Expression of Annexin A1 in Esophageal and Esophagogastric Junction Adenocarcinomas: Association with Poor Outcome


Abstract

Purpose: Annexin A1 (ANXA1) is a calcium-binding protein involved in arachidonic acid metabolism and epidermal growth factor receptor tyrosine kinase pathway. ANXA1 has been implicated in early squamous cell carcinogenesis of esophagus and correlates with degree of tumor differentiation. However, the role of ANXA1 in esophageal adenocarcinoma is unclear. Our goal was to evaluate ANXA1 expression and determine its prognostic significance in adenocarcinoma of the esophagus and esophagogastric junction.

Experimental Design: This study included 104 consecutive patients with primary resected esophageal and esophagogastric junction adenocarcinomas (11 stage I, 24 stage II, 53 stage III, and 16 stage IV). ANXA1 protein expression in each tumor was assessed by immunohistochemical staining of tissue microarrays. ANXA1 expression level was classified as high (≥25% of tumor cells with cytoplasmic staining), low (<25% of tumor cells with cytoplasmic staining), or negative; and was correlated with clinicopathologic features and patients’ outcomes.

Results: High ANXA1 expression was present in 39% (41 of 104) of tumors and was associated with higher pathologic T stage (P = 0.03) and distant metastasis (P = 0.04). High ANXA1 expression correlated with increased recurrence rate (P = 0.004) and decreased overall survival (P = 0.003) in univariate analysis. In multivariate analysis, ANXA1 expression and pN stage significantly correlated with recurrence rate (P = 0.008 and P < 0.001, respectively) and overall survival (P = 0.02 and P < 0.001, respectively) independent of T stage.

Conclusion: Our results indicate that high ANXA1 expression is frequent in esophageal and esophagogastric junction adenocarcinomas, correlates with more advanced pathologic T stage and the presence of distant metastasis, and is an independent prognostic factor for patient survival.

The incidence of esophageal adenocarcinoma has significantly increased in the past three decades in the United States as well as parts of western Europe. In the 1970s, esophageal adenocarcinoma accounted for only 5% of esophageal cancers in the United States, whereas squamous cell carcinoma was the dominant type. Currently, esophageal adenocarcinoma accounts for ~60% of esophageal cancers (1, 2). The rise in incidence of esophageal adenocarcinoma is thought to represent a real increase in disease burden and not the result of detection bias or misclassification bias (3, 4).

The survival of patients with esophageal adenocarcinoma has improved slightly in the United States over the past 25 years (5). The two most important prognostic indicators for patients with esophageal cancer continue to be depth of tumor invasion and nodal involvement (6–9). Other useful prognostic factors that relate to clinical outcome and survival in these patients are poorly defined.

A promising candidate is annexin A1 (ANXA1), a protein that has been implicated in early squamous cell carcinogenesis of the esophagus. The annexins comprise a family of calcium- and phospholipid-binding proteins. Structurally, annexins consist of a COOH-terminal domain that allows the binding of calcium and phospholipids and an NH$_2$-terminal domain that is variable in both length and sequence and imparts upon the family its functional diversity (10). The exact function of ANXA1 remains unknown. ANXA1 was initially cloned as a phospholipase A2 inhibitor (11) and is a critical mediator of apoptosis (12–14). ANXA1 also serves as a substrate for epidermal growth factor receptor (15) and is a steroid-regulated protein; thus, it is implicated in the actions of glucocorticoids, including inhibition of cell proliferation and regulation of cell migration (16–18).

Alterations in ANXA1 expression have been observed in a variety of tumors, but the biological consequence of these findings is unclear. Previous studies have shown increased...
ANXA1 expression in pancreatic (19), breast (16, 20), hepatic (21), glial (22), and stomach cancers (23). In contrast, reduced expression of ANXA1 has been seen in prostate cancers (24–26) and esophageal squamous cell carcinomas (27–30). Thus, the role of ANXA1 in carcinogenesis may occur in a tissue-specific manner.

In this study, we evaluated the expression of ANXA1 in resected adenocarcinomas of the esophagus and esophagogastric junction and determined its prognostic significance by correlating ANXA1 expression with clinicopathologic features and survival.

