Overexpression of Stromelysin-3, BM-40/SPARC, and MET Genes in Human Esophageal Carcinoma: Implications for Prognosis


Unité d'Institut National de la Santé et de la Recherche Médicale (INSERM) U 482, Equipe Cancérologie et Différenciation de l’Epithélium Gastro-Intestinal [H. P., L. K., Y. D., C. G., E. C.], Unité de Biostatistiques et d’Informatique Médicale [F. C.], Service Central d’Anatomie et de Cytologie Pathologiques [S. P.], and Centre de Chirurgie Digestive et Unité INSERM 402 [B. N.], Hôpital Saint-Antoine, 75571 Paris Cedex 12, France; Clinique Chirurgicale, Hôpital Albert Calmettes [H. P., A. W.], and Service de Chirurgie Digestive et Endocrinienne, Hôpital Claude Huriez [J. P. T.], Centre Hospitalier Régional Universitaire de Lille, 59037 Lille Cedex, France; and Institute for Cancer Research, University of Torino Medical School, Torino 10126, Italy [P. C.]

ABSTRACT

Molecular markers can improve staging and predict aggressive clinical behavior in esophageal cancer, thus helping to define appropriate therapeutic protocols and to identify patients who will benefit from surgery. We therefore characterized, by Northern blot and/or immunohistochemistry, the relative expression of three effectors involved in the invasion, angiogenesis, and dissemination of tumor cells in esophageal cancer versus nontumoral mucosae: (a) stromelysin-3 (ST3), a member of the metalloproteinase family; (b) basement membrane 40/secreted protein acidic and rich in cysteine (BM-40/SPARC), an extracellular matrix-associated protein involved in angiogenesis; and (c) the hepatocyte growth factor receptor, MET, which triggers the scattering of epithelial cells. Results were analyzed in relation to clinicopathological parameters (cpTNE) including tumor size (T), lymph node status (N), periesophageal tissue invasion (E), disease recurrence, and overall survival.

The ST3, BM-40/SPARC, and MET genes were found to be overexpressed in tumor samples compared to control mucosa. BM-40/SPARC and MET mRNA levels were not linked to any one of the cpTNE, indicating that this overexpression occurs at an early stage of neoplastic progression. In contrast, ST3 expression, identified by immunohistochemistry in fibroblastic cells surrounding neoplastic islets, correlated with tumor size and periesophageal tissue invasion. Of the 36 patients studied, those with high ST3 levels had shorter disease-free survival than those with low levels, but there was no relationship between the cpTNE and disease recurrence or survival.

Our study demonstrates that ST3, BM-40/SPARC, and MET are involved in different steps of esophageal carcinogenesis and that ST3 overexpression is a marker of aggressive clinical behavior. We conclude that in esophageal cancer, ST3 might help to assess survival and the risk of recurrence after surgical resection.

INTRODUCTION

The surgical treatment of esophageal cancer is associated with high postoperative morbidity and is almost a palliative, due to the very high rate of local and distant recurrence. Consequently, 5-year overall survival after potentially curative surgical resection is about 7% (1–3). The clinicopathological criteria known as cpTNE2 relating to the prognosis of esophageal cancer include tumor size, lymph node status, invasion in the adventitia, and residual disease after surgery. However, these criteria do not define the individual risk of recurrence after surgical resection (4–6). For this purpose, identification of the molecular markers of the neoplastic progression would provide new insight into tumor biology by making it possible to define a subgroup of patients with operable esophageal cancers who would benefit from surgery in terms of survival and quality of life.

Carcinogenesis is the result of a complex accumulation of genetic and cellular defects involving multiple interactions between neoplastic cells and the stromal compartment, including mesenchymal, endothelial, and inflammatory cells (7, 8). Molecular studies of human esophageal tumors have revealed a series of frequent genetic abnormalities, such as deletions and mutations of p53, p105th, p15- and pl6-cyclin kinase inhibitors, amplification of c-myc and cyclin D1 (9–11), and activation of the tyrosine kinase pp60src (12). However, most studies have only involved a few patients or have not explored the link between the molecular defects and recurrence or survival.

