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ORIGINAL ARTICLE

Serum level changes of matrix metalloproteinases 2 and 9, vascular endothelial growth factor and epidermal growth factor receptor during platinum-based chemotherapy in advanced non-small cell lung cancer patients

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Summary

Purpose: *To evaluate serum changes of matrix metalloproteinases (MMPs) 2 and 9, vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) levels in patients with advanced non-small cell lung cancer (NSCLC) and their association with main clinicopathological parameters during chemotherapy with cisplatin and gemcitabine.*

Patients and methods: *In this prospective study, consecutive patients with stage III and IV NSCLC were enrolled. Serum MMP2 and 9, VEGF and EGFR levels were monitored in blood samples taken on day 1 of starting chemotherapy (baseline 1st), and after 3 cycles of chemotherapy (2nd) using commercial sandwich ELISA method.*

Results: *116 patients were evaluated. Males / females 100 / 6, ECOG performance status (PS) 0/1/2: 47/65/4, stage III / IV: 49/67, squamous /adeno/large cell carcinoma 41/31/19. Forty-two (36%) patients achieved partial response (PR), 32 (28%) stable disease (SD) and 42 (36%) showed progressive disease (PD). Mean serum values ± standard deviation (SD) of the analyzed markers at baseline/at response evaluation were: EGFR 86±87/96±47 fmol/ml; MMP9 236 ± 156/162 ± 133 ng/ml ; MMP2 525 ± 189/569 ± 201 ng/ml; VEGF 555±476/599±611 pg/ml; VEGF adjusted for platelets (PLT) 1.9 ± 1.45/2.4 ± 2.78 pg/10^6. In logistic regression model for response rate adjusted for stage, the increase in MMP9 levels during chemotherapy (mean = 74 ng/ml ± SD 140) was predictive for progression (p=0.041) with 5% increase in the odds of progression for an increase of 10 ng.*

Conclusion: *MMP9 level increase was found to be predictive of disease progression. EGFR levels could reflect extracellular domain (ECD) loss from resistant cells and its shedding into the circulation.*

Key words: advanced non small cell lung cancer, chemotherapy, epidermal growth factor receptor, metalloproteinases 2 and 9, vascular endothelial growth factor

Introduction

Lung cancer is a biologically aggressive tumor and despite advances in treatment, survival rate is still unsatisfactory. This is probably due to the complex biological background of this disease. Chemotherapy is still the milestone of treatment of advanced NSCLC, but now the benefits of currently used regimens have reached a plateau. The molecular approach to the treatment of lung cancer is being developing, but the initial results of combining targeted therapies with chemotherapy are rather disappointing. Recently published clinical trials of adding antiangiogenic and anti-EGFR therapies to chemotherapy resulted in improved patient outcome with higher benefit for selected patient populations. It might be that chemotherapy causes tumor cells phenotype modifications with some impact on

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disease biological profile and on treatment clinical outcomes. The hypothesis that angiogenic factors production and their changes during chemotherapy might reflect the clinical behavior of the disease is based on the knowledge that tumor growth and metastasis are angiogenesis-dependent [1]. The circulating levels of angiogenic factors could reflect the overall angiogenic activity of the tumor [2], but since they are multifunctional proteins their clinical significance is still unclear.

We focused on MMP2 and 9, VEGF and EGFR, which are the most important molecules involved in angiogenesis. MMPs belong to the endopeptidases family, capable of degrading extracellular matrix. MMP2 and MMP9 degrade type IV collagen of the basement membrane. Their enhanced expression and/or secretion has been related to tumor progression and poor prognosis [3]. In patients with NSCLC elevated serum levels of MMP2 and 9 correlate with advanced disease [4] and poor survival [5].

VEGF is a key regulator of angiogenesis, encoded in 7 molecular isoforms; VEGF 165 is the predominant secreted isoform [6]. Some studies revealed an association between circulating VEGF levels and patients' outcome [7]. VEGF could influence treatment effectiveness through modulation of drug delivery and chemotherapy-induced apoptosis; high VEGF levels are associated with both response [8-10] and resistance to chemotherapy [11, 12].

EGFR is a 170-kDa transmembrane glycoprotein, involved in the process of cancer cells proliferation and their protection from apoptosis. Activation of EGFR-signalling pathway promotes angiogenesis including MMPs and VEGF overexpression [13]. The short form of EGFR (soluble EGFR) which can scavenge EGF, has also been found in some cancers [14]. Data concerning serum levels of EGFR in NSCLC are contradictory [15] and their clinical utility remains an open question.

