CD97, but Not Its Closely Related EGF-TM7 Family Member EMR2, Is Expressed on Gastric, Pancreatic, and Esophageal Carcinomas

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Abstract

CD97 expression is related closely to the dedifferentiation and tumor stage in thyroid carcinomas. We systematically examined the role of CD97 and its closest relative, EMR2, in normal and malignant gastric, esophageal, and pancreatic tissue.

The normal tissues were EMR2−, whereas CD97 was expressed slightly in the parietal cells of gastric mucosa and in exocrine pancreatic cells. Interestingly, intralobular and interlobular pancreatic ducts were CD97+.

All tumors were EMR2−. CD97 was expressed by 44 of 50 gastric, 14 of 18 pancreatic, and 10 of 13 esophageal carcinomas. Of the 44 gastric cancers, 27 showed disseminated or scattered tumor cells at the invasion front with stronger CD97 expression than tumor cells located in solid tumor formations. There was no correlation between CD97 levels in the tumors or soluble CD97 in the serum samples and the clinicopathologic features of the patients. Taken together, significant numbers of gastric, esophageal, and pancreatic carcinomas are CD97+, whereas its homolog, EMR2, does not have any role in such tumors.

Among the hundreds of known G protein–coupled receptors, only CD97,1 a member of the epidermal growth factor–7-transmembrane (EGF-TM7) family, has been shown to have a cellular ligand, CD55, that distinguishes CD97 as a potential adhesion molecule.2,3 The EGF-TM7 family is characterized by an extended extracellular region with a varying number and/or combination of N-terminal EGF-like domains4,5 coupled to a TM7 domain by a stalk region.6 Apart from CD97, the human EGF-TM7 family comprises 4 other members: EMR1,7 EMR2,8 EMR3,9 and ETL.10

Among them, CD97 and EMR2 share the highest homology. The EGF-like domains of CD97, necessary for its interaction with CD55, differ from those of EMR2 by only a few amino acids.5 However, although there are only minor differences in the binding region, EMR2 is unable to interact with CD55.8 The significant amino acid sequence homology between CD97 and EMR2 also extends to the spacer and TM7 region. In contrast with the high structural similarity, EMR2 and CD97 have distinct expression profiles. EMR2 seems to be restricted to myeloid cells,5 whereas CD97 is distributed broadly and can be found on several hematopoietic cells and muscle cells.11 In addition, CD97 is strongly present in dedifferentiated thyroid carcinomas.12,13 CD97 immunostaining suggests that its expression parallels the aggressiveness and lymph node involvement of thyroid tumors and that CD97-permitted adhesion has a role in the interaction between the tumor cell and the surrounding stroma. CD97 expression in other carcinomas has not been studied systematically, and no information is available on EMR2 in tumors.

The aim of the present study was to gain insight into the distribution pattern of CD97 and its highly homologous
family member, EMR2, in gastric, pancreatic, and esophageal carcinomas. First, cancer cell lines were examined for CD97 and EMR2 expression at the messenger RNA (mRNA) and protein levels by reverse transcriptase–polymerase chain reaction (RT-PCR) and flow cytometry, respectively. Second, we screened malignant tumors and the corresponding normal tissues by immunohistologic methods and determined soluble CD97 (sCD97) in the serum samples to correlate the results with the pathomorphologic criteria and with clinical data for the patients.

Materials and Methods

Specimens

The study comprised a series of gastrointestinal carcinomas obtained from patients undergoing surgery at the Department of Surgery, University of Leipzig, Leipzig, Germany, between October 1999 and July 2001. Normal mucosal specimens as far away as possible from carcinoma lesions were obtained from the same patients. The study was approved by the local committees of medical ethics, and all patients gave their written consent. No patient had received chemotherapy or radiotherapy before surgery. Samples were cryopreserved in liquid nitrogen. Histologic diagnosis and staging following the TNM system proposed by the UICC (International Union Against Cancer) were carried out according to standard criteria (Table 1). All carcinomas were examined for differentiation, according to the predominant growth, and for the presence of blood and lymphoid vessel invasion. Gastric carcinomas were further classified to the histologic tumor type according to Lauren and tumor cell dissemination (TCD) at the invasion front as described by Gabbert et al. TCD was judged semiquantitatively in H&E-stained sections (TCD0, compact tumor glands or solid tumor complexes without any TCD; TCD1, few dissociated tumor cells; TCD2, partly dissolved tumor glands or tumor complexes; TCD3, mostly dissociated cells at the invasion front).

Serum samples were obtained from all patients on the day before operation and stored in aliquots at –80°C. Age- and sex-matched healthy blood donors were used as control subjects (n = 26).

