

## Protein Expression Profiling in Esophageal Adenocarcinoma Patients Indicates Association of Heat-Shock Protein 27 Expression and Chemotherapy Response

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**Abstract Purpose:** To identify pretherapeutic predictive biomarkers in tumor biopsies of patients with locally advanced esophageal adenocarcinomas treated with neoadjuvant chemotherapy, we used an explorative proteomic approach to correlate pretherapeutic protein expression profiles with tumor response to neoadjuvant chemotherapy.

**Experimental Design:** Thirty-four patients with locally advanced esophageal adenocarcinomas who received neoadjuvant platin/5-fluorouracil – based chemotherapy before surgical resection were enrolled in this study. Response to chemotherapy was determined (a) by the amount of decline of [<sup>18</sup>F]fluorodeoxyglucose tumor uptake 2 weeks after the start of chemotherapy measured by positron emission tomography and (b) by histopathologic evaluation of tumor regression after surgical resection. Explorative quantitative and qualitative protein expression analysis was done through a quantitative differential protein expression analysis that used dual-isotope radioactive labeling of protein extracts. Selected identified biomarkers were validated by immunohistochemistry and quantitative real time reverse transcription-PCR.

**Results:** Proteomic analysis revealed four cellular stress response – associated proteins [heat-shock protein (HSP) 27, HSP60, glucose-regulated protein (GRP) 94, GRP78] and a number of cytoskeletal proteins whose pretherapeutic abundance was significantly different ( $P < 0.001$ ) between responders and nonresponders. Immunohistochemistry and gene expression analysis confirmed these data, showing a significant association between low HSP27 expression and nonresponse to neoadjuvant chemotherapy ( $P = 0.049$  and  $P = 0.032$ , respectively).

**Conclusions:** Albeit preliminary, our encouraging data suggest that protein expression profiling may distinguish cancers with a different response to chemotherapy. Our results suggest that response to chemotherapy may be related to a different activation of stress response and inflammatory biology in general. Moreover, the potential of HSPs and GRPs as biomarkers of chemotherapy response warrants further validation.

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The incidence of adenocarcinomas of the esophagus has increased faster than any other malignancy worldwide (1, 2). Most patients have locally advanced or metastatic disease, and their prognosis is poor with a 5-year survival rate of <20%. The most common therapeutic approach for locally advanced esophageal adenocarcinomas is a multimodal treatment with preoperative cisplatin/5-fluorouracil – based chemotherapy or radiochemotherapy followed by resection (3). Recent studies have shown a survival benefit for patients with perioperative chemotherapy compared with surgery alone for these tumors (4, 5). Moreover, patients with upper gastrointestinal tumors who respond to neoadjuvant chemotherapy show a significantly improved prognosis compared with patients who do not respond (6). However, because only 30% to 50% of the patients respond to induction therapy, the pretherapeutic identification of nonresponding patients would be of utmost interest to avoid inefficient therapy, toxic side effects, and cost.

Many studies have been directed toward understanding the mechanism of chemotherapy response and resistance, with

### Translational Relevance

In the present study, a novel proteomic procedure that uses dual-isotope radioactive labeling of protein extracts for quantitative differential protein expression analysis was applied to attend a clinical question of high relevance: pretherapeutic biopsies of esophageal adenocarcinoma patients treated by a neoadjuvant, 5-fluorouracil/cisplatin – based chemotherapy before surgery were investigated to identify differentially expressed proteins in responders and nonresponders to chemotherapy. For selected markers, the data of proteomic analysis could be confirmed by immunohistochemistry and quantitative, real-time reverse transcription-PCR applied on routine formalin-fixed biopsy tissue. The results of the present study, highlighting the effect of tumor cellular stress activation with regard to chemotherapy response, may be highly valuable for (a) response prediction to chemotherapy, thus optimizing individualized therapy of patients, and (b) a better understanding of the complex mechanism of chemotherapy response in human tumors in general.

the ultimate goal of identifying molecular markers that allow the prediction of chemotherapy response. For esophageal adenocarcinoma, several of these studies have concentrated on the expression analysis of the chemotherapy-associated genes (7, 8) of biomarkers p53 and p21 with known prognostic effect (9), and some have done genome-wide screening and gene expression profiling to identify expression signatures with potential predictive value (10).

Proteomic analysis has emerged as a valuable tool in scientific medicine (11–13), albeit with considerable quantitative and statistical challenges (14). Because the chemoresistance of tumors is a complex and multifactorial event, we did differential quantitative protein expression analysis in an explorative setting by using the recently developed and established radioactive ProteoTope clinical proteomics platform (15–17). In the present study, we compared protein expression patterns in pretherapeutic biopsies of patients with locally advanced esophageal adenocarcinomas who showed a response to preoperative chemotherapy with those of patients who did not respond. On the basis of quantitative differential data, selected protein biomarker candidates were validated with the use of immunohistochemistry and quantitative real-time reverse transcription-PCR (RT-PCR).

### Materials and Methods

#### Patients and tissue specimens

We selected 34 patients with locally advanced (uT<sub>3</sub>cN<sub>0</sub>-1) esophageal adenocarcinomas who underwent neoadjuvant chemotherapy between 2002 and 2005 at the Department of Surgery of the Technische Universität München (see Table 1). Sufficient frozen biopsy material for ProteoTope Analysis (see below) was available from 20 patients. Formalin-fixed paraffin-embedded tissue of pretherapeutic biopsies and surgical resection specimens were available from 34 patients. Twenty-eight patients were treated according to the MUNICON protocol (Metabolic response evaluation for individualization of neoadjuvant chemotherapy in esophageal and esophagogastric adenocarcinoma; ref. 18). All patients gave written informed consent. The use of the

human tissue samples was approved by the local ethics committee (Ethikkommission der Fakultät für Medizin der Technischen Universität München, No. 1928/07).

