

ORIGINAL ARTICLE

## Determination of matrix metalloproteinase 9 (MMP-9) protein expression in laryngeal squamous cell carcinomas based on digital image analysis

T. Ath. Papadas<sup>1\*</sup>, S. S. Naxakis<sup>1</sup>, N. S. Mastronikolis<sup>1</sup>, T. Stathas<sup>1</sup>, N. Ch. Karabekos<sup>1</sup>, E. Tsiambas<sup>2\*</sup>

<sup>1</sup>ENT Department, University Hospital of Patras, Patras; <sup>2</sup>Cytopathology Department, 417 VA Hospital, Athens, Greece

\*Equal contributors to this study

### Summary

**Purpose:** Matrix metalloproteinases (MMPs) is a super-family of proteins involved in angiogenesis and metastatic tissue invasion in many cancers. Overexpression of MMP-9 has been detected in significant proportions of laryngeal squamous cell carcinomas (LSCCs), but its prognostic impact remains unclear. In this study we performed a digital image analysis for analyzing MMP-9 protein expression in a series of LSCCs correlating them with clinicopathological factors.

**Methods:** MMP-9 protein expression level was determined immunohistochemically in 30 tissue sections surgically derived from patients (21 male and 9 female) with LSCC. Using digital image analysis, we measured their corresponding protein expression levels (staining intensity/S.I. range values 0-255).

**Results:** Moderate and high MMP-9 protein expression levels (grouping as 2+/3+ overexpression) were detected in 19/30 (63.3%) cases. Statistical significance was observed correlating stage with SI ( $p=0.02$ ), whereas a borderline association with differentiation grade of the examined tumors was also registered ( $p=0.05$ ). Interestingly, high levels of MMP-9 expression were observed in cases that demonstrated a significant level of inflammatory (predominantly lymphocytic) infiltration.

**Conclusion:** MMP-9 protein overactivation is a frequent and significant genetic event in LSCC, correlating with its biological behavior (increased TNM stage). MMP-9 seems to mediate an epithelial-stromal intra-reaction correlating also with induction of specific inflammation pathways.

**Key words:** digital image analysis, larynx, matrix metalloproteinases, squamous cell carcinoma

### Introduction

Squamous cell carcinomas (SCCs) are the major category of epithelial malignancies that arise in larynx (LSCCs) [1]. During carcinogenesis, normal squamous epithelia accumulate a variety of genetic alterations as a result of viral infections or exposure to carcinogens such as tobacco and alcohol [2,3]. Hyperplasia, dysplasia, carcinoma *in situ* and finally invasive carcinoma occur as progressive stages in this process [4,5]. Deregulated pathways - including signaling transduction to nucleus mediated by growth factor receptors, neo-angiogenesis or tissue metastasis - affect the progression of the disease, modifying also the response level to specific novel targeted

therapeutic agents, such as monoclonal antibodies in these patients [6-9]. Recently published studies have shown that specific protein families such as MMPs influence epithelial-stromal intra-reaction promoting metastatic tissue invasion and angiogenesis in cancers, including LSCCs [10-12].

MMP family is involved in normal embryonic development, reproduction, and tissue remodelling [13]. MMPs are secreted as inactive proproteins which are activated when cleaved by extracellular proteinases [14]. Due to their enzymatic activity, type IV and V collagens are degraded [15]. MMP-9 - a 92kDa enzyme - acts as gelatinase - specifically type IV collagenase - mediating also tumor invasion, angiogenesis, carcinogenesis and apoptosis [16-18].

**Table 1.** Clinicopathological data and MMP-9 IHC analysis

Variables Cases: N=30	MMP-9 expression*			p-value
	No (0) (N=0)	Low (1+) (N=11)	Moderate/High (2+/3+) (N=19)	
Gender (mean age 63.3±4.96 yrs)				NS
Male (mean age 67 yrs)		6/11	15/19	
Female (mean age 58 yrs)		5/11	4/19	
Stage				0.02
I-II		2/11	1/19	
III		8/11	2/19	
IV		1/11	16/19	
Grade				0.05
1		3/11	4/19	
2		6/11	5/19	
3		2/11	10/19	
Anatomic origin				NS
Glottis		7/11	8/19	
Subglottis		4/11	11/19	

\*Based on image analysis (value range at RGB protocol: 0-255) the groups demonstrate the corresponding staining intensity values: Score 0: >159, Score 1+: 132-149, Score 2+: 115-129, Score 3+: 81-112  
NS: non significant, yrs:years

In the current study we digitally analyzed (computerized image analysis/CIA) MMP-9 protein expression levels in a series of LSCCs correlating them with clinicopathological data.

