INTRODUCTION

Pancreatic cancer (PC) is the fourth most common cancer cause of death. The incidence of PC depends on world region analyzed, due to the strong impact of environmental and behavioral factors (1). PC is characterized by a rapid course, fatal prognosis and short survival time. The mean survival from the time of the diagnosis is about half a year (2). Average 5-year survival rate is the lowest of all gastrointestinal tumors (3). Chronic pancreatitis (CP) is a progressive disease leading to fibrosis of the pancreas. Recently an increase of the incidence and hospitalization due to this disease has been shown (4).

Metalloproteinases (MMPs) play a significant role in the pathogenesis of PC and may have impact on CP course, however there is no sufficient data to confirm this statement. MMPs belong to a group of zinc-dependent proteases-ENDopeptidases. These enzymes are responsible for the basement membrane (BM) and extracellular matrix (ECM) degradation (5). MMPs and their inhibitors (TIMPs- tissue inhibitors of matrix metalloproteinases) are involved in many physiological and immunological processes (e.g. organogenesis, angiogenesis, fertilization, cell migration, wound healing, scar formation, inflammatory response). Their participation was also described in numerous pathologies: autoimmune diseases, gastric and duodenal ulcers, hepatic cirrhosis, Crohn's disease, myocardial infarction, atherosclerosis, aneurysms, CNS degenerative diseases, CNS hypoxic diseases and cancer (6-14).

The degradation of BM and ECM plays a key role in tumors progression and severity of inflammation. Gelatinases and their inhibitors play important role in the pathogenesis of the CP as well as PC. The activity and concentration of gelatinases and the concentration of their inhibitors were all significantly higher in the PC group.

Key words: chronic pancreatitis, pancreatic cancer, metalloproteinases, gelatinases, tissue inhibitors of metalloproteinases
The aim of this study was a comparative analysis of the activity and concentrations of MMP2 and MMP9 and the concentrations of their inhibitors (TIMP 1 and 2) in the PC tissue samples as compared to CP. This knowledge may help to understand the pathogenesis of these severe and common diseases and may have important diagnostic or therapeutic implications in the future.

MATERIAL AND METHODS

Patients

The study has been performed in a group of 63 patients including 25 (39.68%) women and 38 (60.32%) men with pancreatic cancer or chronic pancreatitis selected for resection at the Department of Gastrointestinal Surgery, Medical University of Silesia in Katowice (Poland) between 2006-2011. There was no coincidence of pancreatic cancer in CP group. Patients from CP group were included into this study only when the tumor was observed in the course of the disease. They were all considered for resection procedures. There were also several types of resection extents in both groups (Table 1). The only exclusion criterion was coincidence of PC in CP group.

Groups

CP group consisted of 31 patients (F:M=10:21), PC group consisted of 32 patients (F:M=15:17). The mean age of PC patients was 60.25±10.72 years, and the CP patients was 45.64±12.9 years. Average BMI index was 23.84±2.94 kg/m² in PC group and 21.78±3.88 kg/m² in CP. Median of tumor diameter was 4(2-6) cm in CP and 3(1.2-8) cm in PC group (Table 1). Additional divisions of PC group were performed to evaluate relationships between MMPs and TIMPs concentrations and characteristics of PC group was shown in Table 3. Stage and differentiation of the cancer, pathological blood vessels and nerve fibers infiltration were also analyzed in terms of MMPs and TIMPs concentrations. All histopathological examinations were performed by means of routine methods.

Tissue collection and processing

A Local Ethics Committee approved the study protocol (NN-6501-163/07). The pancreatic tumor tissue samples were taken within 10 minutes after surgical resection with a mass about 0.5 g and were immediately frozen at -80°C. They have been then homogenized by means of the ultrasonic homogenizer (Ultrasonic Processor, Cole-Palmer, USA) for 1 minute at a temperature of 40°C. The homogenate was centrifuged for 30 minutes at 6000g. Supernatants were subjected for further analyses.

