# The Prognostic Value of Molecular Marker Analysis in Patients Treated with Trimodality Therapy for Esophageal Cancer

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## ABSTRACT

**The purpose of this study was to define the prognostic value of a group of molecular tumor markers in a well-staged population of patients treated with trimodality therapy for esophageal cancer. The original pretreatment paraffin-embedded endoscopic esophageal tumor biopsy material was obtained from 118 patients treated with concurrent cisplatin + 5-fluorouracil (5-FU) + 45 Gy radiation followed by resection from 1986 until 1997 at the Duke University Comprehensive Cancer Center. Three markers of possible platinum chemotherapy association [metallothionein (MT), glutathione *S*-transferase-π (GST-π), P-glycoprotein (P-gp or multidrug resistance)] and one marker of possible 5-FU association [thymidylate synthase (TS)] were measured using immunohistochemistry. The median cancer-free survival was 25.0 months, with a significantly improved survival for the 38 patients who had a complete response ($P < 0.001$). High-level expression of GST-π, P-gp, and TS were associated with a decreased survival. MT was not significant in this population. Multivariate analysis identified high-level expression in two of the platinum markers (GST-π and P-gp) and the 5-FU marker TS as independent predictors of early recurrence and death. In conclusion, this investigation measured three possible markers associated with platinum and one possible marker associated with 5-FU in a cohort of esophageal cancer patients. Independent prognostic significance was observed, which suggests that it may be possible to predict which patients may benefit most from trimodality therapy. These data need to be reproduced in a prospective investigation.**

## INTRODUCTION

Esophageal cancer occurs in approximately 14,000 patients in the United States annually and has a dismal overall survival (10%). Recent advances in chemotherapy and radiotherapy have demonstrated a slight improvement (1). Prospective, Phase II, single-institution data suggest that the trimodality therapy (chemotherapy + radiotherapy + resection) results in a 20–30% rate of no viable tumor in the surgical specimen (pathological complete response). These patients enjoy an improved survival (2). Unfortunately, one cannot predict who will be in this subset of patients, and those without a complete response have a similar survival to resection-alone patients. There have been several recent prospective randomized trials comparing chemotherapy with or without radiation followed by resection compared with resection alone. All but one (3) have demonstrated 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 (4–7). Therefore, if one could identify the patients likely to respond to a particular regimen, one could tailor the subsequent therapy accordingly.

Our objective in this study was to show that a group of molecular tissue markers can be identified which may have prognostic significance for patients undergoing chemotherapy and/or radiotherapy in a population of patients with esophageal cancer.

## MATERIALS AND METHODS

Recent advances have refined the understanding of the mechanisms of action and resistance to many of the commonly used cancer therapies. Table 1 lists the proposed mechanisms of action and resistance to the antimetabolite 5-FU,[2] the alkylating agent cisplatin, and external beam radiation therapy. These are the treatments most often used in Phase II and randomized Phase III esophageal cancer trials. Using these defined mechanisms, we have reviewed the literature with respect to molecular markers of treatment response or prognosis in both animal models and human trials. There were few data on more than two markers and all of the human data were acquired on small patient series with short follow-up intervals (8–19). Therefore, we designed an investigation using tumor tissue from our experience with trimodality therapy for esophageal cancer.

### Population

The esophageal cancer registry was accessed for patients who underwent trimodality therapy for esophageal cancer from 1986 until 1997 at the Duke University Comprehensive Cancer

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[2] The abbreviations used are: 5 FU, 5-fluorouracil; TS, thymidylate synthase; GST-π, glutathione *S*-transferase-π; P-gp, P-glycoprotein; MT, metallothionein; MDR, multidrug resistance.