The initial steps of invasiveness and metastasis involve the breakdown of cell-cell junctions, dissociation of tumor cells, focal proteolysis of the ECM, and dissemination through neovascularization (7, 8, 13). General features of ECM degradation in carcinomas include the secretion of metalloproteinases (MMPs) by the peritumoral stroma (8, 13). Among MMPs, ST3

Received 8/14/97; revised 2/25/98; accepted 3/4/98.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom requests for reprints should be addressed, at INSERM U 482, Hôpital Saint-Antoine, 75571 Paris Cedex 12, France. Phone: 33-1-49-28-46-84; Fax: 33-1-49-28-46-94; E-mail: chastre@st-antoine.insERM.fr.

2 The abbreviations used are: cpTNE, clinicopathological criteria including tumor size, node status, and esophageal tissue invasion (TNM classification); BM-40, basement membrane 40; ECM, extracellular matrix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IEF-1, human elongation factor 1; HGF, hepatocyte growth factor; MMP, matrix-degrading metalloproteinase; RT-PCR, reverse transcription-PCR; SPARC, secreted protein acidic and rich in cysteine; ST3, stromelysin-3.
has been identified in several types of invasive carcinoma (14–19). Its particular advantage as a biological marker of tumor aggressiveness is its low level of expression in normal tissues, except during embryonic development and wound healing (14, 19, 20).

Besides ECM proteolysis, the ectopic expression of ECM components, such as BM-40/SPARC, participates in the neoplastic process (15, 21–24). BM-40/SPARC is a M, 43,000 glycoprotein that interacts with ECM components in connective tissues, including collagen types I, III, IV, and V (21). BM-40/SPARC was found to reduce endothelial cell and fibroblast spreading and is associated with tissue remodeling and de novo formation of basement membranes (25, 26). It also regulates both endothelial cell proliferation and angiogenesis (21, 27, 28).

Secreted factors, such as HGF/scatter factor also play a key role in tumor angiogenesis and metastasis (29). HGF disrupts cell-cell junctions and promotes motility and invasiveness of epithelial and endothelial cells through the activation of its receptor, the tyrosine kinase MET (30–32). The overexpression and subsequent autophosphorylation of the MET receptor result in constitutive activation of the kinase (33–35).

The present study was undertaken to assess the validity of ST3, BM-40/SPARC, MET, and HGF as prognostic markers of esophageal carcinoma aggressiveness. Consequently, we investigated the expression of these molecular markers in esophageal cancers and in distant control mucosa by Northern blot, RT-PCR, and immunohistochemistry, in relation to (a) the cpTNE classification and (b) disease recurrence and survival.

PATIENTS, MATERIALS, AND METHODS

Patients. From October 1, 1992, to February 1, 1996, 36 patients (mean age, 60 years; range, 43–78 years) who underwent esophagectomy for squamous cell carcinoma or adenocarcinoma of the thoracic esophagus were enrolled in this study (Table 1). None of these patients had induction chemotherapy. There was no selection criterion relating to the pathological nature of the lesion or the type of surgery. Patients with a metastatic spread or incomplete tumor resection, or those who died of surgical complications during the postoperative course, were excluded from the study. We also excluded patients with hypopharyngeal tumors, tumors of the cervical esophagus, and adenocarcinomas of the gastroesophageal junction (less than 3 cm above the cardia) because of the different prognoses and treatments of these lesions. The sampling of lymph nodes included the celiac axis and the diaphragmatic and posterior mediastinal nodes. Patients were followed up by a clinical examination every 3 months, and by thoracic and abdominal CT scan and endoscopic examination every 6 months. Twenty of the 36 patients died of their recurrent esophageal cancer, after a median survival of 24 months. The pathological characteristics of the tumors and the recurrence and survival rates of the patients are reported in Table 1. All of the pathological slides were reviewed by one of us (S. P.) without knowledge of previous findings. In case of disagreement, slides were again reviewed with the initial pathologist.

