Increased EpCAM expression in malignant insulinoma: potential clinical implications

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Abstract

Objective: EpCAM (CD326) is overexpressed in progenitor cells of endocrine pancreatic islands of Langerhans during fetal development and was suggested to act as a morphoregulatory molecule in pancreatic island ontogeny. We tested whether EpCAM overexpression is reactivated in insulinomas, endocrine tumors arising in the pancreas.

Design/method: We used monoclonal anti-EpCAM antibody Ber-Ep4 for immunohistochemistry on formalin-fixed and paraffin-embedded tumor material. We analyzed 53 insulinomas: 40 benign (disease stage IIa) and 13 malignant tumors (disease stage IIIb/IV). Disease stage disposition followed new TNM classification of the European Neuroendocrine Tumor Society (ENETS) for foregut neuroendocrine tumors (2006). Additionally, ten insulinoma metastases were analyzed. Clinical follow-up was available for overall survival analysis from 49 patients. The EpCAM expression of the islands of Langerhans was classified as 2C in healthy pancreatic tissue.

Results: In 38% of the benign insulinomas (disease stage IIa), we found strong (3C) EpCAM expression. In contrast, malignant insulinomas (disease stage IIIb/IV) and their metastases exhibited a strong (3C) EpCAM expression with 78 and 80% respectively, significantly more frequent (P<0.01). The malignant tissue was characterized by a significantly lower number of unstained cells and significantly higher number of 3C stained cells. Quantitative PCR for EpCAM mRNA validated strong EpCAM expression in malignant insulinoma. Kaplan–Meier curves indicated survival disadvantage for EpCAM 3C insulinomas, but this was not statistically significant (log-rank test).

Conclusion: This first EpCAM expression study in benign/malignant insulinomas indicates that strong EpCAM expression could help to identify patients at risk for malignant disease and might be used as a therapeutic target for antibody-based therapies in patients with metastatic insulinoma.

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Introduction

Insulinomas are rare pancreatic endocrine tumors with an incidence estimated at 1–3 new cases per million persons per year (1). The histogenesis of these neoplasms is still under debate. Pancreatic island cell tumors show marked cytological similarity with pancreatic islands and are therefore believed to originate from the endocrine pancreas. In contrast is the hypothesis that island cell tumors originate within the pancreatic ductal/acinar system, because of morphological identification of putative precursor lesions in the pancreatic/ductal compartment (2). Insulinomas retain the ability to synthesize and secrete insulin autonomously, resulting in severe hypoglycemia, the major leading symptom of this disease (1). Insulinomas are the major cause of organic hyperinsulinism and may occur sporadically, or as part of the syndromes multiple endocrine neoplasia type I (OMIM 131100), tuberous sclerosis (OMIM 191092), or von Hippel-Lindau (OMIM 193300). It is estimated that 5–30% of the insulinomas are malignant, with liver and lymph nodes as main targets for metastasis (3–5). The clinical presentation of the malignant form is indistinguishable from that of the benign tumors that are cured by surgery alone. In contrast, multimodal therapies – including surgery, chemotherapy, radiofrequency ablation, chemoembolization, and somatostatin analogs – are necessary to treat malignant insulinomas (6). The prognosis of patients with malignant insulinomas remains, with a cumulative median survival of ∼2 years, poor (3, 7). Since treatment and prognosis of the benign and the malignant form differ fundamentally, the absence of any histological criteria or other markers to predict malignant disease is one of the most tormenting problems managing insulinoma patients. Therefore, markers for the identification of patients at risk for malignant disease are urgently needed.
In this context, the epithelial adhesion molecule EpCAM (CD326) represents a potential marker molecule for malignant progression in insulinoma patients. The physiological expression of this 40-kDa transmembrane glycoprotein, encoded by the gene EPCAM located at 2p21 (8, 9), is restricted to the basolateral cell membrane of glandular, pseudo-stratified, and transitional epithelia (8). The endocrine islands of Langerhans cells in the adult pancreas exhibit an intermediate membranous EpCAM expression (10–12). In the developing fetal pancreas, however, very high EpCAM expression levels were noted at island-like cell clusters budding from the ductal epithelium, a cell compartment thought to comprise endocrine progenitor cells (10). Upon pancreatic island ontogeny, experimental evidence suggested EpCAM as a morphoregulatory molecule. Furthermore, EpCAM is overexpressed in numerous cancers, and in some cancer types, its overexpression predicts prognosis. Therefore, we hypothesized that EpCAM expression changes with the malignant potential and might be a useful biomarker for malignancy in insulinomas. According to our hypothesis, benign and stationary insulinoma cells would express EpCAM at levels comparable to the islands of Langerhans, whereas a progenitor-like phenotype would be reactivated in malignant and potentially motile insulinoma cells that could be reflected in high EpCAM expression levels. To test this hypothesis, we investigated a large collective – for this rare tumor entity – of benign and malignant insulinomas with immunohistochemistry (IHC) for EpCAM expression.

