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Expression of CEA, CA19-9, CA125, and EpCAM in pseudomyxoma peritonei

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Keywords: Pseudomyxoma peritonei; CEA; CA19-9; CA125; EpCAM; Immunohistochemistry

Summary Pseudomyxoma peritonei is a fatal clinical syndrome with mucinous tumor cells disseminated into peritoneal cavity and secreting abundant mucinous ascites. The serum tumor markers CEA, CA19-9, and CA125 are used to monitor pseudomyxoma peritonei remission, but their expression at tissue level has not been well characterized. Herein, we analyzed expression of these proteins and the adenocarcinoma marker EpCAM in 92 appendix-derived pseudomyxoma peritonei tumors by immunohistochemistry. All tumors were found to ubiquitously express CEA and EpCAM. In the majority of the tumors (94.6%), CEA showed polarized immunostaining, but in 5 aggressive high-grade tumors containing numerous signet ring cells, a nonpolarized staining was detected. We found preoperative CEA serum values to correlate with peritoneal cancer index. However, the serum values of the advanced cases with nonpolarized staining pattern were normal, and the patients died within 5 years after diagnosis. Thus, serum CEA measurements did not reflect aggressiveness of these tumors. CA19-9 showed strong immunopositivity in most of the tumors (91.3%), and mutated enzyme FUT3 was demonstrated from the cases showing negative or weak staining. CA125 was infrequently expressed by tumor cells (focal staining in 6.5% of the cases), but in most of the cases (79.3%), adjacent nonneoplastic mesothelial cells showed immunopositivity. As a conclusion, CEA and EpCAM are invariably expressed by pseudomyxoma peritonei tumor cells and could be exploited to targeted therapies against this malignancy.

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Abbreviations CA125, carbohydrate antigen 125; CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; CRC, colorectal carcinoma; EpCAM, epithelial cell adhesion molecule; FUT3, fucosyl transferase 3; HG, high-grade; LG, low-grade; PCI, peritoneal cancer index; PMP, pseudomyxoma peritonei.

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1. Introduction

PMP is a rare malignancy characterized with mucinous tumor cells growing in the peritoneal cavity. Most often, these cells originate from LG mucinous tumors of the appendix, which leak neoplastic cells into the peritoneal cavity. The number of tumor cells in PMP lesions is usually relatively low, but the abundance of secreted mucinous ascites leads to progressive obstruction, which is fatal. In contrast to the relatively slowly progressing LG tumors, HG tumors are able to invade surrounding tissues and to metastasize, leading to reduced survival [1]. The current standard treatment of PMP is complete cytoreductive surgery followed by hyperthermic intraperitoneal chemotherapy [2]. This combinatory therapy is, however, only amenable to 60%-70% of the cases [3,4]. At the moment, no effective targeted therapies are available for the treatment of PMP.

CEA, CA19-9, and CA125 are cell membrane glycoproteins, which are used as serum markers to monitor tumor progression or remission. Furthermore, the preoperative serum levels help in estimation of prognosis. CEA is one of the most widely used tumor markers and is the most commonly used marker of CRC [5]. Serum CEA is elevated in more than 60% of patients with advanced CRC, and patients with high CEA levels have poorer prognosis [6,7]. CA19-9 is a marker commonly used in pancreatic cancer, but it is also elevated in about 45% of patients with advanced CRC [7,8]. Patients having mutated FUT3 gene (approximately 5% of the population) cannot express CA19-9 because of the deficiency of this biosynthetic enzyme [9,10]. CA125, a repeating peptide epitope of the mucin MUC16 [11], is used as a serum marker in ovarian cancer, where it shows elevated serum levels in more than 80% of the patients [12].

EpCAM is a transmembrane glycoprotein mediating epithelium-specific, homotypic cell-cell adhesion [13,14]. EpCAM is strongly expressed in the majority of epithelial cancers [13,15], and especially CRC nearly invariably shows high EpCAM immunopositivity. Because of this widespread overexpression in epithelial cancers, EpCAM has been studied as a target for antitumor immunotherapy [14].

