**MMP-10, MMP-7, TIMP-1 and TIMP-2 mRNA expression in esophageal cancer**

Agnieszka Juchniewicz¹, Oksana Kowalczuk¹, Robert Milewski², Wojciech Laudaniński³, Piotr Dziegielewski³, Mirosław Kozłowski³ and Jacek Nikliński¹,³

¹Department of Clinical Molecular Biology, Medical University of Białystok, Białystok, Poland; ²Department of Statistic and Medical Informatics, Medical University of Białystok, Białystok, Poland; ³Department of Thoracic Surgery, Medical University of Białystok, Białystok, Poland

**Introduction:** Tissue inhibitors of metalloproteinases (TIMP) and the matrix metalloproteinases (MMP) are involved in the spread of cancer. Methods: We have evaluated the matrix metalloproteinases’ (MMP-10, MMP-7) and their inhibitors’ (tissue inhibitors of metalloproteinases – TIMP-1, TIMP-2) mRNA expression in 61 esophageal cancer samples from patients who had undergone surgery, by using real-time quantitative RT-PCR, and correlated the results with the patient clinicopathologic features. Results: MMP-10, MMP-7, TIMP-1, TIMP-2 were overexpressed in 73%, 85%, 55% and 42% of esophageal cancer samples, respectively. The expression of MMP-10, TIMP-1, and TIMP-2 correlated with the tumor size. The MMP-7 overexpression was associated with the tumor stage (I, II vs III, p=0.05) and lymph node metastasis (N0 vs N1, p=0.037). Conclusions: We conclude that in the resected esophageal cancer an increased mRNA expression of MMP-7, MMP-10 and TIMP-1 correlated with clinicopathologic features. We suggest that these genes may play a role during progression of the disease.

**Key words:** Esophageal cancer, gene expression, metalloproteinases

**METHODS**

Esophageal carcinoma (ESCC) is one of the leading causes of cancer death worldwide and causes an estimated 400,000 deaths per year all over the world (Lozano et al., 2012). ESCC is characterized by an extensive local invasion, poor prognosis, and rapid clinical progression with a high frequency of lymph node metastasis and recurrence after treatment (Vallböhmer et al., 2010). Due to this aggressive nature and the lack of a screening strategy to detect an early stage of the disease, the prognosis of ESCC is even worse than other digestive tract cancers (Bollschweiler & Hölscher, 2007; Kollarova et al., 2007).

One of the key steps during invasion and metastasis of ESCC, are structural changes in the extracellular matrix and degradation of the connective tissue surrounding the tumor cells, mediated by the matrix metalloproteinases (MMP) (Lynch & Matrisian, 2002). Matrix metalloproteinases are a family of zinc-containing enzymes which are associated with tissue destruction under various pathologic conditions (Deryugina & Quigley, 2006; Rydlova et al., 2008). MMP have been found to be overexpressed in a variety of epithelial and mesenchymal tumors, such as liver, renal, colon and esophagus (Leeman et al., 2003; Zhang et al., 2005; Miyata et al., 2006). High expression level of MMPs has been correlated with the tumor aggressiveness and unfavorable prognosis (Szarvas et al., 2010). MMPs are produced by the tumor cells themselves, and exhibit a broad and strong proteolytic activity against a variety of components of the ECM, such as collagens, vitronectin, gelatin, lamin, fibronectin, and proteoglycans (Visse & Nagase, 2003).

Tissue inhibitors of metalloproteinases (TIMP) are well-studied inhibitors of metalloproteinases which play an important role in tumor invasion and metastasis (Sato et al., 1992). TIMP consist of a family of four structurally related proteins and have direct effects on cellular behaviors, such as cell growth, migration, differentiation, and apoptosis (Baker et al., 2002; Jian et al., 2002). An increased expression of matrix metalloproteinases and their tissue inhibitors is related to tumor aggressiveness and overall survival in various human malignancies (Stetler-Stevenson, 2001).

In the study presented here, we have evaluated the relationship between the matrix metalloproteinases’ (MMP-10, MMP-7) and their inhibitors’ (tissue inhibitors of metalloproteinases – TIMP-1, TIMP-2) mRNA expression, and the clinicopathological features of patients with esophageal carcinoma.