Zymography

Examined pancreatic tissue was placed in homogenizing buffer (0.01 M CaCl₂ and 0.25% Triton 100) (0.5 grams of tissue per 10 mL of buffer) and then homogenized by means of ultrasonic homogenizer (Ultrasonic Processor, Cole-Palmer, USA) for one minute at a temperature of 40°C. The homogenate was centrifuged for 30 minutes at 6000 g. After centrifugation, supernatant of Triton-soluble fraction was obtained, collected and frozen in -70°C in 100 µL aliquots. Zymography was carried out according to Kleiner and Stetler-Stevenson (1994). Electrophoresis according to the Laemmli (1970) was carried out on SDS-polyacrylamide slab gels with gelatin (concentration of 2 mg/mL per gel) in vertical apparatus (BioRad, USA) filled with conductive buffer (3 g Tris, 14.4 g glycene, 0.1 g SDS per 1 liter of buffer). Before application of the samples, 190 min “pre-run” of the gel was performed using direct current (DC) of 10 mA. The sample was mixed with protein loading buffer (0.02 M Tris pH 8.8, 0.002 M EDTA, 5% SDS, 10% 2-mercaptoethanol, 0.2% bromophenol blue) in a 2:1 ratio. Each sample well was loaded with 40 µL of protein solution. The samples were concentrated in 5% thickener gel (4.875% acrylamide, 0.125% N,N'methylene-bis-acrylaldehyde, 0.0125 M Tris pH 6.8, 0.1% sodium dodecyl sulfate (SDS), 0.1% ammonium persulfate (APS), 0.2% N,N,N',N' methylene tyleno-ethylene-diamine (TEMED)) and then separated in 12.5% separating of gel (12.1875% acrylamide, 0.3125% N,N'methylene-bis-acrylamide, 0.225 M Tris pH 8.8, 0.1% SDS, 0.04 % APS, 0.075% TEMED) (all reagents by Sigma). In each gel, mixture of MMP-2 and MMP-9 proteins (Sigma, USA) was applied as standards. After electrophoresis, the gels were washed twice for 15 minutes in 2.5% Triton. Then, they were placed in the incubation buffer at 37°C degrees for 48 hours. After that, gels were stained for one hour in 0.1% Coomassie R-250 Blue in 40% 2-propanol. Gelatinases digested gelatin from polyacrylamide gels leaving a colorless band. To visualize these bands, gels were destained in mixture of methanol and acetic acid (concentrations 40% and 10%, respectively). This process was carried out, until the maximum contrast between the transparent stripes and a blue background was observed. Then, gels were dried and photographed. The final analysis was carried out after completion of all data. Presence and activity of MMP-2 and MMP-9 in examined samples was stated via comparison of the position of etched bands with the appropriate proteinase standard. Matching position of the bands proved presence of the enzyme, and discoloration of the band - enzyme proteolytic activity.

Table 1. Patients and groups (CP - chronic pancreatitis; PC - pancreatic carcinoma).

<table>
<thead>
<tr>
<th></th>
<th>CP (n=31)</th>
<th>PC (n=32)</th>
<th>Both (n=63)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female/Male</td>
<td>10/21</td>
<td>15/17</td>
<td>25/38</td>
<td>NS</td>
</tr>
<tr>
<td>Age (years) (average, sd)</td>
<td>45.6±12.9</td>
<td>60.2±10.7</td>
<td></td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.78±3.88</td>
<td>23.8±2.9</td>
<td></td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Extent of the resection</td>
<td></td>
<td></td>
<td></td>
<td>0.08</td>
</tr>
<tr>
<td>Proximal</td>
<td>24 (74.2%)</td>
<td>24 (75%)</td>
<td>48 (76.2%)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>22 (69%)</td>
<td>22 (69%)</td>
<td>44 (69%)</td>
<td></td>
</tr>
<tr>
<td>Extended distal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distal</td>
<td>4 (12.9%)</td>
<td>0</td>
<td>4 (6.35%)</td>
<td></td>
</tr>
<tr>
<td>Local tumor excision</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extended proximal</td>
<td>1 (3.23%)</td>
<td>0</td>
<td>1 (1.59%)</td>
<td></td>
</tr>
<tr>
<td>Tumor diameter (cm) (median, interquartile range)</td>
<td>4 (2–6)</td>
<td>3 (1.2–8)</td>
<td></td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
ELISA

Quantitative analysis with use of the spectrophotometric ELISA method according to the manufacturer's instructions was performed (USCN Life Science Inc ®). To determine the concentration of MMP2 and MMP9 and their inhibitors TIMP1 and TIMP2 appropriate ELISA tests were used (USCN Life Science Inc ®).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Median (IQR)</th>
<th>PC (Median, interquartile range)</th>
<th>CP (Median, interquartile range)</th>
<th>p (U Mann Whitney)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP2 (ng/ml)</td>
<td>0.65 (0.34; 0.71)</td>
<td>0.87 (0.8; 1.29)</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>MMP9 (ng/ml)</td>
<td>1.02 (0.33; 1.72)</td>
<td>8.1 (6.02; 9.02)</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>TIMP1 (pg/ml)</td>
<td>52 (35; 76)</td>
<td>400 (297; 680)</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>TIMP2 (pg/ml)</td>
<td>360 (313; 495)</td>
<td>3078 (963; 3305)</td>
<td>&lt;0.01</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Electrophoresis example (CP - chronic pancreatitis, PC - pancreatic carcinoma, SN - standard). MMP2 - metalloproteinase 2 (72kDa), MMP9 - metalloproteinase 9 (84kDa), proMMP2 - proenzyme MMP2 (55kDa), proMMP9 - proenzyme MMP9 (92kDa)

MMP2. Activity of MMP2 alike MMP9 in all tissue samples (chronic pancreatitis and pancreatic cancer) was confirmed.

