they are important for maintaining and creating a microenvironment facilitating angiogenesis and growth of the tumour (10).

Table 2. Correlation between several clinical features and circulating MMP-9 levels.

	< 89.04	> 89.04	p
Stage			
IB	1	3	> 0.05
IIA	1	0	
IIIA	4	1	
IIIB	4	5	
IV	4	2	
T			
1	1	1	> 0.05
2	5	4	
3	3	7	
4	5	9	
N			
0	2	5	> 0.05
1	1	1	
2	11	15	
M			
0	10	10	> 0.05
1	4	11	
Histologic cell type			
Squamous cell cancer	12	16	> 0.05
Adenocancer	2	5	

The role of circulating MMP-9 in cancer patients is controversial. Zucker et al, did not observe the elevation of MMP-9 activity in the plasma of patients (11). Hrabec et al, have observed that the levels of serum or plasma MMP-9 in lung cancer patients were significantly higher than those of healthy subjects (3). In our study cancer patients had significantly higher MMP-9 levels compared to control patients.

In order to evaluate the source of circulating MMP-9 levels in our lung cancer patients we evaluated the correlation of serum levels with staging. The levels were higher in stage intravenous (IV) patients however this did not reveal any statistical significance. Therefore we performed another analysis regarding tumour status, nodal status, and metastatic status. Nodal status was not suggestive. But metastatic group had more patients with higher MMP-9 levels though this was not statistically significant. MMPs have been reported as one of the type IV collagenases which destroys the basement membranes, which may be the first barrier to tumour metastasis. There are some reports suggesting that the expression of MMPs may play crucial role in tumour cell invasion and metastasis in several cancers (4). Expression of MMP-9 has been detected in osteoclasts which suggests that MMP-9 plays a role in bone resorption and is also involved in tissue destruction (12). This may suggest whether the circulating MMP levels be a marker for tumour metastasis. Arkona et al have shown that tumours metastasized to bone expressed high levels of MMP-9 (13). In Ylisirnio's study patients with bone metastasis presented higher plasma and serum levels of MMP-9. This is speculated as the possibility that high expression of MMP-9 in tumour tissue or in the lytic process in bone metastasis could reflect the higher levels (14).

In our study patients with T4 status seems to have higher MMP-9 levels compared to other groups though this was not statistically significant.

lizasa et al have found out that the concentration of plasma MMP-9 was not associated with the expression of MMP-9 in tumour samples or with tumour size (4). We believe that our result needs to be confirmed with further studies.

The contribution of the histological type to circulating MMP-9 levels remains controversial also. Iizasa et al have shown that MMP-9 levels were higher in large cell and squamous cell cancer and the plasma concentrations of MMP-9 decreased to levels within the normal range 4-8 weeks after tumour resection. In our study there was no statistically significant difference among the groups in terms of histology. However the small sample size in the adenocancer group may be a factor.

Several reports have observed that MMP-9 levels were associated with the clinical course of the disease. The prognostic value regarding the serum levels in lung cancer needs to be defined also. In our study the overall mortality of patients with high serum levels were higher but this did not reach statistical significance. In the six-month follow-up among eight patients who had lower MMP-9 levels, seven were survivors at the end of six months. Immunoexpression of MMP-9 is reported to be associated with a worse outcome than lack of expression in T1 lung adenocancer. Besides Kodate et al have shown that patients with lung cancer expressing MMP-9 showed unfavorable survival when compared to patients with tumours negative for MMPs (15).

In conclusion, elevated plasma MMP-9 levels were observed in 60% of the cancer patients compared with healthy controls. Our results reveal that staging, nodal status, tumour status or histology do not correlate with the circulating MMP-9 levels. However our data shows that metastatic group had more patients with higher MMP-9 levels and also circulating. MMP-9 levels may have prognostic value. These promising findings need to be confirmed with further studies with larger sample size since the identification of the mechanisms of the MMPs may lead to a better comprehension of the natural history of lung cancer leading to new insights for therapeutic modalities.

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