**Material and methods**

**Patients and tumor tissue**

Formalin-fixed and paraffin-embedded tissue blocks were selected from the archive of the Institute of Pathology of the University Hospital at the Heinrich-Heine University Düsseldorf from 53 patients who were operated for symptomatic organic hyperinsulinism at the department for General, Visceral and Pediatric Surgery of the University Hospital at the Heinrich-Heine University Düsseldorf. In addition, fresh tumor tissue that was snap-frozen in liquid nitrogen immediately after resection and stored in liquid nitrogen until use was used for EpCAM mRNA expression analysis. The ethics committee of the Heinrich-Heine University Düsseldorf approved the collection of fresh tumor tissue, and written informed consent was obtained from all patients. Patients were classified or reclassified according to the new ENETS/TNM classification for neuroendocrine foregut tumors from 2006 (13).

For EpCAM protein expression, tissue blocks of 40 benign insulinomas (disease stage ≤ IIa) and 13 cases with malignant insulinoma (disease stage IIIb/IV) were available for this study. Furthermore, we analyzed autologous metastatic tissue of eight patients, where the primary tumor was also evaluated for EpCAM expression, and from two additional cases, only metastatic tissue was available. The definition of malignant insulinomas was restricted to cases with diagnosed metastases, e.g. in lymph nodes, liver, or lung (disease stage IIIa/IV). A mean follow-up for overall survival analysis was obtained in 49 cases of primary organic hyperinsulinism (37 benign insulinomas and 12 malignant insulinomas) with a mean follow-up of 238 months (range: 4–314 months). Four patients were lost to follow-up. For patients with benign insulinoma, the mean follow-up was 280 months (range: 47–314 months) and 102 months (range: 4–299 months) for patients with malignant insulinomas. The clinicopathological data are listed in Table 1 (for the group investigated for protein expression) and in Table 2 (for the group investigated for mRNA expression).

**Immunohistochemistry**

Serial 4 μm sections were cut from each formalin-fixed and paraffin-embedded tumor sample. The immunohistochemical staining was performed using the Vectastain.
Table 2 Patient and tumor characteristics of the 26 patients with diagnosed primary organic hyperinsulism investigated with quantitative real-time reverse transcriptase-PCR.

<table>
<thead>
<tr>
<th></th>
<th>Benign (n=8)</th>
<th>Malignant (n=18)</th>
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<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;65</td>
<td>n=3</td>
<td>n=11</td>
</tr>
<tr>
<td>≥65</td>
<td>n=5</td>
<td>n=7</td>
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<tr>
<td><strong>Sex</strong></td>
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<tr>
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</tr>
<tr>
<td>No</td>
<td>n=7</td>
<td>n=17</td>
</tr>
<tr>
<td><strong>Tumor size</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤2 cm</td>
<td>n=5</td>
<td>n=0</td>
</tr>
<tr>
<td>&gt;2 cm</td>
<td>n=3</td>
<td>n=18</td>
</tr>
<tr>
<td><strong>Disease stage</strong></td>
<td></td>
<td></td>
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<tr>
<td>Stage I (T1N0M0)</td>
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<td>n=0</td>
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<tr>
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<td>n=3</td>
<td>n=0</td>
</tr>
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<td>Stage IIb (T3N0M0)</td>
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<td>n=0</td>
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<td>Stage IIIa (4T4N0M0)</td>
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<tr>
<td>Stage IIIb (any TN1M0)</td>
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<td>n=5</td>
</tr>
<tr>
<td>Stage IV (any TNM1)</td>
<td>n=0</td>
<td>n=13</td>
</tr>
</tbody>
</table>

