Prognostic Impact of Blood Biomarkers TS and DPD in Neoadjuvant-treated Esophageal Cancer Patients

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Abstract. *Background/Aim: The prognostic value of TS (thymidylate synthase) and DPD (dihydropyrimidine dehydrogenase) RNA expression in the blood of patients with esophageal cancer is not known. The aim of the present study was to evaluate the significance of these molecular alterations in the blood as a prognostic marker for patients with neoadjuvant-treated esophageal cancer. Patients and Methods: A total of 29 patients with locally advanced esophageal cancer (cT3-T4, Nx, M0) were enrolled in this prospective study. All patients received neoadjuvant chemoradiation followed by a transthoracic resection (curative transthoracic en bloc esophagectomy, RO). Peripheral blood samples were drawn before initiation of therapy. The analysis was performed using quantitative real-time-polymerase chain reaction (RT-PCR). The histomorphological regressions grading after neoadjuvant therapy was defined as follows: major response (MaR)=less than 10% vital tumor tissue, minor response (MiR)=more than 10% vital tumor tissue. Results: Nineteen out of 29 patients (65.5%) had a MiR and 10 (34.5%) had a MaR. The median survival of patients was 2.08 years (range=0.15-4.53). Among the tested genes, the RNA expression of TS was significantly associated with prognosis of patients. Patients with TS expression above 0.78 had a median survival of 1.1 years (range=0.21-3.96) compared to 2.6 years (range=0.15 to 4.53) in patients with TS expression lower than 0.78 (p=0.031, log rank test). There was no association between clinical variables (e.g., tumor stage, gender, age, etc.) and the RNA expression of TS in the serum. Conclusion: The RNA expression of TS in the blood is a potential prognostic marker in patients with neoadjuvant-treated esophageal cancer. The significance of these molecular alterations as non-invasive prognostic marker for esophageal cancer should be evaluated in prospective studies.*

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Esophageal cancer is the eighth most common malignant tumor in the USA. In 2011, an estimated 16,980 new cases (13,450 in men and 3,530 in women) and 14,710 deaths (11,910 in men and 2,800 in women) will occur in men (28). Moreover, esophageal adenocarcinoma is currently the most rapidly increasing cancer in the USA and Western Europe (22, 33). The overall survival (OS) of esophageal cancer remains low with 5-year survival rates from 15 to 39% (1, 23, 34), which is a reason for the development of neoadjuvant treatment strategies to improve patients' survival (29). In the past years, several studies revealed that only patients with major histopathological response benefit from neoadjuvant therapy, but not patients with minor histopathologic response (24, 30-32). Patients with minor response seem to have a disadvantage of neoadjuvant strategies in terms of survival (5, 19). Therefore, markers, which allow to predict pathological response to neoadjuvant treatment and prognosis, are of great interest.

Previously, several blood biomarkers associated with outcome or response to neoadjuvant treatment have been described in miscellaneous tumors (3, 4, 14).

Thymidylate synthase (*TS*) is one gene which has been reported by our group to be a predictive marker for esophageal cancer patients treated with fluoropyrimidine-based therapy. TS is a critical rate-limiting enzyme of DNA synthesis and has been linked to response prediction and prognosis in other cancers, such as colorectal (9), gastric (20) and lung cancers (8, 25).

Dihydropyrimidine dehydrogenase (DPD) reduces the 5,6 double bond of uracil (as well as that of 5-fluorouracil (5-FU)) and is the rate-limiting enzyme for 5-FU catabolism (11). It has been also shown to play a predictive and/or prognostic role in several cancers (8, 16, 18).

The goal of our prospective trial was to determine the prognostic value of *TS* and *DPD* mRNA expression in the peripheral blood of neoadjuvant-treated esophageal patients. Therefore, we present results of the long-term follow-up of previously published data (3).

