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Assessment of ErbB2 (Her2) in oesophageal adenocarcinomas: summary of a revised immunohistochemical evaluation system, bright field double *in situ* hybridisation and fluorescence *in situ* hybridisation

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Amplification and overexpression of *ErbB2 (Her2)* is a frequent event in oesophageal adenocarcinomas. Assessment of ErbB2 status is crucial for identifying patients who are likely to benefit from treatment with trastuzumab. In this study, we performed a comprehensive analysis of *ErbB2* amplification and expression in 142 oesophageal adenocarcinomas by comparing the most commonly used methods for *ErbB2* assessment: ErbB2 expression was determined by immunohistochemistry and was scored (0, 1+, 2+ and 3+) according to a recently described modified scoring system for gastric cancer. *ErbB2* amplification was evaluated by bright field double *in situ* hybridisation. The results were compared with pathologic features, patients' survival and previously published data from fluorescence *in situ* hybridisation analysis. On the basis of immunohistochemistry, which was applicable in 110 cores of the cases, 83 tumours (75%) had a score of 0 or 1+ (immunohistochemistry negative), 13 tumours (12%) were scored as 2+ and 14 tumours (13%) were scored as 3+. *In situ* hybridisation data were obtained from 142 cases. There was a highly significant correlation of immunohistochemistry, bright field *in situ* hybridisation and fluorescent *in situ* hybridisation ($P<0.001$ each). In total, 41 tumours (29%) were categorised as *ErbB2* positive, which was defined as immunohistochemistry 3+ and/or an *ErbB2/Chr17* quotient of ≥ 2 as assessed by either bright field double *in situ* hybridisation or fluorescence *in situ* hybridisation. ErbB2 positivity was observed more frequently in tumours with lower differentiation grades ($P=0.029$). Patients with ErbB2-positive tumours had a significantly worse prognosis, both in univariate analysis ($P=0.004$) and in multivariate analysis ($P=0.03$). In conclusion, we demonstrate that a significant number of oesophageal adenocarcinomas are positive for ErbB2. Assessment of *ErbB2* amplification can be equivalently performed by conventional fluorescence *in situ* hybridisation or other light-microscopy-based methods, such as the novel bright field double *in situ* hybridisation technique.

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ErbB2 (Her2) amplification or overexpression occurs frequently in adenocarcinomas of the upper gastrointestinal tract. Most studies have been conducted in gastric cancer, which shows ErbB2-positivity rates that vary from 10 to 30%.[1–5] *ErbB2* amplification or overexpression also seems to be associated with poorer prognosis, more aggressive disease and shorter survival in gastric cancer.[5–7] Preclinical and clinical studies of upper gastrointestinal adenocarcinomas,[2] and in particular, the large ToGA trial, which included adenocarcinomas of the stomach

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and gastro–oesophageal junction, have demonstrated that trastuzumab exerts antitumour activity on ErbB2-overexpressing tumours.[8] Most recently, this has led to the approval of the addition of trastuzumab to fluoropyrimidine/platinum-based therapies for patients with ErbB2-positive advanced gastric cancer by the European Medicines Agency (EMEA), the FDA and Health Canada.

Similar to breast cancer,[9] determination of ErbB2 status is performed by immunohistochemistry as the primary method of choice. *In situ* hybridisation, which is mostly performed as fluorescence *in situ* hybridisation, is restricted to those cases that have equivocal (ie, immunohistochemistry 2+) ErbB2 expression results. There is evidence that compared to breast cancer, ErbB2 immunohistochemistry in gastrointestinal carcinomas has a few substantial differences; in particular, these differences involve cellular staining patterns and intratumoral heterogeneity. Therefore, for gastric cancer, a modified ErbB2 scoring system for immunohistochemistry has been proposed.[3,10]

Data on ErbB2 in oesophageal adenocarcinomas exist to a lesser extent than gastric cancer. The rate of ErbB2 overexpression/amplification in these cancers has been reported at approximately 20–25%, depending on the method of evaluation and the definition used.[11–15] In terms of the relatively high rate of chemotherapy resistance in oesophageal adenocarcinomas,[16,17] additional therapeutic options, such as ErbB2-targeting are of high demand for patients with advanced or metastatic disease.