Materials and Methods

Case selection. This study included 104 consecutive patients with clinically localized esophageal and esophagogastric junction adenocarcinoma (11 stage I, 24 stage II, 53 stage III, and 16 stage IV) who had undergone esophagectomy without preoperative chemotherapy or radiotherapy between the years 1986 and 1998 at The University of Texas M.D. Anderson Cancer Center. One patient who died from postoperative complications was included in the survival analysis. The median age at time of surgery was 63 years (range, 28-82 years). Fifty-four patients underwent transthoracic (Ivor Lewis) esophagectomy, 39 transhiatal esophagectomy, 7 total (three-field technique) esophagectomy, and 4 esophagectomy not otherwise specified. Sixteen patients received postoperative chemotherapy and/or radiation therapy. Eight patients received both chemotherapy and radiation, two received postoperative chemotherapy alone, and six received postoperative radiotherapy alone. Eight of the 16 patients underwent transthoracic (Ivor Lewis) esophagectomy, six underwent transhiatal esophagectomy, and two underwent total (three-field technique) esophagectomy.

Tissue microarrays. For each patient, the tumor was identified on H&E-stained slides, and the corresponding formalin-fixed, paraffin-embedded tissue blocks were obtained. One-millimeter tissue cores in triplicate were obtained from each tumor and arrayed onto a recipient paraffin block using a tissue arrayer (Beecher Instruments, Sun Prairie, WI) as previously described (31, 32). Duplicate 1.0 mm tissue cores from nonneoplastic esophageal squamous mucosa (n = 6) and gastric body mucosa (n = 6) were also included in the tissue microarray.

Immunohistochemistry for ANXA1. Immunohistochemical staining was done on 4-μm paraffin sections obtained from the tissue microarray blocks. Briefly, sections were deparaffinized in xylene and rehydrated in graded concentrations of ethanol. Antigen retrieval was carried out in citrate buffer [10 mmol/L (pH 6)] for 15 minutes at 100°C in a microwave oven. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide for 10 minutes. Slides were incubated with anti-ANXA1 monoclonal antibody (BD Biosciences Pharmingen, San Jose, CA) at a 1:100 dilution overnight at 4°C, followed by addition of biotinylated anti-mouse secondary antibody and streptavidin-horseradish peroxidase (Zymed Laboratories, South San Francisco, CA). Immunoreactive proteins were visualized with 3,3′-diaminobenzidine solution followed by counterstaining with hematoxylin. Normal esophageal squamous epithelium was used as a positive control. Appropriate negative controls for immunostaining were prepared using nonimmune rabbit IgG.

Because tumors can be heterogeneous, representative tissue cores of the tumor were obtained in triplicate. The immunostaining of the tissue cores were graded in a semiquantitative manner and the percentage of positive cells was agreed upon by two independent pathologists. Immunoreactivity was evaluated by determining the percentage of positive cells in each core and then taking the average of three cores: high expression of ANXA1 was defined as cytoplasmic staining of ≥25% of tumor cells, low expression was defined as cytoplasmic staining of <25% of tumor cells, and negative staining was defined as no cytoplasmic staining of any tumor cells. We used 25% as the cutoff based on the 25% trimmed mean calculated by discarding 12.5% of the lowest and the highest scores and then computing the mean of the remaining scores. A trimmed mean is less susceptible to the effects of extreme scores than is the arithmetic mean. It is therefore less susceptible to sampling fluctuation than the mean for extremely skewed distributions.

Statistical analysis. χ² analysis or Fisher’s exact tests were used to compare categorical data. Overall survival and recurrence-free probability curves were constructed using the Kaplan-Meier method, and the log-rank test was used to evaluate the statistical significance of differences. The mortality data is updated using the latest information on the vital status provided by the institution, checked on the Social Security Death Index, by telephone follow-up and the Thoracic Surgery database. The recurrence information is updated every time the patient comes for a follow-up visit. To date, 90 patients have died, 11 patients are still alive, and 3 patients are lost to follow-up. Two of the three patients lost to follow-up are from Mexico and one patient is from Texas. Overall survival was calculated from time of surgery to time of death from any cause or to time of last follow-up, at which point the data were censored. Cumulative recurrence-free probability was calculated using the time between date of surgery and the date of first recurrence after surgery (if the patient recurred) or last follow-up date (if the patient did not recur).

