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Frequent homogeneous *HER-2* amplification in primary and metastatic adenocarcinoma of the esophagus

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***HER-2* is the target for antibody based treatment of breast cancer (Herceptin®). In order to evaluate the potential role of such a treatment in esophageal cancers, *HER-2* amplification and overexpression was investigated in primary and metastatic cancers of the esophagus. A tissue microarray was constructed from 255 primary esophageal cancers (110 adenocarcinomas and 145 squamous cell carcinomas), 89 nodal and 33 distant metastases. Slides were analyzed by immunohistochemistry (HercepTest™; DAKO) and fluorescence *in situ* hybridization (FISH; PathVysion™; Vysis-Abbott) for *HER-2* amplification and overexpression. Amplification was seen in 16/110 (15%) adenocarcinomas and in 7/145 (5%) squamous cell carcinomas. There was a strong association between *HER-2* amplification and overexpression, especially in adenocarcinomas ($P<0.0001$, log rank). There was a 100% concordance of the *HER-2* results in primary tumor and corresponding metastases in 84 analyzed pairs. Amplification was typically high-level with more than 10–15 *HER-2* copies per tumor cell. Amplification was unrelated to survival, grading, pT, pN, pM or UICC stage. We conclude that esophageal adenocarcinomas belong to those cancer types with relevant frequency high-level *HER-2* gene amplification clinical trials or individual case studies investigating the response of metastatic *HER-2*-positive esophageal cancers to Herceptin® should be undertaken. The strong concordance of the *HER-2* status in primary and metastatic cancers argues for a possible response of metastases from patients with *HER-2*-positive primary tumors to Herceptin®.**
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The *HER-2* oncogene is involved in the development of numerous types of human cancer and has been intensely evaluated as a therapeutic target.[1–3] *HER-2* gene amplification and protein overexpression occurs in about 20% of breast cancers [4] and is linked to poor prognosis within these tumors.[5] Even more importantly, *HER-2* is the target of an antibody based therapy (trastuzumab; Herceptin®), which is routinely used in metastatic *HER-2*-positive breast cancer.[6–8] More recently, adjuvant trastuzumab application was also shown to be dramatically effective in *HER-2*-positive breast cancer patients.[9] Remarkably, several studies have suggested considerable heterogeneity between primary breast cancer and their metastases suggesting that the *HER-2* status of the metastases rather than the primary tumor findings may determine the effect of Herceptin® therapy.[10,11]

The potential benefit of trastuzumab in other tumor entities is largely unknown. *HER-2* positivity has been described in most human tumor types but with a highly variable frequency.[12–15] This especially applies for immunohistochemical (IHC) studies where different reagents and definitions of positivity resulted in an extremely wide range of *HER-2* positivity in almost all tumor types. Despite of this, there is evidence for a possible response of *HER-2*-positive nonbreast cancers to trastuzumab.[16–18] Applying Herceptin® as additional treatment option would be of particular interest in tumors with a notoriously poor prognosis such as esophageal

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cancer. Several studies indeed suggested that *HER-2* amplification/overexpression may be relevant for these tumor entities. *HER-2* overexpression was reported in 0–83% of esophageal cancer, with a tendency towards higher rates of positivity in adenocarcinoma (10–83%)[19–34] compared to squamous cell carcinomas (0–56%).[31,32,35–40] A similar variability was observed in amplification analyses. Different methods for analysis (Southern blot or FISH) and definitions of amplification have resulted in amplification frequencies ranging from 15–100% in adenocarcinomas[19–22,41–43] and from 0 to 25% in squamous cell carcinomas of the esophagus.[31,35,39,44,45]

In an attempt to clarify the significance of *HER-2* gene amplifications in esophageal cancers and their metastases, FDA (US Food and Drug Administration) approved methods and scoring criteria for IHC (HercepTest™; DAKO) and fluorescent *in situ* hybridization (PathVysion™; Vysis-Abbott) were used in this study. In order to obtain most reliable data, a tissue microarray was utilized.[46] With this method, 255 primary tumors and 112 corresponding metastases could be analyzed on one slide on one day in one set of reagents thus allowing maximal assay standardization. Our data suggest an important role of *HER-2* in esophageal adenocarcinoma.

