Abstract. Background: The aim of this study was to clarify the relationship between the expression of MMP-9 and Cathepsin B (CathB) at the main tumor mass and lymph node metastases and the presence of tumor budding and vascular invasion at the invasion front, as well as lymph node involvement. Patients and Methods: The immunohistochemical expressions of MMP-9 and CathB were studied in 55 specimens of colorectal adenocarcinomas (pT3, G2). A standard avidin-biotin immunoperoxidase staining method (ABCu-NCL) was performed on 4-μm paraffin-embedded tissue sections with a mouse anti-human MMP-9 and CathB monoclonal antibody. Results: Positive immunostaining for MMP-9 in the primary tumor was observed in 30/55 (54.6%) cases and in 29 cases (52.7%) for CathB. A statistically significant association was found between the expressions of MMP-9 and CathB in the primary tumor and lymph node involvement (p<0.01). The expression of CathB in the primary tumor was associated with the presence of tumor budding and vascular invasion (p<0.01); but no such association was found for MMP-9. The expressions of MMP-9 and CathB in buds were strongly associated with lymph node involvement (p<0.01). However, the presence of vascular invasion was significantly associated only with positive expression of CathB in the buds (p<0.01).

Conclusion: If the presence of budding in the colorectal cancer invasive front indicates higher invasive potential, the present results seem to suggest the existence of a strong relationship between MMP-9 and CathB expressions in the buds and a more aggressive tumor phenotype.

Recent studies concerning the prognostic factors in colorectal cancer (CRC) have described tumor budding (TB) as a potential prognostic marker (1-8). Morodomi et al., in 1989 (1), defined TB as bundles of 5 or more cancer cells occurring in a well-differentiated region (mainly in the actively invasive area), which showed a tubular structure and were classified as microtubular cancer nests. However, isolated cancer cells without a distinct structure were classified as undifferentiated cells. Since both of these appeared as if budding out from a large cancer gland, the researchers called them "budding". A few reports indicated that TB is associated with metastases (2, 3, 6-9). Masaki et al. (10) reported that tumor budding is more useful than such accepted criteria as massive submucosal invasion, vascular invasion or poorly-differentiated histology in T1 CRC. Okuyama et al. (6) found that the combination of lymphovascular invasion and budding predicted lymph node metastasis in pT1 or pT2 tumors more accurately than lymphovascular invasion alone. They also suggested that the combination of lymphovascular invasion and budding of pT1 or pT2 tumors might provide useful information for a postoperative follow-up and for determining the need for adjuvant treatment, such as chemotherapy.

In 1993, Stetler-Stevenson et al. (11), developed the model system for tumor invasion of the extracellular matrix (ECM) barriers. The model, consisting of tumor cell adhesion, ECM proteolysis and cell migration, is still under investigation. Cathepsin B (CathB) and matrix metalloproteinase-9 (MMP-9) play an important role in cancer invasion and metastases by degrading ECM components and the basement membrane. Rempel et al. (12) revealed that CathB expression is often increased specifically at the invasive edge of tumor cells.

It has been hypothesized that proteolytic enzymes and MMPs facilitate metastatic tumor spread in the surrounding tissue by allowing neoplastic cells to detach from the solid tumor mass, by clearing the way for the migrating cells in the ECM and, ultimately, by allowing the cells to nest (13,
In order to nest, the neoplastic cells have to overcome the surrounding basement membrane and ECM. Electron microscopic studies revealed that the basement membrane becomes degraded at the site of its contact with infiltrating neoplastic cells and that neoplastic cells permeate through the membrane (14). A study on the role of proteolytic enzymes and the MMP family in carcinogenesis indicated their involvement in the progression of the neoplastic process. It is, thus, suggested that degradation of the basement membrane and ECM by CathB and MMP-9 at the site of infiltration is the first stage of neoplastic infiltration. Moreover, it can be assumed that the "budding" observed by researchers at the invasive front is associated with CathB and MMP-9 expression in the tumor. Therefore, the aim of the current study was to assess the expressions of CathB and MMP-9 in primary and metastatic tumors, and to determine their relationship with the chosen parameters, especially with tumor budding.