**INTRODUCTION**

**Patients and tissues.** Tumor specimens were obtained from 61 patients with primary esophageal cancer (29 – esophageal squamous cell carcinoma, 32 – adenocarcinoma of the esophagus), who underwent an esophagectomy at the Department of Thoracic Surgery of the Medical University of Białystok. None of them had received a preoperative chemorotherapy or radiotherapy. The study population consisted of 56 men (91.8%) and 5 women (8.2%). The average age at the time of diagnosis was 64.0 years (ranged from 44 to 82 years). The pathological stage was based on AJCC TNM classification (7th edition) (Edge et al., 2010). All patients were followed up clinically after the surgery. Evaluations were performed every 3–6 months by means of a clinical history, physical examination, laboratory analysis, fiber-optic esophagoscopys, ultrasound examination of the neck and abdomen, barium esophagram, computed tomography, EUS, PET-CT and EBUS, if necessary. The average follow-up time was 33 months (ranged from 5 to 101 months). Nonmalignant esophageal tissues were collected as control specimens (Juchniewicz A. et al., 2015).
RNA extraction and cDNA synthesis. Tissue samples were collected intraoperatively. After the macroscopic visual assessment, the pieces of tumor tissue and unaffected esophageal tissue were frozen in liquid nitrogen, followed by storage at −80°C. Sections (4 μm) of frozen tissue specimens were cut and stained with hematoxylin and eosin for verification of the presence of carcinoma cells (Cryotome FSE, Thermo Scientific, UK). These tissue samples were assessed by experienced pathologists. Only the tumor samples which contained at least 50% of tumor cells in a microscopic section were used for further processing.

Total RNA from tissue specimens was isolated and purified using a mirVana™ miRNA Isolation Kit (Ambion®, Austin, TX, USA) following the manufacturer’s protocols. The resulting RNA extracts were stored at −80°C before further processing. RNA quantity was assessed using a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). RNA quality, both 28S/18S ratio and RNA integrity number (RIN), was measured by using an Agilent 2,100 Bioanalyzer (Serial No DE72905449, Agilent Technologies Inc., CA, USA) and an RNA 6,000 Nano Assay Kit (Agilent Technologies Inc., CA, USA), according the manufacturer’s recommendations. One microgram of total RNA was reverse-transcribed into cDNA. cDNA synthesis was performed using a High Capacity RNA-to-cDNA Master Mix with No RT Control (Applied Biosystems™, Foster City, CA, USA), according to the manufacturer’s instructions, and the Labcytcr thermocycler (Sensosquet GmbH, Göttingen, Germany) (Juchniewicz A. et al, 2015).

mRNA Expression level. An mRNA expression level of the matrix metalloproteinases (MMP-10, MMP-7) and their receptor genes (TIMP-1, TIMP-2) were evaluated in the tumor and the corresponding unaffected esophageal tissues, by the comparative real-time PCR (RT-PCR) method using commercially available TaqMan Gene Expression assays (Applied Biosystems™) (Table 1). Amplification was performed with a 20 μl reaction mixture containing 10 μl TaqMan Gene Expression Master Mix (Applied Biosystems™, Foster City, CA, USA), 1 μl of the appropriate TaqMan Gene Expression assay solution and 2 μl of the cDNA solution. Cycle conditions were as follows: 50°C for 2 min, followed by 95°C for 10 min, hold and 40 cycles of 95°C at 15 s and 60°C for 1 min. Each sample was analyzed in triplicate. The reaction was conducted with an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) equipped with the SDS v.2.4 software for baseline and cycle threshold (Ct) calculations. Gene transcript levels were quantified as Ct values, normalized for the differences in the input cDNA amount by subtraction of the Ct value of the 18S rRNA reference gene (ΔCt=Ct gene−Ct 18S). Gene expression levels were inversely proportional to the ΔCt values and were based on a log-scale. The reaction mixture and cycle conditions for the 18S rRNA cDNA amplification were the same as those described for the matrix metalloproteinases (MMP-10, MMP-7) and their receptors’ (TIMP-1, TIMP-2) amplification.

Tumor-associated fold-change (FC) in the mRNA level was calculated as FC=2ΔΔCt, where ΔΔCt equals the difference between the normalized expression of the gene in the tumor (Ct gene) and its normalized expression in the corresponding nonmalignant esophageal tissue (Ct tissue) from the same patient (Schmittgen & Livak, 2008). For the statistical analysis, logarithmically transformed FC values were used (log2(FC)). The log2(FC) value equaled 1.0 was used as a threshold to categorize samples into two groups with low (log2(FC)<1.0) and high (log2(FC)>1.0) gene expression.

Statistical analysis. Due to asymmetric data distribution (Shapiro-Wilk tests) non-parametric tests were performed. Categorical data was compared by the χ² or Fisher’s exact probability test. The p value of less than 0.05 was considered to be significant. Statistical analyses were carried out using the Statistica 10.0 PL program (StatSoft Inc., Tulsa, OK, USA) and the Stata/IC 12.1 (StataCorp LP, Texas, USA).

