Abstract. Background/Aim: Mucin 4 (MUC4) is a cell surface glycoprotein that is overexpressed in most pancreatic tumors. The aim of the present study was to characterize MUC4 expression in experimental pancreatic cancer in order to clarify the correlation between MUC4 and pancreatic cancer histology in vivo. Materials and Methods: Pancreatic xenograft tumors were generated in immunodeficient mice (n=15) by subcutaneous injection of MUC4+ human pancreatic cancer cell lines Capan-1, HPAF-II or CD18/HPAF. MUC4 immunoreactivity was compared between the cancer models. Alpha-smooth muscle actin (α-SMA) was used to identify cancer-associated fibroblasts and the amount of collagen fibers was quantified with sirius red. Results: Tumor incidence was 100%. Tumor size showed no difference across groups (p=0.796). The median MUC4 count was highest in Capan-1 tumors (p=0.002). α-SMA and collagen extent were also highest in Capan-1 tumors (p=0.018). Conclusion: The Capan-1 xenograft model could serve as a valuable resource to test new therapeutic strategies targeting MUC4 in pancreatic cancer.

Pancreatic cancer is recognized as one of the most therapy-resistant human malignancies (1). To date, no effective tissue or blood biomarker, gene signatures, or molecular targets exist for pancreatic cancer (2). There is an urgent need to develop new treatment strategies to reduce the mortality of pancreatic cancer patients. The recent advancements in the understanding of the pathogenesis of pancreatic cancer on a molecular level may provide new targets for drug discovery and development. It has been found that in many human adenocarcinomas, including pancreatic cancer, mucins are aberrantly expressed and therefore represent potential targets for novel therapeutic approaches (3).

Membrane-bound mucins belong to a large family of O-glycoproteins (4). They are implicated in cell-cell/matrix interactions and also modulate oncogenic properties of cancer cells. Among the mucins, mucin 4 (MUC4) is a promising therapeutic target, especially considering the lack of MUC4 expression in normal pancreas and the de novo expression in precursor lesions with progressively increased expression until invasive carcinoma (5). MUC4 overexpression is associated with more aggressive disease and poor prognosis (6, 7). The frequency of MUC4 positivity in pancreatic cancer tumors has been reported to be above 80% (5, 8, 9). A previous study from our laboratory indicates that MUC4 expression is maintained in metastatic lymph nodes, emphasizing the importance of MUC4 as a potential therapeutic target also in a metastatic disease scenario (8).

In order to evaluate the potential of MUC4 as an adequate therapeutic target as well as to investigate novel MUC4-directed therapies, biologically-relevant pre-clinical models are necessary. The standard method for generating human solid tumors in mice is by inoculation of cancer cells. A concern has been raised that this approach might lead to exclusion of the unique tumor desmoplasia, which is a characteristic feature of the majority of pancreatic cancers (10). On the other hand, it has been demonstrated that human pancreatic cancer cells are capable of creating their own desmoplastic microenvironment, beneficial for the tumor survival and progression (11, 12). The stromal development in xenografts is, however, dependent on specific conditions and properties of inoculated pancreatic cancer cells.

The objective of the present study was to compare and evaluate the pre-clinical and biological relevance of three pancreatic cancer xenograft models established from MUC4+ human pancreatic cancer cell lines Capan-1, HPAF-II and CD18/HPAF implanted subcutaneously in immunodeficient mice. The expression extent of the cell surface antigen...
MUC4 was analyzed with the specific 8G7 antibody. Of particular interest, the stromal microenvironment was also examined using alpha-smooth muscle actin (α-SMA) as a marker of myofibroblast-like cell phenotype and sirius red for collagen accumulation.

Materials and Methods

Cell lines. Three MUC4⁺ human pancreatic carcinoma cell lines were used: Capan-1 and CD18/HPAF provided by Professor Surinder Batra (University of Nebraska Medical Center, Omaha, Nebraska, USA) and HPAF-II obtained from ATCC® (Manassas, VA, USA). Capan-1 was maintained in Iscove’s modified Dulbecco’s medium (Gibco, Life technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (100 U/ml). The HPAF cell lines were maintained in Iscove’s modified Dulbecco’s medium (Gibco, Life technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (100 U/ml). The HPAF cell lines were harvested and pelleted at 1,200 rpm for 3 min. After pellet dissociation by repeated gentle pipetting, cell concentration and viability were determined using 0.06% trypsin blue. 1x10⁶ cells from the respective cell lines were resuspended in 100 μl serum free Dulbecco’s Modified Eagle’s medium/Hams Nutrient Mix F12 medium (Gibco) and inoculated within 2 h.

Human xenograft model. Fifteen immunodeficient mice (Charles River, Sulzfeld, Germany and Barrier Department at Lund University, Sweden) were housed in standardized pathogen-free conditions in individually ventilated cages and provided free access to standard rodent chow, tap water and nesting material ad libitum. All procedures were performed in a dedicated animal operating room in accordance with the guidelines of the Swedish Government and Lund University, Sweden and were approved by the regional ethics committee.

