Adenocarcinoma of the esophagus has shown an increasing incidence in the last three decades, but survival rates remain poor (1, 2). An increased understanding of the molecular events in this disease may provide future therapeutic targets.

Esophageal adenocarcinoma mostly arises from Barrett’s metaplasia (3). The progression from Barrett’s metaplasia involves a sequence of changes from the development of low-grade to high-grade dysplasia and adenocarcinoma (4). This sequence is characterized by alterations in the expression of adhesion molecules, growth factors, and tyrosine kinase receptors (5).

The Met receptor is a tyrosine kinase receptor, the product of a proto-oncogene (6). It acts as a receptor for hepatocyte growth factor (HGF), a potent mitogen and pro-motility agent (7, 8). HGF is primarily produced by mesenchymal cells to act on Met-expressing epithelial cells in a paracrine fashion (9).

Met has roles in gastrointestinal development (10), mucosal healing (11), and epithelial cell migration (12), processes that require disassembly of cell-cell adhesions. The predominant adhesion protein of epithelial tissue is E-cadherin (13), and this is down-regulated in esophageal cancer (14). E-cadherin binds to β-catenin at the cell membrane and is linked to the control of β-catenin–regulated transcription (15, 16). The β-catenin protein is found in three cellular pools: membranous, cytoplasmic, and nuclear. The translocation among these is tightly regulated (17), and the dynamic equilibrium determines the signaling role (18).

Nuclear β-catenin is seen in esophageal tumorigenesis (19), and many catenin target genes show increased expression (20–22). Studies have shown an association between HGF/Met stimulation and increased phosphorylation of β-catenin in cell lines (23–25).
Studies of the expression of Met in esophageal malignancy showed increased expression in tumors compared with normal mucosa (26–28). In breast cancer, high Met expression has been correlated with lower survival rates (29, 30). The situation in esophageal adenocarcinomas remains to be addressed.

Here, we describe the effect of HGF/Met signaling in esophageal adenocarcinoma cell lines and show a down-regulation of E-cadherin and an increase in β-catenin nuclear signaling. We assess the effect of HGF/Met activation on the growth of esophageal cells in an anchorage-independent medium. Finally, we investigate the survival rates of patients with esophageal adenocarcinoma when stratified according to levels of different tyrosine kinase (Met, Erb-B2, and Src) expression.

Our data illustrate that the Met receptor is associated with poorer prognosis and as such is a potential key therapeutic target.

Materials and Methods

**Cell culture.** Three cell lines (OE33, SEG-1, and TE-7), derived from human esophageal adenocarcinoma, were cultured as previously described (31–33). Cells were grown to 60% to 80% confluence and then supplemented with recombinant HGF (Peprotech, London, United Kingdom) at the concentrations and time points specified below. Control groups of unstimulated cells were given fresh media only.

**Quantitative real-time PCR.** RNA isolation was done using Trizol reagent (Invitrogen, Paisley, United Kingdom). cDNA was synthesized from RNA (1 μg/mL) using a cDNA reverse transcription kit (Promega, Southampton, United Kingdom). All reactions were done on a real-time PCR PE7700 cycler (ABI prism). 18S rRNA was assessed as an internal standard control using 18S-specific VIC-labeled probes (PE Biosystems, Warrington, United Kingdom). The gene-specific primer and probe sequences were as follows: E-cadherin probe, FAM- AAATTCACCTGCCCAGGACGCGG-3′; E-cadherin forward primer, 5′-GGCCCACTCCTGAGAGA-3′; E-cadherin reverse primer, 5′-TGTCGACCGGTGCAATCTT-3′.

The real-time PCR reaction mix was made up in 1× Mastermix (PE Biosystems).

Each reaction was replicated thrice. Cycle threshold values for E-cadherin and 18S were obtained graphically. Gene expression was normalized to the 18S value. Relative gene expression was then tabulated and expressed as a ratio of the value for unstimulated cells (given a value of 1).

**Agar colony assay.** To assess the anchorage-independent growth of the cell lines, the ability to form colonies in agar was measured, using methods as previously described (39).

