Abstract. Aim: The aims of this study were i) to assess a new and more detailed histopathological classification and to analyze concordance between pathologists in the histopathological classification of pseudomyxoma peritonei (PMP); ii) to analyze the expression in the stroma of the particularly interesting new cysteine-histidine (PINCH) protein and its prognostic importance in PMP. Materials and Methods: Surgical specimens from 81 patients, classified according to the Ronnett et al histopathological classification were compared to a new system with four groups ranging from indolent to aggressive growth patterns. PINCH protein expression was analyzed and was related to clinical variables. Results: The new four-group classification provided better prognostic information than the classification according to Ronnett et al. (p=0.04). Expression of the PINCH protein in the stroma was found in 83% of the cases and was associated with high tumor burden (p=0.002) and a poor prognosis (p=0.04). Conclusion: The proposed new PMP classification system may provide additional prognostic information. PINCH protein is expressed in PMP and has prognostic information.

Pseudomyxoma peritonei (PMP) is a rare neoplastic disease, with an incidence of approximately two per million per year (1). It is characterized by disseminated intraperitoneal mucus, associated with mucinous implants on the peritoneal surfaces, the omentum and in the subdiaphragmatic spaces (2). The tumor deposits contain mucous-producing epithelial cells (3). Recent studies incorporating morphological, immunohistochemical and molecular genetic techniques strongly support the notion that almost all cases of PMP originate from primary appendiceal neoplasms (4, 5).

Cytoreductive surgery (CRS) combined with intraperitoneal chemotherapy (IPC) is the preferred treatment for PMP (6-8). However, histopathological features are among the factors that influence prognosis (3, 9). Ronnett et al. (3) found a 5-year survival rate of 84% in patients with disseminated peritoneal adenomucinosis (DPAM), but only 7% in patients with peritoneal mucinous carcinoadenomatosis (PMCA). Assessment is complicated by the fact that the intermediate type, i.e. PMCA-I, includes patients with quite variable prognosis, thus indicating a need to subdivide this group further. Due to the rarity of PMP, most pathologists have very limited experience of this disease, making the histopathological classification difficult. Furthermore, there are no biomarkers known to add prognostic information that may indicate the need for adjuvant chemotherapy.

The particularly interesting new cysteine-histidine (PINCH) protein was originally identified by Rearden and functions as an LIM adapter protein, a double zinc finger domain named from the LIN-11, ISL-1 and MEC-3 genes, for signal transduction in the integrin and growth factor pathways (10). Recently, the PINCH protein is shown to be markedly up-regulated in the tumor-associated stroma of many common types of cancer, including breast, prostate, lung, skin and colon (11). Furthermore, stromal expression for the PINCH protein is an independent prognostic factor for colorectal cancer (12).

With this background, the aims of this study were to assess a new, more detailed, histopathological classification,
in order to analyze concordance between pathologists in the histopathological classification of PMP, and to analyze the expression in the stroma of the PINCH protein and its prognostic importance.

Materials and Methods

The study consisted of all 81 patients with PMP (47 men, 34 women, mean age 55 years, range 24-77 years, 66 patients <65 years), scheduled for CRS and IPC at Uppsala University Hospital, Uppsala, Sweden, between 1993 and 2005. Data on patients’ characteristics are summarized in Table I. The eligibility requirements for treatment were: clinically and histologically confirmed diagnosis of PMP; no distant metastasis; adequate renal, hematopoietic and liver functions; and a WHO performance status of ≤2. Data were obtained from a prospective database of clinical records and surgical reports. The study was approved by the Regional Ethical Review Board in Uppsala (nos.2007/073).

