Loss of Heterozygosity and Immunohistochemistry of Adenocarcinomas of the Esophagus and Gastric Cardia

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ABSTRACT

Purpose: Adenocarcinomas of the distal esophagus and gastric cardia are two tumors that have many features in common. They have similar prognoses, treatment modalities, and patterns of dissemination. The etiology is different, with gastroesophageal reflux disease playing a major role for esophageal adenocarcinoma, in contrast to adenocarcinoma of the gastric cardia. In the present study, we investigated several genetic and immunohistochemical features of adenocarcinomas of the distal esophagus and gastric cardia.

Experimental Design: Sixty-two resection specimens of either adenocarcinoma of the esophagus or adenocarcinoma of the gastric cardia were carefully selected. The genetic analysis included loss of heterozygosity of several tumor suppressor genes known to be involved in esophageal cancer carcinogenesis. Immunohistochemical studies included the analysis of p53, c-Met, c-erbB-2, β-catenin, and cyclooxygenase-2. In addition, a mutation analysis of the Tcf1 gene was done by direct sequencing.

Results: Patients with cardiac carcinoma had a significantly worse tumor stage and poorer differentiation on histology. Loss of heterozygosity analysis did not reveal significant differences between esophageal adenocarcinoma and cardiac adenocarcinoma. Immunohistochemical analysis revealed significantly more nuclear accumulation of β-catenin and overexpression of cyclooxygenase-2 in patients with esophageal adenocarcinoma, compared with patients with cardiac carcinoma. No mutation was found in the Tcf1 gene in either tumor type.

Conclusions: Although adenocarcinomas of the distal esophagus and gastric cardia have many features in common, we have found some evidence that they might form two different entities.

INTRODUCTION

Tumors of the esophagogastric junction include two types of adenocarcinoma: distal esophageal adenocarcinoma and adenocarcinoma of the gastric cardia. The incidence of both tumors has increased rapidly over the last two decades (1, 2). The reason for this rising incidence is unknown. The etiology of distal esophageal adenocarcinoma is related to gastroesophageal reflux disease and the development of Barrett’s esophagus (3). The etiology of adenocarcinoma of the gastric cardia is less well understood.

Both tumor types have many features in common. Apart from their rising incidence, they both are most commonly diagnosed in white males, with obesity as an additional risk factor (4, 5). Morphologic criteria such as pattern of growth, degree of differentiation, depth of invasion, likelihood of angioinvasion, and lymphatic dissemination are also similar for both tumors (6). It has also been suggested that cardiac-type columnar mucosa extends into the esophagus and then is prone to the development of esophageal cancer by a stepwise process of intestinal metaplasia and dysplasia, implicating that cardiac and esophageal cancer have an identical origin, namely, cardiac mucosa (7). Finally, treatment for both tumors consists of subtotal esophagectomy and proximal gastrectomy, with a similar prognosis according to tumor stage (8, 9).

Some differences, however, do exist between adenocarcinomas of the esophagus and gastric cardia. Whereas esophageal adenocarcinoma is clearly related to gastroesophageal reflux disease, this is not the case for cardiac carcinoma (3). Intestinal metaplasia in the esophagus is a well-known risk factor and precursor lesion for the development of esophageal adenocarcinoma. Surveillance of these patients is advised (10). The presence of intestinal metaplasia at the gastric cardia is not a strong predictor for the development of cardiac carcinoma, and these patients are not advised to undergo surveillance at this time (11). Intestinal metaplasia in the esophagus has also been found to have a different mucin and cytokeratin histochemistry as compared with intestinal metaplasia in the gastric cardia (12, 13). Finally, our group recently showed that cyclooxygenase-2(COX-2) expression is higher in esophageal adenocarcinoma compared with cardiac carcinoma (14, 15). In addition, COX-2 was a significant prognostic factor for esophageal adenocarcinoma but not for cardiac carcinoma.