**Preoperative chemotherapy.** Neoadjuvant chemotherapy consisted of two cycles of cisplatin (50 mg/m<sup>2</sup>) given on days 1, 15, and 29 (1-h infusion time) plus folinic acid (500 mg/m<sup>2</sup> over 2 h) and 5-fluorouracil (2,000 mg/m<sup>2</sup> over 24 h) given on days 1, 8, 15, 22, 29, and 36, and repeated on day 49. For patients with a glomerular filtration rate <60 mL/kg/min, oxaliplatin (85 mg/m<sup>2</sup> over 2 h) replaced cisplatin. Seventeen patients ages 60 y or younger with good health status were additionally given paclitaxel (80 mg/m<sup>2</sup> over 3 h) on days 0, 14, and 28 (18).

**Positron emission tomography evaluation.** The patients underwent a baseline [<sup>18</sup>F]fluorodeoxyglucose-positron emission tomography (PET) evaluation within 1 wk before the start of neoadjuvant chemotherapy. The patients were eligible for inclusion only if the PET scans showed sufficient contrast between tumor and surrounding tissues:  $SUV_{tumor} > 1.35 \times SUV_{liver} + 2 \times SD\ SUV_{liver}$ . PET was repeated 14 d after the start of chemotherapy. Patients whose tumor SUV had decreased by  $\geq 35\%$  were defined as metabolic responders (19, 20). They continued to receive chemotherapy for a maximum duration of 12 wk before undergoing surgery ( $n = 17$ ). Metabolic nonresponders discontinued chemotherapy and proceeded to surgery ( $n = 11$ ; ref. 18).

**Surgery.** A right abdominothoracic approach with an i.t. anastomosis and 2-field lymphadenectomy (Ivor Lewis procedure) was done. Surgery was conducted within 2 wk after the last administration of chemotherapy in PET nonresponders and within 4 wk of the same in responders.

**Histopathologic evaluation of tumor regression.** To assess histopathologic tumor regression in response to chemotherapy, the resected tumors were evaluated by two pathologists (K.B. and H.H.) according to a 3-grade score based on an estimation of the percentage of vital tumor tissue in relation to the previous tumor (the tumor bed; ref. 21): tumor regression grade 1, no or <10% residual tumor/tumor bed (10 patients); tumor regression score 2, 10% to 50% residual tumor/tumor bed (4 patients); and tumor regression score 3, >50% residual tumor/tumor bed (19 patients). One patient did not undergo surgical resection due to progressive disease during chemotherapy.

**Response evaluation.** Response to chemotherapy was defined as either (a) metabolic response assessed by PET evaluation according to Lordick et al. (18) or (b) histopathologic tumor regression (score 1). Nonresponse to chemotherapy was defined as neither metabolic response nor histopathologic regression.

**Follow-up.** Overall survival was calculated from the day of resection to death. Event-free survival was calculated up to the time of death or relapse, whichever occurred first. No patient was lost to follow-up. Two patients showed immediate postoperative complications and were excluded from survival analysis due to an event-free survival <1 month.

#### Two-dimensional differential proteomic analysis

ProteoTope analysis was done on freshly frozen pretherapeutic biopsies ~2 mm in diameter. After verification of sufficient cancer tissue on a fresh frozen section and standard H&E staining, protein analysis was done on 6 to 10 frozen sections of 20  $\mu$ mol/L thickness in each case; the tissue samples were then transferred into sterile tubes. Only tumor samples with >60% carcinoma content compared with stroma or necrosis were included. The mean carcinoma content of responder samples was 70% (range, 60-90%), comparable with that of nonresponder samples (mean, 75%; range, 60-90%). ProteoTope analysis was also done on a reference sample of pooled, freshly frozen surgical tissue (see below).

**Protein extraction from biopsies.** Sectioned frozen biopsies were sent to ProteoSys, Mainz, Germany, on frozen carbon dioxide and stored at -80°C until processing. The cryosectioning medium constituted >90% of each sample and had to be removed. Each tube was filled with 1.5 mL of 80% acetone (-20°C). Slices adhering to the walls of the tube were scraped into suspension with a small spatula. The tubes were

**Table 1.** Patients' characteristics

Age (y)/Gender	CTX	G	ypT	ypN	c/pM	TRG	PET	EFS	Event	OS	Status
62/F	PFL	3	1	0	0	1	R	0.9*	+	4.6	D
43/M	T/PLF	2	2	0	0	1	R	14.4	+	21.9	A
60/M	PLF	3	3	0	0	3	R	20.7	+	26.6	D
67/M	PFL	4	0	0	0	1	R	13.1	+	35.9	A
64/M	PFL	2	0	0	0	1	R	35.3	-	39.2	A
52/M	T/PLF	3	2	1	0	3	NR	7.7	+	35.5	A
53/M	PLF	2	0	0	0	1	No PET	0.3*	+	3.8	D
36/M	T/PLF	3	2	1	0	1	R	13.5	+	50.5	A
39/M	T/PLF	3	2	0	0	3	NR	5.7	+	32.4	A
50/M	T/PLF	2	3	1	0	3	NR	26	+	26.0	A
59/F	PLF	3	3	0	0	3	R	4.8	+	14.1	D
71/M	PLF	2	3	0	0	3	NR	18.8	+	21.1	A
63/M	T/PLF	2	3	1	0	3	NR	7.9	+	14.1	A
65/M	PLF	2	3	0	0	3	R	34.3	-	34.3	A
52/M	T/PLF	3	3	1	0	3	NR	3.7	+	17.0	D
47/M	T/PLF	3	3	1	0	3	NR	5.5	+	16.0	D
77/M	PLF	2	3	1	0	2	No PET	9.9	+	23.1	A
48/M	PLF	3	3	1	0	3	No PET	3.2	+	6.7	D
71/M	T/PLF	3	2	0	0	3	R	6	+	24.1	A
67/M	PLF	3	0	1	0	3	R	38	-	41.7	A
58/M	T/PLF	2	2	0	0	1	No PET	4.5	+	16.3	A
53/M	PLF	3	3	1	0	3	R	18.4	+	31.0	A
63/M	PLF	2	1	0	0	1	R	12	-	15.5	A
64/M	T/PLF	2	1	0	0	1	R	13.8	-	17.8	A
52/M	T/PLF	3	3	0	0	2	R	26.8	-	30.8	A
65/M	PLF	3	3	1	0	3	NR	9.2	+	19.7	D
41/M	T/PLF	3	1	0	0	1	R	23.8	-	27.3	A
62/M	PLF	3	2	1	0	2	NR	20	-	23.2	A
48/M	T/PLF	2	2	0	0	3	NR	9.9	-	12.6	A
56/M	T/PLF	3					No PET	8	+	12.0	D
67/M	PLF	3	3	0	0	2	No PET	21.9	-	26.2	A
67/M	PLF	3	2	0	0	3	R	21	-	25.1	A
54/M	T/PLF	3	3	1	0	3	R	2.9	+	12.6	D
48/M	T/PLF	3	1	0	0	3	NR	10.4	+	17.0	A