## Methods

### Patients/Tissue samples

For the purposes of this study we used 30 formalin-fixed and paraffin-embedded archival tissue samples of histologically confirmed LSCCs. The local ethics committee of the Medical School, University of Patras, gave permission to use these tissues for research purposes, conforming to the ethical guidelines of the "World Medical Association Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects" adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964, as revised in Tokyo 2004. All of the specimens derived from local or extended laryngeal surgical resections. All corresponding Hematoxylin and Eosin (H&E)-stained slides were reviewed by two pathologists for the confirmation of diagnosis and classification according to World Health Organization (WHO 2000) grading and staging criteria. The tissue samples came from 21 male and 9 female (mean age: 63.3±1.96) patients.

### Immunohistochemistry (IHC)

IHC for MMP-9 antigen was carried out on 4 µm serial sections of the corresponding tissue blocks. Ready-to-use rabbit polyclonal anti-MMP-9 (clone

A0150, DAKO Corp, Denmark) was applied in the corresponding slides (dilution 1:70). Slides were deparaffinised and rehydrated. Following incubation with the secondary antibody for 15 min, diaminobenzidine-tetrahydrochloride-DAB (0.03%) containing 0.1% hydrogen peroxide was applied as a chromogen and incubated for 5 min. Slides were counterstained, dehydrated and cover-slipped. The IHC protocol was performed via an automated staining system (I 6000 – Biogenex, San Ramon, CA, USA). Diffuse cytoplasmic/perinuclear and focally dense nuclear staining pattern was observed in epithelial/stromal cells regarding MMP-9 expression. Inflammatory infiltration was also detected in stromal and peritumoral areas in conventional H&E staining and also in the corresponding immunostained slides. For negative control slides, the primary antibody was omitted. Breast cancer epithelial and stromal tissue sections expressing MMP-9 protein and normal-appearing laryngeal epithelia were used as control staining pattern. Protein expression levels were evaluated quantitatively using an image analysis macro.

### Computerized image analysis assay (CIA)

MMP-9 protein expression levels were evaluated quantitatively by estimating SI levels. CIA was performed using a semi-automated system (Microscope CX-31, Olympus, Menville, NY, USA, Windows XP/NIS-Elements Software AR v3.0, Nikon Corp, Tokyo, Japan). Areas of interest were identified (10 optical fields per slide at 200x magnification) and filed in a digital base. A macro was implemented for measuring the amount of MMP-9 protein expression. Grouping the extracted staining intensity values (value range at

RGB protocol: 0-255; values increasing to 255 correlated with loss of expression, whereas values decreasing to 0 demonstrate overexpression of the molecule), we considered complete absence of stain regarding MMP-9 as 0 score (identified only in negative control immunostained slides), moderate expression levels as 1+, whereas moderate/strong expression validated as 2+/3+ score.