In brief, trypsinized cells (5 × 10⁴/mL) were seeded on the top layer of an agar coated six-well insert and were cultured in DMEM, with or without HGF. HGF was supplemented to the bottom agar layer so that when this had diffused into both layers it would give a final concentration of 100 ng/mL.

The number of colonies per well were counted at day 10 after seeding. Results were expressed as mean number ± SD of colonies per well of triplicates. Wells supplemented with HGF were compared with unstimulated control wells.

**ELISA assay.** A commercially available kit (Human HGF Immunoassay DGH00, Quantikine, R&D Systems, Abingdon, United Kingdom) was used according to manufacturer’s instructions. HGF concentration was measured in duplicate tissue biopsies from matched fundic, Barrett’s, and normal esophageal mucosa from 10 different subjects. Briefly, biopsies were collected and immediately snap-frozen in ornithine carbamyl transferase embedding compound (R.A. Lamb, Eastbourne, United Kingdom) and isopentane (Fisher Scientific, Leicester, United Kingdom). Each biopsy was then sectioned using a cryostat into 100 μL of total cell lysis buffer and assayed for HGF according to the manufacturer’s instructions. The final HGF concentration was calculated to the total cellular protein content for each lyzed sample.

**Human tissues.** To assess the expression of tyrosine kinases along the metaplasia-dysplasia-adenocarcinoma sequence, paraffin-embedded biopsies were identified from a patient database at University Hospital Birmingham NHS Trust. In all patients, the samples of Barrett’s metaplasia showed intestinal-type metaplasia. The samples of normal gastric mucosa, normal esophageal mucosa, dysplastic Barrett’s (confirmed as high grade by two pathologists), and esophageal adenocarcinoma had been confirmed histologically. Ten different samples of each tissue type were used for immunohistochemistry.

To assess the prognostic implication of different kinase expression profiles, samples were obtained from a case series of 72 patients who underwent curative resection of adenocarcinoma of the esophagus at Birmingham Heartlands Hospital between December 1990 and July 1998. No patient received preoperative chemotherapy or radiotherapy. Thoracic esophagectomy was done with standard two-field resection of regional lymph nodes. Macroscopic tumor was judged to be completely resected in all patients. All specimens were fixed in formalin and processed into paraffin. Three-micrometer sections of the primary cancer were cut for use in immunohistochemistry.
Tumours were staged according to the 1987 Union Internationale Contra Cancrum Classification based on pTNM subsets. Survival times were calculated as date of operation to date of death or date of censor if still alive. All dying patients died of their primary cancer. Causes of death were obtained from hospital records or from the West Midlands Cancer Intelligence Unit. Permission to use the series has been passed by the East Birmingham Local Research Ethics Committee.

**Immunohistochemistry.** The streptavidin-biotin indirect immunoperoxidase method was used for immunohistochemistry as previously described (34). Slides were incubated with the following primary antibodies: ErbB-2 rabbit polyclonal A00485 (10 μg/mL; DAKO, Ely, United Kingdom), Met rabbit polyclonal SC-161 (67 ng/mL; Santa Cruz Biotechnology), and Src mouse monoclonal SC-5266 (6.7 μg/mL; Santa Cruz Biotechnology).

A slide of human breast adenocarcinoma was simultaneously processed as a positive control. Negative controls consisted of the same tissue but for which the primary antibody was omitted. To further assess the specificity of the primary antibodies, peptide absorption controls were done. The primary antibody was incubated first with a 5-fold excess solution of the specific blocking peptide (Blocking peptides sc-161 P for Met and sc-5266 P for Src; Autogen, Calne, United Kingdom). For the ErbB-2 antibody, absorption blocking was carried out using a lysate of cell line MCF-7 known to express ErbB-2 (40). The specificity of the antibody was determined by the absence of immunoreactivity on sections previously known to be positive.

All sections were scored by three independent observers (R. Harrison, S. Chowdary, and M. Anderson). Each slide was classed as positive for expression only if strong staining was seen in more than two thirds of cells. All observers were blind to the clinical data from the prognostic series. A concordance rate of 86% was reached (percentage of the slides on which all three opinions were the same).