Table 2. Comparison of MMP2, MMP9, TIMP1 and TIMP2 concentrations in CP and PC tissue samples.

Fig. 2. Comparison of MMP2 (A) MMP9 (B) TIMP1 (C) and TIMP2 (D) concentrations in CP and PC tissue samples.
As a result of above procedures the activity of MMP2 and MMP9 as well as the concentrations of MMP2, MMP9, TIMP1, TIMP2 were obtained.

**Statistical analysis**

Results are expressed as the mean ± standard deviation (S.D.) or median and interquartile range. Statistical analysis of the results was performed with use of the following tests: Student's t test, Mann Whitney U test, chi-square test, ANOVA, Kruskal Wallis test. Differences were considered significant for p<0.05. Statistical analysis was performed using STATISTICA 9.0 software (Statsoft, Poland).

**RESULTS**

Patients with PC were statistically significantly older compared to patients with CP (p<0.01). In both groups, males were prevalent (PC - M:F=17:15; CP - M:F=21:10), but this difference was not statistically significant. PC patients presented also significantly higher BMI compared to CP patients (p<0.05) (Table 1).

All patients in our study underwent different pancreatic resection procedures. In PC group, there were no total pancreatic resections and in the CP group, distal resection did not occur, thus the groups were statistically homogeneous in terms of resection range of the pancreas (p=0.08). Proximal pancreatic resection was the most frequently performed surgical procedure in both groups. CP group - 24 (77.42%) patients PC - 24 (75%) patients. Other pancreatic resection types are presented in Table 1.

Enzymatic activity of both gelatinases in all tissue samples was confirmed in PC and CP group. An example result of gelatinases enzymatic activity was shown on Fig. 1.

Concentrations of MMP2, MMP9, TIMP1, and TIMP2 were compared in both groups. Significantly higher concentrations of all proteins were observed in a PC group (Fig. 2).

MMP2 levels were compared with MMP9 and the concentrations of inhibitors TIMP1 and TIMP2 within groups. The concentrations of each enzyme and each inhibitor were compared between CP and PC groups. In CP and PC groups, similar statistically significant differences in the concentrations of the enzymes and their inhibitors were observed. MMP9 and TIMP2 concentrations were significantly higher in both groups (Table 2 and Fig. 2).

Analysis of correlations between the concentrations of inhibitors and the corresponding matrix metalloproteinases was also done (MMP2 vs. TIMP2 and MMP9 vs. TIMP1). Correlation was analyzed for each group separately. There was a significant correlation between MMP2 and TIMP2 in PC group, as well as MMP9 and TIMP1 in CP group respectively (Fig. 3).

Beside of foregoing direct analysis of concentrations and activity of MMPs and concentration of TIMPs, additional comparative analysis based on the histopathological examination was performed. The pathological examination confirmed finally chronic pancreatitis in 31 samples and...
pancreatic adenocarcinoma in 32. Median of the tumor diameter was 4 (2-6) cm in CP group, and 3 (1.2-8) cm in PC group (Table 1). Positive correlation of tumor size with the concentration of MMP9 (r=0.52), TIMP1 (r=0.42) and TIMP2 (r=0.49) was found only in CP group (Fig. 4). There was no correlation between the concentrations of MMPs as well as their inhibitors in PC group.

Based upon the TNM classification system, four clinical stages of PC were distinguished. The 2nd and 3rd stages (total 62.5%) were predominantly observed (Table 3). Lymph node metastases occurred in 17 (53.12%) patients. An average number of 16±6 (range 8-33) lymph nodes were removed during a single resection procedure in patients with PC. Analysis of concentration of MMPs and TIMPs according to the TNM stage revealed no statistically significant differences in concentrations of MMP2 and 9 and TIMP1 and 2 depending on the clinical stage of cancer, however concentrations of MMP9 and TIMP2 were higher if tumor stage was greater. The concentrations of MMP9 and TIMP2 were also significantly higher if lymph node metastases were present (Fig. 5).

