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Expression of matrix metalloproteinase-9 (gelatinase B) in benign, premalignant and malignant laryngeal lesions

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Summary. The matrix metalloproteinases (MMPs) are a family of proteolytic zinc-containing enzymes, which are responsible for the breakdown of the extracellular matrix components in pathological and physiological conditions. They are involved in basement membrane disruption, stroma and blood vessel penetration, metastasis and more recently there is evidence that they participate in tumor growth and angiogenic events. Matrix metalloproteinase 2 and 9 (MMP 2 and 9) belong to the gelatinases, a subgroup of MMPs, and have the capacity to degrade the triple helix type IV collagen of basal lamina of the basement membrane. With the present study, we tried to demonstrate the expression of MMP-9 immunohistochemically, comparatively in benign, premalignant and malignant lesions of the larynx. We studied 154 laryngeal lesions including 55 squamous cell carcinomas, 8 in situ carcinomas, 54 cases of dysplasia (of low and intermediate grade), 13 papillomas and 24 cases of keratosis. Overexpression of MMP 9 was observed in 74.4% and 50% in invasive and in situ squamous cell carcinomas respectively. In dysplastic cases, in papillomas and in keratoses the percentage of overexpression was 62.9%, 61.53% and 54.16% respectively and the expression of MMP-9 was significantly higher in invasive squamous cell carcinomas compared to dysplasias (p=0.000004). Also significantly higher was the expression of MMP-9 in dysplastic cases compared to papillomas (p=0.023). The MMP-9 expression was related neither to survival nor to the other available clinicopathological parameters (tumor size, grade, clinical stage, lymph node status and patient age). In conclusion, our study indicates that the expression of MMP-9 is up-regulated in a stepwise fashion, with two main steps, the first one, when a dysplastic lesion evolves and the next one, when the dysplasia progresses to invasive carcinoma.

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Introduction

Local growth, spread and metastasis are features that are shared among all malignant tumors, and require the presence of the appropriate microenvironment of the host. The matrix metalloproteinases (MMPs) are a family of proteolytic zinc-containing enzymes, which are responsible for the breakdown of the extracellular matrix components in pathological and physiological conditions (Jones et al., 1999). Matrix metalloproteinases (MMPs) are involved in basement membrane disruption, stroma and blood vessel penetration, metastasis and more recently there is evidence that they participate in tumor growth and angiogenic events (Nelson et al., 2000). Additionally, they participate in normal extracellular matrix remodeling (Brenner et al., 1989), as well as in wound healing and bone resorption (Delaisse and Vaes, 1992; Wolf et al., 1992). The basement membrane is composed of type IV collagen, laminin, entactin, proteoglycans, and glycosaminoglycans (Nelson et al., 2000), but among all these components, type IV collagen predominates (Leblond and Inoue, 1989) and it is thought to act as a barrier to the penetration of carcinoma cells into the matrix (Krecicki et al., 2001). The MMPs comprise a large family of proteolytic enzymes, which is divided according to their substrate specificity in four groups: the collagenases (MMP 1, 8 and 13), the gelatinases (MMP 2 and 9), the stromelysins (MMP 3, 10, 11 and 19) and the membrane-bound MMPs (MT-MMP 1, 4) (Murphy et al., 1991). The gelatinases degrade the triple helix type IV collagen of basal lamina of the basement membrane (Nethery and O'Grady, 1989). Additionally, they have the capacity to degrade collagen types V, VII, IX and X, fibronectin and elastin (Senior et al., 1991). The tissue inhibitors of matrix metalloproteinases,

known as TIMPs inhibit the catalytic activity of MMPs by binding to the active MMP and also they inhibit the conversion of the inactive proenzyme to the active enzyme (Magary et al., 2000). Increased expression of MMP-9 has been showed in various tumors (Coussens and Werb, 1996; Westermarck and Kahari, 1999; Mc Cawley and Matrisian, 2000). With the present study, we try to demonstrate the expression of MMP-9 immunohistochemically, comparatively in benign, premalignant and malignant lesions of the larynx and to highlight any possible relation to survival in invasive squamous cell carcinomas. Also emphasis is placed on the regulatory mechanisms that control the production and activation of MMP-9.

