

Concordance of HER2 Status in Primary Tumour and Lymph Node Metastases in Patients with Esophageal Carcinoma

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Abstract. *Background:* Human epidermal growth factor receptor 2 (HER2) is an important prognostic factor in several types of solid tumours. Although HER2 seems not to influence survival in esophageal carcinomas, an impact of the HER2 status of disseminated tumour cells (DTCs) on survival has been shown. The aim of our study was to investigate the significance of the HER2 status in primary esophageal carcinomas and matched lymph node metastases. *Materials and Methods:* The HER2 status of primary tumours and matched lymph node metastases were analysed for 158 patients with esophageal carcinoma using immunohistochemistry (IHC) and fluorescence *in situ* hybridisation (FISH). *Results:* The study specimen included 90 adenocarcinomas (AC) and 68 squamous cell carcinomas (SCC). HER2 amplification was found in 12% and overexpression in 8.9% of all primary tumours. HER2 amplification was identical in the primary tumour and lymph node metastases in all AC and in 75% of SCC. Discordant-positive HER2 lymph node status and negative primary tumour status was found in 4.4% of AC and 1.5% of SCC in FISH analyses. No significant associations were found between HER2 amplification/overexpression and overall survival. *Conclusion:* HER2 gene status remains highly conserved in metastatic esophageal carcinoma. Discrepancies occur rarely between primary tumour and lymph node metastases and might be due to heterogeneity of the HER2 status of the primary tumour. This could be the reason for heterogeneity of DTCs and may result in metastasis of only a subset of tumour cells.

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Carcinoma of the esophagus, despite technical advances, still has a dismal prognosis. In Western countries, the incidence of esophageal carcinoma, especially of adenocarcinomas, is increasing (6). Investigation of protein and gene alterations that occur in tumours and their corresponding lymph node metastases can provide with clues for the development of novel targets for an individualized therapeutic approach (1, 10, 12).

Activation of human epidermal growth factor receptor-2 (HER2) is an important factor for initiation and progression of malignancies (9). Several studies have verified that the HER2 oncogene, located on chromosome 17, is amplified in many tumour entities (3, 4, 17-19, 21). HER2 status has been described as an important prognostic factor in breast cancer. The humanized antibody HER2, trastuzumab, has been established in the targeted therapy of breast cancer. The role of HER2 in esophageal cancer is still controversial. Several studies reported amplification and overexpression rates of 11-73% (5, 23, 28, 34). A multicenter randomized phase III trial described a survival benefit for patients with esophagogastric carcinoma treated with trastuzumab (2).

HER2 status is usually determined in the primary tumour because metastatic sites are rarely biopsied before treatment. Therefore it is still unknown if the HER2 status differs between primary tumours and their lymph node metastases.

The aim of our study was to investigate the significance of the HER2 status determined by fluorescence *in situ* hybridisation (FISH) and immunohistochemistry (IHC) in primary esophageal carcinomas and matched lymph node metastases. The nodal HER2 status might, as has been shown for disseminated tumour cells (DTCs), influence the metastatic process and determine the course of the disease.

Materials and Methods

Patients. A collective of 187 consecutive patients that underwent operation for esophageal cancer at the University Medical Centre Hamburg-Eppendorf between 1994 and 2007 was included in this study. None of the patients had neoadjuvant therapy and all patients were operated on with curative intent and had *en bloc* esophageal

resection with radical lymph node dissection. All lymph nodes were mapped by the surgeon according to the system of the American Thoracic Society (20) as modified by Casson *et al.* (8). Tumour stage and grade were classified according to the seventh edition of the tumour-node-metastasis classification (TNM) of the International Union against Cancer (29), by routine pathological staging. The use of tissue samples in this study was approved by the Ethics Committee of the chamber of physicians at Hamburg, Germany (approval number 1045) and written informed consent was obtained from the patients to use the resected tumour samples. Continuous follow-up data were available from 158 patients. In case of death during the follow-up period, the cause of death was determined as cancer-related or not cancer-related. Patients who died within 30 days of surgery were excluded from the analysis.

Tissue microarray (TMA) construction. Tissue samples were fixed in buffered 4% formalin, embedded in paraffin, and used to construct TMAs as described previously (16). Sections stained with haematoxylin-eosin were made from each selected primary tumour block (donor blocks) to define representative tumour regions. Tissue cylinders (0.6 mm in diameter) were then punched from that region of the donor block using a custom-made precision instrument (Beecher Instruments, Silver Spring, MD, USA). Tissue cylinders from each of the 158 lymph node-positive primary tumours and from every available lymph node metastasis of each of the lymph node-positive primary tumour (969 metastases in total) were distributed among three 25-mm × 35-mm paraffin blocks to produce the TMAs used for our study. In total, sections from 158 patients were available for analysis on the TMA. The resulting TMA blocks were cut into 3 µm sections that were transferred to glass slides using the Paraffin Sectioning Aid System (Instrumedics, Hackensack, NJ, USA). Two separate sets of TMA blocks were used for FISH and for immunohistochemical analysis.

