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A specific expression profile of heat-shock proteins and glucose-regulated proteins is associated with response to neoadjuvant chemotherapy in oesophageal adenocarcinomas

J Slotta-Huspenina^{*1,7}, C Wolff^{1,7}, E Drecoll¹, M Feith², M Bettstetter³, K Malinowsky¹, L Bauer¹, K Becker¹, K Ott⁴, H Höfler^{1,3,5}, K-F Becker¹ and R Langer^{1,6}

¹Institute of Pathology, Technische Universität München, Munich, Germany; ²Department of Surgery, Technische Universität München, Munich, Germany; ³Teilgemeinschaftspraxis Molekularpathologie Südbayern, Munich, Germany; ⁴Department of Surgery, Universität Heidelberg, Heidelberg, Germany; ⁵Institute of Pathology, Helmholtz-Zentrum München, Oberschleissheim, Germany and ⁶Institute of Pathology, Universität Bern, Bern, Switzerland

Background: Oesophageal adenocarcinomas often show resistances to chemotherapy (CTX), therefore, it would be of high interest to better understand the mechanisms of resistance. We examined the expression of heat-shock proteins (HSPs) and glucose-regulated proteins (GRPs) in pretherapeutic biopsies of oesophageal adenocarcinomas to assess their potential role in CTX response.

Methods: Ninety biopsies of locally advanced adenocarcinomas before platin/5-fluorouracil (FU)-based CTX were investigated by reverse phase protein arrays (RPPAs), immunohistochemistry (IHC) and quantitative RT-PCR.

Results: CTX response strongly correlated with survival ($P=0.001$). Two groups of tumours with specific protein expression patterns were identified by RPPA: Group A was characterised by low expression of HSP90, HSP27 and p-HSP27^(Ser15, Ser78, Ser82) and high expression of GRP78, GRP94, HSP70 and HSP60; Group B exhibited the inverse pattern. Tumours of Group A were more likely to respond to CTX, resulting in histopathological tumour regression ($P=0.041$) and post-therapeutic down-categorisation from cT3 to ypT0–T2 ($P=0.040$). High HSP60 protein (IHC) and mRNA expression were also associated with tumour down-categorisation ($P=0.016$ and $P=0.004$).

Conclusion: Our findings may enhance the understanding of CTX response mechanisms, might be helpful to predict CTX response and might have translational relevance as they highlight the role of potentially targetable cellular stress proteins in the context of CTX response.

Adenocarcinoma of the oesophagus is a very aggressive tumour with increasing incidence, particularly in countries of the Western world. For locally advanced tumours, neoadjuvant

chemo(radio)therapy is now frequently utilised as an integral part of a multimodal treatment regimen, and this treatment provides a survival benefit when compared with surgery alone (Lordick *et al*,

*Correspondence: Dr J Slotta-Huspenina; E-mail: slotta.huspenina@lrz.tum.de

⁷These authors contributed equally to this work.

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2004a; Sjoquist *et al*, 2011; van Hagen *et al*, 2012). However, approximately half of the patients show no obvious response to chemotherapy (CTX). Therefore, the pretherapeutic identification of non-responding patients would be valuable and help patients avoid inefficient therapy, toxic side effects and costs. For oesophageal adenocarcinoma, several studies have concentrated on the analysis of CTX-related markers (Fareed *et al*, 2009). Despite increasing knowledge regarding the molecular background of this cancer entity, no valid biomarkers have been identified to predict prognosis or CTX response in clinical routine.

Previously, we demonstrated that the regulation and expression of several molecular chaperones, such as heat-shock proteins (HSPs) and glucose-regulated proteins (GRPs), may have an important impact on the biology of this tumour with respect to prognosis (Langer *et al*, 2008a; Slotta-Huspenina *et al*, 2012a) and response to CTX (Langer *et al*, 2008b). In this study, we further elucidated the role of these regulatory proteins in the context of CTX response.

We performed a tissue-based, comprehensive expression analysis of the HSPs HSP60, HSP70, HSP90 and HSP27, including the phosphorylated forms p-HSP27^(Ser15), p-HSP27^(Ser78) and p-HSP27^(Ser82), and the GRPs GRP94 and GRP78 in a well-characterised collection of pretherapeutic biopsies of patients with oesophageal adenocarcinoma. These patients were treated with platinum/5-FU neoadjuvant CTX before surgery (Lordick *et al*, 2004a; Bader *et al*, 2008). We analysed the expression of these molecules using reverse phase protein array (RPPA) technology, which allows quantitative analysis of protein expression from formalin-fixed, paraffin-embedded tissue and, in addition, by immunohistochemistry (IHC) and quantitative real-time PCR (qRT-PCR). The results of the expression studies were then correlated with tumour response to neoadjuvant CTX treatment.

MATERIALS AND METHODS

Ethics Statement. All patients provided informed written consent, and the study was approved by the Ethics Committee of the Technische Universität München, Munich, Germany (No. 2056/08).

Patient characteristics and tissue specimens. Formalin-fixed and paraffin-embedded tumour samples from 90 patients with locally advanced oesophageal adenocarcinomas (cT3-T4) were investigated. These patients were treated between 1995 and 2009 in the Department of Surgery at Klinikum Rechts der Isar der Technischen Universität München, Germany. The median age of the patients was 62 (range 33–82) years. Eight female (8.9%) and 82 male patients (91.1%) were included in the study. Histopathological examination of the diagnostic biopsies revealed adenocarcinoma with a tumour differentiation grade two (G2, moderately differentiated) in 40 cases (46%) and three (G3, poorly differentiated) in 47 cases (54%). For three cases, no valid tumour differentiation grade could be assigned due to unrepresentative material.

