Prognostication in esophageal cancer

Lagarde, S.M.

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Met expression is an independent prognostic risk factor in patients with esophageal adenocarcinoma

Jurriaan B Tuynman
Sjoerd M Lagarde
Fiebo JW ten Kate
Dick J Richel
J Jan B van Lanschot
Abstract

Introduction
Esophageal adenocarcinoma is an aggressive malignancy with propensity for early lymphatic and hematogenous dissemination. Since conventional TNM-staging does not provide accurate prognostic information, novel molecular prognostic markers and potential therapeutic targets are subject of intense research. The aim of the present study was to study the prognostic significance of Met, the hepatic growth factor (HGF) receptor and a possible target for therapy in comparison to cyclooxygenase-2 (COX-2).

Patients and Methods
Tumor sections from 145 consecutive patients undergoing intentionally curative surgery for esophageal adenocarcinoma were immunohistochemically analyzed for Met and COX-2 expression. Clinicopathological data were prospectively collected for all patients.

Results
Patients with high Met expression had significantly reduced overall and disease specific 5-year survival rates (p≤0.001 and p≤0.001, respectively) and were more likely to develop distant metastases (p=0.002) and local recurrences (p=0.004) compared to patients with low Met expression. High COX-2 expression tended to be correlated with poor long term survival but this did not reach statistical significance. Expression of Met was recognized as an independent prognostic factor by multivariate analysis (relative risk, 2.3; 95% CI, 1.3 –4.1).

Conclusions
These findings support the importance of Met in esophageal adenocarcinoma and support the concept of Met tyrosine kinase inhibition as (neo-) adjuvant treatment.
Introduction

Esophageal adenocarcinoma is a highly aggressive malignancy with early lymphatic and hematogenous dissemination. The incidence of adenocarcinoma of the esophagus is increasing rapidly in the Western World. Despite advances in diagnosis and treatment of the disease, overall 5-year survival of all patients suffering from esophageal adenocarcinoma remains approximately 15%. Even after potentially curative surgery the overall 5-year survival rate rarely exceeds 35%. For adenocarcinoma of the esophagus the most important conventional prognostic factors are summarized in the pTNM-stage of the esophagus. Also other pathological aspects such as extracapsular lymph node involvement and number of positive nodes have prognostic impact. However, these conventional prognostic factors have limited accuracy. Therefore, molecular prognostic markers which can serve as targets for therapy are subject of intense research. For esophageal adenocarcinoma only few molecular prognostic factors have been identified and molecular events responsible for the development of lymphatic and hematogenous dissemination are still poorly understood. Identification of growth factor receptors with tyrosine kinase activity, highly expressed in advanced cancer, has been shown to provide both prognostic information and potential molecular targets for (neo-)adjuvant therapy. A promising development in cancer therapy is the combination of surgery with potent selective growth factor receptor inhibitors as (neo-)adjuvant therapy resulting in improved overall and disease specific survival. Therapeutic usage of small molecules selectively inhibiting c-KIT, a growth factor receptor present in gastrointestinal stromal cell tumors (GIST), has resulted in remarkable responses and has enhanced prognosis for patients with GIST to a great extent. Other examples of targeted (neo-)adjuvant therapy are the inhibition of vascular endothelial growth factor receptor (VEGFr) in patients with advanced colorectal cancer, the inhibition of HER2-Neu, an epidermal growth factor receptor in patients with breast cancer and the inhibition of both VEGFr and platelet derived growth factor receptor (PDGFr) in patients with renal cell carcinoma.

Growth factor receptors have been identified in esophageal adenocarcinoma and some show higher expression in later stages of cancer development. However, the prognostic significance of growth factors expressed in adenocarcinoma of the esophagus has only been investigated in relatively small patient cohorts and no significance in multivariate analysis was demonstrated so far. The only independent molecular prognostic factor demonstrated for adenocarcinoma of the esophagus is cyclooxygenase-2 (COX-2) expression as published by our group. Recently, we have reported a clinical study in which neo-adjuvant selective COX-2 inhibition downregulates Met expression in conjunction with COX-2 expression in patients with adenocarcinoma of the esophagus. Met is the hepatocyte growth factor (HGF) receptor and is identified in esophageal adenocarcinoma. Overexpression of Met and/or its ligands has been shown to contribute to progression and dissemination of several malignancies including lung, colorectal, gastric, breast, prostate, thyroid, pancreas, and esophageal cancer. In experimental models, activation of Met (endogenously
by mutations in its tyrosine kinase domain, or exogenously by HGF and prostaglandins produced by COX-2) causes decreased apoptosis and enhanced proliferation, angiogenesis, and invasion\textsuperscript{22-24}.

