Predictive Biomarkers and Personalized Medicine

Thymidylate Synthase and Excision Repair Cross-Complementing Group-1 as Predictors of Responsiveness in Mesothelioma Patients Treated with Pemetrexed/Carboplatin

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Abstract

Purpose: The pemetrexed/platinum agent combination represents the standard of care in first-line treatment for malignant pleural mesothelioma (MPM). However, there are no established indicators of responsiveness that can be used to optimize the treatment. This retrospective study aimed to assess the role of excision repair cross-complementing group-1 (ERCC1) and thymidylate synthase (TS) in tumors, and correlate expression levels and polymorphisms of these key determinants of drug activity with the outcome of MPM patients treated with carboplatin/pemetrexed in first-line setting.

Experimental design: Analysis of TS and ERCC1 polymorphisms, mRNA and protein expression was done by PCR and immunohistochemistry (with the H-score [histologic score]) in tumor specimens from 126 MPM patients, including 99 carboplatin-/pemetrexed-treated patients.

Results: A significant correlation between low TS protein expression and disease control (DC) to carboplatin/pemetrexed therapy ($P=0.027$), longer progression-free survival (PFS; $P=0.017$), and longer overall survival (OS; $P=0.022$) was found when patients were categorized according to median H-score. However, patients with the higher tertile of TS mRNA expression correlated with higher risk of developing progressive disease ($P=0.022$), shorter PFS ($P<0.001$), and shorter OS ($P<0.001$). At multivariate analysis, the higher tertile of TS mRNA level and TS H-score confirmed their independent prognostic role for DC, PFS, and OS. No significant associations were found among ERCC1 protein expression, TS and ERCC1 polymorphisms, and clinical outcome.

Conclusions: In our series of carboplatin-/pemetrexed-treated MPM patients, low TS protein and mRNA levels were significantly associated to DC, improved PFS, and OS. Prospective trials for the validation of the prognostic/predictive role of TS in MPM patients treated with pemetrexed-based regimens are warranted.

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Introduction

Malignant pleural mesothelioma (MPM) is an aggressive tumor with a poor prognosis. Its incidence is increasing throughout the world and is predicted to increase in the next 10 to 15 years in the Western European countries (1).

The majority of patients (up to 80%) are diagnosed in stage III/IV, and systemic therapy represents the only potential treatment option for most cases (2). Two prognostic scoring systems have been reported (3, 4), but there are no clinical/biological markers that can be used to optimize the treatment.

The combination of cisplatin and pemetrexed has recently become the standard of care in the first-line treatment of MPM; it has improved the response rate (RR; 41.3% vs. 16.7%; $P<0.0001$), time to progression (TTP; 5.7 vs. 3.9 months; $P=0.001$), overall survival (OS; 12.1 vs. 9.3 months; $P=0.020$), and quality of life compared with cisplatin alone (3). Many patients with MPM are unfit to receive the cisplatin-based chemotherapy due to the increasing incidence of the disease in the elderly population and the poor performance status often observed at onset. Pemetrexed alone (6) or combined with carboplatin (7, 8) has been proposed as alternative treatment choices for these patients.

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Translational Relevance

The identification of predictors of effective therapy that can be easily detectable in the clinical routine is crucial for maximizing therapeutic efficacy and minimizing useless treatment in patients with cancer. In this study, the analysis of thymidylate synthase (TS) and excision repair cross-complementing group-1 polymorphisms, mRNA, and protein expression was done by PCR and immunohistochemistry (with the histologic score) in tumor specimens from 126 malignant pleural mesothelioma patients, including 99 carboplatin-/pemetrexed-treated patients. This is the first study showing that the immunohistochemical detection of TS expression is a predictor of clinical outcome. In fact, compared with patients with high TS expression, the patients with low TS expression had a significantly higher probability to achieve disease control to carboplatin/pemetrexed chemotherapy, a significantly longer progression-free survival, and a significantly longer overall survival. Moreover, the TS mRNA analysis confirmed immunohistochemical data.

