Correlation between Tumor-associated Proteins and Response to Neoadjuvant Treatment in Patients with Advanced Squamous-cell Esophageal Cancer

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Abstract. Background: Possible predictive markers of response to neoadjuvant radiochemotherapy (NRCT) of esophageal cancer have been identified. Patients and Methods: Patient biopsies were obtained from both tumor and normal tissue before the NRCT of locally advanced esophageal squamous cell carcinoma. Protein solutions were separated and immunoblot analysis was performed with heat shock protein (Hsp)16.2, heme-binding protein 2 (SOUL), BCL2-associated X protein (Bax), B-cell-associated leukemia protein 2 (Bcl-2) and heat shock protein 90 (Hsp90) antibodies. Following NRCT, the patients were restaged according to the Response Evaluation Criteria In Solid Tumors (RECIST). Following resections the pathological down-staging was evaluated. Results: Clinical restaging revealed a response rate of 65%. Pathological examination revealed down-staging in 30% and 25% of the cases for the T and N categories respectively. Compared to the normal esophageal mucosa, a decreased expression of Hsp16.2, Hsp90 and SOUL proteins and an increased Bax/Bcl-2 ratio was found in the responding tumors. Conclusion: Hsp16.2, Hsp90 and SOUL expression and Bax/Bcl-2 ratio correlates to the efficacy of NRCT and predict outcome in patients with locally advanced squamous-cell esophageal cancer.

Esophageal cancer is the eighth most frequent cancer in the world, carrying a poor prognosis, with a 3-30% overall 5-year survival rate (1, 2). Dysphagia, the classical symptom of esophageal cancer, usually appears when the tumor has obstructed over 50% of the lumen, usually meaning that the tumor is irresectable (1). Squamous-cell cancer is mostly found in the upper two-thirds of the esophagus and is associated with a bad socioeconomic environment, nicotine- and alcohol abuse (1, 3).

The prognosis of a particular cancer patient is very important in the individualization of treatment, to plan the patient’s follow-up and to inform the patient about the probable outcome. The standard therapy of esophageal cancer in the I-II stages is surgery although two out of three patients are inoperable at diagnosis (1, 4). For locally advanced cancer neoadjuvant radiochemotherapy (NRCT) has become the accepted modality of treatment (4, 5). Preoperative RCT increases long-term survival by decreasing the original tumor size and metastatic potential as well as by increasing the resection rate (4-6). Recent meta-analyses have proved that multimodal treatment improves survival (2, 5, 7, 8), but significant improvement in the long term survival can only be expected, if patients have pathological complete response (pCR), which occurs in 20-30% of the cases (9, 10). Unfortunately, not all the tumors are sensitive to neoadjuvant therapy thus, in the group of non-responder patients, this may lead to unnecessary overtreatment with cytotoxic drugs. Consequently, the pretherapeutic identification of those squamous-cell carcinoma cases that would benefit from neoadjuvant treatment has become an important task in order to avoid preventable toxicity, to lengthen survival and to ameliorate life quality (11, 12).

A number of recent studies have attempted to identify markers that could be used to predict clinical response to neoadjuvant therapy. FDG-PET imaging, which shows alterations in tissue metabolism, was thought to prognosticate response, however, results have proved equivocal (13-15). Serum markers were also examined with the same purpose. Kim et al. analyzed serum carcinoembryonic antigen (CEA) levels and found that an increased CEA level predicted relapse and correlated well with visceral involvement, while clinical response correlated with decreased CEA values (16). In another study, elevated plasma DNA was demonstrated to be a more reliable marker than CEA as an indicator of the
presence of recurrent disease (17). Gene expression arrays have been used on the basis that cancer is the consequence of a malfunction of gene expression. A study by Duong et al. produced a 32-gene panel classifier that was identified as a predictor of response (18).

No clinically useful predictors of response to neoadjuvant therapy in squamous-cell esophageal cancer have yet been found. The aim of this study was to investigate certain molecular-biological markers which characterize the two major cell death pathways induced by oncological treatment as possible clinically useful predictors of response. The expression of anti-apoptotic heat shock protein 90 (Hsp90), small heat shock protein 16.2 (Hsp16.2) due to its weight of 16.2 kDaltons (19), also referred to as HSPB11 (20), B-cell-associated leukemia protein 2 (Bcl-2) and proapoptotic BCL2-associated X protein (Bax) as well as the expression of necrosis-inducing heme-binding protein 2 (SOUL) (21) were examined in esophageal tumor specimens prior to RCT, to assess their possible association with favorable or unfavorable response to treatment.

Patients and Methods

Patients and tumor specimens. Twenty patients with esophageal cancer, candidates for NRKT, were enrolled in the study between 2005 and 2006. All the patients had squamous-cell cancer, with stages cT3-4, cN0-1, cM0, located in the upper two-thirds of the esophagus (Table I). All the patients signed informed consent, which was approved by the Local Ethics Committee.

