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mRNA profiling of the cancer degradome in oesophago–gastric adenocarcinoma

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BACKGROUND: Degradation of the extracellular matrix is fundamental to tumour development, invasion and metastasis. Several protease families have been implicated in the development of a broad range of tumour types, including oesophago–gastric (OG) adenocarcinoma. The aim of this study was to analyse the expression levels of all core members of the cancer degradome in OG adenocarcinoma and to investigate the relationship between expression levels and tumour/patient variables associated with poor prognosis.

METHODS: Comprehensive expression profiling of the protease families (matrix metalloproteinases (MMPs), members of the ADAM metalloproteinase-disintegrin family (ADAMs)), their inhibitors (tissue inhibitors of metalloproteinase), and molecules involved in the c-Met signalling pathway, was performed using quantitative real-time reverse transcription polymerase chain reaction in a cohort of matched malignant and benign peri-tumoural OG tissue (n = 25 patients). Data were analysed with respect to clinicopathological variables (tumour stage and grade, age, sex and pre-operative plasma C-reactive protein level).

RESULTS: Gene expression of MMP1, 3, 7, 9, 10, 11, 12, 16 and 24 was upregulated by factors >4-fold in OG adenocarcinoma samples compared with matched benign tissue (P < 0.01). Expression of ADAM8 and ADAM15 correlated significantly with tumour stage (P = 0.048 and P = 0.044), and ADAM12 expression correlated with tumour grade (P = 0.011).

CONCLUSION: This study represents the first comprehensive quantitative analysis of the expression of proteases and their inhibitors in human OG adenocarcinoma. These findings implicate elevated ADAM8, 12 and 15 mRNA expression as potential prognostic molecular markers.

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Keywords: MMPs; ADAMs; degradome; gastric cancer; oesophageal cancer; adenocarcinoma

Oesophageal and junctional adenocarcinomas continue to increase in incidence and gastric adenocarcinoma remains the fourth commonest form of cancer worldwide (Pera, 2003). Oesophago–gastric (OG) adenocarcinoma causes more than a million deaths per annum (Parkin et al, 2005). Survival is generally poor with 5-year survival rates in the United Kingdom of 13% and 7.5% for gastric and oesophageal cancer, respectively (Quinn, 2003). The identification of biomarkers for diagnosis, treatment and prognosis is therefore an unmet clinical need that requires urgent attention.

Degradation of the extracellular matrix (ECM) is fundamental to tumour development and invasion (Egeblad and Werb, 2002; Bourboulia and Stetler-Stevenson, 2010). The 21 members of the human MMP (MMPs) family cleave a wide range of substrates including ECM molecules (Egeblad and Werb, 2002). Examples of such enzyme systems include the matrix metalloproteinases (MMPs), their endogenous inhibitors (the tissue inhibitors of metalloproteinases (TIMPs)) (Bourboulia and Stetler-Stevenson, 2010), and the ADAM (A Disintegrin And Metalloproteinase) family. Matrix metalloproteinases are a subfamily of zinc-dependent endopeptidases consisting of 24 members divided into five main groups according to their structure and substrate specificity: collagenases, gelatinases, membrane type, stromelysins and matrilysins (Egeblad and Werb, 2002; Bourboulia and Stetler-Stevenson, 2010; Kessenbrock et al, 2010). Most MMPs are secreted into plasma and are inhibited by 2-macroglobulin secreted by the liver (Bourboulia and Stetler-Stevenson, 2010). The MMPs are regulated by the four members of the TIMP family, which each bind reversibly to MMPs with some specificity; for example, TIMP1 binds preferentially to MMP1, 3, 7 and 9 and is a relatively poor inhibitor of membrane-type MMPs (Baker et al, 2002; Bourboulia and Stetler-Stevenson, 2010). The 21 members of the human ADAM family are transmembrane and secreted metalloproteinases that are relatives of the MMPs, as both families belong to the...
metzincin superfamily (Edwards et al., 2008; Rocks et al., 2008; Duffy et al., 2009). In addition to proteolysis, the ADAMs have roles in cell adhesion mediated by interaction of their disintegrin and Cys-rich domains with integrins and other receptors (Rocks et al., 2008; Duffy et al., 2009). Approximately 50% of ADAMs contain the catalytic consensus sequence HEXXH in their protease domain (Duffy et al., 2009), and it is thought that only these ADAMs possess protease activity (Duffy et al., 2009). The activity of selected ADAMs can also be inhibited by certain TIMPs, principally TIMP3 (Amour et al., 1998; 2000; Kashwagi et al., 2001). There is some evidence supporting a role for the earliest identified proteases (MMP2, 7, 9 and TIMP1, 2) in the development of OG cancer (Duffy et al., 2009; Riddick et al., 2005). Most studies have used immunohistochemical methods to assess protease expression, and some have identified correlations with tumour progression and clinical outcome (Brown, 1998; Duffy et al., 2009; Bourbouila and Stetter-Stevenson, 2010). However, diverse techniques and a lack of reliable anti-protease antibodies have contributed to some contradictory observations (Bourbouila and Stetter-Stevenson, 2010).

