Combined GADD45A and Thymidine Phosphorylase Expression Levels Predict Response and Survival of Neoadjuvant-Treated Gastric Cancer Patients

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Abstract

Purpose: We evaluated the expression of seven therapy-related genes to predict the clinical outcome of advanced gastric cancer patients treated with a neoadjuvant chemotherapeutic protocol. Experimental Design: Pretherapeutic, formalin-fixed, and paraffin-embedded biopsies of 61 patients, who received a 5-fluorouracil (5-FU)– and cisplatin-based chemotherapy were studied. The expressions of the 5-FU– related genes TS, DPD, and TP and of the cisplatin-related genes ERCC1, ERCC4, KU80, and GADD45A were analyzed by quantitative real-time PCR. The expression levels of single genes and of various combinations were tested for an association with response and overall survival. Results: High DPD levels were more frequently found in nonresponding patients and were associated with worse survival. GADD45A and TP levels showed weak associations with response, but GADD45A expression correlated with survival. There was no association with response for TS expression, but tumors with a high TS level were associated with worse survival. The combination of GADD45A and TP revealed the strongest predictive effect. High expression values of TP and/or GADD45A were exclusively found in nonresponding patients (P = 0.002) and were associated with a significantly poorer survival (P = 0.04). Conclusions: Combined gene expression levels of TP and GADD45A represent a new variable to predict the clinical outcome after neoadjuvant chemotherapy in gastric cancer. The association of DPD expression with response and survival underlines a predominant role of DPD to predict 5-FU sensitivity. The association of TS expression levels with survival but not with response suggests an importance of this gene for tumor progression.

Gastric carcinoma is characterized by a high mortality rate that is mainly due to its diagnosis at late, advanced stages. Neoadjuvant chemotherapy has been used since 1989, but only 30% of the patients respond to treatment and the majority of patients undergo an expensive and potential harmful therapy without having any benefit (1,2). Thus, the identification of molecular genetic variables in pretherapeutic biopsies that could predict response is essential for the future development of neoadjuvant chemotherapy for gastric cancer patients.

5-Fluorouracil (5-FU) and cisplatin are major chemotherapeutic components used in the neoadjuvant treatment of advanced gastric carcinoma. 5-FU is an inhibitor of thymidylate synthase (TS), a key enzyme in nucleotide metabolism. In addition, dihydロプロミド dehydrogenase (DPD) and thymidine phosphorylase (TP) are important regulatory enzymes involved in 5-FU metabolism (3). Low levels of DPD have been shown to predict response in gastric carcinoma patients (4,5), but conflicting results have been reported for the expression of TS and TP (4–11).

The main effect of cisplatin is an induction of intrastrand and interstrand DNA cross-links (12). This type of DNA damage is mainly thought to be repaired by the nucleotide excision pathway; however, recent findings indicate that complex interactions between recombinatorial repair mechanisms involved in the repair of DNA double-strand breaks exist (13). Expression of ERCC1, which is mainly involved in nucleotide excision repair has been shown associated with response to cisplatin- and 5-FU–based chemotherapy in gastric carcinoma (8). Furthermore, expression of KU80, an enzyme involved in nonhomologous end joining repair as well as expression of GADD45A, which regulates G2-M transition and also stimulates nucleotide excision pathway, have been found increased in cell lines exhibiting resistance to cisplatin, indicating that they are
potential candidates for response prediction in cisplatin-based chemotherapeutic regimens (14, 15).

The goal of our study was to test the expression of three genes involved in 5-FU metabolism (TS, DPD, and TP) and four genes that function in DNA repair of cisplatin-induced DNA damage (ERCC1, ERCC4, KU80, and GADD45A) in pretherapeutic biopsies of patients receiving 5-FU- and cisplatin-based neoadjuvant chemotherapy for their potential use as predictive therapeutic markers. In addition, we tested, if any combination of the expression levels of the analyzed genes would have an increased effect for tumor response and patient survival prediction.