Preoperative CTX. Patients were treated with a cisplatin/oxaliplatin- and 5-FU-based CTX (PLF/OLF scheme) without additional radiation or postoperative CTX according to previously published protocols, including the MUNICON trial (Lordick *et al*, 2004a, 2007). Thirty patients with an age of ≤ 60 years and good health status were also given paclitaxel (T-PLF/OLF; Lordick *et al*, 2007; Bader *et al*, 2008). Briefly, a total of 50 mg m^{-2} cisplatin was scheduled to be given as a 1-h intravenous (i.v.) infusion on days 2, 16 and 30. For patients with a glomerular filtration rate of $< 60 \text{ ml kg}^{-1} \text{ min}^{-1}$, oxaliplatin (85 mg m^{-2} over 2 h) replaced cisplatin. Moreover, 500 mg m^{-2} leucovorin was applied i.v. over 2 h on days 2, 9, 16, 23, 30 and 37, followed by a 2000 mg m^{-2} i.v.

infusion of 5-FU over 24 h. Paclitaxel consisted of 80 mg m^{-2} , given as a 3-h i.v. infusion on days 1, 15 and 29. Seventy-one patients received at least one cycle (61 patients two cycles, 10 patients one cycle). Following the schedule of the MUNICON trial, 19 patients had early therapy abort after 2 weeks due to metabolic non-response.

Surgery. A right abdominothoracic approach using an intra-thoracic anastomosis and two-field lymphadenectomy (Ivor Lewis procedure) or a transhiatal approach with cervical anastomosis was performed. Surgery was conducted 2–3 weeks after CTX completion.

Histopathological evaluation of tumour regression. The histopathological response following neoadjuvant CTX was investigated, and the procedure was conducted as previously described with a highly standardised work up of the corresponding resection specimens (Becker *et al*, 2003; Langer *et al*, 2009). The tumour regression grading (TRG = tumour regression grade) was based on an estimation of the percentage of vital tumour tissue in relation to the macroscopically determined tumour bed, which was completely worked up histologically. The following system of tumour regression was used: grade 1, complete (0% residual tumour); grade 2, subtotal and partial tumour regression (1–50% residual tumour per tumour bed); and grade 3, minimal or no tumour regression ($> 50\%$ residual tumour per tumour bed). The slides were reviewed separately by the two pathologists (KB and RL). In case of disagreement, both pathologists reviewed the specimen under a double-headed microscope and reached a consensus diagnosis. According to this grading system, 7 patients (7.8%) had complete tumour regression (TRG 1), and 30 patients (33.3%) had subtotal or partial tumour regression (TRG 2). Fifty-three patients (58.9%) demonstrated no response to neoadjuvant CTX (TRG 3), among them 18 patients with metabolic non-response and early therapy abort. Postoperative tumours were classified as ypT0 in 7 cases (7.8%), ypT1 in 12 cases (13%) and ypT2 in 19 cases (21.1%). Therefore, a tumour down-categorisation from cT3 to ypT0–T2 was observed in 38 cases (42%). Postoperative histopathological findings (TRG, UICC classification and tumour differentiation) are presented in Table 1. Regarding subgroups of patients according to the CTX scheme applied (PLF/OLF vs T-PLF/OLF), there was no difference between the groups in terms of response rate (37% vs 41%) and down-categorisation (40% vs 41%).

Follow-up. Overall survival (OS) was calculated from the day of surgery to death. One patient died within 1 month after surgery and was excluded from survival analysis. Three patients were lost to follow-up. Forty-three of the 86 remaining patients (50%) died with a median OS of 33 months (95% CI 23–42). The median follow-up time for surviving patients was 40.2 months (range 5.7–134.6).

Protein extraction and antibodies. Protein extractions were performed as previously described (Wolff *et al*, 2011b). Briefly, tumour tissue from three 10- μm sections was deparaffinised (addition of xylene twice for 10 min) and rehydrated (using 100%, 90% and 70% ethanol for 5 min each). The tumour tissue was microdissected to obtain a percentage of tumour tissue of at least 80%; haemorrhagic and necrotic areas were excluded. Approximately 0.5 cm^2 of tissue from three 10- μm -thick sections was processed in $100 \mu\text{l}$ of extraction buffer (EXB Plus, Qiagen GmbH, Hilden, Germany). Protein concentrations were determined using the Bradford protein assay according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). Lysates were probed for β -actin by western blot to verify protein extraction success and the suitability of the material for RPPA analysis. Before performing RPPA for HSP/GRP expression analysis, all antibodies were validated by western blot using protein extracts of formalin-fixed

paraffin-embedded tissues. A detailed list of the antibodies used is provided in Table 2.