NOTE: Tumor grade 1, well differentiated (no patient); tumor grade 2, differentiated; tumor grade 3, poorly differentiated; tumor grade 4, anaplastic.

Abbreviations: CTX, chemotherapy; G, tumor grading; TRG, tumor regression grade; PET, positron emission tomography response; R, responder; NR, nonresponder; EFS, event-free survival; OS, overall survival; M, male; F, female; PLF, cisplatin (oxaliplatin)/folinic acid/5-fluorouracil; T, paclitaxel; D, dead; A, alive.

\*Patients were excluded from survival analysis due to an event-free survival <1 month (immediate postoperative complications).

centrifuged for 1 min at 12,000 × g, the acetone was removed, and 1 mL of 50% acetone was added. The pellet containing tumor tissue was washed for 15 min by occasional vortexing. In the intervening periods, the samples were kept on ice. The washing procedure was repeated 5 times.

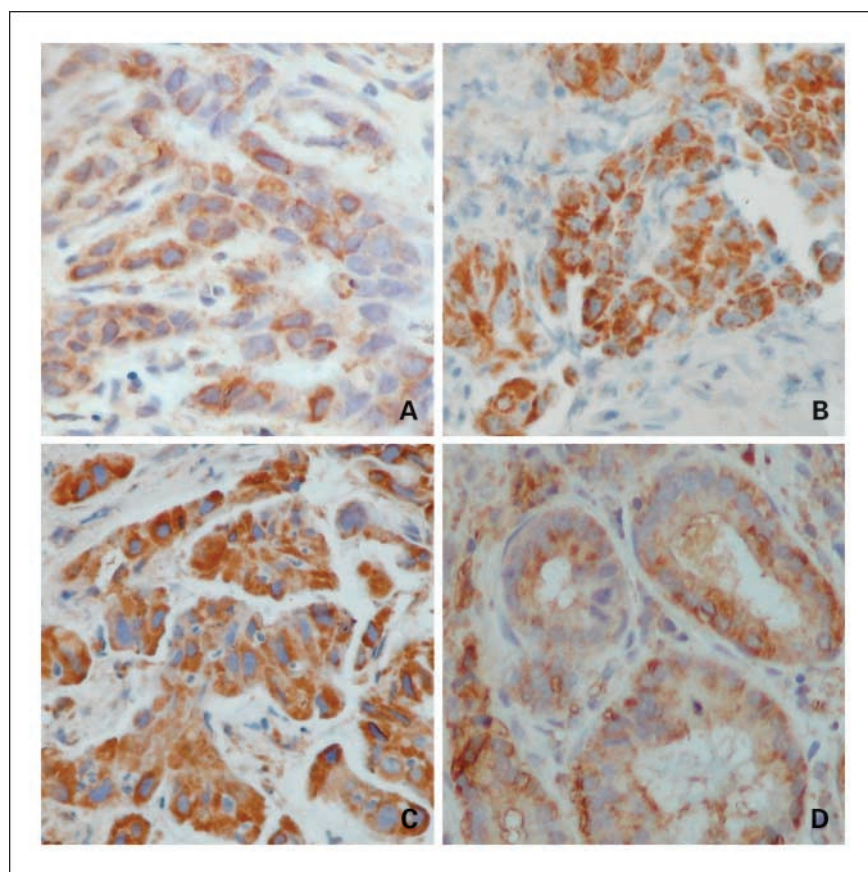
After washing, the samples were centrifuged for 30 min at a relative centrifugal force of 30,000. The acetone was removed, and the remaining 50% acetone was evaporated in a speed vac for 15 min. Dry pellets of biopsy samples were dissolved in 35 μL of preheated (95°C) 100 mmol/L Tris (pH 7.4), and 2% SDS (iodination buffer) and incubated for 5 min at 95°C. Protein determinations by the BCA method ranged from 35 to 109 μg per biopsy (15).