Cell Lines

The human gastric cell lines AGS and Kato-III and the pancreatic cell lines AsPC-1, HS 766T, and PANC-1 were obtained from the American Type Culture Collection. HM0215 and St 3051 were provided by Siegfried Wagner (Hannover Medical School, Hannover, Germany) and Hans Konrad Müller-Hermelink (University of Würzburg, Würzburg, Germany), respectively. The cells were maintained in Dulbecco modified Eagle medium or RPMI 1640 (Invitrogen, Karlsruhe, Germany) containing 10% fetal calf serum, except for AGS cells, which were grown in Hams F12/10% fetal calf serum.

Detection of CD97 and EMR2 mRNA Isoforms by RT-PCR

Total cellular RNA was isolated from cell cultures with the QIAGEN total RNA isolation kit (QIAGEN, Hilden, Germany). Complementary DNA was synthesized using the first-strand complementary DNA synthesis kit from Amersham Pharmacia Biotech (Freiburg, Germany). The primer pair PhCD97\textsuperscript{EFG}\textsubscript{5} (5'-TCC TGC CGG CAG CTC CAA CC-3')/r1 (5'-GGC AGC GGC AGC TGT ATG AAC-3') over- spans the alternatively spliced EGF-like domain coding region resulting in a 452-base-pair (bp) PCR product. The primer pair PhCD97\textsuperscript{T}\textsuperscript{TM7}\textsubscript{5} (5'-CTG GCC GCC TTC TGC TGG ATG AG-3')/r1 (5'-CTG GCC GGC AGC GAT CGT GAT GG-3') amplifies the transmembrane coding region in
CD97 resulting in a 356-bp product. The primer pair PhEMR2EGF s1 (5’-CCC GGT GGT GCC CTC AGG ACT CC-3’) r1 (5’-AGG GGA TCC TCT TGC ACG TAC TAG TGG GCC-3’) overspans the alternatively spliced EGF-like domain coding region, resulting in various bands that were analyzed further by sequencing (not shown). The primer pair PhEMR2TM7s2 (5’-AGG TGC TCT GTG TCT TCT GGG A-3’) r2 (5’-TTC CAC CGG CAAG AAG GGG AAT TTA TTC-3’) amplifies the transmembrane region of EMR2.

Monoclonal Antibodies

MEM-180 (provided by Václav Horejsi, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague) and CLB-CD97/317 bind to the stalk region of CD97. CLB-CD97/1 binds to the N-terminal EGF-domain of CD97; this monoclonal antibody was used only for the sCD97 enzyme-linked immunosorbent assay (ELISA). The EMR2-specific 2A1 monoclonal antibody was developed recently. The CD55 monoclonal antibody was purchased from BD Biosciences (Heidelberg, Germany).

Flow Cytometry

Cells were phenotyped with the desired monoclonal antibody by indirect immunofluorescence using an F(ab’2) of goat antimouse immunoglobulin (fluorescein isothiocyanate–goat-antimouse, DAKO, Hamburg, Germany) with a flow cytometer (FACScan, Becton Dickinson, Mountain View, CA). Expression levels of CD97, EMR2, and CD55 were determined as the mean fluorescence intensity of stained cells compared with cells stained with an isotype-matched but irrelevant monoclonal antibody.

CD97 and EMR2 Immunostaining of Tissue Samples

Serial frozen sections were cut at 5 µm, fixed in ice-cold methanol for 10 minutes followed by a short rinse in a 0.2-mol/L concentration of phosphate-buffered saline, and incubated with the monoclonal antibodies for CD97 or EMR2. In negative control sections, an irrelevant isotype-matched monoclonal antibody was applied at the same concentrations as the primary antibody. Following incubation with the monoclonal antibody (4°C overnight), the bound monoclonal antibody was detected with a supersensitive detection kit (BioGenex, San Ramon, CA), including biotinylated antimouse immunoglobulin and horseradish peroxidase–conjugated streptavidin.

Scoring

The immunoreactivity was scored semiquantitatively using a light microscope (Axioskop, Zeiss, Jena, Germany) by 2 independent investigators (G.A. and M.S.) who were unaware of the histologic results. The percentage of positive cells was judged ranging from 0 to 4 (0, none; 1, <10%; 2, 10%–50%; 3, 51%–80%; 4, >80% positive tumor cells); the level of staining intensity was given as a level of estimation between 0 and 3 (0, negative; 1, weak; 2, moderate; 3, strong staining). The product of positive cells and staining intensity describes the score (0–12). We considered samples positive if the score was higher than 2. A score of 3 to 6 was regarded as moderate and more than 6 as strong.