Materials and Methods

Patients and specimens. The patients' characteristics have been described previously (4): All patients were recruited from an ongoing clinical trial on neoadjuvant chemoradiation for esophageal cancer. None of the patients had prior radio- and/or chemotherapy. Twenty-nine consecutive patients (median age=61 years; age range=41-71 years; gender, 23 men and six women, 18 adenocarcinomas and 11 squamous cell carcinomas) with locally advanced, resectable esophageal cancers (cT2-4, Nx, M0) were offered standardized neoadjuvant chemoradiation. Clinical staging was based on barium swallow, endoscopic ultrasound and computed tomography of chest and abdomen. Cisplatin (CDDP) (20 mg m^{-2} day^{-1}) was administered as a short-term infusion on days 1-5, while 5-FU (1,000 mg m^{-2} day^{-1}) was administered as a continuous infusion over 24 h on days 1-5. Radiation therapy was administered by linear accelerators with 10- to 15-MV photons. Radiation was delivered in daily fractions of 1.8 Gy (days 1-5, 8-12, 15-19 and 22-26) to a total dose of 36 Gy using a multiple-field technique. Surgical resection was performed 4-5 weeks after completion of chemoradiation after clinical restaging using the same procedures as for primary staging. Standardized transthoracic en bloc esophagectomy with two-field lymphadenectomy and reconstruction by gastric tube interposition with high intrathoracic anastomosis was performed in all patients (27). Informed consent was obtained from each patient and the scientific protocol was approved by the local ethics committee.

The degree of histomorphological regression was classified into four categories according to Schneider *et al*. (26): (i) grade I, >50% vital residual tumor cells; (ii) grade II, 10-50% vital residual tumor cells; (iii) grade III, nearly complete response with <10% vital residual tumor cells; and (iv) grade IV, complete response (pCR, ypT0). Regression grades III and IV were considered as major histomorphological response (MaHR) compared with grades I and II constituting minor histopathological response (MiHR). The median follow-up of the patients was 3.42 years.

Blood procurement, tumor cell enrichment and RNA extraction. Twenty milliliters of whole blood were drawn prior to the start of the neoadjuvant therapy using 5-ml citrate tubes (Sarstedt, Numbrecht, Germany). Blood samples were immediately further processed for the enrichment of disseminated circulating tumor cells by density gradient centrifugation using a kit (OncoQuick1; Hexal, Frickenhausen, Germany) as reported by Hoffmann *et al*. (15). In brief, 20 ml of whole blood was transferred in supplied 50 ml polypropylene tubes containing a porous barrier and separation medium and centrifuged for 20 min (1,600 × *g*, 48˚C). Cells were separated according to their different buoyant densities and the circulating tumor cells became enriched in a layer formed between plasma and separation medium. This cell fraction was transferred into polypropylene tubes containing 50 ml washing buffer followed by centrifugation for 10 min to eliminate contaminating platelets. This washing step was repeated once and, according to the manufacturer's protocol, a detection limit of 1.46 tumor cells in 20 ml of whole blood could be achieved. Total cellular RNA from this pellet of enriched tumor cells was extracted using the Purescript1 kit (Gentra1, Hamburg, Germany) according to the manufacturer's recommendation.

Quantification by real-time polymerase chain reaction (RT-PCR). Generation of cDNA was performed using oligo (dT)18 primers and Moloney Murine Leucemia Virus (MMLV) reverse transcriptase (Clontech Advantage™ Kit, Palo Alto, CA, USA). Direct quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) (TaqMan™, ABI PRISM 7900 HT Sequence Detection System; Applied Biosystems™, Darmstadt, Germany) assays were performed in triplicates to determine *TS*, *ERCC1* and *DPD* mRNA expression levels in relation to the internal reference gene β-actin (7, 12). The primers and probes for *TS*, *ERCC-1* and *DPD* mRNA detection were designed as reported by our group (4, 14): The primers and probe sequences for *TS* were as follows: 5'-ACG TAC ATG ATT GCG CAC ATC-3' and 5'-ATG TGA TTC AGG TAA ATA TGT GCA TCT-3'; probe 6FAM-5'-CCT GAA GCC AGG TGA CTT TAT ACA CAC TTT GG-3'-TAMRA. The primers and probe sequences for *ERCC-1* were as follows: 5'-AGC CGC CCA TGG ATG TAG T-3', reverse primer: 5'-TGG GAA TTT GGC GAC GTA A-3', TaqMan Probe: 5'-CCC TGT TCC TCA GCC TCC GCT ACC-3'. The primers and probe sequences for *DPD* were as follows: 5'-AGG ACG CAA GGA GGG TTT G-3' and 5'-GTC CGC CGA GTC CTT ACT GA-3'; probe 6FAM-5'-CAG TGC CTA CAG TCT CGA GTC TGC CAG TG-3'-TAMRA. For β-actin (*ACTB*), the primers and probe sequences were as follows: 5'-TGA GCG CGG CTA CAG CTT-3' and 5'-TCC TTA ATG TCA CGC ACG ATT T-3'; probe 6FAM-5'-ACC ACC ACG GCC GAG CGG-3'-TAMRA.