Surgical treatment. One or more of the following surgical procedures were conducted depending on the extent of the disease: greater omentectomy with/without splenectomy, parietal peritonectomy, right and left upper quadrant peritonectomy, colon and small bowel resection, pelvic peritonectomy with/without rectosigmoid resection with/without hysterectomy, and cholecystectomy with/without lesser omentectomy and dissection of the duodenal-hepatic ligament. The tumor load was recorded immediately after surgery using the Sugarbaker’s peritoneal cancer index (PCI) (13) and completeness of cytoreduction was recorded as no residual macroscopic tumor (R1) or macroscopic residual tumor (R2), according to the International Union Against Cancer (15). The PCI (range 1-39) was calculated by summing the lesion size scores (0-3) in 13 different regions of the abdomen. For the purposes of this analysis, the PCI score was simplified as follows: 1-10 as PCI-I; 11-20 as PCI-II; and 21-39 as PCI-III. PCI-I was found in 11 patients, PCI-II in 23 patients and PCI-III in 47. R1 was achieved in 37 patients (46%) and R2 in 44 (54%) (Table I). The mean operating time was 9.2 hours, (range 4-15 hours) with a mean blood loss of 2100 ml (range 50-13500 ml).

Intraperitoneal chemotherapy. Between September 1993 and October 2003, 31 patients were scheduled for repeated sequential intraperitoneal chemotherapy (SPIC) and from October 2003 to October 2003, 31 patients were scheduled for repeated sequential intraperitoneal chemotherapy treatment (EPIC).

Sequential intraperitoneal chemotherapy. In order to administer SPIC treatment, a Port-a-Cath (No. 21-2000-04, SIMS Deltec Inc., St. Paul, MN, USA) was implanted at the end of surgery, as previously described (16). The day after surgery, 5-fluorouracil (5-FU; 550 mg/m²/day) dissolved in 500 ml of 0.9% saline was administered as IPC. Sixty minutes after the start of the IPC infusion, an intravenous (i.v.) infusion of leucovorin (60 mg/m²) was administered.

SPIC treatment was given sequentially for six days at four to six week intervals for eight courses, provided there was acceptable tolerance and no clinical tumor progression. The treatment was administered as an outpatient procedure, except for the first course which was given directly after surgery.

Hyperthermic and early postoperative intraperitoneal chemotherapy. Mitomycin C (12 mg/m²) was given as HIPEC in accordance with the Coliseum technique (17) and was followed by five days of EPIC (5-FU 550 mg/m²/day) and i.v. leucovorin (60 mg/m²).

Histopathology. In all cases, the origin of PMP was judged to be from appendiceal neoplasm. Surgical specimens were prepared in a routine fashion, fixed in 4% buffered formaldehyde, imbedded in paraffin, sliced into 3-4 μm sections and stained with hematoxylin-eosin (HE). Alcian Blue PAS was used to illustrate neutral and acid mucin. In order to more easily identify tumor cells, immunohistochemistry was carried out with antibodies for cytokeratins 7 and 20. The proliferative activity was estimated by Ki-67 expression.

Tissue samples were obtained from different areas of metastases, and the most aggressive area was decisive for histopathological scoring. The sections were microscopically examined and scored independently by two pathologists (AW, RW) with a special interest in gastrointestinal malignancies. This examination was performed without any clinicopathological information. Histopathology was classified as DPAM, PMCA-I (PMCA – intermediate) or PMCA according to Ronnett et al. (3). Furthermore, for the purpose of this study, the histopathological classification was also divided into one of the four following categories: PMP group I: No clear evidence of viable tumor cells; only granulation tissue and mucin were found, despite widespread sampling. PMP group II: Presence of mucin and simple single-layer epithelium with no or with low-grade cellular atypia (on a three-scaled grading system low-moderate-high), and without any or with only a few mitoses in HE or Ki-67 immunostaining. PMP group III: Presence of mucinous neoplastic epithelium with moderate cellular atypia, or more complex epithelial features, such as stratified epithelium frequently combined with micropapillary growth configurations; HE stains shows mitosis with proliferating cells of up to 5%. PMP group IV: Presence of epithelium with high-grade dysplasia; solid growth pattern in small islands with cribriform growth pattern, as well as single cell invasion; signet ring cell carcinoma was included in this group.