Assays for sCD97 and Soluble Tumor Markers

sCD97 ELISA has been described elsewhere. Briefly, ELISA plates were coated with 5 µg/mL of the CD97 CLB-CD97/3 monoclonal antibody at 4°C and left overnight. After washing the plates, undiluted serum was applied to the plates for 60 minutes at room temperature. After washing, the wells were incubated with 1 µg/mL of biotinylated monoclonal antibody CLB-CD97/1. After that, streptavidin peroxidase and, subsequently, tetramethylbenzidine were added to the plates, and the optical densities were measured in an ELISA reader. Results are expressed as arbitrary units (U/mL) of a standard amount of CD97 (supernatant of COS-7 cells transfected with CD97 mRNA coding the extracellular part) in duplicate measurements. Carcinoembryonic antigen (CEA; IRMA-coat CEA; Byk-Sangtec Diagnostica, Dietzenbach, Germany), CA15-3 (IRMA-mat CA15-3; Byk-Sangtec), CA19-9 (IRMA-mat CA19-9; Byk-Sangtec), and CA72-4 (ELSA-CA72-4, CISbio International, Gif-sur-Yvette, France) were determined preoperatively according to the manufacturer’s protocol.

| Table 11  |
| Patient Characteristics | Gastric Adenocarcinoma (n = 50) | Pancreatic Ductal Adenocarcinoma (n = 17) | Esophageal Carcinoma (n = 13) |
| Mean ± SD age (y) | 66.2 ± 12.7 | 66.4 ± 8.8 | 58.2 ± 6.7 |
| Male/female | 30/20 | 9/8 | 10/3 |
| TNM stage* | 1 | 15 | 2 | 1 |
| 2 | 10 | 2 | 5 |
| 3 | 3 | 6 | 6 |
| 4 | 22 | 7 | 1 |

* TNM stage was determined in accordance with the 1997 TNM classification system of the International Union Against Cancer (UICC).

Results

Expression of CD97 and EMR2 on Tumor Cell Lines

All cell lines expressed CD97, although intensities varied considerably.
was also present on all cell lines. There was no correlation between CD55 and CD97 staining intensity ($P = .91$; Spearman). The results of CD97 mRNA analysis by RT-PCR with primers flanking the coding region for the EGF-like domains indicated that all 3 known isoform mRNAs containing 3 (EGF 1, 2, 5), 4 (EGF 1, 2, 3, 5), or 5 (EGF 1, 2, 3, 4, 5) domains were present in the cell lines Image I. All cell lines displayed the strongest expression value for the shortest CD97 isoform mRNA and only slight appearance for the longest isoform, as known from other cell types. In analysis of the stalk (not shown) and TM7 regions, only 1 band could be amplified, indicating no splice variants in these regions.

Only 2 of the cell lines, HM0-2 and HS 766T, expressed EMR2 in flow cytometry. RT-PCR analysis confirmed this result. In analysis of the TM7 region, a clear band was obtained from both cell lines. Amplifying the EGF and part of the stalk region of EMR2 mRNA and sequencing the obtained PCR products (not shown) revealed a unique pattern of isoforms in both cell lines and in peripheral blood mononuclear cells used as a positive control. HM02 cells expressed only 1 isoform (EGF 1, 2, 3, 5), whereas HS 766T cells were positive for 5 different forms: (EGF 1, 2, 3, 5), (EGF 1, 2, 5), (EGF 1, 2), (EGF 1, 2, 3, 5) delta exon 12, and (EGF 1, 2, 5) delta exon 12. The last 2 splice variants resulting from the bypass of exon 12 predicted to encode soluble EMR2 due to a frameshift.5

CD97 and EMR2 Expression in Gastric Tissue Samples

The cell-surface epithelium of normal gastric mucosa did not express EMR2 and was negative or slightly positive for CD97 (not shown). We found a subpopulation of cells, especially in normal gastric samples obtained from the corpus region located within the middle parts of the gastric glands, that showed staining for CD97 Image 2A. Serial sections were used to characterize these CD97+ cells. Localization of pepsinogen and CD97 and comparison with H&E-stained sections showed that these were parietal cells (not shown). Smooth muscle cells of the lamina propria mucosae or the muscular coat of the stomach were strongly CD97+ (not shown).