The aim of this study was to perform a concise analysis of ErbB2 expression in a well-characterised series of oesophageal adenocarcinomas. For this purpose, we applied the revised ErbB2 immunohistochemistry scoring recommendations for gastric cancer[3,10] to a tissue microarray that comprised a large number of primary resected oesophageal adenocarcinomas. In addition, *in situ* hybridisation was performed using the recently developed bright field double *in situ* hybridisation.[18] Results were compared with fluorescence *in situ* hybridisation data from a previously published study by our group to assess the reliability of both the modified scoring system for immunohistochemistry and the novel *in situ* hybridisation technique. Furthermore, the expression and amplification data were correlated with histopathological parameters and patients' survival.

Materials and methods

Patients

Immunohistochemistry and *in situ* hybridisation were performed on paraffin-embedded formalin-fixed tumour samples from a total of 142 patients with oesophageal adenocarcinomas who were treated between 1991 and 2006 in the Department of Surgery at the Klinikum Rechts der Isar der Technischen Universität München. All patients provided consent for additional molecular analyses at the time of their original operation. Mean patient age was 64 years, and ages ranged from 33 to 83 years. The female/male ratio was 12/130. Median disease-free survival was 44 months (95% CI; 29–58 months). Median overall survival was 49 months (95% CI; 31–67 months). All patients had been treated by radical surgical resection—either transthoracic or transhiatal oesophagectomy—without neoadjuvant chemotherapy or radiochemotherapy. The pT category of the tumours (according to UICC 2010[19]) was as follows: pT1, 63 cases (44%); pT2, 28 cases (20%); and pT3–pT, 4–51 cases (36%). Lymph node metastases were present in 59 cases (42%). Tumour grading was G1 (well differentiated) in 12 cases (9%), G2 (moderately differentiated) in 62 cases (44%) and G3–G4 (poorly differentiated) in 68 cases (48%; Table 1).

Tissue Microarrays

Tissue microarrays constructed at two different time points were used. Fluorescence *in situ* hybridisation analysis was done on the first tissue microarray, which contained cores from 124 tumours[11,12] with three cores per tumour. Results of this study were originally published in 2006 and 2007. For the purpose of this study, a second tissue microarray was constructed, which contained samples of 110 with two cores per tumour. Overall, 94 tumours of

Table 1 ErbB2 (Her2) status in oesophageal adenocarciomas and pathological parameters

	ErbB2 status		*Total*	P-*value* (χ^2)
	Negative	*Positive*		
pT Category				
pT1	50	13	63	0.154
pT2	18	10	28	
pT3–4	33	18	51	
Total	101	41	142	
Lymph node metastases				
Absent	64	19	83	0.090
Present	37	22	59	
Total	101	41	142	
Distant metastases				
Absent	94	38	132	1.00
Present	7	3	10	
Total	101	41	142	
Grading				
G1	12	0	12	0.029
G2	39	23	62	
G3–4	50	18	68	
Total	101	41	142	

ErbB2 positivity is defined as immunohistochemistry 3+ and/or an *ErbB2/Chr17* quotient of $\geqslant 2$ as assessed by *in situ* hybridisation.

the first tissue microarray could be included in replicate, whereas 30 tumours could not be included in the second tissue microarray due to the decreased tumour content in the donor paraffin blocks, which particularly occurred with the early carcinomas. Instead, 16 other tumours were added to the second tissue microarray.

Immunohistochemistry

The paraffin blocks of the tissue microarrays were freshly cut into 3 μm sections that were incubated with antibodies against ErbB2 (clone 4B5, Ventana Medical Systems Inc., Tucson, Arizona) according to the manufacturer's recommendations. The method of ErbB2 scoring was essentially based on previously described recommendations, which was also applied in the ToGA study for gastric cancer and is described in detail elsewhere.[3,10] Briefly, the major features of the modification that are different from the ErbB2 scoring in breast cancer are as follows: a positive reaction is considered a membranous staining pattern that can be unequivocally assessed as linear staining at cell–cell contact sites and includes baso-lateral or lateral without restriction on the circularity of staining; the degree of microscopic magnification (x-fold) at which the membranous (linear intercellular) staining is clearly visible for the estimation of ErbB2 staining intensity is considered; the 10% minimum number of stained tumour cells that could reliably be assessed for ErbB2 staining in biopsies is eliminated. In this study, at least five cohesive, unequivocally stained evaluable cells are considered to be the limit for ErbB2 evaluation (Figure 1). In this study, we followed this recommendation because the tumour content of a tissue microarray core may actually represent that of a biopsy rather than a slice of a resection specimen.