**Materials and Methods**

**Patient characteristics.** Pretherapeutic biopsies from 1993 to 2002 of 61 patients with locally advanced gastric cancer (tumor category cT3 or cT4), who were treated at the Department of Surgery with a combined preoperative chemotherapy containing cisplatin, leucovorin, and 5-FU (PLF regimen) were analyzed. Of the 61 patients, 48 (79%) had a complete tumor resection (R0), 10 (16%) had a residual resection (R1) after chemotherapy and three patients were not resected due to tumor progression. All 61 patients were evaluated clinically for response and were included in this study (none was lost to follow-up). The mean age of the patients was 55.1 years (range, 29.8-72.4), and there were 12 (20%) females and 49 (80%) males. In respect to the histopathologic classification, 29 (48%) were of the intestinal and 32 (52%) were of the nonintestinal type. In respect to tumor location, 46 (75%) were located in the proximal third, 10 (16%) in the middle, two (3%) in the distal third of the stomach, and three (5%) tumors encompassed the total stomach. The median follow-up of the patients was 27.2 months (range, 9.3-107.5), the median survival was 38.1 months (range, 5.5-107.5).

**Preoperative chemotherapy.** The preoperative chemotherapy protocol consisted of 50 mg/m² body surface area cisplatin at weeks 1, 3, and 5; 500 mg/m² body surface area leucovorin; and 2000 mg/m² body surface area 5-FU at weeks 1, 2, 3, 4, 5, and 6. The inclusion and exclusion criteria for chemotherapy were as published elsewhere (16).

**Response evaluation.** Response evaluation was done by measuring the size of the primary tumor by computer tomography scan, endoluminal ultrasound, and endoscopy. Responders were defined by an at least 50% reduction in the size of the primary tumor. Specifically, the definition of response required that the maximal wall thickness and length had decreased by ≥50% in computer tomography. No amount or only minimal amounts of residual tumor tissue could be present in endoscopy. Only when all these criteria were met was the tumor classified as responding. Patients with a tumor reduction of <50% or with newly detected metastatic lesions were classified as nonresponders (16, 20). According to clinical evaluation, there were 19 (31%) responders and 42 (69%) nonresponders.

For the initial analysis of the gene expression levels in association with response, patients with a congruent evaluation by both methods (n = 54) were used. This included 15 (28%) responders and 39 (72%) nonresponders.

Patient survival showed a statistically significant association with response, irrespective of the response evaluation method (histopathologic response, P = 0.0002; clinical response, P = 0.0005; congruent classification, P = 0.0001). Analysis of the gene expression levels looking for an association with survival was done for all 61 patients that were analyzed.

The study protocol was approved by the Institutional Review Board at the Technische Universität München (Munich, Germany).

**RNA extraction from archival tissues and reverse transcription.** Total RNA from gastric cancer specimens was extracted from formalin-fixed, paraffin-embedded tissue using 5-μm sections. For the isolation of RNA from formalin-fixed and paraffin-embedded tissues, we used the standard method published by Poustka et al. (21) with minor modifications. Microdissected sections were digested with proteinase K and RNA was purified by phenol and chloroform extraction and reversed transcribed as published (21, 22).

**Real-time quantitative reverse transcription-PCR.** Real-time quantitative reverse transcription-PCR was done using the ABI PRISM 7700 Sequence Detection System instrument and software (Applied Biosystems, Inc., Foster City, CA). Primers and probes were designed with the primer express software (Applied Biosystems), with the exception of GAPDH, which were synthesized according to published sequences (22). Primer and probe sequences are available from the authors on request. PCR was done in a final volume of 30 μL with the Taqman Universal PCR Master Mix (Applied Biosystems) using 5 μL cDNA, 300 nmol/L of each primer, and 200 nmol/L probe for the respective genes (except for GADD45A, for which a probe concentration of 300 nmol/L was used). Cycling conditions were 50°C for 10 seconds and 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute.

**Quantitation of expression.** Relative gene expression levels were determined by the standard curve method using a standard cDNA solution from the cell line Hsc45 for TS, DPD, ERCC1, ERCC4, and KU80 and from HT29 carcinoma cell line for TP and GADD45A. At least 5-fold dilution series (100-0.16 ng) were analyzed in duplicates for the genes of interest and were adjusted for variances in the amount of input of cDNA using GAPDH as an endogenous reference. GAPDH has been used as a reference gene in various studies analyzing gene expression levels in gastrointestinal carcinomas such as colorectal cancer (24). All standard curves were linear in the analyzed range with a correlation coefficient (R²) between 0.9903 and 0.9972.

Analyses of all tumor samples were done in at least two replicates, and the mean value was calculated. Only samples with values corresponding to SD < 20% were included in the study.