Subjects and methods

Patients

A consecutive set of 292 patients operated for esophageal cancer between 1992 and 2004 of the University Medical Center Hamburg-Eppendorf was analyzed including 129 adenocarcinomas and 163 squamous cell carcinomas from 228 males and 64 females, ranging in age from 34 to 92 years (median 62 years). All patients had curative en bloc esophageal resection and radical lymph node dissection. Continuous follow-up data were recorded from 287 patients (125 adenocarcinomas, 162 squamous cell carcinomas). For each patient who died during the follow-up period, the cause of death was determined as cancer-related or not cancer-related. Patients that died within 1 month after surgery were excluded as perioperative failures. Four patients with adenocarcinoma and one with squamous cell carcinoma were lost to follow-up. Median follow-up time for all patients was 23 months (95% CI: 18.4–27.6). All tumors were reclassified for this study according to the WHO 2000 classification.[47]

Tissue Microarray Construction

Tissue samples were fixed in 4% buffered formalin, paraffin embedded, and used for tissue microarray construction as described.[46] Haematoxylin-eosin stained sections were made from selected primary tumor blocks (donor blocks) to define representative tumor regions. Tissue cylinders (0.6 mm in diameter) were then punched from that region of the donor block using a homemade semiautomated tissue arrayer. Control samples included normal esophagus mucosa ($n=10$), endometrium ($n=2$), skin ($n=2$), skeletal muscle ($n=2$), heart muscle ($n=2$), colon mucosa ($n=2$), lung ($n=2$), lymph node ($n=2$), prostate ($n=2$) and kidney ($n=2$). An overview of the complete tissue microarray with a total of 510 tissue samples is shown in Figure 1a, a single tissue spot of each adenocarcinoma and squamous cell carcinoma stained with haematoxylin-eosin is shown in Figure 1b and c. Three μm sections were made by use of the Paraffin Sectioning Aid System (Instrumentics, Hackensack, NJ, USA). Consecutive sections were used for fluorescence *in situ* hybridization (FISH) and IHC analysis.

Fluorescence *In Situ* Hybridization

For proteolytic slide pretreatment a commercial kit was utilized (Paraffin pretreatment reagent kit, Vysis, Downers Grove, IL, USA). Spectrum-Orange-labeled *HER-2* probes were used together with Spectrum-Green-labeled centromer 17 reference probes (PathVysion™, Vysis-Abbott). Before hybridization, sections were deparaffinized, air dried, dehydrated and then denaturized for 5 min at 74°C in 70% formamide-2 X SSC solution. After overnight hybridization at 37°C in a humid chamber, slides were washed and counterstained with 0.2 μM DAPI in an antifade solution. The mean numbers of *HER-2* and centromer 17 signals were estimated for each tumor sample as described before.[48,49] Our criteria for *HER-2* gene amplification were a *HER-2*/centromer 17 signal ratio ≥ 2. Low-level amplification ratio was defined as *HER-2*/centromer 17 at ≥ 2 to <3, high-level amplification was defined as a *HER-2*/centromer 17 ratio of ≥ 3.

Immunohistochemistry

The HercepTest™ (DAKO, Glostrup, Denmark) was used according to the protocol of the manufacturer. Antigen retrieval of the deparaffinized tissue sections was performed in a waterbath at 95°–99°C for 50 min followed by peroxidase blocking and incubation with the prediluted primary antibody. Cell line test slides provided by the manufacturer were used as positive and negative controls. Immunostaining was scored by one pathologist (UR), following a four-step scale (0, 1+, 2+, 3+) according the manufacturer's directions.

Statistical Analysis

Contingency table analysis and Fisher's exact test were used to study the relationship between *HER-2* alterations and categorical parameters. Survival