Tissue Samples. All of the specimens of esophageal carcinomas were supplied by the departments of surgery of the Hôpital Claude Huriez (Lille) and Hôpital Saint-Antoine (Paris). The corresponding control mucosa was dissected out at the resection margin. Tissue samples weighing 0.5–1 g were snap frozen in liquid nitrogen and stored at −80°C until use. The relative amount of stromal tissue in tumor specimens, assessed by histological analysis of the tissue section, ranged from 15 to 20% of the sample. Twenty-five of the 36 tumors sampled were matched with corresponding distant control mucosa.

RNA Isolation and Northern Blot Analysis. Frozen tissue samples were homogenized with a Polytron apparatus in 4.7 M guanidinium isothiocyanate lysis buffer (10% w/v) and centrifuged for 20 h through a 5.7 M guanidinium isothiocyanate/0.1 M sodium citrate/30 mSM cesium chloride solution at 180,000 × g and 20°C. RNA samples (15 μg) were denatured and then under-went electrophoresis in 1% agarose-2.2 M formaldehyde gels and were subsequently transferred to nylon membranes (Hybond N+, Amersham, Les Ulis, France). The relative loading and quality of the RNAs were evaluated by methylene blue staining of the rRNA bands. The membranes were hybridized overnight with the 32P-labeled probes specified below (Megaprime; Amersham); washed twice for 15 min at room temperature in 2× SSC, which comprised 0.3 M NaCl and 30 mm sodium citrate (pH 7), and 0.1% SDS; and incubated for 60 min at 56°C in 0.1× SSC and 0.1% SDS. The ST3, BM-40/SPARC, and MET probes were respectively the 1.7-kb cDNA extending from nucleotides 346-2105 and the 1-kb and 4.5-kb cDNAs encompassing the entire coding sequences of BM-40/SPARC and MET kinase (14, 35, 36). Autoradiography was performed for 16 h at −70°C using Kodak X-OMAT AR films (Rochester, NY) and the Chromex Quanta III intensifying screen (Dupont de Nemours, Les Ulis, France). Accumulation of the ST3, BM 40/SPARC, and MET transcripts was quantified by direct counting of the radioactivity on the blots (Instant Imager; Packard). The ST3, BM-40/SPARC, and MET signals were then normalized for the level of the RNA encoding hEF-1, the expression pattern of which resembles the pattern revealed by methylene blue staining of

| Table 1 Tumor characteristics and the recurrence and survival rate in 36 patients with esophageal cancer |
|---|---|
| Tumor location | No. of patients |
| Upper thoracic | 3 |
| Middle thoracic | 12 |
| Lower thoracic | 21 |
| Clinicopathological parameters | |
| Squamous cell carcinoma | 27 |
| Adenocarcinoma | 9 |
| Tumor size | |
| T1 | 1 |
| T2 | 13 |
| T3 | 21 |
| T4 | 1 |
| Nodal status | |
| Negative | 11 |
| Positive | 25 |
| Periesophageal involvement | |
| Negative | 11 |
| Positive | 25 |
| Survival and recurrence rate | |
| Alive without recurrent esophageal cancer | 5 |
| Alive with recurrent esophageal cancer | 11 |
| Death from recurrent esophageal cancer | 20 |
the rRNA (35). One RNA sample was included as internal control for each series of Northern blots.

**Expression of the HGF Gene by RT-PCR and Southern Blot.** For RT-PCR analysis, RNA samples (2 μg) were reverse transcribed for 60 min at 37°C, using 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Cergy Pontoise, France). The cDNAs (500 ng) were digested in 25 μl of 20 mM Tris-HCl buffer (pH 8.5) containing 16 mM (NH₄)₂SO₄, 2.5 mM MgCl₂, 150 μg/ml BSA, 12.5 pmol of each primer, 100 μM deoxyribonucleotide triphosphates, and 1.25 units of Bio-Taq polymerase (Bioprobe Systems, France). Amplification consisted of 28–30 denaturation cycles at 94°C for HGF or 20–22 cycles for GAPDH, annealing for 1 min at 58°C, and a 1-min extension at 72°C in an automated thermal cycler (Robocycler Gradient 96; Stratagene, CA). The reaction was initiated by a 5-min incubation at 94°C and ended after a 7-min extension at 72°C. PCR products were run on 1.5% agarose gels stained with ethidium bromide.