Thus COX-2 and Met seem functionally connected. In cancer development, COX-2 is present in early stages of dysplasia, initiating cancer growth and progression whereas Met is an important key regulator of molecular processes in later stages of cancer development and progression\textsuperscript{16}. Small molecules selectively inhibiting Met have been shown to inhibit dissemination and cancer growth both in vitro as in animal studies\textsuperscript{16,23,25-29}. Consequently, inhibition of Met as (neo-) adjuvant therapy for esophageal adenocarcinoma seems a promising strategy.

A relation between Met expression and stage of disease has been previously described\textsuperscript{17}. However the potential value of Met expression in esophageal adenocarcinoma as an independent prognosticator calculated by multivariate analysis has not yet been addressed in a large consecutive cohort. Therefore, the aim of the present study was to further characterize the prognostic significance of Met expression in a large consecutive cohort of patients with esophageal adenocarcinoma.

### Patients and Methods

#### Patients

A consecutive series of 306 patients who underwent potentially curative esophagectomy at the Department of Surgery at the Academic Medical Centre at the University of Amsterdam, The Netherlands, for adenocarcinoma of the mid-/distal esophagus between January 1993 and December 2000 was selected. Preoperative workup included endoscopy with histological biopsy, external ultrasonography of the abdomen and neck, CT scan of the abdomen and chest, radiography of the chest, esophageal endosonography, and indirect laryngoscopy. Lymph node metastases at the celiac trunk were a contraindication for resection only when considered non-resectable (i.e. larger than 2 cm in diameter) and confirmed by cytological puncture. Patients did not receive additional (neo-) adjuvant chemo- and/or radiotherapy. Clinicopathological data from all operated patients were permanently prospectively collected. Follow-up was complete for all patients and extended until July 2006, ensuring a minimal potential follow-up of 5.5 years. Recurrence of disease was diagnosed on clinical grounds; only upon suspicion of locoregional recurrence or distant metastases, further investigations were performed. Recurrences were classified as locoregional recurrence (including all lymphatic recurrence), and hematogenous recurrence. All pathology reports were reviewed to identify those patients in whom the adenocarcinoma had developed in a histologically proven Barrett’s segment (defined by the presence of goblet cells). Patients with an adenocarcinoma of the cardia or gastroesophageal junction without a clear Barrett’s segment were excluded (n=161). This careful selection of patients has been described in
our previous report. Archival materials of the remaining 145 patients were re-evaluated to obtain the sample with deepest invasion of each tumor.

Surgical tissue specimens
All 145 patients were treated with subtotal esophagectomy and resection of the lesser curvature of the stomach. In 95 patients (65.5%), resection was performed by a transhiatal approach without thoracotomy. Lymphadenectomy comprised en bloc removal of all lymphatic tissue in the lower posterior mediastinum, along the cardia and the lesser curvature of the stomach. Fifty patients (34.5%) underwent esophagectomy through a right-sided thoracotomy followed by a laparotomy in combination with 2-field lymph node dissection. This procedure included an abdominal lymphadenectomy as described plus the removal of lymph nodes along the common hepatic artery, the splenic artery, and the celiac trunk as well as an extended lymph node dissection in the chest (i.e., including the right paratracheal, infra-aortic arch, and subcarinal lymph nodes).