The prognostic significance of clinical and pathologic characteristics was determined using univariate Cox regression analysis. Cox proportional hazards models were fitted for multivariate analysis. After interactions between variables were examined, a backward stepwise procedure was used to derive the best-fitting model.

Statistical analysis was done using SPSS software (version 13 for Windows; SPSS, Chicago, IL). Kaplan-Meier survival curves were drawn with GraphPad Prism (version 4 for Windows; GraphPad Software, San Diego, CA). We used two-sided significance level of 0.05 for all statistical analyses.

Results

Clinicopathologic features. The clinicopathologic characteristics of the study population are summarized in Table 1. There were 94 men and 10 women ranging in age from 28 to 82 years old. In accordance with the WHO classification standards (33), 3% (3 of 104) of cases were well-differentiated adenocarcinoma, 43% (45 of 104) were moderately differentiated, and 54% (57 of 104) were poorly differentiated. Ninety-eight of 104 tumors are from the lower esophagus/gastroesophageal junction and only 6 of 104 tumors are from the upper and mid esophagus. The majority (75%, 78 of 104) of tumors were more advanced stage (pathologic T3), and lymph node metastasis was observed in 73% (76 of 104) of cases. Sixteen patients (15%) underwent postoperative therapy. Thirteen of the 16 had negative/low ANXA1 expression. The pN stage in patients with postoperative therapy (15 of 16 were N1) showed no statistical difference compared with patients without postoperative therapy (61 of 88 were N1; P = 0.063). There was also no statistical difference between the pT stage in patients with postoperative therapy (15 of 16 were T1) versus patients without postoperative therapy (63 of 88 were T1; P = 0.067).

ANXA1 expression in esophageal adenocarcinoma. In Fig. 1, the nonneoplastic gastric mucosa showed no ANXA1 immunoreactivity. In contrast, the normal esophageal squamous mucosa showed diffuse cytoplasmic staining for ANXA1.
The majority (61%, 63 of 104) of esophageal and esophagogastric junction adenocarcinomas showed negative (n = 44) or low (n = 19) expression of ANXA1. In the 44 negative cases, no ANXA1 expression was detected in any of the tissue cores examined. A subset (39%, 41 of 104) of esophageal and esophagogastric junction adenocarcinomas showed high expression of ANXA1 with ≥25% of cancer cells staining positively (Fig. 2). Heterogeneity in the staining pattern of the triplicate cores was seen in 5 of 19 low staining cases and 17 of 41 high staining cases.

**Clinicopathologic correlation.** High ANXA1 expression was associated with higher pathologic tumor (pT) stage (P = 0.03) and distant metastasis (P = 0.04; Table 1). There was no association between ANXA1 expression and other clinicopathologic features, including gender, age, tumor differentiation, lymph node metastasis, and pathologic stage (pTNM).

**Survival analysis.** The median follow-up time was 18 months postesophagectomy and the mean follow-up time was 36 months. Only three patients were lost to follow-up and so this should not have a significant effect on survival analysis. In patients with high ANXA1 expression in tumors, the mean follow-up was 21 versus 46 months in patients with negative/low ANXA1-expressing tumors (P = 0.002; Table 1). Median follow-up time was 14 months in high ANXA1 tumors versus 26 months in negative/low ANXA1 tumors.

In univariate analysis (Fig. 3; Table 2), there is increased risk of recurrence with increasing pT stage (T2 versus T1, P = 0.03; T3 versus T2, P = 0.002) and increasing pathologic stage (II versus I, P = 0.03; III versus II, P = 0.002; IV versus III, P < 0.001). There is also increased risk of recurrence with distant metastasis (P < 0.001), lymph node metastasis (P < 0.001), or high ANXA1 expression (P = 0.004). Patients with high tumor expression of ANXA1 recurred earlier (median 10.6 months, 6.4-14.8 months) than patients with negative/low ANXA1 expression in tumor (median 21.2 months, 10.9-31.5 months; P = 0.003). Between patients with negative tumor expression of ANXA1 and low tumor expression of ANXA1, there was no statistical difference in recurrence risk (P = 0.052). In Fig. 3C, Kaplan-Meier curves obtained with two categories (negative/low versus high ANXA1 expression) resulted in statistically significant correlation of high ANXA1 expression with increased tumor recurrence-free probability (P = 0.003). The correlation remained significant (P = 0.003) when three categories (negative, low, and high ANXA1 expression) of ANXA1 expression were used. Sixteen of 104 cases received postoperative treatment and when these 16 patients were excluded, high ANXA1 expression remained significantly correlated with
ANXA1 expression in esophageal adenocarcinomas. A, negative ANXA1 expression (no tumor cells show cytoplasmic staining). B, low ANXA1 expression (25% of tumor cells show cytoplasmic staining). C, high ANXA1 expression (>25% of tumor cells show cytoplasmic staining).