CEA, CA19-9, and CA125 serum markers are used to monitor the remission of PMP tumors, and their levels have also been related to the prognosis of PMP patients [4,16,17]. The tissue expression patterns of these proteins have, however, not been well characterized in PMP specimens, the previous reports analyzing mostly overall positivity in a limited number of specimens [18–20]. The aim of this study was to investigate by immunohistochemistry the expression of CEA, CA19-9, and CA125 in a larger series of PMP tumors (n = 92) and to evaluate their relevancy as PMP serum markers and their possible prognostic value. Furthermore, we studied expression of the epithelial cell marker and an immunotherapy target EpCAM in PMP.

2. Materials and methods

2.1. Tissue samples

Ninety-two formalin-fixed, paraffin-embedded tumor specimens of appendix-derived PMPs (52 LG and 40 HG) were analyzed using immunohistochemistry. The appendix origin was judged by reviewing the appendix tissue block when possible and otherwise trusting the surgery report. Grading of the tumors was done according to the WHO 2010 classification [21]. All the tissue samples were processed at the Meilahti Pathology Division, HUSLAB, Helsinki University Central Hospital, between 2006 and 2014. This study was approved by the Ethics Committee of the Helsinki University Central Hospital (DNRO 239/13/03/02/2011 and 265/13/03/02/2011).

2.2. Immunohistochemistry

Three-micrometer tissue sections were immunostained with CEA, CA19-9, CA125, EpCAM, and E-cadherin specific mouse monoclonal antibodies CEA31 (Cell Marque, Rocklin, CA), NCL-L-CA19-9 (Leica Biosystems, Nussloch GmbH, Germany), NCL-L-CA125 (Leica Biosystems), VU-1D9 (Abcam, Cambridge, UK), and HECDC-1 (Thermo Fischer Scientific, Kalamazoo, MI), respectively. CEA and EpCAM stainings were performed with Ventana BenchMark XT immunostainer (Ventana Medical Systems, Tucson, AZ) using UltraView DABv3 kit (Ventana), and CA19-9, CA125, and E-cadherin stainings were performed with LabVision (Thermo Fisher Scientific) using EnVision kit (Dako, Glostrup, Denmark). The chromogen was 3,3′-diaminobenzidine in all the stainings. Positive controls were colorectal carcinoma for CEA and EpCAM, pancreatic cancer for CA19-9, and ovarian cancer for CA125. To inspect the expression pattern of the used markers in normal appendix epithelium, 5 normal appendix samples were stained.

2.3. CEA serum levels and peritoneal cancer index

The preoperative serum CEA levels were determined immunochromeluminometrically (HUSLAB, Helsinki, Finland) from 91 patients (1 patient had palliative emergency operation without serum measurements). PCI [22], estimating the extent of the disease, was determined for the patients attempted to treat with cytoreductive surgery and hyperthermic intraperitoneal chemotherapy (n = 81; PCI range, 5-39).

2.4. Sequencing of the mutational hotspots of FUT3 gene

Genomic DNA was extracted from the formalin-fixed, paraffin-embedded samples of all CA19-9–negative/low-expressing patients and 3 CA19-9–immunopositive patients as previously reported [23]. To determine the germline FUT3
mutations of positions 59, 314, and 1067, we amplified the flanking DNA sequences by polymerase chain reaction using 1 mol/L betaine (Affymetrix, Santa Clara, CA), primers (Sigma-Aldrich, St Louis, MO), and variables shown in Supplementary Table 1 and protocol reported previously [24]. Each sample was then sequenced in both directions as reported [24]. As mutations in FUT3 positions 202 and 314 are known to be coupled [10], we decided to analyze only position 314 for which more specific primers could be designed.

2.5. Statistical analyses

The statistical analyses were performed with IBM SPSS Statistics software (Version 20; IBM Corporation, Armonk, NY). Spearman rank-order correlation was performed to assess the correlation between serum CEA value and PCI score (calculated from the 81 cases having both values), and Pearson χ² analysis was used to measure the association between tumor grade and serum CEA value (91 cases having CEA value). A P value of less than .05 was considered statistically significant.

