High Gene Expression of TS1, GSTP1, and ERCC1 Are Risk Factors for Survival in Patients Treated with Trimodality Therapy for Esophageal Cancer

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ABSTRACT

Purpose: To assess the relationship between molecular markers associated with chemotherapy resistance and survival in esophageal cancer patients treated with trimodality therapy.

Experimental Design: The original pretreatment formalin-fixed, paraffin-embedded endoscopic esophageal tumor biopsy material was obtained from 99 patients treated with concurrent cisplatin plus 5-fluorouracil plus 45 Gy radiation followed by resection at Duke University Medical Center (Durham, NC) from 1986 to 1997. cDNA was derived from the biopsy and analyzed to determine mRNA expression relative to an internal reference gene (β-actin) using fluorescence-based, real-time reverse transcription-PCR. Possible markers of platinum chemotherapy association [glutathione-S-transferase π (GSTP1) and excision cross-complementing gene 1 (ERCC1)] and 5-fluorouracil association [thymidylate synthase 1 (TS1)] were measured.

Results: Cox proportional hazards model revealed a significant inverse, linear effect for TS1 with respect to survival (P = 0.007). An inverse relationship between TS1 expression and treatment response was also detected (P ≤ 0.001). Univariate analysis identified an association with decreased survival for GSTP1 ≥ 3.0 (P = 0.05). In multivariate analyses, TS1 >6.0, ERCC1 >3, and GSTP1 >3 were statistically significant predictors of decreased survival (P = 0.007). Additionally, the presence of ERCC1 >3.0 or TS1 >6.0 was associated with an ~2-fold increase in the risk of cancer recurrence (P = 0.086 and 0.003, respectively).

Conclusion: The measurement of relative gene expression of molecular markers associated with chemoresistance in endoscopic esophageal tumor biopsies may be a useful tool in assessing outcome in patients with trimodality-treated esophageal cancer. These data should be validated further in larger prospective studies.

INTRODUCTION

About 14,000 new cases of esophageal cancer are diagnosed each year in the United States with an overall survival rate of ~10%. Those patients who have undergone surgical resection with curative intent recognize only a limited increase in survival at 2 years (20-30% survival). Such a poor outcome is most likely attributed to the presence of locally advanced and undetected metastatic disease at the time of diagnosis (1, 2).

Previous studies in the treatment of esophageal cancer have yielded conflicting results regarding the benefit of neoadjuvant chemotherapy with or without radiation followed by resection. This may be due to problems with the selection criteria of the subjects. These problems may be a lack of consistent pretreatment staging (no computed tomography or endoesophageal ultrasound requirement), inadequate treatment dose secondary to either the study design or the limited patient tolerance and poor performance status, or use of agents that have shown limited activity in esophageal cancer.

More recent advances in chemotherapy and radiotherapy have shown a slight improvement (2). Prospective, phase II, single institution data suggest that the neoadjuvant therapy (chemotherapy followed by resection) results in a 20% to 30% rate pathologic complete response. Only these patients enjoy an improved survival, whereas those without a pathologic complete response have a similar survival as patients treated with resection alone (3). Unfortunately, one cannot predict which patients will fall into which subset. There have been several recent prospective, randomized trials comparing chemotherapy with or without radiation followed by resection compared with resection alone. All but one has shown no significant difference in survival, but each has shown a similar complete response rate for the treatment arm (15-30%). These patients make up the only significant group of long-term survivors (4–8). Therefore, if one could identify the patients likely to respond to a particular regimen, therapy could subsequently be tailored accordingly and the survival rate could therefore be improved.

Advances in molecular pharmacology have refined the understanding of the mechanisms of action of drugs and resistance to chemotherapy. Several mechanisms of resistance have been identified among the most commonly used agents in the treatment of patients with esophageal cancer. Assessment of the probability of chemotherapy resistance by molecular marker expression may allow for the selection of a more effective chemotherapeutic regimen. The authors of the current study have...
Risk Factors in Trimodal-Treated Esophageal Cancer

previously published immunohistochemically measured protein expression for several possible markers of chemoresistance in trimodality-treated patients with esophageal cancer. Associations with decreased survival were obtained for relative overexpression of markers of platinum resistance [glutathione S-transferase π (GSTP1) and multidrug resistance] and 5-fluorouracil [5-FU; thymidylate synthase 1 (TS1); ref. 9]. However, immunohistochemical determination of protein expression may have limited predictive value because it is subjective and semiquantitative. Due to these limitations, reverse transcription-PCR (RT-PCR) measuring gene expression may be a more accurate method.