In Situ Hybridisation

Bright field *in situ* hybridisation was performed using the BenchMark® XT automated slide processing system (Ventana Medical Systems Inc.), which involves a one-step fully automated assay that consists of baking, deparaffinisation, pretreatment, hybridisation, stringency wash, signal detection and counterstaining as described in detail in a recent publication.[18] For *ErbB2* gene detection, the Inform® *ErbB2* DNA Probe (Ventana Medical Systems Inc.) was used, and for *Chr 17* detection, the Inform Chromosome 17 Probe (Ventana Medical Systems Inc.) was used (Figure 1).

Additional data from conventional fluorescence *in situ* hybridisation using 4 μm tissue microarray sections and three-dimensional (3D) fluorescence *in situ* hybridisation using 16 μm tissue microarray sections were obtained from a previously published study by our group.[12]

For the purpose of this study, cases with an *ErbB2/Chr17* ratio of ≥ 2 based on either conventional or 3D fluorescence *in situ* hybridisation were classified as *ErbB2* amplified. *ErbB2* amplification was further subclassified into low-level amplification (*ErbB2/Chr17* ratio 2–3) and high-level amplification (*ErbB2/Chr17* ratio >3), consistent with published studies.[13]

Statistical Analysis

PASW 18.0 statistical software 11.5 (SPSS Inc., Chicago, IL) was used for statistical analysis. The associations between immunohistochemical expression patterns, the results from *in situ* hybridisation and the pathologic features were entered into 2×2 tables and evaluated with the χ^2-test. Survival analysis was performed using Kaplan–Meier estimates, log-rank tests and Cox's proportional hazards regression analysis. All tests were two-sided, and the significance level was set at 5%.

Results

Immunohistochemistry

On the basis of the immunohistochemistry, which was applicable in 110 cores of the tissue microarray, 83 tumours (75%) had a score of 0 or 1+ (immunohistochemistry negative), 13 tumours (12%) were scored as 2+ and 14 tumours (13%) were scored as 3+. In two cases, there was an obvious intratumoral heterogeneity with divergent staining patterns (negative *vs* 2+ or 3+) in adjacent tumour areas. We also had the opportunity to compare the breast cancer scoring system, which we used for the ErbB2 assessment in oesophageal adenocarcinomas in a previous study, with the proposed scoring system for gastric cancer: 94 tumours were contained in both tissue microarrays, and significant ($P<0.001$) concordant scoring that ranged from negative to 3+ was observed in 56 cases (60%), or in 77 cases (82%), if negative and 1+ cases were grouped together (Table 2).

In Situ Hybridisation

In situ hybridisation data were obtained from a total of 142 tumours on tissue microarrays as indicated above. Bright field double *in situ* hybridisation showed a low-level amplification of *ErbB2* (*ErbB2/Chr17* ratio 2–3) in 11 cases (10%) and a high-level amplification (*ErbB2/Chr17* ratio >3) in 15 cases (14%). No amplification of *ErbB2* (*ErbB2/Chr17* ratio <2) was observed in 81 cases (76%). There was a significant correlation between bright field double *in situ* hybridisation data, both with conventional fluorescence *in situ* hybridisation and 3D fluorescence *in situ* hybridisation ($P<0.001$ each). In three cases, there was an intratumoral heterogeneity

