Expression of MMP2, MMP9, TIMP1 and TIMP2 could predict lymph node metastases in oropharyngeal squamous cell carcinoma.

ARTICLE *in* HEAD & NECK · JANUARY 2014

Impact Factor: 2.64 · DOI: 10.1002/hed.23618 · Source: PubMed

Expression of metalloproteinases 2 and 9 and tissue inhibitors 1 and 2 as predictors of lymph node metastases in oropharyngeal squamous cell carcinoma

Pawel K. Burduk, PhD,[1*] Magdalena Bodnar, PhD,[2] Piotr Sawicki, MD,[1] Łukasz Szylberg, MD,[2] Ewa Wiśniewska, PhD,[2] Wojciech Kaźmierczak, PhD,[1] Maria Martyńska, MD,[3] Andrzej Marszałek, PhD[2,4]

[1]Department of Otolaryngology and Clinical Oncology Chair and Clinic of Otolaryngology and Department of Pathophysiology of Hearing and Balance System Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University of Torun, Bydgoszcz, Poland, [2]Department of Pathomorphology, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University of Torun, Bydgoszcz, Poland, [3]Department of Otolaryngology, Oncology Centre, Bydgoszcz, Poland, [4]Department of Oncologic Pathology, Poznan University of Medical Sciences and Greater Poland, Oncology Center, Poznan, Poland.

Accepted 10 January 2014
Published online 00 Month 2014 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/hed.23618

ABSTRACT: *Background.* The matrix metalloproteinases (MMPs) and their tissue inhibitor of metalloproteinases (TIMPs) can decompose extracellular matrix (ECM) components and brake down basement membranes and, thus, promote tumor local invasion and metastasis.

Methods. We studied 41 patients with squamous cell carcinoma (SCC) of the oropharynx who underwent surgical treatment. Material was diagnosed by routine procedure and examined by immunohistochemical analysis and dot blot technique. We searched for correlations between expressions of MMPs, such as MMP-2 and MMP-9 and their tissue inhibitors TIMP-1 and TIMP-2 and treatment outcome.

Results. We found cytoplasmic expression of analyzed proteins, both in cancer cells and tumor stroma. The expression of analyzed antigens was higher in patients with lymph node metastases comparing patients without lymph node involvement.

Conclusion. Our data suggest that microenvironment changes are one of key factors in tumor progression. Divergent expression of MMPs and their inhibitors might be used as prognostic factor of oropharyngeal carcinoma progression. © 2014 Wiley Periodicals, Inc. *Head Neck* **00:** 000–000, 2014

KEY WORDS: metalloproteinases, tissue inhibitors of matrix metalloproteinases, oropharyngeal cancer, metastases, tumor progression

INTRODUCTION

Oropharyngeal squamous cell carcinoma (SCC) presents a high risk of local recurrence and lymph node metastases. Treatment regimens for SCC are dependent according to tumor size and presence of metastasis at initial diagnosis.[1–3] The prognosis is poor with the 5-year survival rate of 40% to 50%.[1,4] Even tumors at the early stage of disease may collapse in treatment response with quick development of recurrence or metastasize.[1,4–7] Aggressive surgery and radiation or radiochemotherapy are not always sufficient for all oropharyngeal SCC cases because it could be physically and emotionally debilitating for patients.[1] It becomes clear that working on the identification of risk factors to predict lymphogenic metastatic spread and local aggressiveness of the tumor is essential.

Tumor progression is characterized by its growth, invasion, and metastasis.[1] The extracellular matrix (ECM) remodeling is essential for tumor invasion, neoangiogenesis, and metastasis.[1,4,6,8,9] The matrix degradation requires matrix metalloproteinases (MMPs) proteolytic enzymes.[1,4–6,8,10] The gelatinases MMP-2 and MMP-9 could play a significant role in SCC invasion by degrading type IV collagen.[1,4,6,10] The activity of these enzymes is regulated at several levels: transcription, translation, extracellular activation, and by specific tissue inhibitor matrix metalloproteinases (TIMPs).[8] By some authors, it was proven that changes in expression of MMP-2, MMP-9, TIMP-1, and TIMP-2 in SCC is responsible for invasion and tumor progression.[1–4,6,8,11]

The purpose of this study was to assess the prognostic impact of MMP-2, MMP-9, TIMP-1, and TIMP-2 expression in the process of invasion and metastasis in patients with SCC of the oropharynx.