The esophagogastric tumors can also be characterized by genetic changes to identify tumor-specific characteristics. Loss
of heterozygosity (LOH) is a common mechanism responsible for carcinogenesis. Several groups studied LOH in patients with distal esophageal adenocarcinoma, whereas studies in cardiac carcinoma are more limited (16–19). Only one study directly compared the presence of LOH in patients with adenocarcinoma of either the esophagus or gastric cardia (20). For a better understanding of potentially different genetic changes in esophagogastric tumors, we studied several losses of heterozygosity in 62 carefully selected patients with adenocarcinoma of either the esophagus or gastric cardia. Tcf1 is a novel potential tumor suppressor gene, which has been implicated in the development of colorectal carcinomas and might also play a role in esophageal carcinoma (21). We studied LOH of two microsatellite markers in the vicinity of the Tcf1 gene and screened for mutations in Tcf1 in those patients with LOH at its locus. Tumors can also be characterized by phenotypic changes, which can be detected by immunohistochemistry. To study potential phenotypic differences between adenocarcinomas of either the esophagus or gastric cardia, we investigated the expression of p53, c-Met, c-erbB-2, β-catenin, and COX-2 in the resection specimens of the selected patients.

**PATIENTS AND METHODS**

**Patients and Tissue Samples.** Sixty-two esophageal resection specimens of patients with adenocarcinomas of the distal esophagus or gastric cardia were retrieved from the archives of the pathological department. All patients underwent a surgical resection between 1993 and 1998 at the Academic Medical Center (Amsterdam, the Netherlands). Thirty-one resection specimens contained esophageal adenocarcinoma, and the other 31 resection specimens contained cardiac carcinoma (Table 1). Distal esophageal adenocarcinoma was defined as a tumor with predominant localization in the esophagus and association with Barrett’s intestinal metaplasia. Adenocarcinoma of the gastric cardia was defined as a tumor with predominant localization below the esophagogastric junction and without an associated Barrett’s intestinal metaplasia. All other tumors without a clear origin were excluded from this analysis. From each resection specimen, a sample of tumor tissue and normal tissue was available in paraffin-embedded slides. Two pathologists historically assessed the tissue samples, and differentiation was categorized as good, moderate, or poor.

**DNA Extraction.** Tumor tissue was carefully microdissected from deparaffinized hematoxylin and eosin-stained 5-μm slides. Areas of tumor containing a minimal amount of stromal cells were microdissected using a surgical blade or needle directly under a microscope. For each case, matching nontumor tissue was obtained from a lymph node without metastases. DNA was isolated from the tissue using a standard proteinase K digestion, as described previously (22). Briefly, the microdissected tissue was collected in a DNA isolation buffer containing 1 mg/mL proteinase K. This was incubated overnight at 56°C, and thereafter proteinase K was inactivated by an incubation of the samples at 96°C for 10 minutes.

**Loss of Heterozygosity Analysis.** Thirteen microsatellite markers representing nine autosomal arms were selected for LOH analysis. The microsatellite markers were selected for their location at tumor suppressor loci, comprising MSH2, FHIT, APC, p16, PTEN, E-cadherin, p53, and DCC. All these loci are known to be involved in the development of esophagogastric junctional tumors (Table 2). D5S210 and D5S500 were selected based on their proximity to the Tcf1 gene. D14S68, which is in the vicinity of TSIRh, was chosen because a previous study has shown significantly more loss of 14q31 in patients with esophageal cancer compared with patients with cardiac cancer (23). The microsatellite marker sequences, size, and their corresponding locations on the chromosomes were obtained from the Genome Database, the Cooperative Human Linkage Center, and Genethon. Optimal MgCl2 and deoxynucleotide triphosphate concentrations were determined for each primer set at an annealing temperature of 55°C using control human DNA. The polymerase chain reaction (PCR) consisted of 40 cycles in a PTC-100 thermal cycler (MJ Research, Inc., Waltham, MA). The PCR mix contained 40 ng of each primer, 0.1 mg/mL bovine serum albumin, 1.5 μL of genomic DNA, and 1.0 unit of platinum Taq polymerase (Life Technologies, Inc., Rockville, MD). The PCR products were analyzed with an automated ABI 377 sequencer and Genescan 2.1 software (PE Biosystems, Foster City, CA).