**Statistics and analysis.** Statistical analysis was done using InStat (GraphPad Software, San Diego, CA). Significance was taken at the 5% level. Advise on statistical tests was obtained from R. Joshi and Dr. H. Ross (University of Birmingham).

Unpaired t test was used to determine if statistical significance existed between the means of two data sets. For dose response curves, an initial ANOVA test was done to insure against false positives from multiple t tests. The log-rank test was used to compare overall survival distribution data, and Fisher’s exact test was used to assess the proportions of patients surviving to set time points.

### Results

**Met expression in esophageal tissues and cell lines.** To assess the relationship between Met expression and disease progression, we assessed a panel of esophageal tissues. Met showed no membranous expression in any of the normal squamous epithelium. Strong membranous expression was seen in 4 of 10 Barrett’s metaplasia cases, 5 of 10 high-grade dysplasia cases, and 7 of 10 adenocarcinomas. Some weak cytoplasmic staining was also noted in the cases of dysplasia and adenocarcinoma. Photomicrographs are shown in Fig. 1. These results suggest that the expression of Met is seen in areas of columnar epithelium but not squamous epithelium. The overexpression of Met becomes more common as dysplasia develops and is seen in the majority of adenocarcinomas (ANOVA of overall results, P = 0.009; t tests: squamous versus Barrett’s, P = 0.025; squamous versus dysplasia, P = 0.008; squamous versus carcinoma, P = 0.001). The increased expression of Met seems to be an early and common event.

To develop an in vitro model, we assessed three cell lines derived from esophageal adenocarcinoma (OE33, SEG-1, and TE-7 cells).

The cell lines were assessed for the expression of the Met receptor by Western blotting, the results of which are shown in Fig. 2 compared with a housekeeping gene cytoplasm-19. Two of the cell lines (OE33 and SEG-1) expressed the Met receptor, but the TE-7 cell line did not. This resulted in the TE-7 cell line acting as an additional negative control group to show that any observed effects of HGF in the other cells were dependent on Met receptor expression.

**HGF levels in human tissue.** To ascertain the presence of HGF in tissues, we used matched biopsies (Fundus, Barrett’s, and normal esophageal mucosa) and measured HGF levels in duplicate from 10 different subjects. Biopsies were homogenized into assay media and assessed with an ELISA. Mean values of HGF were 929 ng/mL per microgram total protein in Barrett’s metaplasia [95% confidence interval (95% CI), 331-1,527], 715 ng/mL in Gastric fundus (95% CI, 536-894), and 391 ng/mL in squamous esophagus (95% CI, 140-642). Overall, when comparing the mean values, the Barrett’s and the fundic epithelium contained significantly more HGF (929 and 715 ng/mL per microgram of total protein respectively) compared with the normal (squamous) epithelium (391 ng/mL per microgram of total protein, P < 0.05). The data also indicated that there was more HGF in Barrett’s epithelium compared with Fundic epithelium, although this was not significant (P > 0.05).

**Effect of Met activation on E-cadherin expression: assessment by real-time reverse transcription-PCR.** To investigate the functional effects of Met signaling, the effect of HGF stimulation on E-cadherin expression was explored using quantitative real-time reverse transcription-PCR. Cells were stimulated with HGF at 100 ng/mL. RNA was harvested after 30 minutes, 2 hours, and 24 hours. Results were compared with control groups of unstimulated cells for which the media had been changed at the same time points. Levels of E-cadherin mRNA were calculated relative to those for 18S which served as an internal control.

The results are shown in Fig. 3.

OE33 cells showed a mean 38% reduction (95% CI, 19-57% reduction; P = 0.0052) in mRNA levels after 30 minutes of stimulation and a mean 31% reduction (95% CI, 18-44% reduction; P = 0.0015) by 2 hours. Levels had begun to increase by 24 hours.

SEG-1 cells showed a mean 68% reduction (95% CI, 55-81% reduction; P = 0.008) in mRNA levels after 30 minutes of stimulation, and levels were returning to normal by 2 and 24 hours after stimulation. This dramatic decrease and quick recovery was consistent with the fact that they were also the fastest growing cell line.

TE-7 cells (which lack the Met receptor) showed no significant response to HGF stimulation.