Materials and methods

Patients and study design

Formalin-fixed and paraffin-embedded tissues from 154 laryngeal lesions including 55 squamous cell carcinomas, 8 in situ carcinomas, 54 cases of dysplasia (20 of low, 25 of intermediate and 9 of severe grade), 13 papillomas and 24 cases of keratosis were retrieved from the archives of the Pathology Department of University Hospital of Ioannina. Detailed clinical and laboratory data, along with follow-up data were available for 46 patients with invasive squamous cell carcinoma. All relevant data such as age, clinical stage and number of extranodal disease sites were recorded. The mean age was 58.5 and they were followed up for a period of 4 years (range 1-7). Thirteen patients died during the follow-up period (six died as a result of therapy), while twenty four presented complete remission of the disease and four presented partial remission. To assess the advancement of the disease, stage classification according to the American Joint Committee on Cancer was used. Main clinical and pathological characteristics are analyzed in Table 1. All tumor samples were obtained by biopsy or surgery before any particular therapy and were fixed in 10% buffered formalin and embedded in paraffin for immunohistochemical analysis.

Immunohistochemistry

Immunohistochemistry was performed after selecting one representative paraffin block, for each case, on 4 μm thick tissue sections placed on poly-L-lysine-coated glass slides. Tissue sections were deparaffinized in xylene and rehydrated to distilled water. A step of immersion in sodium citrate buffer (0.01M, pH 6.0) in plastic coplin jars and subjection to microwave irradiation twice for 15 minutes was used. Sections were then placed in 1.5% hydrogen peroxide/methanol for 10 minutes in order to block the endogenous peroxidase activity. Monoclonal antibody directed against matrix metalloproteinase 9 (clone 2C3, Novocastra, dilution 1:50), was applied for 60 minutes at temperature 25°C. Subsequently the tissues were incubated with the secondary antibody for 30 minutes. We used the method involving the avidin-biotin-peroxidase complex and developed the chromogen with immersion of the slides in a diaminobenzidine-H_2O_2 substrate for 5 min. The slides were counterstained in Harris' haematoxylin, dehydrated and mounted. To assess the specificity of the reaction, positive, as well as negative control slides were used in all cases.

Immunohistochemical evaluation

At least 10 high power fields (x 400) were counted independently by two observers (pathologists) and the percentage of positive staining of neoplastic, dysplastic or hyperplastic squamous epithelium was recorded. Whenever there was a disagreement between the two observers with a difference in the percentage levels >5%, the sections were reassessed simultaneously by the two pathologists. For statisistical analysis of the survival data we established, as described by other authors (Franchi et al., 2002) a three-scaled system on the basis of the percentage of positive cells: +, low expression (<10% positive cells); ++, moderate expression (10-50% positive cells) and +++, diffuse expression (>50% positive cells). For practical reasons we defined as overexpression the presence of more than 50% positive cells for MMP-9. Non-specific immunostainings were omitted from the study.

Statistical analysis

The program SPSS for Windows Release 10.0 was used for statistical analysis. Pearson's and Spearman's correlation coefficients were used for the assessment of correlation between continuous variables. The results

Table 1. Patients' characteristics.

CHARACTERISTIC	VALUE
Age	
Mean	58.5
Sex	
Male	55
Female	2
Tumor stage	
T1	9
T2	10
T3	16
T4	6
Tumor differentiation	
Well	12
Moderately	9
Poorly	2
Lymph node metastasis	
Absent (pN0)	26
Present (pN+)	10
Distant metastasis	
M1	2

were considered as statistically significant when p<0.05.

Results

Details on the expression levels of MMP-9 for all lesions are shown in Table 2

Invasive carcinomas

Overexpression of MMP-9 (>50% positive cells) was observed in 47/55 cases (75.5%) of invasive squamous cell carcinoma. The immunoreactivity of MMP 9 was significantly higher in invasive carcinomas than in dysplasias (p=0.000004). The MMP-9 expression was related neither to survival nor to the other clinicopathological parameters that were available (tumor size, grade, clinical stage, lymph node status, patient age). MMP-9 was predominantly localized to epithelial cells, although it was also evident in the stroma, to varying degrees. Stromal cells surrounding the tumors stained in general more intensely. Leukocytes and especially macrophages showed stain reactivity as well; the same was the case for endothelial cells around the tumors. Staining results are shown at Figure 1. No significant correlation was seen between the three subgroups of MMP-9 protein expression and prognosis. (Table 3).