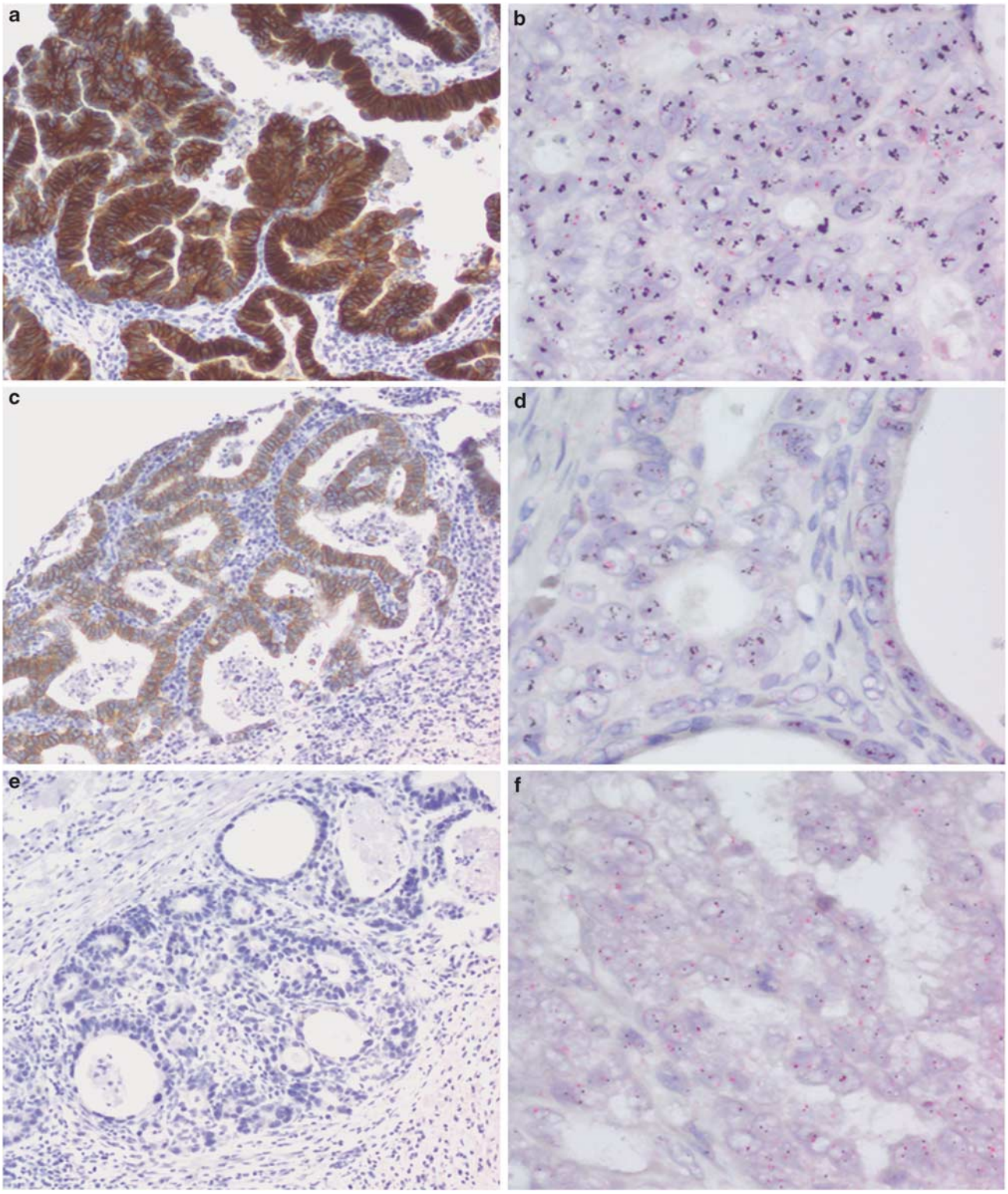

Figure 1 Examples of immunohistochemical stainings and bright field *in situ* hybridisation for ErbB2 (Her2) in oesophageal adenocarcinomas. (**a**) ErbB2 immunohistochemistry score 3+ (× 200); (**b**) same case, *ErbB2* high-level amplification with clustering of *ErbB2* signals. *ErbB2* signals are black; *Chr17* signals are red (× 400). (**c**) ErbB2 immunohistochemistry score 2+ (× 200); (**d**) same case *ErbB2* low-level amplification with a ratio of *ErbB2/Chr17* 2.6 (× 400). (**e**) ErbB2 immunohistochemistry score 0 (× 200); (**f**) same case, *ErbB2* non-amplified (× 400).

(no amplification *vs* high-level amplification in adjacent tumour areas) within the tissue microarray cores. This was congruent to immunohistochemical findings in two of these cases as described above. In addition, there was discordance in the evaluation of high-level amplification (present *vs* absent) in five

cases (5.6%) for the comparison between bright field double *in situ* hybridisation and fluorescence *in situ* hybridisation and in six cases (7%) for bright field *in situ* hybridisation and 3D fluorescence *in situ* hybridisation (Table 3). However, this finding may also be caused by intratumoral heterogeneity as discussed below.

Correlation between Immunohistochemistry and *In Situ* Hybridisation

There was a highly significant correlation between immunohistochemistry and *in situ* hybridisation ($P<0.001$; for bright field double *in situ* hybridisation, fluorescence *in situ* hybridisation, 3D fluorescence *in situ* hybridisation and *in situ* hybridisation in total): 12/13 ErbB2 2+ cases and 13/13 ErbB2 3+ cases were amplified with *in situ* hybridisation. However, 10 cases with a ErbB2 immunohistochemical score of 0 and 3 cases with a ErbB2 score of 1+ were also amplified, showing low-level amplification in the majority of cases (11/13 cases). Four ErbB2 3+ cases were not amplified by fluorescence *in situ* hybridisation analysis. However, bright field double *in situ* hybridisation detected amplification in all of those cases, indicating intratumoral heterogeneity of ErbB2. In contrast, 12 cases that were negative by bright field double *in situ* hybridisation showed amplification with either conventional fluorescence *in situ* hybridisation or 3D fluorescence *in situ* hybridisation. In addition, one ErbB2 3+ case that had been added to the second tissue microarray and had not been included in the fluorescence *in situ* hybridisation collective could not be analysed by bright field double *in situ* hybridisation due to technical reasons (Table 4).