For Southern blot analysis, PCR products were transferred to Hybond N* membranes by alkali blotting and hybridized overnight with the internal probes end labeled with [γ-32P]ATP. Membranes were washed twice at room temperature in 6× saline-sodium phosphate-EDTA [1.08 mM NaCl, 60 mM NaPO₄, and 6 mM EDTA (pH 7.7)] and 0.1% SDS, followed by 45 min of incubation at 55°C. Autoradiography was performed for 3–8 h at −70°C using Kodak Biomax MR films and a Chromex Quanta III intensifying screen.

To identify the full-length and truncated variants of the HGF transcripts that contain the first kringle domes, we used the following sense and antisense oligonucleotides, spanning exons I–III: 5’-ATCTCCTCTCGTCCTCCATC-3’ (nucleotides 53–72) and 5’-CTCCACTGAGATGCTATTG-3’ (nucleotides 303–322). The expected size of the PCR product was 270 bp (37). The cDNA fragment extending from the K3 domain (exon VIII) to the 5’ portion of the HGF sequence β chain (exon XV) was amplified using the sense primer 5’-GGAATGGAATFTC-3’ (nucleotides 962–985) and the antisense primer 5’-TCAAGTCTCAGGAGAAGAA-3’ (nucleotides 1603–1624). The expected size of the PCR product was 663 bp (37). The sequences of the corresponding internal probes for the Southern blots were 5’-GGGAACCAGGAGGATTTCAGCT-3’ (nucleotides 1403–1425). To evaluate the integrity and relative amounts of RNA samples, a 574-bp sequence of the GAPDH mRNA was amplified in the presence of 0.5 μCi of [α-32P]dATP, resolved by agarose gel electrophoresis, transferred to nylon membranes, and subjected to autoradiography (37).

**Immunohistological Studies of ST3.** Immunohistochemical analysis was performed on formalin-fixed paraffin-embedded sections of esophageal tumors. Adjacent sections were incubated overnight in a 1:400 dilution of the STT4A9 monoclonal antibody (ascites fluid) directed against the COOH-terminal portion of the hemopexin-like domain of ST-3, as described previously (38), and probed using streptavidin-biotin peroxidase (kit K 5007; DAKO Corp., Carpinteria, CA) and 3-amino-9-ethyl carbazole as detection agent (kit K 681; DAKO). The specificity of immunostaining was checked by omitting the 5 STT4A9 antibody.

**Statistical Studies.** Time variables were defined as the time from the date of surgery. In the analysis of overall survival, the end point was defined as death, from whatever cause. Data concerning the surviving patients were censored at the last follow-up evaluation. Disease-free interval was defined as the time from complete resection to the discovery of the recurrence in the course of regular clinical and biological examinations every 3 months and endoscopy, thoracic, and abdominal CT scan every 6 months.

The Mann-Whitney nonparametric U test was used to compare ST3, BM-40/SPARC, and MET mRNA levels according to the cpTNE criteria, and Wilcoxon’s signed rank test was used to compare the expression of the molecular markers on paired samples. Correlations were computed using the Spearman rank coefficient. The links between ST3, BM-40/SPARC, and MET mRNA levels; the cpTNE; and recurrence or survival were estimated using the Wald and the log-rank tests. Statistical significance was defined as P < 0.05. All tests were two tailed.