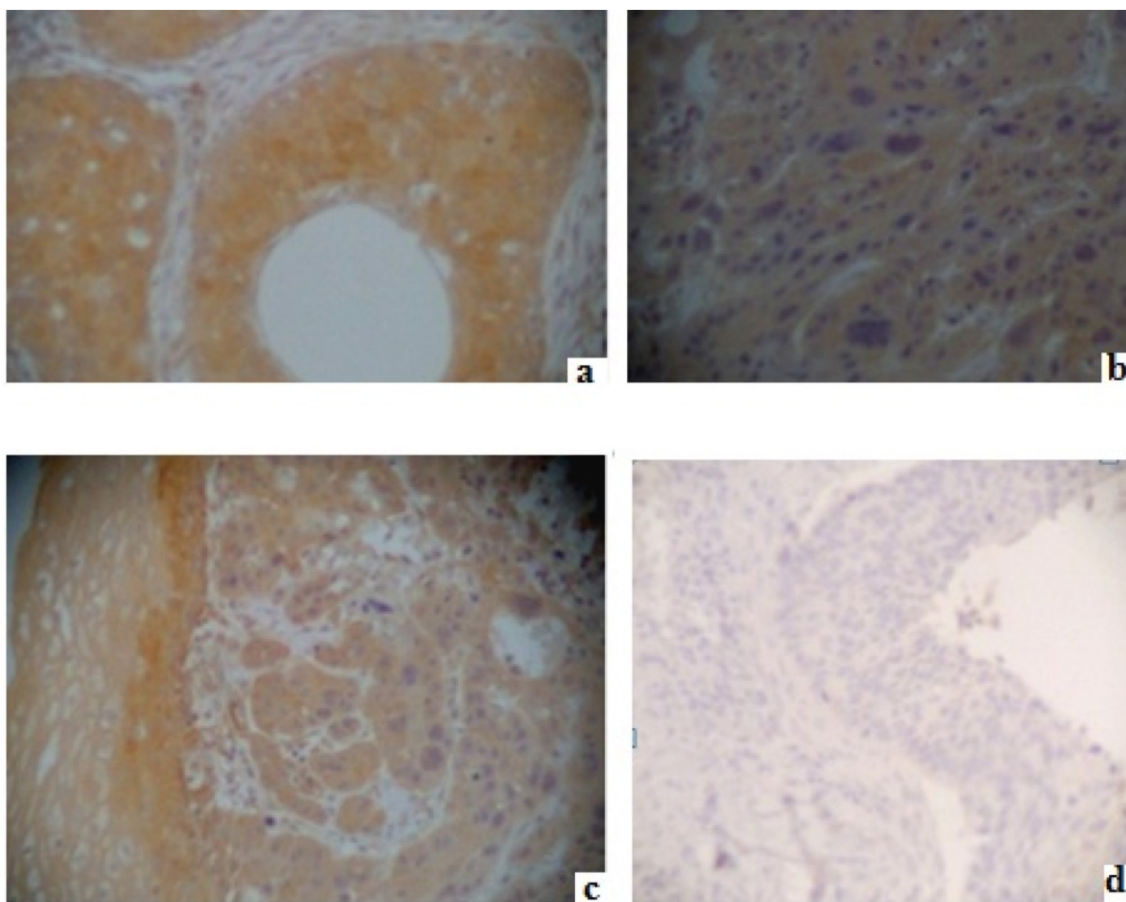
#### Statistics

Several variables were examined including gender, stage, anatomic origin, and grade. Chi square test for categorical data and Fisher's exact test for categorical data with limited number of frequencies were used. The significance level was set at  $p=0.05$ . The SPSS v11 (SPSS Inc, Chicago, IL, USA) statistical package was used to analyze the data.

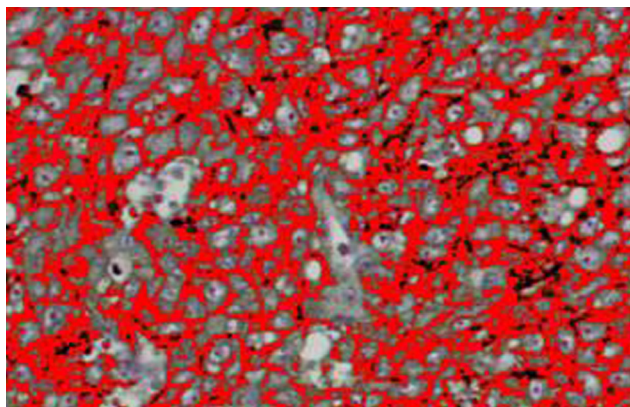
#### Results

Diffuse cytoplasmic/perinuclear and focally dense nuclear staining pattern of MMP-9 expression was observed in epithelial and stromal cells