In accordance with the Declaration of Helsinki, the study protocol was approved by the local Ethics Committee (No R-1-002/28/2010) and written informed consent was obtained from all participants prior to analysis.

RESULTS

An increased expression of MMP-10 mRNA was observed in tumor samples from 45 (73.77%) patients, and MMP-7 mRNA level was increased in 52 (85.25%) patients (Table 2). Expression of MMP-10 correlated with the patients’ age and tumor size. There were no associations between the MMP-10 mRNA expression level and sex, histological type, tumor stage, lymph node metastasis, depth of tumor invasion and location, histological grade and residual tumor. The MMP-7 overexpression was associated with the tumor stage and lymph node metastasis. In cancerous tissues, high expression level of TIMP-1 mRNA was observed in 55.74% of patients, and TIMP-2 mRNA in 42.62% of patients. The TIMP-1 and TIMP-2 expression level had a significant relationship with tumor size.

DISCUSSION

The development of malignant neoplasms is a multistep process which results in rapid growth and invasion of the tumor cells into lymphatic and blood vessels. The first step of the tumor development and metastasis is associated with degradation of the extracellular matrix, which leads to proteolysis of microvessel basement membranes, invasion of endothelium and migration of malignant cells (Samantaray et al, 2004). Some studies indicate that MMP and their inhibitors have been reported to appear closely associated with the tumor invasion,
metastasis and prognosis in patients with esophageal cancer (Yamashita et al., 2000; Gu et al., 2005). The aim of the study presented here was to analyse the mRNA expression levels of MMP-7, MMP-10, TIMP-1, TIMP-2, and the clinicopathological features of patients with esophageal cancer.

We have observed overexpression of MMP-7, MMP-10, TIMP-1, TIMP-2 in the tumor tissues. Our findings are in accordance with the findings of Yamashita et al., Zhou et al. who have shown through immunohistochemistry, western blot, and RT-PCR assays that MMP-7 was overexpressed in the esophageal tumor cells (Yamashita et al., 2000; Zhou et al., 2011). Mukherjee et al. had shown by using quantitative RT-PCR and gelatin zymography that levels of the MMP-10 protein, and its mRNA expression were higher in the esophageal cancer cells than in the corresponding normal tissues (Mukherjee et al., 2010). Studies indicate that some stromal-derived factor might stimulate the MMP expression. Borchers et al. have shown through immunohistochemistry that levels of the MMP-10 protein and its mRNA expression were higher in the esophageal cancer cells.

Here, we have found that the MMP-7 mRNA overexpression associated with the tumor stage and lymph node metastasis. This is in accordance with studies by Yamashita et al. and Tanioka et al. who have reported that the MMP-7 mRNA expression was related to the nodal metastasis and prognosis (Yamashita et al., 2000; Tanioka et al., 2003). Yamanoto et al. had demonstrated by immunohistochemical techniques that the MMP-7 mRNA expression level was related to an advanced tumor stage and a worse prognosis (Yamamoto et al., 1999).

The effects of TIMPs on esophageal tumorigenesis are multifunctional and paradoxical. TIMPs are considered to be inhibitors of tumor development, as well as growth factors. The imbalance between MMPs and their inhibitors may facilitate progression of cancer cells (Kähäri et al., 1999; Nabeshima et al., 2002). In the study presented here, the TIMP-1 mRNA expression level was observed to be increased in 55.7% of ESCC cases, whereas TIMP-2 was increased in 42.6%. Mroczko et al. had shown that patients with gastric cancer had higher TIMP-1 serum levels than the healthy subjects (Mroczko et al., 2009). Salmela et al. had shown that TIMP-1 was overexpressed in 80% of esophageal tumor cells (Salmela et al., 2001).

Mroczko et al. had found that the serum TIMP-1 significantly correlated with the depth of the tumor invasion, lymph node metastases, and distant metastases in gastric cancer and pancreatic cancer (Mroczko et al.,