**RESULTS**

**Expression of the ST3, BM-40/SPARC, and MET Genes.** As shown in Fig. 1, ST3 and MET transcripts were respectively identified as single autoradiographic bands of 2.4 and 6.5 kb, whereas the BM-40/SPARC transcript was a doublet of 2.2 and 3 kb. This doublet originates from alternative polyadenylation
Table 2  Relationships between the levels of ST3, BM-40/SPARC, and MET mRNA and clinicopathological features

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>ST3 Median</th>
<th>Range</th>
<th>P</th>
<th>BM-40 Median</th>
<th>Range</th>
<th>P</th>
<th>MET Median</th>
<th>Range</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esophageal tissues</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neoplasms</td>
<td>36</td>
<td>0.5</td>
<td>0.04–23.68</td>
<td>&gt;0.1%b</td>
<td>1.06</td>
<td>0.12–5.44</td>
<td>1.03</td>
<td>0.02–1.14</td>
<td>&lt;1%b</td>
</tr>
<tr>
<td>Control mucosa</td>
<td>25</td>
<td>0.16</td>
<td>0.02–2.07</td>
<td>&lt;0.1%b</td>
<td>0.93</td>
<td>0.12–1.76</td>
<td>0.55</td>
<td>0.02–3.81</td>
<td>&lt;1%b</td>
</tr>
<tr>
<td>Pathological parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1–T2</td>
<td>14</td>
<td>0.28</td>
<td>0.04–23.68</td>
<td>1.06</td>
<td>0.12–5.44</td>
<td>1.03</td>
<td>0.02–1.14</td>
<td>&lt;1%b</td>
<td></td>
</tr>
<tr>
<td>T2–T4</td>
<td>22</td>
<td>0.74</td>
<td>0.04–3.13</td>
<td>&lt;1%b</td>
<td>1.01</td>
<td>0.13–4.32</td>
<td>0.95</td>
<td>0.02–1.14</td>
<td>&lt;1%b</td>
</tr>
<tr>
<td>Nodal status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>11</td>
<td>0.34</td>
<td>0.04–23.68</td>
<td>1.06</td>
<td>0.12–5.44</td>
<td>1.03</td>
<td>0.02–1.14</td>
<td>&lt;1%b</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>25</td>
<td>0.67</td>
<td>0.04–3.13</td>
<td>1.01</td>
<td>0.13–4.32</td>
<td>0.95</td>
<td>0.02–1.14</td>
<td>&lt;1%b</td>
<td></td>
</tr>
<tr>
<td>Periesophageal involvement</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>11</td>
<td>0.12</td>
<td>0.04–0.5</td>
<td>1.07</td>
<td>0.43–5.44</td>
<td>1.36</td>
<td>0.07–1.14</td>
<td>&lt;1%b</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>25</td>
<td>0.81</td>
<td>0.04–23.68</td>
<td>1.07</td>
<td>0.43–5.44</td>
<td>1.36</td>
<td>0.07–1.14</td>
<td>&lt;1%b</td>
<td></td>
</tr>
</tbody>
</table>

* The relative accumulation of ST3, BM-40, and MET transcripts was determined by quantifying the corresponding radioactive signals on the blots, which were normalized to hEF-1 signal. The results for each blot were adjusted using an internal control sample.

b Wilcoxon’s signed rank test, performed on the 25 paired samples.

c Mann-Whitney U test. NS, not significant.

The relative accumulation of the ST3, BM-40/SPARC, and MET transcripts in esophageal neoplasms and their control mucosa was quantified by direct measurement of radioactivity on the membranes and then normalized for hEF-1 expression (Fig. 1; Table 2). The degree of variation in ST3, BM-40/SPARC, and MET gene expression in neoplastic and control tissues is illustrated in Fig. 2. In the 25 tumor samples, expression of these genes was found to be significantly greater than in the corresponding mucosal control tissue (P < 0.001–0.01), suggesting the involvement of these three genes in the neoplastic transformation of esophageal mucosae (Table 2).

The level of ST3 gene expression in tumors correlated with the levels of MET and BM-40 gene expression (P < 0.002). The levels of ST3 and MET gene expression were also correlated in the 25 samples of nontumoral tissue investigated (Spearman correlation; P < 0.001).