RPPAs and quantitative protein analysis. RPPAs were generated using the Calligrapher MiniArrayer (Bio-Rad) (Gulmann *et al.*,

2006). Three replicates per lysate and dilution (undiluted, 1:2, 1:4, 1:8, 1:16, buffer) were applied to a nitrocellulose-coated glass slide (Grace Bio-Labs, Bend, OR, USA), yielding a total of 18 data points per sample. Peroxidase blocking was performed according to the manufacturer's instructions (Dako, Glostrup, Denmark). Immunodetection was conducted similar to western blot analysis. To estimate the total protein content, arrays were stained in parallel with Sypro Ruby Protein Blot Stain (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. The TIFF images of the antibody-stained slides and Sypro Ruby-stained slides were analysed with MicroVigene 3.5.0.0 software (VigeneTech, Carlisle, MA, USA). The MicroVigene signal-intensity points were calculated by the integral of a logistic four-point fit model that was matched optimally to the 18 data points.

IHC. IHC staining included antigen retrieval, antibody incubation and detection and was performed as described previously (Bauer *et al.*, 2012). A detailed list of the antibodies and dilutions is provided in Table 2. According to the results of a previously published study (Slota-Huspenina *et al.*, 2012a), we only analysed p-HSP27^{Ser15} from the group of pHSPs; p-HSP27^{Ser78} and p-HSP27^{Ser82} had been shown to exhibit only very focal immunoreaction, which cannot be considered as representative of whole tumoural expression. We counted at least 500 unequivocally identifiable tumour cells. Expression was assessed based on the intensity of cytoplasmic immunostaining and the percentage of stained tumour cells. The intensity was scored as 0 (negative), 1 (weak staining), 2 (moderate staining) or 3 (strong staining). The percentage of positive tumour cells was scored as 0 (none), 1 (<10%), 2 (10–50%), 3 (51–80%) or 4 (>80%). Multiplication of the scores for intensity and percentage resulted in a semiquantitative immunoreactive score (IRS) ranging from 0 to 12. Two independent observers (JSH and ED) evaluated the tumour staining. Differences were discussed at a double-header microscope to achieve a final consensus.

Quantitative real-time PCR. Microdissection, RNA extraction and cDNA synthesis were performed as described previously with minor modifications (Specht *et al.*, 2001). Following tissue preparation, the microdissected tumour tissue was transferred into a sterile 1.5-ml tube containing RNA lysis buffer. Lysis was conducted at 60 °C for 24 h until the tissue was completely solubilised. RNA was purified by phenol and chloroform extraction, followed by precipitation with an equal volume of isopropanol in the presence of 20 µl of 2 M sodium acetate (pH 4.0) and 2 µl of 10 mg ml⁻¹ glycogen at -20 °C. The RNA pellet was washed once in 70% ethanol, dried and resuspended in 20 µl of RNase-free water. One microgram of RNA was transcribed into cDNA using Superscript II reverse transcriptase (Invitrogen,

Table 1. Postoperative histopathology findings following neoadjuvant chemotherapy

Factor	Number of patients	%
	90	100
Tumour regression grade (TRG)		
TRG 1	7	7.8
TRG 2	30	33.3
TRG 3	53	58.9
UICC ypT category		
ypT0	7	7.8
ypT1	12	13.3
ypT2	19	21.1
ypT3	52	57.8
Lymph node metastases		
Absent	31	34.4
Present	59	65.6
Distant Metastases		
Absent	75	83.3
Present	15	16.7
Tumour grading		
G2	40	44.4
G3	47	52.2
Resection status		
R0	74	82.2
R1	16	17.8

Abbreviation: uicc = Union for International Cancer Control.

Table 2. Antibodies used for immunohistochemistry (IHC) and western blot (WB)/RPPA analysis

Protein	Antibody	Distributor	IHC Dilution	WB/RPPA
HSP27	#2402	Cell Signaling, Danvers, MA, USA	1:250	1:1000
Phospho-HSP ^(Ser15)	#ab39399	Abcam, Cambridge, UK	1:500	1:1000
Phospho-HSP ^(Ser78)	#2405	Cell Signaling, Danvers, MA, USA	ND	1:1000
Phospho-HSP ^(Ser82)	#2401	Cell Signaling, Danvers, MA, USA	ND	1:1000
HSP60	#ab46798	Abcam, Cambridge, UK	1:2000	1:2000
HSP70	#ab17850	Abcam, Cambridge, UK	1:1	1:50
HSP90	#ab1429	Abcam, Cambridge, UK	1:100	1:200
GRP78	#ab32618	Abcam, Cambridge, UK	1:1000	1:1000
GRP94	#sc1794	Santa Cruz Biotechnology Inc., CA, USA	1:5000	1:500

Abbreviations: GRP = glucose-related protein; HSP = heat-shock protein; ND = not done; RPPA = reverse phase protein array.