**Statistical analysis.** To test for an association of gene expression with response, dichotomization of the gene expression values as equal/above or below and above the median expression value of the respective genes was done and tested by Fisher’s exact test (two sided). Furthermore, we searched for an optimal cutoff value by categorizing the expression values into high and low values and tested by Fisher’s exact test for all possible cut points available from the data that guaranteed at least five patients per group.

If there was at least a trend for an association (P ≤ 0.1) in the group of patients with a congruent classification by clinical and histopathologic response evaluation, the respective cutoff values were also analyzed separately with respect to clinical and histopathologic response and for an association with survival by the log-rank test. If no cutoff value with P ≤ 0.1 was found for response, possible cut points were tested for an association with survival by log-rank analysis. For the
Expression of the 5-fluorouracil–related genes TS, DPD, and TP and association with response. For the initial analysis of gene expression in association with response, patients with a congruent evaluation by histopathologic and clinical response evaluation (n = 54) were used. The TS:GAPDH ratio varied from 0.14 to 1.06 in the 61 analyzed pretherapeutic biopsies. There was no significant association of the TS expression level with response (Fig. 1A; Table 1). The DPD:GAPDH ratio ranged from 1.49 × 10⁻³ to 48.88 × 10⁻³. Segregation of the tumors according to the median value of the DPD mRNA level (8.55 × 10⁻³) revealed a trend of an association with response (P = 0.07). An optimized cutoff value of ≤7.49 × 10⁻³ showed a clear association with response. Gene expression levels below or equal to this value were found more frequently in tumors of responding patients (P = 0.006; P_adj = 0.10; Fig. 1B; Table 1). This cutoff value revealed similar results when considered separately with respect to clinical (P = 0.003) and histopathologic (P = 0.09) response evaluation. The TP:GAPDH ratio ranged from 8.59 × 10⁻³ to 1539.2 × 10⁻³. Analysis of TP expression according to the median TP mRNA level (137.26 × 10⁻³) revealed no association with response. Categorizing TP into high and low expression values, all responding patients showed an expression level ≤347.71 × 10⁻³ (P = 0.05; P_adj > 0.1; Fig. 1C; Table 1). Results were similar with respect to clinical response evaluation (P = 0.01) but revealed no significant association with histopathologic response (P > 0.1).

Association between gene expression of the cisplatin-related genes ERCC1, ERCC4, KU80, and GADD45A with survival. For the initial analysis of gene expression in association with survival, patients with a congruent evaluation by histopathologic and clinical response evaluation (n = 54) were used. The GADD45A:GAPDH ratio varied from 10.66 × 10⁻³ to 139.22 × 10⁻³ and the KU80:GAPDH ratio ranged from 66.43 × 10⁻³ to 283.38 × 10⁻³. There was no correlation between expression and response for either gene (Fig. 1D; Table 1). The ERCC4:GAPDH ratio ranged from 4.28 × 10⁻³ to 71.65 × 10⁻³. Analysis of the median ERCC4 mRNA level (19.5 × 10⁻³) revealed no association with response. A weak association with response was observed for patients with an expression level ≤11.24 × 10⁻³ in their tumors and were more frequently found among responders (P = 0.07; P_adj > 0.1; Fig. 1E; Table 1). This cutoff value revealed a similar association for clinical response (P = 0.09) but not for histopathologic response (P > 0.1). The GADD45A:GAPDH expression ratio ranged from 3.83 × 10⁻³ to 188.56 × 10⁻³. The median GADD45 expression value (47.49 × 10⁻³) showed no association with response. However, all responding patients showed an expression value of ≤82.18 × 10⁻³ (P = 0.09; P_adj > 0.1; Fig. 1F; Table 1). This cutoff value was weakly associated with clinical response (P = 0.05) but not with histopathologic response (P > 0.1).