The samples were scored for LOH as described previously (24). Briefly, a microsatellite marker was considered “informative” if two distinct alleles were found in the normal tissue of a patient. For the informative markers, the allelic imbalance was calculated as described by Cawkwell et al. (25). A tumor had LOH for a certain marker if the allelic imbalance factor was >1.6 or <0.63. A finding of LOH had to be confirmed at least once to ensure reproducibility. Allelic loss was not scored for markers that showed microsatellite instability. A marker was scored as microsatellite unstable when an additional peak was seen in the PCR amplification product of tumor DNA compared

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**Table 1** Patient and tumor characteristics

<table>
<thead>
<tr>
<th></th>
<th>Distal esophageal adenocarcinoma (n = 31)</th>
<th>Gastric cardia adenocarcinoma (n = 31)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>22</td>
<td>23</td>
</tr>
<tr>
<td>Female</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td><strong>Age (y)</strong></td>
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<td></td>
</tr>
<tr>
<td>Mean</td>
<td>63.8</td>
<td>64.8</td>
</tr>
<tr>
<td>SD</td>
<td>7.26</td>
<td>8.42</td>
</tr>
<tr>
<td><strong>Differentiation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Good/moderate</td>
<td>16</td>
<td>7</td>
</tr>
<tr>
<td>Poor</td>
<td>15</td>
<td>24</td>
</tr>
<tr>
<td><strong>Tumor stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II A</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>II B</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>III</td>
<td>12</td>
<td>28</td>
</tr>
<tr>
<td>IV</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

* P < 0.05 according to Fisher’s exact test.
† P < 0.05 according to χ² test.

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5 http://gai.nci.nih.gov/CHLC.
with the respective normal sample. The fractional allelic loss (FAL) was calculated as the ratio of LOH-positive cases to the total number of informative cases of that marker.

**Immunohistochemistry for p53, c-Met, c-erbB-2, β-Catenin, and Cyclooxygenase-2.** Immunohistochemistry was performed on formalin-fixed and paraffin-embedded specimens. The p53 immunostaining was done as described previously with DO7 (Dako, Glostrup, Denmark) used as a primary antibody (26). For c-Met immunostaining, DO24, a monoclonal antibody (clone 14; Transduction Laboratories, Lexington, KY) was used (27). Immunostaining for c-erbB-2 was done with an anti-c-neu antibody (Ab-3, clone 3B5) monoclonal antibody (Oncogene Science, Fremont, CA) was used to visualize the antibody-binding sites with 3,3'-diaminobenzidine as a chromogen.

The slides were scored by an experienced gastrointestinal pathologist (G. J. A. O.; Fig. 1). P53 staining was considered positive if >30% of the nuclei of tumor cells stained positive (26). Immunostaining for c-Met was mainly localized on the cell membrane and categorized as negative, weakly positive, positive, and strongly positive (27). COX-2 expression was categorized on a scale ranging from 0 to +++ (0, no expression; +, weak staining; ++, moderate staining; ++++, strong staining). For c-erbB-2, a distinct staining pattern along the cell membrane and categorized as negative, weak staining; +, moderate staining; ++, strong staining).

**RESULTS**

**Patients and Tumor Characteristics.** Of the 62 selected patients, 31 had distal esophageal adenocarcinoma, and 31 had adenocarcinoma of the gastric cardia. The mean age of patients was 64 years, and there was a majority of males (Table 1).
histologic differentiation and more advanced tumor stage compared with esophageal adenocarcinoma.