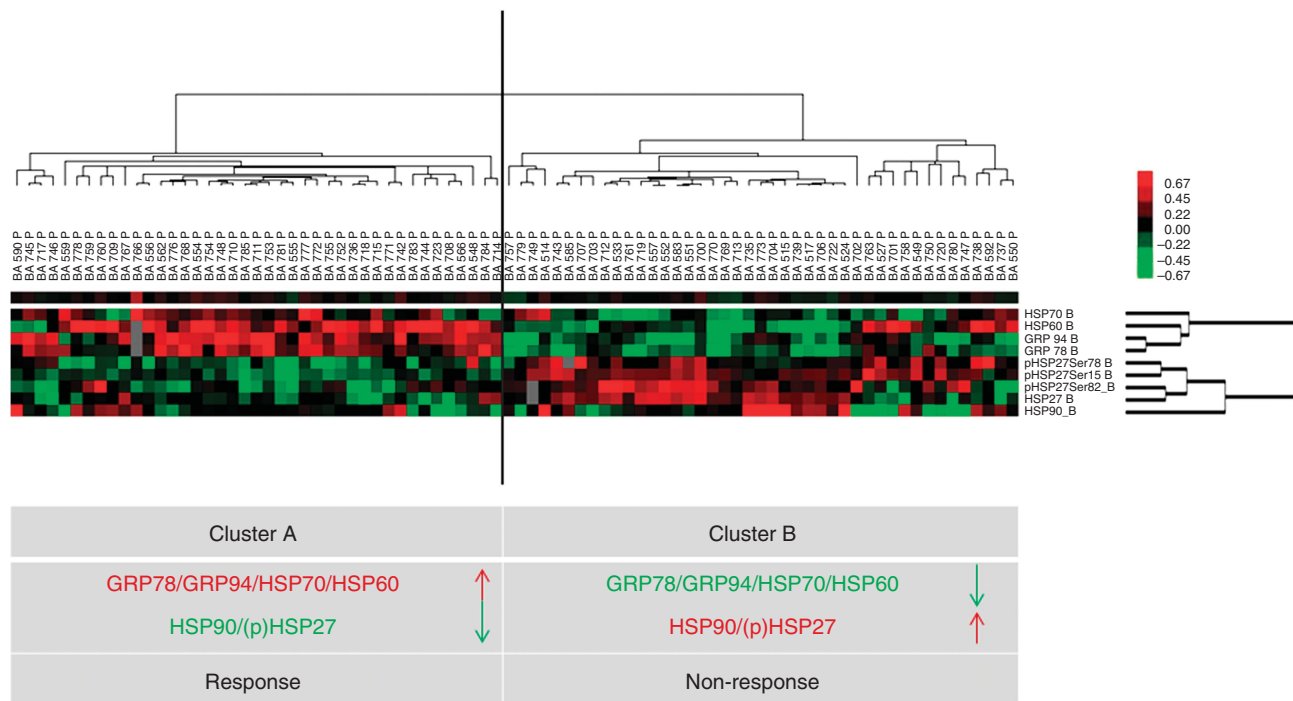


Figure 1. Unsupervised hierarchical cluster analysis of 81 oesophageal adenocarcinomas based on the expression of HSP27, HSP60, HSP70, HSP90, phosphorylated forms of HSP27 (Ser78, Ser82, Ser15), GRP78 and GRP94 measured by RPPAs. This analysis identified two groups of tumours with specific HSP/GRP protein expression patterns (A and B). The hallmark of Group A was high expression levels of HSP60, HSP70, GRP78 and GRP94 and low expression of HSP27, including its phosphorylated form (p-HSP27) and HSP90. Group B showed the inverse of Group A. Cluster colour key: ed – upregulated; green – downregulated; black – unchanged; grey – missing.

Darmstadt, Germany) and 250 ng of random hexamers (Roche Diagnostics, Penzberg, Germany) in accordance with the manufacturer's recommendations; the final volume of the reaction was 20 μ l. Gene expression was quantified using RealTime ready single assays (Roche Diagnostics) for the target genes *GRP94* (ID 100489), *HSP27* (ID 100497), *HSP70* (ID 110730), *GRP78* (ID 110805), *HSP90* (ID 138013) and *HSP60* (ID 137175) and for the housekeeping genes *PPIA* (ID 102088), *ALAS1* (ID 102108) and *ACTB* (ID 101125), as described previously (Slotta-Huspenina *et al.*, 2012a). Housekeeping genes were selected in a previous study using the RealTime ready Reference Gene Panel (Roche Diagnostics), which contains 19 different reference genes to facilitate the identification of the most suitable genes from 8 different carcinoma samples. Using GeNorm software (Biogazelle, Zwijnaarde, Belgium), the reference genes *PPIA*, *ALAS1* and *ACTB* were demonstrated to be stably expressed in all the analysed tissues. qRT-PCR was performed in triplicate with the LightCycler 480 Instrument using LightCycler 480 Probes Master (Roche Diagnostics) and 10 ng of cDNA per well. Thermal cycler conditions included 45 cycles at 95 °C for 10 s, 60 °C for 30 s and 72 °C for 1 s. Relative mRNA expression was calculated by the $\Delta\Delta$ Ct method using the LightCycler 480 Software and an efficiency-corrected algorithm with standard curves and triple normalisation to the *PPIA*, *ALAS1* and *ACTB* reference genes.

Statistical analysis. Unsupervised hierarchical clustering was performed using Cluster and TreeView software. Following log transformation and center to median calculations, average hierarchical clustering was performed using Spearman rank correlation (Eisen *et al.*, 1998). SPSS statistical software (IBM SPSS statistics version 21, Ehningen, Deutschland) was used for additional statistical analyses. Associations between the groups of patients are provided in cross tabs, and differences were determined using the χ^2 -test. Comparisons between groups were

performed using non-parametric tests (Mann-Whitney *U* test, Kruskal Wallis test and Spearman rank correlation). Survival analyses were performed using Kaplan-Meier estimates and log-rank tests. All tests were two-sided, and the significance level was set to $P < 0.05$.