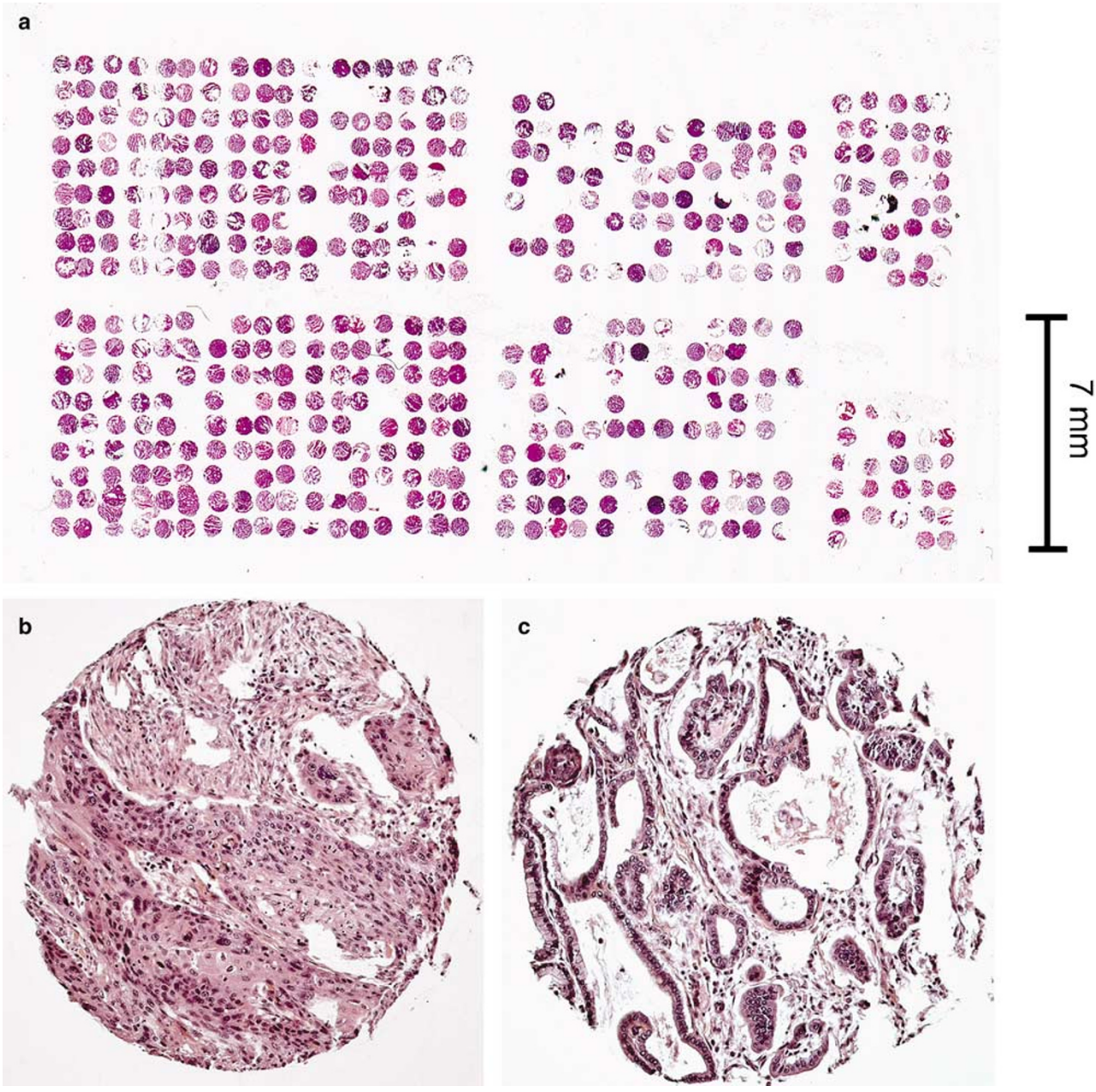

Figure 1 Esophageal cancer array. (**a**) Complete esophageal cancer array, stained with hematoxylin-eosin, consisting of one paraffin block (25 × 30 mm). (**b**) Example of a single hematoxylin-eosin stained tissue spot of an esophagus squamous cell carcinoma (magnification × 100). (**c**) Example of a single hematoxylin-eosin stained tissue spot of an esophageal adenocarcinoma (magnification × 100). (**d**) Example of a single tissue spot of an esophageal adenocarcinoma showing strong (3 +) *HER-2* protein expression (magnification × 100). (**e**) Example of a single tissue spot of an esophageal adenocarcinoma showing *HER-2*-negative immunohistochemistry (magnification × 100). (**f**) Example of a single *HER-2*-amplified tissue spot of an esophageal adenocarcinoma (magnification × 1000). (**g**) Example of a single *HER-2*-nonamplified tissue spot of an esophagus adenocarcinoma (magnification × 1000).

curves were plotted according to Kaplan–Meier. A log rank test was applied to examine the relationship between molecular or histological data and tumor-specific survival. Tumor-specific survival was scheduled as our clinical end point. Analysis was performed using the SPSS statistical software package for Windows (version 13.0, SPSS Inc., Chicago, IL, USA).