*Table 1* Mechanisms of action and resistance to therapies

1. 5-FU: An antimetabolite (interferes with DNA/RNA synthesis) that is cell-cycle specific (synthetic periods) because its metabolite, FdUMP,[a] binds/inhibits TS. It can be blocked by:
   Enzyme deregulation/poor activation
   Alternative thymidine synthetic pathways
   Alterations in the binding site of TS
   Enzyme gene amplification (increased levels of TS)
   Increased pool of competing base-precursors (dUMP)
   Increased degradation of FdUMP (dihydropyrimidine de-hydrogenase)
2. Platinum-containing drugs: An alkylating agent that is non-cell-cycle specific ($G_1$-M $\gg$ $G_0$). Cisplatin and carboplatin irreversibly cross-links DNA strands with platinum and blocks DNA repair after damage, signaling apoptosis. Cisplatin resistance:
   Improved DNA repair
   Mismatch repair proteins that recognize DNA damage and attempt repairs; if unable to repair, they signal the apoptosis cascade.
   hMSH-2 (human MutS homolog-2)
   hMLH-1 (human MutL homolog-1)
   ERCC-1 (excision repair −1)
   Increased intracellular levels of heavy metal chelators:
   MT
   Increased intracellular glutathione levels
   GST-π binds to platinum and allows exportation from cytosol using a MDR (P-gp) energy-dependent efflux pump mechanism.
3. Irradiation: Irradiation photons release excited electrons: $H_2O + O_2$ to form $O_3$-OH-free radicals. These cause DNA double-strand breaks "reproductive death" and activate the apoptotic pathway. Radiation is blocked by:
   Oxygen free radical scavengers (*i.e.*, glutathione, reactivated by GST-π)
   Lack of available substrates (hypoxia)

[a] FdUMP, 5-fluoro-dUMP.

*Table 2* Pretreatment clinical stage by computed tomography (CT)[a]

| TNM Stage | (n) |
|---|---|
| $T_1N_0$ | 6 |
| $T_2N_0$ | 32 |
| $T_3N_0$ | 50 |
| $T_2N_1$ | 7 |
| $T_3N_1$ | 16 |
| $T_3/T_4N_0$ | 7 |

[a] Since 1994, Endo-esophageal ultrasound has been added to CT staging, and no patient was treated on this protocol with documented $T_4$ invasion prior to therapy. Prior to that, if the CT scan suggested that the tumor abutted the aorta or bronchus without invasion, it was called $T_3/T_4$.

Center. The regimen included two cycles of 5-FU given as 800 mg/m$^2$ per day for 5 days or 1000 mg/m$^2$ per day for 4 days plus 75 mg/m$^2$ cisplatin with concurrent 45 Gy radiation followed by resection. For entry, each patient had to have completed the chemotherapy and the prescribed radiotherapy, undergone a gross complete resection, and lived at least 30 days after the surgery. All of the patients had their pathology reviewed to verify malignancy and had double-contrast chest/abdominal/pelvic computed tomography for clinical staging (Table 2). One hundred fifty-six patients were identified who met the study criteria. The original pretreatment paraffin-embedded, endoscopic esophageal tumor biopsy material was obtained from 126 of these patients. The original tumor blocks were recut and evaluated for viable tumor. One hundred eighteen patients had the tumor identified and were designated as the study population (mean age, 59 ± 11 years). There were 82 adenocarcinoma and 36 squamous carcinoma tumors (Table 3).

### Immunohistochemical Technique

**Antibodies.** Antibodies used for the immunohistochemical staining were as follows: mouse monoclonal IgG: anti-P-gp (JSB-1), anti-GST-π (both from BioGenex Laboratories, Inc., San Ramon, CA) and anti-MT (E9; DAKO, Carpinteria, CA). The anti-TS antibody is a monoclonal antibody from the National Cancer Institute laboratory of Dr. Allegra (20).

**Immunohistology.** The study material was formalin-fixed, paraffin-embedded tissue that was serially sectioned at 4–5 μm, deparaffinized in three changes of xylene, and then rehydrated in graded alcohols. After quenching endogenous peroxidase, the slides were gradually brought to water, then placed in citrate buffer (pH 6.0), and underwent Antigen Retrieval (United States Patent no. 5,244,787) or were incubated in pepsin at 37°C for 10 min. The slides were placed on the OptiMax PLUS automated slide stainer (BioGenex Laboratories, Inc.), and the following procedure was carried out. The sections were rinsed in three washes of PBS, preincubated in Power Block (BioGenex Laboratories, Inc.) for 8 min, and then incubated in a humidity chamber with primary antibodies. The reaction product was developed using the peroxidase-anti-peroxidase (PAP) method of detection, using the BioGenex BS-A (biotin streptavidin amplified) HRP (horseradish peroxidase) kit. This procedure includes a 20 min incubation with a biotinylated, affinity-purified secondary antibody, followed by a 20-min incubation with Avidin DH (biotinylated horseradish peroxidase H complex). The slides were developed with the chromogen diaminobenzidine. Finally, the slides are counterstained with hematoxylin. TS immunostaining and slide evaluation were performed by Dr. Carmen Allegra of the National Cancer Institute (21): each slide was assigned a score for intensity and staining pattern. Intensity scores ranged from 0 to 3+, and the staining pattern was either F (focal) or D (diffuse). For intensity, the scale was as follows: 0, no staining; 1+, light or trace staining; 2+, definite staining of light to moderate intensity; and 3+, bright and/or dark intensity. Slides with 50% or fewer of malignant cells stained at the assigned intensity level