MATERIALS AND METHODS

We studied 41 patients with SCC of the oropharynx who underwent surgical treatment of the primary tumor and with neck dissection at the Department of Otolaryngology and Laryngological Oncology, at Collegium Medicum Nicolaus Copernicus University.

The approval for those studies was obtained from the Ethics Commission of Collegium Medicum in Bydgoszcz in decisions: KB 497/2009, KB 417/2010, and KB589/2011.

The patients were divided into 2 groups: group 1 consisted of 21 patients without lymph node metastasis N0 (mean age, 55.1 years; 18 men, 3 women) and group 2

***Corresponding author:** P. K. Burduk, Department of Otolaryngology and Laryngological Oncology, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University of Torun, Poland, Sklodowska-Curie 9 Str, 85-094 Bydgoszcz, Poland. E-mail: pburduk@wp.pl

This work was presented at the 5th European Conference on Head and Neck Oncology in Poznan, Poland, April 18–21, 2012.

Pawel K. Burduk and Magdalena Bodnar contributed equally to this work.

TABLE 1. The positive control.

Researched antigen	Positive control tissue
MMP-2	Colon – endothelial cells
MMP-9	Salivary glands – glandular cells
TIMP-1	Colon – glandular cells
TIMP-2	Colon cancer – cancer cells

Abbreviations: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases.

which consisted of 20 patients with neck metastasis N+ (mean age, 56 years; 15 men, 5 women). The T classification in group 1 was classified for 7 patients as T2 (33.3%), for 9 as T3 (42.9%), and for 5 as T4 (23.8%). Respectively, T classification in group 2 was classified for 1 patient as T1 (5%), for 8 as T2 (40%), for 4 as T3 (20%), and for 7 as T4 (35%). A total of 20 patients with lymph node metastasis was classified in 3 cases as N1 (15%), in 12 cases as N2 (60%), and in 5 cases as N3 (25%).

Immunohistochemistry was performed with standard protocol described elsewhere. To establish immunohistochemical procedures, a series of positive control reactions were performed. The positive controls were performed on a model tissue, for which in reference sources (The Human Protein Atlas, http://www.proteinatlas.org, as well as in antibodies datasheet) the presence of the analyzed antigens was indicated (Table 1). The negative control reactions were performed on additional tissue sections during proper immunohistochemical staining, by substituting the primary antibody by the solution of diluted 1% bovine serum albumin (BSA) in phosphate buffered saline. Briefly, in this study, we used mouse monoclonal antibody against: MMP-2 (HPA001939; Sigma–Aldrich, Poznan, Poland; dilution 1:100), MMP-9 (ab58803; Abcam, Cambridge, UK; clone: 56-2A4; dilution 1:100), TIMP-1 (M7293; Dako, Glostrup, Denmark; clone: VT7, dilution 1:50), TIMP-2 (ab1828; Abcam; clone 3A4, dilution 1:50). Epitopes were unmasked by Epitope Retrieval Solution high-pH (Dako) and then slides were incubated with primary antibody overnight in 4°C. Detection of evaluated antibody complex was done with EnVision Anti-Mouse/Rabbit HRP Labeled Polymer (Dako). Then antigens complex was localized according to the presence of brown product of reaction by using diaminobenzidine as chromogen (Dako).

Results were performed at ×20 original objective magnification in the light microscope ECLIPSE E800 (Nikon Instruments Europe, Amsterdam, The Netherlands) and the level of antigens expression was estimated using morphometric principles using the Remmele–Stegner scale[12] according to the intensity of expression and the number of positively expressed tissue area. The intensity of staining was scored as follows: 0 – negative; 1 – low staining; 2 – moderate staining; and 3 – strong staining. The number of positive immunoreactive area was categorized as follows: 0 – negative; 1 – <10% positive area; 2 – 10% to 50% positive area; 3 – 50% to 80% positive area; and 4 – ≥80% positive area. The total immunoreactivity score was defined according to the scale obtained from the grade of intensity multiplied by the score of expression area, total score (0–12). The pathologists (A.M. and L.S.) who were evaluating the immunohistochemical expression of examined antigens, worked independently. Moreover, they were blinded from the patients' clinical data (including TNM), and they have been blinded from the study results.