Immunohistochemistry
Of all patients, 5μm-thick sections of paraffin and formaldehyde fixed tissue of the resection specimens were cut. For immunohistochemical staining sections were incubated overnight at 37 °C and subsequently deparaffinised in xylene, rehydrated, and treated with 3% H2O2 in methanol for ten minutes to block endogenous peroxidase activity. All specimens were subjected to heat-induced antigen retrieval in 10mM sodium citrate buffer (pH 6.0) for ten minutes at 95°C. To block aspecific binding the slides were incubated with tris-buffered saline (TBS) supplemented with 5% goat serum. Sections were incubated with the primary antibodies anti-Met c-Met (3D4; Zymed, San Francisco, CA, USA) (1:100), and anti human COX-2 (160112; Cayman Chemical Co., Ann Arbor, MI, USA) (1:200) diluted in TBS with 1% bovine serum albumin overnight at 4°C. For the Met staining the sections were incubated after washing steps with anti-mouse/rabbit-peroxidase polymer for thirty minutes at room temperature (Powervision; Immunovision, Inc., Daly City, CA, USA). Diaminobenzidine chromogen (Sigma, St. Louis, MO, USA) was used for visualization. For the COX-2 staining the sections were treated with biotinylated horse anti-mouse immunoglobulin (1:200; Vector Laboratories Inc., Burlingame, CA) and avidin-biotin peroxidase complex (Vectastain ABCComplex;Vector Laboratories). After these steps for Met and COX-2 staining the sections were counterstained with hematoxylin and embedded. Specificity of the antibodies was confirmed by controls using irrelevant immunoglobulins instead of primary antibodies. Colon cancer tissue was included as a positive control. The analysis of all tissue sections was performed independently by three different investigators (JBT, SML, and FJWTK) without patient identification parameters. In cases of disagreement consensus was reached after re-evaluation using a multiheaded microscope. For Met and COX-2 immunohistochemical staining, the following scoring criteria of tumor cells were agreed upon before the analysis: 0, no staining or equal to background; 1, weak diffuse cytoplasmic staining (may contain stronger intensity in less than 10% of cancer cells); 2, moderate to strong granular cytoplasmic
staining in 10–90% of cancer cells; 3, over 90% of tumor cells stained with strong intensity. (Figure 1) These scoring criteria have been described previously. Data regarding COX-2 staining intensity are equal as previously described and used for the present analysis\textsuperscript{13,16}. Areas of diffuse hemorrhage or necrosis were neglected.

Statistics
Statistical calculations were performed using SPSS version 14.0 (Statistical Package for the Social Sciences, Chicago, IL, USA). The association between demographic and clinicopathological features and protein expression was analyzed using Student-t-test (continuous data) and \( \chi^2 \) test (categorical data). Overall and disease specific 5-year survival rates were estimated according to the Kaplan–Meier method and compared between groups using the log-rank test. Overall survival was calculated using deaths since time of surgery irrespective of cause. For disease specific survival all non-disease related deaths were excluded including in-hospital death within 90 days of surgery, since we assumed that these patients had died because of co-morbidity and surgery related causes. The Cox proportional hazards regression model was used to identify prognostic factors. To identify independent prognostic factors multivariate Cox regression analysis was carried out. Variables with multiple categories were recoded into dichotomous variables by combining categories with a comparable prognosis (differentiation grade, good versus moderate and poor (poor); Tumor T stage, stage 1 and 2 versus 3; Met expression, no or weak staining (low) versus moderate to strong staining (high); COX-2 expression, no or weak staining (low) versus moderate to strong staining (high).

Results
A total of 145 consecutive patients with OA were included for immunohistochemical analysis. Of these patients 120 were men (83%) and 25 were women (17%) with a median age of 67 years (range, 35–85). (Table 1). The majority of patients (N= 83, 57%) had a T3 tumor and 80 patients (55%) had positive lymph nodes. The overall 5-year survival in the included group was 35% and the disease specific 5-year survival was 48%. Two patients (1.4%) died within 90 days due to postoperative complications (myocardial and respiratory failure in one patient and cerebrovascular event in one other patient).

High Met staining (as opposed to low Met staining) was observed in 78 cases (54%). Of these 78 patients 28 cases were scored as strong Met expression and 50 as moderate Met expression. In 67 patients (46%) Met expression was classified as low; 56 patients had weak Met expression en 11 patients had no or equal to background staining of Met. Met expression was mainly localized in neoplastic cells (Figure 1 A and B) but was also weakly identified in non-neoplastic epithelial cells (both squamous and columnar epithelium) and in stromal cells. Interobserver variation was 8% for Met expression. All specimens that were discrepant (n=13) were re-evaluated and the consensus score was used for further analysis.
Results of COX-2 expression have been described previously in this cohort of patients. Briefly, COX-2 expression was negative to weak in 21% (COX-2 low) and moderate to strong in 79% (COX-2 high) of the carcinomas\textsuperscript{13,16}.