In 78.1% of cases moderately differentiated (G2) tumor was observed. Poorly differentiated cancers (G3) occurred in 6.3% and well differentiated (G1) in 15.6% cases respectively (Table 3). Well differentiated tumors (G1) were characterized by a

<table>
<thead>
<tr>
<th>Histopathology of the PC</th>
<th>N</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I</td>
<td>7</td>
<td>21.88%</td>
</tr>
<tr>
<td>Stage II</td>
<td>6</td>
<td>18.75%</td>
</tr>
<tr>
<td>Stage III</td>
<td>14</td>
<td>43.75%</td>
</tr>
<tr>
<td>Stage IVa</td>
<td>3</td>
<td>9.38%</td>
</tr>
<tr>
<td>Stage IVb</td>
<td>2</td>
<td>6.25%</td>
</tr>
<tr>
<td>Tumor differentiation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate (G2)</td>
<td>25</td>
<td>78.1%</td>
</tr>
<tr>
<td>Well (G1)</td>
<td>5</td>
<td>15.6%</td>
</tr>
<tr>
<td>Poor (G3)</td>
<td>2</td>
<td>6.3%</td>
</tr>
<tr>
<td>Additional data</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infiltrated pancreatic resection margin</td>
<td>9</td>
<td>28.13%</td>
</tr>
<tr>
<td>Blood vessels infiltration</td>
<td>13</td>
<td>40.63%</td>
</tr>
<tr>
<td>Nerve fibers infiltration</td>
<td>14</td>
<td>43.75%</td>
</tr>
</tbody>
</table>

Table 3. Histopathology - characteristics of the PC tissue samples.

Fig. 4. Significant positive correlation of tumor diameter in CP group with A) MMP9; B) TIMP1; C) TIMP2 concentrations.
lower MMP2 and higher TIMP2 concentrations compared with poorly differentiated ones (G3) (Table 4). However, in the case of MMP9 or TIMP1 concentrations and tumor differentiation inverse relationship has been revealed. Nevertheless no statistically significant differences were found.

The presence of cancer cells in the resection margins was confirmed in 9 (28.13%) cases. Pathological infiltration of blood vessels was found in 13 (40.6%) samples whereas the nerve fibers infiltration in 14 (43.7%) (Table 3). No statistically significant difference between the concentrations of metalloproteinases and their inhibitors where demonstrated if cancer cells were present in the distal pancreatic resection margin or malignant infiltration of blood vessels was observed, compared to samples with no invasion. Similar concentrations of MMP2, MMP9, and TIMP1 were found in samples with or without nerve fibers infiltration, however significantly lower concentration of TIMP2 has been shown if nerve fibers infiltration was present (Fig. 6).

Table 4. MMPs and TIMPs concentrations in PC group depending on cancer differentiation.

<table>
<thead>
<tr>
<th>Cancer Differentiation</th>
<th>MMP2 (Median, range)</th>
<th>MMP9 (Median, range)</th>
<th>TIMP1 (Median, range)</th>
<th>TIMP2 (Median, range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well (G1)</td>
<td>1.3 (0.8-1.9)</td>
<td>7.1 (5.03-9)</td>
<td>616.7 (355.1-873.5)</td>
<td>2170 (1908-3121)</td>
</tr>
<tr>
<td>Moderate (G2)</td>
<td>0.9 (0.8-1.3)</td>
<td>8.1 (6.08-9)</td>
<td>401.2 (295.1-673.8)</td>
<td>3036 (962-3305)</td>
</tr>
<tr>
<td>Poor (G3)</td>
<td>0.8 (0.8-0.9)</td>
<td>8.6 (8.15-8.9)</td>
<td>216.6 (132.9-300.2)</td>
<td>3384 (3367-3400)</td>
</tr>
</tbody>
</table>

Kruskal Wallis test NS NS NS NS

Fig. 5. Comparison of MMP9 - A) and TIMP2 - B) concentrations. depending on the characteristics of N attribute (according to TNM classification system) in PC group (N0 - no lymph node metastases present. N1 - lymph node metastases present).

Fig. 6. TIMP2 concentrations in PC tissue samples depending on the presence of nerve fibers cancer infiltration.
cancer suspicion are the main indications for CP surgical treatment total, and central pancreatectomies (22, 23). Pain without relief literature: proximal, extended proximal, distal, extended distal, location. There are several ranges of the pancreatic resection in the ratio for PC was 16.5 compared to the CP - 13.5, however, showed no statistically significant difference (Table 5, Fig. 7).