Results

Clinical Data

Among all 251 patients with available follow-up data (four adenocarcinomas were lost to follow-up) there were 134 tumor-related deaths (53%), 27 perioperative deaths (11%) which were excluded

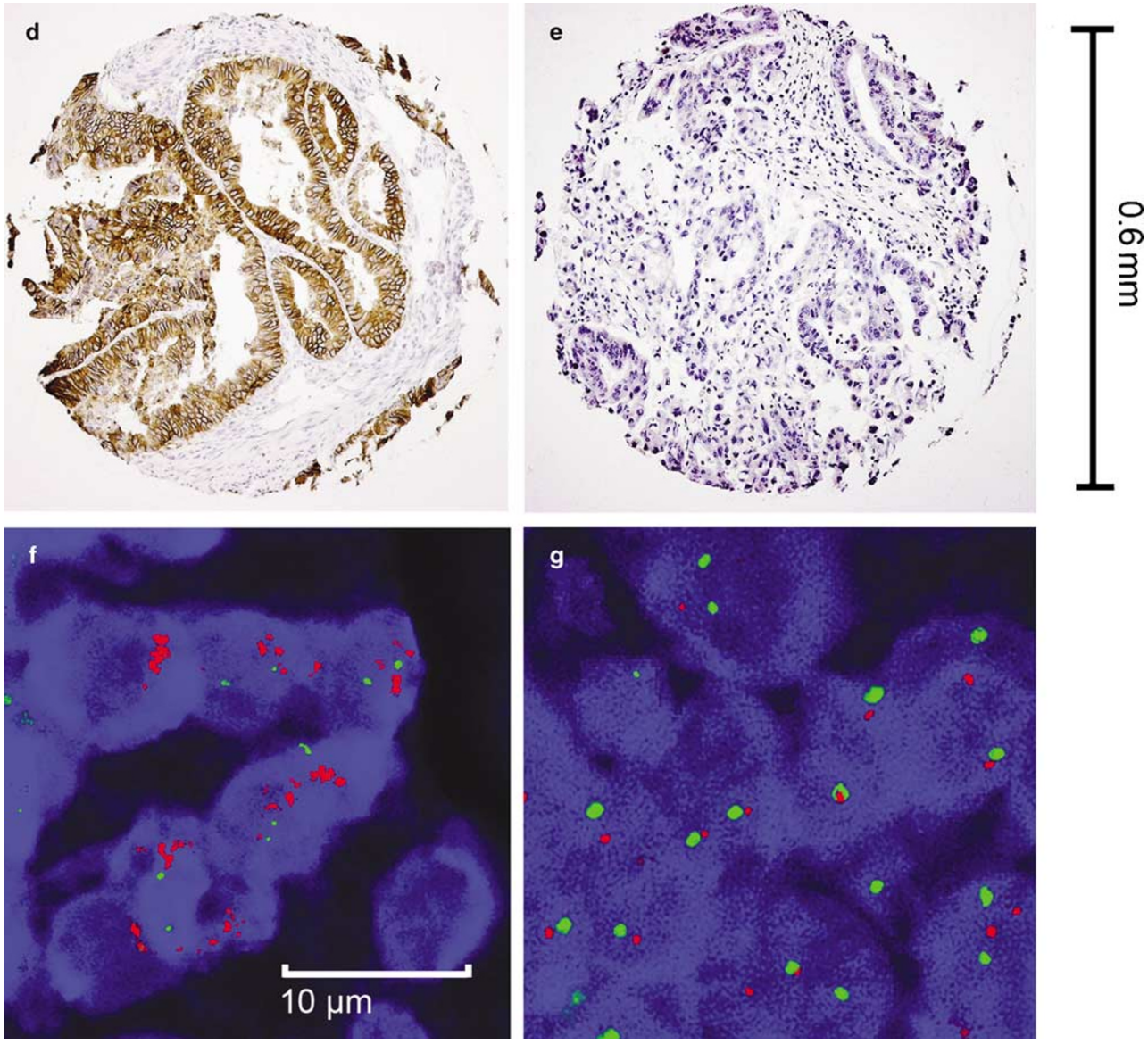

Figure 1 Continued.

from survival analysis and 23 tumor-unrelated deaths (9%). The remaining 67 patients were alive at the time of their last clinical control. This includes 54 (22%) disease-free patients. Average disease-specific survival was 25 months (95% CI: 18.7–31.3) for adenocarcinomas and 19 months (95% CI: 14–24) for squamous cell carcinomas. pT ($P<0.0001$, log rank; Figure 2a), pN ($P<0.0001$, log rank), pM ($P<0.0001$, log rank) and UICC stage ($P<0.0001$, log rank), but not histologic grade and tumor type were significantly related to survival time.

