Pre-treatment gelatinases’ serum levels and post-treatment changes in laryngeal cancer patients

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Abstract
Introduction: Laryngeal cancer, especially in the advanced stages, is a highly devastating disease, characterized by increased invasiveness and high rates of metastasis. Gelatinases A and B (MMP-2 and -9 respectively) are of particular interest due to their contribution to various stages of carcinogenesis. There is a growing body of evidence with regard to the prognostic value of certain MMPs and their possible role as tumour markers.

Aim: To identify the pattern of alteration of serum gelatinases A and B in patients with laryngeal cancer following treatment, and a possible correlation with various clinicopathological parameters.

Materials and methods: Forty nine patients were included in this study. Pre-treatment and post-treatment serum samples were collected and processed by gelatin zymography and western blotting.

Results: Only the latent forms of MMP-2 and -9 were identified. Both gelatinases were increased in the serum of laryngeal cancer patients compared to healthy individuals. Patients with supraglottic tumours and active smokers had significantly higher pre-treatment levels of proMMP-2 than patients with glottic tumours (p < 0.05) and ex-smokers (p < 0.05), respectively. Patients with primary disease and patients with lymph node involvement showed lower serum proMMP-9 pre-treatment levels than patients with recurrence (p < 0.05) and patients without neck disease (p < 0.1), respectively. During the follow-up period the proMMP-2 serum levels increased significantly in the first ten to fifteen days after treatment, gradually decreasing over the following months. The proMMP-9 serum levels showed a gradual decrease after treatment, which was statistically significant (p<0.05).

Conclusions: The post-treatment alteration pattern of proMMP-9 serum levels shows a possible role of this molecule as a tumour marker in laryngeal cancer. Further research is necessary to clarify the contribution of both gelatinases to the disease progress and determine their role as prognostic factors and tumour markers. Hippokratia 2013; 17 (3): 220-227

Keywords: MMP-2, MMP-9, matrix metalloproteinases, gelatinases, serum, head and neck cancer, laryngeal cancer

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Introduction
Head and neck cancer is the sixth most common cancer in the world, accounting for about 300,000 deaths every year1. Laryngeal cancer accounts for about 1.2% of all cancers and 1.1% of all deaths due to cancer, with about 150,677 new cases in a year [2.2 ASR (W): Age-world-standardized incidence rate] and a ratio of men to women of 6.2:1 worldwide2. Laryngeal cancer appears most commonly in glottis (60%) and supraglottis (30%), the vast majority being squamous cell carcinoma. The 5-year survival rate of head and neck cancer patients varies in Europe (26 to 63%) depending on the subsite3. Especially for laryngeal cancer, survival rates depend on the anatomical site and stage of the disease. The five-year survival rate is 59-90% in stage I cancer and decreases at higher stages to 34-59%, 44-74% and 32-56% for supraglottic, glottic and subglottic cancers respectively4.

The major risk factors for head and neck cancer are tobacco smoking and alcohol consumption5. Additional risk factors include human papillomavirus (HPV) infection6, passive smoking7, low body mass index8, poor diet9, and family history of cancer10.

The regular post-treatment follow-up of laryngeal cancer patients is based on clinical examination and imaging. It is critical to identify a locoregional recurrence as early as possible, as the advanced disease is characterized by increased invasiveness and high rate of metastasis. The lack of a reliable tumour marker renders the identification of such a molecule highly desirable. Nevertheless, recent research has shown that matrix metalloproteinases (MMPs) may play a role towards that direction.

MMPs are a family of zinc-dependent extracellular enzymes capable of degrading many extracellular matrix (ECM) proteins11. One particular group of MMPs,
the matrix metalloproteinase-2 and -9 (structure group: gelatin-binding) known as 72 and 92 kDa type IV collagenase, respectively, is of particular interest with respect to the development and progression of cancer. Elevated levels of serum or plasma MMPs have been found in a variety of malignant tumours, such as breast cancer\textsuperscript{12}, colonic cancer\textsuperscript{12}, lung cancer\textsuperscript{13}, head and neck squamous cell carcinoma\textsuperscript{14}, hepatocellular carcinoma\textsuperscript{15}, and gastric cancer\textsuperscript{16-18}. Proteolytic activity of MMP-9 is overexpressed in serum of gastric cancer in stages 3 and 4\textsuperscript{19}.