One of the most widely used first-line chemotherapy regimens in advanced NSCLC is cisplatin and gemcitabine combination [16]. We hypothesized that the presence of high amount of angiogenesis-related factors in circulation and/or their increase during chemotherapy could represent a resistant tumor phenotype based on escaping apoptosis. In fact, Holmgren et al. [17] demonstrated that tumors exhibiting marked angiogenesis are resistant to apoptosis.

The aim of this study was to evaluate the serum changes of MMP2, MMP9, VEGF and EGFR levels during chemotherapy in patients with advanced NSCLC.

Patients and methods

Patient selection and study design

Previously untreated NSCLC patients with locally advanced and metastatic disease entered this prospective observational study. Eligible were patients with histologically or cytologically proven NSCLC, with normal renal and hepatic functions and no previous cancer diagnosis. Patients received gemcitabine 1250 mg/m^2 diluted in 300 ml N/S as 30 min infusion on days 1 and 8 in combination with cisplatin 80 mg/m^2 diluted in 500 ml N/S as 60 min infusion with adequate hydration and forced diuresis on day 2. All patients received antiemetic premedication with granisetron and dexamethasone in standard doses. Patients treated with blood transfusions, recombinant erythropoietin or low-molecular weight heparin (LWMH) were excluded from the study. Pretreatment evaluation included medical history and physical examination with evaluation of the ECOG PS score, complete blood cell count, serum biochemistry, ECG and computed tomography (CT) scan of the thorax and abdomen. Written informed consent was obtained from each patient before entering the study.

The clinical stage and patient response were defined according to the accepted standard criteria [18,19].

Response to chemotherapy was assessed by the RECIST criteria after 3 cycles. Blood samples were collected as follows: baseline-before chemotherapy (1st) and at the time of response evaluation (2nd; after the 3rd cycle) to assay the serum biological factors levels. The difference (Δ) between serum concentrations of biological factors obtained at the time of response evaluation and baseline was also determined.

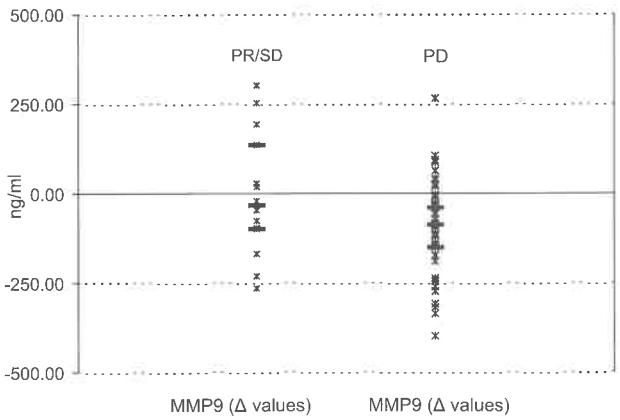


Figure 1. Difference (Δ) between MMP9 levels at baseline and at the time of response evaluation, PR: partial response, SD: stable disease, PD: progressive disease

Methods

Ten millilitres of venous blood were taken after overnight fasting from patients before starting chemotherapy and were centrifuged within 30 min from sampling at 2500 RPM for 10 min at 4° C. The supernatants were aliquoted and stored at –80° C until analysis.

Serum samples were analyzed for MMP2, MMP9, VEGF and EGFR by quantitative enzyme-linked immunosorbent assay (ELISA) technique. All samples were measured in duplicate. MMP2 and MMP9 serum levels were assayed using commercially available ELISA Kit (BIOTRAK™, Amersham Pharmacia Biotech), that provides a simple, specific and precise quantitative determination of the total levels of the active form in the sample. The concentration of total active MMP2 and MMP9 in a sample was determined by interpolation from a standard curve [20]. The sensitivity of the assay for MMP2 and MMP9 was 0.19 ng/ml and 0.125 ng/ml, respectively. The intra and inter-assay coefficients of variation for MMP2 and MMP9 were 7.0 %, 12% and 4.3%, 10%, respectively.

VEGF serum concentration was determined with Quantikine™, Human VEGF Immunoassay (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The lower detection limit was 9.0 pg/ml. The intra and inter-assay coefficients of variation were below 8%. To correct for variation in PLT counts among patients, the concentration of VEGF per PLT (pg/10^6) was calculated by dividing the serum VEGF concentration (pg/ml) by the PLT count $\times 10^6$/ml [21].

Levels of EGFR ECD were assayed using a mouse monoclonal capture antibody against EGFR precoated onto a microtiter plate and a biotinylated detector antibody against EGFR, according to the recommendation of the manufacturer for human EGFR quantitative ELISA assay KIT (Oncogene Research Product; CN Bioscience Inc). The minimum detectable level was 0.3 fmol/ml and the intra and inter-assay coefficients of variation were below 10%.