PINCH protein determination. The preparation of PINCH antibodies was performed as described elsewhere (11, 18). Five micrometer sections were de-paraffinized and rehydrated, treated by high pressure cooking with 0.01 M Tris-EDTA buffer (pH 9.0) and kept at room
temperature (RT) for 30 minutes. The sections were incubated with 3% H₂O₂-methanol for 20 minutes and then washed with phosphate-buffered saline (PBS, pH 7.4). The sections were further treated with protein block solution (Dako, Carpinteria, CA, USA) for 10 minutes. After removing the solution, the sections were incubated with a primary antibody (Dako), followed by rinsing with PBS. Subsequently, the sections were incubated with a secondary antibody, coupled with peroxidase provided with the Dako ChemMate™EnVision™ Detection Kit, and were washed with PBS. For peroxidase reaction 3,3’-diaminobenzidinetetrahydrochloride (Dako, A/S, Denmark) was used. Sections known to stain positively were included as positive controls. The negative control used PBS instead of the primary antibody. Cases with no or weak staining were classified as PINCH absence group (negative staining group) and cases with a staining as the PINCH occurrence group (positive staining group).

Statistical methods. The pathologists’ concordance, i.e. inter-rater agreement (κ-value), was compared with 95% confidence intervals (CI). To test for differences between the groups, the Mann-Whitney U-test was used for quantitative variables. The Cox proportional hazard ratio was used to assess the effect of the histopathological classification on survival, as well as to assess the effect of the expression of PINCH protein staining on survival. Survival differences between the groups were evaluated with the log-rank test. The Spearman rank correlation test was used for analyses of correlations between the histopathological classifications and PINCH protein staining. A two-tailed p-value <0.05 was considered statistically significant.

Results

Inter-rater agreement and histopathology classification. Table II summarizes histopathology classification. According to Ronnett et al classification, the inter-rater agreement between the two independent pathologists in categorizing the histopathology was very good (κ-value=0.88; 95% confidence intervals (CI)=0.80-0.95, Table II). Moreover, the histopathological classification that was set up for the purpose of this study (the ‘PMP group classification’) also revealed a very good correlation between the pathologists in categorizing the histopathology (κ-value=0.82; 95% CI=0.73-0.92, Table II).
Eleven out of the 27 patients classified as DPAM by Ronnett et al classification fulfilled the new proposed PMP group classification criteria for PMP group I disease, whereas 16 were categorized as PMP group II (Table III).

Seven out of the 34 patients classified as PMCA-I according to the Ronnett et al system, were categorized as PMP group II, 20 patients as PMP group III and seven as PMP group IV. When analyzing the survival of these 34 patients with the Cox proportional hazard analysis, histopathological classification according to Ronnett et al., classification showed no statistical differences [hazard ratio HR=0.62 (95% CI=0.25-1.49), p=0.3]. However, survival rate differences were observed according to the new proposed PMP group classification [HR=1.95 (95% CI=1.00-3.77), p=0.04] (Table III).

All 20 patients classified as PMCA according to the Ronnett et al. system were categorized as having PMP group IV disease according to the new proposed PMP group classification (Table III).

Relationship between PINCH expression and survival. PINCH protein expression in the tumor stroma was absent in 14 patients (17%) and present in 67 (83%). The presence of PINCH protein expression in tumor stroma tended to be associated with poorer survival (HR=0.26; 95% CI=0.06-1.08; p=0.04, Figure 1). Survival was found to be better in patients below the age of 65 years and with low PINCH protein expression than in older patients with PINCH protein expression (HR=0.24; 95% CI=0.06-1.00; p=0.05). No statistically significant correlations were found between PINCH protein expression and Ronnett et al. classification (p=0.14), nor between PINCH protein expression and the new proposed PMP group classification (p=0.17). However, PINCH protein expression correlated to PCI (p=0.002). No clear five-year survival differences were seen in multivariate analysis between the three PCI groups and the PINCH expression (Table IV).

Irrespective of histopathology, patients with a low PCI score survived longer than patients with a high PCI score (p=0.001). However, the extent of tumor burden, i.e. PCI, did not correlate with the histopathological subtypes. Macroscopically radical surgery was associated with longer survival (p=0.001).