Extraction of total protein from formalin-fixed, paraffin-embedded tissues for dot blot reactions were processed by using the Qproteome formalin-fixed, paraffin-embedded tissue kit (Qiagen, Duesseldorf, Germany), in accord with the provided protocol. All protein concentrations were determined with the Bio-Rad Laboratories DC protein assay kit, based on the method of Lowry (Bio-Rad Laboratories, Hercules, CA). In short, proteins were diluted to 50 μg/μL and 2 μl each, were slowly spotted onto 0.45 μm nitrocellulose membrane (Bio-Rad Laboratories), and air-dried. To block nonspecific binding sites, membranes were incubated with 5% BSA in TRIS-buffered saline-Tween-20 (TBST) buffer (10 m*M* Tris-HCl; 150 m*M* NaCl; 0.05% Tween 20; pH 7.6) for 1 hour at 25°C. Then they were incubated with appropriate antibodies recognizing MMP-2, MMP-9, TIMP-1, and TIMP-2 (described above, in immunohistochemical section) and β-actin (product no. A1978; Sigma–Aldrich, St. Louis, MO) at 4°C for 12 hours. All antibodies were diluted 1:250 in TBST supplemented with 1.6% BSA. Then membranes were washed 4 times in TBST for 15 minutes each and incubated with appropriate secondary antibody – Rabbit Anti-Mouse Alkaline Phosphatase Conjugated (product no. A4312; Sigma–Aldrich) or Mouse Anti-Rabbit Alkaline Phosphatase Conjugated (product no. A2306; Sigma–Aldrich) both at a dilution of 1:4000 in 1% BSA in TBST for 1 hour at 25°C. After several washings in TBST buffer and AP buffer (100 m*M* Tris-HCl; 100 m*M* NaCl; pH 8.5) all blots were visualized using BCIP/NBT Phosphatase Substrate Systems (product no. B3679; Sigma–Aldrich) for 10 minutes. The reaction was stopped by dipping membranes in distilled water. Densitometric analyses of the membranes were performed using GeneTools analysis software (Syngene, Cambridge, UK). Expression of MMP-2, MMP-9, TIMP-1, and TIMP-2 were comparable to that seen for β-actin.

Statistical analysis

The Statistica 10 (StatSoft Sp. z o.o. Krakow, Poland) program was used for statistical analyses. Because the distribution of the results differed from the normal distribution (checked according to Kolmogorov–Smirnov test with Lilliefors significance correction), further statistical analyses were performed using nonparametric tests. In the course of statistical analysis, we applied the Mann–Whitney *U* test to analyze the correlation between lymph node involvements in analyzed groups. A value of $p < .05$ was considered as statistically significant.

RESULTS

We found cytoplasmic expression of analyzed proteins both in cancer cells and tumor stroma (Figure 1).

Detection of matrix metalloproteinase-2 and matrix metalloproteinase-9 signals in cancer cells and stroma in group 1

The immunohistochemical examination of 21 oropharyngeal SCCs showed MMP-2 expression in 62% and