High Met expression was observed more often in patients with higher T stage ($p=0.003$), in patients with positive lymph nodes ($p\leq 0.001$) and a poor differentiation grade ($p=0.003$). (Table 1) Met expression was not correlated with COX-2 expression ($p=0.839$).

Table 1: Correlation of clinicopathological findings and Met expression

<table>
<thead>
<tr>
<th>patient characteristics (n=145)</th>
<th>Overall</th>
<th>low Met expression (N=67)</th>
<th>high Met expression (N=78)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>median age (range)</td>
<td>67 (35-85)</td>
<td>67 (35-83)</td>
<td>68 (44-85)</td>
<td>0.493</td>
</tr>
<tr>
<td>sex male (%)</td>
<td>120 (83%)</td>
<td>63 (94%)</td>
<td>57 (73%)</td>
<td>0.435</td>
</tr>
<tr>
<td>tumor characteristics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>44 (30 %)</td>
<td>29 (43%)</td>
<td>15 (19%)</td>
<td>0.003</td>
</tr>
<tr>
<td>T2</td>
<td>18 (12 %)</td>
<td>8 (12%)</td>
<td>10 (13%)</td>
<td>0.546</td>
</tr>
<tr>
<td>T3</td>
<td>83 (57 %)</td>
<td>30 (45%)</td>
<td>53 (68%)</td>
<td>0.078</td>
</tr>
<tr>
<td>N stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>65 (45 %)</td>
<td>38 (57%)</td>
<td>27 (35%)</td>
<td>0.000</td>
</tr>
<tr>
<td>N1</td>
<td>80 (55 %)</td>
<td>29 (43%)</td>
<td>51 (65%)</td>
<td>0.000</td>
</tr>
<tr>
<td>M stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>122 (84 %)</td>
<td>58 (87%)</td>
<td>64 (82%)</td>
<td>0.086</td>
</tr>
<tr>
<td>M1a</td>
<td>23 (16 %)</td>
<td>9 (13%)</td>
<td>14 (18%)</td>
<td>0.086</td>
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<td>differentiation grade</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>good</td>
<td>11 (8 %)</td>
<td>5 (7%)</td>
<td>6 (8%)</td>
<td>0.231</td>
</tr>
<tr>
<td>moderate</td>
<td>56 (39 %)</td>
<td>35 (52%)</td>
<td>21 (27%)</td>
<td>0.041</td>
</tr>
<tr>
<td>poor</td>
<td>78 (54 %)</td>
<td>27 (40%)</td>
<td>51 (65%)</td>
<td>0.003</td>
</tr>
<tr>
<td>overall 5-year survival</td>
<td>35%</td>
<td>57%</td>
<td>16%</td>
<td>0.000</td>
</tr>
<tr>
<td>disease specific 5-year survival</td>
<td>48%</td>
<td>66%</td>
<td>33%</td>
<td>0.000</td>
</tr>
</tbody>
</table>

\textsuperscript{a} T1, tumor limited to (sub)mucosa; T2, tumor infiltrates muscularis propria; T3, tumor infiltrates adventitia layer; as determined in the pathological resection specimens. \textsuperscript{b} N0, no tumor positive locoregional lymph nodes; N1 locoregional lymph node metastasis. \textsuperscript{c} M0, no distant metastasis, M1a, metastasis in celiac lymph nodes.

Figure 1: Representative samples of immunohistochemical staining of Met (A) and COX-2 (B), (Str=stroma, T=tumor).
During five year follow-up, 92 patients died: 17 patients died of unrelated causes and 75 patients died of recurrent disease. Of these patients, 23 had locoregional recurrences, 39 patients had hematogenous recurrences and 13 patients had both locoregional and hematogenous recurrences.

After a complete follow-up, overall 5-year survival was significantly lower in patients with high Met expression as compared to patients with low Met expression; 16% versus 57% (p ≤ 0.001). Furthermore, disease specific 5-year survival was significantly lower in patients with high Met expression as compared to patients with low Met expression; 33% versus 83% (p ≤ 0.001).