**Protein extraction from reference samples.** A reference sample was prepared for this study to be coelectrophoresed during two-dimensional PAGE with each respective test sample and to provide comigrating reference proteins in sufficient abundance to permit protein identification by mass spectrometry (MS). The pooled reference sample contained proteins from nine control samples of normal cardia and esophageal mucosa as well as of tumor tissue of esophageal adenocarcinoma. The reference samples were dissolved with different amounts of buffer according to estimated pellet sizes. Extracts were centrifuged for 5 min at 12,000 rpm, and 6 μL were used to prepare a 1:10 dilution for protein determination. This was done with the protein

assay reagent method (Bio-Rad). Protein yields measured by the protein assay reagent method ranged from 400 to 800 μg per control sample. The reference sample for proteomics analysis was constructed by pooling together 170 μg of protein from each respective control sample, providing a reference sample of 1.5 mg protein in 203 μL iodination buffer.

**Protein iodination.** Proteins were iodinated by the chloramine-T method and transferred to a urea-based isoelectric focusing buffer as recently described in detail (17).

**Radioactive two-dimensional gels.** Protein samples in isoelectric focusing buffer (7 mol/L urea, 1% Triton X-100, 10% glycerol, 2 mol/L thiourea, 4% CHAPS, 1% DTT, 0.8% immobilized pH gradient buffer from Amersham Pharmacia) were loaded onto the immobilized pH gradient strips (pH 4-7). For all gels, the first dimension focusing was done over an effective separation distance of 18 cm. Each first dimension focusing was afterwards loaded onto a second dimension SDS-PAGE gel, and radioactive proteins were imaged by ProteoTope as described (16, 22, 23). Gels loaded with I-125 and I-131 labeled proteins were dried after electrophoresis, laminated in 0.07 mm plastic, and exposed to routine ProteoTope imaging for each isotope for between 20 and 24 h per exposition. Protein iodination reactions with either <sup>125</sup>I or <sup>131</sup>I were conducted with identical chemical iodine concentration, providing two independent series of duplicates of



**Fig. 1.** Immunohistochemical staining for HSP27 (A), HSP60 (B), GRP78 (C), and GRP94 (D) in esophageal adenocarcinoma biopsies. Examples of strong staining reactions are shown. ( $\times 20$ ).

inversely labeled sample pairs. For each pair of samples, reverse replicate gels were prepared [i.e., the labels ( $^{125}\text{I}$  and  $^{131}\text{I}$ ) used on sample 1 and sample 2 were inverted]. A total of two individual samples per group entered the study, so the statistical evaluation was based on four data points in each group. Details of quantitative treatment of dual-radioisotope labeling and underlying statistics can be found online.<sup>9</sup>

**Data analysis.** Comparisons between the two groups of responders and nonresponders were done in the following manner: for each spot on each gel, the ratio of the abundances of the pertinent protein in the respective individual over its abundance in the reference pool was computed. The sets of these ratios, originating from responders and from nonresponders, were formed and compared with each other through the *t* test for unpaired samples based on the null hypothesis of identical volume ratios. Spot volumes were rated to be significantly different between responders and nonresponders if the *t* test resulted in a *P* value of  $<0.01$ .

Spots found to be different in responders and nonresponders by ProteoTope analysis were given unique labels (selSpotID). Radioactive images of preparative gels were warped to the same target as the ProteoTope images through the Delta2D software package by DECODON.<sup>10</sup> The above labels were transferred to the corresponding silver-stained images of the preparative gels, and the spot coordinates of the labeled spots were exported as pick lists for automatic excision and processing as described (14, 23).

**Protein identification by MS.** For MS-based identification of proteins, preparative tracer-control enrichment gels (tracer gels) were used, in which a trace of radioactively labeled protein sample corresponding to the sample used for analytic two-color gels was

coelectrophoresed with a vast excess of nonradioactively labeled protein from the pooled reference sample ( $\sim 200\ \mu\text{g}$ ) to provide preparative amounts of protein for identification (22, 24). After electrophoresis gels are silver stained according to Shevchenko et al. (25). Protein identification was based on different mass spectrometric methods: an automated procedure that allows a very quick and reliable identification of higher abundant proteins [peptide mass fingerprinting with matrix-assisted laser desorption ionization-time of flight (MALDI-TOF)-MS] but also allows the identification of very low abundant proteins with more time-consuming procedures (liquid chromatography-electrospray-IonTrap-MS/MS or MALDI TOF-TOF). Briefly, the gel plugs of selected protein spots are excised, and the proteins contained in the gel plugs are digested with the use of trypsin. The resulting solution is analyzed first with a high throughput peptide mass fingerprint procedure based on MALDI-TOF-MS. Mass spectra were internally mass calibrated with the use of trypsin autodigestion peptide signals as reference values. Mass measurement accuracies were typically 50 parts per million (22, 24). For the identification of the proteins, the peptide masses extracted from the mass spectra were searched against the National Center for Biotechnology Information nonredundant protein database<sup>11</sup> with the use of the MASCOT software version 1.9 (Matrix Science). The resulting scores (peptide mass fingerprinting or molecular weight search scores) provide the most widely used and conventional validity measure for database identification of peptides and proteins by MS/MS data. Briefly, these scoring algorithms compare the calculated peptide masses for each entry in the sequence database with the set of experimental data, resulting in an expression of the statistical probability of the identification: scores  $>65$  are considered significant (26).

<sup>9</sup> <http://www.proteosys.com>

<sup>10</sup> <http://www.decodon.com/>

<sup>11</sup> <http://www.ncbi.nlm.nih.gov>

### Validation of differential proteomic data

For validation of prospective biomarkers from the differential proteomic part, we selected four proteins [heat-shock protein (HSP) 27, HSP60, glucose-regulated protein (GRP) 78, GRP94] with a significantly differential expression ( $P < 0.001$ ) between the two patient groups. The selection was done after considerations about the biological role of the proteins in relation to response to chemotherapy. Immunohistochemistry and mRNA-gene expression analysis by quantitative real-time RT-PCR were done on preoperative, paraffin-embedded and formalin-fixed biopsies from all 34 patients.