RESULTS

Tumour regression correlates with patients' survival. According to the CTX response classification described above, TRG was prognostic ($P = 0.001$), and complete responders (TRG 1, $N = 7$) and partial responders (TRG 2, $N = 30$) had improved survival (median not reached in both the groups) compared with non-responders (TRG 3, $N = 53$), who had a median OS of 22 months (95% CI 11–33 months). Therefore, patients with complete and partial tumour regression (TRG 1 and 2) were classified as responders, and patients without tumour regression (TRG 3) were classified as non-responders according to their prognosis. Response was associated with postoperative tumour down-categorisation from cT3 to ypT0–T2 ($P < 0.001$), which was observed in 38 patients (42.2%). Patients with tumour down-categorisation had significantly improved outcome (median not reached) compared with patients with ypT3 tumours ($N = 52$); the median OS of patients with ypT3 tumours was only 25 months (95% CI 17–34 months; $P = 0.007$). A better tumour differentiation grade (G2 vs G3) in pretherapeutic biopsies was significantly associated with tumour down-categorisation ($P = 0.001$). Regarding the subgroups of patients according to the CTX scheme applied (PLF/OLF vs T-PLF/OLF), the association between histopathological response/down-categorisation and OS hold true for the PLF/OLF ($P = 0.002/0.020$) and in trend for the smaller T-PLF/OLF-treated patients ($P = 0.017/0.20$).

Expression analysis of HSPs and GRPs by RPPA in pretherapeutic biopsies identifies two patient groups that differ in response to CTX. RPPA was performed on 81 pretherapeutic biopsies; in 9 cases, protein lysates could not be further analysed due to limited amounts of extracted proteins. Representative RPPA results for the proteins analysed are provided in Supplementary file S1. All HSP and GRP proteins, including the phosphorylated forms of HSP27, could be detected and were expressed at various levels in tumour biopsies. The median quantitative protein expression (protein/SyproRuby) levels were as follows: HSP27: 164 (range 55–462), p-HSP27^(Ser15): 224 (range 52–848), p-HSP27^(Ser78): 319 (range 105–1274), p-HSP27^(Ser82): 121 (range 32–477), HSP60: 346 (range 87–151), HSP70, 298 (range 110–670), HSP90: 162 (range 75–360), GRP94: 316 (range 94–897), and GRP78: 491 (range 122–1444). In general, there was a significant positive correlation between the protein expression levels of HSP60, HSP70, GRP78 and GRP94 ($P < 0.001$) and between HSP90, HSP27 and p-HSP27^(Ser15, Ser78, Ser82) ($P < 0.001$). Unsupervised hierarchical clustering was conducted to generate a tumour-specific protein expression pattern for better understanding of the relationship between the HSP/GRP proteins. This approach is similar to one that was recently published by our group (Slotta-Huspenina *et al.*, 2012a). Two groups of tumours (Group A and Group B) could be clearly distinguished by their specific HSP/GRP protein expression patterns (Figure 1). Group A consisted of 38 tumours characterised by low expression of HSP90, HSP27 and p-HSP27^(Ser15, Ser78, Ser82) and high expression of HSP60, HSP70, GRP78 and GRP94. Group B consisted of 43 tumours and displayed an inverse pattern; these cases had low expression levels of HSP60, HSP70, GRP78 and GRP94 and high expression levels of HSP90, HSP27 and p-HSP27^(Ser15, Ser78, Ser82). The median expression levels of the single proteins in the two tumour groups are listed in Table 3. Next, we determined whether the identified HSP/GRP protein patterns correlated with CTX responses, particularly with respect to histopathological tumour regression and tumour down-categorisation. Tumours with HSP/GRP-pattern A were more likely to respond to neoadjuvant CTX (Table 4). There was a significant association with better tumour regression ($P = 0.041$) as well as a post-treatment down-categorisation ($P = 0.040$). Moreover, patients with pretherapeutic HSP/GRP-pattern A were more likely to be free of distant metastases ($P = 0.023$). No significant association was observed between the HSP/GRP protein expression patterns and other histopathological characteristics, which included lymph node status ($P = 0.075$) and tumour differentiation ($P = 0.132$). Analysing subgroups of patients according to the CTX

regime applied, significant association between HSP/GRP expression pattern and down-categorisation after CTX hold true for the PLF/OLF-treated patients ($P = 0.035$); however, regarding the small subgroup of patients who additionally were treated with Paclitaxel (T-PLF/OLF), there was only a trend similar between GSP/GRP patterns and response/down-categorisation, which did not reach statistical significance (see Supplementary file 2).

Immunoreactivity scores for HSP60 and HSP27 correlate with tumour down-categorisation. IHC analysis was performed on 82 biopsies according to the criteria given above. For eight cases, tumour cell content was not sufficient for reliable interpretation. Staining intensity scores (IRS) ranged from 0 to 12 for HSP27, HSP60, HSP90, GRP94 and GRP78 and from 0 to 9 for p-HSP27^(Ser15) and HSP70. The expression of the single proteins was compared in responders and non-responders. High HSP60 immunoreactivity (cutoff = median = IRS 9) in pretherapeutic biopsies was associated with tumour down-categorisation ($P = 0.016$), and low HSP27 immunoreactivity (cutoff = median = IRS 3) was associated with both down-categorisation ($P = 0.043$) and tumour regression ($P = 0.012$). For the other proteins, no associations were observed between IHC and CTX response. Similar results were obtained when analysing the subgroups of

Table 4. Association of HSP/GRP patterns A and B (RPPA) with histopathological response (TRG) and ypT category

	HSP/GRP-pattern A	HSP/GRP-pattern B	Total	P-value
Response	20	13	33	0.041
Non-response	18	30	48	
Total	38	43	81	
Down-categorisation	21	14	35	0.040
No down-categorisation	17	29	46	
Total	38	43	81	

Abbreviations: GRP = glucose-related protein; HSP = heat-shock protein; RPPA = reverse phase protein array; TRG = tumour regression grade. Responder: TRG 1/2, Non-responder: TRG 3; Down-categorisation: ypT0–T2, no down-categorisation: ypT3.