Several studies have shown methodological issues affecting the determination of MMPs\textquotesingle activities\textsuperscript{20-22}. Many commercial kits, such as ELISA, fluorometric, and zymographic methods are being used for the determination of MMPs in blood and urine. Serum samples have increased MMP-9 levels, as compared with plasma samples. The higher MMP concentrations that are commonly found in serum probably result from MMPs release by platelets or leukocytes during platelet activation or during the process of collection\textsuperscript{23}. MMP-9 levels in serum correlate with plasma MMP-9 levels, even though serum samples have artificially higher MMP-9 levels\textsuperscript{24}.

In this study, we examined the presence of gelatinases (MMP-2 and MMP-9) in the serum of patients with laryngeal cancer by SDS-PAGE zymography and we investigated a possible correlation between MMPs and tumour stage, tumour grade, anatomical site, lymph node metastasis, type of disease (primary or recurrence), lifestyle of patients and treatment modality. Furthermore we aimed at identifying the pattern of changes observed in the serum following treatment, in association with the aforementioned clinicopathological parameters.

Materials and Methods

Patients

Forty-nine patients from the Academic Otolaryngology Departments of the University Hospital of Patras and of AHEPA University Hospital of the Aristotle University of Thessaloniki were included in the study, the disease characteristics of which are shown in Table I. Serum samples were planned to be obtained on the day of histological diagnosis (pre-treatment levels), ten to fifteen days post-treatment, and then every month up to one year post-treatment. Eight healthy volunteers, of the same age range, were also included. The study design was approved by the Ethical Committee of the University Hospital of Patras and was in accordance with the Helsinki Declaration of 1975, as revised in 1983.

Gelatin zymography

Gelatinolytic activities were detected in SDS-polyacrylamide gels (T: 10%, C: 2.7%), containing 1 mg/ml gelatin (Sigma, USA). Samples from each serum (1 μl) were mixed with 10 μl of 0.0625 M Tris-HCl pH 6.8, containing 2% (w/v) SDS and 10% (v/v) glycerol, heated at 100°C for 3 min and then subjected to SDS-PAGE\textsuperscript{25}. Electrophoresis was carried out in a mini Protean apparatus (Bio-Rad, USA) at a constant voltage of 200 V under cooling. After electrophoresis, the gels were washed with 5% (v/v) Triton X-100 (20 ml for each gel) for 3x20 min periods at room temperature and were finally incubated in 50 mM Tris-HCl pH 7.4 containing 5 mM CaCl\textsubscript{2}, 1 μM ZnCl\textsubscript{2}, 0.02% (w/v) sodium azide and 0.1% (v/v) Triton X-100 for 20 h at 37°C.

Western blotting

The samples were subjected to SDS-PAGE (T: 10%, C: 2.7%) according to Laemmli\textsuperscript{25}. After electrophoresis, the protein bands were electrotransferred to polyvinylidene difluoride (PVDF) membranes at constant current of 80 mA at 4°C for 20 h in 0.05 M Tris-HCl pH 8.3. The membranes were washed with PBS containing 0.1% Tween 20 (PBS-T) and blocked with 5% dry skimmed milk in PBS-T. They were then incubated with the respective polyclonal antibody in an appropriate dilution in PBS-T for 1 h at room temperature. After repeated washings with PBS-T, the membranes were incubated with second antibody (goat anti-rabbit IgG) peroxidase-conjugated (1:5,000) in PBS-T, for 1 h at room temperature and washed exhaustively with PBS-T. The immuno-reacting bands were visualized by enhanced chemiluminescence method (ECL), according to the manufacturer\textquotesingle s instructions (Amersham, UK) and by exposure to Agfa Curix X-ray film.

Statistical analyses

The zymograms were scanned by a digital scanner and the lysis bands were quantified by Scion Image PC. Analysis of the quantitative results was performed by using SPSS 17 (SPSS Inc, Chicago, IL, USA).