Discussion

In this study, we examined the expression of ANXA1 in 104 cases of primary resected esophageal and esophagogastric junction adenocarcinomas. We have shown that high ANXA1 expression in esophageal or esophagogastric junction adenocarcinomas was associated with higher tumor stage and the presence of distant metastasis. Patients with high ANXA1 expression in tumor had an increased tumor recurrence and worse overall survival in comparison with patients with negative/low ANXA1 expression in tumor. In addition, high ANXA1 expression in tumor was an independent prognostic marker for both worse overall survival and increased tumor recurrence. Although there have been multiple studies on ANXA1 expression and esophageal squamous cell carcinomas (27, 30, 34, 35), to the best of our knowledge this is the first reported study of ANXA1 in adenocarcinomas of the esophagus. Previous studies have shown both up-regulation and down-regulation of ANXA1 in a variety of malignancies; thus, ANXA1 may have a tissue-specific effect in carcinogenesis. ANXA1 expression has been correlated with clinicopathologic features traditionally associated with poor prognosis, such as histologic differentiation in pancreatic cancers (19), and head and neck squamous cell carcinomas (36) and as a metastasis-associated protein in head and neck squamous cell carcinoma (37), gastric adenocarcinoma (23), and breast carcinoma (20). Our data confirm that pT stage and lymph node status are prognostic indicators for esophageal and esophagogastric junction adenocarcinoma treated with surgery alone. However, because most patients present with locally advanced disease, secondary prognostic markers may be of use. Several molecular markers have been identified as possible poor prognostic indicators for esophageal adenocarcinoma, including increased tumor expression of p53 (38), cyclin D1 (39), c-erbB2 (40), cyclooxygenase-2 (41), and e-cadherin (42), as well as hypermethylated plasma adenomatous polyposis coli DNA (40, 43). However, very few of these studies have been reproducible and none of these markers have seen widespread clinical use.

In this study, we have shown that expression of ANXA1 can be used as a potential molecular marker to predict patient outcome in patients treated with esophagectomy alone. Our data showed that high ANXA1 expression has a strong correlation with poor survival and increased tumor recurrence both in univariate and multivariate analyses. Multivariate analysis indicated that ANXA1 expression is an independent prognostic factor for poor survival. Thus, tumor levels of ANXA1 regardless of nodal status and T stage can be used to determine patient outcome. In addition, ANXA1 expression level can potentially be used as a prognostic factor before surgery can be done. Our findings suggest that ANXA1 plays an important role in tumor growth, progression, and metastasis in esophageal and esophagogastric junction adenocarcinoma.
Whether ANXA1 can provide a potential target for therapy is not known. Support for this hypothesis stems from the known biological role of ANXA1 in the inhibition of phospholipase A2, a key regulator in arachidonic acid metabolism (11). Carcinogenesis in Barrett’s esophagus is associated with increased expression of cyclooxygenase 2 (44–46), which, in turn, is associated with significantly reduced survival of patients undergoing surgery for esophageal adenocarcinoma (41, 44–48). Thus, the role of ANXA1 in esophageal carcinogenesis may be similar to the role of cyclooxygenases in esophageal carcinogenesis, i.e., through the deregulation of arachidonic acid metabolism. Alternatively, ANXA1 has been shown to be a substrate for epidermal growth factor receptor (15) and may exert its effect on esophageal adenocarcinoma by promoting the role of epidermal growth factor receptor in cellular proliferation and differentiation. Interestingly, epidermal growth factor receptor is also a proposed indicator of poor prognosis in esophageal adenocarcinomas (49).