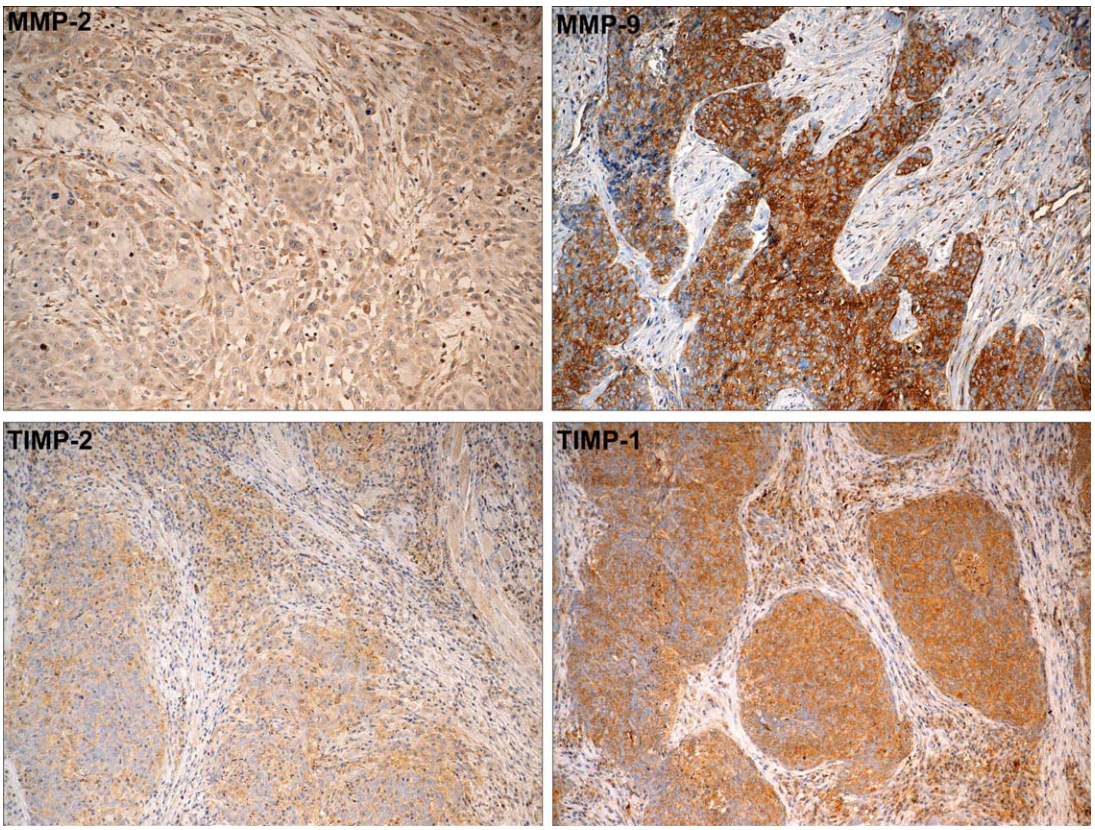

FIGURE 1. Representative immunohistochemical staining of matrix metalloproteinase (MMP)-2, MMP-9, tissue inhibitor of metalloproteinases (TIMP)-1, and TIMP-2 in oropharyngeal cancer. Positive cytoplasmic staining (brown; original magnification ×10). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

MMP-9 in 86% of the cases. The intensity of MMP-2 and MMP-9 expression with <50% of positive tumor cells or the stroma tissue was found in 17 cases for MMP-2 and 16 cases for MMP-9, whereas the expression of over 50% of the tumor cells or stroma area was found in 4 cases for MMP-2 and 5 cases for MMP-9.

Detection of matrix metalloproteinase-2 and matrix metalloproteinase-9 signals in cancer cells and stroma in group 2

Immunohistochemical examination of 20 oropharyngeal SCCs showed MMP-2 expression in 75% and MMP-9 in 90% of the cases, respectively. The intensity of MMP-2 and MMP-9 expression was <50% of the tumor cells or the stroma tissue was found in all cases for MMP-2 and 14 cases for MMP-9, whereas it was over 50% of the tumor cells or stroma area in 6 cases for MMP-9.

Detection of tissue inhibitor of metalloproteinases-1 and tissue inhibitor of metalloproteinases-2 signals in cancer cells and stroma in group 1

The immunohistochemical examination of 21 oropharyngeal SCCs showed TIMP-1 expression in 76% and TIMP-2 in 45% (5 of 11) of the cases. The intensity of inhibitors of MMP expression within <50% of the tumor cells or the stroma tissue was found in 20 cases for TIMP-1 and all cases for TIMP-2, over 50% only in 1 case for TIMP-1.

Detection of tissue inhibitor of metalloproteinases-1 and tissue inhibitor of metalloproteinases-2 expression in cancer cells and stroma in group 2

Immunohistochemical examination of 20 oropharyngeal SCCs showed TIMP-1 expression in 85% and TIMP-2 in 79% (3 of 14) of the cases, respectively. The intensity of inhibitors of MMP expression in <50% of the tumor cells or the stroma tissue was found in 17 cases for TIMP-1 and all cases for TIMP-2, expression over 50% was found in 3 cases for TIMP-1.