For simplicity, monthly serum measurements were replaced by mean values in five time intervals as follows: 1\textsuperscript{st} measurement - pre-treatment levels, 2\textsuperscript{nd} measurement - ten to fifteen days post-treatment, 3\textsuperscript{rd} measurement - one to two months post-treatment, 4\textsuperscript{th} measurement - three to four months post-treatment, and 5\textsuperscript{th} measurement - five to eight months post-treatment.

Missing values were replaced by the average of that particular time interval. Taking into account a medium effect size (i.e., .5 based on Cohen, 1992)\textsuperscript{26} and power =80%, we adopted a level of significance equal to 5% (for a one-tailed test) in order to identify such effects.

Results

The present study was undertaken to examine whether serum gelatinases of laryngeal cancer patients are affected by different factors related to either disease, such as anatomical site, grade and cancer stage, type of disease (primary or recurrence), lymph node metastasis and type of intervention, or lifestyle of patients, such as smoking and alcohol intake. In addition, we included the examination of the gelatinases’ levels in a follow-up study up to eight months after treatment.

The samples were first subjected to gelatin zymography (Figure 1) and the MMP-9 and MMP-2 lysis bands were obtained. Only the latent forms (proforms) of the enzymes were observed. The presence of gelatinases was verified by western blotting and a typical result is shown in figure 2.

After the quantification of the lysis bands, the results obtained for day zero, i.e., the day where histological diag-
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N: code number of individual patient. *G: glottic; SG: supraglottic; TG: transglottic. **NO: refers in all cases to previously smokers. ***NO: drinking alcoholic beverages randomly; D: drinking alcoholic beverages daily; H: drinking heavy quantity of alcoholic beverages daily. †Follow-up information up to 43 months post treatment. R1: Patients included in the study after recurrence of an older primary cancer. R2: Patients included in the study for a primary cancer, treated and then had a recurrence. M: Patients included in the study for a primary cancer, treated and then metastasized. P2: Second primary.
nosis of laryngeal cancer was made, were classified according to the parameters described in “materials and methods” section. Table II shows the mean values of proMMP-2 and -9 lysis bands in patients and mean values in healthy individuals.

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The mean pretreatment serum levels of proMMP-2 were significantly higher in patients than in healthy individuals, when all patients were considered together [t (48) = 7.716, p < 0.001)] (Figure 3). No significant differences were demonstrated between patients of different stage, grade, type of disease (primary or recurrence), lymph node involvement, and alcohol consumption. Patients with supraglottic tumours had significantly higher levels than patients with glottic ones (p < 0.05) (Figure 4). Similarly active smokers had significantly higher levels than ex-smokers (p < 0.05) (Figure 5).

With regard to the role of proMMP-9, the mean pretreatment serum levels were significantly higher in patients than in healthy individuals [t (48) = 6.594, p < 0.001)] (Figure 3). Stage, grade, anatomical site, and lifestyle (smoking and alcohol consumption) did not affect the levels of the circulating enzyme in patients of different subgroups. Primary disease was associated with significantly lower levels of circulating enzyme compared to recurrent disease (p < 0.05) (Figure 6). Patients with lymph node involvement showed lower levels than patients without neck disease (p < 0.1) (Figure 7).

The gelatinases’ levels were quantified in a follow-up term. MMP-2 levels significantly increased in the first fortnight after treatment [t (48) = -2.360, p < 0.05], and thereafter decreased over the next eight months (Figure 3), but not significantly compared to pretreatment levels. Smokers had a very high decrease rate of proMMP-2 levels during the follow-up period, whereas in ex-smokers, proMMP-2 levels significantly increased (Figure 5). On the other hand, alcohol consumption as well as grade,
type of disease (primary or recurrence) and lymph node involvement seemed not to affect proMMP-2 variations during the follow-up period. Stage II patients showed significantly lower levels of circulating enzyme compared to patients with more advanced disease five to eight months post treatment ($p < 0.05$) (Figure 8). Similarly, conservative (non-surgical) management was associated with lower levels of serum proMMP-2 compared to surgical management five to eight months following treatment ($p < 0.05$) (Figure 9). Significant differences which developed among groups with regard to anatomical location were not maintained three to four months after treatment.