**Loss of Heterozygosity Analysis.** The overall frequency of LOH as indicated by the mean FAL values was 0.33 (SD = 0.19) for esophageal adenocarcinoma and 0.31 (SD = 0.21) for cardiac carcinoma. The specific losses of heterozygosity identified for the two groups are described in Table 2. LOH was most frequently found at markers surrounding p53 in 15 of 26 (58%) and 14 of 22 (64%) informative cases for esophageal and cardiac carcinomas, respectively. The markers surrounding APC and DCC also exhibited LOH in up to 60% of informative cases for both carcinomas. The markers surrounding APC exhibited LOH in 9 of 29 (31%) and 7 of 31 (23%) informative cases of esophageal adenocarcinoma and cardiac carcinoma, respectively. LOH for marker D5S500 (Tcf1) was more frequently observed in patients with esophageal adenocarcinoma compared with patients with cardiac carcinoma (48% versus 27%), but this difference did not reach statistical significance. For the remaining markers, there was a similar occurrence of LOH in the two groups.

**Immunohistochemistry.** Overexpression for p53 was found in up to 50% of both patients with esophageal adenocarcinoma and cardiac carcinoma (Table 4). Immunostaining for c-Met was positive in roughly 80% of both patient groups. This included weakly positive, positive, and strongly positive staining for c-Met, and these scores were evenly distributed between the two groups. Positive staining for c-erbB-2 was also similar in both groups. Nuclear accumulation of β-catenin was more frequently observed in patients with esophageal adenocarcinoma compared with patients with cardiac carcinoma (81% versus 48%; Fisher’s exact test, \( P = 0.01 \)). Moderate to strong COX-2 expression was observed in 11 patients (39%) with esophageal adenocarcinoma and 3 patients (10%) with cardiac carcinoma. COX-2 expression was significantly higher in patients with distal esophageal adenocarcinoma according to \( \chi^2 \) analysis (\( P < 0.05 \)). COX-2 overexpression was higher in patients with nuclear β-catenin compared with patients with normal β-catenin staining (37% versus 13%), but this difference

![Fig. 1 Immunohistochemistry of adenocarcinomas of the gastric cardia and distal esophagus.](image)

**Table 3** Primer sets and sequences for 10 different exons of the Tcf1 gene

<table>
<thead>
<tr>
<th>Exon no.</th>
<th>Sequence</th>
<th>PCR product (bp)</th>
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<tr>
<td>1</td>
<td>TGGAGTAACGAGACCCCAGC</td>
<td>226</td>
</tr>
<tr>
<td>2</td>
<td>CTTGACAGCTGGATGCTGGG</td>
<td>207</td>
</tr>
<tr>
<td>3</td>
<td>GTCACCTGCTGAGGAGGCTG</td>
<td>216</td>
</tr>
<tr>
<td>4</td>
<td>CTGCCAGTCCAGCCACTCACT</td>
<td>187</td>
</tr>
<tr>
<td>5</td>
<td>AACAGAGGCTGGCCGGCTGA</td>
<td>262</td>
</tr>
<tr>
<td>6</td>
<td>GAGGAGGCTGCTGGTTCTGG</td>
<td>211</td>
</tr>
<tr>
<td>7</td>
<td>TCTCTCCTGTCCTGGGAG</td>
<td>257</td>
</tr>
<tr>
<td>8</td>
<td>GCCTGCTTCTGAGCAGTAG</td>
<td>235</td>
</tr>
<tr>
<td>9</td>
<td>GCCGAGGCTGGCTGCTGCTAT</td>
<td>271</td>
</tr>
<tr>
<td>10a</td>
<td>GGAAGGAACACCTGTGTTCCA</td>
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</tr>
<tr>
<td>10b</td>
<td>CAAAACATCCCTGAGAGGCTCA</td>
<td>250</td>
</tr>
<tr>
<td>10c</td>
<td>AGGCCTGGCTGATCCTCCTCG</td>
<td>287</td>
</tr>
</tbody>
</table>
did not reach statistical significance. There was no significant correlation between tumor stage and expression of β-catenin or COX-2 (data not shown). In a limited number of patients, immunostaining could not be evaluated due to technical problems or lack of suitable material. None of the LOH markers could be correlated to one of the five different immunohistochemical findings, patient characteristics, tumor stage, or degree of differentiation (data not shown).