3. Results

3.1. CEA shows ubiquitous expression in PMP tumor cells

All PMP tumors (n = 92) showed CEA immunopositivity in all tumor cells. However, 2 different staining patterns were observed. In most of the tumors (94.6%), intense staining of the apical membrane and less intense staining of the cytoplasm were detected (polarized staining pattern; Fig. 1A and B), resembling the staining of normal appendiceal epithelium (Supplementary Fig. 1A). Especially nests of HG tumor cells showed altered cell polarity, “inverse orientation,” characterized by staining of the stroma-facing membrane (Fig. 1B). Five aggressive HG tumors containing also numerous signet ring cells showed instead a nonpolarized staining pattern composed of more homogeneous staining of the membranes and the cytoplasm (Fig. 1C). The LG structures found in 1 of these aggressive HG tumors, in turn, showed polarized staining. The mucus pools of PMP tumors also stained moderately with CEA antibody. As E-cadherin expression is known to be essential for epithelial cell polarization [25,26], we stained this marker from the 5 nonpolarized CEA staining pattern–possessing tumors and 5 polarized ones. Indeed, a remarkable decrease or complete absence of E-cadherin immunopositivity was detected in all the nonpolarized CEA staining–possessing tumors (Supplementary Fig. 2).

3.2. CEA serum levels correlate with PCI values but do not always reveal the poor prognosis

Preoperative serum CEA levels were measured from all patients except 1 (n = 91; Supplementary Table 2), and in 56.0% of the cases, it was higher than the cutoff value (>5 μg/L). No significant difference (P = .546) existed between LG and HG forms of the disease, the percentages of elevated values being 57.7% and 53.8%, respectively. Serum CEA levels were, however, found to correlate with PCI values (P < .001), and increasing percentages of elevated CEA levels were thus observed with more extensive disease (Table 1). Serum CEA levels of all the 5 cases of aggressive HG tumors showing nonpolarized staining pattern were normal despite quite widespread disease. For the 3 cases with PCI value determined, the values were 19, 29, and 31 (maximum, 39). For 2 of the cases, PCI was not determined because radical surgery was not achievable. All 5 patients died within 5 years after diagnosis (4 within 2½ years).

3.3. CA19-9 is strongly expressed by the majority of PMP tumors

Whereas most of the PMP tumors (84/92, 91.3%) were strongly positive for CA19-9 (Fig. 2A), 6 tumors (6.5%) were negative (Fig. 2B) and 2 (2.2%) showed weak immunoreaction (Fig. 2C). In contrast to CEA, mostly showing a thin rim of intense staining at the apical membrane of tumor cells, CA19-9 showed strong staining of the secretory vesicles of tumor cells and secreted mucus pools (Fig. 2A), implicating abundant secretion of CA19-9 into the mucus. As compared with normal appendiceal epithelium (Supplementary Fig. 1B), the staining of tumor cells was more intense and universal. By sequencing of the mutational hotspot positions 59, 314, and 1067 of FUT3, the cases showing negative or weak CA19-9 staining were found to contain mutated FUT3 enzyme (Table 2).

3.4. CA125 is seldom expressed by PMP tumor cells but more often by adjacent mesothelial cells

In most of the studied tumors (93.5%), all tumor cells were found to be CA125 negative (Fig. 3A), and only 6 cases (6.5%) showed focal CA125 positivity in a small proportion of the tumor cells (Fig. 3B). In line with this, all the 5 normal appendix samples were immunonegative (Supplementary Fig. 1C). In most of the PMP cases (73/92, 79.3%), however, mesothelial cells showed immunopositivity (Fig. 3C). Ovarian carcinomas used as positive controls showed intense staining of the apical membrane and less intense staining of the cytoplasm of the tumor cells (Fig. 3D).

3.5. EpCAM is strongly expressed by PMP tumor cells

All PMP samples showed strong membranous immunostaining of EpCAM in all tumor cells (Fig. 4). In most of the tumors and in the normal control appendices (Supplementary Fig. 1D), the staining was basolateral, but in the 5 nonpolarized tumors, a circumferential membranous staining was observed (Fig. 4B-C). Overall, the staining was epithelial cell
specific, as surrounding stromal cells did not stain. In addition, with EpCAM staining, single invading PMP cells were very easily detectable (Fig. 4B).