Table 2 Comparison between the ErbB2 (Her2) gastric cancer grading system and the breast cancer grading system

	ErbB2 IHC gastric cancer grading				*Total*
	0	*1+*	*2+*	*3+*	
ErbB2 IHC breast cancer grading					
0	42	16	3	3	64
1+	5	3	4	0	12
2+	3	1	3	1	8
3+	1	0	1	8	10
Total	51	20	11	12	94

IHC, immunohistochemistry.
Significant correlation between both grading systems (χ^2-test; $P<0.001$).

Table 3 Comparison between for bright field double *in situ* hybridisation, fluorescence *in situ* hybridisation and 3D fluorescence *in situ* hybridisation

	FISH (ErbB2/Chr17)			*Total*	*3D-FISH (ErbB2/Chr17)*			*Total*
	<2	*2–3*	*>3*		*<2*	*2–3*	*>3*	
BDISH (ErbB2/Chr17)								
<2	64	3	1	68	62	7	1	70
2–3	8	1	1	10	6	2	2	10
>3	4	0	8	12	5	0	7	12
Total	76	4	10	90	73	9	10	92

BDISH, bright field double *in situ* hybridisation; FISH, fluorescence *in situ* hybridisation.
Significant correlation between the results of BDISH and FISH ($P<0.001$; χ^2-test) and BDISH and 3D-FISH ($P<0.001$; χ^2-test).

Correlation between ErbB2 Status and Pathological Parameters

In total, 41 tumours (29%) were categorised as ErbB2 positive, which was defined as 3+ by immunohistochemistry and/or a *ErbB2/Chr17* quotient of ≥ 2 as assessed by either bright field double *in situ* hybridisation or fluorescence *in situ* hybridisation. ErbB2 positivity was observed more frequently in tumours with a lower differentiation grade ($P=0.029$). There was no correlation between ErbB2 status and pT and pN category (Table 1).

ErbB2 Status and Survival

Patients with ErbB2-positive tumours had a significantly worse clinical outcome, with a significantly shorter disease-free survival and a significantly decreased overall survival: the median disease-free survival for patients with ErbB2-negative tumours was 60 months (95% CI; 22–97 months), and the

Table 4 Comparison between ErbB2 immunohistochemistry and *in situ* hybridisation

	Total	*ISH total*		*FISH (ErbB2/Chr17)*			*3D FISH (ErbB2/Chr17)*			*B-DISH (ErbB2/Chr17)*		
		Not amplified	*Amplified*	*<2*	*2–3*	*>3*	*<2*	*2–3*	*>3*	*<2*	*2–3*	*>3*
IHC												
Negative	58	48	10	47	1	1	46	4	1	56	2	0
1+	25	22	3	18	1	0	17	3	0	23	0	0
2+	13	1	12	7	2	1	8	1	2	2	6	5
3+	14	1	13	4	0	8	4	1	7	0	3	10
Total	110	72	38	76	4	10	75	9	10	81	11	15

BDISH, bright field double *in situ* hybridisation; FISH, fluorescence *in situ* hybridisation; ISH, *in situ* hybridisation.
Significant correlation between IHC and FISH, 3D-FISH and BDISH (χ^2-testing; $P<0.001$ each).