Technical Aspects of *HER-2* Analysis

A total of 255 primary tumors, 89 nodal and 33 distant metastases were interpretable by FISH (79% of all samples). The remaining 102 samples were noninformative because of absence of tissue on the tissue microarray, lack of unequivocal tumor cells in the arrayed samples or insufficient hybridization ($n=377$). Only one tissue microarray section was hybridized. No attempts were made to increase the number of informative cases by additional experiments under different conditions because the absolute number of interpretable cases was considered large enough for the purpose of this study. Examples of interpretable *HER-2* amplified and nonamplified cancers are shown in Figure 1f and g. For IHC, 378 of our samples were interpretable while 101 were not interpretable due to the absence of tissue on the tissue microarray or a lack of unequivocal tumor cells in the arrayed samples. Examples of interpretable tumor tissue samples of positive and negative *HER-2* IHC are shown in

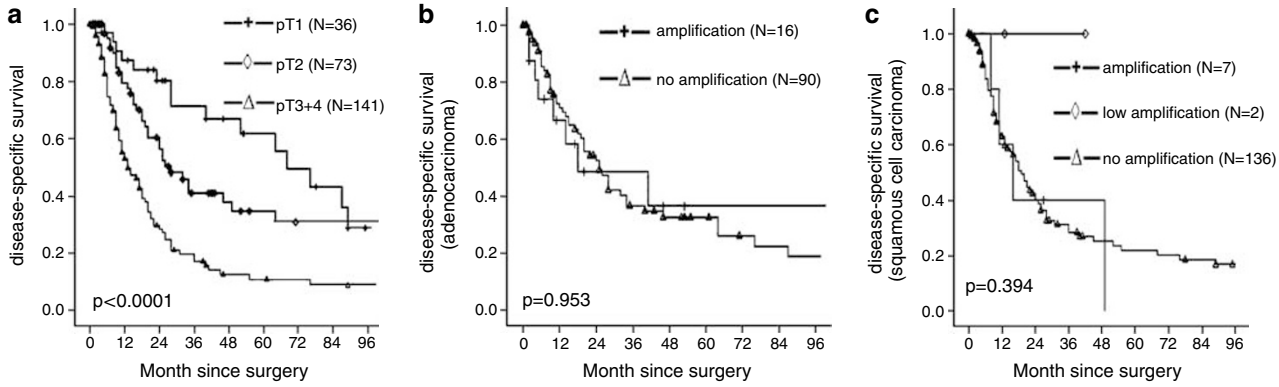

Figure 2 (**a**) Kaplan–Meier curve for disease-specific survival and pT-stage in esophageal cancer. *P*-values were calculated using log rank test. (**b**) Kaplan–Meier curve for disease-specific survival and *HER-2* amplification in esophageal adenocarcinomas. *P*-values were calculated using log rank test. (**c**) Kaplan–Meier curve for disease-specific survival and *HER-2* amplification in esophageal squamous cell carcinomas. *P*-values were calculated using log rank test.

Table 1a *HER*-2 amplification and overexpression in primary adenocarcinoma (subgroup analysis)

	FISH					*Immunohistochemistry*					
	N	*Ratio≤2 (%)*	*Ratio≥2 to <3 (%)*	*Ratio >3 (%)*	P-*value*	N	*Neg. (%)*	*+1 (%)*	*+2 (%)*	*+3 (%)*	P-*value*
pT											
1	17	77	0	23		18	50	28	11	11	
2	41	90	0	10		42	71	19	5	5	
3+4	53	85	0	15	0.351	53	60	26	6	8	0.702
Grade											
1	0	0	0	0		0	0	0	0	0	
2	41	80	0	20		41	56	24	10	10	
3	68	88	0	12	0.278	71	68	23	4	5	0.445
pN											
0	27	78	0	22		28	54	21	14	11	
1–3	82	88	0	12	0.219	84	67	24	4	6	0.158
R											
0	87	86	0	14		90	63	23	7	7	
1	12	83	0	17		12	67	17	8	8	
2	8	88	0	12	0.870	8	63	38	0	0	0.965
pM											
0	80	86	0	14		82	62	24	6	7	
1+2	30	83	0	17	0.763	31	65	23	7	7	1.000
UICC status											
1	19	79	0	21		20	50	25	15	10	
2	32	91	0	9		33	73	18	3	6	
3	30	83	0	17		30	53	33	3	10	
4	29	86	0	14	0.671	30	70	20	7	3	0.542

P-values were calculated using Fisher's exact test.