In situ carcinomas

As expected, a lower percentage of in situ carcinomas 5/8 (62.5%), demonstrated strong immunoreactivity for MMP 9, compared to invasive carcinomas, although this difference was not statistically significant (p=0.13).

Dysplasias

37/54 dysplastic cases (68.5%) presented high levels of MMP-9 expression. Staining results are shown in Figure 2. The expression of MMP-9 was significantly higher in dysplasias, when compared to papillomas (p=0,023) and similarly the dysplastic cases exhibited significantly higher levels of MMP-9 in comparison with the keratotic lesions (p=0.000036). In addition, no differences in MMP-9 expression was observed between groups of low, moderate and severe dysplasia, using the chi square test (p=0.219).

Table 2. Expression levels of MMP-9 in different lesions.

MMP-9 %	N	Mean	Std. Deviation	Minimum	Maximum
Cancer	55	74,1091	20,02191	10,00	96,00
Dysplasia	54	62,9630	23,32135	5,00	95,00
In situ carcinoma	8	52,5000	18,51640	30,00	85,00
Papilloma	13	59,2308	22,25292	15,00	90,00
Keratosis	24	55,8333	24,30185	5,00	90,00

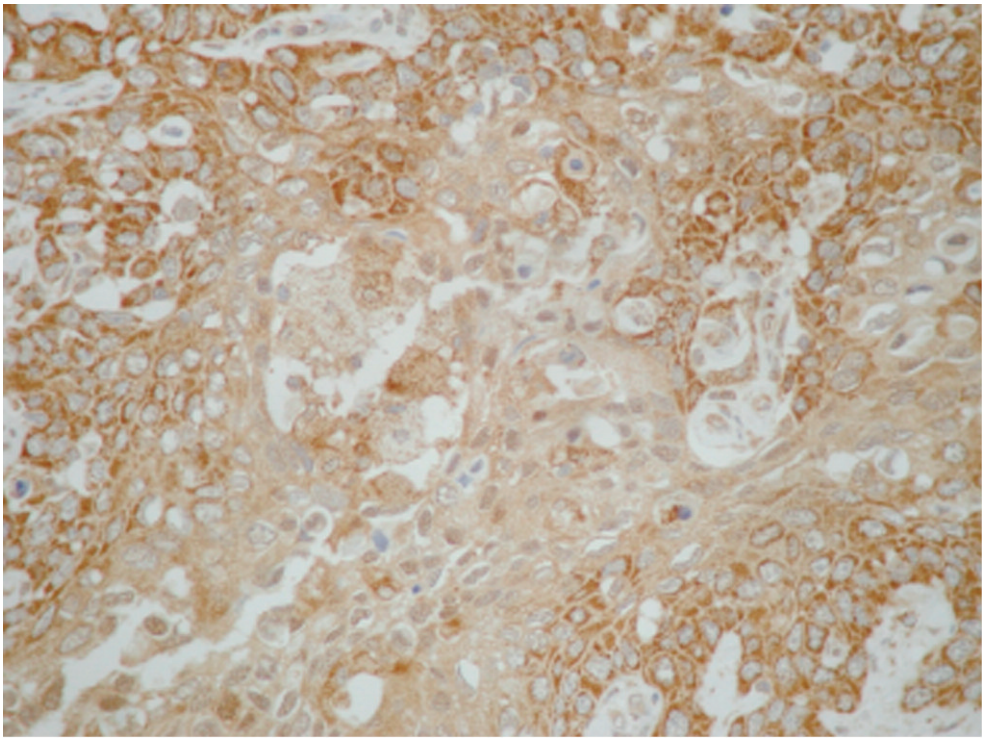

Fig. 1. High expression of MMP-9 in invasive squamous cell carcinoma, moderately differentiated (ABC, x 400).

Papillomas

Concerning papillomas, 9/13 (63.2%) showed high MMP-9 expression.

Keratoses

For keratotic lesions 12/24 cases (50%) presented strong immunoreactivity for MMP-9.