Thermal cycling conditions were 2 min at 50˚C and 10 min at 95˚C for initial denaturation followed by 40 cycles of 95˚C for 15 s and 60˚C for 60 s. One microgram of human placental total cellular RNA (Clontech™ Lab, Palo Alto, CA, USA) was used to control each run of reverse transcription. This cDNA was used in serial dilutions as standard for the quantitative real-time RT-PCR. Triplicates of the blood samples were assayed in each run. The tested gene levels were standardized for β-actin (ratio to β-actin) to account for loading differences as extensively described (15).

Statistical analyses. OS was calculated as the time from the operation until death from any cause, except perioperative death, or until the date of the last follow-up. The SPSS Statistics version 17.0 (SPCC Inc., Chicago, IL, USA) was used for all statistical analyses. TaqMan analyses yield values that are expressed as ratios between two absolute measurements (gene of interest/internal reference gene). The Mann-Whitney *U*-test was used to test for significant associations between the continuous test variable gene expression and dichotomous variables. The Kruskal-Wallis test was used to test for significant differences in gene expression within multiple groups. The maximal χ^2 method of Miller and Sigmund (21) and Halpern (10) was adapted to determine which expression value best segregated patients into poor- and good-prognosis subgroups (in terms of likelihood of surviving), with the log-rank test as the statistics used to measure the strength of the grouping. Correlations between the analyzed genes were calculated using the two-tailed Spearman's rho test. The level of significance was set to $p<0.05$.

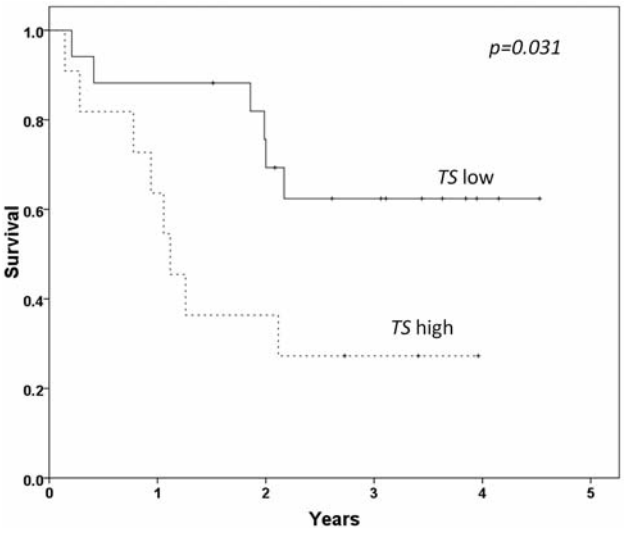

Figure 1. *Kaplan-Meier survival curves for patients with high thymidylate synthase (TS) and low TS mRNA expression in blood serum (p=0.031, log-rank test, cut-off=0.78).*