**DISCUSSION**

Diagnosis and treatment of PC and CP are still a great challenge. PC is the fourth leading cause of cancer death in men and fifth in women all over the world (1). Our study revealed that elder, overweight patients with comorbidities are characteristic as a PC group but young healthy male as a CP patients. This is in accordance with other papers (1, 4, 19, 20). There are several treatment strategies in PC. Surgical resection is still thought to be the best one (21). The resection range depends on the tumor location. There are several ranges of the pancreatic resection in the literature: proximal, extended proximal, distal, extended distal, total, and central pancreatectomies (22, 23). Pain without relief and with no response to pharmacological treatment, as well as cancer suspicion are the main indications for CP surgical treatment (24). It is commonly believed that pancreatic resection is the most popular method of surgical CP treatment (25, 26). According to other authors drainage procedures were superior to the resections (e.g. Frey, Partington and Rochelle procedure) (24, 27). Each type of therapy has its proponents and opponents, and the discussions concerning the selection of the optimal treatment are ongoing (21, 24-26). However, the most recommended surgical treatment currently is in fact a combination of the drainage procedures with partial resection of pancreatic head (27).

In our material, all patients underwent resection procedures. Thus patients with CP should be obviously considered as patients who underwent resection procedures only. The most commonly performed surgery in both groups was proximal or extended proximal resection (PC: CP=81.2%; 80.6%). Total pancreatic resection was performed in 13% of patients. Such procedure was not performed in the PC group. Statistical analysis showed groups homogeneity in terms of performed surgery. All PC tumors were found resectable. Only 40.6% of resected tumors were diagnosed as 1st and 2nd stage according to the TNM classification system. 43.7% were classified as the 3rd stage. In the 9.37% cases of palliative resections (R2), distant metastases were also found. Data presented above showed that the ratio of R0 resections was about 40%, R1 - 50%, and R2 - 10%. An average number of removed lymph nodes during the surgery and percentage of metastases to them as well as tumors differentiation in our study were in consent with the literature (28). We found considerable difference in the ratio of R0 resections when compared with other authors (28, 29) who used similar methods. However ratio of patients with lymph nodes involvement, tumors differentiation grade, presence of malignant cells in the resection margin were similar to above mentioned data (28-30).

Gelatinases play a significant role in the PC and CP pathogenesis because of their substrates affinity (decomposition of the ECM and BM components). The degradation of both structures involving these enzymes is regulated at several levels. Excessive degradation of these structures is prevented by the tissue inhibitors of metalloproteinases (TIMPs). Imbalance between enzymes and their inhibitors leads to excessive BM and ECM degradation what allows cancer cells spreading and facilitates neoangiogenesis (5, 8, 10, 11). In the presented material enzymatic activity of gelatinases has been demonstrated in PC as well as CP tissue samples with use of the zymography method. These results correspond with many papers (5, 9, 31).

Whenever gelatinases activity was confirmed in the zymography, tissue samples were analyzed by means of quantitative ELISA immunoassay. Zymography to determine gelatinases enzymatic activity, as well as ELISA immunoassay tests to determine concentrations of enzymes and their tissue inhibitors are both commonly used (32). However, there are contradictory data concerning activity and concentrations of gelatinases and their inhibitors in the PC and CP (33, 34). Many authors described the increased activity of MMP2 in PC as well as CP tissue samples with use of the zymography. These results correspond with many papers (5, 9, 31).

**Table 5.** MMP2/TIMP2 ratio and MMP9/TIMP1 ratio describing a degradative environment.

<table>
<thead>
<tr>
<th>RATIO</th>
<th>PC Median (interquartile range)</th>
<th>CP Median (interquartile range)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP2/TIMP2</td>
<td>0.31 (0.24; 1.04)</td>
<td>1.48 (0.62; 2.14)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>MMP9/TIMP1</td>
<td>16.51 (6.76; 28.80)</td>
<td>13.50 (8.43; 29.48)</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Fig. 7.** MMP2/TIMP2 ratio (A) and MMP9/TIMP1 ratio (B).
pancreatic juice (33-36). In 1997, Koshiha analyzed gelatinases activity in PC and normal pancreatic tissue homogenates using zymography method. The activity of MMP9 in both groups was revealed, while the active form of MMP2 was detected only in the PC samples in this study (33). In the study at Mroczko et al., the levels of MMP9 and TIMP1 in serum of patients with PC and CP were assessed. Significantly higher level of MMP9 and TIMP1 in PC compared with those with CP was shown in this study. It should be underlined that elevated level of MMP9 was an independent survival predictor (34). However, according to other authors (35) MT1-MMP, one of the membrane type metalloproteinases, is most responsible for the cancer progression by excessive MMP2 activation. Significant correlation between MT1-MMP expression and MMP2 expression and activity was demonstrated in PC tissue homogenates (35). Estimation of activity of MMP2 in pancreatic juice samples were examined by Yokoyama et al. (36). Authors compared the presence and activity of proMMP2 and active MMP2 in pancreatic juice in three groups of patients: PC, CP and without pathology of the pancreas. Their study shown greater MMP2 activation ratio (ratio of active form of MMP2 to the total MMP2) in pancreatic juice of PC patients compared to those with CP (36). In our study, the levels of gelatinases and their inhibitors were compared between the CP and PC group. Statistically significantly higher levels of both gelatinases and their inhibitors were observed in PC group. These results are compliant with the literature data (33-36).