In this study, we measured gene expressions of TS1, GSTP1, and excision cross-complementing gene 1 (ERCC1) by quantitative RT-PCR in pretreatment biopsies of esophageal tumor tissue specimens from patients who were scheduled to undergo neoadjuvant therapy with a regimen that included 5-FU, cisplatin, and radiation. High expressions of these genes were found to be significantly associated with patients’ survival.

MATERIALS AND METHODS

Patient Selection and Tissue Sample Collection. The esophageal cancer registry was accessed for patients who underwent trimodality therapy for esophageal cancer from 1986 to 1997 at the Duke University Comprehensive Cancer Center. The regimen included two cycles of 5-FU given as 800 mg/m²/d × 5 or 1,000 mg/m²/d × 4 plus 75 mg/m² cisplatin with concurrent 45 Gy radiation followed by resection. The radiation field included the celiac axis. For entry, each patient was required to meet the following criteria: (a) pathologically positive endoscopic esophageal biopsy, (b) complete the prescribed chemotherapy and radiotherapy, (c) gross complete resection [all patients had lymph node resected in the chest and abdomen, with >10 per resection; operations included Ivor Lewis (lower third, 85%) and three incision (middle third lesions, 15%), (d) lived at least 30 days after operation, (e) have pretreatment pathology available for analysis, and (f) have undergone double contrasted chest/abdominal/pelvis computed tomography for clinical staging. The preclinical staging of the study population ranged from T₁N₀ to T₃N₁ (9), with most being either T₃ or N₁ positive.

RNA Extraction and cDNA Synthesis. RNA was isolated from formalin-fixed, paraffin-embedded specimens using a novel, proprietary procedure (Response Genetics, Los Angeles, CA, U.S. Patent No. 6,248,535). After RNA isolation, cDNA was derived from each sample according to a previously described procedure (10).

PCR Quantification of mRNA Expression. Target cDNA sequences were amplified by quantitative PCR using a fluorescence-based real-time detection method [ABI PRISM 7700 Sequence Detection System (Taqrman), Applied Biosystems, Foster City, CA] as described previously (11, 12). The 25 μL PCR reaction mixture contained 600 nmol/L of each primer (Table 1); 200 nmol/L each of dATP, dCTP, and dGTP; 400 μmol/L dUTP; 5.5 mmol/L MgCl₂; and 1× Taqman buffer A containing a reference dye (all reagents were supplied by Applied Biosystems). PCR conditions were 50°C for 10 seconds and 95°C for 10 minutes followed by 42 cycles at 95°C for 15 seconds and 60°C for 1 minute.

Table 1  PCR primer and probe sequences of the analyzed genes

<table>
<thead>
<tr>
<th>Primer/probe</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERCC1-504F (21 bp)</td>
<td>GGGAATTTGCGGACGTAATTCC</td>
</tr>
<tr>
<td>ERCC1-574R (18 bp)</td>
<td>GCGGAAGCTGAGGAACCGA</td>
</tr>
<tr>
<td>Probe ERCC1-530 (25 bp)</td>
<td>CACAGGTGCTTCGGCACCACATA</td>
</tr>
<tr>
<td>TSI-763F (17 bp)</td>
<td>GGCTCTGGTGGTCCCTTT</td>
</tr>
<tr>
<td>TSI-825R (21 bp)</td>
<td>GAATGCGCAATGATGACGT</td>
</tr>
<tr>
<td>Probe TSI-781 (22 bp)</td>
<td>AACATCGGCAACGCTACCGTCG</td>
</tr>
<tr>
<td>GST2-192F (24bp)</td>
<td>CCTGATACGCTTGCAATACCTC</td>
</tr>
<tr>
<td>GST2-263R (20 bp)</td>
<td>TCTCCTGCTGCTTCCTCCTTA</td>
</tr>
<tr>
<td>Probe GST2-219T (20 bp)</td>
<td>TGACCGCGCTACAGCTTT</td>
</tr>
<tr>
<td>β-actin-592F (18 bp)</td>
<td>TCGACTGCGGATCGATCGT</td>
</tr>
<tr>
<td>β-actin-651R (22 bp)</td>
<td>TCGTAAATGTCACGACAGATT</td>
</tr>
<tr>
<td>Probe β-actin-611 (18 bp)</td>
<td>ACCACACGCCGAC</td>
</tr>
</tbody>
</table>