The staging procedures included endoscopy with biopsy, endoscopic ultrasound, computed tomography (CT) scan of chest and abdomen and bronchoscopy. From each patient one biopsy was taken from the tumor and one biopsy from the intact part of the esophagus to serve as control. The biopsy from the tumor was divided into two parts. One tumor sample and the normal tissue sample were immediately frozen in liquid nitrogen and the other tumor sample and the normal tissue sample were formalin-fixed for pathological examination. The biochemical examinations were carried out on fresh frozen samples. The patients then received external-beam radiotherapy (total of 36 to 40 Gy, fraction dose: 1.8 Gy) and concomitant chemotherapy during the first week of irradiation: cisplatin (100 mg/m² intravenously on day 1) and 5-fluourouracil (1000 mg/m²/day, continuous intravenous infusion through days 1-5). Four weeks after the completion of RCT, the clinical response to treatment was assessed according to the Response Evaluation Criteria In Solid Tumors (RECIST; control CT scan and endoscopy with biopsy) (22). Six to nine weeks after the neoadjuvant therapy the patients underwent definitive surgical resection. Pathological response to treatment was determined by the histological evaluation of the resected specimen. Side-effects were documented in conformity with the Common Terminology Criteria for Adverse Events, Version 3.0. (http://ctep.cancer.gov).

Preparation of polyclonal antibodies against Hsp 16.2 and SOUL. Rabbits were immunized subcutaneously at multiple sites with 100 pg of recombinant Hsp16.2/Glutathione S-transferase (GST) or SOUL/GST fusion proteins dissolved in Freund’s complete adjuvant, as described before (19, 23). Then four subsequent booster injections of 50 pg doses at 4-week intervals were given. Blood was collected 10 days after the last boosting, and the antisera were stored at −20°C. IgGs were affinity purified from the sera by protein G-Sepharose chromatography (Sigma-Aldrich, Munich, Germany) according to the manufacturer’s protocol.

Immunoblot analysis. The tissue specimens were homogenized in chilled lysis buffer of 0.5 mM sodium metavanadate, 1 mM EDTA and protease inhibitor mixture in phosphate-buffered saline in a Teflon/glass homogenizer, then centrifuged for 10 minutes. Isolation of the cytosol and nuclear fractions was carried out by standard laboratory protocols described previously (24). The samples were equalized to 1 mg/ml total protein concentration using Biuret’s method and subjected to SDS-PAGE. The proteins (20 μg/lane) were separated on 15% gels and then transferred to nitrocellulose membranes. The membranes were blocked in 5% low fat milk for 1 h at room temperature, then exposed to the primary antibodies at a dilution of 1:2,000 at 4°C overnight in blocking solution. The primary antibodies used were: anti-Hsp 16.2, anti-SOUL, anti-Hsp 90 (Cell Signaling, Danvers, MA, USA), anti-Bax (Cell Signaling, Danvers, MA, USA) and anti-Bcl-2 (Cell Signaling, Danvers, MA, USA) antibodies. Appropriate horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich, Munich, Germany) were used for 2 h at room temperature and at 1:5,000 dilution. Peroxidase labeling was visualized with enhanced chemiluminescence (ECL).
using an ECL Western blotting detection system (GE Healthcare, Uppsala, Sweden). The developed films were scanned, and the pixel volumes of the bands were determined using NIH Image J software (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). All the experiments were repeated four times.

**Statistical analysis.** Statistical analysis was performed by analysis of variance followed by Student’s t-test and the Mann-Whitney U-test. Statistical significance was set at \( p<0.05 \). The analyses were performed using the statistical software SPSS for Windows (version 11.5; SPSS Inc., Chicago, IL, USA).

**Results**

**Clinical outcome.** A 65% clinical response rate was found. One patient had complete remission (5%), 12 patients had partial remission (60%), 5 patients had stable disease (25%), 1 patient had progressive disease (5%) and 1 patient died during the treatment (5%) (Table II). The patients with complete or partial remission underwent definitive surgery. The following histological response was observed: no residual tumor tissue in 2 patients (10%), down-staging of the tumor size (T) or lymph node involvement (N) in 6 (30%) and 5 (25%) cases, respectively. Complete (R0) resection was possible in 9 cases (70%) and no perioperative mortality occurred. Grade 3 or 4 gastrointestinal, hematological and pulmonary side-effects occurred, one patient died due to severe sepsis (Table III).

**Detection of possible new markers by Western-blot.** All twenty squamous-cell esophageal cancer and corresponding normal esophageal tissue samples were examined by Western-blot. The tumor samples from the patients with no clinical response contained approximately double the level of Hsp 90 and Hsp 16.2, significantly higher than responding tumors (\( p=0.049 \) and \( p=0.019 \) respectively). They also expressed SOUL at a higher level and had a lower Bax/Bcl-2 ratio than those with good clinical response, but these results were not significant (\( p=0.247 \) and \( p=0.883 \)) (Figure 1).

The results of the pathological examination were similar to the clinical results. The tumors with no histological response expressed twice as much Hsp90 (\( p=0.0005 \)) and Hsp16.2 (\( p=0.002 \)) and 1.5 times more SOUL (\( p=0.218 \)) than the responders. On the other hand, a lower Bax/Bcl-2 ratio was seen in the non-responders compared to the responders, but as SOUL this result was not significant (\( p=0.499 \)) (Figure 2).