4. Discussion

CEA, CA19-9, and CA125 are used as serum tumor markers in PMP, mostly based on their use in other tumor types, especially colorectal, pancreatic, and ovarian cancer. However, their tissue expression patterns are poorly known in PMPs. Furthermore, the utilization of CEA in tumor-targeting mechanisms of novel cancer treatment strategies makes CEA expression in PMP tumors even more interesting. Similarly, the tissue expression of the potential immunotherapy target EpCAM has not been studied in PMP before. To cast light into these issues, we analyzed these markers by immunohistochemistry in 92 appendix-derived PMPs.

Ubiquitous CEA immunoreactivity was detected in the tumor cells of all PMP specimens. Previously, Ronnett et al [18] have reported CEA positivity in all 13 studied appendiceal mucinous neoplasms related to PMP; and Bibi et al [19], in 25 of 26 of PMP tumors. We found CEA immunoreactivity also in the mucinous material, implying that this glycosylphosphatidylinositol-anchored glycoprotein is probably shed from the surface of PMP cells into the mucinous ascites. Similarly, excessive CEA in mucin pool has been reported in pulmonary mucinous (colloid) adenocarcinoma [27]. We found 2 patterns of CEA immunostaining: the predominant pattern characterized by intense staining of the apical membranes (polarized staining pattern), detected in the majority of the tumors, in contrast to a more homogenous staining (nonpolarized staining pattern) detected in aggressive HG tumors containing numerous signet ring cells. This latter kind of membranous staining pattern has previously been reported in 1.6% of large bowel carcinomas, and similarly, those tumors tended to be poorly differentiated and more aggressive [28]. Indeed, already in 1982, Ahnen et al [29] demonstrated by immunoelectron microscopy that poorly differentiated colon cancer cells display CEA immunoreactivity over the entire cell surface. In addition to the lumen-facing apical membrane, also areas showing altered cell polarity, “inverse orientation,” in HG cell nests revealed by staining of the stroma-facing membrane. C, Signet ring cells of aggressive HG tumor showing nonpolarized staining pattern. Original magnification ×100.

Fig. 1 Immunohistochemical staining of CEA in PMP tissues. A, LG tumor and (B) HG tumor showing intense staining of the apical membrane and weaker staining of the cytoplasm (polarized staining pattern). Note the altered cell polarity, “inverse orientation,” in HG cell nests revealed by staining of the stroma-facing membrane. C, Signet ring cells of aggressive HG tumor showing nonpolarized staining pattern. Original magnification ×100.
predict the probability of reaching complete cytoreduction in the surgery. Between LG and HG forms of the disease, we could not demonstrate any significant difference in the percentages of elevated CEA levels, similarly to that reported by Canbay et al [17]. In the aggressive HG tumors showing nonpolarized staining pattern, the serum CEA levels were normal, albeit with high tissue content of immunoreactive CEA and PCI values higher than 18. It might thus be that these tumors release less CEA than the polarized ones. As all these 5 patients died within 5 years after diagnosis despite normal serum CEA levels, normal serum levels do not seem to predict better follow-up in all cases of PMP. Indeed, HG PMP tumors with tissue-invading signet ring cells have been shown to have the worst survival [31]. Most importantly, as CEA was found to be ubiquitously expressed by all the PMP tumors, novel therapies using anti-CEA antibodies for increased tumor cell specificity might be good options to treat these tumors [32].

By applying CEA-targeted therapy intraperitoneally, more efficient growth suppression of PMP cells could be obtained because of higher local concentrations. Delivery of the agent directly into the target site, peritoneal cavity, could also reduce adverse effects experienced after systemic delivery.

The second serum marker, CA19-9, showed strong immunopositivity in about 90% of the PMP tumors, but negative/weak cases were also detected. These cases were explained by defective CA19-9 biosynthesis, which occurs in individuals with mutation-caused inactivation of the biosynthetic FUT3 enzyme (5% of population) [9,10]. For using serum CA19-9 levels for estimation of the prognosis of PMP patients, as well as other cancer patients, the FUT3 genetic status of the patient should be known.