Statistical Analyses. Cancer-specific survival was calculated from the date of endoscopic esophageal biopsy until date of death or last follow-up. Survival was censored for patients who died without disease progression. Data derived from ABI PRISM 7700 Sequence Detection System (Taqrman) are expressed in ratios between the two absolute values (target gene/internal reference gene). To determine the cutoff value that best dichotomized patients into low and high expression, the maximal $\chi^2$ method of Miller and Siegmund (13) and Halpern (14) was used along with defined quartiles of relative gene expression values. The Cox proportional hazards model was used to assess the prognostic importance of each marker on survival as well as any linear relationship. Variables with $P$ < 0.2 were used for further multivariate analysis.

RESULTS

Patient Population. Patients ($n = 156$) were identified and found to meet the study criteria. The original pretreatment formalin-fixed, paraffin-embedded endoscopic esophageal tumor biopsy material was available from 126 of these patients. This material was re-cut and evaluated for viable tumor. In some cases, the depth of the tumor sample had been exhausted during the cutting process of previous studies, leaving insufficient tumor for evaluation. Therefore, 99 patients had adequate identifiable areas of tumor cells and were designated as the study population. The median age of the study population was 60 years (range, 28-79 years). There were 70 adenocarcinoma and 29 squamous carcinoma tumors (Table 2). The evaluation of all of the markers could not be completed on every sample because the original endoscopic biopsies were small and a limited amount of tumor sample remained in the paraffin block. However, there was enough material available for the analyses of the internal reference gene β-actin and at least one gene of interest (Tables 3 and 4).

Survival versus Treatment Response. Treatment response was based on the determination of pathologic complete resection or no pathologic complete resection at the post-treatment resection. A log-rank test was used to determine any significant correlation between survival and pathologic response to treatment (Table 5; Fig. 1). The investigators determined a significant correlation between increased survival and a pathologic complete response ($P = 0.007$).

Survival versus Level of Gene Expression. TS1 mRNA expression was detectable by quantitative RT-PCR in all of the...
69 samples in which material was available for analysis. The median TS1 mRNA expression was 2.98 (range, 0.9-14.09). Fig. 2A shows that levels of TS1 >6 are significantly associated with decreased survival ($P = 0.003$). A significant association with decreased survival was also found using a TS1 level >3.32 ($P = 0.026$). These results led to further investigation into the linear effect of TS1 expression. Using the Cox model analyses, we found a significant linear relationship between an increase in TS1 expression and a decrease in survival ($P = 0.007$). To determine a relationship between response and gene expression, contingency tables were generated. Fisher’s exact test was used to determine if any statistically significant relationship existed between variables. Statistical significance was not reached for any of the variables. A significant association between an increase in the number of markers expressed and a decrease in survival ($P = 0.00035$) was observed.