These results show that Met-expressing cells show a reduction in E-cadherin mRNA in response to stimulation with HGF at 100 ng/mL.

In all cell lines, any response to HGF was lost by 24 hours after stimulation. To ensure that this was not simply due to breakdown of the recombinant HGF, a stimulation was done using HGF-supplemented media that had been made up 24 hours before and incubated at 37°C. This still produced
a down-regulation and therefore had a half-life of >24 hours; thus, deterioration of the HGF was not a confounding factor.

To assess the effect of different concentrations of HGF, a dose response curve was produced by stimulating OE33 cells with different doses of HGF (1, 10, 50, 100, and 500 ng/mL) and measuring E-cadherin mRNA levels after 2 hours. The minimum effective dose to cause down-regulation was found to be between 50 and 100 ng/mL.

However, these were cells that were routinely cultured in 10% FCS. To investigate whether the amount of calf serum influenced the dose response, the experiment was repeated with OE33 cells grown in only 1% FCS. This showed that reducing the amount of calf serum altered the dose response curve by shifting it to the left, and that 50 ng/mL of HGF was now enough to cause a significant down-regulation of E-cadherin mRNA levels ($P = 0.006$).

No significant alterations in E-cadherin mRNA levels were seen at any dose used on TE-7 cells, showing that these were truly unresponsive and not simply dose dependent.

To see if the TE-7 cells lack of response was due to lack of the receptor, we induced Met expression using transient transfection with a Met-encoding plasmid and then stimulated the cells with HGF. The baseline level of E-cadherin mRNA was decreased by 64% in Met-TE-7 cells compared with wild-type TE-7 cells ($P < 0.0001$). A similar decrease was still seen after adding HGF at 100 ng/mL ($P < 0.0001$).

**Effect of met activation on E-cadherin expression: assessment by Western blotting.** To assess whether the reductions in mRNA levels led to actual changes in E-cadherin protein expression, the technique of Western blotting was used.

OE33 and TE-7 cell lines were stimulated with HGF at 100 ng/mL, and the cells were then lysed at 12, 24, and 48 hours after stimulation to allow time for any effect on protein expression to be detected. Control cells were given fresh media only and were lysed at the same time points. These represented a positive control group for normal E-cadherin expression in the cell line at each particular time point.

The results for OE33 are shown in Fig. 4. Only the principal bands are displayed in this diagram. A reduction in E-cadherin expression was evident at 12 hours, but the difference between unstimulated cells and cells receiving HGF was most pronounced at 24 hours. By 48 hours, the cells had recovered the normal level of expression, and no difference was seen between control cells and stimulated cells.
TE-7 cells displayed no significant difference at any time point between the level of expression in control cells and the level in stimulated cells.

**Effect of Met activation on β-catenin signaling.** To investigate whether the down-regulation of E-cadherin was linked to a subsequent increase in nuclear signaling via β-catenin–mediated transcription, we used the transient transfection of reporter construct.

The construct termed TOPFLASH is a reporter in which luciferase expression is controlled by a c-fos promoter containing multiple Tcf-binding sites. Only the binding of β-catenin/Tcf complexes activates luciferase transcription; thus, an assay of luciferase activity is a quantitative report of the level of transcriptionally active nuclear β-catenin/Tcf complexes.

Cells with TOPFLASH were stimulated with HGF at 100 ng/mL for 2 hours and compared with control cells given only fresh normal media. A separate set of cells were cotransfected with wild-type APC in addition to the TOPFLASH plasmid and underwent the same stimulation and control experiments. All experiments were done thrice, and the results are displayed as the mean ± 95% CI.

Results for OE33 cells are shown in Fig. 5A. A significant 2-fold increase in β-catenin–mediated transcription was seen in cells stimulated with HGF for 2 hours. This increase could be partially abrogated by cotransfection with wild-type APC, which increases β-catenin breakdown in the cytoplasm.

Results for SEG-1 cells are shown in Fig. 5B. A significant 45% increase in β-catenin–mediated transcription was seen in cells stimulated with HGF for 2 hours. This increase was not significantly affected by cotransfection with wild-type APC.