Patients and Methods

Sample collection. Fifty-five patients with CRC, treated at the Department of Surgery, the J. Sniadecki Memorial Hospital (Bialystok, Poland), were studied. The median age was 69 years with a range of 43 to 89 years. Twenty-one patients were men and 34 were women. The tissue specimens were collected immediately after tumor removal, fixed in 10% formaldehyde and embedded in paraffin. Hematoxylin-eosin-stained sections were examined according to the TNM classification. The present study paid special attention to the following histological features at the invasive front: a) Vascular invasion – lymphatic and venous invasion were examined and assessed together as vascular invasion. b) Tumor budding according to the criteria of Morodomi et al. (1).

Only cases with pT3 stage and G2 grade of histological differentiation were included in the study.

Immunohistochemistry. Slides of 4-μm-thick serial sections of the primary tumor and regional lymph nodes were prepared from each patient. A standard avidin-biotin immunoperoxidase method (Novostain Super ABC Kit Universal) was used for the detection of MMP-9 and CathB expression. Briefly, the slides were dewaxed using xylene, transferred to alcohol, placed in citric acid buffer (pH=6.0) and heated in a microwave oven (700W) for 10 min to expose antigens. Endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxide in methanol for 10 min. The slides were then washed 3 times in phosphate-buffered saline (PBS) and incubated in normal horse serum for 15 min to reduce nonspecific antibody binding. After washing with PBS, the slides were incubated overnight at room temperature with monoclonal antibodies. Mouse anti-human MMP-9 monoclonal antibody (Novocastra/NCL-MMP9, dilution 1:40, Biokom, Poland) was used for one slide and mouse anti-human CathB monoclonal antibody (Novocastra/NCL-CATH-B, dilution 1:40) was used for another slide. Nonspecific mouse IgG was used as a negative control. The reaction products were visualized with diaminobenzidine DAB (DAKO S3000, Dako, Poland). Appropriate positive and negative controls were used.

Evaluation of samples. Cytoplasmic immunostaining (Figures 1 and 2) for both MMP-9 and CathB was observed. Expressions were semi-quantitatively assessed in neoplastic cells of the primary tumor and lymph node metastases and were defined as follows: MMP-9, CathB-negative (lack of staining, or staining reaction present in less than 30% of tumor cells) and MMP-9, CathB-positive (staining reaction present in more than 30% of tumor cells). The percentages of MMP-9 and CathB-positive cells were calculated in at least 500 neoplastic cells per each sample using a light microscope (x400).

Method of determining budding (microtubular cancer clusters or undifferentiated cancer cells). Budding was evaluated according to the criteria of Morodomi et al. (1). The invasive front was observed in 500 μm x 2500 μm square visual fields at 4 locations in each slide: tumor budding was classified as negative when no bud was observed and positive when at least one bud was observed. Similar conditions were used for evaluating MMP-9 and CathB protein expressions in tumor budding; i.e., negative when no reaction was observed and positive when protein expression was present in the buds.

Statistical analysis. The associations between MMP-9 and CathB expressions and clinicopathological parameters were examined using the $\chi^2$ test and Fisher’s exact test for statistical analysis. A p-value of <0.05 was considered statistically significant.

Results

In the current study, budding at the invasive front of the investigated colorectal adenocarcinomas was strongly associated with lymph node involvement and vascular invasion. However, no such relationship was found with tumor location or age of the patients (Table I). The expressions of CathB and MMP-9 were observed in the cytoplasm of cancer cells, MMP-9 expression in the main...
Figure 1. *Strong cytoplasmic expression of MMP-9 in neoplastic cells.*

Figure 2. *Representative photograph of cathepsin B expression in buds.*
mass of tumor was observed in 30/55 (54.6%) cases. A statistically significant association was found between the expression of MMP-9 and lymph node metastases, but not with vascular invasion and tumor budding. CathB expression in the main tumor mass was present in 29/55 cases and a statistically significant association was observed between positive expression of CathB and lymph node metastases, vascular invasion and tumor budding (Table II). In 28 cases out of 43 with tumor budding-positive CRCs MMP-9 and CathB expressions were observed in the buds. Statistical analysis revealed a strong association between the expressions of MMP-9 and CathB in buds and lymph node metastases and the expressions of both proteins in the main mass of the tumor (Table III). Vascular invasion was strongly associated only with the presence of CathB in the buds, but not with MMP-9.