Table 2: Patient characteristics and survival

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>Median survival (mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>85</td>
<td>20</td>
</tr>
<tr>
<td>Female</td>
<td>14</td>
<td>47</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>70</td>
<td>22</td>
</tr>
<tr>
<td>Squamous</td>
<td>29</td>
<td>19</td>
</tr>
<tr>
<td>Clinical stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I (T1N0)</td>
<td>4</td>
<td>60</td>
</tr>
<tr>
<td>IIA (T2N0, T3N0)</td>
<td>68</td>
<td>22</td>
</tr>
<tr>
<td>IIB (T1N1)</td>
<td>6</td>
<td>18</td>
</tr>
<tr>
<td>III (T2N1)</td>
<td>21</td>
<td>14</td>
</tr>
<tr>
<td>I-IIIB (T1N1)</td>
<td>72</td>
<td>26</td>
</tr>
<tr>
<td>III</td>
<td>27</td>
<td>15</td>
</tr>
</tbody>
</table>

NOTE. Survival based on clinical characteristics. There were no significant survival differences among these clinicopathologic characteristics.

DISCUSSION

Several randomized studies have investigated the utility of neoadjuvant chemotherapy with or without radiation followed by resection. These studies have consisted of small population sizes and the results reported have been conflicting. This may be due to issues regarding the selection criteria, lack of uniform pretreatment staging, inadequate treatment dose secondary to either the study design or the limited patient tolerance and poor performance status, or use of agents that have shown limited activity in esophageal cancer (7, 8, 15–19). Recently, a multiinstitutional study conducted in Europe investigated the effect of preoperative chemotherapy on survival. In this study, 802 patients were randomized to either chemotherapy (cisplatin plus 5-FU) followed by surgery or surgery alone. These investigators reported a significant increase in 2-year survival in the population that received preoperative chemotherapy ($P < 0.0001$). However, 9% of the study population were removed before analysis because these patients were also treated with preoperative radiation. An analysis of the effect of radiation on survival between those patients receiving radiation plus chemotherapy and those receiving chemotherapy alone was not reported (1).
and carboplatin, react with cellular DNA. This results in platinum-DNA adducts. By binding to DNA, cisplatin inhibits replication and can lead to arrest in the G2 phase of the cell cycle, thus resulting in apoptosis (20). There are two main mechanisms of cisplatin resistance. One leads to the reduction in the formation of cytotoxic DNA lesions and the other minimizes their impact. GSTP1 not only aids in detoxification of oxygen free radicals (one method of radiation injury) but also actively binds to platinum and allows it to be removed from the cytosol. In a previous study, the current authors observed an association between the overexpression of GSTP1 protein, measured by immunohistochemistry, with a significant decrease in survival ($P = 0.02$) in a similar population of esophageal cancer patients treated with trimodality therapy. In the current study, univariate analysis indicates that patients with a relative GSTP1 gene expression level of $>3$ had a decrease in survival ($P = 0.05$).

The nucleoside excision repair system is one of the major DNA repair systems. It consists of integrated recognition and excision of a broad variety of DNA damage, including UV lesions and bulky chemical adducts, such as those that are the result of cross-linking by platinum agents. The damaged DNA strand is incised at both sides of the lesion and is removed. The gap is filled by DNA synthesis and the remaining nick is ligated (20). To determine the underlying mechanism of the increase in nucleoside excision repair tumors resistant to platinum chemotherapy, the genes and gene products involved in nucleoside excision repair have to be analyzed. All of the nucleoside excision repair genes have been cloned and named with the xeroderma pigmentosa complementation group, with the exception of the ERCC1 gene. Thus, mutations in the xeroderma pigmentosa genes and ERCC1 are used as markers for nucleoside excision repair deficiency and treatment resistance (21, 22). Although not statistically significant, univariate analysis of relative ERCC1 gene expression in relation to survival showed that those patients with a level of $>3$ tended to have a decrease in survival ($P = 0.086$).

5-FU is an antimetabolite that interferes with DNA and RNA synthesis during the cell cycle. Its metabolite (5-fluoro-dUMP) was simply determined using the pathologic diagnosis at the time of resection and was defined as pathologic complete resection or no pathologic complete resection. Using this method to indicate response to therapy, the investigators determined a significant correlation between increased survival and a pathologic complete response ($P = 0.007$). Due to the number of deaths in the follow-up interval of causes unrelated to esophageal cancer $>60$ days after discharge ($n = 25$), mortality by any cause was not used as an end point. Therefore, the end point of this study was cancer-specific survival.