ABC Mouse IgG Kit (Vector Laboratories, Burlingame, CA, USA). After deparaffinization in xylol for 20 min, the sections were rehydrated in sequentially graded alcohol baths. Antigen retrieval was performed using the DAKO retrieval solution (pH=6.0, Dako, Hamburg, Germany) at 95 °C for 20 min and cooled down by room temperature for 20 min. Antigen retrieval was followed by incubation with 0.3% hydrogen peroxide for 30 min at room temperature. Then, the Vectastain kit was applied according to the manufacturer’s instruction. As primary antibody for EpCAM detection, we used the monoclonal mouse anti-human IgG1 antibody clone Ber-EP4 (Dako) at a concentration of 2 μg/ml. The antibody was applied for 30 min at room temperature. We performed IgG1 isotype controls (MOPC21; 2 μg/ml, Dako) for nonspecific antibody binding in a serial section of every tissue sample. For the visualization of specific antigen detection, we used the dianaminobenzidine (DAB) chromogen (Dako). Subsequently, all sections were counterstained with hematoxylin for 15 s and mounted.

**Evaluation**

For evaluation of the EpCAM staining, we classified the brown membranous DAB staining from 0 to 3+ with 0 as absent staining, 1+ as faint staining, 2+ as weak to moderate staining, and 3+ as strong staining (14). Then, we determined the percentage of positive tumor cells according to their staining intensity for each tumor. For the final categorization of the EpCAM expression level of each tumor, we used the standardized criteria for membranous cell staining, specified by Dako for the HercepTest with a scoring system ranking from 0 to 3 (14). Staining was assessed independently by two investigators blinded to all clinical and pathological parameters. The evaluation included cell membrane staining of the acinar pancreas, the islands of Langerhans, benign and malignant insulinoma, lymph node and liver metastases.

**Total RNA extraction and quantitative real-time reverse transcriptase-PCR**

Frozen tumor samples were cut into 20-μm thick sections with a cryostat. Ten sections were immediately placed in an Eppendorf tube, and total RNA was extracted by using the TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The RNA concentration was verified spectrophotometrically (BioPhotometer, Eppendorf, Hamburg, Germany) by using the OD260 method. RT was performed in a volume of 20 μl by using random hexamere primer, 2 μg RNA, and transcriptor reverse transcriptase in 5× RT buffer (all: Roche). PCR with cDNA was performed by using the following primers and probes (all: MWG-Biotech, Ebersberg, Germany): for EpCAM, primers and probe were (forward) 5'-CAG TTG GTG CAC AAA ATA CTG TC-3' and (reverse) 5'-TTT CCT CCA TCA CCT GCA AAC-3', and probe 5'-FAM-ATT TGC TCA AAG CTG GCT G TAMRA. To normalize the EpCAM expression, we used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal reference gene. The primers for GAPDH were (forward) 5'-CGT TTA CAC GAT CAT GAC CA-3' and (reverse) 5'-GCT ATT ACG CCA CAG TTT C-3', and the probe was 5'-FAM-CAG AAG ACT GTG GAT GCC CCC TTC TAMRA. For the PCR, 11.25 μl (1 ng/μl) cDNA template (or water as negative control) was mixed with 12.5 μl iQ Supermix mastermix (Bio-Rad) and 1.25 μl primer-mix (10 μM each primer, 4 μM probe) and 75 μl H2O. All samples were run in duplicate. Quantitative real-time reverse transcriptase-PCR (qPCR) was carried out by using the DyadDisciple Chromo 4 (Bio-Rad) with the following conditions: 95 °C for 10 min followed by 40 cycles each comprising denaturation for 15 s at 95 °C, annealing and extension for 1 min at 60 °C. Gene expression was quantified by determining ΔCt values. The ΔCt value for EpCAM is the difference between the Ct for EpCAM and for GAPDH as internal reference control gene. High ΔCt values are correlated with low levels of gene expression, whereas low ΔCt values are correlated with high levels of gene expression. Since the amplification efficiencies of both genes were close to 100% (data not shown), ΔCt values essentially correspond to a log-2 scale. For example, ΔCt values that differ by 2 correspond to a 2× or fourfold change in gene expression levels. Differences in expression between sample groups were calculated via the 2-ΔΔCt method.