The concentration levels of MMP2 vs. MMP9 and TIMP1 vs. TIMP2 were also compared separately for PC and CP group. Statistically significantly higher levels of MMP9 concentrations compared to MMP2 were observed in both groups. Higher levels of TIMP2 concentrations compared to TIMP1 were observed also in both groups.

In many articles, the higher expression and activity of MMP9 in the PC tissue samples in comparison with CP or normal pancreas were confirmed (33, 35, 36). Some authors suggest that higher activity of MMP9 in the PC is associated with increased invasive potential. This manifested with the intensity of angiogenesis, or distant metastases formation (37). Other authors studying the activity of gelatinases in PC and CP tissue samples revealed a importance of MMP2 activity in the PC compared to the CP (33, 35). This statement supported the studies assessing the activity of MMP2 in pancreatic juice of PC patients (36), however there are also reports about increased MMP9 expression in PC pancreatic juice in comparison with CP, in immunostaining and Western blot (38). Some authors also assessed the concentration of MMP9 inhibitors. Giamnopoulos and colleagues studied the expression of MMP2, MMP9 and TIMP2 in PC immunohistochemically. The results of this study indicated that PC of poor differentiation were characterized by decreased TIMP2 expression. High TIMP2 concentrations correlated with lymphatic vessels infiltration (39). Other paper demonstrated higher levels of TIMP1 in PC group serum compared with the CP (34). These results were also confirmed in 1996 by Bramhall et al. studies. (40).

The analysis of correlation between the concentrations of inhibitors and the corresponding matrix metalloproteinases was also performed in our study (MMP2 levels correlated with TIMP1 and MMP9 with TIMP1) separately for each group (CP and PC). Two statistically significant correlations were found: one in CP and one in CP group. MMP9 concentration correlated positively with the concentration of TIMP1 in the CP group. MMP2 concentration correlated negatively with the concentration of TIMP2 in the case of PC. There were no other significant correlations between gelatinases and their inhibitors in both groups. This result may indicate that the enzymatic activity of MMP9 prevails in the PC tissue samples, because with increasing concentrations of MMP9, the corresponding inhibitor concentration did not increase at all. With increasing MMP2 concentration the corresponding inhibitor concentration decreased significantly. It may be explained by the MMP2 activation and inhibition pathways. For the activation of MMP2 one molecule of TIMP2 is needed, while the second molecule of TIMP2 deactivates the enzyme. Furthermore, MMP2 can play a major role in the CP, since the increase in its concentration did not follow increasing concentration of its inhibitor, as it is in case of MMP9 and TIMP1. Putting together the above analysis, correlation between the concentrations of the gelatinases, inhibitors and previously shown difference in their concentrations in both groups (MMP2>MMP9 and TIMP1<TIMP2), one can conclude that MMP9 plays an important role in the pathogenesis of PC while MMP2 in the CP. These results are supported by the earlier papers (33, 35, 36, 39, 40). It should be also noted that the activity of MMP2 in CP was also the subject of animal studies by Yamaguchi et al. in 2005. In their experiment conducted on rats with induced chronic pancreatitis, it was demonstrated that long-term increase in MMP2 activity in the CP causes disease progression, while the short-term episodes of increased MMP2 activity allow the pancreas remodeling and accelerate healing process (41).

Tumor size showed positive correlation with the concentration of MMP9 (r=0.52), TIMP1 (r=0.42) and TIMP2 (r=0.49) only in the CP group. In our material, the average tumor size in the CP group was statistically significantly bigger than in PC. CP tumor of bigger diameter showed an increase in both MMP9 and its inhibitor TIMP1 concentrations, what may indicate a balance in this system. The increasing TIMP2 concentration with increasing tumor diameter, with no increase in MMP2 concentration was discussed above.