In 94% of PMP specimens, no CA125 immunoreactivity was found in the PMP tumor cells themselves. Previously, Guo et al [33] have similarly reported CA125 immunonegativity in all 35 studied PMP specimens. However, in most of our tissue specimens, variable numbers of mesothelial cells showed immunopositivity, as previously described [34]. It is further known that peritoneal irritation caused by intraperitoneal catheter [35], as well as abdominal surgery [36], can increase serum CA125 levels, and in light of our study, the elevated CA125 serum levels of PMP patients may originate mostly from adjacent nonneoplastic mesothelium irritated by advancing PMP. Serum CA125 may thus reflect the extension of PMP tumor dissemination, as reported by Baratti et al [16], but it seems not to be a PMP tumor cell–specific marker. In line with this, Di Fabio et al [37] recently reported a significant

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<th>PCI</th>
<th>S-CEA &gt;5 μg/L (%)</th>
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<tr>
<td>&lt;10</td>
<td>0/6 (0%)</td>
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<tr>
<td>10-19</td>
<td>7/15 (46.7%)</td>
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<tr>
<td>20-29</td>
<td>20/37 (54.1%)</td>
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<td>≥30</td>
<td>19/23 (82.6%)</td>
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Table 1  Elevated serum CEA values in patient groups with increasing PCI values

Fig. 2  Immunohistochemical staining of CA19-9 in PMP. A, LG tumor showing intense staining of the secretory vesicles and secreted mucus. FUT3-mutated tumors are (B) totally negative or (C) only weakly positive. Original magnification ×100 in A-B, ×200 in C.

drop in the serum CEA and CA19-9 levels of PMP patients after complete tumor removal, whereas only a nonsignificant decrease was detected in CA125 levels.
We found ubiquitous EpCAM immunopositivity in all the PMP tumors studied, raising the possibility to use also EpCAM to target treatments to PMP tumor cells. Indeed, EpCAM-targeted therapy exploiting immunotoxin has been studied in an experimental mouse model of mucinous peritoneal surface malignancies and was found to show some degree of growth inhibition, especially in combination with mitomycin C [38]. Interestingly, a more sophisticated system using tri-functional antibody catumaxomab has been developed and approved for the intraperitoneal treatment of EpCAM-positive tumors giving rise to malignant ascites in patients to whom standard therapy is not available or no longer feasible [39]. The basis of this method is the ability of the antibody to bind to EpCAM on the surface of tumor cells, CD3 antigen on T cells, and also Fc receptors on accessory cells (eg, natural killer cells, dendritic cells, and macrophages), thereby leading to tumor cell killing by T cell–mediated lysis, antibody-

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<th>Patient</th>
<th>Hotspot location</th>
<th>CA19-9</th>
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Abbreviation: wt, wild type.

Table 2  FUT3 mutations detected by DNA sequencing and corresponding CA19-9 immunostainings

Fig. 3  Immunohistochemical staining of CA125 in PMP. A, LG PMP tumor without CA125 immunoreactivity; (B) LG PMP tumor showing focal positivity in PMP cells (arrows); (C) mesothelial cells showing immunopositivity; and (D) positive control staining of ovarian carcinoma. Original magnification × 100.
dependent cell-mediated cytotoxicity, and phagocytosis. At the moment, the targeted peritoneal tumor cells are mostly originating from gastric, ovarian, or breast carcinoma, but the agent has been reported to be effective also against colon-originating tumors [40]. Based on our results, catumaxomab might be a potential therapy for PMP tumors as well. Moreover, because of ubiquitous EpCAM expression in PMP, EpCAM immunostaining can be used to identify PMP tumor cells when diagnosing cases with low tumor cell content and to differentiate PMP tumor cells from mesothelial cells.

In conclusion, of the 3 studied serum markers, CEA seems to be the most uniform marker for PMP, even if it may miss some of the most aggressive PMP tumors. Most importantly, ubiquitous expression of CEA and EpCAM in PMP tumor cells suggests their potential for targeted therapy of this disease.

Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.humpath.2016.02.022.

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