Of 50 of the gastric adenocarcinomas, 44 expressed CD97 (mean ± SEM score, 5.8 ± 2.7). Of the 44 CD97+ tumors, 27 contained areas with a varying presence of the antigen. Two locations of stronger CD97+ tumor cells could be observed. First, the strongest staining with CD97 showed isolated, disseminated or scattered tumor cell clusters or single tumor cells located in the stroma (n = 25; score, 11.0 ± 2.0) compared with cells grown in glands or trabecula of the same tumor (score, 5.6 ± 2.4; $P < .0001$) Image 2B and Image 2C. This phenomenon could be detected only in carcinomas with TCD greater than 0 at the invasion front. Not in every gastric carcinoma were the disseminated or scattered tumor cells observed in H&E-stained sections more strongly CD97+ than tumor cells within solid formations. The percentage of tumors containing disseminated or scattered tumor cells that were more strongly CD97+ increased in relation to TCD (TCD1, 39%; TCD2, 50%; TCD3, 64%). Second, in 2 of 44 tumors, strong CD97 expression was found most prominently at the margin or invasion front of the tumor glands or trabecula. Larger homogeneous tumor areas showed weaker CD97 expression.

None of the gastric tumors expressed EMR2. A subpopulation of tumor-infiltrating leukocytes, namely monocytes and macrophages as judged by morphologic features, was EMR2+ (not shown).

### Table 2

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Origin</th>
<th>CD97</th>
<th>EMR2</th>
<th>CD55</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGS</td>
<td>Stomach</td>
<td>90-100</td>
<td>4.1 ± 0.8</td>
<td>0-5</td>
</tr>
<tr>
<td>HM02</td>
<td>Stomach</td>
<td>90-100</td>
<td>35.1 ± 12.7</td>
<td>50-90</td>
</tr>
<tr>
<td>Kato-III</td>
<td>Stomach</td>
<td>90-100</td>
<td>7.5 ± 2.4</td>
<td>0-5</td>
</tr>
<tr>
<td>St 3051</td>
<td>Stomach</td>
<td>90-100</td>
<td>11.0 ± 6.4</td>
<td>0-5</td>
</tr>
<tr>
<td>AsPC-1</td>
<td>Pancreas</td>
<td>90-100</td>
<td>16.7 ± 0.2</td>
<td>0-5</td>
</tr>
<tr>
<td>HS 766T</td>
<td>Pancreas</td>
<td>90-100</td>
<td>12.3 ± 1.4</td>
<td>90-100</td>
</tr>
<tr>
<td>PANC-1</td>
<td>Pancreas</td>
<td>90-100</td>
<td>55.5 ± 10.3</td>
<td>0-5</td>
</tr>
</tbody>
</table>

* The percentage is the percentage of labeled cells, and the intensity is the mean fluorescence intensity (specific labeled cells–isotype-matched control cells; mean ± SD, n = 3).
of the pancreas, 14 expressed CD97 (score, 7.1 ± 3.6) but none expressed EMR2.

Cells of the normal squamous epithelium of the esophagus were EMR2–. CD97 was not or was only slightly expressed. In a few cases (2/13), the basal membrane below the normal squamous epithelium was CD97+. Smooth muscle cells were CD97+ (Image 3C).

Eight of 8 squamous cell carcinomas and 2 of 5 adenocarcinomas of the esophagus expressed CD97 (score, 5.0 ± 2.3) but all were EMR2–.

**Clinicopathologic Features**

Nearly all gastric carcinomas were CD97+. Thus, we compared only the clinicopathologic features of patients with carcinomas with more strongly CD97+ disseminated or scattered tumor cells with the results of the other gastric carcinomas. None of the following variables correlated with the presence of strongly disseminated or scattered CD97+ tumor cells: age, sex, localization, local extent of tumor (pT), number of lymph node metastases (pN), or distant metastases (pM) assessed pathologically by clinical stage, blood and lymphoid vessel infiltration, histologic examination, or the Lauren classification. The percentage of carcinomas with strongly CD97+ cells was higher in high-grade carcinomas (grades III and IV, 72%) compared with low-grade carcinomas (grades I and II, 45%; P = .35). The mean levels (± SE) of sCD97 in serum were not higher in patients with gastric (0.13 ± 0.13 U/mL), pancreatic (0.13 ± 0.06 U/mL), or esophageal (0.12 ± 0.03 U/mL) carcinomas than in the control group (0.16 ± 0.01 U/mL). The levels of sCD97 varied considerably in gastric carcinomas; however, there was no correlation between sCD97 serum levels and CD97 expression in tumor tissues or any other clinicopathologic variable studied, including CEA, CA15-3, CA19-9, and CA72-4 serum levels (not shown).