Table 2. Association between mRNA expression and clinicopathological criteria

<table>
<thead>
<tr>
<th>Clinicopathological Criteria</th>
<th>MMP-10</th>
<th></th>
<th>MMP-7</th>
<th></th>
<th>TIMP-1</th>
<th></th>
<th>TIMP-2</th>
<th></th>
<th>P value</th>
</tr>
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<tr>
<td>Low</td>
<td>16(26.33)</td>
<td></td>
<td>45(73.77)</td>
<td></td>
<td>9(14.76)</td>
<td></td>
<td>52(85.25)</td>
<td></td>
<td>27(44.26)</td>
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<tr>
<td>High</td>
<td>11(39.3)</td>
<td></td>
<td>17(60.7)</td>
<td></td>
<td>6(21.4)</td>
<td></td>
<td>22(78.6)</td>
<td></td>
<td>0.032</td>
</tr>
<tr>
<td>Age &lt;64</td>
<td>5(15.2)</td>
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<td>28(84.8)</td>
<td></td>
<td>3(9.1)</td>
<td></td>
<td>30(90.9)</td>
<td></td>
<td>0.161</td>
</tr>
<tr>
<td>Age &gt;=64</td>
<td>5(16.1)</td>
<td></td>
<td>47(83.9)</td>
<td></td>
<td>3(60.0)</td>
<td></td>
<td>2(40.0)</td>
<td></td>
<td>0.390</td>
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<tr>
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<td>20(69.0)</td>
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<td>6(20.7)</td>
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<td>23(79.3)</td>
<td></td>
<td>0.301</td>
</tr>
<tr>
<td>Type</td>
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<td></td>
<td>25(78.1)</td>
<td></td>
<td>3(9.4)</td>
<td></td>
<td>29(90.6)</td>
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<td>0.005</td>
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<tr>
<td>Location</td>
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<td>25(69.4)</td>
<td></td>
<td>5(13.9)</td>
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<td>31(86.1)</td>
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<td>Tumor size</td>
<td>5(21.7)</td>
<td></td>
<td>18(78.3)</td>
<td></td>
<td>4(17.4)</td>
<td></td>
<td>19(82.6)</td>
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<tr>
<td>Stage</td>
<td>6(19.4)</td>
<td></td>
<td>25(80.6)</td>
<td></td>
<td>4(12.9)</td>
<td></td>
<td>27(87.1)</td>
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<tr>
<td>Histological grade</td>
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<td>17(63.0)</td>
<td></td>
<td>5(18.5)</td>
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<td>22(81.5)</td>
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<tr>
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<td></td>
<td>25(73.5)</td>
<td></td>
<td>1(2.9)</td>
<td></td>
<td>33(97.1)</td>
<td></td>
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</tr>
<tr>
<td>Histological grade</td>
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<td></td>
<td>15(71.4)</td>
<td></td>
<td>6(28.6)</td>
<td></td>
<td>15(71.4)</td>
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<td>0.496</td>
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<tr>
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<td>41(77.4)</td>
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<td>6(11.3)</td>
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<td>47(88.7)</td>
<td></td>
<td>0.116</td>
</tr>
<tr>
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<td>4(50.0)</td>
<td></td>
<td>4(50.0)</td>
<td></td>
<td>3(37.5)</td>
<td></td>
<td>5(62.5)</td>
<td></td>
<td>0.087</td>
</tr>
</tbody>
</table>
2009; Mroczko et al., 2009). Moreover, Mimori et al had demonstrated that the primary gastric carcinomas with high TIMP-1 expression have shown a higher grade malignant potential (Mimori et al., 1997). In esophageal cancer, Mori et al observed a higher frequency of lymph node metastasis and advanced disease stage with poorer prognosis in high TIMP-1 mRNA expression cases (Mori et al., 2000). Kozlowski et al had shown a significant association between the tumor depth, advanced stage, lymph node metastasis and serum concentration of TIMP-1 in the esophageal cancer patients (Kozlowski et al., 2013). A multivariate analysis expression of TIMP-1 was an independent prognostic factor for overall survival in resected EC patients (Mori et al., 2000). Sharma et al had shown the prognostic significance of TIMP-1 and TIMP-2 mRNA expression in ESCC in relation to invasion, tumor progression, and metastasis (Sharma et al., 2004).

Patients with TIMP-2 negative carcinoma had a significantly shorter disease free survival in comparison with the TIMP-2 positive tumors (Sharma et al., 2004). Similar results were obtained by Alakus et al who had suggested that aggressive forms of gastric cancer are associated with low TIMP-2 expression. In their study, the TIMP-2 levels were lower in more advanced tumor stages than in the early stages I+II (Alakus et al., 2008). In the study presented here, the TIMP-1 and TIMP-2 expression were not found to be a significantly independent prognostic factor (data not shown).

In summary, the study presented here demonstrates that in the resected esophageal cancer, an increased mRNA expression of MMP-7, MMP-10 and TIMP-1 is correlated with clinicopathological features. We suggest that these genes may play a role during the progression of the disease and this issue requires further investigation.

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