The markers analyzed have been associated previously with the biological pathway of the two chemotherapy drugs administered (5-FU and cisplatin) and data exist with respect to likely drug resistance in other tumor models, such as colorectal and gastric carcinoma. Platinum compounds, such as cisplatin and carboplatin, react with cellular DNA. This results in

### Table 5 Response to therapy

<table>
<thead>
<tr>
<th>Response</th>
<th>$n$</th>
<th>No. dead</th>
<th>Median survival (95% confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathologic complete resection</td>
<td>30</td>
<td>9</td>
<td>NE</td>
</tr>
<tr>
<td>No pathologic complete resection</td>
<td>69</td>
<td>42</td>
<td>19.2 (14.3-28.3)</td>
</tr>
</tbody>
</table>

NOTE. Survival based on response to therapy in terms of pathologic complete response or no pathologic complete response.

The method of action and treatment resistance for many therapies in various tumor types have been described by many investigators. These include cisplatin, 5-FU, and radiotherapy. However, this information is limited in esophageal cancer. This study assessed the possible relationship of molecular markers of chemotherapy resistance to survival. To date, it is the largest of its type and was done on pretreatment tissue collected from a well-defined patient population. Before therapy, all patients underwent clinical staging that included at least a chest/abdomen/pelvic computed tomogram with p.o. and i.v. contrast. Table 2 shows the stages observed before therapy. Note, the majority of the patients were T1 or N1 positive. Treatment consisted of two cycles of 5-FU given as 800 mg/m²/d $\times$ 5 or 1,000 mg/m²/d $\times$ 4 plus 75 mg/m² cisplatin with concurrent $45$ Gy radiation (radiation field included the celiac axis) followed by resection.

In the present study, we attempted to determine an association between gene expression and response to therapy. However, quantifying response to treatment of patients enrolled in a clinical phase II trial can be difficult. Radiological and clinical staging before treatment is often inaccurate, in that the presence of locally advanced and metastatic disease are not detected at the time of diagnosis, thus making it difficult to assess a clinical response when compared with the pathologic results after therapy. Although for this project we used the best clinical staging modalities available for esophageal cancer, the task was even more problematic due to a lack of a standard scale to quantify a therapy response in esophageal cancer. For example, a patient with a large T3N1 tumor may be downsized to a small T1N1 after therapy and resection but recur within 12 months of the initiation of therapy. (Thus, any measured response would have little clinical relevance.) Therefore, response was simply determined using the pathologic diagnosis at the time of resection and was defined as pathologic complete resection or no pathologic complete resection. Using this method to indicate response to therapy, the investigators determined a significant correlation between increased survival and a pathologic complete response ($P = 0.007$). Due to the number of deaths in the follow-up interval of causes unrelated to esophageal cancer $>60$ days after discharge ($n = 25$), mortality by any cause was not used as an end point. Therefore, the end point of this study was cancer-specific survival.
is a tight-binding inhibitor of TS1. Overexpression of TS1 seems to be a major method of resistance to 5-FU and data from colorectal cancer patients suggest an association with TS1 and resistance to 5-FU (23, 24). Previously, we showed that overexpression of TS1 protein using immunohistochemistry was associated with a decreased survival in esophageal cancer ($P = 0.04$). In the current study, univariate analysis of relative TS1 gene expression and survival showed that patients with TS1 expression levels of >3.32 and >6.0 had an increase in survival ($P = 0.026$ and 0.003, respectively). These results prompted further investigation into a possible linear effect of an increase in gene expression of each gene with survival as a continuous variable. Significant correlation between increasing TS1 expression and decreasing survival ($P = 0.007$) was observed, whereas a linear effect approaching statistical significance was also noted, indicating that decreasing survival was associated with increasing gene expression of $GST1$ ($P = 0.064$) and $ERCC1$ ($P = 0.071$).

To determine a relationship between response and gene expression, contingency tables were generated. Fisher’s exact test was used to determine if any statistically significant relationship existed between variables. There was not a statistically significant relationship between response and GSTP1 or ERCC1. However, the relationship between response and TS1 expression was approaching statistical significance ($P = 0.07$). On further analysis, an inverse relationship between levels of TS1 expression and response to therapy ($P \leq 0.001$) was detected.