PC group was analyzed separately. The concentration of gelatinases and their inhibitors, depending on the attributes of the TNM classification system were examined. The relationship between MMPs and TIMPs concentrations and the degree of tumor differentiation, the presence of cancer cells in the pancreatic resection margin, as well as nerve fibers invasion and tumor stage were also analyzed in the same manner. No statistically significant difference between MMP2 and TIMP1 levels depending on the characteristics of TNM attributes were observed. In contrast, significantly higher levels of MMP9 and TIMP2 in the presence of lymph nodes metastases (N1) were observed as compared to the group N0 (no lymphatic nodes metastases). This showed a significant MMP9 and TIMP2 influence on cancer progression that results in the increased invasiveness potential. There are also other studies confirming this hypothesis (39, 42). In 2007, Pryczynicz et al. showed a significant effect of increased MMP9 expression in tissue samples on the ability of the cancer cells to metastasize to adjacent lymph nodes as well as to the distant organs (42). According to other authors, increased TIMP2 concentration in the PC correlates with the degree of lymphatic vessels infiltration, what ultimately leads to the lymphatic node metastases formation (39). On the other hand, there are also studies based on PC tissue samples demonstrating higher TIMP1 levels in the presence of lymphatic node metastases (40). In our study, cancers of higher invasiveness potential, characterized by a low degree of differentiation (G3), had higher MMP2 levels and lower TIMP2 levels. Lower MMP9 levels and higher TIMP1 levels were observed as well. However, no statistically significant difference in the concentrations of MMPs and TIMPs between cancers of various degrees of differentiation were found.

The degree of tumor differentiation was also examined in above mentioned Bramhall et al. paper (40). According to them, high concentration of MMP2 and TIMP1 was characteristic for lower degree of cancer differentiation and for the so-called
invasive phenotype. Our data presented above led to similar conclusion. Experiment carried out in cell cultures demonstrated the ability of synthetic protease inhibitors to reduce the degree of invasiveness of PC tumors what seems to be additional confirmation of our results. Treatment with gabexate mesilate (GM) was reducing the MMP2 expression. This resulted in inhibition of tumor cell invasiveness (43). According Giannopoulos and colleagues (39) low levels of TIMP2 in PC tissue samples, is parallel with low degree of cancer differentiation.

If pancreatic surgical resection margin was found to be infiltrated by cancer cells, higher concentrations of both enzymes and their inhibitors were revealed in comparison with cancers with no such infiltration. Nevertheless, these differences were not statistically significant. There was also no statistically significant difference between the concentrations of MMPs and their inhibitors in cases with pathological infiltration of blood vessels, compared to these with no such infiltration. There was no statistically significant difference between concentrations of MMP2, MMP9, and TIMP1 in cases of nerve fibers infiltration compared to the absence of such infiltration as well. Only TIMP2 concentration was statistically lower when infiltration of nerve fibers was observed. Among others, Nagakawa et al. examined tissue expression of gelatinases in PC depending on the malignant infiltration of blood vessels and distant metastases formation. According to these authors, increased MMP2 and MMP9 expression characterized tumors infiltrating large vessels, with presence of liver metastases as well (44). According to another authors, an increased MMP9 activity was observed in the locally advanced tumors, infiltrating blood vessels, and giving distant metastases (42). Otherwise, according to Harvey et al. (45) MMP9 is one of the factors responsible for the angiogenesis induction, what also has great impact on the tumor growth and cell invasion by facilitating blood vessels penetration into the tumor. Comparison of the gelatinases and their inhibitors concentrations, depending on the clinical stage of cancer was also performed in our study. Though there was no statistically significant difference in the concentrations of MMPs and TIMPs according to the clinical stage of cancer, there was an rising trend for MMP9 and TIMP2 levels with increasing tumor stage. Other authors (45, 46) did not found a correlation between the severity of PC and the concentration of gelatinases and their tissue inhibitors either. However, the existence of such correlation was found with respect to the degree of cancer differentiation (34, 39), and the presence of lymphatic node or distant metastases as well (42, 45).

MMP2/TIMP2 ratio in PC group was significantly different from the ratio in the CP interquartile range for the CP was in the (1/2, 1) range (the interval of high activity). Range for CP ratio goes beyond the value of 2. There is an excess of enzyme but is may not be active in the absence of inhibitor required for activation. Its activity is determined by the presence of the inhibitor. MMP9/TIMP1 ratio for PC was greater to the CP however, showed no statistically significant difference. There is theoretically straightforward forward correlation between MMP9/TIMP1 ratio and MMP9 activity. The higher the ratio, the greater the activity.