Figure 1d and e. Both FISH and IHC were interpretable on the same tissue spot in 110 primary adenocarcinomas and in 144 primary squamous cell carcinomas. All control samples were always *HER-2* negative both by IHC and FISH.

HER-2 in Primary Tumors

Frequency and type of *HER-2* alterations varied considerably between adenocarcinomas and squamous cell carcinomas (Tables 1a and b). In adenocarcinomas, high-level amplification (ratio *HER-2*/centromer 17≥3) was markedly more frequent (15%; 16/110) than in squamous cell carcinomas (5%; 7/145; $P<0.009$, Fisher's exact test). High-level amplifications were strongly associated with protein overexpression in both tumor types (adenocarcinoma: $P<0.0001$; squamous cell carcinoma: $P=0.002$, Fisher's exact test). Lower-level amplifications, defined as ratio *HER-2*/centromer 17 between 2 and 3, were not observed in adenocarcinomas.

Table 1b *HER-2* amplification and overexpression in primary squamous cell carcinoma (subgroup analysis)

	FISH					*Immunohistochemistry*					
	N	*Ratio <2 (%)*	*Ratio≥2 to <3 (%)*	*Ratio≥3 (%)*	P-*value*	N	*Neg. (%)*	*+1 (%)*	*+2 (%)*	*+3 (%)*	P-*value*
pT											
1	19	100	0	0		19	68	16	16	0	
2	34	82	3	15		34	65	24	6	6	
3+4	91	97	1	2	0.035	92	70	27	2	1	0.130
Grade											
1	4	100	0	0		4	75	25	0	0	
2	98	92	2	6		99	70	23	6	1	
3	42	98	0	2	0.771	42	64	29	2	5	0.632
pN											
0	55	91	2	7		55	71	20	7	2	
1–3	90	96	1.1	3	0.509	91	67	28	3	2	0.563
R											
0	115	94	0	6		116	70	22	6	3	
1	24	96	4	0		24	59	38	4	0	
2	6	83	17	0	0.034	6	83	17	0	0	0.739
pM											
0	125	94	1	5		126	71	23	5	2	
1+2	19	90	5	5	0.285	19	53	37	5	5	0.188
UICC status											
1	15	100	0	0		15	67	20	13	0	
2	54	91	2	7		54	70	20	6	4	
3	49	98	0	2		50	72	26	3	0	
4	26	89	4	8	0.474	26	58	35	4	4	0.505

P-values were calculated using Fisher's exact test.

The vast majority of amplified adenocarcinomas (14/16; 88%) showed a strong *HER-2* protein expression (2+/3+) on corresponding IHC stained sections. One of 94 (1%) nonamplified adenocarcinomas (ratio <2.0) showed a positive *HER-2* protein expression (2+) and two highly amplified tumors showed no (0) or just a low positive *HER-2* protein expression (1+) (Figure 3a).

HER-2 amplification was less frequent in squamous cell carcinoma than in adenocarcinoma. In these tumors, the average *HER-2* gene copy number per amplified tumor cells (3.2; 95% CI: 2.529–3.885) was significantly lower than in adenocarcinomas (6.4; 95% CI: 3.838–8.944; $P=0.008$; ANOVA). Most 2+ tumors (5/7) and one of three 3+ tumors had no *HER-2* amplification by FISH. Accordingly, the relationship with *HER-2* protein overexpression was much less stringent than in adenocarcinoma but still significant ($P=0.002$, log rank; Figure 3b).

Both *HER-2* amplification and overexpression were generally unrelated to pT, pN, pM, grade, UICC-status and resection margin (Tables 1a and b). This holds true for all tumors as well as for the subgroups of adenocarcinomas and squamous cell carcinomas, with two exceptions in squamous cell carcinomas. Here, an inverse correlation was found between amplification and resection margin ($P=0.034$, Fisher's exact test) and a positive association between pT and *HER-2* amplification ($P=0.035$, Fisher's exact test, Table 1b).