GSTP1 not only actively binds to platinum and allows it to be removed from the cytosol but also aids in detoxification of oxygen free radicals, which is one method of radiation injury (25). Because all of these patients were treated with concurrent 45 Gy radiation, the effect GSTP1 expression on radiotherapy should be addressed. According to our findings, increased expression is associated with decreased survival. Due to the design of this study, it is difficult to determine if GSTP1 expression affects the effectiveness of the chemotherapy or radiation alone or in combination. The literature addressing this issue is contradictory. Preclinical studies have reported that decreased expression of the GSTP1 protein GSTP indicate radiosensitivity (26). However, in studies of resected breast and uterine cervical cancer followed by radiotherapy, overexpression of GSTP indicate a increased benefit of radiotherapy (27, 28). These contradictions indicate that the complexity of cellular detoxification mechanisms requires further investigation.

Data obtained from the previous immunohistochemistry and current relative gene expression RT-PCR experiments done on formalin-fixed, paraffin-embedded tissue samples indicate that the measurement of TS1, GSTP1, and ERCC1...
could be a potential tool for predicting those patients that will most likely respond to neoadjuvant therapy. Immunohistochemistry and RT-PCR are important tools in clinical diagnostics as well as in research. Although easily done on fresh, frozen tissue samples, immunohistochemistry done on formalin-fixed, paraffin-embedded tissue samples is met with some obstacles. The major factors that effect staining results are (a) tissue fixation, (b) antigen unmasking, (c) sensitivity of the detection system, (d) quality control of each assay done, and (e) standardization (29, 30). Some of these same obstacles are also faced when working with RNA extracted from formalin-fixed, paraffin-embedded tissue, but the main difference between these two methodologies is the data that are obtained. Immunohistochemistry allows the location of defined tissue antigens to be visualized through the binding of antibodies to small and unique regions on the antigens (29). Although immunohistochemistry is useful in determining the location of the marker of interest, the scoring of the slides is based on a semiquantitative scale that is not referenced to a known internal protein concentration but to a known positive control tissue. This scale measures the number of tumor cells stained but not the intensity of the staining. This may vary with the age of the paraffin blocks (30). Advances in image cytometry technology may provide a way to further standardize the scoring process allowing for improved quantification. Currently, determination of relative gene expression through quantitative real-time PCR is considered to be a more sensitive and more quantitative methodology than immunohistochemistry (31). This method uses an internal reference gene as a control for DNA quality for each sample assayed. It also acts as internal baseline measurement of the amount of DNA present, thus allowing the ability to differentiate smaller differences between samples and making these results more precise than immunohistochemistry-measured protein expression. However, the universal application of RT-PCR has not arrived in the clinical setting due to the significant cost of reagents, technology, and skilled labor.

In conclusion, our data suggest an increase in gene expression of \textit{GSTP1}, \textit{ERCC1}, and \textit{TS1} and that the increase in the number of the genes expressed is associated with a decrease in survival. These results seem to define a subset of patients who would gain the most benefit from neoadjuvant chemotherapy. The investigators are currently testing this conclusion in a comparison study of gene expression (of \textit{GSTP1}, \textit{ERCC1}, and \textit{TS1}) in patients treated with neoadjuvant chemotherapy plus radiation plus surgery and those treated with surgery alone. These data should be validated in a large prospective cohort of patients.

\begin{table}
\centering
\begin{tabular}{|l|c|c|c|c|}
\hline
Marker & Hazard ratio & (95\% confidence interval) & SE & Z & P \\
\hline
ERCC1 & 9.39 (0.902-3.58) & 0.68 & 3.28 & 0.001 \\
TS1 & 4.48 (0.232-2.77) & 0.65 & 2.32 & 0.020 \\
GSTP1 & 2.5 (0.224-1.81) & 0.46 & 2.01 & 0.045 \\
\hline
\end{tabular}
\caption{Multivariate model for survival}
\end{table}

\textbf{Table 6.} Multivariate model for survival.

\textbf{REFERENCES}


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