Immunohistopathological Studies. The cellular compartment involved in the overexpression of ST3 was investigated by immunohistochemistry on three esophageal squamous cell carcinomas. ST3 immunoreactivity was detected in two of them. The immunoreactivity was located in the cytoplasm of the stromal fibroblasts surrounding the carcinomatous islets (Fig. 3). These results confirm the stromal origin of ST3, previously documented in carcinomas of the breast, colon and rectum, lung, head and neck, and basal cells (14–19).

Expression of the HGF Gene Revealed by RT-PCR and Southern Blot. To assess the functional significance of MET overexpression in esophageal neoplasms, we evaluated by RT-PCR the expression of the MET receptor ligand HGF (scatter factor). As shown in Fig. 4, large amounts of PCR products encoding the amino acid sequence upstream of the first kringle domain were identified in esophageal neoplasms and nontumoral mucosae. Because truncated isoforms of HGF, NK1, and NK2 extend through the first and second kringle domains and behave like HGF/SF partial antagonists (39, 40), we conducted amplification with a set of primers that encompassed the third
for recurrence, and \( P = 0.11, 0.57, \) and 0.13 for survival, for cpTNE, respectively).

Accumulation of the ST3, BM-40/SPARC, and MET transcripts did not correlate with disease recurrence or survival (Wald test; \( P = 0.1, 0.17, \) and 0.67, respectively, for recurrence). However, as shown in Fig. 5, patients with tumoral ST3 levels above the median value had a shorter disease-free interval than patients with lower ST3 levels (log-rank test, \( P = 0.03 \)). The half-times for recurrence were 10 ± 1.9 and 16 ± 4.9 months, for the high and low ST3 levels, respectively.

**DISCUSSION**

In the present study, we demonstrated that the neoplastic progression of esophageal cancer is associated with the induction of ST3, BM-40/SPARC, and MET gene expression. Overexpression of the ST3 gene was related to tumor size and periesophageal invasion, whereas BM-40/SPARC and MET gene expression was not related to these clinicopathological parameters. Therefore, the BM-40/SPARC and MET genes are probably induced at an early stage of esophageal carcinogenesis. In agreement with this hypothesis, we previously reported high levels of SPARC and MET genes in the preneoplastic lesions of colorectal mucosa (15, 35).

BM-40/SPARC exerts pleiotropic biological functions, including the enhancement of endothelial cell permeability and the reduction of focal adhesion and endothelial cell proliferation. It is also involved in ECM remodeling via the induction of matrix proteinases and their inhibitors, such as collagenase, transin, plasminogen activator inhibitor, and collagen I (21, 25, 26). This broad spectrum of activities is triggered by distinct domains of BM-40/SPARC that can be released through proteolysis by serine proteases. BM-40/SPARC is a source of copper-binding peptides that stimulate angiogenesis (41). Because a tumor can only develop if it is supplied with a vascular system, neoangiogenesis is certainly one of the most important factors involved in the initiation and progression of solid tumors. Because angiogenesis has a prognostic significance for metastasis and survival (42), SPARC may be involved in both the early and late stages of esophageal carcinogenesis. Down-regulation of BM-40/SPARC by an antisense expression vector was recently demonstrated to suppress the tumorigenic potential of human melanoma cells (23). This down-regulation was associated with reduced adhesive and invasive capacities in vitro, whereas cell growth remained unchanged. Furthermore, the rejection of melanoma cells was associated with the presence of inflammatory infiltrates, indicating that changes in matrix deposition and interactions between tumor cells and matrix components not only act on the adhesive and invasive properties of neoplastic and stromal cells, but also alter immunological responses (23).

Another interesting result of our study concerns the overexpression of the tyrosine kinase MET that transduces the motility, proliferation, morphogenic, and invasive signals of HGF in epithelial cells. Although MET overexpression transforms NIH-3T3 fibroblasts, the recent demonstration that activating germ-line mutation of this kinase occurs in patients with papillary renal carcinoma proves that interactions with other signaling pathways are required for the completion of neoplastic transformation (43). The overexpression of MET established in the present study may originate either from gene amplification