No correlation was observed between survival and *HER-2* amplification (Figure 2b and c) or overexpression (data not shown), neither in adenocarcinoma ($P=0.953$, Fisher's exact test) nor in squamous cell carcinoma ($P=0.394$, Fisher's exact test).

HER-2 in Metastases

HER-2 status of lymph nodes and distant metastases was strongly correlated to the *HER-2* findings in the respective primary tumors. For FISH, the findings in lymph node metastases corresponded exactly to the primary tumor data in all 84 (adenocarcinoma: $n=43$; squamous cell carcinoma: $n=41$) successfully analyzed pairs (100%; $P<0.0001$, Fisher's exact test) and in distant metastasis in all 30 (adenocarcinoma: $n=21$; squamous cell carcinoma: $n=9$) analyzed pairs. These pairs included 16 patients (adenocarcinoma: $n=11$; squamous cell carcinoma: $n=5$) from which the primary tumor, the corresponding lymph node metastasis and the distant metastasis could be analyzed, all with 100% concordance.

The concordance between primary tumors and lymph node metastasis was still good but less perfect for IHC. In adenocarcinomas a complete concordance in the scoring results was seen in 23 (54%, $P=0.001$, Fisher's exact test), in squamous cell carcinomas in 20 (50%, $P=0.036$, Fisher's exact

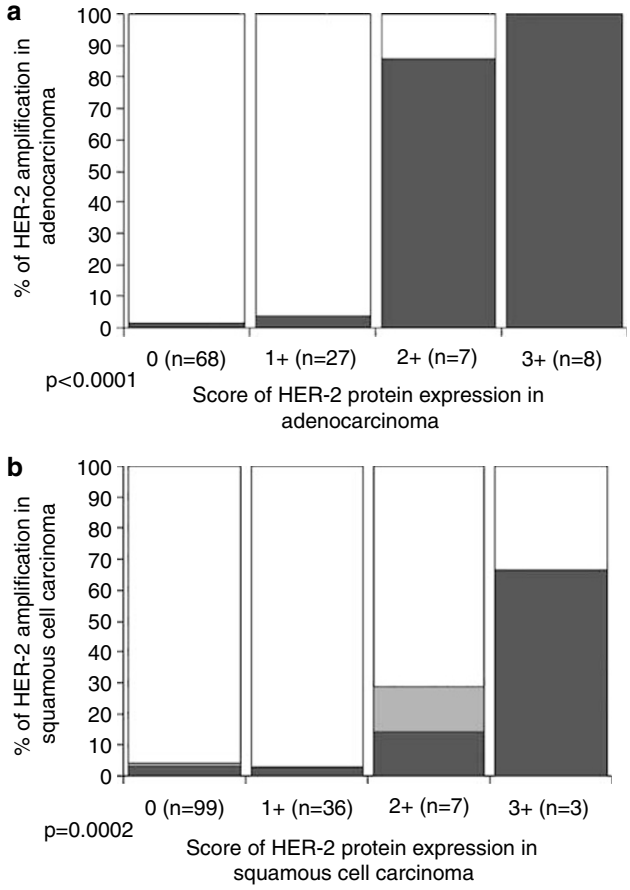

Figure 3 (**a**) *HER-2* status in primary tumor samples. Comparison of *HER-2* expression determined by IHC and *HER-2* amplification determined by FISH in esophageal adenocarcinomas. Black area = percentage of tumors with high-level *HER-2* amplification; white area = percentage of tumors with no *HER-2* amplification. (**b**) *HER-2* status in primary tumor samples. Comparison of *HER-2* expression determined by IHC and *HER-2* amplification determined by FISH in esophagus squamous cell carcinomas. Black area = percentage of tumors with high-level *HER-2* amplification; gray area = percentage of tumors with low-level *HER-2* amplification; white area = percentage of tumors with no *HER-2* amplification.

test) of pairs. A difference of only one point was seen in 18 (42%) adenocarcinomas and in 19 (48%) squamous cell carcinomas while a difference of two or more points occurred in only two (5%) adenocarcinomas and in one (2%) squamous cell carcinoma.