**Tcf1 Mutation Analysis.** The microsatellites DSS210 and DSSS00 were both located near the Tcf1 gene. Overall, there were 10 patients (18%) with LOH at locus DSS210 and 17 patients (36%) with LOH at marker DSSS00. Seven patients had LOH for both markers, and 20 patients had LOH for either DSS210 or DSSS00. All 20 of these patients were analyzed for a mutation in the Tcf1 gene. In this analysis, no mutation in the Tcf1 gene was identified.

**DISCUSSION**

We investigated several genetic and biological characteristics of patients with distal esophageal adenocarcinoma and adenocarcinoma of the gastric cardia. The genetic analysis included a LOH analysis using several microsatellite markers surrounding well-known tumor suppressor genes. The Tcf1 gene was analyzed for mutations in tumors that showed LOH at chromosomal arm 5q. With the genetic analysis, no differences could be detected between the two tumors, and no mutations were identified in the Tcf1 gene. In addition, several biological markers known to be involved in the development of esophagogastric tumors were studied immunohistochemically. This analysis included the expression of p53, c-Met, c-erbB-2, β-catenin, and COX-2. β-Catenin expression was significantly different. More patients with esophageal adenocarcinoma had nuclear localization of β-catenin compared with patients with cardiac carcinoma. In addition, expression of COX-2 was significantly higher in esophageal adenocarcinoma compared with cardiac carcinoma.

Esophageal adenocarcinoma originates from intestinal metaplasia in the esophagus, the so-called Barrett’s esophagus. A segment of intestinal metaplasia is prone to the development of low-grade dysplasia, which can further develop into high-grade dysplasia and eventually develop into adenocarcinoma. This sequence of events is accompanied by accumulation of genetic changes. Common genetic changes in the development of cancer are characterized by the loss of chromosomal regions, identified as LOH. When LOH occurs at a tumor suppressor gene that already has a genetic alteration on its remaining allele, this will lead to loss of function of this tumor suppressor gene, which can contribute to the development of cancer. Early genetic changes in the development of metaplasia to dysplasia and adenocarcinoma include chromosomal losses at 5q (APC), 9p (p16), and 18q (DCC; refs. 33 and 34). Loss of p53 (17p) also plays an important role in the development of esophageal adenocarcinoma. In both dysplastic and metaplastic tissue, identical mutations of p53 were found, suggesting that loss of p53 plays a role in the early stages of development of esophageal adenocarcinoma (35). In established esophageal adenocarcinoma, many additional chromosomal losses have been described. Dolan et al. (18) tested 120 microsatellite markers representing 39 autosomal arms in 23 patients with esophageal adenocarcinoma. They found LOH for 3p (VHL) in 64% of patients, LOH for 5q11 (MSH3) in 50% of patients, LOH for 9q21 (APC) in 33% of patients, LOH for 9p21 (p16) in 50% of patients, and LOH for 13q (Rb) in 30% of patients. The most common deletion was found at the locus of p53 (17p) in 22 of 23 informative patients (96%).

It is unclear whether distal esophageal adenocarcinoma and adenocarcinoma of the gastric cardia are tumors of the same kind or whether they represent different entities. Tumors can be characterized by genetic changes, and this could provide more insight in the development and characteristics of esophageal and cardiac adenocarcinoma. Several previous studies used comparative genomic hybridization (CGH) to quickly compare all of the chromosomal alleles and this did not show significant changes (36–38). One study found significantly more loss for locus 14q in patients with esophageal adenocarcinoma using CGH, which could not be confirmed in a subsequent study by LOH analysis (20, 23). LOH analysis gives more specific information about changes near putative tumor suppressor genes, as compared with CGH. Only one study directly compared the occurrence of LOH in patients with adenocarcinomas of the distal esophagus and gastric cardia, but no differences were found (20). In the present study, we compared the prevalence of LOH in patients with distal esophageal adenocarcinoma and adenocarcinoma of the gastric cardia. Thirteen microsatellite markers surrounding 10 tumor suppressor genes were selected. These tumor suppressor genes included MSH2, FHIT, APC, p16, PTEN, E-cadherin, p53, and DCC, which have been related to esophageal and gastric carcinoma previously. No differences were found in the occurrence of the different losses of heterozygosity in patients with esophageal adenocarcinoma or cardiac carcinoma.