In an attempt to improve the activity and efficacy of current chemotherapy regimens, a pharmacogenetic approach has been advocated. Platinum compounds act through the formation of platinum-DNA adducts. Removal of these adducts, which leads to chemoresistance, is mainly carried out by the nucleotide excision repair (NER) system that consists of at least 30 identified polypeptides, including the pivotal protein excision repair cross-complementing group-1 (ERCC1; ref. 9). It is hypothesized that low expression of ERCC1 might predict increased sensitivity to platinum-based chemotherapy, possibly due to the saturation of the enzyme complex; conversely, high levels of ERCC1 may predict a resistance to platinum-based chemotherapy. Low ERCC1 protein expression was related to improved outcomes for adjuvant platinum–based chemotherapies in non–small-cell lung cancer (NSCLC) patients (10). Other studies have shown a correlation between increased gene and/or protein ERCC1 expression and unresponsiveness and/or decreased survival after platinum-based chemotherapy in different tumor types (11–14). However, a high ERCC1 level might also be a positive prognostic variable because it may increase the removal of carcinogenic DNA lesions. In NSCLC patients that received only surgical treatment, high expression levels of ERCC1 protein were significantly associated with longer disease-free survival (DFS) and OS (15). In a small population of platinum-treated MPM patients, patients in the lower tertile of protein ERCC1 expression had a significantly shorter survival (HR, 3.06; 95% CI, 1.08–8.69; \( P = 0.035 \); ref. 16).

Pemetrexed is a multitarget agent that is converted to a series of active polyglutamate derivatives by folylpolyglutamate synthetase. These metabolites inhibit 3 folate-dependent enzymes, thymidylate synthase (TS), dihydrofolate reductase (DHFR), and glycaminide ribonucleotide formyltransferase (GARFT; ref. 17). However, pemetrexed is a weak inhibitor of GARFT, and when TS is inhibited, tetrahydrofolate oxidation stops and there is no longer a need for DHFR activity (18). Therefore, most studies have focused on pemetrexed effects on TS. TS mRNA levels were inversely correlated with pemetrexed activity in different tumor cells (19, 20), whereas other studies suggested a correlation between high levels of TS protein expression and reduced sensitivity to pemetrexed in colon and lung cancer cells (21, 22). Furthermore, TS mRNA and protein expression were predictive of responsiveness in patients with advanced breast cancer treated with pemetrexed alone and in NSCLC patients treated with pemetrexed/gemcitabine neoadjuvant therapy, respectively (23). In a recent observational study, Righi and colleagues observed that low TS protein levels in MPM were predictive of longer TTP and OS. Conversely, they did not find a significant correlation between TS expression and outcome in MPM patients who were not treated with pemetrexed. However, TS mRNA and protein expression levels might also play a prognostic role, as reported in patients with NSCLC (24–26), whereas the sensitivity of pemetrexed was not predicted by the expression levels of TS in gastric cancers (27).

Furthermore, functional polymorphisms in both TS and ERCC1 gene may modulate their expression/activity and contribute to individual variations in chemotherapy response. In particular, NSCLC patients harbouring the ERCC1-C118C genotype had significantly longer OS after platinum combination chemotherapies (28), whereas colorectal cancer patients carrying the TS enhancer region (TSER) 28-bp tandem repeat TSER-2R/2R polymorphism had longer OS after TS inhibitors (e.g., 5-fluorouracil/capcitabine-based chemotherapy; refs. 29–31). However, recent meta-analysis showed that data on the predictive value of ERCC1 polymorphisms in advanced NSCLC, and on TSER genotypes in colorectal cancer patients receiving platinum-based chemotherapy are inconclusive (32, 33). These conflicting results suggest that pharmacogenetic associations may not always be reproducible when explored in small-size series, without standardized methods, and in different settings for tumor type, stage, and treatment. Further studies in larger homogeneous populations are warranted to understand the utility of candidate biomarkers (at DNA and/or RNA/protein level) in the prediction of outcome in specific clinical settings.