The expression level for MMP-9, TIMP-1, and TIMP-2 was higher in patients with lymph node metastases (N+) compared with patients without lymph node metastasis (N0; Figures 2 and 3). We found that oropharyngeal cancer without metastasis showed lower MMP-9, TIMP-1, and TIMP-2 expression levels both in cancer cells and stroma. Moreover, we have noticed loss of MMP-2 expression in cancer cells, with parallel higher expression of this protein in tumor stroma in patients with lymph node involvement (N+). However, we found no statistical significant differences in MMP-2, MMP-9, TIMP-2, and TIMP-1 immunoexpression according to lymph node involvement.

Expression of MMPs and their inhibitors evaluated by dot-blots revealed stronger signal in almost all studied

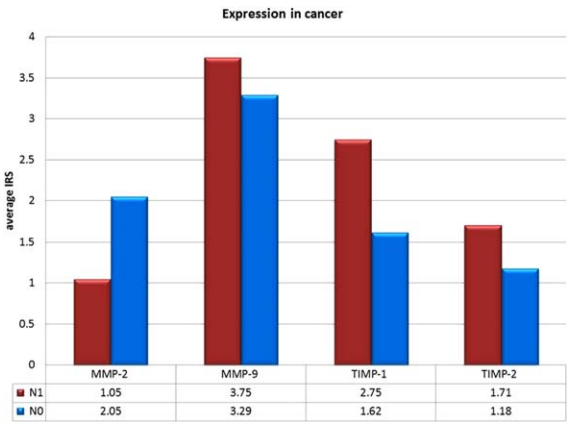

FIGURE 2. Average expression of matrix metalloproteinase (MMP)-2, MMP-9, tissue inhibitor of metalloproteinases (TIMP)-1, and TIMP-2 in neoplastic cells in patients with oropharyngeal squamous cell carcinoma (SCC). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

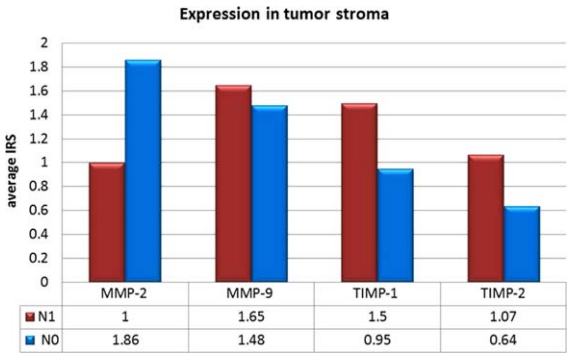

FIGURE 3. Average expression of matrix metalloproteinase (MMP)-2, MMP-9, tissue inhibitor of metalloproteinases (TIMP)-1, and TIMP-2 in tumor stroma in patients with oropharyngeal squamous cell carcinoma (SCC). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

proteins extracted from patients with lymph node metastases (N1) then those observed in proteins derived from patients' tissues without lymph node involvement (N0; Figure 4).

DISCUSSION

The ECM degradation, including the basement membrane of epithelial lining and blood vessels, is critical for tumor invasion and both regional and distant metastases.[1,13] SCC cells are able to secrete several matrix enzymes (MMPs), serine proteases, and plasminogen activator. However, most important for tumor progression are MMPs, especially MMP-2 and MMP-9 which degrade type IV collagen.[1,6] The overexpression of these 2 metalloproteinases could be associated with tumor progression and survival rate of the patients.[1,5,6,14] It could be observed that high expression of MMP-9 is detected in patients with lymph node metastasis and this can promote recurrence or second primary tumors.[1,3,5,8,15] In our study, the high expression of MMP-9 both in cancer cells and stroma correlated with more aggressive tumor behavior, namely the presence of lymph node involvement. This was also observed in other studies.[1,5,14] The high expression of MMP-9 degrading of ECM could also act as a promoter for growth of new blood vessels. The present studies, as well as data presented by others, prove that MMP-9 regulates an angiogenesis process leading to recurrence or metastasis.[5,8]

The overexpression of MMP-2 is considered as another poor prognostic factor. Its high expression is correlated with local recurrence and metastasis.[5–7,14] In our study, we found a higher expression of MMP-2 in stroma than in cancer cells among patients with lymph