With regard to proMMP-9, serum levels significantly decreased post treatment (Figure 3). Social alcohol consumption, i.e., consumption on special occasions, was associated with an initial increase of the serum levels post treatment followed by a decrease similar to that of heavy drinkers and alcoholics. The pre-treatment differences between patients with lymph node metastasis and patients without neck disease do not exist five to eight months post treatment (Figure 7). Similarly, the initial significant difference between patients with primary disease and patients with recurrent disease was not maintained one month post treatment (Figure 6). Stage II patients showed slower rates of decrease in the early post-treatment period which, nevertheless, equalized the rate of decrease in more advanced stages of disease five to eight months past treatment (Figure 10). Differences which developed between groups with regard to anatomical location and grade were not maintained three to four months past treatment. Smoking did not affect the rate of decrease of proMMP-9 in serum.

**Discussion**

Laryngeal cancer is the second most common head and neck cancer, taking into account cutaneous malignancies. The early stages of the disease carry an excellent prognosis, whereas the advanced disease is highly devastating, characterized by increased invasiveness and high rates of metastasis.

Over the last two decades extensive research has been carried out in order to elucidate the role of MMPs in the process of invasion and metastasis. Gelatinases (MMP-2 and -9) are of particular interest as they are capable of degrading type IV collagen, an important component of the basement membranes. Overexpression of these two enzymes in the cancerous tissue has been identified in various types of malignancies, including head and neck cancer. In this context, both gelatinases have been associated with neck lymph node metastatic disease27-31, increased invasiveness32,33 and prognosis34-37 in head and neck cancer.

Our study aimed at identifying possible associations between pre-treatment circulating MMP-2 and MMP-9 and various clinicopathological parameters in laryngeal cancer patients, i.e. stage, grade, location, lymph node metastasis, lifestyle, as well as the progress of serum levels of both enzymes following therapeutic intervention in association with the aforementioned parameters.

Both enzymes were increased in serum of laryngeal cancer patients, as compared with healthy individuals. This
finding is in line with other reports with regard to MMP-9. However previous reports on MMP-2 plasma levels found no difference between head and neck cancer patients and healthy individuals. On the contrary, high serum levels of MMP-2 have been reported by other authors in lung cancer and cystic adenocarcinoma of the ovaries.

It is possible that the increased levels of both gelatinases in serum in their latent forms represent the over-expression of these enzymes in cancerous tissue. Identification of the active form of both enzymes would shed light on the microenvironment with regard to the enzymatic activity. No correlation has been demonstrated between the pretreatment levels of MMP-2 and stage, grade, type of disease (primary or recurrence) and alcohol consumption. This is in line with previous reports by other authors. An interesting finding in our study was the increased level of proMMP-2 in ex-smokers compared with active ones. A better appreciation of the enzymatic environment would be achieved by quantifying the active form of both enzymes in these groups of patients. Furthermore, increased levels of proMMP-2 were found in supraglottic carcinomas compared with the glottic ones. The latter finding may be the result of delayed diagnosis of the supraglottic disease and consequently of the increased tumour burden. We could not demonstrate any association between serum levels of proMMP-2 and lymph node metastasis, although previous studies have shown a positive correlation between overexpression of proMMP-2 in the malignant tissue and metastatic neck disease. This discrepancy may be explained by the lack of correlation between the circulating enzyme and the expression of its tissue counterpart as it has been reported on by Ruokolainen et al.

With regard to proMMP-9 we found no correlation between serum levels of the enzyme and stage, grade, location of the disease, and lifestyle. An interesting finding in our study was the decreased level of proMMP-9 in the serum of patients with metastatic neck disease. Previous authors found no correlation between pretreatment levels of proMMP-9 in serum and lymph node metastasis. In our study this group of patients comprised of clinically N positive as well as clinically N negative but pathologically N positive patients. Quantification of the active form of MMP-9 would shed light on the enzymatic microenvironment and would produce a more comprehensive picture of this activity. The answer may lie in the regulatory mechanisms of activation and deactivation of the enzyme. Another interesting finding in our study was the increased level of proMMP-9 in patients with recurrence compared to those with primary disease. Assuming that the recurrent disease is diagnosed at an early stage as a consequence of a regular follow-up, the increased levels of the enzyme are in line with the tendency for higher levels –although not statistically significant - of proMMP-9 in early stage tumours (figure 10). A more comprehensive picture of the enzymatic activity would be achieved by quantifying the active form of the enzyme as well. The high levels of proMMP-9 might be the result of increased synthesis in early stages, followed by increased activation as the disease progresses to more advanced stages.