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Statistical analysis

For statistical analysis, contingency tables were tested with Fisher’s exact test and when appropriate with the χ² test. Significance of differences between groups with a nonparametric data distribution was analyzed with the Mann–Whitney U test for two independent groups. We used log-rank test for the univariate survival analysis. Primary end point was survival, as measured from first operation time to last follow-up or death. Data of patients who were still alive at the end of the study were censored. The threshold for statistical significance was chosen at P<0.05. The program SPSS 11.0 (SPSS, Chicago, IL, USA) for Windows was used for all statistical analysis.

Results

Immunohistochemical analysis of EpCAM protein expression

First, we evaluated the EpCAM immunostaining with the antibody Ber-EP4 in the normal pancreatic tissue. The acini showed a weak to moderate positive staining, while the islands of Langerhans, as previously reported (10, 12), exhibited a homogeneous and stronger EpCAM staining in nearly 100% of all investigated cases (Fig. 1A). In most cases, the membranous staining of the islands of Langerhans was categorized as 2+ . In contrast, the analysis of EpCAM expression in insulinomas revealed a different distribution pattern (Table 3). While 10% of the cases (n=4) showed a lower expression compared to the physiological EpCAM expression of the islands of Langerhans, the majority of the cases (52%, n=21) showed a faint to moderate staining and were classified as 1–2+ (Fig. 2, Table 3). Tumors classified as 3+ were observed in 38% (n=15; Table 3). This was significantly different in malignant insulinomas (disease stage IIIb/IV) and in their metastatic tissues, where 78% (n=10, P=0.026, Fisher’s exact test) and 80% (n=8, P=0.0073, Fisher’s exact test) respectively were classified as 3+. The increase of cells with 3+ membranous staining intensity in primary malignant insulinoma and in the metastatic tissue compared to the benign tumors was statistically significant (P=0.051 and P=0.047 respectively, Mann–Whitney test) as well as the lower number of unstained cells in metastatic tissue compared to benign and malignant insulinomas (P=0.0013 and P=0.006 respectively, Mann–Whitney test; Fig. 1A).

Quantitative real-time RT-PCR analysis of EpCAM mRNA expression

In order to validate our protein expression study on the transcripational level, we performed qPCR for EpCAM mRNA expression. In only six of the cases (five malignant primaries and one metastasis), in which we investigated the formalin-fixed and paraffin-embedded tumor with IHC, fresh frozen tissue was available for RNA analysis. Therefore, we added 20 cases in which sufficient fresh frozen tumor material was available. In fact, the EpCAM mRNA expression analysis yielded comparable data to the EpCAM protein expression analysis of the formalin-fixed and paraffin-embedded tumors (Fig. 1B). With a mean ΔCt value of −0.51±2.82, the EpCAM mRNA expression in the benign insulinomas was significantly lower compared to that in the malignant insulinomas (both, the primary tumors and the metastases) with a mean ΔCt value of −3.86±2.14 (P=0.012, Mann–Whitney test). Thus, the mean EpCAM mRNA expression was 10.2-fold higher in the malignant insulinomas than in the benign tumors. While we observed a heterogeneous EpCAM mRNA expression between the different benign insulinomas with a wide range from very low expression to high expression, the malignant primary insulinomas clustered all, except one outlier, at a very high expression level with a mean ΔCt value of −5.10±2.82 (difference to benign insulinomas: P=0.012, Mann–Whitney test; Fig. 1B). Compared to the primary malignant insulinomas with an almost uniform EpCAM expression level, the metastatic tissue exhibited a more heterogeneous EpCAM mRNA expression with a significantly lower mean ΔCt value of −3.21±1.71 (P=0.024, Mann–Whitney test; Fig. 1B). However, the EpCAM
mRNA expression level of the metastases was significantly higher than that of the benign tumors \((P = 0.043, \text{ Mann–Whitney test; Fig. 1B})\). Compared to the benign insulinomas, the metastases displayed a 6.5-fold increased EpCAM mRNA expression.