### Discussion

The current study demonstrated an association between tumor budding in the invasive frontal region and lymph node metastases. Budding at the invasive front was observed in 26 out of 27 cases of lymph node involvement (96.3%). At the same time, a strong correlation was found between budding and vascular invasion. The presence of poorly-differentiated cells at the CRC invasive front seems to indicate that the tumor is highly aggressive, which is reflected in lymph node involvement and distant metastases (1, 4, 5-10).

The loss of basement membrane integrity may correlate with an increased probability of distant metastases and poor prognosis. Therefore, overexpressions of CathB and MMP-9 at the invasion front of CRCs may be a part of the multistep...
process by which the neoplastic cell can proliferate and metastasize. The proteolytic degradation of the ECM by MMPs and CathB has been shown to be one of the essential events in tumor invasion and metastasis (15). Cancer cells are characterized by overexpression and secretion of a large proportion of lysosomal proteases-cathepsins, especially CathB. CathB takes part in the degradation of ECM components, enhancing this process by activation of metalloproteinases (15, 16). Murnane et al. (16), who investigated activity assays for cathepsins B, L, H and the matrix metalloproteinases, MMP-2 and MMP-9 in CRC, postulated that the proteolytic profiles may prove useful in exploring differences in tumor behavior among cancers of the same stage or in identifying similarities in tumor behavior among cancers diagnosed at different stages. In the present study, in which only pT3, G2 colorectal adenocarcinomas were investigated, out of the 27 cases with lymph node metastases, the expressions of MMP-9 and CathB in the main mass of the tumor were positive in 21 (77.8%) and 27 (100%), respectively. Talieri et al. (17) found that immunostaining for cathepsins B and D increased from adenoma to adenocarcinoma and that the degree of staining for cathepsins B and D were associated with differentiation grade, Dukes’ stage and lymph node involvement. In the present study, budding at the tumor invasive front was strongly correlated with CathB expression in the primary tumor.

Most researchers have confirmed the involvement of cathepsins in the degradation of the ECM and in promoting infiltration – invasion or active metastatic spread in CRC. Some articles describe the relationship between the overexpression of CathB and poor prognosis (18). In our previous study, we observed the association between the CathB activity in tumor cell tissue and the presence of tumor budding (7). Emmert-Buck et al. (19), using a microdissection technique to determine the levels of enzyme activity in specific microscopic areas of invasive human colon cancer, indicated that gelatinase A and CathB activity were up-regulated in fields of invasive colon tumors. Hirai et al.(20) observed that the percentage of CathB-positive cases was significantly higher in the group with liver metastases than in the group without them. Waas et al. suggested a role for MMPs in CRC liver metastasis (21). Masaki et al. (22), in their investigation of interactions among MMP-7, MMP-2 and MT1-MMP at the invasive front in early CRC, suggested that MMP-7 and MMP-2 or MT1-MMP may contribute compensationally to tumor invasion and metastases in T1 CRC. Heslin et al. (23) concluded that the overexpression of MMP-7 is an early event in the adenoma-to-carcinoma pathway and that its expression does not appear to increase further in carcinomas. MMP-2 and MMP-9 appeared to be primarily overexpressed in carcinomas.

However, little is known about CathB or MMP-9 expression in the buds themselves. The current study demonstrated a strong correlation between CathB and MMP-9 expressions in the buds and lymph node involvement.

In conclusion, if the presence of budding in the CRC invasive front indicates higher invasive potential, the present results seem to suggest the existence of a strong correlation between MMP-9 and CathB expressions in the buds and a more aggressive phenotype of tumor.

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References