*Table 3* Univariate survival estimates

| Variable | (*n*) | Survival | | *P* |
|---|---|---|---|---|
| | | Median | 2-year | |
| Gender | | | | |
| Male | 103 | 21 mo | 48% | |
| Female | 15 | Not reached[a] | 76% | 0.13 |
| Age | | | | |
| ≤60 years | 62 | 19 mo | 35% | |
| >60 years | 56 | Not reached | 70% | 0.001 |
| Histology | | | | |
| Adenocarcinoma | 82 | 25 mo | 53% | |
| Squamous | 36 | 21 mo | 48% | 0.69 |
| Clinical stage | | | | |
| Stage 1 | 6 | Not reached | 80% | |
| Stage 2A | 82 | 27 mo | 51% | |
| Stage 2B | 7 | Not reached | 57% | |
| Stage 3 | 23 | 17 mo | 37% | 0.45 |
| Stage 1–2B | 95 | 31 mo | 53% | |
| Stage 3 | 23 | 17 mo | 37% | 0.21 |
| Clinical nodes | | | | |
| Negative | 89 | 32 mo | 54% | |
| Positive | 29 | 17 mo | 37% | 0.18 |
| Treatment response | | | | |
| None | 50 | 17 mo | 41% | |
| Partial | 30 | 20 mo | 40% | |
| Complete | 38 | Not reached | 78% | 0.0001 |
| Cisplatin marker overexpression | | | | |
| GST-π | | | | |
| Present | 85 | 21 mo | 46% | |
| Absent | 32 | Not reached | 60% | 0.02 |
| P-gp | | | | |
| Present | 68 | 21 mo | 45% | |
| Absent | 50 | Not reached | 61% | 0.07 |
| MT | | | | |
| Present | 86 | 25 mo | 51% | |
| Absent | 31 | 24 mo | 52% | 0.45 |
| 5-FU marker overexpression | | | | |
| TS | | | | |
| Present | 64 | 21.1 mo | 48% | |
| Absent | 50 | 47.1 mo | 58% | 0.04 |

[a] Not reached, median survival not obtained.

were considered focal, whereas those with >50% stained were scored as diffuse, with the final score being adjusted for the amount of tumor stained. All of the specimens were analyzed by three separate investigators (A. P., I. L., C. A.) blinded to clinical information. Discrepant scores were resolved by consensus.

**Slide Evaluation.** Known positive blocks were simultaneously prepared with each tissue assay, as well as IgG-negative control slides. Individual slides were read by three independent observers (D. H. H., M-B. M., T. A. S.) blinded to clinical information and classified as either positive or negative for the respective antibody on a semiquantitative scale: 0+, none; 1+, 1–20%, 2+, 21–50%; and 3+, >50%. This reproducible scale measures the number of tumor cells stained, not the intensity of stain present, which may vary with the age of the paraffin blocks. Discrepant scores were resolved by consensus.

### Statistical Considerations

Times were calculated from the date of tissue diagnosis until death or date of last follow-up. (The date of tissue diagnosis to the date of the institution of treatment was 14 days or less.) Cancer-free survival was defined as the time between diagnosis and first recurrence. Cancer-free survival was censored for patients who died without recurrence of their disease. The markers tested were dichotomized with the median score: GST-π, P-gp, and TS with 0 to 1+ *versus* 2 to 3+, and MT with 0 *versus* 1 to 3+. High-level expression was defined as the higher values for each marker. The log-rank test and Cox's proportional hazards model were used to examine the effect of various markers of (P-gp, MT, GST-π, TS) on the end points of cancer-free survival. The assumption of proportional hazards was assessed using rescaled Schoenfeld residuals (22). Kaplan-Meier's product limit estimator was used to graphically display cancer-free survival within strata defined by the potential prognostic variables. Response to therapy was defined as: none for gross residual tumor, partial response for microscopic tumor in the original mass with negative surgical margins and lymph nodes, and complete response for a pathologically negative specimen.