in all cases (Figure 1). Among them, 19 (63.3%) cases overexpressed the protein (2+/3+ categories), whereas the rest demonstrated low levels of expression (1+ category; Figure 2). Statistical significance was observed correlating stage with SI levels ( $p=0.02$ ), whereas a borderline association with grade of differentiation of the examined tumors was also registered ( $p=0.05$ ). Concerning the other examined parameters (gender, anatomic origin of the neoplasm), no statistical significance was noted. In fact, MMP-9 overexpression was identified in cancerous tissues with an aggressive phenotype (increased stage). Interestingly, high levels of MMP-9 expression were also observed in cases with a significant level of inflammatory (predominantly lymphocytic) infiltration. This type of reaction was detected in stromal and peritumoral areas of the corresponding cases, enhancing the connection between MMPs enzymatic activity and mechanisms that induced lymphocytic production and tumor penetration.



**Figure 1.** Different MMP-9 protein expression patterns in LSCC cases. Note a diffuse epithelial immunostaining, focally perinuclear/nuclear (a), an epithelial/stromal strong expression (b), a moderate epithelial-strong stromal expression with elevated inflammatory reaction (c), and a control negative expression (d). Original magnification 100x, in a,d and 200x in b,c).



**Figure 2.** Performance of digital image analysis protocol for evaluating MMP-9 protein expression in LSCC cases. Red labeled areas represent diffuse/focal MMP-9 expression.

## Discussion

MMPs act as proteases targeting a variety of extracellular proteins such as membrane receptors and growth factors [19,20]. Interestingly, a strong reaction with galectins - especially galectin-7 - has been already identified *in situ* correlating also with prognosis in LSCC [21]. Galectins is a family of adhesion molecules (endogenous lectins) that act as mediators in signaling pathways regarding epithelial-stromal intra-reaction [22]. In the same study, a negative correlation between MMP-9 and galectin -1 and -3 was observed, although the latter is a substrate for MMP-9 activity. Moreover, a strong correlation between MMP-9 and fibronectin has been recently detected [23]. Fibronectin stimulates MMP-9 secretion inducing cancer cell tissue metastasis and cancer growth. This metastatic process seems to be mediated by the integrin receptor  $\alpha 5\beta 1$  involving activation of multiple signaling pathways and finally leading to the transactivation of the MMP-9. Furthermore, co-expression of MMP-9 and CD44 in LSCCs seems to correlate with neck metastasis especially in the supraglottic cases (increased metastatic lymph node pN+ status) [24].

In the current study we measured MMP-9 protein expression levels in LSCC cancerous tissue sections based on a CIA protocol. To our knowledge this is the second study based on this procedure regarding this marker evaluation [21]. Using this method we estimated objectively and accurately the corresponding levels of MMP-9 expression as immunostaining intensity values, grouping them in subcategories (1+/2+/3+) for

statistical reasons. We observed a statistical association between overall expression and tumor stage, and also a borderline correlation with the grade of differentiation. Interestingly, high levels of MMP-9 expression were detected in cases that demonstrated a significant level of inflammatory (predominantly lymphocytic) infiltration. Other study groups have already identified an increased degree of inflammation in MMP-9 stromal over expression in skin carcinomas [25].

In addition, elevated expression of MMP-9 also correlates with the depth of tissue metastatic penetration (invasion) in oesophageal squamous cell carcinomas, evidence of an aggressive phenotype [26]. Furthermore, the significance of MMP-9/MMP-2 overexpression in LSCCs including different expression patterns of tissue inhibitor of metalloproteinase (TIMP)-1 and -2 in those tumors is under investigation [27,28]. Based on these studies, TIMP-1 strong stromal expression is a frequent event in normal tissues. In contrast, TIMP-2 diffuse cytoplasmic expression in epithelia and stromal immunolocalization are associated with advanced stage (stage IV) in LSCCs. Another important published observation regarding the role of MMP-9 in these tumors is related with angiogenesis and blood vessel density [29]. Overexpression of the marker in the stroma of the cancerous tissues - especially in inflammatory cells - was strongly correlated with higher density of blood vessels and also with grade of differentiation, similarly to our analysis.