An additional pathway hypothesised to play a role in the expression of ECM-degrading proteases during malignancy is the c-Met signalling pathway (Peruzzi and Bottaro, 2006; Gentile et al., 2008). On binding to the tyrosine kinase Met cell surface receptor, hepatocyte growth factor (HGF) activates a programme of cell dissociation and motility coupled with increased protease production, promoting cellular invasion and metastasis (Birchmeier et al., 2003). At present, however, there are little data regarding the role of the c-Met pathway in OG tumorigenesis.

In the present study, we aimed to investigate the expression of the entire MMP and TIMP families, a subset of the ADAMs, and the c-Met signalling pathway, in a cohort of samples of matched malignant and benign peri-tumoural OG tissue, using quantitative real-time polymerase chain reaction (PCR). Furthermore, we aimed to investigate the relationship between gene expression levels and tumour/patient variables associated with poor prognosis, including age, sex, tumour grade, stage and plasma C-reactive protein (CRP) concentration (Skipworth et al., 2010).

**MATERIALS AND METHODS**

**Study patients**

Patients with a histological diagnosis of OG adenocarcinoma undergoing surgical resection with curative intent (n = 25) were recruited. Patients were staged according to the AJCC/International Union Against Cancer (Sobin and Wittekind, 2002) (UICC) criteria before surgical resection. All patients provided written informed consent and the study was approved by the Lothian Research Ethics Committee.

**Tissue collection**

A Consultant Pathologist dissected matched samples of tumour tissue and peri-tumoural normal tissue from the resected specimens within 20 min of the blood supply of the resection specimen being interrupted. Samples were frozen immediately in liquid nitrogen using liquid nitrogen-resistant tubes (Corning BV, Amsterdam, The Netherlands), and stored at −80°C until analysis.

**RNA extraction and reverse transcription**

Total RNA was isolated from the tissue samples by homogenisation in RNA Bee (Biogenesis Ltd, Poole, UK) using the TissueLyser (Quiagen, Crawley, UK) followed by the SV Total RNA isolation kit as described previously (Porter et al., 2004; Riddick et al., 2005). The RNA quality and concentration were determined using the NanoDrop ND-1000 UV-Vis spectrophotometer (Labtech, Ringmer, UK). Total RNA (1 µg) was reverse transcribed with 2 µg random hexamers (Amersham Pharmacia Biotech, Little Chalfont, UK) and 200 units of SuperScript II reverse transcriptase (Invitrogen, Cambridge, UK) according to the manufacturer’s instructions using a GRI DNA Engine (GRI Ltd, Braintree, UK). The cDNA was diluted 1:100 with PCR grade water and stored at −20°C.

**Quantitative real-time PCR**

Specific primers and probes for selected human MMPs, TIMPs, ADAMs, c-Met and HGF were designed as detailed previously (Nuttall et al., 2003; Porter et al., 2004; Riddick et al., 2005). The 18S ribosomal RNA (rRNA) was used as an endogenous control to normalise for differences in the amount of total RNA in each sample, using previously validated procedures (Wall and Edwards, 2002). Polymerase chain reaction reactions were carried out as described previously (Nuttall et al., 2003) using the ABI 7700 real-time PCR machine (Applied Biosystems, Carlsbad, CA, USA) with each reaction containing 5 ng of reverse-transcribed RNA (1 ng for 18S) in a 25-µl reaction. The RNA levels (compared with 18S rRNA) in each sample were determined by performing standard curves for all target genes covering 1–0.0625 ng of RNA for 18S and 20–0.5 ng RNA for all other genes. Genes with cycle threshold (Ct) of 40 were excluded from the analysis as there was no PCR amplification.