The aim of this study was to retrospectively determine whether ERCC1 and TS mRNA and protein expression as well as their functional polymorphisms can predict clinical outcome in a series of MPM patients treated with carboplatin/pemetrexed chemotherapy.

Material and Methods

Patients and samples

Patient selection was based on the following inclusion criteria: a diagnosis of advanced MPM, treatment with...
pemetrexed plus carboplatin in a first-line setting, and the availability of tumor tissue. The paraffin-embedded surgical or biopsy specimens were collected from the pathology files of the Istituto Clinico Humanitas, Rozzano, Milan, Italy. All patients received pemetrexed at 500 mg/m² and a carboplatin infusion with an area under the plasma concentration–time curve of 5 mg/mL/minutes, administered intravenously every 21 days. All patients received folic acid and vitamin B12 supplementation. Tumor staging and response evaluations were assessed according to the tumor node metastasis (TNM) staging system proposed by the International Mesothelioma Interest Group (34) and the modified Response Evaluation Criteria in Solid Tumors (RECIST; ref. 35), respectively. This study was conducted in agreement with the Declaration of Helsinki and approved by the appropriate ethical review board (ClinicalTrials.gov ID: NCT00867711).

Immunohistochemistry

Immunohistochemical studies were carried out with specific monoclonal mouse antihuman antibodies for ERCC1 (clone 8F1, dilution 1:50, Santa Cruz Biotechnology) and TS (clone 106, dilution 1:100, DAKO). The specimens were formalin-fixed, paraffin-embedded, and sliced into 2-µm-thick tissue sections.

To enhance the immunoreactivity, antigen retrieval was done for 30 minutes at room temperature in W-CAP pH 8.0 buffer (Bio-Optica) for both ERCC1 and TS. The endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 30 minutes, and the primary antibody incubation was carried out for 1 hour at room temperature. Negative controls were prepared by replacing the primary antibodies with buffer. The immunoreaction was revealed with a biotin-free detection system on the basis of a dextran polymer chain linked to the secondary antibody and peroxidase activity and denature the DNA, 50 cycles of denaturation at 95°C for 15 seconds followed by annealing and extension at 60℃ for 1 minute. Specific forward and reverse primers and probe were designed by Primer Express 2.0 software. The sections were reviewed and scored by 2 pathologists that were blinded to patient identity and clinical outcome. In agreement with previous studies (36), the results were interpreted by a system on the basis of staining intensity and the number of stained cells. Staining for ERCC1 was considered positive when tumor cells showed nuclear and cytoplasmic reactivity.

The percentage of positive tumor cells and the staining intensity were analyzed by a semiquantitative histologic score (H-score). In particular, the staining intensity of tumors—ranging from low (score 0) to moderate and high (scores 2 and 3)—was multiplied by the percentage of positive neoplastic cells (thus obtaining values from 0 to 300).

Endothelial cells from the tonsils served as an external positive control, and lymphocytes were used as an intratumoral-positive control.

DNA and RNA isolation

DNA and RNA were isolated from paraffin-embedded tumor samples, verified by the study pathologist (E. Thunnissen) to contain at least 50% of tumor cells. DNA was isolated by the microDNA Kit (Qiagen), as described previously (37), whereas RNA was isolated by the RecoverAll Total Nucleic Acid Isolation Kit (Ambion, Applied Biosystems) according to the manufacturer’s instructions. DNA and RNA yields and integrity were checked at 260 to 280 nm with NanoDrop-1000 Detector (NanoDrop-Technologies).