Table 3. Median protein expression levels of reverse phase protein arrays (RPPAs) in tumours with HSP/GRP-patterns A and B identified by unsupervised hierarchical cluster analysis

Protein	HSP/GRP-pattern A		HSP/GRP-pattern B		P-value
Median protein expression level (min–max)					
HSP60	560 (188–1508)	↑	290 (87–749)	↓	<0.001
HSP70	331 (109–597)	↑	283 (143–671)	↓	0.069
HSP90	148 (77–286)	↓	184 (75–361)	↑	0.003
GRP78	621 (149–1444)	↑	402 (123–941)	↓	<0.001
GRP94	429 (115–899)	↑	253 (95–810)	↓	<0.001
HSP27	122 (56–255)	↓	223 (65–463)	↑	<0.001
p-HSP27 ^(Ser15)	142 (52–357)	↓	312 (109–766)	↑	<0.001
p-HSP27 ^(Ser78)	216 (106–566)	↓	392 (172–1275)	↑	<0.001
p-HSP27 ^(Ser82)	82 (33–239)	↓	190 (52–477)	↑	<0.001

Abbreviations: GRP = glucose-related protein; HSP = heat-shock protein.

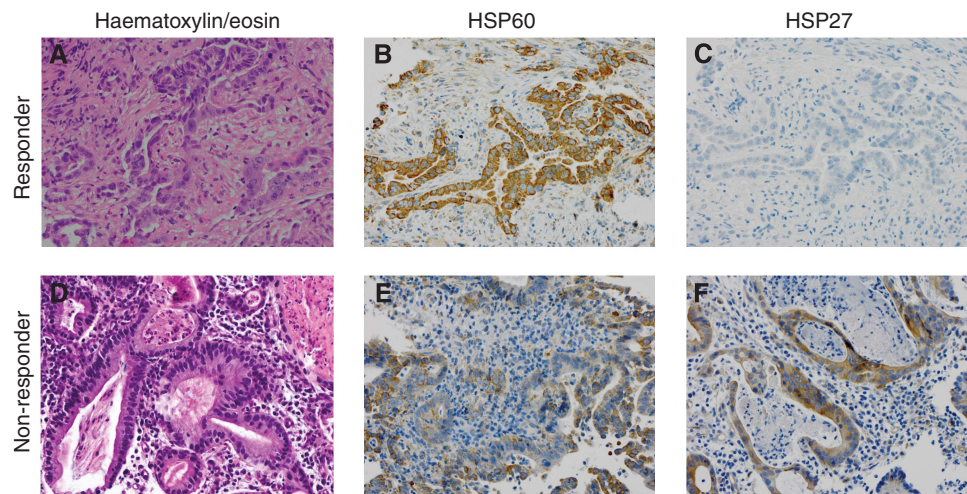


Figure 2. IHC staining for HSP60 and HSP27 in pretherapeutic biopsies of two oesophageal adenocarcinoma cases. (A–C) a CTX responder (No.745, TRG 1) and (D–F) a non-responder (No.772, TRG 3), are shown ($\times 200$): (A) haematoxylin/eosin; (B) very strong HSP60 expression; (C) no HSP27 expression; (D) haematoxylin/eosin; (E) weak HSP60 expression; and (F) strong HSP27 expression.

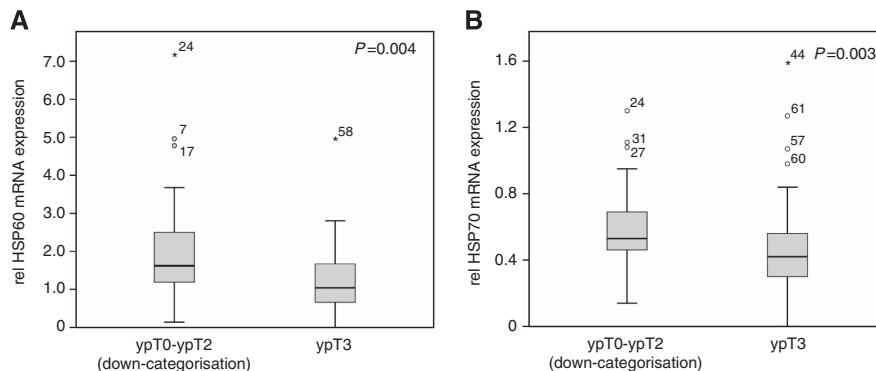


Figure 3. Box plot analysis of HSP60 and HSP70 mRNA expression in pretherapeutic biopsies of oesophageal adenocarcinoma. Box plots are illustrating the relative mRNA expression of (A) HSP60 and (B) HSP70 in 88 pretherapeutic biopsies of patients with tumour down-categorisation (ypT0–T2, $N = 38$) and without down-categorisation (ypT3, $N = 50$) after neoadjuvant chemotherapy.