**Immunohistochemistry and scoring.** For immunohistochemistry, primary antibodies HSP27 (Abcam; monoclonal) diluted at 1:250, HSP60 (Abcam; monoclonal) diluted at 1:250, GRP78 (Abcam; monoclonal) diluted at 1:100, and GRP94 (Santa Cruz Biotechnology, Inc.; polyclonal) diluted at 1:2,000 were used. Positive controls were human breast carcinoma tissue samples for HSP27, HSP60, GRP98, and GRP94 according to the manufacturer's recommendations. Depending on the percentage of positive cells and intensity, the staining results were classified into one of three groups: negative/weak, moderate, and strong. Staining examples are given in Fig. 1.

**Real-time quantitative RT-PCR.** Microdissection, RNA extraction, cDNA synthesis, and RT-PCR from pooled tumor tissue from at least two biopsies per case were done as described previously (7, 27). PCR reactions were done in at least two replicates. Predeveloped primer-probe sets (Applied Biosystems) were used for HSP27 (assay ID Hs00356629\_g1), HSP60 (assay ID Hs01036747\_m1), GRP78 (assay ID Hs99999174\_m1), and GRP94 (assay ID Hs00427665\_g1). The relative expression levels of target genes were determined by the relative standard curve method with *glyceraldehyde-3-phosphate dehydrogenase* as normalizing housekeeping gene. The primer and probe sequences of *glyceraldehyde-3-phosphate dehydrogenase* are available from the authors on request.

**Statistics (nonproteomic).** Validation statistical analysis was done with the use of nonparametric methods: comparisons between the two groups of responders and nonresponders were made through the Mann-Whitney  $U$  test. Associations in  $2 \times 2$  tables were evaluated by  $\chi^2$  tests and Fisher's exact tests. Survival analysis was done with the use of Kaplan-Meier estimates, log rank tests, and Cox proportional hazards regression analysis. Correlations between proteomic data and quantitative real-time RT-PCR were made by the determination of the Spearman's rank correlation coefficient ( $r$ ). The association between immunohistochemical staining patterns and proteomic data were determined through post-hoc, one-way ANOVA with least significant difference testing. All tests were 2-sided, and the significance level was set at 5%. For all validation statistical procedures, SPSS 15 software (SPSS, Inc.) was used.

### Results

The patients' mean age was 56 years (range, 36-77 years), with a male to female ratio of 32:2. According to the chemotherapy-response classification described above, 20 patients were responders and 14 were nonresponders. The mean age was 60 years for responders (range, 36-71 years) and 52 years for nonresponders (range, 39-77 years). There was no difference in tumor differentiation (grading) assessed in pretherapeutic biopsies between responders and nonresponders: 7 responding patients had moderately differentiated tumors and 13 patients had poorly differentiated or anaplastic tumors whereas 5 nonresponding patients had moderately differentiated tumors and 9 nonresponders had poorly differentiated tumors. ypT<sub>0</sub> and ypT<sub>1</sub> stages were more frequently seen in responders ( $P = 0.11$ ) as were ypN<sub>0</sub> stages ( $P < 0.01$ ). The median disease-free survival was 12 months (range, 2.9-38.0 months), and the median overall survival was 23.0 months

(range, 6.7-50.5 months). The outcome of responders was significantly better than that of nonresponders: the median disease-free survival was 16.4 months (range, 2.9-38.0 months) for responders and 8.6 months (range, 3.2-26.0 months) for nonresponders ( $P = 0.015$ ). The mean overall survival for responders was 26.4 months (range, 12.6-50.5 months) and 18.35 months (range, 6.7-35.5 months) for nonresponders ( $P = 0.169$ ). For more detailed descriptions, see Table 1.

**Proteomics analysis.** ProteoTope analysis was done on freshly frozen pretherapeutic biopsies of 20 patients. Proteins found to be differentially abundant ( $P < 0.001$ ) between responders and nonresponders classed over the entire data set are depicted in Supplementary Table S1. All proteins designated as "not identified" were subjected to at least two separate MS/MS measurements to improve the certainty of identification. The ion intensities generated by peptides from these proteins were too weak to permit suitable MS/MS identifications. A notable feature of Supplementary Table S1 is that there are no proteins that were identified to be unambiguously and systematically more abundant in the nonresponder biopsies, with the exception of only one unidentified spot (R3\_3685), which was at the low molecular weight basic region (pH ~ 6.7) of the gels. This was not due to systematically weaker signal intensities for nonresponder gels, which was manually verified by the inspection of total gel intensities for all samples. The protein spots from nonresponders exhibited signal intensities similar to those of responders. This situation is unusual in proteomic studies and, as such, it is noteworthy and is consistent with the response to therapy putatively requiring the pretherapeutic underlying susceptibility to an increased induction of a biological stress response that we will discuss below.

Differentially expressed and identified proteins found by ProteoTope Analysis could be subdivided into two major groups: one group includes cytoskeletal proteins, such as actin

**Table 2.** Immunohistochemistry: staining patterns of HSP27, HSP60, GRP78, and GRP94 in formalin-fixed paraffin-embedded biopsies

	Immunohistochemical staining patterns				P
	n	Negative/ weak	Moderate	Strong	
HSP27	31	10	14	7	
Responders	20	4	11	5	
Nonresponders	11	6	3	2	0.137
					0.049*
HSP60	34	17	10	7	
Responders	20	8	8	4	
Nonresponders	14	7	4	3	0.771
GRP78	27	3	8	16	
Responders	17	3	5	9	
Nonresponders	10	0	3	7	0.354
GRP94	34	7	8	19	
Responders	20	4	5	11	
Nonresponders	14	3	3	8	0.971

NOTE: The first lines show staining results for all patients; the 2nd and 3rd lines show results for responders and nonresponders to neoadjuvant CTX.  $P$  values are given for  $\chi^2$  and Fisher's exact test, respectively.