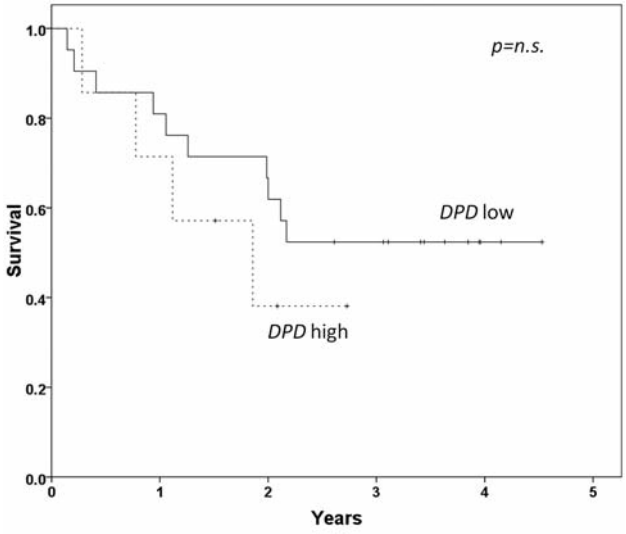

Figure 2. *Kaplan-Meier survival curves of patients with high dihydropyrimidine dehydrogenase (DPD) and low DPD mRNA expression in peripheral blood serum (p=0.376, log-rank test, cut-off=19.46).*

Results

Gene expression results. RNA expression from peripheral blood of patients was detectable for TS in 100% (29 of 29), for *DPD* 100% (29 of 29) and 100% (29 of 29) for β-actin (*ACTB*).

The median relative *TS* mRNA expression level standardized for β-actin (*ACTB*) was 0.376 (0-29.739) and for *DPD* mRNA expression 12.05 (range=0-282.06), as reported before (3).

Histopathological response. The response frequencies for the 29 resected tumors were as follows: 34.5% (ten of 29) of the tumors demonstrated major (grades III and IV) histopathologic response and 65.5% (19 of 29) of the tumors demonstrated minor histopathologic response (grades I and II) to our neoadjuvant treatment regimen. The median expression levels of *TS* and *DPD* in responders and non-responders were published previously by Brabender *et al*. (3). The median *TS* RNA expression level was 0.37 (minimum 0.00, maximum 29.7) and the median *DPD* RNA expression level was 12.1 (range=0-282.2). The median *TS* RNA expression was 0.61 (minimum, 0.00; maximum, 29.7) in minor responders and 0.23 (minimum, 0.00; maximum, 4.9) in major responders and the median *DPD* mRNA expression was 16.95 (minimum 0.00, maximum 282.26) in minor responders and 5.54 (minimum 1.68, maximum 85.1) in major responders (p=0.18, Mann–Whitney test) (3).

Survival analysis. The median survival of patients was 2.08 years (range=0.15-4.53) with a median follow-up of 3.42 (range=1.51-4.53) years. Among the tested genes, the RNA expression of *TS* was significantly associated with prognosis of patients (Figure 1). Patients with *TS* mRNA expression above 0.78 had a median survival of 1.1 years (range=0.21-3.96) compared to 2.6 years (range=0.15 to 4.53) in patients with *TS* expression lower than 0.78 (p=0.031, log-rank test). High *DPD* mRNA expression was not significantly associated with reduced survival (p=0.376, log-rank test) (Figure 2). There was no association between clinical variables (*e.g.*, tumor stage, gender, age, *etc.*) and *TS* and *DPD* mRNA expression in the serum. Major responders had a median survival of 2.6 years (range=0.15-4.15), which was not significantly higher than in minor responders with 1.8 years (range=0.21-4.53) (p=0.228, log-rank test) (Figure 3).

Discussion

The goal of this study was to update the already published data of *TS* and *DPD* expression levels and histopathological response with outcome. As previously reported, the expression of *TS* and *DPD* in peripheral blood can be detected in a high frequency. Interestingly we found peripheral blood TS expression to be significantly associated with prognosis in neoadjuvant-treated and curative-resected esophageal cancer patients. *DPD* was not significantly associated with outcome. We complement previous reported results, where a significant association of peripheral blood