Reverse-transcription and quantitative PCR

RNA (10–500 ng) was reverse-transcribed with the DyNAmo cDNA Synthesis Kit (Finzymes Oy), and the resulting cDNA was amplified by the 7500HT Sequence Detection System (Applied Biosystems). PCR reactions were done in triplicate with 5 µL of cDNA, 12.5 µL of TaqMan Universal PCR Master Mix, 2.5 µL of probe, and 2.5 µL of forward and reverse primers in a final volume of 25 µL. Samples were amplified by the following thermal profile: an initial incubation at 50°C for 5 minutes to prevent the reamplification of carry-over PCR products by AmpErase uracil-N-glycosylase (UNG), followed by incubation at 95°C for 10 minutes to suppress AmpErase UNG activity and denature the DNA, 50 cycles of denaturation at 95°C for 15 seconds followed by annealing and extension at 60°C for 1 minute. Specific forward and reverse primers and probe were designed by Primer Express 2.0 (Applied Biosystems) on the basis of TS (NM_001071) and ERCC1 (NM_001983) gene sequence obtained from the GenBank, whereas primers and probe for β-actin were obtained from Applied Biosystems Assay-on-Demand products, as described previously (38). After validation experiments showing that efficiencies of the target (TS) and reference (β-actin) were approximately equal, gene expression quantification was performed by standard curves obtained with dilutions of cDNA from Quantitative-PCR Human-Reference Total-RNA (Stratagene). All the samples were amplified in triplicate with appropriate nontemplate controls, and the CV (coefficient of variance) was less than 2%.

Polymorphisms analysis

The ERCC1 C118T (rs11615) polymorphism was studied with TaqMan-probes–based assays by using the ABI PRISM-7500HT instrument. Specific primer and probe for ERCC1 C118T polymorphisms were obtained from Applied Biosystems (C__2532959_1). The PCR reactions were done with 10 ng of DNA diluted in 5.94 µL DNAse–RNAse-free water, 6.25 µL of TaqMan Universal PCR Master Mix, with AmpliTaq Gold, and 0.31 µL of the assay mix, including the specific primers and probes, in a total volume of 12.5 µL. After thermal cycling, the 7500HT instrument determined the allelic content of each sample in the plate by reading the generated fluorescence, using the SDS version 2.0 software. The TSER genotype was assessed by PCR amplification as described previously (39) Genomic DNA was amplified in
the enhancer region (5′UTR) of the TS gene by using a forward primer (5′-GTG GCT CCT GCG TTT CCT CC-3′) and a reverse primer (5′-GCT CCG AGC CCG CCA CAG GCA TGG CGC GG-3′). The reaction mixture for PCR contained magnesium-free buffer, 2.5 units of Taq DNA Polymerase, 1.0 mmol/L MgCl2, 0.2 mmol/L dNTPs (deoxynucleotide triphosphate), 10% DMSO (dimethyl sulfoxide), 0.2 μmol/L of each primer, and 2 μL of DNA. The mixture was overlaid with mineral oil and transferred to a thermal cycler for 30 cycles of PCR. Each cycle consisted of 1 minute at 94°C, 1 minute at 60°C, and 2 minutes at 72°C, followed by 5 minutes at 72°C after the last cycle. The amplified DNA fragments were separated by electrophoresis on a 3% agarose gel with ethidium bromide. PCR fragments of 220 (2R/2R), 248 bp (3R/3R), or both (2R/3R) were obtained. Genomic DNA from the heterozygous colon cancer cell line LS174T was used as a control in each experiment.

**Statistical analysis**

The objective of this study was to assess the role of 2 selected biomarkers (TS and ERCC1 protein expression) in the outcome of patients with MPM. Patient characteristics were described in terms of number and percentage, or median and range, when appropriate.

Modified RECIST criteria were used to classify the tumor response to treatment as complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD). The patients who showed disease control (DC), including CR, PR, and SD, were compared with patients with PD.

Logistic regression was carried out to determine the association between biomarker expression and therapy responsiveness, calculating the OR with 95% CIs. When a significant P value was found, receiver operating characteristic (ROC) curves were computed to find a cutoff value and obtain corresponding estimates of sensitivity and specificity. In the presence of an unsatisfactory area under the curve (AUC) value (lower than 0.70), the population was categorized according to median or tertile values of the considered marker.

Progression-free survival (PFS) was calculated from the first day of chemotherapy treatment until progression, death from any cause, or the last visit where a patient was alive without progression. OS was defined as the time between the start of treatment until patient death or last contact. OS and PFS were evaluated with the Kaplan–Meier method and groups were compared with the log-rank test. HRs with 95% CI were calculated with the Cox proportional-hazards regression model. Statistical significance was set at P < 0.05 for each evaluation. All statistical analyses were done with the R software package.