\* $P = 0.049$  for negative/weak HSP27 expression versus grouped moderate and strong.

$\alpha$ -1,  $\beta$ , and  $\gamma$ -1, as well as tubulin, desmin, tropomyosin, and vimentin.

The second group includes HSP27, HSP60, GRP78, and GRP94, belonging to the molecular chaperone family. We chose the latter for further validation due to (a) the conclusion that the difference between responders and nonresponders may involve the differential activation of a stress response and (b) recent reports about the role of molecular chaperones in tumor resistance to chemotherapeutics (28, 29). Additional statistical analysis comparing patients with histopathologic tumor regression grade 1 versus tumor regression grade 2 to grade 3, irrespective of metabolic response, revealed significantly differential expression of the abovementioned cytoskeletal proteins and of HSP60 and GRP78 as well (data not shown).

**Immunohistochemistry.** Immunohistochemical stainings of formalin-fixed paraffin-embedded biopsy samples were available from 31 patients for HSP27, from 34 patients for HSP60, from 27 patients for GRP78, and from 34 patients for GRP94.

Comparison between immunohistochemical staining and ProteoTope Analysis revealed only trends not reaching statistical significance for 15 of 20 patients with ProteoTope data and sufficient biopsy material available for immunohistochemical analysis (Supplementary Table 2).

By comparing responders to nonresponders, negative and weak staining for HSP27 was associated with nonresponse to neoadjuvant chemotherapy ( $P = 0.049$ ). However, a survival disadvantage for patients with negative/weak HSP27 staining could not be shown. For HSP60, GRP78, and GRP94, no association of immunohistochemical staining patterns and response or prognosis, respectively, could be shown (for details, see Table 2). With regard to histopathologic tumor regression only, none of the investigated markers showed significant association between staining patterns and tumor regression grade.

**Gene expression analysis (quantitative real-time RT-PCR).** The expression of the four selected genes was detectable in formalin-fixed paraffin-embedded biopsy samples of 31 patients.

There was no significant correlation between ProteoTope data and mRNA expression levels of the corresponding patients for HSP27 ( $r = 0.10$ ;  $P = 0.72$ ), HSP60 ( $r = 0.35$ ;  $P = 0.20$ ), GRP78 ( $r = 0.28$ ;  $P = 0.32$ ), and GRP94 ( $r = 0.136$ ;  $P = 0.63$ ; 15 of 20 patients with proteomic data examined).

With respect to response to chemotherapy, nonresponders showed significantly lower HSP27 gene expression levels compared with responders ( $P = 0.032$ ). In addition, patients with higher HSP27 gene expression levels (cutoff, median) showed a trend to better clinical outcome (disease-free survival;  $P = 0.131$ ). For HSP60, GRP78, and GRP94, no significant difference was noted between responders and nonresponders (for details, see Table 3; Fig. 2). However, all patients with an overexpression of GRP78 (relative GRP78 expression level  $>20$ ) were responders, and this result showed marginal statistical significance ( $P = 0.052$ ). An association between patients' outcome and gene expression signatures of HSP60, GRP78, and GRP94 could not be shown. Comparable with immunohistochemical investigations, none of the investigated genes showed significant association between expression levels and histopathologic tumor regression considering histopathologic tumor regression only.

**Table 3.** RT-PCR analysis: median gene expression levels with 95% CIs (gene/GAPDH) of HSP27, HSP60, GRP78, and GRP94 in formalin-fixed paraffin-embedded biopsies of responders and nonresponders to neoadjuvant CTX

	Relative gene expression levels (median; 95% CI)			
	n	Responders	Nonresponders	P
HSP27*	31	6.36 (5.78-12.0)	3.87 (2.50-6.26)	0.032*
HSP60	31	0.61 (0.58-0.92)	0.80 (0.55-0.99)	0.412
GRP78	31	2.06 (2.42-20.02)	4.07 (2.09-9.96)	0.921
GRP94	31	0.67 (0.21-5.95)	0.49 (0.26-1.98)	0.589

Abbreviations: 95% CI, 95% confidence interval; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

## Discussion

Proteomics has been increasingly applied to cancer research with intense interest in an improved understanding of cancer development, advances in diagnostics, response prediction to various therapy regimens, and targeted therapeutic approaches. A differential quantification of potential cancer markers has to consider the resolution of the analytic procedure and dynamic range of the detection method. For complex samples, radioactive labeling and high-resolution two-dimensional electrophoresis offer nearly the only alternative to obtain quantitative data that can be treated in the necessary statistical way (14, 23). The approach has been successfully applied for the detection of differentially expressed proteins in cancer and nonneoplastic tissue of the prostate (16), breast (15), and kidney (30). Moreover, biomarkers found in this way have successfully been validated with the use of large cancer tissue arrays (31). In the present study, we show similar performance when analyzing relative protein abundances from single, small tumor biopsy particles, achieving excellent differential protein abundance quantifications between cancer patients. We conclude that this methodology is suitable for analyzing small amounts of freshly frozen tissue to address important clinical questions, such as the response prediction to certain therapy regimens.

In the present study, we have applied this proteomic procedure to study the pretherapeutic protein expression pattern in biopsies of patients with locally advanced adenocarcinoma of the esophagus, treated by a neoadjuvant, 5-fluorouracil/cisplatin-based chemotherapy. Our aim was to identify differentially expressed proteins in responders and nonresponders to chemotherapy. As in the very recent past, [ $^{18}\text{F}$ ]fluorodeoxyglucose-PET has been introduced in the early response evaluation of neoadjuvant chemotherapy in adenocarcinomas of the esophagogastric junction (18), showing survival advantages for PET responders (19, 20); metabolic (PET) response was also included in chemotherapy response evaluation in the present study: chemotherapy nonresponse was defined as neither metabolic response nor histopathologic regression in contrast to response to chemotherapy, defined as either metabolic response or histopathologic tumor regression.