Particularly interesting results were observed when the samples were analyzed according to the tumor location. The upper tract tumors expressed the Hsp proteins in significantly lower quantities than the tumors located in the lower-third of the esophagus (Hsp90 upper vs. middle-third \( p=0.006 \) and Hsp16.2 upper vs. middle-third \( p=0.012 \)). The SOUL protein was also expressed in significantly smaller quantities in the upper-third of the esophagus (\( p=0.047 \)). Although the Bax/Bcl-2 ratio seemed to be lower in the middle-third tumors, the difference was not significant (\( p>0.05 \)) (Figure 3 and Figure 4).

**Discussion**

Bcl-2 and its family members influence cell behavior in response to genotoxic stress (25). While Bcl-2 inhibits apoptosis by regulating the release of certain proteins such
as cytochrome c from the mitochondria, Bax shows proapoptotic activity by permeabilizing the outer mitochondrial membrane (26, 27). In the present study the patients with clinical and histological response to neoadjuvant therapy seemed to have a higher Bax/Bcl-2 ratio, whereas seemingly lower Bax/Bcl-2 ratios were found in the non-responders, although this trend was statistically not significant. Similarly better survival of patients with proapoptotic p21 positive esophageal tumors treated with RCT compared to those with no p21 expression has also been demonstrated (28), while elevated levels of survivin, an apoptosis inhibitor and key factor in resistance to RCT, predicted a significantly reduced median survival in patients receiving preoperative therapy (29, 30).

Hsps are a group of proteins that are present in all cells in all life forms. Their production is induced when a cell undergoes various types of environmental stress such as heat, cold and hypoxia. The anti-apoptotic activity of Hsps including small Hsps has been reported previously (31, 32). These proteins are molecular chaperones helping to preserve original protein function and activity by protecting cells against various stress stimuli (e.g. hydrogen peroxide, taxol) (19, 33). Hsps are highly expressed in cancer cells and are essential to their survival. Hsp90 plays a particularly versatile role in cell regulation, forming complexes with a large number of cellular kinases, transcription factors and other molecules. Wu and coworkers demonstrated that Hsp90 was selectively expressed in esophageal cancer tissue compared to the corresponding normal tissue, and the inhibition of Hsp90 resulted in decreased proliferation and viability as well as radiosensitisation of esophageal cancer cells (34). Hsp16.2 forms self-aggregates and binds to Hsp90, thus promoting the effect of the latter protein. The over-expression of Hsp16.2 inhibits cell death via the stabilization of the mitochondrial membrane, activation of Hsp90, stabilization of lipid rafts and by the activation of the Phosphatidylinositol-3-kinases-Akt cytoprotective pathway (19). Both Hsps and small Hsps were confirmed as playing a role in the development of tumors, for example malignant brain tumors (35, 36). Furthermore, their overexpression in cancer cells has been linked to increased tumor growth and resistance to RCT (31, 32). Elevated Hsp expression in malignant cells plays a key role in protection against spontaneous apoptosis associated with malignancy, as well as against apoptosis generated by therapy. These are mechanisms which may underlie the role of Hsp in tumor progression and resistance to treatment (37). Hence, the observation that the upper esophageal tumors expressed the Hsps at significantly lower levels than the middle-third tumors, is of particular importance, since it may be the possible explanation of the widely known fact, that cervical esophageal cancer has a superior sensitivity to multimodal

Figure 2. Tumor-associated proteins and pathological response (*statistically significant difference between responder and non-responder tumor tissue).
therapy. As expected, the samples from the non-responding esophageal tumors expressed Hsp90 and Hsp16.2 at twice the levels of the responding tumors.

Response to stress may not only result in apoptosis, but also in necrotic cell death. The recently identified heme-binding protein SOUL sensitizes cells to necrosis by promoting the opening of mitochondrial permeability transition pores under stress (38). SOUL was also observed at a higher level in non-responders compared to the responding tumors. Overall the results were almost identical for both the clinical and pathological response. The results also suggested that the response to preoperative RCT may be related to the activation of stress mechanisms which act through different signal transduction pathways.

In conclusion, the evaluation of tumor-associated protein expression in biopsy specimens could serve as a good predictor of response to RCT and could contribute to better patient selection. However, the potential of these proteins as biomarkers of response warrants further validation.

![Figure 3. Tumor-associated proteins and tumor location (p-values, upper vs. middle-third tumors).](image)

![Figure 4. Western blot showing protein expression in tumors of the Up (upper third) or Mid (middle third) of esophagus compared to C (control, normal tissue) from the same patients.](image)
Acknowledgements

This work was supported by a grant from the Hungarian Science Foundation 68469, by Research Grants from the Ministry of Health (2006-2008; 01/270), by the Janos Bolyai Research Scholarship of the Hungarian Academy of Sciences and Research Grants from AOKKA-34039-1004/2010.

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Received January 10, 2011
Revised March 30, 2011
Accepted April 1, 2011