PLF/OLF- and T-PLF/OLF-treated patients (see Supplementary file S2). HSP60 and HSP27 staining are shown for two representative cases (responder and non-responder) in Figure 2.

mRNA levels of HSP60 and HSP70 correlate with tumour down-categorisation. Quantitative real-time PCR analysis was performed on 88 biopsies; in two cases, mRNA extraction was not successful. mRNA from the target genes was detectable at various levels in cancer biopsies from all analysed patients. The relative median mRNA expression levels (ratio of target gene/housekeeping genes) were as follows: *HSP27*: 0.47 (range 0.1–0.75), *HSP60*: 1.26 (0.01–7.12), *HSP70*: 0.48 (0.01–1.59), *HSP90*: 0.45 (0.01–2.39), *GRP78*: 0.24 (0.01–1.85), and *GRP94*: 0.47 (0.01–1.24). The expression level of single HSP/GRP mRNAs was compared between responders and non-responders, but we found no association between any of the mRNAs and histopathological tumour regression. However, higher pretherapeutic HSP60 mRNA levels ($P = 0.004$) and higher HSP70 mRNA levels ($P = 0.003$) were significantly associated with tumour down-categorisation (median HSP60 in yT0–2 tumours: 1.62 vs yT3: 1.02, median HSP70 in yT0–2 tumours: 0.53 vs yT3: 0.42) (Figure 3). This holds true for the T-PLF/OLF subgroup of patients ($P = 0.019$ and 0.035; see Supplementary file S2).

Correlation between RPPA analysis, qRT-PCR and IHC. The two groups identified by RPPA analysis (Groups A and B) exhibited

significantly different mRNA expression levels for GRP78 (median relative expression for Group A: 0.30 and for Group B: 0.22, $P = 0.034$) and GRP94 (median relative expression for Group A: 0.56 and Group B: 0.41, $P = 0.050$). HSP60 may also be differentially expressed in these two groups (median relative expression for Group A: 1.45 and for Group B: 1.23, $P = 0.077$). For HSP27, a significant correlation was observed between protein levels (RPPA) and mRNA levels ($r = 0.335$; $P = 0.002$). For HSP70 and HSP90, no correlation was observed between RPPA and qRT-PCR results.

With IHC, tumours with HSP/GRP protein expression pattern A (Group A) had significantly higher IRs for HSP60 ($P = 0.010$) and a trend for lower p-HSP27^(Ser15) scores ($P = 0.083$). For HSP90, HSP70, GRP94 and GRP78, no correlation was observed between RPPA and IHC.

Between IHC reactive scores and mRNA levels, a significant correlation was detected for HSP90 ($P = 0.007$). For all other analysed proteins, no correlation was observed between IHC staining intensity and mRNA levels.

DISCUSSION

Expression of HSPs and GRPs can be induced in cells following exposure to different insults, allowing cells to survive stress

conditions. These proteins act as molecular chaperones either by helping refold damaged proteins or by assisting in their elimination. HSPs and GRPs also have an important role in the regulation of apoptosis (Garrido *et al*, 2006; Powers *et al*, 2009). Recently, studies have reported an association of HSPs and GRPs with cancer and demonstrated their important roles in cancer biology. Molecular chaperones have been suggested to influence tumour growth and differentiation and may have a major impact on the clinical outcome of patients (Ciocca and Calderwood, 2005; Calderwood *et al*, 2006; Fu and Lee, 2006; Lee, 2007; Khalil *et al*, 2011). Given the important cytoprotective role of HSPs in cancer cell physiology, it is not surprising that HSP expression also has been linked to radio- and CTX resistance (Fu and Lee, 2006; Kuramitsu *et al*, 2012).

Here, we present a tissue-based study reporting the role of HSPs and GRPs in the CTX response of oesophageal adenocarcinomas. We investigated the expression of the best-known HSPs and GRPs at the protein level by IHC, at mRNA level and by RPPA. We analysed pretherapeutic biopsies of a well-characterised sample collection of oesophageal adenocarcinoma patients. These patients were homogeneously treated by neoadjuvant CTX and tumour resection according to the standardised protocols (Lordick *et al*, 2004a, 2007). Tumour response to neoadjuvant treatment was assessed in a highly standardised and elaborated manner by determination of histopathological tumour regression grade (TRG) and tumour stage (Becker *et al*, 2003; Langer *et al*, 2009). We identified a specific protein expression pattern by RPPA exhibiting high levels of GRP78, GRP94, HSP70 and HSP60 and low levels of HSP90, HSP27 and p-HSP27^(Ser15, Ser78, Ser82); this expression pattern was significantly associated with response to neoadjuvant CTX. In a recently published study on primary resected oesophageal adenocarcinomas, we described a similar HSP/GRP expression pattern (high levels of GRP78, GRP94, HSP60 and a low level of p-HSP27) that was associated with poor prognosis and clinical outcome (Slotta-Huspenina *et al*, 2012a). This underlines the relevance of the findings of the current study. Both results taken together suggest that the combination of highly expressed GRP78, GRP94, HSP70 and HSP60 and low p-HSP27 expression may, on the one hand, be preferentially found in tumours with an aggressive biological behavior and, on the other hand, in tumours with a higher degree of sensitivity to CTX. However, for the cases analysed in the present study, we were unable to demonstrate that this HSP/GRP expression pattern impacted the prognosis, as the most important and best prognostic factor for OS was TRG, which was histomorphologically assessed in tumour resection specimens after neoadjuvant CTX. Another morphological-based criterium, better tumour differentiation grade using the grading system outlined by the WHO classification of tumours Sobin *et al*. (2010), highly correlated with post-treatment down-categorisation from cT3 to ypT0–T2 in our cohort. Although there are certain limitations regarding representativity and objectivity in the estimation of histopathological grading (Dikken *et al*, 2012), currently there are no validated molecular biomarkers in oesophageal adenocarcinomas to predict CTX response. Nevertheless, there is more evidence that the revealed protein pattern is of high relevance in this context. Previously, our group used a proteomic screening approach to explore potential predictive proteins in a small cohort of oesophageal adenocarcinoma patients treated with neoadjuvant CTX (Langer *et al*, 2008b). In that study, high pretherapeutic expression levels of GRP78, GRP94, HSP60 and HSP27 were associated with response to neoadjuvant CTX. This protein combination coincided with the pattern of the current study (high GRP78, GRP94, HSP70, HSP60 and low HSP90, (p)HSP27) except for one protein – HSP27. The discrepancy of the prediction potential of this marker might be explained by several points: (1) The present study was conducted on a larger sample collection of 90 patients, in contrast to the small sample set of 34