In conclusion, MMP-9 overexpression seems to play a critical role in LSCCs aggressive phenotype due to advanced stage and grade of differentiation. Because deregulated epithelial/stromal intra-reaction is involved in the carcinogenetic process and biological behavior of tumors, development of MMPs inhibition strategies for disrupting tumor neovascularization and decreasing tumor tolerance - associated to the induction of tolerogenic dendritic cells - is a very promising field of investigation [30]. Concerning targeted therapeutic strategies in handling LSCCs patients, upregulation of a recently identified tumor suppressor gene TSLC1 (tumor suppressor in lung cancer 1) was also correlated with decreased levels of MMP-2 and MMP-9 proteins [31]. In addition, based on an experimental animal model, lentivirus-mediated RNA interference can inhibit invasion and growth of LSCCs by providing MMP-9 gene silence [32].

## References

1. Greenlee RT, Murray T, Bolden S, Wingo PA. Cancer statistics, 2000. *CA Cancer J Clin* 2000;50:7-33.
2. Albertson D, Collins C, McCormick F, Gray J. Chromosome aberrations in solid tumours. *Nat Genet* 2003;34:369-376.
3. Veltman JA, Bot FJ, Huynen FC, Ramaekers FC, Manni JJ, Hopman AH. Chromosome instability as an indicator of malignant progression in laryngeal mucosa. *J Clin Oncol* 2000;18:1644-1651.
4. Gale N, Kambic V, Poljak M, Cor A, Velkavrh D, Mlacak B. Chromosomes 7,17 polysomies and overexpression of epidermal growth factor receptor and p53 protein in epithelial hyperplastic laryngeal lesions. *Oncology* 2000;58:117-125.
5. Quon H, Liu FF, Cummings BJ. Potential molecular prognostic markers in head and neck squamous cell carcinomas. *Head Neck* 2001;23:147-159.
6. Shin DM, Donato NJ, Perez-Soler R et al. Epidermal growth factor receptor-targeted therapy with C225 and cisplatin in patients with head and neck cancer. *Clin Cancer Res* 2001;7:1204-1213.
7. Robert F, Ezekiel MP, Spencer SA et al. Phase I study of anti-epidermal growth factor receptor antibody cetuximab in combination with radiation therapy in patients with advanced head and neck cancer. *J Clin Oncol* 2001;19:3234-3243.
8. Koynova DK, Tsenova VS, Jankova RS, Gurov PB, Toncheva DI. Tissue microarray analysis of EGFR and HER2 oncogene copy number alterations in squamous cell carcinoma of the larynx. *J Cancer Res Clin Oncol* 2005;131:199-203.
9. Ang K, Berkey X. Impact of epidermal growth factor receptor expression on survival and pattern of relapse in patients with advanced head and neck carcinoma. *Cancer Res* 2002;62:7350-7356.
10. Riedel F, Gotte K, Schwalb J, Bergler W, Hormann K. Expression of 92-kDa type IV collagenase correlates with angiogenic markers and poor survival in head and neck squamous cell carcinoma. *Int J Oncol* 2000;17:1099-1105.
11. Franchi A, Santucci M, Masini E, Sardi I, Paglierani M, Gallo O. Expression of matrix metalloproteinase-1, matrix metalloproteinase-2 and matrix metalloproteinase-9 in carcinoma of the head and neck. *Cancer* 2002;95:1902-1910.
12. Ruokolainen H, Paakko P, Turpeenniemi-Hujanen T. Expression of matrix metalloproteinase-9 in head and neck squamous cell carcinoma: a potential marker for prognosis. *Clin Cancer Res* 2004;10:3110-3116.
13. Sternlicht MD, Werb Z. How matrix metalloproteinases regulate cell behavior. *Annu Rev Cell Dev Biol* 2001;17:463-516.
14. Folgueras AR, Pendas AM, Sanchez LM, Lopez-Otin C. Matrix metalloproteinases in cancer: from new functions to improved inhibition strategies. *Int J Dev Biol* 2004;48:411-424.
15. Villalobo A, Nogales-Gonzalez A, Gabius HJ. A guide to signaling pathways connecting protein-glycan interaction with the emerging versatile effector functionality of mammalian lectins. *Trends Glycosci Glycotechnol* 2006;18:1-37.