Several attempts were made to create a scale of treatment response in this population. As long as the pathological result had gross tumor irrespective of the change in size with treatment, the cancer-free survival was poor. Therefore, our statisticians thought that cancer-free survival was the only clinical relevant outcome variable. After multiple reanalyses, a subset of patients was defined as having an improved outcome using response on a 0-to-2 scale (0, persistent gross tumor; 1, nodes negative with only microscopic tumor in esophageal wall $T_2$ or less; 2, pathological complete response). This scale was significantly associated with cancer-free survival. Therefore, stepwise regression analysis was used to define any association between marker expression and treatment response as the end point.

## RESULTS

The follow-up after esophageal resection was complete in all of the patients for a median 35 months (range, 40–157 months). The median cancer-free survival was 25.0 months (Fig. 1*A*). Survival times were significantly different for the 38 patients who had a complete response, the 30 patients with a partial response, and the remainder with no response ($P < 0.001$; Fig. 1*B*). Table 3 demonstrates the univariate survival estimate for patient demographics, histopathology, and pretreatment clinical stage. Only increased patient age was significantly associated with an improved prognosis.

Table 3 also demonstrates the immunohistochemical results for the three markers of possible platinum association (MT, GST-p, P-gp) and for the possible 5-FU association marker (TS). High-level expression of P-gp, GST-π, and TS were associated with poor cancer-free survival (Fig. 2, *A–C*). MT was not significant in this population. Cross-tabulations were examined between the four markers. A significant relationship was identified for GST-π and MDR ($P = 0.05$), such that high and low scores were more likely to be observed in the same patients. Cox proportional hazards regression analysis was performed on the four markers separately and with the platinum markers combined. Each demonstrated significant independent associations with a decreased cancer-free survival (Table 4; Fig. 3, *A*