Fig. 3. Kaplan-Meier curves of recurrence-free probability and overall survival among patients with esophageal adenocarcinoma treated by primary esophagectomy. The recurrence rate increased and overall survival rates were significantly worse in patients with higher overall pathologic stage (P < 0.001 and P < 0.001; A and B). In addition, patients with high expression of ANXA1 in tumor had increased recurrence rate and decreased overall survival compared with patients with negative/low ANXA1 expression in tumor (P = 0.003 and P = 0.003; C and D). Bottom of each graph, corresponding censor tables.
In summary, our results showed high ANXA1 expression was present in a subset of esophageal adenocarcinoma. High ANXA1 expression was associated with higher tumor stage, increased distant metastasis, increased tumor recurrence, and poor overall survival in patients treated with esophagectomy. These findings suggest that ANXA1 expression may be used to predict patient outcome and can serve as a potential target for directed therapy in esophageal adenocarcinoma.

### Table 2. Univariate analysis of recurrence-free and overall survivals

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No.</th>
<th>Recurrence-free probability</th>
<th>Overall survival</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td>HR (95% CI)</td>
<td>P</td>
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<td>Age (y)</td>
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<td>0.98 (0.10-1.00)</td>
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<tr>
<td>Gender</td>
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<tr>
<td>Male</td>
<td>94</td>
<td>0.83 (0.36-1.95)</td>
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<tr>
<td>Female (reference)</td>
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<tr>
<td>pT stage</td>
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<td></td>
</tr>
<tr>
<td>T1 (reference)</td>
<td>12</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>14</td>
<td>5.77 (1.24-26.78)</td>
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<tr>
<td>T3</td>
<td>78</td>
<td>9.59 (2.31-39.79)</td>
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<tr>
<td>pN stage</td>
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<td>N0 (reference)</td>
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<tr>
<td>N1</td>
<td>76</td>
<td>5.83 (2.79-12.21)</td>
<td>&lt;0.001</td>
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<tr>
<td>pM stage</td>
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<td></td>
<td></td>
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<tr>
<td>M0 (reference)</td>
<td>88</td>
<td>1.00</td>
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<tr>
<td>M1</td>
<td>16</td>
<td>3.82 (1.80-8.13)</td>
<td>&lt;0.001</td>
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<td>Pathologic stage (pTNM stage)</td>
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<tr>
<td>I (reference)</td>
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<tr>
<td>II</td>
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<td>9.74 (1.28-74.19)</td>
<td>0.03</td>
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<tr>
<td>III</td>
<td>53</td>
<td>23.16 (3.12-171.83)</td>
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<tr>
<td>IV</td>
<td>16</td>
<td>53.72 (6.58-438.49)</td>
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<td>Postoperative treatment</td>
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<td>Yes (reference)</td>
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<td>Negative/low (reference)</td>
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<tr>
<td>High</td>
<td>41</td>
<td>2.30 (1.31-4.04)</td>
<td>0.004</td>
</tr>
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</table>

Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval.

*Overall P value for pT stage.

**Table 3. Multivariate Cox regression analysis of recurrence-free and overall survivals**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No.</th>
<th>Recurrence-free probability</th>
<th>Overall survival</th>
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<tr>
<td></td>
<td></td>
<td>HR (95% CI)</td>
<td>P</td>
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<td>pT stage</td>
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<tr>
<td>T1 (reference)</td>
<td>12</td>
<td>1.00</td>
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<tr>
<td>T2</td>
<td>14</td>
<td>3.21 (0.64-16.21)</td>
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<td>T3</td>
<td>78</td>
<td>3.31 (0.69-15.83)</td>
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<td>pN stage</td>
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<tr>
<td>N0 (reference)</td>
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<td>1.00</td>
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<tr>
<td>N1</td>
<td>76</td>
<td>3.99 (1.77-9.02)</td>
<td>&lt;0.001</td>
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<td>ANXA1 expression</td>
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<tr>
<td>Negative/low (reference)</td>
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<td>1.00</td>
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<tr>
<td>High</td>
<td>41</td>
<td>2.20 (1.23-3.95)</td>
<td>0.008</td>
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*Overall P value for pT stage.
References

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