**Assessment of plasma CRP concentration**

Plasma CRP was assayed using automated methods on an Olympus AU2700 analyser (Olympus Diagnostica GmbH, Lismeehan, Ireland), in the Department of Clinical Chemistry, Royal Infirmary of Edinburgh (fully accredited by Clinical Pathology Accreditation (UK) Ltd). Appropriate internal quality controls were included, with CV’s typically 3.4% at concentrations <15 mg l⁻¹ and 1.6% at 80 mg l⁻¹.

**Statistical analysis**

All statistical analyses were performed using Statistical Package for Social Services version 17 (SPSS, Chicago, IL, USA) and GraphPad Prism version 5 (GraphPad Software Inc., La Jolla, CA, USA). Malignant gene expression is expressed as a relative level compared with gene expression in matched benign peri-tumoural samples. Matched-pair analysis was performed using the Wilcoxon signed-rank test, whereas non-parametric comparisons were performed using the Mann–Whitney U-test and Kruskall–Wallis test. Correlation analysis was performed using Spearman’s Rank Correlation Coefficient. Box-and-whisker plots of the gene expression levels (relative to 18S rRNA) in malignant compared with benign OG tissue are also shown. Statistical significance was set at P < 0.05.

**RESULTS**

**Study patients**

The median age of the recruited patients (n = 25) was 64 years, and 19 (76%) patients were male. Primary tumour sites were oesophageal (n = 16, 64%) and gastric (n = 9, 36%). Histological tumour subtype was adenocarcinoma in all cases. A summary of patient demographics is shown in Table 1.

**Protease expression in matched malignant and benign OG tissue samples**

Expression data for all protease and inhibitor genes are summarised in Table 2. In total, expression levels of 13 different MMPs, 2 TIMPs, 6 ADAMs, c-Met and HGF were upregulated significantly in malignant OG tissue compared with matched
relative increase in expression was MMP11, which had a median and Supplementary Figures 1 and 2. The protease with the largest expression levels compared with 18S rRNA are shown in Figure 1. The protease with the largest relative increase in expression in malignant tissue was significantly greater in women than men (P = 0.015) (Table 3). No significant relationships were demonstrated between relative mRNA expression and pre-operative plasma CRP level or patient age.

**DISCUSSION**

The ECM is a complex structure consisting of many different proteins, and thus its degradation requires a combination of proteases (Bourboula and Steller-Stevenson, 2010). In order to understand the complex processes underpinning tumour development and invasion, it is important to study the complete range of proteases that may be responsible. This study is the first comprehensive analysis of the expression of the MMP, TIMP and ADAM families in a series of human OG adenocarcinoma samples.
c-Met signalling pathway in the pathogenesis of OG cancer (Lee et al., 2007), a concept that is supported by the increased malignant expression of HGF and c-Met in the present study. Of the proteases that were highly upregulated in the present study, MMP1, 3, 7, 10 and 12 are located in a cluster on chromosome 11q22.3 (Jackson et al., 2010), raising the possibility of co-ordinated expression of these genes. Immunohistochemistry has demonstrated increased MMP1 (Inoue et al., 1999), MMP10 (Aung et al., 2006), MMP11 (Zhao et al., 2010) and MMP12 (Salmela et al., 2001) expression in gastric adenocarcinoma, whereas MMP3 is associated with increased risk of oesophageal adenocarcinoma (Bradbury et al., 2009). These MMPs share similar promoter conformation, with TATA boxes at around −30 bp and activator protein-1 transcription factor sites around −70 bp (Clark et al., 2008). It has been suggested that these MMPs can be co-regulated by a variety of chemical stimuli, including IL-1 and TNF-α (Clark et al., 2008).

The MMP with the greatest upregulation in the present study is MMP11 (Table 2), with a 20-fold increase in expression in malignant compared with matched benign OG tissue. Zhao et al. (2010) have shown using RT–PCR that MMP11 expression is elevated in gastric adenocarcinoma and correlated with TNM stage. Unlike other members of the MMP family, MMP11 does not degrade classical ECM proteins and instead facilitates the breakdown of the serine protease inhibitors α1-antitrypsin and insulin-like growth factor binding protein-1 (IGFBP-1) (Nedic et al., 2007). Insulin-like growth factor-binding protein-1 proteolysis leads to the release of IGF-1, inhibited apoptosis, and the progression to a more invasive stage of malignancy (Kasper et al., 2007).