Discussion

Matrix metalloproteinases (MMPs) are believed to participate in complex processes during the development of malignant tumors, acting at several points. Various studies have been carried out in order to elucidate the role of MMPs in malignant tumors and to highlight their possible prognostic significance. Our study failed to document any significant correlation between the expression of MMP-9 in invasive squamous cell carcinomas and clinicopathological parameters, such as tumor stage, lymph node status, age and grade. Nor the expression of MMP-9 was related to survival. This is in agreement with other studies. Bogusiewicz et al. found no correlation between the activity of MMP-9 and clinical stage or tumor size of laryngeal carcinomas (Bogusiewicz et al., 2003). Similarly, the study of Franchi et al. revealed no significant correlation between MMP-9 expression and clinical parameters (Franchi et al., 2002). Also in gastric cancers, Murray et al. demonstrated no association between the expression of MMP-9 and tumor stage or lymph node status, nor to survival (Murray et al., 1998). Although for members of the MMP family there is evidence of a stepwise increase of their expression levels, our results failed to reveal significant differences in the expression of MMP-9 between invasive and in situ squamous cell carcinomas. (Campo et al., 1992; Garzetti et al., 1996; Sutinen et al.,

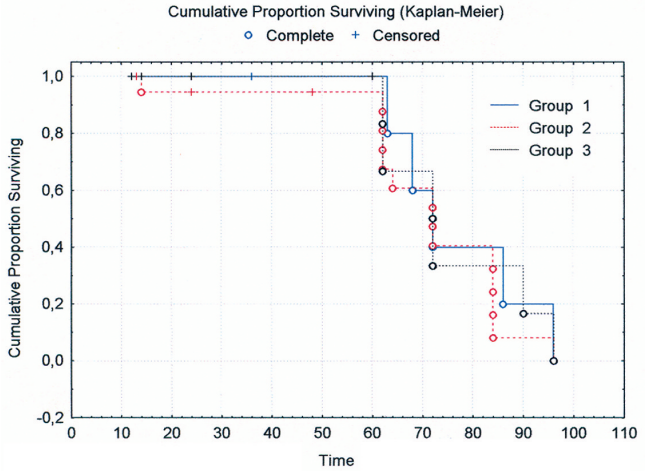

Table 3. Survival analysis and MMP-9 expression (Group1: <10%, Group2: 10-50%, Group 3: >50%).

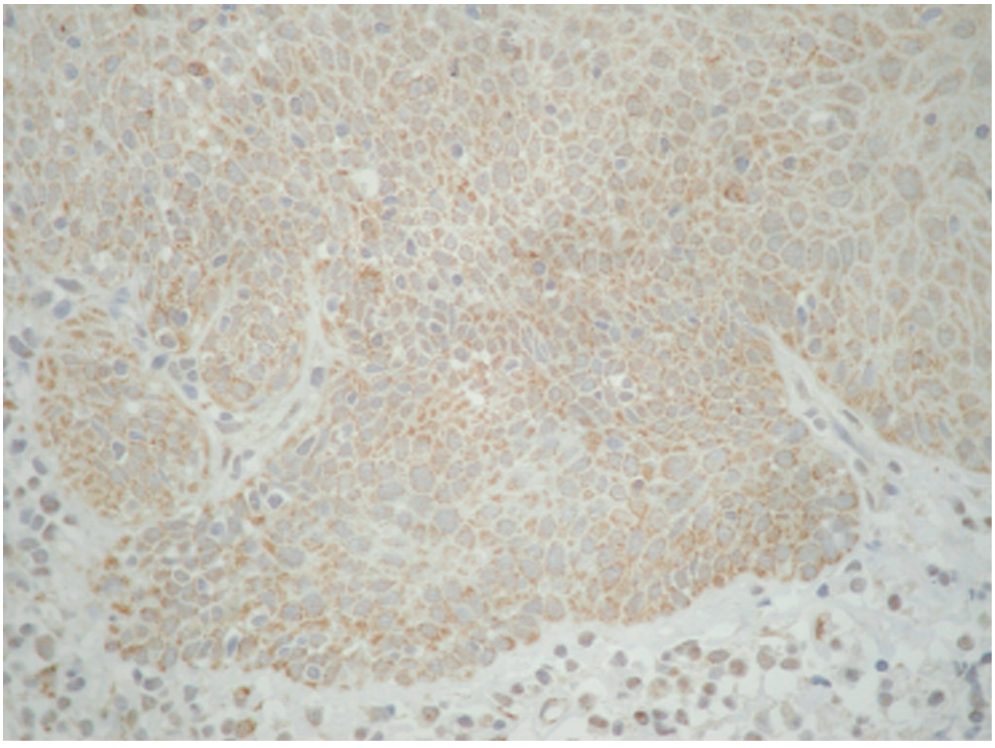

Fig. 2. High expression of MMP-9 in squamous dysplasia (ABC, x 400).