Statistical analysis

The distributions of the studied parameters in each group and by selected clinical characteristics were compared with Wilcoxon test for continuous and with the Mantel-Haenszel test for categorical variables. The correlations among biological, clinico-pathological and haematological factors were assessed with the Kendall Tau Correlation Coefficient.

The relationships among each of the baseline serum level (1st), at the time of response evaluation (2nd), as well as their changes during chemotherapy (Δ = 2nd - 1st) and response, have been evaluated using a logistic regression model, adjusting for stage. The results were reported as Odds Ratio (OR) estimates with the corresponding 95% confidence intervals (95% CIs). An OR of 1 denoted no evidence of different response between the two groups, whereas an OR >1 or <1 denoted, respectively, an increased or decreased risk of not responding in a given patients' group with respect to the reference one. Overall survival (OS) was defined as the time from pathological diagnosis to death from any cause; patients known to be still alive at the time of analysis were censored at the time of their last contact. Progression-free survival (PFS) was defined as the time from pathological diagnosis to the first appearance of PD or death from any cause; patients known to be alive and without PD at the time of analysis were censored at the time of their last contact. Kaplan-Meier curves for OS and PFS were compared using the Mantel-Cox version of the log-rank test [22]. Linear proportional hazards regression model [23] was also used for PFS and OS to estimate the relative hazards (HR) for each variable, while adjusting for all the others. All analyses were done on an intention-to-treat basis. All p-values were two-sided. Analyses were carried out using SAS software Version 8.20.

Results

Patient characteristics

From January 2002 to September 2003, a total of 116 patients were prospectively enrolled at the Division of Medical Oncology, Policlinico Monteluce, Perugia, Italy. Characteristics of the eligible patients are provided in Table 1.

The vast majority of the patients were male (86 %) with 60% having ECOG PS > 0. Their median age was 63 years (range 25-79). Squamous cell carcinoma was the most frequent histological subtype (35%). Forty-nine (42%) patients had locally advanced disease (stage IIIA+IIIB), and 67 (58%) patients had metastatic disease (stage IV). The prevalent metastatic site was bone (16%), adrenals (14%) and liver (11%).

The mean dose of cisplatin + gemcitabine administered for 1st / 2nd / 3rd cycles was 90/85/75%, respectively. Five (4%) patients continued with gemcitabine monotherapy after the 1st cycle. Forty-two (36%) patients achieved PR, 32 (28%) SD and 42 showed (36%) PD. Sixteen (14%) patients who died before response evaluation were included in the analysis as progressed.

Table 1. Association between pretreatment patient characteristics and response rate

Patient data	*Total number of patients*		*Responders*		*Non-responders*		*p-value*
	n	*%*	*n*	*%*	*n*	*%*	
Age (years)							
≤ 63	52	45	27	52	25	48	0.016
> 63	64	55	47	73	17	27	
Sex							
Male	100	86	64	64	36	36	0.9
Female	16	14	10	63	6	37	
Smoking status							
Smokers	107	92	67	63	40	37	0.39
Non-smokers	9	8	7	78	2	22	
ECOG PS							
0	47	40	37	79	10	21	0.005
1+2	69	60	37	54	32	46	
Weigth loss							
No	103	89	68	66	35	34	0.15
Yes	13	11	5	40	8	60	
Stage							
IIIA+IIIB without pleural effusion	33	28	24	73	9	27	0.21
IIIB with pleural effussion+IV	83	72	50	60	33	40	
Histology							
Squamous cell	41	35	26	35	15	36	
Adeno	31	27	18	25	13	31	0.85
Large cell	19	16	15	20	4	9	
NOS	25	22	15	20	10	24	
Hb (g/dl)*							
≤ 13.5	58	50	31	54	26	46	0.045
>13.5	59	50	43	73	16	27	

NOS: not otherwise specified; *median value

Six (5%) patients with stage IIIA and 3 (2%) with IIIB underwent surgery after chemotherapy. Twenty-two (55%) patients with PD shifted to taxane-containing 2nd line chemotherapy.

All 116 patients were available for serum analyses at baseline. Mean serum values ± SD at baseline were: MMP9 236 ± 156 ng/ml; MMP2 525 ± 189 ng/ml; VEGF 555 ± 476 pg/ml; VEGF/PLT 1.9 ± 1.45 pg/10^6 and EGFR 86 ± 87 fmol/ml. Mean values of serum biological markers at response evaluation (2nd) and the difference (Δ) between 2nd and 1st evaluation are shown in Table 2.