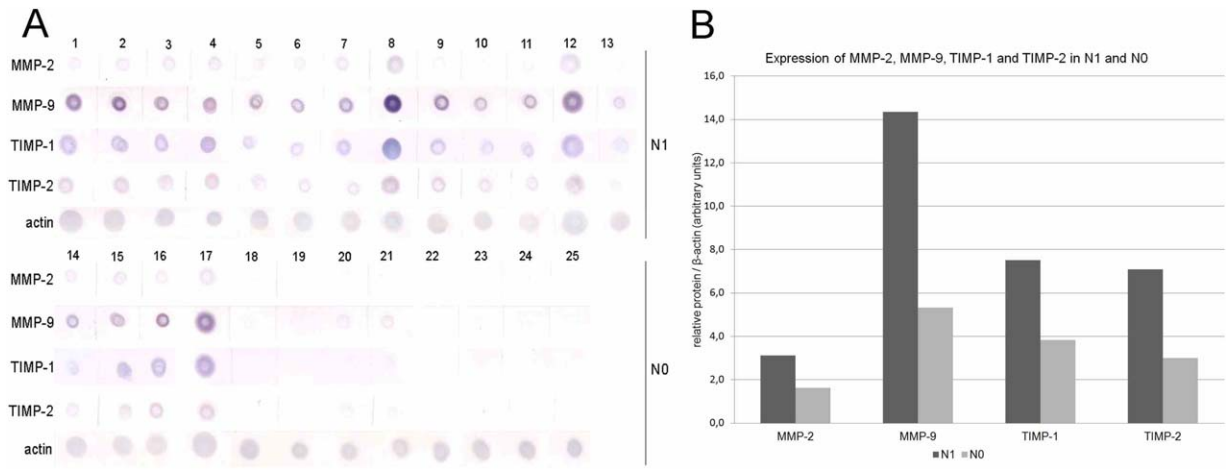

FIGURE 4. Immunoblots assays of matrix metalloproteinase (MMP)-2, MMP-9, tissue inhibitor of metalloproteinases (TIMP)-1, TIMP-2, and β-actin (as internal control) revealed stronger signal in almost all studied proteins extracted from patients with lymph node metastases (N1) than those observed in proteins derived from patients' tissues without lymph node involvement (N0). Lines 1 to 13 correspond to proteins from N1 and lines 14 to 25 to N0 (A). Densitometric analyses of dot-blots were performed (B). Data were normalized to the housekeeping protein β-actin. Values are presented as means ± SD. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

node metastasis. Such an observation allows for suggestion that increasing expression of MMP-2 in tumor stroma may be a prognostic factor for lymph node metastasis development. Such results of additional studies in clinical material could be taken into consideration of modification of adjuvant oncologic therapy after surgery treatment.

The activity of MMPs is regulated by gene expression and also by stimulation of their secretion. The secreted enzyme activity is regulated by propeptide or by tissue inhibitors of metalloproteinases.[1] SCC of the oropharynx with lymph node metastasis are characterized with high expression by TIMP-1 and TIMP-2 compared with patients without metastases.[1–3,6] Patients with lymph node metastasis had higher levels of TIMP-1 expression both in cancer cells and stroma.[1,3,6] TIMP-2 presents less expression than TIMP-1 both in cancer cells and stroma with moderate increase of expression in patients with lymph node metastasis.[3,6] Our study confirmed the previous observations.

The new direction according to cancer progression indicates that the metastases formation during cancer progression is the result of interactions between the tumor cells and the microenvironment. The understanding of multilateral interactions between the tumor and its microenvironment might allow for a better understanding of tumor biology, which was one of the purposes of those studies. Moreover, we emphasize the importance of the microenvironment during cancer progression, which may have an impact for new therapeutic strategies.

In conclusion, our data suggest that changing of metalloproteinase and TIMP activity are one of the key factors in tumor progression by microenvironment degradation. The increase expression of MMP-9, TIMP-1, and TIMP-2 in cancer cells are characteristic for lymph node metastasis development. On the other hand, the increased expression of MMP-2 in tumor stroma may be a prognostic factor associated with the node metastasis.

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