Enzyme (MMP) combines with the inhibitor (TIMP) in a stoichiometric ratio. For the activation of MMP2 one molecule of TIMP2 is needed, while the second molecule of TIMP2 deactivates the enzyme. The situation is different in the case of MMP9, where enzyme remains active only until it is not connected to TIMP1 molecule (for the deactivation of MMP9 only one molecule of TIMP1 is required) (10, 11). When MMP9/TIMP1 ratio is less than or equal to 1, this may indicate that the inhibitor concentration is equal or greater than the enzyme in the environment and may lead to the fact that the enzyme can be locked in its entirety. If the ratio is greater than 1, it could mean that the concentration of the enzyme is greater than the inhibitor. Theoretically, this could manifest itself in higher MMP9 activity. In the case of MMP2/TIMP2 ratio, situation is different and more complicated because two TIMP2 proteins are required for the enzyme inactivation, while for the activation one TIMP2 protein is needed. Therefore, if MMP2/TIMP2 ratio is in the (0, 1/2) range, inhibitor may be superior to the enzyme (the entire enzyme can be blocked and inactive). If the value of the ratio is equal to 1, this theoretically means the greater enzyme activity. If, however, the ratio is in the (1/2, 1) range, it could mean that part of the enzyme is active and part is locked by inhibitor. Above described situation is theoretical. Real environmental reactions between enzymes and inhibitors are accidental. Thus conclusions from this analysis should not be the main ones. In this paper enzymes activity was confirmed in the zymography (Fig. 1).

Analyzing own study results, some inaccuracies should be also certainly considered. The number of samples in analyzed groups (30 and 31) could cause a uncertain statistical inference although multiple statistical analyses performed in our study may reduce this uncertainty. Although some results leave no illusions as to their significance, some only show trends, and some conclusions could be more certain if the group sizes were larger. Another drawback of used methodology was the lability of the biological material causing difficulty in obtaining valuable tissue samples, without enzymatic degradation of proteins. In a certain percentage of cases, a prolonged period of ischemia caused for example by preparation during surgery induced the biological material breakdown, which resulted in the inability to determine enzymatic activity. It should be noted that the activity of the enzyme determined by zymography may be associated also with some uncertainty. It may arise from the fact that it is never quite clear, whether in case of low enzymatic activity in a tissue sample, there is really a small amount of the enzyme, or on the other hand it is really present in large amount, but unfortunately blocked by inhibitors present in the same sample. Part of those doubts were dispelled by MMPs/TIMPs ratios and immunoenzymatic analysis of concentrations of both gelatinases and their tissue inhibitors. Comparison of CP and PC tissue samples brings more imperfections that may be reflected in the appropriate inference and may follow to improper conclusions. This follows from the fact that some cases of pancreatic cancer may be associated with inflammation as well (47). In correspondence with the arguments cited above our results may be saddled with a certain, though small error resulting from the methodology imperfections. However, it should be emphasized that the main hypotheses posed at the outset have been clearly confirmed. (47).

In conclusion, it should be unfortunately highlighted that the differentiation between pancreatic cancer and chronic pancreatitis remains still a major challenge for science. The pathogeneses of both diseases are still not completely understood. New markers of differentiation between these diseases are still being investigated. Gelatinases were also examined in this matter (34, 40). Literature data shows that so far used differentiating serum markers (CEA, CRP, CA19-9) give way to new ones, such as IL-6 (47), neopterin (48), IgG4 (49), IgE (50), VEGF (51). A deeper knowledge of the pathogenesis of PC and CP allows better differentiation of these diseases, which often have a similar clinical course. Precise diagnosis allows also immediate commencement of appropriate treatment. Research for new PC invasiveness parameters, as well as an understanding of this phenomenon can also lead ultimately to new drugs development which may be more effective in the treatment of this more frequently occurring cancer. Unfortunately advances made recently in PC diagnostics and therapy have not significantly improve the survival rates of patients suffering from pancreatic cancer (52). Perhaps thorough
analysis of the genetic disturbances or genome polymorphisms of proteins involved in the disease should be the appropriate direction in PC diagnostics (51). Although prevention of cancer could be possible when disease inducing factors would be known. That could lead to new drugs revealing or emphasizing the unknown value of some nutritional factors that could protect us from cancer. An interesting example was demonstrated in the Calluna Vulgaris (known as common heather) extract. Multidirectional approach is essential to achieve improvement in the PC diagnosis and treatment.

Conflict of interests: None declared.

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