The post-treatment levels of both gelatinases have been
quantified over an 8-month follow-up period. Our study demonstrated an initial significant increase of proMMP-2, followed by a gradual decrease, which, nevertheless, did not reach the levels of healthy individuals at the end of the follow-up period. The initial increase of the enzyme might be attributed to the inflammatory process, which ensued from the therapeutic intervention. A previous study by Tutton et al, on colonic cancer reported on significant decreases of circulating MMP-2 to the levels of healthy individuals six to twelve months following treatment.

An interesting finding in our study was the interaction between smoking and the circulating proMMP-2 in time. The levels of the enzyme decreased significantly in the subset of active smokers as time goes by, whereas the opposite occurred in the group of ex-smokers. The mechanism to explain the aforementioned interaction is not clear. The answer may lie in the processes of activation and deactivation of the enzyme. The significant decrease of proMMP-2 five to eight months post treatment in the non-surgically treated group, as compared to the other treatment groups, may reflect the absence of expression of the enzyme in the irradiated tissues. Similarly, the significantly lower levels of proMMP-2 five to eight months post treatment in stage II tumours, as compared to more advanced disease, may be due to decreased expression or possibly to decreased load of tumour.

With regard to proMMP-9, we demonstrated a significant decrease to the levels of the enzyme in serum over time. This finding is in line with reports by other authors. However, serum proMMP-9 did not reach the levels of healthy individuals at the end of the 8-month follow-up period. A more extended follow-up would be required in order to investigate further progress of the enzymes’ serum levels. This gradual decrease of both enzymes in the post-treatment period may reflect their limited synthesis in the absence of active malignant disease. The significantly lower pretreatment levels demonstrated in cases of patients with positive neck disease were not maintained five to eight months past treatment. Similarly the higher pre-treatment levels found in recurrent tumours seem to equalize the primary ones, one month post treatment.

In general, it seems that the rate of post-treatment decrease of both enzymes in serum was not affected by the known clinicopathological parameters in the long term, with the exception of proMMP-2 in stage II disease, in irradiated patients, and in ex-smokers. In this latter group of patients the proMMP-2 serum levels increased significantly.

Although not proven as yet, the post-treatment pattern of alteration of serum proMMP-9 shows that this enzyme might play a role as a tumour marker in laryngeal cancer. Furthermore, a longer period of follow-up would be necessary to determine the role of both gelatinases as prognostic factors, as well as tumour markers. Furthermore, a longer period of follow-up would be necessary to determine the time range within which both enzymes equalize the levels of healthy individuals, if they do so. Further elucidation of the molecular mechanisms of regulation of both gelatinases and their interaction with other molecules would shed light on important aspects of carcinogenesis and disease progress, i.e. invasion and metastasis.

Conclusions

Pretreatment serum levels of proMMP-2 and proMMP-9 are significantly higher in laryngeal cancer patients than in healthy individuals. The serum levels of proMMP-2 at pre-treatment are higher in supraglottic tumours than in glottic ones. ProMMP-9 pretreatment serum levels are significantly lower in patients with metastatic neck disease and in patients with primary tumours, compared to patients with N(-) neck and recurrent disease, respectively.

Both gelatinases’ levels in serum decrease in the post-treatment period, although proMMP-2 shows an initial significant increase, possibly due to an inflammatory process. It seems that the rate of post-treatment decrease of both proenzymes in serum is not affected by the known clinicopathological parameters in the long term, with the exception of proMMP-2 in stage II disease, in irradiated patients, and in ex-smokers. The post-treatment pattern of alteration of serum proMMP-9 indicates that this enzyme might play a role as a tumour marker in laryngeal cancer disease. Further research is required in order to elucidate the molecular mechanisms by which gelatinases contribute to the disease progress and determine their role as prognostic factors and tumour markers in laryngeal cancer.

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Conflict of interest

There is no conflict of interest.

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