**Results**

**Pemetrexed-/carboplatin-treated patients**

Ninety-nine paraffin-embedded specimens were collected (70 specimens from biopsy and 29 from surgical treatment). Most tumor specimens (N = 87) analyzed were collected before carboplatin/pemetrexed chemotherapy in 87 patients, whereas 12 samples were collected after chemotherapy. The median follow-up of this population was 24 months (range 0–54 months). The median PFS and OS were 7 and 13 months, respectively. Patient characteristics and outcomes of study population according to the clinical characteristics are listed in Tables 1 and 2.

**TS protein and gene expression**

TS protein was detected by immunohistochemistry in 96 patients resulting in a median H-score of 21 (range 0–240; Fig. 1). Gene expression analyses from paraffin tumor tissues were successfully done in 67 cases (47 specimens from biopsies obtained during thorascopy and 20 from surgical treatment), and TS mRNA median level was 21.7 (range 2.8–88.4).

Both expression data were analyzed continuously, but by considering the unsatisfactory AUC values (TS protein = 0.67; TS mRNA = 0.69), the cutoff values were not identified. Then, the population was categorized according to median or tertile values. In a univariate analysis (Table 3), a significant correlation between low TS protein expression and DC to chemotherapy (P = 0.027), longer PFS (P = 0.017), and OS (P = 0.022) was found when patients were categorized according to median H-score (Fig. 2). Similarly, patients with TS mRNA level below the median had significantly longer PFS (P < 0.001) and OS (P < 0.001). In contrast, no significant association was found between TS mRNA levels and PFS or OS. A similar trend was observed for histologic subtype.

### Table 1. Characteristics of patients with MPM treated with pemetrexed plus carboplatin

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Number of patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>99 (100)</td>
</tr>
<tr>
<td>Age, years (median, range)</td>
<td>63 (35–84)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>74 (75.0)</td>
</tr>
<tr>
<td>Female</td>
<td>25 (25.0)</td>
</tr>
<tr>
<td>EORTC prognostic score</td>
<td></td>
</tr>
<tr>
<td>Good</td>
<td>43 (43.4)</td>
</tr>
<tr>
<td>Poor</td>
<td>55 (55.6)</td>
</tr>
<tr>
<td>Missing</td>
<td>1 (1.0)</td>
</tr>
<tr>
<td>Histologic subtype</td>
<td></td>
</tr>
<tr>
<td>Epithelial</td>
<td>91 (92.0)</td>
</tr>
<tr>
<td>Not epithelial</td>
<td>7 (7.0)</td>
</tr>
<tr>
<td>Unspecified</td>
<td>1 (1.0)</td>
</tr>
<tr>
<td>Response to treatment</td>
<td></td>
</tr>
<tr>
<td>CR</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>PR</td>
<td>28 (28.3)</td>
</tr>
<tr>
<td>SD</td>
<td>50 (50.5)</td>
</tr>
<tr>
<td>PD</td>
<td>16 (16.2)</td>
</tr>
<tr>
<td>Missing</td>
<td>5 (5.0)</td>
</tr>
</tbody>
</table>

Abbreviation: EORTC, European Organization for Research and Treatment of Cancer.
mRNA levels and DC ($P = 0.11$), but the higher tertile of TS mRNA expression correlated with higher risk of developing PD ($P = 0.022$), shorter PFS ($P < 0.001$), and shorter OS ($P < 0.001$; Fig. 3). At a multivariate analysis, TS mRNA and TS H-score confirmed their independent prognostic role for DC, PFS, and OS. In particular, patients in the higher tertile of TS mRNA expression presented a higher risk of developing PD (OR: 6.66; 95% CI, 1.47–33.33; $P = 0.014$), a shorter PFS (HR 3.09; 95% CI, 1.70–5.64; $P < 0.001$), and a shorter OS (HR 5.63; 95% CI, 2.84–11.16; $P < 0.001$), whereas patients with low TS protein expression had a higher probability to achieve a DC (OR 3.85; 95% CI, 0.37–0.93; $P = 0.025$), and a longer OS (HR 0.60; 95% CI, 0.37–0.98; $P = 0.041$). Interestingly, by considering patients with nonepithelial histology, 4 out of 6 patients had a high TS protein level (from 40 to 120) according to median H-score, while 5 of 5 patients had a high TS mRNA level (from 27.5 to 63.9) according to median value.