patients included in the former screening study. (2) Different methods for protein and mRNA expression analysis were used in the previous study, and (3) the definition of response was based on the metabolic response assessed by PET – whereas in the current study, it was based on histopathologic tumour regression and down-categorisation (T-stage), which have been shown to be of greater prognostic value in the past few years. However, the divergent findings with respect to HSP27 of the proteomic analysis cannot be fully explained. Moreover, we are aware that both histopathological regression and metabolic response are only surrogate markers associated with improved survival and cannot guarantee recurrence-free long-term survival, because it is supposed to be influenced by multiple clinical, histopathological and molecular factors.

In accordance with our observations in primary resected oesophageal adenocarcinomas, the most striking result from this study was that the specific prognostic protein expression pattern could only be detected by RPPA and subsequent clustering analysis. However, expression levels of single HSP and GRP proteins were not predictive when analysed individually.

RPPA is a new, high-throughput technology and a powerful tool for the molecular characterisation of paucicellular material. RPPA can also be used for the challenging analysis of small numbers of cells of human biopsies (Paweletz *et al*, 2001; Agari *et al*, 2012) and samples from formalin-fixed, paraffin-embedded tissue (Wolff *et al*, 2011a). A benefit of RPPA is the ability to simultaneously quantify different proteins in several biological samples at the same time and under the same experimental conditions, generating patient-specific ‘protein patterns’ and signaling networks. Hierarchical cluster analysis of expression data then allows to identify similarities in overall protein expression patterns and to stratify patients based on their molecular level. However, this approach also has some disadvantages: expression of individual proteins becomes less relevant as the clustering process progresses and an incorrect assignment made early in the process cannot be corrected (Tamayo *et al*, 1999). Using RPPA and cluster analysis, we identified a prognostic HSP/GRP pattern in oesophageal adenocarcinomas in a former study (Slotta-Huspenina *et al*, 2012a). Now we found a similar HSP/GRP expression pattern that was associated with response to CTX.

Interestingly, high HSP60 and low HSP27 expression assessed by IHC were also significantly associated with CTX response. Thus, HSP60 and HSP27 may serve as surrogate markers for the underlying protein expression profile and may serve as clinically useful predictors for CTX response.

Moreover, as for high HSP60 mRNA expression an association to CTX response could be demonstrated as well, these additional results underline the possible important role of HSP60 in the context of CTX response in oesophageal adenocarcinomas. However, the use of HSP/GRP expression as a molecular predictor for CTX response is not clinically applicable in its current form yet and needs to be validated in independent case collections or in prospective studies.

Although the sample size of the present study is appropriate for the identification of predictive biomarkers and is comparable with other publications in this field, we also face the problem with regard to a homogenous cytotoxic treatment, as others do (Fareed *et al*, 2009). Most anticancer regimes consist of a combination of various drugs, thus hampering the analysis of substance-specific resistance mechanism *in vivo*. In this study, we investigated a collective of patients who were all treated with a platin/5-FU-based CTX (PLF/OLF) according to standardised protocols (Lordick *et al*, 2004b, 2007); additional application of paclitaxel in a subgroup of patients did not show a significant bias with regards to the outcome of the patients, the rate of CTX response and the impact on the molecular findings. We suppose that these findings support the idea of a general cellular sensitivity towards cytotoxic therapy

that is unspecific, substance independent and is mainly caused by differential expression profiles of molecular chaperones. The result of our study may not only improve the molecular understanding of CTX resistance but may also impact the development of new molecular targeted substances in the era of personalised treatment. Recently, novel therapeutic agents that inhibit HSPs have been developed and have already emerged as powerful anti-tumoural agents in preclinical settings alone or in combination with other drugs in gastrointestinal and non-gastrointestinal malignancies (Moser *et al*, 2009; Jego *et al*, 2010; Dickson *et al*, 2013).

In summary, using RPPA technology we were able to detect a specific HSP/GRP protein expression pattern in oesophageal adenocarcinomas that was associated with response to neoadjuvant CTX but not with prognosis. Additional results from IHC and qRT-PCR experiments suggest an important role for HSP60 in CTX response. Our findings may be helpful to predict CTX response. Moreover, these findings may facilitate our understanding of CTX response mechanisms and may also serve as a foundation for the development of strategies to overcome CTX resistance.

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