Results for TE-7 cells are shown in Fig. 5C. Stimulation with HGF did not cause a significant change in the level of β-catenin–mediated transcription in any TE-7 group. However, it was noted that the addition of wild-type APC reduced the basal level of β-catenin–mediated transcription by 50% but was unaffected by the presence of HGF.

**Effect of Met activation on anchorage-independent growth.** To investigate whether Met activation could influence cell invasion or early metastasis, we assessed the ability of the cell lines to form colonies in an anchorage-independent medium. We used an assay of colony numbers in agar gels with and without HGF supplementation. The concentration of HGF in supplemented agar was 100 ng/mL. The concentration of cells added to the agar when first plated was 5 × 10^5/mL. Colonies were counted after 10 to 14 days. The means of three experiments are given.

The TE-7 cell line that lacked the Met receptor was unable to form any colonies in the agar gels, with or without the presence of HGF.

The OE33 cells and the SEG-1 cells were able to form colonies in agar. An increase in colony formation was seen in response to HGF supplementation in both cell lines. OE33 showed a 37% increase in mean colony numbers per well from 68 (range, 54-78) to 93 (range, 79-105; P < 0.05). SEG-1 showed a 20% increase in mean colony numbers per well from 275 (range, 240-324) to 331 (range, 318-342; P < 0.05).

Therefore, HGF provides an additional survival signal in anchorage-independent media to cells that express the Met receptor.

**Association between Met expression and clinical variables.** To investigate whether this *in vitro* data may hold clinical relevance, we assessed the relationship between expression of three different tyrosine kinases and the survival of patients with esophageal adenocarcinoma.
The expression of three tyrosine kinases (ErbB-2, Met, and Src), known to be present in a proportion of esophageal adenocarcinomas and previously linked to β-catenin signaling, were assessed using immunohistochemistry on 72 cases of esophageal adenocarcinoma for which tumor-node-metastasis staging data and long-term survival figures after surgical resection were known.

There were 60 cases (83%) that were positive for Met expression. The median survival was 12 months in those that were positive and 16 months in those that were negative (not significant). The number of patients alive by the 6-month time point was 38 of 60 positive cases (63%) compared with 11 of 12 negative cases (92%). This difference in short-term survival was statistically significant (Fisher’s exact test, \( P < 0.05 \)).

The proportion of patients that had lymph node spread in the resection specimen (stage N1) was not significantly different in those that were positive for Met (62%) compared with those that were negative (42%). The survival distributions of the two groups are shown in Fig. 6.

There were 16 of the 72 cases (22%) that were positive for ErbB-2 expression. The median survival in the positive cases was 13.5 months compared with 13 months in the negative (not significant). The survival rates at measured time points were not significantly different. The proportion of patients that had lymph node spread in the resection specimen (stage N1) was not significantly different in those that were positive for ErbB-2 (62%) compared with those that were negative (42%). The survival distributions of the two groups are shown in Fig. 6.

There were 61 of the 72 cases (85%) that were strongly positive for Src expression. The median survival and the survival rates at different time points were not significantly different. The proportion of patients that had lymph node spread was not significantly different in those that were positive for Src (59%) compared with those that were negative (55%). Thus, neither ErbB-2 nor Src expression was shown to correlate significantly with prognosis.

Thus, Met expression was the only tyrosine kinase shown to be significantly associated with poorer short-term survival.

**Discussion**

We have shown here that the expression of Met in esophageal adenocarcinoma is associated with a poorer prognosis in vivo. Data here suggest that the Met receptor and its ligand HGF show increased expression along the metaplasia to adenocarcinoma sequence. We have shown functional mechanisms that may explain how Met signaling can influence cell behavior and exert clinical effects.

For a protein not only to be expressed early in a pathogenesis but to also have an influence on later survival may seem paradoxical. One reason behind this may be that although the
Met receptor is present, the ligands to activate it are expressed at higher levels further along the sequence and may thus exert an influence on later metastasis. The conventional ligand for Met is HGF, and a recent study using reverse transcription-PCR has shown higher levels of HGF expression in Barrett’s epithelium compared with squamous mucosa (41), consistent with our findings here. In addition, another study has shown that Met may also be activated by the presence of the transforming growth factor-α and the epidermal growth factor receptor (42).