**ERCC1 protein expression**

The ERCC1 protein was detected in 67 patients with a median H-score of 60 (range 0–270), whereas the ERCC1 mRNA expression was detected in 43 patients with a median level of 8 (range 0–33).

### Table 2. Outcome of study population according to clinical characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>DCR (%)</th>
<th>Median PFS (months)</th>
<th>Median OS (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>82.9</td>
<td>7.0</td>
<td>13.0</td>
</tr>
<tr>
<td>Female</td>
<td>83.3</td>
<td>6.0</td>
<td>9.0</td>
</tr>
<tr>
<td>(P)</td>
<td>(1.00)</td>
<td>(0.92)</td>
<td>(0.64)</td>
</tr>
<tr>
<td><strong>Histology</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelial</td>
<td>84.9</td>
<td>7.0</td>
<td>13.0</td>
</tr>
<tr>
<td>Nonepithelial</td>
<td>57.1</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>(P)</td>
<td>(0.01)</td>
<td>(0.52)</td>
<td>(0.66)</td>
</tr>
<tr>
<td><strong>ECOG performance status</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>85.7</td>
<td>9.0</td>
<td>23.0</td>
</tr>
<tr>
<td>$\geq 1$</td>
<td>75.0</td>
<td>5.0</td>
<td>9.0</td>
</tr>
<tr>
<td>(P)</td>
<td>(0.359)</td>
<td>(0.013)</td>
<td>(0.013)</td>
</tr>
<tr>
<td><strong>EORTC Score</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Good</td>
<td>92.7</td>
<td>8.0</td>
<td>14.0</td>
</tr>
<tr>
<td>Poor</td>
<td>75.5</td>
<td>6.0</td>
<td>12.0</td>
</tr>
<tr>
<td>(P)</td>
<td>(0.03)</td>
<td>(0.41)</td>
<td>(0.88)</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;65 years</td>
<td>82.2</td>
<td>8.0</td>
<td>14.0</td>
</tr>
<tr>
<td>$\geq 65$ years</td>
<td>83.7</td>
<td>6.0</td>
<td>9.0</td>
</tr>
<tr>
<td>(P)</td>
<td>(0.85)</td>
<td>(0.15)</td>
<td>(0.03)</td>
</tr>
</tbody>
</table>

Abbreviations: DCR, DC rate; ECOG, Eastern Cooperative Oncology Group; EORTC, European Organization for Research and Treatment of Cancer; SL, second line.

![Figure 1. Examples of TS expression in an MPM tumor section stained with anti-TS antibody with an H-score below (A) the median value and with an H-score above (B) the median value (40×).](image)
Both expression data were analyzed continuously, but by considering the unsatisfactory AUC values (ERCC1 protein = 0.57; ERCC1 mRNA < 0.5), the cutoff values were not identified. No significant correlation was found among ERCC1 protein expression and PD (OR, 2.4, 95% CI, 0.68–8.39; P = 0.21), PFS (6.0 vs. 7.0 months; P = 0.675), and OS (12.0 vs. 18.0 months; P = 0.666) when patients were categorized according to median H-score (Table 3) or tertile values (data not shown). No correlations were also found for ERCC1 mRNA expression (data not shown).

### Polymorphisms analysis

The genotyping was successfully performed in DNA extracted from 91 tumors. The frequencies of ERCC1 C118T T/T, C/C, and C/T genotypes were 25.3%, 38.5%, and 36.2%, respectively. The TSER 2R/2R genotype was detected in 27.5%, whereas the TSER 3R/3R and TSER 2R/3R were reported in 29.7% and 42.8% of the patients, respectively. Both polymorphisms followed Hardy–Weinberg’s equilibrium and their allelic frequencies were comparable with those reported in previous studies in Caucasian population and the National Center for Biotechnology Information (NCBI) and National Cancer Institute (NCI)-SNP500 databases.