### Table 3: Relationship between relative gene expression in malignant oesophago–gastric tissue and clinico-pathological parameters

<table>
<thead>
<tr>
<th>Gene</th>
<th>Clinico-pathological variable</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAM8</td>
<td>Tumour stage</td>
<td>0.048</td>
</tr>
<tr>
<td>ADAM15</td>
<td>Tumour stage</td>
<td>0.044</td>
</tr>
<tr>
<td>ADAM12</td>
<td>Tumour grade</td>
<td>0.011</td>
</tr>
<tr>
<td>MMP19</td>
<td>Gender</td>
<td>0.015</td>
</tr>
</tbody>
</table>

Abbreviations: ADAM = a disintegrin and metalloproteinase; MMP = matrix metalloproteinase; PCR = polymerase chain reaction. Statistical analysis was completed using Kruskal–Wallis tests to investigate the relationship between protease expression and tumour stage and grade, and a Mann–Whitney U-test for relationships between gene expression and gender. Samples with a Ct value of 40 denoting no PCR amplification were excluded before statistical analysis. Values are given to three decimal places.
Of the remaining MMPs, expression of the gelatinases MMP2 and MMP9 has been shown to be elevated in OG adenocarcinoma (Sier et al, 1997; Brew and Nagase, 2010). Our data show that the expression of TIMP1 and 3 were elevated in OG adenocarcinoma. Classically, it was thought that TIMPs protected against malignancy by inhibiting the proteases responsible for ECM degradation (Bourboulia and Stetler-Stevenson, 2010), and studies in various cancers have shown reduced expression (Ko(Bourboulia and Stetler-Stevenson, 2010), and thus it is possible that high TIMP3 expression was the cause of the lower MMP2 expression observed.

Tissue inhibitors of metalloproteinases are key regulators of MMPs, and have an important role in tumourigenesis (Mimori et al, 1997; Brew and Nagase, 2010). We data show that the expression of TIMP1 and 3 were elevated in OG adenocarcinoma. However, other researchers have revealed increased TIMP expression in malignant tissue (Bourboulia and Stetler-Stevenson, 2010), and thus it is possible that high TIMP3 expression was the cause of the lower MMP2 expression observed.

Of the remaining MMPs, expression of the gelatinases MMP2 and MMP9 has been shown to be elevated in OG adenocarcinoma (Sier et al, 1997; Murray et al, 1998; Parsons et al, 1998; Shim et al, 2007; Sampieri et al, 2010). In the present study, MMP9 levels were increased ~2-fold in malignant OG tissue compared with benign, but MMP2 expression was not significantly different, mirroring work by Sampieri et al (2010). Tissue inhibitors of metalloprotease 3, which was elevated in malignant tissue in the current study, may indirectly influence the expression of MMP2 (Bourboulia and Stetler-Stevenson, 2010), and thus it is possible that high TIMP3 expression was the cause of the lower MMP2 expression observed.

Tissue inhibitors of metalloproteinases are key regulators of MMPs, and have an important role in tumourigenesis (Mimori et al, 1997; Brew and Nagase, 2010). Our data show that the expression of TIMP1 and 3 were elevated in OG adenocarcinoma. Classically, it was thought that TIMPs protected against malignancy by inhibiting the proteases responsible for ECM degradation (Bourboulia and Stetler-Stevenson, 2010), and studies in various cancers have shown reduced expression (Ko et al, 1998; Riddick et al, 2005). Gu et al (2008) demonstrated downregulation of TIMP3 in OG adenocarcinoma and suggested that TIMP3 acts as a tumour suppressor, inhibiting growth, angiogenesis and invasion. However, other researchers have revealed increased TIMP expression in malignancy (Joo et al, 2000; Shim et al, 2007; Brew and Nagase, 2010), including oesophageal cancer (Salmela et al, 2000). Tissue inhibitors of metalloproteinase 3 is proposed to induce apoptosis through inhibition of ADAM17 (TNF-α converting enzyme), with subsequent stabilisation of TNF receptors (Brew and Nagase, 2010), supporting the results of this study. The present study also supports previous investigations that have demonstrated elevated TIMP1 expression in gastric cancer (Mimori et al, 1997; Murray et al, 1998; Joo et al, 2000). Possible mechanisms of action for TIMP1 in tumourigenesis involve a combination of cell-cycle arrest and anti-apoptotic activity, leading to cellular transformation and invasion (Brew and Nagase, 2010).