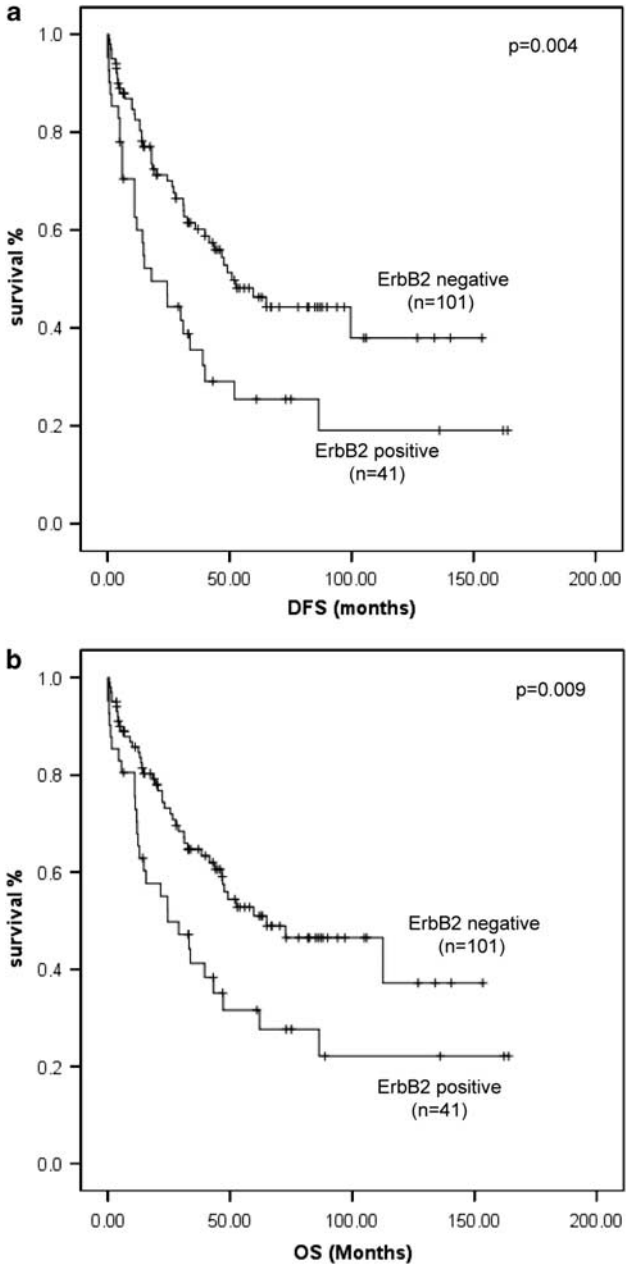

Figure 2 Survival analysis for ErbB2 in oesophageal adenocarcinomas. (**a**) ErbB2 and disease-free survival (DFS); (**b**) ErbB2 and overall survival (OS). ErbB2 positivity is defined as immunohistochemistry 3+ and/or an *ErbB2/Chr17* quotient of >2 as assessed by *in situ* hybridisation.

median overall survival was 73 months (95% CI; 26–120 months). In contrast, the median disease-free survival for patients with ErbB2-positive tumours was 18 months (95% CI; 6–30 months), and the median overall survival was 25 months (95% CI; 7–41 months). These differences were significant, both in univariate analysis ($P=0.004$ for disease-free survival and $P=0.002$ for overall survival, respectively) and in multivariate analysis ($P=0.019$ and 0.032, respectively) that included the factors of pT category, presence of lymph node metastases, tumour differentiation, resection status and presence of distant metastasis at the time of surgery (Figure 2; Table 5).

Table 5 ErbB2 status and disease-free survival (a) and overall survival (b) (multivariate analysis)

	Exp(B)	*95.0% CI*		*Sign.*
		Min	*Max*	
(a)				
pT-Category pT1–pT2–pT3/4	1.318	0.918	1.891	0.134
Lymph node metastases absent/present	1.914	1.050	3.486	0.034
Distant metastases absent/present	2.174	1.000	4.725	0.050
Grading G1–G2–G3/4	1.335	0.874	2.040	0.181
Resection-status R0–R1/2	2.556	1.329	4.916	0.005
ErbB2 (Her2) positive/negative	1.771	1.098	2.856	0.019
(b)				
pT-Category pT1–pT2–pT3/4	1.238	0.847	1.810	0.271
Lymph node metastases absent/present	2.142	1.153	3.982	0.016
Distant metastases absent/present	1.499	0.681	3.303	0.315
Grading G1–G2–G3/4	1.358	0.875	2.109	0.173
Resection-status R0–R1/2	3.123	1.608	6.064	0.001
ErbB2 (Her2) positive/negative	1.709	1.046	2.792	0.032

ErbB2 positivity is defined as immunohistochemistry 3+ and/or an *ErbB2/Chr17* quotient of ⩾2 as assessed by *in situ* hybridisation.

Discussion

Previous studies examining ErbB2 (Her2) status in oesophageal adenocarcinomas observed a prevalence of ErbB2 protein overexpression or gene amplification in approximately 20–25% of cases with conflicting data about the prognostic significance of the amplification or overexpression.[11,13,15] In this study, we tested ErbB2 status in oesophageal adenocarcinomas by *in situ* hybridisation (amplification) and immunohistochemistry (expression). A rate of ErbB2 positivity, which was defined 3+ by immunohistochemistry and/or an *ErbB2/Chr17* quotient of ⩾2 as assessed by *in situ* hybridisation, could be detected in 29% of the cases. Furthermore, ErbB2 positivity was associated with a worse clinical outcome in both the univariate and multivariate analyses.