No significant correlations were found among these genotypes and clinical characteristics. By considering the histology, 3 patients with nonepithelial histology carried the TSER 2R/3R, 2 patients the 3R/3R, and 2 patients the 2R/2R genotype, whereas 4 patients with nonepithelial histology harbored the ERCC1 C118T, 2 patients the C/C, and 1 patient the T/T genotype.
None of the different variants of ERCC1-C118T and TSER was related to DC, PFS, and OS (Table 3).

Control population

The potential prognostic role of TS and ERCC1 was further investigated in a series of 37 MPM patients not CP (cyclophosphamide)-treated. The median follow-up of this population was 93 months (range 0–100 months). The median OS was 13 months.

No significant correlation was found among OS and TS protein expression categorized according to median H-score \((P = 0.697)\), ERCC1 (data not shown), and TS mRNA level (categorized both according to median value, \(P = 0.560\); and considering the higher tertile of TS mRNA expression, \(P = 0.194\) ) ERCC1 C118T \((all: P = 0.891; T/T vs. C/T+ C/C: P = 0.696; C/C vs. C/T+T/T: P = 0.995)\), and TSER 2R/3R polymorphisms \((all: P = 0.265; 2R/2R vs. 2R/3R+3R/3R: P = 0.996; 3R/3R vs. 2R/3R+2R/2R: P = 0.129)\).

Discussion

This study evaluated the differential expression of key determinants of drug activity in MPM specimens and shows the relation among TS expression and response, TTP, and OS in a large series of patients treated with a pemetrexed/carboplatin regimen.

The identification of predictors of effective therapy that can be easily detectable in the clinical routine is crucial for maximizing therapeutic efficacy and minimizing useless treatment in patients with cancer. It is now recognized that the way a patient responds to chemotherapy is a complex
trait, influenced by the tumor characteristics and individual genetic constitution. Therefore, the search of different predictive biomarkers to define optimal treatment regimens represents an intriguing modern challenge (40, 41). This approach could help in the selection of drugs best suited to the individual patient and allow physicians to avoid unnecessary toxicity and hospitalization, preserving economical and human resources.

The combination of cisplatin/carboplatin and pemetrexed represents the standard of care in the first-line treatment of MPM. However, more than one third of patients do not respond to this schedule and are exposed to useless toxicity. Almost no data are available on possible predictors of responsiveness in this clinical setting. Recently, Righi and colleagues (15) reported that a long-term survivor of MPM with an excellent clinical outcome had low TS expression and protein expression in 1 patient who experienced a long-term survival and a continuous effect of pemetrexed, whereas high TS expression was detected in the most aggressive case. However, the treatment schedule and the correlation with patient responsiveness were not reported (42).

In our study, we retrospectively evaluated whether the expression of ERCC1 or TS and their main functional polymorphisms could predict clinical outcome in a homogeneous population of 99 MPM patients treated with the carboplatin/pemetrexed regimen described in our previous studies (7, 8).

This is the first study showing that the immunohistochemical detection of TS expression is a predictor of clinical outcome. By comparing the patients with high TS expression, the patients with low TS expression had a significantly higher probability to achieve DC to carboplatin/pemetrexed chemotherapy. In particular, when the median H-score value was considered, the possibility to achieve a DC of progression compared with patients with high TS expression, and similar results were observed for OS. Although Righi and colleagues (15) did not find a correlation between TS mRNA expression and outcome, suggesting that the poor results obtained by mRNA analysis were determined by technical limits due to limited amounts of tumor cells in MPM specimens; in our study, the analysis of TS mRNA confirmed immunohistochemical data. Patients with TS mRNA level below the median had significantly longer PFS and OS. Moreover, the higher tertile of TS mRNA expression correlated with a higher risk of developing PD, shorter PFS, and shorter OS. At multivariate analysis, both TS mRNA level and TS H-score confirmed their independent prognostic role for DC, PFS, and OS.