**Expression of the ST3, BM-40, and MET Genes in Relation to Clinicopathological Parameters and Clinical Outcome.** There was no difference with regard to recurrence; survival; and the levels of ST3, BM-40, and MET gene expression between the subgroups of patients with squamous cell carcinomas or adenocarcinomas. The relationships between ST3, BM-40, and MET gene expression and the conventional clinicopathological parameters with prognostic significance in esophageal cancers (cpTNE) were therefore analyzed in the entire series of tumor samples (Table 2). Accumulation of the ST3 transcripts correlated with tumor size (\( P = 0.025 \)) and esophageal tissue invasion (\( P = 0.002 \)), but not with node status, *i.e.*, lymph node metastasis (\( P = 0.1 \)). In contrast, expression of the BM-40/SPARC and MET genes was not related to any of these clinicopathological parameters. In a univariate analysis, none of the cpTNE parameters displayed any association with either disease recurrence or reduced overall survival in our series of patients (log-rank test, \( P = 0.62, 0.73, \) and 0.19

---

**Fig. 3** Immunohistochemistry of ST3 in an esophageal carcinoma. Paraffin-embedded tissue sections were incubated with monoclonal antibody ST4A9 (ascites fluid diluted 1:1000), probed using streptavidin-biotin peroxidase and 3-amino-9-ethyl-carbazole (brown staining), and stained with hematoxylin (blue staining). ci, carcinomatous islet; f, fibroblast. Original magnification: top, ×200; bottom, ×400.

kringle domain and the 5' part of the β chain. The resulting amplification pattern was unchanged (data not shown), indicating that full-length HGF transcripts were coexpressed with the MET receptor in both control mucosae and esophageal tumors.

**Fig. 3** Immunohistochemistry of ST3 in an esophageal carcinoma. Paraffin-embedded tissue sections were incubated with monoclonal antibody ST4A9 (ascites fluid diluted 1:1000), probed using streptavidin-biotin peroxidase and 3-amino-9-ethyl-carbazole (brown staining), and stained with hematoxylin (blue staining). ci, carcinomatous islet; f, fibroblast. Original magnification: top, ×200; bottom, ×400.
or from activation of the ets, ras, or pp60Src signaling pathways (35, 37, 44, 45). Accordingly, we demonstrated previously that oncogenic activation of pp60Src kinase triggered MET overexpression and rendered human colonic adenoma PC-9src cells susceptible to HGF-induced invasiveness (37). Because increased pp60Src activity was evidenced in premalignant Barrett’s esophagus (12), one may justifiably postulate that pp60Src and MET cooperate in the early steps of esophageal carcinogenesis. The implication of MET in esophageal cancers was further substantiated in the present study by the identification of HGF transcripts in tumoral tissue and control esophageal mucosae. Greater HGF immunoreactivity was previously reported in esophageal squamous cell carcinomas than in control mucosae (46). HGF is secreted as the inactive single-chain precursor pro-HGF, which is converted into an active heterodimer by specific serine proteases, the HGF activator, and urokinase (47, 48). The changes in the balance of HGF activator and its inhibitor (49), and the release of the cytokine sequestered in the ECM during tissue injury, might allow subtle regulation of HGF bioavailability during physiopathological processes, including wound healing and angiogenesis. HGF exerts angiogenic effects by stimulating proliferation, protease production, invasion, and the organization of endothelial cells into capillary-like tubes (50). HGF overexpression in tumors may therefore exert convergent actions on both cancer cells and endothelial compartments. Taken together, our results indicate that the early induction of BM-40/SPARC and MET in esophageal carcinomas might constitute a reliable marker in the survey of preneoplastic lesions in esophageal mucosae.