Discussion

In addition to previously known prognostic factors (6-8), low tumor burden (i.e. low PCI), macroscopically radical surgery and DPAM or PMP group I and II histopathological classification according to the new proposed PMP group scale, were found to be favorable prognostic factors. PMP is a rare disease (1) and because of this, the likelihood of pathologists, outside a peritoneal carcinomatosis center, acquiring enough experience to assess PMP histopathology is limited. The pathologists’ concordance in our study was very good in both classification settings and this might reflect the body of experience of pathologists at peritoneal carcinomatosis centers, despite the rarity of PMP. Thus, the pathologists’ inter-rater result from this study may not be applicable in settings other than peritoneal carcinomatosis centers. However, in a peritoneal carcinomatosis referral center, the histopathological classification of PMP according to the new proposed PMP group classification may work equally robustly and may supply additional prognostic information, especially for the PMCA-I subgroup.

A simple and robust PMP histopathology classification is needed in order to improve histopathological examination and reproducibility. Contrary to previous proposals (3, 9) and in order to minimize misunderstanding, four subgroups of PMP are proposed. PMP groups I and II comprise mucinous tumors of uncertain malignancy potential, i.e. lacking both significant cellular dysplasia and invasive growth pattern but with mucin within or outside of the appendiceal wall. PMP groups III and IV often derive from mucinous carcinomas with moderate or high-grade dysplasia and invasive growth pattern. There is no difference between the Ronnett et al. system and the new proposed PMP group classification for defining and categorizing the extremes of the pathological subtype, i.e. PMP group I versus DPAM and PMP group IV versus PMCA. All 27 patients categorized as having DPAM by the Ronnett et al. classification fulfilled our grouping

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<tr>
<th>Ronnett’s classification</th>
<th>DPAM</th>
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<tr>
<td>DPAM</td>
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<tr>
<td>PMCA-I</td>
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<td>5</td>
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(K-value=0.88; 95% CI=0.80-0.95)

DPAM: Disseminated peritoneal adenomucinosis; PMCA-I: peritoneal mucinous carcinoadenomatosis – intermediate; PMCA: peritoneal mucinous carcinoadenomatosis.

<table>
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<th>PMP group classification</th>
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<td>Group I</td>
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(K-value=0.82; 95% CI=0.73-0.92)
criteria for PMP group I and group II disease. Furthermore, all 20 patients categorized by the Ronnett et al. classification as having PMCA were categorized as having PMP group IV disease in our classification (Table III). The difficulty occurs in the Ronnett et al. PMCA-I group, with patients being categorized according to the new proposed PMP group classification into PMP group II, group III and also in group IV (seven patients as PMP group II, 20 in group III and seven patients in group IV). Therefore, PMCA-I as the intermediate group consisted of nearly 40% of the cases; they should be clearly defined so that reproducibility can be maintained in order to improve the accuracy of prognosis.

PINCH protein staining in PMP. Previous studies of PINCH protein expression have been conducted in, for example, invasive colon and rectal cancer and those studies revealed that stromal staining for PINCH protein was an independent prognostic factor for colorectal cancer (12). There are well-established clinical and surgical prognostic indicators for PMP (13, 14). However, there is a lack of prognostic factors based on immunohistochemistry staining for PMP. PINCH protein may be able to provide additional prognostic information. In this study, the five-year survival for patients with no PINCH protein stroma expression was 85% compared with a 56% five-year survival for patients in whom PINCH protein expression occurred in the stroma. The reason for the higher PCI score being correlated to the occurrence of PINCH protein in the tumor stroma is not clear but may relate to the time of tumor growth rather than the implicated biology since PINCH expression did not differ between histopathological subtypes.

Younger patients with low PINCH protein expression in the tumor had slightly better survival rates than older patients with high PINCH expression. A confounding mechanism that could influence this finding is the delay before diagnosis of PMP. However, this finding warrants further exploration before using PINCH protein staining as an additional prognostic indicator, especially in younger patients.

In conclusion, histopathological classification of PMP shows low inter-pathologist variation irrespective of the classification system used. The proposed new four-group PMP classification system and stromal PINCH protein expression may provide additional prognostic information.

Disclosure Statement
The Authors declare no conflict of interest.

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