Association between combined gene expression of cisplatin- and 5-fluorouracil–related genes with response. Various combina-

Discussion
In this study, we analyzed mRNA expression of seven genes involved in 5-FU metabolism or DNA repair in pretherapeutic biopsies of 61 gastric cancer patients after preoperative 5-FU– and cisplatin-based chemotherapy to see if it was associated with therapy response and survival. The most interesting
finding of our study was the high predictive effect of the combined TP and GADD45A expression by the tumor. A significant association was seen with response, irrespective of the response evaluation method (histopathologic or clinical). Patients that had high expression levels of TP and/or GADD45A were nonresponders and had a significantly worse survival. To the best of our knowledge, this is the first report that has shown that the combined gene expression of TP and GADD45A may serve as a molecular genetic variable to predict the clinical outcome of gastric cancer patients treated by a neoadjuvant protocol. TP and GADD45A mRNA levels did not correlate with one other (data not shown), which may point to an additive effect of two independent mechanisms. TP is a key enzyme involved in 5-FU metabolism and catalyzes the conversion of 5-FU to FdUMP, which inhibits TS. Due to this function, TP expression has been studied as a possible predictive marker for 5-FU–based chemotherapeutic regimens in various cancers, but the data have been inconsistent (26, 27). In gastric cancer, no correlation between TP expression and response was found in the adjuvant setting (11). Others have reported correlation of high TP levels with response and high expression levels have been found to predict a more favorable prognosis after a postoperative chemotherapy (10, 28, 29). In contrast, high mRNA levels of TP correlated with nonresponse to 5-FU–based therapy in colorectal cancer patients (30), which is similar to the finding in our study. Based on the function of TP in 5-FU metabolism, one would expect a high TP expression associated with response. However, besides this function, TP has been shown to play an important role in angiogenesis, in cancer invasiveness and

![Fig. 1. Gene expression of 5-FU ^ and cisplatin-related genes grouped according to response (♦) and nonresponse (▲). Respective cutoff values for gene expression (dotted lines). A, TS; B, DPD; C, TP; D, KU80; E, ERCC4; F, GADD45A; G, TP and GADD45A.](https://www.aacrjournals.org)
metastasis and can inhibit apoptosis under hypoxic conditions (31–33). GADD45A is involved in cell cycle control and stimulates DNA repair after DNA damage (34). Thus, a high level of tumor GADD45A might counteract the effect of chemotherapeutic agents, which mainly induce DNA damage, as it is the case for cisplatin. Thus, our result suggests that, at least in a subset of patients, higher expression of GADD45A might contribute to nonresponse by enhancing DNA repair. This is in line with the finding that a colon carcinoma cell line that overexpresses GADD45A shows higher resistance to cisplatin (15).

Regarding the gene expression levels of the other genes, an association with response and survival was observed for DPD, with a low DPD level being associated with response and prolonged survival. This is in accordance to other studies demonstrating that a low DPD activity or low DPD mRNA levels were more frequently found in tumors of responders in gastric, colorectal as well as carcinomas of other organs (4, 30, 35, 36).

We found no correlation between TS gene expression and response. Despite this, patients with high TS tumor expression did have a worse prognosis. This suggests that TS expression has a role in tumor progression that is independent of its interaction with 5-FU. TS has been widely studied as a response predictor in 5-FU–containing regimens, but for gastric cancer, inconsistent results exist. High TS expression has been found associated with nonresponse by some authors (7–9), whereas a lack of association between TS expression and response has been reported by other (6, 37). In addition, high TS expression has been reported in nonchemotherapeutic settings to be correlated with worse prognosis in gastric carcinoma and in carcinomas of other organs (38, 39). Our results underline the findings of a previous study in gastric cancer, which suggested that TS has a more important role for tumor progression and DPD may be the dominant predictor of 5-FU sensitivity (37).

Genes involved in nucleotide excision repair have been suggested to be important for the susceptibility to treatment with cisplatin and in particular, ERCC1 mRNA levels showed a significant relationship to response in a cisplatin- and 5-FU–based neoadjuvant treatment of gastric cancer (8). Although we did not find a correlation of ERCC1 expression neither with response nor with survival in our study, a weak association with response and survival was observed for ERCC4, pointing to a more predominant role of this repair gene in our patient group. Conflicting results between different studies may be related to biological variations of the analyzed tumors or to variations in respect to the chemotherapeutic protocol or to response evaluation. In addition, sampling errors with a variation of stromal cells may contribute to an inconsistency of the results, in particular when analyzing tumors with a predominantly scattered growth pattern, which is typically for diffuse-type gastric carcinoma. However, in our study, we used laser-based microdissection in these cases to ensure that the tumor cell content for RNA extraction was at least 60% and corresponded to the criteria used for the isolation done by manual microdissection.