1998; Talvensaari-Mattila et al., 1999; Zeng et al., 1999). Sarioglu et al. demonstrated such a sequential rise in the MMP-2 expression, with carcinomas exhibiting the highest expression; atypical hyperplasias lower expression and in situ carcinomas showing intermediate scores between the two former entities (Sarioglu et al., 2001). During the process of malignant transformation, where low grade dysplasia and invasive carcinoma represent the two ends of the same spectrum, we demonstrated only one step of increase in the values of MMP-9, namely in the transition of dysplasia to invasive carcinoma. We suggest that this is the crucial point at which there is a rise of MMP-9 and that for the transition from in situ to invasive carcinoma, only a slight increase of MMP-9 takes place. Significant differences occurred in the expression of MMP-9 between dysplasias and the frankly benign lesions (papillomas, keratoses), a fact that indicates that already from the evolution of a premalignant lesion, MMP-9 is up regulated. It is well established that MMP activity is regulated at several levels, including the induction by different cytokines and growth factors, such as EGF, bFGF and TNFα (Goldberg et al., 1990; Schmitt et al., 1992; Rabbani and Xing, 1998), as well as by various oncogenes (Jones et al., 1999). The gene encoding for MMP-9 is one of the target genes of Ets-1 transcriptional factor, which is encoded by the ets 1 protooncogene (Wasylyk et al., 1991; Grevin et al., 1993; Iwasaka et al., 1996; Watabe et al., 1998). Behrens et al demonstrated in his study a significant upregulation of Ets-1 transcripts and protein, as well as of MMP-9 protein in the stroma of invasive sporadic colorectal carcinomas, pointing out at the important role of Ets-1 in the induction of MMP-9 (Behrens et al., 2003). Moreover, the status of tumor suppressor gene p-53 has been linked to the expression of various MMPs (Sun et al., 1999, 2000). Mutant p-53 activates the transcription of MMP genes (Sun et al., 1999, 2000). Overexpression of MMP-9 is correlated strongly with the presence of p53 mutations in head and neck squamous cell carcinomas and this indicates that MMP-9 up regulation is a result of p-53 inactivation (Franchi et al., 2002). Also, activated molecules of MMPs can activate MMP proenzymes by positive feedback mechanisms (Jones et al., 1999). MMP-9 is activated by other MMPs, namely MMP-3 and MMP-2 (Friedmann et al., 1995).

It seems that for further progression of a high grade intraepithelial lesion to invasive carcinoma, other regulatory mechanisms exist. Posttranscriptionally, MMP activity is controlled by proteolytic activation of latent proenzymes and by specific TIMPs (Jones et al., 1999). We believe that in order to precede a precancerous lesion to invasiveness, the ratio of active/inactive form of MMP-9 turns out to be more important rather than the absolute expression levels of MMP-9 themselves. Since immunohistochemical evaluation cannot discriminate latent from the activated enzyme, the enzymatic activity can be measured by substrate gel electrophoresis, named zymography (Kleiner and Stetler-Stevenson, 1994). Laryngeal cancer displays significantly higher MMP-9 activity by zymography, compared with normal mucosa (Bogusiewicz et al., 2003). In addition TIMPs, which are negative regulators of MMPs also play an important role in keeping the balance of MMP levels. Extracellular matrix degradation with concomitant tumor progression has been linked to altered TIMP levels (Di Nezza et al., 2002).

In conclusion, the present study indicates that the expression of MMP-9 is up regulated early, when a dysplastic lesion evolves and the crucial step is when the dysplasia progresses to invasive carcinoma. The observation that the levels of MMP-9 did not differ significantly between in situ and invasive carcinomas, reflects the existence of post-transcriptional mechanisms which act synergistically to the increase of the production of MMP-9.

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