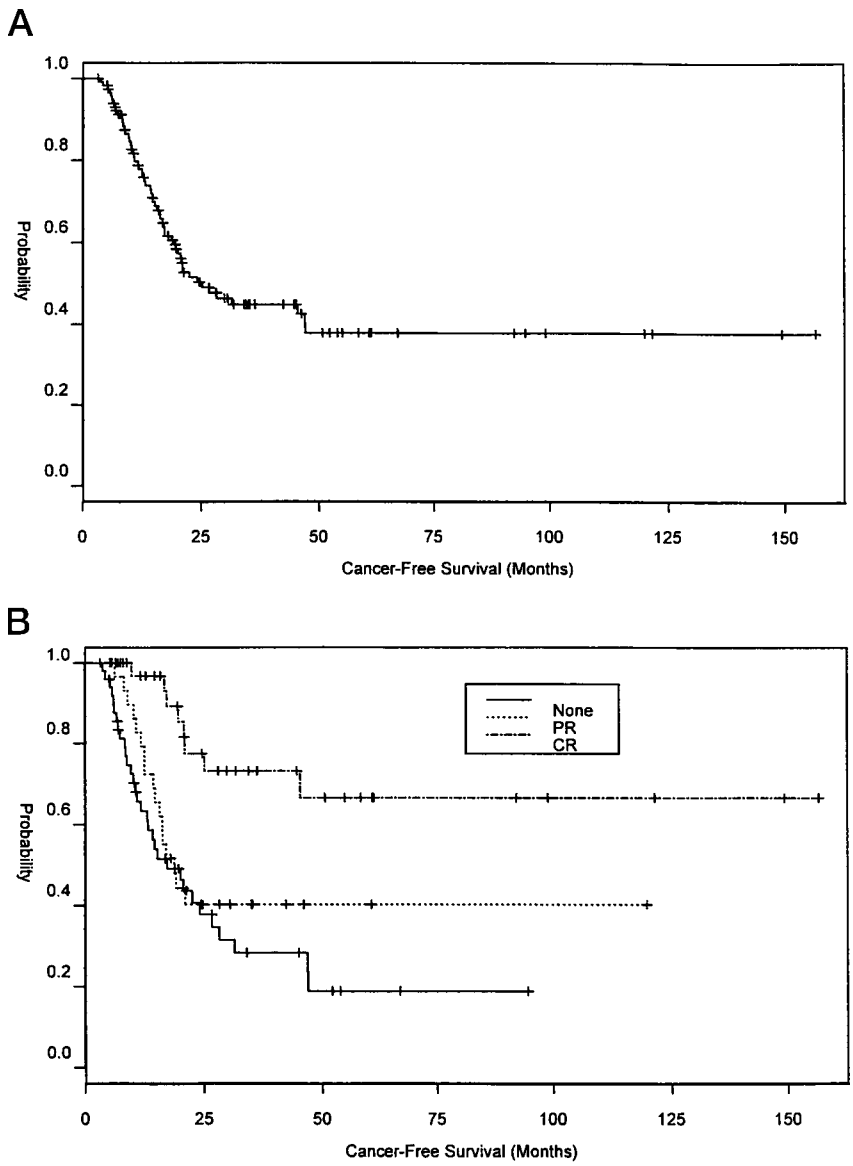


*Fig. 1* Kaplan-Meier survival estimate for the overall cancer-free survival is demonstrated for all of the 118 patients with a median survival of 25 months (*A*). The cancer-free survival estimates are demonstrated with respect to a measured pathological response to therapy at resection [no response ($n = 50$; median, 19 months); partial response ($n = 30$; median, 20 months); and complete response ($n = 38$; median, not reached); $P < 0.001$] (*B*). Kaplan Meier survival estimates are shown for cancer-free survival. *Tic marks on the curves*, censored data. Follow-up was complete for all of the 118 patients, with a median interval of 35 months.

and *B*). Additional multivariate analyses were completed for all of the univariate variables with a significant <0.25 (gender, age, stage). Age >60 years was added to the models from Table 4 (Table 5). Additionally, no first order interaction effects were observed between the markers with respect to recurrence or survival.

Analyses were performed to identify any association between marker expression and response as an end point instead of cancer-free survival. Two scales were used: pathological complete response *versus* all others (Model 1) and the 0-to-2 response scale defined by our statistician (Model 2). Logistic regression analyses was unable to associate any single marker with either response scale. The data were reanalyzed using TS and a variable created by combining the effect of the three platinum markers. An association with response was identified that would likely be significant in a larger population (Model 1: TS, $P = 0.13$; platinum combination, $P = 0.13$; Model 2: TS, $P = 0.24$, platinum combination, $P = 0.10$).

## DISCUSSION

Previous studies in the treatment of esophageal cancer have observed conflicting results with respect to the benefit of neoadjuvant chemotherapy with or without radiation followed by resection, compared with resection alone. These trials may have been flawed because of a number of potential problems. This would include a lack of consistent pretreatment staging (no computed tomography or endo-esophageal ultrasound requirement), too low a planned dose or inability to deliver the prescribed therapy because of poor performance status, and the use of agents that may not be the most active in esophageal cancer. Additionally, there appears to be an increasing incidence in