Under this definition of chemotherapy response, the significantly differentially expressed and identified proteins found by ProteoTope proteomics were cytoskeletal proteins (aktin, tubulin, desmin, tropomyosin, and vimentin), and

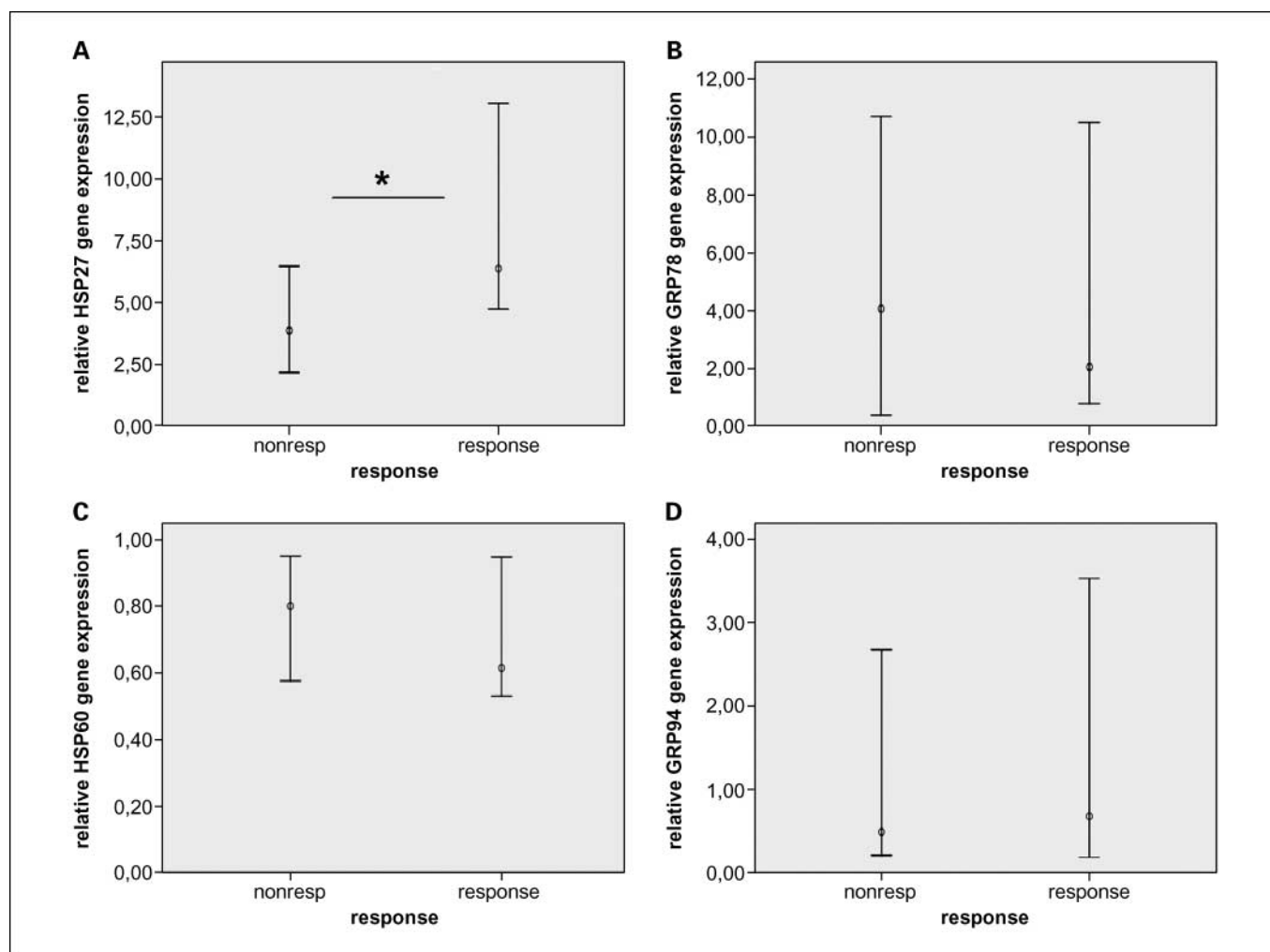
HSP27, HSP60, GRP78, and GRP94, which are members of the molecular group of the chaperone family. In this context, it is also noteworthy that similar results were found considering histopathologic tumor regression irrespective of metabolic response in the proteomic approach although these are not confirmable with immunohistochemistry and gene expression analysis. This phenomenon may support considerations to include both histopathologic and metabolic response behavior in general response evaluation.

With regard to the first, abovementioned group of proteins differentially expressed in responders and nonresponders, it is rather unclear why responders exhibit increased levels of certain cytoskeletal components. Because not all cytoplasmic proteins were detected as differential, it is unlikely that this difference reflects a general enlargement of cytoplasmic mass in responders. Rather, it is possible that these proteins are repressed in nonresponders. One study describes an up-regulation of the proteins actin- $\gamma$ , tropomyosin, and tubulin- $\alpha$  (among many other proteins) in a cervix carcinoma cell line

after cisplatin treatment (32). Another study shows an association between the expression of some tubulin isoforms and response to docetaxel (33). However, only half of the patients investigated in our study were treated by taxanes (paclitaxel), so involvement of the microtubule system alone may not provide an explanation. Therefore, the underlying causes of this phenomenon merit further examination.