**Figure 2:** Kaplan-Meier survival curves of 145 patients with adenocarcinoma of the esophagus. Patients with high Met expression had a significantly worse overall 5-year survival (2A) (p ≤ 0.001) and disease specific 5-year survival (p ≤ 0.001) (2B) as compared to patients with low Met expression. Overall 5-year survival and disease specific 5-year survival tended to be worse in patients with high COX-2 expression (2C and 2D resp.) as compared to patients with low COX-2 expression but this did not reach statistical significance (p=0.180 and p=0.238 resp.).
66% (p≤0.001). Patients with high Met expression were more likely to develop distant metastases (p=0.002) as well as local recurrences (p=0.004). Patients with high COX-2 expression tended to have a poor overall and disease specific 5-year survival as compared to patients with low COX-2 expression but in contrast to previous reports this did not reach statistical significance. (Figure 2) Univariate analysis revealed that T stage, N stage, M1a stage, differentiation grade, and Met expression were all significant prognostic indicators for disease specific 5-year survival. (Table 2) Multivariate analysis of these variables demonstrated that T3 stage (relative risk (rr) 1.9 (1.0-3.5), (p=0.035)), lymph node involvement (rr 2.8, (1.5-5.3) (p=0.001)) and high Met expression (rr 2.3 (1.3-4.1), (p=0.004)) were independent prognostic factors (Table 3).

**Discussion**

This study provides evidence that Met expression level (as detected by immunohistochemical analysis) is an independent prognostic factor in esophageal adenocarcinoma. Overall 5-year
survival after potentially curative resection is significantly worse in patients with tumors expressing high Met levels compared to low Met levels. In literature, lymphatic dissemination as identified on histopathological examination is the single most important prognostic factor in patients with esophageal cancer. Also in the present study, lymph node involvement is a strong independent prognostic factor next to T stage and Met expression level.

Since Met expression appears to be an important independent prognosticator, this might offer an attractive opportunity for targeted therapy. Selective inhibitors of Met have recently become available and successful inhibition of tumor progression, stromal and endothelial adhesion and dissemination has been reported both in vitro and in animal studies. Targeted therapy of growth factor receptors has been shown clinically effective in other cancer types such as chronic myelogenous leukemia, gastrointestinal stromal tumors, HER-2/NEU overexpressing breast cancer, colorectal cancer and non-small cell lung cancer.

Surprisingly, COX-2 expression was not a significant prognostic factor in this study. The same cohort of patients was employed for the current analysis of Met expression as reported on earlier for COX-2 expression. In this study, a minimal follow-up of 60 months was available whereas in the previous study the median follow up was only 27 months. Although survival in patients with high COX-2 expression tended to be poorer than that in patients with low COX-2 expression this did not reach statistical significance. Theoretically, the difference between COX-2 expression and Met expression as prognostic indicators can probably be explained by their function. The COX-2 enzyme is enhanced in inflammation and has been shown to be involved in early progression of esophageal metaplasia and dysplasia into (adeno-) carcinoma. Increased COX-2 expression causes activation of several cancer related genes including the HGF receptor Met. Vice-versa COX-2 inhibition causes downregulation of cancer related genes including Met as has been published previously by our group. In comparison to COX-2, Met is involved later in the process of cancer development and has been shown vital in cancer progression. The proto-oncogene Met, also known as the scatter factor, has been shown particularly important in morphogenic differentiation and organization of three-dimensional tubular structures as well as in cell growth and loss of cellular adhesion causing migration (dissemination) of cells. Since esophageal adenocarcinoma is known for its propensity to early lymphatic and hematogenous dissemination, the strong prognostic significance of high Met expression for both overall and disease specific 5-year survival can explain this clinical behavior at least partly. These results suggest that employment of new therapeutic agents targeting Met might be of value as (neo-) adjuvant therapy in patients with esophageal adenocarcinoma, especially if Met expression is high.

In conclusion, our data indicate that high Met expression is a significant independent indicator of poor long term survival in patients after potentially curative resection of esophageal adenocarcinoma. Targeting this receptor by a selective Met kinase inhibitor is an attractive (neo-) adjuvant treatment option that should be tested especially in patients with high tumoral Met expression.
References