Discussion

Fundamental differences in the role of *HER-2* amplification/overexpression were found between adenocarcinomas and squamous cell carcinomas of the esophagus in this study. The findings in adenocarcinomas were very similar to breast cancer, the best examined cancer type for *HER-2* alterations so far.[4,50] Strong overexpression was seen in 13% of cases and almost all of them had high-level *HER-2* amplification with greater than 10–15 *HER-2* copies per cell. It appears possible, that the one adenocarcinoma with overexpression (2+) in the absence of amplification and the two cases with amplification in the absence of overexpression may represent technical errors of IHC based on fixation variability in routinely processed clinical samples.[51] The same reasons may apply for discrepant IHC results between primary tumors and metastases. It is well known, that prolonged formalin fixation can disturb immunohistochemical detectability of *HER-2* protein while insufficient formalin fixation with consecutive tissue exposure to ethanol during technical processing can lead to false positive *HER-2* IHC.[52–54]

Considering the encouraging results of clinical trials in breast cancer, it could be speculated that Herceptin® might also represent a possible option for *HER-2* amplified esophageal adenocarcinomas. The only published clinical Herceptin® trial for esophagus cancer patients was a phase I study testing 30 patients, including six patients with *HER-2* amplified tumors. In this study, only local tumor response was studied after combined radiation, paclitaxel, cisplatin, Herceptin® therapy. Although many responders were reported, the possible contribution of Herceptin® cannot be appreciated in this study.[34] Clinical trials or individual case studies investigating the response of *HER-2* amplified metastatic esophageal cancers to Herceptin® are clearly needed.

Remarkably, *HER-2* results in squamous cell carcinoma were much less unequivocal. Moderate expression (2+) according to the FDA approved IHC criteria were more frequently seen than 3+ positivity. Accordantly the association of IHC positivity with amplification was less strong than in adenocarcinoma. These data fit well with observations in other squamous carcinoma types. *HER-2* amplification is less frequent in squamous cell carcinoma of the lung than in adenocarcinoma of the lung[55–58] and rather infrequent in most prevalent squamous cell carcinomas like head and neck,[59,60] skin[61] or cervical carcinomas.[62,63]

The current use of molecular diagnostics for targeted cancer therapy is depending on the assumption that all metastases will show the same target gene alteration as observed in the primary tumor. To investigate this issue, nodal and distant metastases of our cancers were also included in our tissue microarray. Remarkably, no discrepant finding was observed in any of our 84 patients for which primary tumor and its metastases could be analyzed for *HER-2* amplification. This fits well to the results of a previous FISH study which failed to find discrepant *HER-2* results in eight primary esophageal cancers and corresponding lymph node metastasis.[43] Importantly, this perfect concordance could only be seen after a reanalysis of 10 cases initially discrepant data. All these tumors had a borderline finding (ratio *HER-2*/centromer 17 of 1.8–3.0) they were initially considered amplified in one sample

(primary tumor or metastasis) and nonamplified in the other sample (metastasis or primary tumor). A direct comparison of the *HER-2* findings in these cases including large section analyses revealed no significant differences between primary and metastatic tumors, however. The strong concordance of the *HER-2* status in primary and metastatic carcinomas ($P<0.0001$, Fisher's exact test) provides further support for a possible application of Herceptin®, especially in highly amplified adenocarcinomas. Occasional discrepancies in the IHC results are most likely due to the inherent shortcoming of the IHC dealing with tissues that were processed in a nonstandardized way.

High-quality clinical and experimental data were used in this study. The strong association between clinical data and patient survival provides a perfect indirect validation for quality of our clinical data. The validity of our (FDA approved) assays is indirectly corroborated by the very strong concordance of FISH and IHC data. In spite of this clean data set, no clear association was found between *HER-2* alteration and clinicopathological parameters. In particular, there was no association between *HER-2* alteration and patient prognosis. This result is in line with the results of most previous studies.[28,31,32,36] The existence of several published papers reporting associations of *HER-2* amplification/overexpression with both poor[24,35,42] and improved prognosis[25,30] also rather argue against an important prognostic role of *HER-2* in esophageal cancers. The general absence of an association of *HER-2* amplification or overexpression with clinical parameters of malignancy is in line with previous studies.[19,24,31,37] We cannot offer a biologic explanation for the observed association between *HER-2* status and negative resection margins or pT2 stage in squamous cell carcinoma. It cannot be excluded that these results represent statistical artefacts in a study patterning a high number of statistical analyses.

In conclusion, a thorough analysis of 255 esophageal cancers using FDA approved reagents showed a frequent amplification of *HER-2* in esophageal adenocarcinoma (15%) and a strong concordance of *HER-2* status in primary and metastatic cancer ($P<0.0001$). These results should encourage clinical trials or individual case studies using Herceptin® in metastatic adenocarcinomas of the esophagus.

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