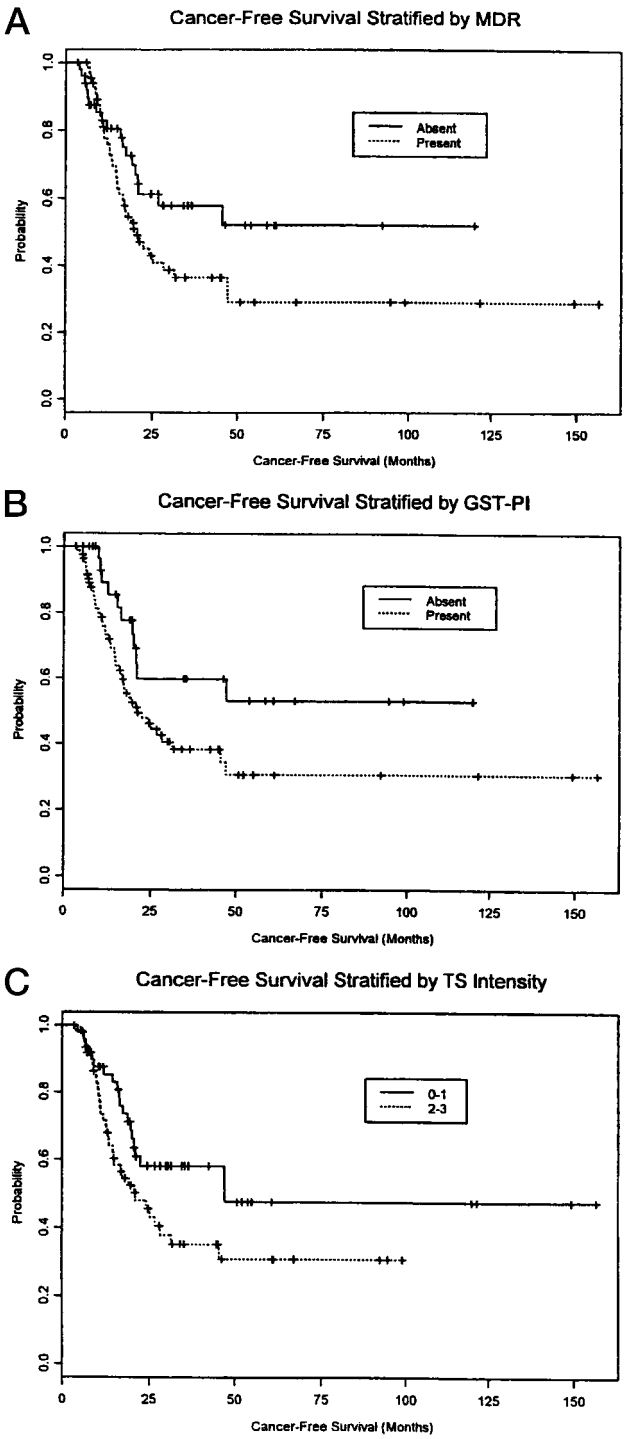


*Fig. 2* Kaplan-Meier cancer-free survival estimates are demonstrated for each of the markers with univariate significance: P-gp (*MDR*); GST-π, and TS (staining intensity score).

adenocarcinoma of the distal esophagus that has changed the demographics of the population of patients with esophageal cancer (23). Recent innovations in molecular biology have refined the method of action and treatment resistance for a number

*Table 4* Cox proportional hazards regression analysis of resistance markers

| Marker | Hazards ratio | SE | z | P |
|---|---|---|---|---|
| P-gp[a] | 1.52 | 0.298 | 1.41 | 0.160 |
| GST-π[a] | 1.82 | 0.357 | 1.68 | 0.093 |
| TS | 1.75 | 0.284 | 1.97 | 0.048 |
| Cisplatin resistance[b] | 1.64 | 0.210 | 2.37 | 0.018 |
| TS | 1.76 | 0.284 | 1.99 | 0.047 |

[a] If the two significant platinum markers were added separately, there was marginal significance for each. However, the total model remained significant at $P = 0.017$.

[b] A variable created as the number of cisplatin markers expressed (range, 0 to 2 for GST-π and P-gp) allowed a multivariate model with significant independent predictive value (total model, $P = 0.006$).

of therapies, including cisplatin, 5-FU, and radiotherapy. However, little data exist with respect to these novel investigations and esophageal cancer. This project was designed to evaluate a possible new methodology for selecting patients for a specific therapy. It is the largest of its type and was performed on tumors from a well-defined patient population. All of the patients underwent clinical staging prior to therapy that included at least a chest/abdomen/pelvic computed tomogram with oral and i.v. contrast. Table 2 demonstrates the stages observed prior to therapy; the majority of the patients were $T_3$- or $N_1$-positive. The treatment plan included the usual accepted doses of chemotherapy and radiation, which included the celiac axis.

We chose cancer-free survival as our major end point of this study for several reasons. First, because it is an end point often chosen for clinical trials and is a relevant outcome for the patients. Second, because the statistical results obtained were identical to those for cancer-specific survival. (All of the patients who recurred died of their malignancy.) Mortality, by any cause, was not used as an end point in the study because of the number of deaths in the follow-up interval who died of cardiac or pulmonary causes more than 60 days after discharge ($n = 26$).

Another possible outcome is treatment response. Quantifying a response to treatment is not easy for patients enrolled in a clinical Phase II trial. Radiological and clinical staging before treatment are often inaccurate, which makes it difficult to assess a clinical response when compared with the pathological results after therapy. Although this project used the best clinical staging modalities available for esophageal cancer, the task was even more problematic because of a lack of a standard scale to quantify a therapy response in esophageal cancer. For example, a patient with a large $T_3N_1$ tumor may be down-sized to a small $T_1N_1$ after therapy and resection but recur within 12 months of the initiation of therapy. (Any measured response would have little clinical relevance.)