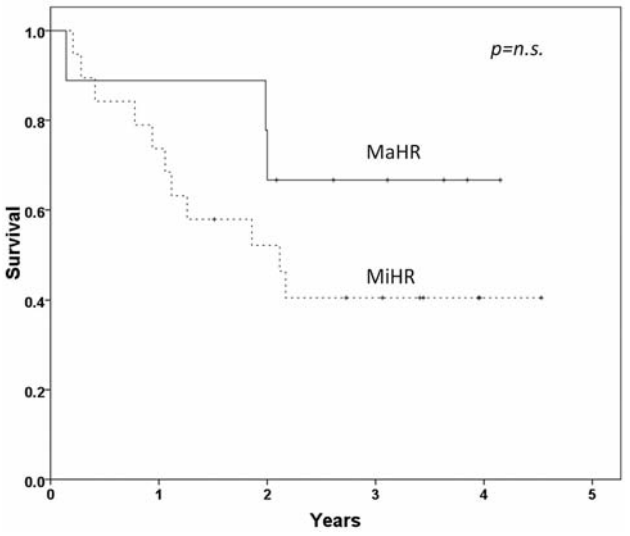

Figure 3. *Kaplan-Meier survival curves of patients with major (MaHR) and minor (MiHR) histopathological response to neoadjuvant treatment (p=0.228, log-rank test).*

TS expression and response to neoadjuvant treatment in esophageal cancer was reported. Using the same cut-off for *TS* and *DPD* as before, high *TS* mRNA expressers, who received neoadjuvant chemoradiation and curative surgery, seem to have a reduced overall survival compared to low-*TS* mRNA expressers. Interestingly the survival benefit of curative-resected esophageal cancer patients with major response to neoadjuvant chemoradiation did not reach a statistical significance compared to patients with minor response. Combined analysis of *TS* and *DPD* in patients' blood has been reported to increase the specificity for response prediction (3); however, for survival analysis, blood-based TS expression seems to be more crucial compared to blood-based *DPD* expression analysis.

The presented study shows the prognostic potential of blood-based molecular markers for the neoadjuvant chemoradiation treatment in locally advanced esophageal cancer. *TS* and *DPD* are key factors in the DNA synthesis and involved in the 5-FU metabolism (13). TS is directly targeted by activated 5-FU and *DPD* is important for the degradation of 5-FU. Our data suggest a potential role of *TS* and *DPD* mRNA expression in peripheral blood for response prediction and outcome.

A limiting factor of our study is the number of patients investigated, which could be one possible reason for the not-significant association of *DPD* expression and outcome. The same reason may also be responsible for the not-significant association of major responders and improved overall survival, since previous studies have reported a significant association of pathological tumor response and survival (2, 26). Also, differences in lymph node metastases could be a reason for the not-significant correlation of response and survival. Due to the limited number of investigated patients a subset analysis was not possible.

Not all patients benefit from neoadjuvant treatment in locally advanced esophageal cancer using the described protocol with 5-FU, CDDP and radiation; however, studies show a better prognosis for patients receiving neoadjuvant chemoradiation compared to those who underwent curative surgery-only (6, 17). More important to know is that patients with histopathologic response to chemoradiation (approximately 20-30% (2)) do benefit from neoadjuvant treatment, while non-responders do not (26). *TS* mRNA expression analysis in peripheral blood seems to be an equivalent marker for the prognosis after neoadjuvant treatment in locally advanced esophageal cancer followed by a curative resection compared to the histomorphological response classification; however, this has to be evaluated in larger trails.

Our results constitute the first report of blood-based predictive and prognostic markers for neoadjuvant-treated esophageal cancer patients. The evaluation of *TS* and *DPD* mRNA expression in peripheral blood would be very interesting for clinical use. Patients, who are diagnosed with locally advanced esophageal cancer could be, additionally to staging examination, tested for *TS* and *DPD* mRNA expression in the peripheral blood, which is drawn anyway. This could help identify the patients who will benefit from neoadjuvant treatment and spare patients from ineffective treatment(s).

Conclusion

Our data confirmed that gene expression levels of *TS* mRNA are associated with clinical outcome. *TS* gene expression levels may allow for selection of patients with esophageal cancer who will likely benefit from neoadjuvant chemoradiation. TS expression seems to be associated with prognosis in these patients and predict the effectiveness of neoadjuvant chemoradiation. Although prospective phase II studies with a high evidence level are warranted, our findings appear important for tailoring treatment strategies in esophageal cancer patients.

Previous Presentation

Parts of these results were presented at the DGCH 2011 Annual Meeting in the poster presentation (Abstract-ID:365).
Parts of these results were published in the Journal of Gastrointestinal Surgery (2008) 12: 1815-1821.

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