The first aim of this study was to apply the modified ErbB2 immunoscoring method for gastric cancer, which was shown to be predictive of the response to trastuzumab-based therapies in patients with advanced gastric cancer in the ToGA study.[3,8,10] For immunohistochemistry, we used the 4B5 assay. This antibody has also been approved by the FDA for ErbB2 testing in breast cancer and appeared to be at least as sensitive and may even show a higher inter-laboratory concordance for ErbB2 scoring and a closer relationship between immunohistochemi-

cally 3+ and ErbB2 gene amplification for gastric cancer.[10]

There is the evidence that the determination of ErbB2 status by transferring the breast cancer immunohistochemistry scoring roles to gastric cancer may lead to a significant number of false-negative ErbB2 tumours.[20] Comparison between studies that have assessed ErbB2 in gastric cancer using the breast cancer evaluation system, on the one hand, and those that used the modified scoring system for gastric cancer, on the other hand, reveals significantly higher ErbB2 rates after the application of the proposed gastric cancer scoring system. Moreover, concordance between fluorescence *in situ* hybridisation and immunohistochemistry is better when using the gastric cancer scoring system; therefore, this evaluation system should be considered the scoring method of choice for immunohistochemical assessment of ErbB2 status in oesophagogastric adenocarcinomas.[10] In a previous study, we also used the breast cancer scoring system (Dako; Hercept-test) for the assessment of ErbB2 in oesophageal adenocarcinomas and found a rate of 7% 2+ cases and 10% 3+ cases.[11] In this study, we demonstrated a significant correlation between the two scoring systems. However, there was an 18.1% discrepancy rate for the evaluation negative/1+ *vs* 2+/3+.

For breast cancer and gastric cancer, *in situ* hybridisation is considered the standard method for the accurate determination of ErbB2 status in cases with equivocal immunohistochemical staining (ie, 2+).[9] In this study, we choose the novel, recently described bright field double *in situ* hybridisation for the assessment of *ErbB2* amplification. This light-microscopy-based *in situ* hybridisation technique may be superior to conventional fluorescence *in situ* hybridisation for the investigation of small amplified tumour cell foci.[18,21,22] This phenomenon frequently occurs in oesophagogastric carcinomas[1,3,23] and can readily be recognised by this method. We have compared bright field double *in situ* hybridisation data with fluorescence *in situ* hybridisation data from a previously published study[12] and found a significant correlation between bright field double *in situ* hybridisation and both conventional fluorescence *in situ* hybridisation and the more complex but more sensitive 3D fluorescence *in situ* hybridisation. For breast cancer, similar observations were reported by others who described a high concordance in the assessment of *ErbB2* amplification between fluorescence *in situ* hybridisation and the light-microscopy-based techniques of silver *in situ* hybridisation and bright field double *in situ* hybridisation, respectively, with advantages in terms of handling, orientation within the slides and in cases of intratumoral heterogeneity for bright field double *in situ* hybridisation.[21]

For the application of immunohistochemical staining and *in situ* hybridisation, we used tissue microarrays, which contained cores from a total of 142 oesophageal adenocarcinomas from which extensive clinico-pathological documentation was available. Unfortunately, we could not use one tissue microarray for the comparison of all methods because tissue microarrays constructed at two different time points were required. Nevertheless, the majority of cases were contained in both tissue microarrays so that correlation between immunohistochemistry, bright field double *in situ* hybridisation and fluorescence *in situ* hybridisation could be performed in approximately 100 cases. Discrepancies between the various *in situ* hybridisation methods and immunohistochemistry in our study may not only be caused by methodical or technical differences or the 'grey zone' of low-level gains[12] but also represent the phenomenon of intratumoral heterogeneity of ErbB2 expression in gastro-oesophageal adenocarcinomas,[23–26] because the cores of the donor blocks were obtained from slightly different tumour areas. Therefore, a heterogeneous distribution of ErbB2-positive cell clones may also be responsible for those divergent findings. This may be the case particularly for those cases with divergent results of high-level amplification. This phenomenon could also be observed in three cases within the same tissue microarray.