The markers chosen in this study have been previously associated with the biological pathway of the two chemotherapy drugs administered (5-FU and cisplatin). Data exists with respect to likely drug resistance in other tumor models, such as colorectal and lung carcinoma. To absolutely rule out that these markers are not natural history prognostic markers would require a similar cohort of nontreated and followed esophageal cancer patients, not part of a Phase II treatment trial or this

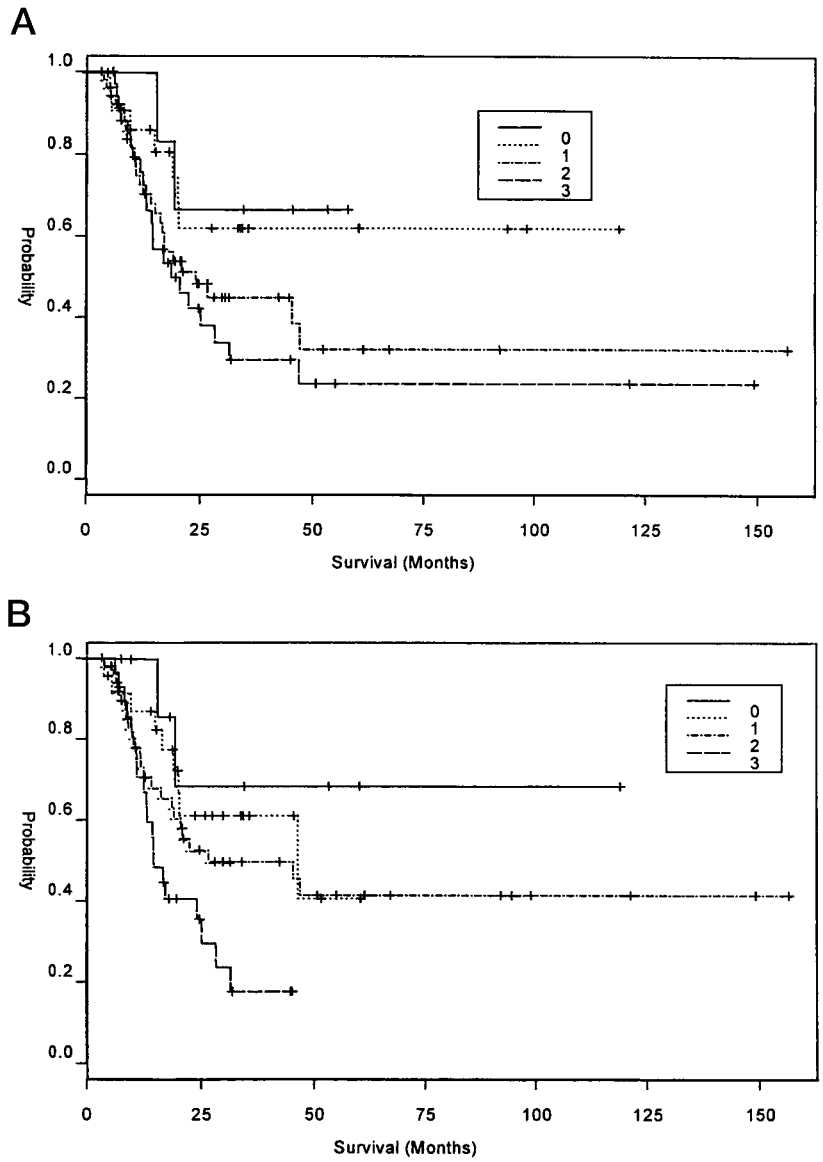


*Fig. 3* Kaplan-Meier cancer-free survival estimates are demonstrated for the multivariate analysis for overexpression all of the three markers of cisplatin [GST-π, MT, P-gp (MDR)] (*A*) and for the overexpression of the significant markers of cisplatin [GST-π, P-gp (MDR)] and 5-FU (TS) (*B*). These data demonstrate a step-wise decrease in survival with each additional positive marker.

project. However, we are presently evaluating expression of these markers in a population of patients with pathological $T_{1-3}N_0$ esophageal cancer treated by resection alone. This nonchemotherapy-treated dataset should help answer whether the prognostic significance is related to trimodality therapy or the natural history of the tumors.

Table 1 briefly lists possible modes of resistance for cisplatin, 5-FU, and radiation. Most of the data have been acquired for platinum agents. For example, once cisplatin is absorbed into the cytosol, it may be bound to MT, a heavy metal chelator that acts to detoxify the cell. Once bound, the platinum is removed. Hishikawa *et al.* (19) observed a significantly increased response rate and survival ($P = 0.02$) for 14 of 43 squamous esophageal cancer patients treated with cisplatin + concurrent radiation who were MT-negative prior to treatment. This project observed immunohistochemical evidence of MT overexpression in 73% of patients. However, the results did not reach statistical significance with respect to survival.