Several previous studies showed that ERCC1 expression was correlated with clinical outcome in patients treated with platinum-based regimens. However, no significant associations between ERCC1 expression and outcome were observed in this study. There may be multiple explanations for this discrepancy. Most published studies are conducted in different tumor types, with a variety of treatment regimens, and different patient populations. In this study, all the patients were treated with the same regime. The inclusion of carboplatin might have produced different results from therapies that included cisplatin, because they have different mechanisms of DNA repair, producing d(GpNpG)Pt and d(GpG)Pt adducts, respectively (43). In this study, all the patients were Caucasian, recruited in 1 center, and treated with the same regimen. This homogeneity was implemented to improve the reliability of tissue sampling, preparation, and immunohistochemical/PCR analysis. Nevertheless, the general high ERCC1 expression observed could explain the low chemosensitivity of patients with MPM to cisplatin as single agents, with an RR < 20% (5, 44). However, due to the increase in dUTP (2'-deoxyuridine 5'-triphosphate) levels caused by TS inhibition, other members of the DNA repair pathways might also have played a role in regulating pemetrexed activity. In particular, dUTP incorporation into the DNA is toxic, but is mainly counteracted by the uracil DNA glycosylase encoded by the CCNO/UNG2 gene. The DNA glycosylase eliminates uracil from the DNA and initiates the base excision repair pathway (45). Of note, a recent study reported that a long-term survivor of MPM with an excellent response to pemetrexed had CCNO mRNA expression levels 40% higher than the patient with the most aggressive disease (42). Therefore, further studies are warranted to identify other possible biomarkers of responsiveness to DNA damage after the combination of carboplatin with other drugs, such as pemetrexed, in patients with MPM.

Similarly, the gene polymorphisms analysis did not show any significant correlation with the outcome of patients. Previous studies reported contradictory conclusions about the impact of TS and ERCC1 polymorphisms on clinical outcome (46). Assessing polymorphisms as predictive markers of drug activity is very appealing because...
a DNA sample is more amenable for collection and storage than a tissue sample, and genotyping analysis can be much more easily done in routine clinical setting than gene expression analysis. FDA has recently approved the analysis of thiopurine S-methyltransferase and uridine diphosphate glucuronosyltransferase polymorphisms to predict the toxicity of 6-mercaptopurine and irinotecan, respectively. However, tumor progression is a dynamic process, whereas the polymorphic germline status is a static portrait whose clinical impact may be more or less influenced by the accumulating genetic changes in the tumor cells. In particular, MPM tissues have a profound increase in chromosomal aberrations and gene copy number alterations that can be explained by chromosomal instability typically associated with MPM. The most common chromosomal aberrations within MPM progression include deletions in chromosomes 22q, 17p, and 19q and gains in 5p, 8q, 17q, and 18p, and the genes coding for ERCC-1 and TS are located on chromosomes 19 and 18, respectively (47). Furthermore, the regulation of TS expression is influenced by multiple events occurring at both the transcriptional, posttranscriptional, and translational level (48). These events are associated to cell-cycle regulation, proliferation, and apoptosis pathways, and these processes are often disrupted in different tumors. Therefore, TS protein levels emerged as the most reliable biomarker to predict the outcome after pemetrexed-based treatment.

In conclusion, TS expression showed significant correlations with responsiveness, PFS, and OS in this retrospective analysis and emerges as a potential predictor of responsiveness to carboplatin/pemetrexed treatment in patients with MPM. Adequate prospective studies to confirm the potential of TS expression as a predictive and/or prognostic biomarker in MPM patients and also to define subgroups of patients with a high risk of developing hematological and nonhematological toxicity are warranted.

Disclosure of Potentials Conflicts of Interest

No potential conflicts of interest were disclosed.

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Thymidylate Synthase and Excision Repair Cross-Complementing Group-1 as Predictors of Responsiveness in Mesothelioma Patients Treated with Pemetrexed/Carboplatin

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