For the purpose of the determination of the prognostic impact of ErbB2 in oesophageal adenocarcinomas, this 'bias' of using two different tissue microarrays for the assessment of ErbB2 may therefore provide more reliable data about the ErbB2 status, because cores from different regions were investigated and false-negative results due to intratumoral heterogeneity may have been eliminated. Moreover, for the determination of ErbB2 status, if any amplification was observed on *in situ* hybridisation, including either fluorescence *in situ* hybridisation, 3D fluorescence *in situ* hybridisation or bright field double *in situ* hybridisation, the case was considered amplified based on the current recommendations for gastro-oesophageal carcinomas.

Compared to gastric cancer, comprehensive data about ErbB2 expression in oesophageal adenocarcinomas are relatively scarce due to the small sample sizes of homogenous collectives. In previous studies, we investigated ErbB2 expression in tissue microarrays of oesophageal adenocarcinomas using immunohistochemistry according to the breast cancer grading system and fluorescence *in situ* hybridisation (conventional and 3D fluorescence *in situ* hybridisation).[11,12] We found a 7% rate of ErbB2 DAKO score 2+ cases and a 10% rate of ErbB2 DAKO score 3+ cases.[11] *ErbB2* overexpression (*ErbB2/CHR17* ≥ 2) was demonstrated in 17% of cases by 3D fluorescence *in situ* hybridisation and in 13.1% of cases by conventional fluorescence *in situ* hybridisation, based on an analysis of at least 50 carcinoma cells per tissue core. Fluorescence *in situ* hybridisation data and immunohistochemistry data showed a significant correlation. However, a

significant association between ErbB2 positivity and increased mortality could only be demonstrated by 3D fluorescence *in situ* hybridisation and not by immunohistochemistry or conventional fluorescence *in situ* hybridisation. Furthermore, a distinct subgroup of patients with a low-level gain of *ErbB2* with unfavourable prognosis could be detected using 3D fluorescence *in situ* hybridisation.[12] In a large study by a different group, the rate of ErbB2 overexpression/amplification was 15%, and no prognostic impact with respect to patients' survival could be demonstrated.[13] Others report similar rates of ErbB2 positivity in oesophageal adenocarcinomas, highlighting the impact of *ErbB2* amplification and ERBB2 overexpression during the malignant progression of Barrett's metaplasia to invasive cancer.[27–29]

The relatively high number of ErbB2-positive cases in our study compared with other reports may be due to the definition of ErbB2 positivity, which was based on the guidelines of the ToGA study and the EMEA for trastuzumab (Herceptin©) treatment for advanced gastric cancer. This recommendation requires a ErbB2 score of 3+ by immunohistochemistry or an amplification level of ≥ 2 (*ErbB2/Chr17*) even in small tumour cell clusters,[10] which is different from the breast carcinoma requirements, in which cases with a *ErbB2/Chr17* ratio between 1.8 and 2.2 are considered as equivocal and ErbB2 expression is less heterogeneous.[9] Furthermore, as mentioned above, false-negative results may have been avoided by using various methods for the assessment of the amplification and intratumoral heterogeneity based on the investigation of tissue microarray cores from the same tumours from two different tissue miroarrays. On the basis of the results of our study, $\sim$30% of oesophageal adenocarcinoma patients would be potential candidates for ErbB2-targeted therapy. The original ToGA trial included metastatic gastric adenocarcinomas and adenocarcinomas of the gastric junction,[8] which are described as distinct entities in the differentiation of oesophageal adenocarcinomas by the previous and most recent WHO classifications.[30,31] However, the FDA and Health Canada approvals for trastuzumab (Herceptin©) treatment include all types of oesophagogastric adenocarcinomas; therefore, strict criteria for ErbB2 assessment in oesophageal adenocarcinomas are of the highest clinical relevance for selecting patients for ErbB2-targeted treatment, which may represent an expensive but promising additional therapeutic option for the advanced stages of this disease.[32–36]

In summary, in this study, we found that a significant number of oesophageal adenocarcinomas are positive for ErbB2. The assessment of ErbB2 by immunohistochemistry in combination with *in situ* hybridisation (bright field double *in situ* hybridisation or fluorescence *in situ* hybridisation) reveals highly relevant prognostic information and may also serve as a basis for targeted trastuzumab therapy. Immunohistochemistry scoring as the primary method of ErbB2 assessment should be performed in accordance with the recommendations for gastric cancer. The method of evaluation of *ErbB2* amplification by *in situ* hybridisation can be equivalently performed by conventional fluorescence *in situ* hybridisation or other light-microscopy-based methods, such as the novel bright field double *in situ* hybridisation technique.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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