16. Noel A, Jost M, Maquoi E. Matrix metalloproteinases at cancer tumor-host interface. *Semin Cell Dev Biol* 2008;19:52-60.
17. Cao XL, Xu RJ, Zheng YY et al. Expression of type IV collagen, metalloproteinase-2, metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 in laryngeal squamous cell carcinomas. *Asian Pac J Cancer Prev* 2011;12:3245-3249.
18. Garavello W, Maggioni D, Nicolini G, Motta L, Tredici G, Gaini R. Association between metalloproteinases 2 and 9 activity and ERK1/2 phosphorylation status in head and neck cancers: an ex vivo study. *Eur J Cancer* 2011;47:600-610.
19. Nelson AR, Fingleton B, Rothenberg ML, Matrisian LM. Matrix metalloproteinases: biological activity and clinical implications. *J Clin Oncol* 2000;18:1135-1149.
20. Uloza V, Liutkevičius V, Pangonytė D, Saferis V, Lesauskaitė V. Expression of matrix metalloproteinases (MMP-2 and MMP-9) in recurrent respiratory papillomas and laryngeal carcinoma: clinical and morphological parallels. *Eur Arch Otorhinolaryngol* 2011;268:871-878.
21. Saussez S, Cludts S, Capouillez A et al. Identification of matrix metalloproteinase-9 as an independent prognostic marker in laryngeal and hypopharyngeal cancer with opposite correlations to adhesion/growth-regulatory galectins-1 and -7. *Int J Oncol* 2009;34:433-439.
22. Demers M, Magnaldo T, St-Pierre Y. A novel function for galectin-7: promoting tumorigenesis by upregulating MMP-9 gene expression. *Cancer Res* 2005;65:5205-5210.
23. Sen T, Dutta A, Maity G, Chatterjee A. Fibronectin induces matrix metalloproteinase-9 (MMP-9) in human laryngeal carcinoma cells by involving multiple signaling pathways. *Biochimie* 2010;92:1422-1434.
24. Yuce I, Bayram A, Cagli S, Canoz O, Bayram S, Guney E. The role of CD44 and matrix metalloproteinase-9 expression in predicting neck metastasis of supraglottic laryngeal carcinoma. *Am J Otolaryngol* 2011;32:141-146.
25. O'Grady A, Dunne C, O'Kelly P, Murphy GM, Leader M, Kay E. Differential expression of matrix metalloproteinase (MMP)-2, MMP-9 and tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 in non-melanoma skin cancer: implications for tumour progression. *Histopathology* 2007;51:793-804.
26. Tanioka Y, Yoshida T, Yagawa T et al. Matrix metalloproteinase-7 and matrix metalloproteinase-9 are associated with unfavourable prognosis in superficial oesophageal cancer. *Br J Cancer* 2003;89:2116-2121.
27. Christopoulos TA, Papageorgakopoulou N, Ravazoula P et al. Expression of metalloproteinases and their tissue inhibitors in squamous cell laryngeal carcinoma. *Oncol Rep* 2007;18:855-860.
28. Christopoulos TA, Papageorgakopoulou N, Theocharis DA et al. Diagnostic and classification value of metalloproteinases in squamous human laryngeal carcinoma. *Int J Oncol* 2004;25:481-485.
29. Wittekindt C, Jovanovic N, Guntinas-Lichius O. Expression of matrix metalloproteinase-9 (MMP-9) and blood vessel density in laryngeal squamous cell carcinomas. *Acta Otolaryngol* 2011;131:101-106.
30. Wang BQ, Zhang CM, Gao W, Wang XF, Zhang HL, Yang PC. Cancer-derived matrix metalloproteinase-9 contributes to tumor tolerance. *J Cancer Res Clin Oncol* 2011;137:1525-1533.
31. Lu B, Di W, Wang H, Ma H, Li J, Zhang Q. Tumor suppressor TSLC1 is implicated in cell proliferation, invasion and apoptosis in laryngeal squamous cell carcinoma by regulating Akt signaling pathway. *Tumour Biol* 2012;33:2007-2017.
32. Sun Y, Liu M, Yang B, Lu J, Li B. Inhibition of laryngeal cancer cell invasion and growth with lentiviral-vector delivered short hairpin RNA targeting human MMP-9 gene. *Cancer Invest* 2008;26:984-989.