Relationships between clinical-pathological factors and baseline serum levels of biological factors

A significant association (p=0.04) between MMP9 and patient ECOG PS was observed. Patients with PS=0 had lower MMP9 levels compared to those with PS >0. MMP9 was positively correlated with white blood cells (WBC) (r=0.19; p=0.0019) and negatively with MMP2 (r = – 0.12; p=0.048). A significant association between MMP2 and patient sex was found (p=0.04); females had higher levels. Positive correlations between VEGF and PLT as well as WBC (r=0.20 p=0.009; r=0.17 p=0.007, respectively) were observed. Moreover, patients with cardiovascular comorbidities were found to have elevated baseline VEGF/PLT pg/10^6 levels (p=0.052).

No relationship between serum levels of the biological markers and other clinical-pathological factors analyzed (age, smoking, weight loss, stage and histology) was observed.

Biological factors, pretreatment parameters, response to chemotherapy and survival

Out of all pretreatment patient parameters studied, statistical significance for response to chemotherapy was found for patient age (≤ 63 vs. > 63 years;

Table 2. Predictive role of biological factors to chemotherapy response

Biological factor	*At response evaluation (2nd)*			*2nd evaluation adjusted for stage*		*Δ evaluation**			*Δ evaluation* adjusted for stage*	
	Patients, n	*Mean*	*SD*	*OR (95% CI)*	*p*	*Patients, n*	*Mean*	*SD*	*OR (95% CI)*	*p*
MMP2 ng/ml	70	569	201	0.97 (0.94-1.01)	0.11	70	70	237	0.99 (0.96-1.02)	0.45
MMP9 ng/ml	68	162	133	1.03 (0.99-1.08)	0.13	68	–74	140	1.05 (1.00-1.10)	0.041
EGFR fmol/ml	62	96	47	0.87 (0.76-1.01)	0.06	61	– 1.7	113	0.98 (0.93-1.03)	0.39
VEGF pg/ml	76	599	611	1.00 (1.00-1.01)	0.24	76	109	591	1.00 (0.99-1.01)	0.44
VEGF/PLT pg/10^6	70	2.4	2.78	1.23 (0.94-1.35)	0.21	70	7.4	38	1.01 (0.98-1.15)	0.88

*Δ: difference between 2nd - 1st; SD: standard deviation; OR: odds ratio; CI: confidence interval

p=0.016), ECOG PS (0 vs. 1+2; p=0.005) and baseline Hb levels (≤ 13.5 vs. > 13.5 g/dl; p=0.045) (Table 1).

Baseline serum levels of all biological factors studied were not associated with chemotherapy response and survival. The results of logistic regression models for response rate adjusted for stage are shown in Table 2. MMP9 Δ value (mean = –74 ng/ml ± SD 140; range -395 to 304) was predictive for no response (p=0.041): for each increase of 10 ng with respect to baseline value a 5% increase in the odds of progression was seen (Figure 1). P-value for EGFR levels at 2nd evaluation was equal to 0.06 for no response, while VEGF and MMP2 were not predictive for response to chemotherapy (Table 2).

At a median follow-up of 11.1 months, 84 patients progressed and 62 patients died; the estimated median survival was 10.3 months (range 1-33).

The disease stage was the only clinico-pathological parameter that reached statistical significance for both PFS (HR =2.2; 95% CI: 1.3-3.7; p=0.0034) and OS (HR=3.2; 95% CI: 1.7-6.2; p=0.0004). Even after adjusting for stage none of the angiogenesis-related factors were found to have prognostic role for patients' survival.

Discussion

To our knowledge, this is the first study in advanced NSCLC where the serum levels of MMP2, MMP9, VEGF and EGFR, pre-treatment and at chemotherapy response evaluation, were simultaneously investigated. Our attempts were to identify the reliability of serum angiogenesis-related markers in order to explain if the response to chemotherapy is dependent on changes in the angiogenic status of the tumor. Study results showed that increase of MMP9 serum levels during chemotherapy was predictive for chemoresistance.

The activation of angiogenesis by MMP9 would explain the inhibited apoptosis [17]. In fact, cytotoxic chemotherapy causes endothelial cell apoptosis which precedes tumor cell apoptosis [24]. Thus, it could be that increased MMP9 levels might contribute in inhibition of endothelial cell apoptosis [25], rending cytotoxic chemotherapy less efficient.