Prognostic impact of EpCAM protein expression

In a last step, we investigated the prognostic impact of EpCAM protein expression and found no significant difference between the groups with differential staining pattern \((0–2⁺ vs 3⁺)\) upon the log-rank test \((P = 0.2173)\). However, the Kaplan–Meier curves (Fig. 3) indicated a survival disadvantage for patients with strong \((3⁺)\) EpCAM expressing tumors according to the HercepTest classification, with a mean survival of 197 months, compared to patients with \(0–2⁺\) EpCAM expressing tumors with a mean survival time of 259 months.

Discussion

Here, we report about the first comprehensive study on EpCAM expression in the rare endocrine tumor entity of benign and malignant insulinomas. While a very heterogeneous EpCAM expression was observed in benign insulinomas with a wide range from low to high expression, a significantly increased EpCAM expression was observed in malignant insulinomas (disease stage IIIb/IV).

In a first step, we performed IHC for the analysis of the EpCAM expression level and categorized the IHC results using the evaluation criteria for the FDA approved HercepTest (Dako) for p185HER², the target for the therapeutic antibody trastuzumab (Herceptin; Roche, Basel, Switzerland). We used these guidelines because, comparable to p185HER², EpCAM is a membranous protein, and the criteria are standardized and are already used to assign patients to antibody-based therapy. The applicability to quantify EpCAM expression with the HercepTest Scoring System was previously demonstrated in squamous cell carcinomas of the esophagus (15). Applying this scoring system, we identified three different levels of EpCAM expression with a significant difference in the distribution between benign and malignant insulinoma tissues. Cells with strong \((3⁺)\) EpCAM expression were significantly more frequent in the malignant tumors. We could validate this finding in an independent insulinoma collective at the transcriptional level, since malignant insulinomas displayed a 10.2-fold higher EpCAM mRNA expression compared to benign insulinomas in a qPCR assay.

Table 3 EpCAM protein expression in organic hyperinsulinism (%).

<table>
<thead>
<tr>
<th>EpCAM expression</th>
<th>0</th>
<th>1+</th>
<th>2+</th>
<th>3+</th>
<th>Sum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign insulinoma (disease stage &lt; IIa)</td>
<td>4 (10)</td>
<td>10 (25)</td>
<td>11 (27)</td>
<td>15 (38)</td>
<td>40 (100)</td>
</tr>
<tr>
<td>Malignant insulinoma (disease stage IIIb/IV)</td>
<td>2 (15)</td>
<td>1 (8)</td>
<td>0 (0)</td>
<td>10 (77)</td>
<td>13 (100)</td>
</tr>
<tr>
<td>Metastatic tissue</td>
<td>0 (0)</td>
<td>1 (10)</td>
<td>1 (10)</td>
<td>8 (80)</td>
<td>10 (100)</td>
</tr>
<tr>
<td>Sum</td>
<td>6</td>
<td>12</td>
<td>12</td>
<td>33</td>
<td>63</td>
</tr>
</tbody>
</table>

Figure 2 Examples for EpCAM expression in organic hyperinsulinism. Scale bar in overview indicates 100 μm and in boxed inlay indicates 40 μm. (A) Acinar pancreas and islands of Langerhans (*), (B) benign insulinoma (0), (C) benign insulinoma (1⁺), (D) benign insulinoma (2⁺), (E) malignant insulinoma (3⁺), (F) malignant insulinoma (3⁺), (G) lymphatic insulinoma metastasis (3⁺), and (H) hepatic insulinoma metastasis (3⁺, note unusual strong cytoplasmatic staining of this case).
Because high EpCAM expression was found to be associated with poor prognosis in several tumor entities (15–18), we checked whether a high (3+) protein expression conferred a poor survival. Although the Kaplan–Meier curves indicated a survival disadvantage for 3+ tumors, the level of significance was not reached probably due to the few death events that occurred during the clinical follow-up period.