GST-π not only aids in the 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. Bai *et al.* (10) observed an increased response rate and a longer median survival ($P = 0.001$) for 38 non-small cell lung cancer patients without overexpression of GST-π measured on pretreatment biopsies. There were no data with respect to esophageal cancer prior to this project. We observed overexpression of GST-π in 73% of patients with a significantly decreased survival.

Other methods of inactivation of cisplatin include the DNA mismatch repair proteins such as ERCC1, MSH, and MLH. Preliminary data have associated overexpression of the proteins (or amplification of the genes) with platinum drug resistance in

*Table 5* Cox proportional hazards regression analysis of significant variables

| Marker | Hazards ratio | SE | *z* | *P* |
|---|---|---|---|---|
| Model Including All Factors | | | | |
| Age increase per yr | 0.394 | 0.289 | −3.12 | 0.0013 |
| MDR[a] | 1.65 | 0.286 | 1.76 | 0.079 |
| GST-π[a] | 1.84 | 0.338 | 1.80 | 0.070 |
| TS[a] | 1.49 | 0.286 | 1.97 | 0.16 |
| Compressed Model | | | | |
| Age increase per yr | 0.385 | 0.306 | −3.12 | 0.0018 |
| Cisplatin resistance[b] | 1.54 | 0.207 | 2.08 | 0.037 |
| TS | 1.56 | 0.288 | 1.54 | 0.12 |

[a] The addition of age to the model reduced the significance of the individual markers. Each additional year of age decreased the risk of death. However, the total overall effect of the three biological markers was statistically significant ($P = 0.03$; three degrees of freedom).

[b] A variable created as the number of cisplatin markers expressed (range, 0 to 2 for GST-π and P-gp) improved the significance of the total model ($P = 0.0001$).

animal models, and we are investigating these markers in esophageal cancer (12–14).

Several transmembrane proteins exist that act as an energy-dependent drug efflux pump. One family of these proteins includes the MDR protein P-gp. Darnton *et al.* (18) observed P-gp overexpression in 7 of 10 patients with adenocarcinoma of the esophagus treated with cisplatin + mitomycin and ifosfamide, but there were no data with respect to P-gp overexpression and survival. This project observed P-gp overexpression in 58% of patients with a significantly decreased survival. Cisplatin and 5-FU are usually not considered to be drugs that are detoxified by P-gp. However, there may be two possible explanations for the significance of overexpression of P-gp and a poor prognosis in our population. First, the antibody used in this project binds to all P-gp-like transmembrane proteins. It is possible that another of the P-gp family of proteins removes cisplatin. Second, the presence of overexpressed P-gp may actually signify a group of tumor cells that are metabolically active and can eliminate the chemotherapy agents by other mechanisms.

Over the last several years, much has been learned about the mechanisms of resistance to 5-FU in colorectal adenocarcinoma. Overexpression of TS appears to be a major method of resistance to 5-FU, and data from colorectal cancer patients suggest an association with TS and resistance to 5-FU (24–25). This project is the first to associate TS overexpression (observed in 56% of patients) with decreased survival in esophageal cancer.

Additional methods of resistance may be found in overexpression of dihydropyrimidine dehydrogenase. This enzyme breaks down 5-FU into inactive metabolites or interferes with thymidylate phosphorylase, which is an enzyme in one of the pathways for the activation of 5-FU. Investigations in our laboratory and others are evaluating these possible resistance markers.

In conclusion, this investigation measured three possible markers associated with platinum and one possible marker associated with 5-FU in a cohort of patients from a single institution. Multivariate analysis suggested an independent association between marker expression and cancer-free survival. These data need to be reproduced in a prospective investigation prior to using these techniques to select patients for specific therapies. The current national esophageal cancer treatment trial prospectively randomizes patients to trimodality therapy using a treatment regimen similar to the Duke protocol or esophageal resection alone. The authors are the principal investigators in a companion correlative science investigation for this trial. Biological markers will be evaluated as possible resistance markers in the trimodality patients ($n = 250$) or as possible natural history prognostic markers in the resection-alone patients ($n = 250$).

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