Due to very recent reports on the interaction between molecular chaperones and tumor resistance to chemotherapy (28, 29), we decided to validate the identified proteins HSP27, HSP60, GRP78, and GRP94 with the use of immunohistochemistry and to analyze them also at the gene expression level through quantitative real-time RT-PCR.

Two members of the GRPs were identified to be differentially expressed in responders and nonresponders. GRP78, also referred to as immunoglobulin heavy-chain binding protein (BiP), and GRP94, also referred to as glycoprotein 96, are constitutively located within the endoplasmic reticulum and do normal physiologic functions under moderate levels of basal



**Fig. 2.** A, relative tumor *HSP27* mRNA levels (*HSP27/GAPDH*) versus response to neoadjuvant chemotherapy. Median gene expression levels are depicted. Error bars, 95% CIs.  $P = 0.032$  for median *HSP27* gene expression and tumor response to chemotherapy (Mann-Whitney *U* test). B, relative tumor *GRP78* mRNA levels (*GRP78/GAPDH*) versus response to neoadjuvant chemotherapy. Median gene expression levels are depicted. Error bars, 95% CIs. C, relative tumor *HSP60* mRNA levels (*HSP60/GAPDH*) versus response to neoadjuvant chemotherapy. Median gene expression levels are depicted. Error bars, 95% CIs. D, relative tumor *GRP94* mRNA levels (*GRP94/GAPDH*) versus response to neoadjuvant chemotherapy. Median gene expression levels are depicted. Error bars, 95% CIs. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; 95% CI, 95% confidence interval.

expression. Because of their ability to assist in protein folding and assembly, the GRPs are referred to as molecular chaperones (34). Synthesis of GRPs is enhanced under pathologic conditions, such as glucose starvation, acidosis, hypoxia, or hypothermia. In human cancers, like breast cancer (35, 36), prostate cancer (37), colorectal cancer, or lung cancer, there has been a general observation that higher GRP levels correlate with higher pathologic grade and aggressive behavior. However, in addition to intratumoral or intracellular conditions, the regulation of GRP expression in tumor cells may be dependent on exposure to various extratumoral stress factors, such as the potency of the immunologic answer of the host, hypoxia, or cytotoxic treatment (38).

We recently have shown an association of GRP78 and GRP94 mRNA and protein expression with tumor stage and behavior in esophageal adenocarcinomas: increased expression of GRP78 was mainly observed in early tumor stages but also in a few patients with advanced tumor stages (39). Tumors of this latter subset of patients with high GRP tumor levels may be exposed to special cellular stress conditions that may lead to increased chemosensitivity. Others have also reported on the relationship between induction of GRPs and tumor resistance against chemotherapy treatment. In contrast to our findings, high GRP78 expression levels were associated with tumor resistance to chemotherapy in breast (40) and prostate (41) cancer. The discrepancies between our results and those findings may reflect a different GRP regulation depending on the tumor type or the heterogeneity of the investigated tumor collectives.

Moreover, the pretherapeutic expression of two HSPs, HSP27 and HSP60, was significantly different in responders and nonresponders. Similar to GRPs, HSPs are chaperones that assist the proteins in their folding, stability, assembly into multiprotein complexes, and transport across cellular membranes. The expression of some of them is highly modulated in response to a wide variety of physiologic and environmental changes (34). Generally, intracellular HSPs have a cellular protective function due to their antiapoptotic properties. In cancer cells, in which the expression of HSPs is frequently abnormally high, they participate in oncogenesis and in resistance to chemotherapy (42, 43): for instance, overexpression of HSP27 has been shown to be associated with resistance to chemotherapy in advanced breast cancer (44), ovarian cancer (45), and in esophageal squamous cell carcinoma (46). Up-regulation of HSP60 was shown to occur after cisplatin treatment of a cervix carcinoma cell line (32). Expression of HSP27 in esophageal adenocarcinoma has been described by Soldes et al. (47) and Doak et al. (48), with both studies

dealing with the expression dynamics from nonneoplastic esophageal mucosa to metaplastic Barrett's mucosa and adenocarcinoma but not with potential response to chemotherapy. In our study, high pretherapeutic HSP27 and HSP60 protein expression were associated with tumor response to neoadjuvant chemotherapy. Similar to the discrepancies of our observations on the association between high GRP expression and chemosensitivity, the difference from other studies may be explained by different regulations depending on the tumor type, the tumor stage, and the investigated collectives, including different therapeutic regimens (e.g., adjuvant or neoadjuvant, combination with radiation, or hormonal therapy). Differences between these studies at the level of general protein abundances could be explained by the presence of redundant posttranslational isoforms of proteins.

However, in case of HSP27, the results of ProteoTope analysis were convincingly confirmed by immunohistochemistry and mRNA abundance. This correlation does not need to be expected because proteomic and genomic information often fail to correlate due to different kinetics of posttranslational modifications or protein turnover (49, 50). This also may explain the lack of correlation between proteomic data and validation experiments for HSP60, GRP78, and GRP94 besides the small number of samples analyzed. A fortiori, our results strongly indicate a potential predictive effect of high HSP27 expression in locally advanced esophageal adenocarcinoma.

In conclusion, we successfully applied a sensitive and precise differential proteomic technology to small pretherapeutic biopsies of patients with locally advanced esophageal adenocarcinoma. Explorative proteomic analysis detected differentially expressed proteins in responding relative to nonresponding patients after neoadjuvant chemotherapy. The results of this pilot study suggest that the difference between responders and nonresponders may involve the differential activation of a stress response. Chemotherapy generally may show a relation to a tumor-associated stress and inflammatory reaction. Validation of the potential of HSPs, especially HSP27, and GRPs as predictive biomarkers of chemotherapy response with a larger cohort of samples is warranted.

### Disclosure of Potential Conflicts of Interest

A. Schrattenholz, M. Cahill: ProteoSys AG, employees.

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