We noted with great interest that the accumulation of ST3 transcripts in the peritumoral stroma correlated with the cpTNE parameters, which are the most important indicators known thus far of the risk of recurrence and death in esophageal carcinoma. In this relatively restricted series of 36 patients, these clinicopathological parameters were not linked to disease recurrence or survival. Accordingly, these parameters yielded little information concerning the prognosis in individual cases (4) and were shown to correlate with recurrence and survival in large series of patients (6). In contrast, high ST3 levels were proved here to be associated with shorter disease-free survival. Our results also suggest that ST3 is implicated in the growth and local invasiveness of esophageal cancer, in agreement with the previously reported high ST3 levels in breast, colorectal, and head and neck tumors with a poor prognosis (14, 15, 17, 51–53). ST3 gene expression was not significantly linked to node status, suggesting that ST3 is not involved in the homing of metastatic cells in lymph nodes. In this connection, high ST3 immunoreactivity did not correlate with axillary lymph-node metastasis in breast cancer, but it did correlate with patient outcome (51). This result confirms the notion that ST3 could become a reliable marker of cancer aggressiveness, independently of the conventional clinicopathological criteria.

One intriguing question concerns the biological significance of ST3 during carcinogenesis. Although ST3 is overexpressed in carcinomas and in remodeling tissues, its substrate specificity is still unknown. It has been proposed that the specificity of ST3 is restricted to the inhibitors of serine proteases, i.e., serpins (54). Subsequent activation of these proteases leads to the MMP cascade and ECM proteolysis. However, it is noteworthy that serpin inactivation is not the hallmark of ST3, given that it is also mediated by the interstitial collagenase MMP-1 (55). Furthermore, ST3 is released as a $M_\text{r}$ 45,000 active enzyme that is further processed to a $M_\text{r}$ 35,000 protein truncated in the catalytic domain via a MMP and basic fibroblast growth factor-dependent mechanism (56). A recent study demonstrated that overexpression of ST3 following the transfer of cDNA into the human breast cancer MCF-7 cell line did not confer a growth advantage or the invasive phenotype in vitro, but it promoted the tumor take in nude mice (57). Conversely,
antisense ST3 reduced NIH-3T3 fibroblast tumorigenicity, indicating that ST3 promotes tumor formation via mesenchymal cells rather than tumor growth via cancer cells. One emerging hypothesis is that ST3 acts as a paracrine factor by contributing to the survival of cancer cells. In agreement with this hypothesis, it was recently shown that ST-3, as well as stromelysin-1 and matrilysin, cleave insulin-like growth factor binding protein-I and restore insulin-like growth factor-induced survival in BAF3 and MCF-7 cell lines (58).

Additional studies are required to establish whether ST3 can help to assess individual survival and the risk of recurrence after surgical resection when used together with other biomarkers known to have prognostic significance, such as epithelial growth factor receptor, cyclin A, and cyclin D1 (59–61). The critical point is to find a simple and reliable approach to the assessment of ST3 gene expression in esophageal cancer in a large-scale prospective study, using techniques available in daily clinical practice. Detection of ST3 overexpression in colorectal cancer by RT-PCR (62) suggests that its use as a molecular marker may become a routine part of the management of patients with esophageal cancer.

ACKNOWLEDGMENTS

We are grateful to Dr. P. Basset and Prof. P. Chambon (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France) for providing the ST3 cDNA and the monoclonal antibody ST-4A9 and to Dr. T. Krieg (Dermatologische Klinik der Universität München, Germany) for providing the BM-40/SPARC cDNA. We are indebted to the staffs of the departments of surgery (Hôpital Claude Huriez, Hôpital Albert Calmette, and Hôpital St-Antoine) for their collaboration. We also thank Y. Issoulié for photographic reproduction.

REFERENCES


Updated version  Access the most recent version of this article at: http://clincancerres.aacrjournals.org/content/4/6/1375

<table>
<thead>
<tr>
<th>E-mail alerts</th>
<th>Sign up to receive free email-alerts related to this article or journal.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reprints and Subscriptions</td>
<td>To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at <a href="mailto:pubs@aacr.org">pubs@aacr.org</a>.</td>
</tr>
<tr>
<td>Permissions</td>
<td>To request permission to re-use all or part of this article, use this link <a href="http://clincancerres.aacrjournals.org/content/4/6/1375">http://clincancerres.aacrjournals.org/content/4/6/1375</a>. Click on &quot;Request Permissions&quot; which will take you to the Copyright Clearance Center's (CCC) Rightslink site.</td>
</tr>
</tbody>
</table>