The expression levels of the majority of the profiled ADAMs, namely ADAM8, 9, 10, 12, 15 and 17, were increased significantly in malignant OG tissue compared with matched benign samples (Table 2). The ADAM12 demonstrated the highest expression levels with a relative increase of ~4-fold in malignant tissue (Figure 2). Previous studies have shown that various members of the ADAM family are increased in several cancer types, including lung, brain and prostate (Rocks et al, 2008; Duffy et al, 2009), but few have investigated the role in OG malignancy. The present study supports the work of Carl-McGrath et al (2005), who used RT–PCR to show that ADAM9, 12 and 15 expression is elevated in malignant compared with matched benign gastric tissue. The authors suggested that these proteases are involved in malignant transformation via the proteolytic shedding of signalling molecules and the consequent transactivation of their receptors, such as the epithelial growth factor receptor and its ligands (Carl-McGrath et al, 2005). Yoshimura et al (2002) demonstrated upregulation of ADAM10 and ADAM17, but not ADAM15, in antral gastric tissue during Helicobacter pylori infection, suggesting that their expression is upregulated by the bacteria. Furthermore, it is believed that ADAM17 is important for the release of active TNF-α, a pro-inflammatory cytokine involved in cancer development via mucosal inflammation and damage (Yoshimura et al, 2002).

Expression of ADAM8 and ADAM15 in malignant OG adenocarcinoma correlated with increasing tumour stage, and ADAM12 expression correlated with tumour grade (Figure 2), implying that these proteases are potential prognostic markers of OG adenocarcinoma. The ADAM8 expression has been shown to correlate significantly with poor prognostic parameters in prostate (Fritzsche et al, 2006) and brain (Wildeboer et al, 2006) tumours. However, this is the first study to report ADAM8 expression in OG malignancy. The ADAM15 is located on chromosome 1 at 1q21.3 (Kuefer et al, 2006), a region known to be amplified in several types of adenocarcinoma (Glinksy et al, 2003). The ADAM15 digests collagen IV and gelatin and is involved in the promotion of cell growth (Mochizuki and Okada, 2007). It has shown to be expressed significantly in gastric adenocarcinoma (Carl-McGrath et al, 2005), and correlation with tumour stage has been demonstrated in breast and prostate cancer (Kuefer et al, 2006). Figure 2 suggests that the expression of ADAM8 and ADAM15 in stage 4 cancers declines to stage 1 levels. Stage 4 disease is associated with distant (or at least significant nodal) metastases, and therefore ADAM8 and 15 levels may be reduced at this time in order to allow the dissociation of tumour cell adhesion and the process of metastasis. Whether this decline in expression reflects a causative initiator of metastasis or simply a small component of an overall metastatic phenomenon remains unclear. Further studies are required to verify this finding in other patient groups.

A similar link between ADAM12 expression and tumour grade has been demonstrated previously in malignancies of other tissues (Kveiborg et al, 2008; Duffy et al, 2009). Frohlich et al (2006) showed that the level of ADAM12 mRNA and protein expression in bladder tumour tissue samples correlated with the tumour grade. Interestingly, the concentration of ADAM12 in the urine of patients with bladder cancer was significantly elevated compared with healthy patients (Frohlich et al, 2006), suggesting that ADAM12 may be secreted by malignant tissue. Furthermore, Roy et al (2004) have also established a positive correlation between urinary ADAM12 levels and breast cancer progression. Thus, patient urine and possibly plasma may be potential biomarker sources in patients with OG cancer.

In conclusion, this study provides the most detailed profile to date of the degradome in OG malignancy. It implicates...
several MMPs, TIMPs, ADAMs and the c-Met signalling pathway in OG tumourigenesis. Larger clinical studies are required to assess the potential predictive and prognostic nature of these RNAs. Studies of both protein expression and function are required to identify if these proteins may represent novel therapeutic targets. However, such studies may be hampered by the unreliability of anti-protease antibodies.

REFERENCES


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