In order to establish the xenograft tumors, fifteen animals were randomly divided in three groups of five animals in each group. Animals were shortly anesthetized by inhalation of Isofluran in at least 30% oxygen and injected subcutaneously through a 27G needle into the right flank with 100-μl fibroblast-free cell suspension containing 1x10⁶ cells from the respective cell lines (13). After cell inoculation, body weight and the tumor development were monitored once a week. When the tumor size reached about 10 mm in diameter, the animals were sacrificed and examined for metastases. The subcutaneous tumors were resected, fixed in 4% paraformaldehyde for at least 24 h and paraffin-embedded for sectioning and staining for histology or antigen expression.

Immunohistochemistry. Paraffin-embedded tumors were sectioned and stained for expression of MUC4 or α-SMA antigen. Briefly, 4-μm sections were deparaffinized in xylene and rehydrated in graded ethanol. The slides were pretreated with 5% normal goat serum in tris-buffered saline (25 mM Tris, 75 mM NaCl, 0.025% TritonX-100 [pH 7.4]) (Sigma-Aldrich, St. Louis, MO, USA) with 2% bovine serum albumin (Millipore, Billerica, MA, USA) to minimize the cross-reaction of the secondary antibody with endogenous immunoglobulins in the tissue. The sections were then incubated overnight at 4°C with the monoclonal antibody 8G7 (Abcam, Cambridge, MA, USA) which recognizes tandem repeat peptide regions of human MUC4 or monoclonal antibody 1A4 (Dako, Glostrup, Denmark) recognizing α-SMA. The primary antibody was diluted 1:50 or 1:100, respectively, in antibody dilution buffer (TBS with 2% BSA). In order to block the endogenous peroxidase activity, the sections were incubated for 15 min with 10% fetal bovine serum (FBS) (Gibco) and trypsinized for 5 min, harvested and pelleted at 1,200 rpm for 3 min. After pellet dissociation by repeated gentle pipetting, cell concentration and viability were determined using 0.06% trypsin blue. 1x10⁶ cells from the respective cell lines were resuspended in 100 μl serum free Dulbecco’s Modified Eagle’s medium/Hams Nutrient Mix F12 medium (Gibco) and inoculated within 2 h.

Table I. Capan-1, HPAF-II and CD18/HPAF tumors, MUC4⁺ cell amount, α-SMA staining extent.

<table>
<thead>
<tr>
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<th>Capan-1 tumors (n=5)</th>
<th>HPAF-II tumors (n=5)</th>
<th>CD18/HPAF tumors (n=5)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor volume (mm³)</td>
<td>540 (290-800)</td>
<td>400 (230-580)</td>
<td>460 (120-650)</td>
<td>0.796</td>
</tr>
<tr>
<td>MUC4⁺ cells</td>
<td>576 (553-599)</td>
<td>94 (62-131)</td>
<td>0</td>
<td>0.002</td>
</tr>
<tr>
<td>α-SMA extent (%)</td>
<td>38.80 (13.26-56.70)</td>
<td>25.62 (20.99-45.01)</td>
<td>8.95 (3.19-16.33)</td>
<td>0.018</td>
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Quantitative analysis. Tumor size was measured with a digital caliper and the volume was calculated using the following formula: Volume (mm³)= (Length x Width²)/2. In multi-lobed tumors, the volume of individual lobes was calculated and summarized to obtain the final volume. Quantitative analysis of expressed MUC4 or α-SMA biomarkers was performed on digital microscope slides created by the ScanScope® microscope slide scanner using the Aperio Image Scope software version 11 (Aperio Technologies Inc., Vista, CA, USA). Briefly, for analysis of MUC4 expression, six optical fields (20x magnification) were randomly selected on the digital slides from specimens stained with the 8G7 antibody. The number of strongly-positive cells was counted in each field and summarized for each specimen in the respective group. The results are presented in Table I as median of the total amount of positively stained cells for each specimen in the respective group. The extent

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of α-SMA expression or sirius red staining was determined as percentage of the strongly positive stained region in relation to the total area of the specimen using Positive Pixel Count 2004-08-11 algorithm, version 8.100 (Aperio Technologies). The results are presented in Table I.

**Statistics.** Values are reported as median with range. The non-parametric Kruskal-Wallis test was used for multiple group comparisons. When significant, post hoc tests were performed using the Dunn’s test. Data were analyzed using SPSS version 22.0 (IBM, Armonk, NY, USA) and GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA). Significance was assumed with a p-value <0.05.

**Results**

All animals developed solid subcutaneous tumors within two weeks after inoculation. Tumor volume varied in a similar manner in all groups with no significant differences between groups (Kruskal-Wallis, \( p = 0.796 \); Table I). No metastases were found.

**Histology.** Capan-1 cells formed poorly-differentiated solid tumors without duct structures. The tumor consisted of epithelial cells with irregular nuclei rich of mitosis and an eosinophil cytoplasm with focal vacuolar de-generation. Cells set in nests were supported by a fibrous stroma. The tumor area had pseudocyst formation with protein-rich liquid in the lumen. CD18/HPAF cells formed solid tumors characterized by epithelial-like structures with a delicate fibrous stroma around and poorly defined necrosis. Moderate pleomorphism, numerous mitosis and an eosinophilic cytoplasm were prominent. Tumors derived from HPAF-II cells were composed of poorly-formed glands infiltrated by poorly-differentiated epithelial cells and abundant fibrous stroma. Necrosis could occasionally be noted in individual large tumors, exceeding a volume of 500 mm³. Examples of histological findings in the respective tumors are presented in Figure 1.