**Discussion**

The presence of CD97 in gastric, pancreatic, esophageal, and, as shown relatively recently, thyroid carcinomas12,19 suggests that the expression of this molecule may be a common feature of tumors. The results obtained from a limited number of other carcinomas confirmed this assumption.11 In thyroid carcinomas, strong immunostaining of CD97 was found exclusively in anaplastic carcinomas, which were highly dedifferentiated (G4), whereas well-differentiated papillary and follicular carcinomas (G1-G2) were CD97– or expressed CD97 at lower levels. CD97 was associated with dedifferentiation and aggressiveness of
thyroid tumors. In contrast, we found no correlation between CD97 staining intensity or number of stained cells and grade of differentiation of the tumor or any other clinicopathologic feature in gastric carcinomas. This may be caused by the lower range of differentiation stages examined within this tumor entity (G2-G3) or the heterogeneity of the patient groups in combination with the small number of cases analyzed.

The presence of CD97 in the carcinomas suggests that the molecule has an unknown function in tumors. CD97 is involved in adhesion processes by binding to the cell surface molecule CD55.2,3 Studies on CD97 in thyroid carcinomas suggest that CD97-permitted adhesion has a role in the interaction of the tumor cell with the surrounding stromal cells and/or the extracellular matrix during invasion.12,13 CD97 may indeed be involved in such interactions since its ligand, CD55, recently has been shown to be overexpressed in the tumor environment of colorectal carcinomas.20 CD55 may be deposited into the extracellular matrix by direct secretion or by cleavage of CD55 from the cell surface of tumor or endothelial cells. Moreover, a modified CD55 isoform expressed in some gastric carcinomas, but not in normal gastric tissue, recently has been demonstrated.21

The possible interaction of CD97 with CD55 located in the extracellular matrix and its relevance in tumor invasion is supported by our results regarding the higher expression of CD97 in disseminated or scattered tumor cells located at the invasion front and surrounded by extracellular matrix compared with tumor cells located within solid formations of the same gastric tumor. TCD represents a strong prognostic factor for gastric carcinomas of the intestinal type.14,22 It is supposed that dissemination of organized tumor cell complexes into isolated tumor cells at the invasion front of gastric carcinomas mobilizes the tumor cells
from the main tumor bulk, enabling them to invade the host tissue by active locomotion. The process is accompanied by a loss of most of the cytologic features of differentiation.23 Such cells at the invasion front showed perturbation in the expression or function of the adhesive E-cadherin–catenin complex.24,25 beta-Catenin, usually expressed on the cell membrane, showed an abnormal accumulation in the cell nuclei, a process that has an important role in the acquisition of invasive potential.26

Whereas CD97 was not or was only slightly expressed in the corresponding normal tissues in most of the tumor entities studied, this is not the case in the pancreas. Here, pancreatic ducts, the origin for most pancreatic adenocarcinomas and containing pancreatic progenitor cells,27 were CD97+. The expression pattern of CD97 in normal pancreas tissue parallels that of Ep-CAM, an epithelial cell adhesion molecule.28 Epithelial cell growth through budding from duct cells induces Ep-CAM up-regulation, whereas
endocrine differentiation is associated with down-regulation of Ep-CAM, indicating features of a morphoregulatory and differentiation-dependent molecule. Whether CD97 acts in a similar way and whether expression of CD97 on pancreatic progenitor cells supports a more general function of CD97 in the differentiation processes remains speculative.

In contrast with CD97, all carcinomas studied were negative for EMR2, and only a few tumor cell lines slightly expressed the molecule. There is greater diversity in the number and/or combination of the EMR2 mRNA isoforms found in single cell lines or types compared with that demonstrated for CD97 mRNA. Because the only available monoclonal antibody against EMR2 binds to the stalk region, nothing is known about the presence of the distinct isoforms at the protein level. Only a subpopulation of tumor infiltrating leukocytes, probably macrophages, was EMR2+ in the tumor tissues, which tallies with the restricted myeloid expression pattern of the molecule in peripheral blood. Whether EMR2 in fact has a role in tumors needs to be evaluated using other tumor entities. The ligand and function of EMR2 are unknown. Although EMR2 (EGF 1, 2, 5) differed only in a few amino acids from CD97 (EGF 1, 2, 5) in the ligand-binding EGF-like domains, the affinity of EMR2 to CD55 is at least 1 order of magnitude lower. Thus, interaction between EMR2 and CD55 might not be involved in signaling of this molecule.

Taken together, this is the first study demonstrating the presence of CD97 in gastric, pancreatic, and esophageal tumors. The higher expression of the molecule in disseminated or scattered tumor cells at the invasion front compared with tumor cells in solid glandular or trabecular structures, especially in gastric carcinomas, revealed a function of CD97 in the invasion of tumor cells, perhaps as a differentiation-dependent or adhesion molecule.

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