Our results have shown a statistically significant association between ECOG PS and pretreatment MMP9 serum levels, with higher MMP9 levels in patients with worse PS, an observation not reported in other studies. The negative correlation found between baseline serum levels of MMP2 and MMP9 is probably due to their different role in advanced NSCLC. To the best of our knowledge this is the first time where a different role of MMP2 and MMP9 in advanced disease is being reported. Based on our observation, a biological hypothesis to explain why targeted therapy that blocks both MMPs has resulted in unsatisfactory clinical outcomes could be developed. Indeed, in a phase III trial the addition of prinomastat to standard chemotherapy showed no survival advantage in advanced NSCLC [26].

The significantly elevated MMP2 levels in female patients could be explained by the role of MMP2 in physiological breast remodelling [27].

At the time of response evaluation serum EGFR levels showed some evidence of association, although not statistically significant, meaning that chemoresistant disease could be associated with an elevation of serum EGFR. Trials adding EGFR tyrosine kinase inhibitors (TKIs) to chemotherapy in non-selected patients were negative on all survival endpoints [28]. At present, analysis of EGFR mutations appears to be the most promising means of predicting sensitivity to EGFR TKIs [29].

Chemotherapy alters EGFR expression [28] and cancer cells with activated EGFR pathway are more chemosensitive, but it is unknown how EGFR expression is correlated with ECD serum levels. Thereby we could speculate that ECD levels probably could reflect EGFR loss from resistant cells and its shedding into the circulation.

Chemotherapy response was dependent on patients' age, ECOG PS and baseline Hb levels. The role of these pretreatment characteristics have been already reported as predictors of response in patients with advanced NSCLC [30]. Anaemia causing tumor hypoxia is associated with poor response, even if our cut-off value for Hb levels was higher than the usually accepted laboratory norms. This could be the reason for the lack of an association between Hb and VEGF levels as we would expect.

The baseline VEGF serum levels correlated positively with PLT and WBC, which is in concordance with the finding that PLT scavenged circulating, presumptively tumor-derived VEGF [21]. Considering this and gemcitabine side effects causing both thrombocytosis and thrombocytopenia [31] we used VEGF/PLT pg/10^6 concentration. The association between the presence of cardiovascular comorbidities and VEGF/PLT has already been reported [32]. In a subgroup of NSCLC patients with normal PLT counts, Brattström et al. [33] found that elevated serum VEGF levels measured after radiotherapy were associated with better prognosis.

Up to now the data concerning a predictive role for VEGF and MMPs levels are conflicting. Kido found in 9 small-cell lung cancer (SCLC) and 20 NSCLC patients that baseline serum VEGF did not differ between responders and non-responders [9]. On the contrary, Salven et al. [10] reported that high pretreatment VEGF serum levels were associated with poor chemoresponse and shorter survival in SCLC patients. Similar results were observed by Gabrisa et al. [4] monitoring serum levels of MMP2 in 7 patients with SCLC. Lissoni et al. [8] showed a normalization of VEGF serum levels in advanced NSCLC and colorectal cancer patients who responded to chemotherapy, but not in non-responders. In our previous study [12] VEGF tissue expression was weakly correlated with regulators of apoptosis like BCL-2 and RB, while Volm et al. [11] concluded that the angiogenic factors are inversely correlated with chemoresistance in NSCLC. In the positive randomized phase II and II/III studies of adding the anti-VEGF bevacizumab monoclonal antibody to chemotherapy improved patients' outcomes in the combined arm were observed [34], but no correlation between pretreatment and on-treatment VEGF levels and response rate and survival were found [35].

Possible explanations of these conflicting results are: first, the lack of standard method of measuring VEGF tissue expression and/or VEGF circulating levels; second, the small numbers of evaluated patients do not permit catching significant difference; third, the capability of cancer cells to produce alternative pro-angiogenic factors during treatment; and fourth, the multifunctional nature of those angiogenic factors and the type of chemotherapy might play a role.

In our study pretreatment levels of VEGF, MMP2, MMP9 and EGFR were not associated with disease stage, histology and survival. In other studies some of those biological factors were correlated with patients' outcome, but usually heterogeneous groups of operated and advanced NSCLC patients or only patients with early disease were enrolled [3,5,30]. Laack et al. [5] reported serum MMP9 levels as an independent prognostic in 118 NSCLC, where 50 patients had early-stage disease.

In conclusion, increasing levels of MMP9 during treatment were found predictive of disease progression. EGFR levels could reflect EGFR loss from resistant cells and its shedding into the circulation. The changes in serum levels of angiogenic factors during treatment could reflect their different role in chemotherapy-induced apoptosis, but further data are required to elucidate these multifunctional interactions in advanced NSCLC patients.

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