**Figure 1.** Representative histological examples. Capan-1 (A), HPAF-II (B) and CD18/HPAF tumors (C) (H&E staining).

**MUC4 expression.** Significant differences between the groups were detected by quantitative analysis of MUC4 + cells. The highest amount of MUC4 + cells was estimated in specimens from Capan-1 xenografts (Kruskal-Wallis, \( p = 0.002 \)). The ratio between the variations in staining intensity was, however, proportionally distributed between Capan-1 and HPAF-II groups. No expression of MUC4 was identified in the xenografts derived from CD18/HPAF cells (Figures 2 and 3).

**α-SMA expression and collagen accumulation.** The extent of α-SMA or picro-sirius red staining differed between groups. A significantly larger extent of α-SMA immunoreactivity, covering up to 38.80% of the tumor area, was uniformly distributed in all sections from Capan-1 xenografts (Kruskal-Wallis, \( p = 0.018 \); Dunn’s post hoc, Capan-1 vs. CD18/HPAF (\( p = 0.027 \)), Capan-1 vs. HPAF-II (\( p = 1.000 \)), HPAF-II vs. CD18/HPAF (\( p = 0.071 \)). No variation in staining intensity was observed between the groups (Figure 4). The staining pattern of α-SMA and sirius red revealed a positive correlation with the grade of α-SMA expression and collagen accumulation in all groups (Figure 4).

**Discussion**

MUC4 is a cell surface marker that is expressed in the majority of pancreatic tumors, but not in normal pancreas (5, 8, 9). It is involved in the invasiveness of pancreatic cancer cells and confers a poor patient prognosis (5-7). Herein we generated subcutaneous pancreatic tumors in mice by inoculation of cells from MUC4-expressing human pancreatic cancer cell lines, Capan-1, HPAF-II or CD18/HPAF. It is important that the *in vivo* model exhibits characteristics translatable to the human pathological scenario of pancreatic cancer with respect to parameters intended to study.

Transplanted MUC4 + tumor cells created well-defined tumor nodules with a similar growth pattern in immunodeficient mice.
Morphologically, the tumor xenografts in general consisted of poorly-differentiated epithelial-like formations surrounded by a more or less pronounced fibrotic tissue stroma and could therefore represent an experimental tool to recapitulate conditions found clinically in pancreatic cancer. MUC4 expression, however, was only detected in Capan-1 and HPAF-II xenografts. These results are indicating that the MUC4 expression is maintained through the in vivo tumor progression process, which is in agreement with and mimics the clinical scenario (8). Even though the amount of labeled MUC4 antigen expressed in both in vivo models well- reflects the human situation, the MUC4 expression in xenografts derived from Capan-1 cells was supreme. Desmoplastic reaction, which is one of the representative histological findings in pancreatic cancer, was present in all tumors to various degrees. Based on the α-SMA staining analysis of tissue specimens, we speculated that local host dermal fibroblasts became activated in response to mechanical tension (15) caused by the growing xenograft, acquiring contractile stress fibres and adopting the migratory myofibroblast-like phenotype (16), which enabled the activated fibroblasts to invade the tumor. De novo expression of α-SMA together with the characteristic spindle-shaped morphology is

Figure 2. Representative examples of the surface MUC4 antigen expression in Capan-1-derived tumor (A, D), HPAF-II tumor (B, E) and CD18/HPAF tumor (C, F).

Figure 3. Capan-1 tumors have the highest MUC4 count. Kruskal-Wallis test, p=0.002; Dunn’s post hoc, Capan-1 vs. CD18/HPAF (p=0.001), Capan-1 vs. HPAF-II (p=0.215), HPAF-II vs. CD18/HPAF (p=0.215). n=5 mice per group.
commonly used for identification of cancer-associated fibroblasts (CAF) (17). CAF represent a source of various types of factors and mediators enhancing the tumor progression, inclusive extracellular matrix (ECM) components, such as various types of collagen. CAF are thought to be responsible for the dense stroma production, which is one of the hallmarks for pancreatic adenocarcinoma (18-20). These findings indicate that xenograft tumors are self-sufficient in creating a favorable microenvironment for further progression, i.e. in agreement with the clinical situation (21, 22).

In conclusion, our results show that a biologically-relevant pre-clinical model of pancreatic cancer with the characteristic desmoplastic microenvironment, expressing MUC4 with a comparable intensity as the human counterpart (8), can be developed from Capan-1 or HPAF-II cell lines. However, on account of the given superior expression of MUC4 in tumors developed from Capan-1 cells, we suggest, that the Capan-1 xenograft model could serve as a valuable resource to test new therapeutic strategies specifically targeting MUC4 in pancreatic cancer.

Acknowledgements

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References