Data presented here have shown that Met receptor activation in esophageal cell lines is associated with increases in β-catenin nuclear signaling, which precede a reduced expression of E-cadherin. Activation of this receptor also imparts a pro-proliferative signal to cells growing in an anchorage-independent medium. The dose of HGF needed to observe such effects in our cancer cell model system was between 50 and 100 ng/mL, varying according to experimental conditions. It is not known what physiologic dose range is found in the microenvironment of an esophageal adenocarcinoma, but HGF is also known what physiologic dose range is found in the microenvironment of an esophageal adenocarcinoma, but HGF is also known to be present in saliva (43), consistent with our findings here. In addition, another study has shown that Met may also be activated by the presence of the transforming growth factor-α and the epidermal growth factor receptor (42).

Data presented here have shown that Met receptor activation in esophageal cell lines is associated with increases in β-catenin nuclear signaling, which precede a reduced expression of E-cadherin. Activation of this receptor also imparts a pro-proliferative signal to cells growing in an anchorage-independent medium. The dose of HGF needed to observe such effects in our cancer cell model system was between 50 and 100 ng/mL, varying according to experimental conditions. It is not known what physiologic dose range is found in the microenvironment of an esophageal adenocarcinoma, but HGF is also known to be present in saliva (43), which constantly reaches the esophagus. A study on the serum level of HGF in patients with gastric carcinoma found that levels were higher in patients with metastases (44). However, it is local and intratumoral concentrations that are likely to be relevant in vivo as these growth factors act by autocrine and paracrine signaling. An important finding of our report is that we have shown that intratumoral HGF levels in Barrett’s metastasis are raised.

The mechanism of E-cadherin down-regulation is not known. However, the response is time dependent, and this suggests that a repressor protein may be used and ultimately used up. Snail and Sip-1 are two proteins that are known to bind to repressor elements in the cadherin gene promoter region (45, 46), and it is possible that one of these is triggered by Met activation. The results from the real-time reverse transcription-PCR suggest that there is a compensatory increase in E-cadherin expression 24 hours after HGF stimulation. This is partly explained by the time-dependent utilization of the HGF and the metabolic breakdown of this growth factor once the receptor-ligand complex is internalized by the cells. Another explanation would be a gradual decrease in repressor element levels following prolonged receptor activation.

Previously, work on lung cancer cell lines has shown a similar effect on colony formation in agar to the one shown here, with an increased colony number following HGF/Met activation (47). It is known that Met can phosphorylate Akt, which provides resistance to anoxia upon loss of matrix contact (48), and this may be of relevance in promoting anchorage-independent growth.

Finally, we have shown that Met shows increasingly frequent expression in Barrett’s dysplasia and adenocarcinoma. It seems to influence patient survival. Those that overexpress Met display worse short-term survival at 6 months after surgery than those that do not express the receptor. It has no demonstrable influence on nodal spread in these patients who underwent resection. It would have been of interest to investigate a link between expression profile and T and M stage, but as all these cases were obtained from operable cases, T1/T2 and M0 was the oncologic grading in the majority of cases at the time of resection. The data presented here need further studies to confirm whether the expression of Met is a robust, independent variable. However, no significant association was seen with the other tyrosine kinases that we assessed, suggesting that this is specific to the Met receptor.

In conclusion, this body of work has increased our understanding of the importance of Met receptor signaling in esophageal adenocarcinoma and has now set the stage for the testing of specific Met receptor inhibitors as potential treatments. Several pharmaceutical companies are currently developing such agents that have selectivity for Met and have been used to reduce cell proliferation or increase apoptosis in vitro (49, 50). It would be timely now to test such drugs in the context of esophageal cell model systems and ultimately in human patients.

We have shown that HGF/Met activity leads to decreased E-cadherin expression, increased β-catenin signaling, and an increased colony formation of esophageal cells in vitro. The expression of Met in esophageal adenocarcinoma is associated with a poorer prognosis in vivo, and this is now a prime area for targeting therapies.

References


Met Receptor Signaling: A Key Effector in Esophageal Adenocarcinoma

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