Although overexpression of EpCAM is present in most cancer types and a correlation of strong EpCAM expression and poor prognosis has been observed in several tumor entities (15–18), the exact mechanisms of EpCAM contributing to the malignant potential of tumor cells are not understood. It is, however, necessary to note that the impact of EpCAM in cancer progression may be different in various cancer types. For example, in renal cell cancer and in gastric cancer (19, 20), observations were made that higher levels of EpCAM expression are correlated with good prognosis. In vitro testing of EpCAM function revealed in different studies reduced as well as increased motility and invasiveness upon EpCAM expression (21, 22). Therefore, EpCAM functions are most probably determined by the different predominant histogenetic molecular background.

In pancreatic tissue with its exocrine and endocrine functions, EpCAM is membrane-boundly expressed at intermediate levels on the endocrine cells of the islands of Langerhans – higher than in the exocrine acinar cells but lower when compared to the exocrine pancreatic ductal cells (10, 12). Both the endocrine and exocrine cells derive most probably from common progenitor cells present in the early fetal ductal epithelium engaging respective differentiation programs at distinct developmental stages. Cirulli and co-workers studied extensively EpCAM expression during the endocrine pancreatic development (10). They observed that EpCAM was strongly expressed in fetal pancreatic endocrine precursor cells at sites of cell–cell contacts, while a reduced expression was found in the adult cells of the islands of Langerhans. Moreover, blockade of cell–cell contacts with anti-EpCAM antibodies in fetal pancreatic cells induced endocrine differentiation. The results of their comprehensive study suggested EpCAM as a morphoregulatory molecule that not only mediates cellular adhesion but also functions as a signaling molecule delivering growths and developmental signals. In fact, it was shown by Olivier Gires’ group that i) EpCAM functions indeed as a signaling molecule involved in nuclear WNT signaling (23), and ii) murine EpCAM is essential for maintenance of the phenotype of murine embryonic stem cells (24). In view of these findings, one possible and intriguing explanation for our observation of the predominantly strong EpCAM expression in malignant insulinomas is the reactivation of fetal pancreatic developmental programs. Hypothetically, as a signaling molecule, EpCAM confers stem-like properties to the cells in malignant insulinomas contributing to their malignant phenotype. However, the strong EpCAM expression levels may also represent a bystander effect of global epigenetic or transcriptomic changes occurring in β-cell neoplasia. Regardless of these two different possible explanations, potentially important clinical aspects arise from our study.

First, EpCAM might be used as an additional marker to diagnose and predict a malignant potential in patients with a primary pancreatic insulinoma. This is an important point, since morphological analysis fails to discriminate between benign and malignant disease. Furthermore, no reliable marker for a malignant potential – occurring in 5–30% of the cases – exists. Currently, a tumor diameter ≥ 2 cm, an increased mitotic index and necrosis are the best indicators for a malignant potential, but are not sufficient for valid diagnosis (1). Our data indicate that EpCAM staining and especially qPCR for EpCAM could improve malignant insulinoma diagnosis and prediction. However, our data also show that a diagnostic decision based on EpCAM alone might be difficult and needs larger prospective studies. As described for pancreatic cancer detection or Barrett’s esophagus (25, 26), a quantitative multimarker assay that includes EpCAM and other candidate genes such as SERPIN1 (27) might be promising to improve the diagnosis of malignant insulinoma and should be investigated in future studies.

The second important aspect derives from the fact that EpCAM represents one of the most extensively investigated immunotherapeutic targets for which several different therapeutic antibodies are already in clinical trials or under development (16). EpCAM-directed therapies represent a realistic chance to improve the poor prognosis of patients suffering from metastatic insulinoma, particularly since metastatic insulinoma tissue expressed EpCAM at the highest levels.
In conclusion, we present the first EpCAM expression study in benign and malignant insulinomas. Our results indicate that strong EpCAM expression could help to identify patients at risk for malignant disease. EpCAM could potentially be evaluated as a therapeutic target for antibody-based therapies in patients with metastatic insulinoma.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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