Allelic loss at locus 5q has often been identified in patients...
with esophageal carcinoma in previous studies and in our current study. LOH at locus 5q is often responsible for inactivation of the tumor suppressor gene APC. Inactivation of the APC gene will lead to accumulation, membranous loss, and nuclear translocation of β-catenin, which will subsequently lead to activation of the Wnt signal transduction pathway. Inactivation of APC and activation of the Wnt pathway are important factors in the development of colorectal cancers (39). In esophageal cancer, the Wnt pathway is also activated, but mutations in APC are rarely found (40). Other factors such as a mutation in β-catenin or AXIN1 have also been studied as a potential cause for Wnt dysregulation in esophageal carcinoma, but mutations were not found in either of these genes (41, 42). Tcf1 is a novel tumor suppressor gene that is located on 5q, near APC, and its inactivation is also associated with activation of the Wnt pathway, which makes it a potential candidate gene involved in the development of esophagogastric tumors (21). We selected two microsatellite markers adjacent to Tcf1 and analyzed losses for these loci. LOH was found in 50% of cases, which were subsequently analyzed for a mutation of the Tcf1 gene by direct sequencing. This analysis did not reveal a mutation. Other tumor suppressor genes at 5q responsible for activation of the Wnt pathway need to be explored in esophagogastric carcinomas.

Immunohistochemical analysis revealed an increased expression of COX-2 and nuclear β-catenin in patients with distal esophageal adenocarcinoma. Overexpression of COX-2 isoforms has been observed in a variety of cancers. The use of nonsteroidal anti-inflammatory drugs is associated with a lower incidence of cancer, especially of the gastrointestinal tract. We previously reported (14) a decreased expression of COX-2 in patients with a gastric cardia adenocarcinoma compared with patients with a distal esophageal adenocarcinoma. The present data, in a different group of patients, confirm our previous observation that COX-2 expression is increased in esophageal adenocarcinoma compared with cardiac carcinoma. The expression of β-catenin also differed for the two patient groups. In esophageal adenocarcinoma, nuclear translocation of β-catenin has been reported to occur in up to 60% of patients (43). In the present study, we observed more nuclear accumulation of β-catenin in patients with esophageal adenocarcinoma (81%) compared with patients with cardiac carcinoma (48%). Conceptually, the nuclear β-catenin and COX-2 overexpression could be related to each other, as has been suggested in other studies (44). However, in the currently studied patients, we could not identify a significant correlation between COX-2 overexpression and nuclear accumulation of β-catenin. For the interpretation of these results, it is important to realize that tumor stage and differentiation of cardiac carcinomas were significantly worse compared with esophageal adenocarcinomas (Table 1). This is most likely due to the surveillance of patients with Barrett’s esophagus, which results in less advanced esophageal adenocarcinomas when these patients undergo surgery (45). In addition, cardiac carcinoma is less frequently accompanied by dysphagia, and therefore patients present with a more advanced tumor stage. A separate analysis on tumor stage and expression of β-catenin and COX-2 could not show a significant correlation.

In conclusion, we studied several genetic and biological markers in patients with adenocarcinoma of the distal esophagus or gastric cardia. The genetic analysis did not reveal significant differences. In addition, mutation analysis for the Tcf1 gene did not identify any mutation. Immunohistochemical analysis revealed a higher COX-2 and nuclear β-catenin expression for patients with distal esophageal adenocarcinoma, although the esophageal cancers had a less advanced stage and better differentiation. These latter results support the hypothesis that adenocarcinomas of the distal esophagus and gastric cardia might form two different entities.

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