Response evaluation in our study was done according to clinical and histopathologic methods. Both methods have been shown highly correlated to the survival of the patients and are accepted methods for response evaluation. Nevertheless, both methods have some limitations. Evaluation of tumor response by endoscopy and imaging techniques has

<table>
<thead>
<tr>
<th>Gene</th>
<th>Relative gene expression levels (×10⁻³)</th>
<th>Responders (%)</th>
<th>Nonresponders (%)</th>
<th>P*</th>
<th>P adj.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS</td>
<td></td>
<td>—</td>
<td>—</td>
<td>&gt;0.1</td>
<td>—</td>
</tr>
<tr>
<td>DPD</td>
<td>≤7.49</td>
<td>11 (73)</td>
<td>12 (31)</td>
<td>0.006</td>
<td>0.103</td>
</tr>
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<td></td>
<td>&gt;7.49</td>
<td>4 (27)</td>
<td>27 (69)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TP</td>
<td>≤347.71</td>
<td>15 (100)</td>
<td>29 (74)</td>
<td>0.05</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td></td>
<td>&gt;347.71</td>
<td>0 (0)</td>
<td>10 (26)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERCC1</td>
<td></td>
<td>—</td>
<td>—</td>
<td>&gt;0.1</td>
<td>—</td>
</tr>
<tr>
<td>ERCC4</td>
<td>≤11.24</td>
<td>6 (40)</td>
<td>6 (15)</td>
<td>0.07</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td></td>
<td>&gt;11.24</td>
<td>9 (50)</td>
<td>33 (74)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GADD45A</td>
<td>≤82.18</td>
<td>15 (100)</td>
<td>31 (79)</td>
<td>0.09</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td></td>
<td>&gt;82.18</td>
<td>0 (0)</td>
<td>8 (21)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KU80</td>
<td></td>
<td>—</td>
<td>—</td>
<td>&gt;0.1</td>
<td>—</td>
</tr>
<tr>
<td>TP and GADD45A</td>
<td>≤82.18 and ≤347.71</td>
<td>15 (100)</td>
<td>22 (56)</td>
<td>0.002</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>&gt;82.18 and/or &gt;347.71</td>
<td>0 (0)</td>
<td>17 (44)</td>
<td></td>
<td></td>
</tr>
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</table>

*Based on Fisher’s exact test (two sided).
†P value after adjustment for multiple testing according to Hilsenbeck and Clark (25).
‡Multiple testing for the combined variables has not been done, as only the respective cutoff values determined by the single gene analysis were tested.
particular limitations in gastric cancer. According to the WHO criteria, gastric cancer is not bidimensionally measurable and the wall thickness may be dependent on the distension of the stomach during examination (40). Furthermore, criteria from the Response Evaluation Criteria in Solid Tumors Group are not validated for neoadjuvant-treated gastric cancer (41). Due to these problems, evaluation of histopathologic tumor regression has been used to determine response in some recent studies (17, 18, 42). However, histopathologic evaluation has also its limitations, in particular when determining the size of the initial tumor bed as in the case of exophytic tumors, which might have been reduced in size due to chemotherapy. The strength of our study is that our patients were evaluated for response by clinical as well as histopathologic methods. Patients classified by both methods as responders and nonresponders, respectively, should represent the most suitable groups for the initial determination of optimal cutoff gene expression values. The optimal statistical method for the evaluation of expression data is a matter of debate, and we are aware that

![Gene expression of 5-FU-related and cisplatin-related genes and survival](https://www.aacrjournals.org/clin-cancer-research/article-pdf/11/8/3029/579167/11005852.pdf)

Fig. 2. Gene expression of 5-FU-related and cisplatin-related genes and survival. Kaplan-Meier plots of overall survival from start of chemotherapy according to the respective cutoff values for gene expression. A, TS; B, DPD; C, TP; D, KU80; E, ERCC4; F, GADD45A; G, TP and GADD45A.
the determined cutoff values have to be evaluated in a subsequent prospective study. Response of a tumor to chemotherapy treatment is a complex biological process. Thus, it seems plausible that the combined evaluation of variables, as it was seen for the combined analysis of TP and GADD45A in our study, might have a higher predictive power than single gene analysis.

In conclusion, our study shows for the first time that the combined gene expression levels of TP and GADD45A are associated with response and overall survival after neoadjuvant chemotherapy in gastric cancer. The association of DPD expression with response and survival underlines a predominant role of DPD to predict 5-FU sensitivity. Furthermore, our result of the association of the gene expression of TS with survival, but not with response, support the suggested predominant role of this gene for tumor progression.

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References

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