Fluorescence in situ hybridization. TMA sections were used for dual-colour FISH analysis. A commercial kit was used for proteolysis slide pre-treatment performed according to the manufacturer's instructions (paraffin pre-treatment reagent kit; Abbott Laboratories, Abbott Park, IL, USA). Commercial FISH probe kits containing both gene-specific and corresponding centromere probes were utilized for copy number detection of *HER2* (PathVysion probe kit, cat. # 2J0136; Abbott, Laboratories). Hybridization and post-hybridization washes were carried out according to the locus-specific identifier (LSI) procedure (Vysis; Abbott, Laboratories) for *HER2*. Slides were then counterstained with 125 ng/ml 4',6-diamino-2-phenylindole in an antifade solution. Copy numbers of gene-specific and the corresponding centromere sequences were estimated for each tissue spot, as previously described (13). The mean numbers of *HER2* and centromere 17 signals were estimated for each tumor sample, as described before (7, 13). The criteria for *HER2* gene amplification were a ratio of greater than 2 for *HER2*/centromere 17 signals (ratio C=2.0). All other tumours were considered not to have amplification of the gene.

Immunohistochemistry. The HercepTest (Dako, Glostrup, Denmark) was used according to the protocol of the manufacturer. Antigen retrieval of the de-paraffinized tissue sections was carried out using a water-bath at 95°C–99°C for 50 min followed by peroxidase blocking and incubation with the pre-diluted primary

antibody. Cell line test slides provided by the manufacturer were used as positive and negative controls. Immunostaining was scored by one pathologist, following a four-step scale (0, 1+, 2+, 3+) according the manufacturer's directions. IHC results were defined as negative when the intensity of staining was lower than 2+ in 10% of tumour cells.

Statistical analysis. Chi-square test was used to study the relationship between *HER2* alterations and categorical parameters. Pearson's correlation test was used to compare the *HER2* status assessed by IHC and FISH. A strong correlation was defined as a correlation coefficient $R \geq 0.8$. The overall survival rates were calculated using the Kaplan–Meier method and the curves were compared using the log-rank test. Tumour-specific survival was defined as the clinical end-point. Differences were considered statistically significant when the *p*-value was ≤ 0.05 . Analysis was performed using the SPSS statistical software package for Windows (version 17.0; SPSS Inc., Chicago, IL, USA).

Results

The TMA comprised tumour samples of 187 primary tumours, including 106 adenocarcinomas, 81 squamous cell carcinomas, and tissues from their respective lymph node metastases. The patient collective included 152 male and 35 female patients. Only cases with interpretable results for FISH and IHC were included in the analysis ($n=158$). Samples that showed a lack of unequivocal tumour cells in the array or insufficient hybridization were excluded. Patients had a median age of 62 years and the median follow-up time for patients was 12.66 months [95% confidence interval (CI)=9.44–15.88]. Patients' characteristics are shown in Table I.

***HER2* gene amplification and overexpression in primary tumour.** FISH analysis of primary tumour samples showed gene amplification of *HER2* in 19 patients (12%) with mostly high level amplifications of 4-50 *HER2* copies/cell, while 139 tumours (88%) exhibited no amplification. Low-level amplification was seen in three tumour samples, with 4-8 *HER2* copies/cell. Examples of homogenous and negative amplification in FISH analysis are shown in Figure 1 C and D. Immunostaining for *HER2* in this patient collective of esophageal carcinomas showed an overexpression rate of 8.9% (Table II). Examples of positive *HER2* staining are given in Figure 1 B. Ninety adenocarcinoma samples were included in the analysis. An overall amplification rate of 13.3% and an overexpression rate of 10% were found (Table II). The analyses of 68 squamous cell carcinoma samples revealed *HER2* gene amplification in 10.3% and *HER2* overexpression in 7.4% of the cases (Table II). Comparison of *HER2* amplification and overexpression in the primary tumours showed high correlation, especially in adenocarcinomas (Pearson's $R=0.850$, $p<0.001$; Table II). All nine primary adenocarcinomas with *HER2* overexpression, revealed *HER2* gene amplification (Table II).

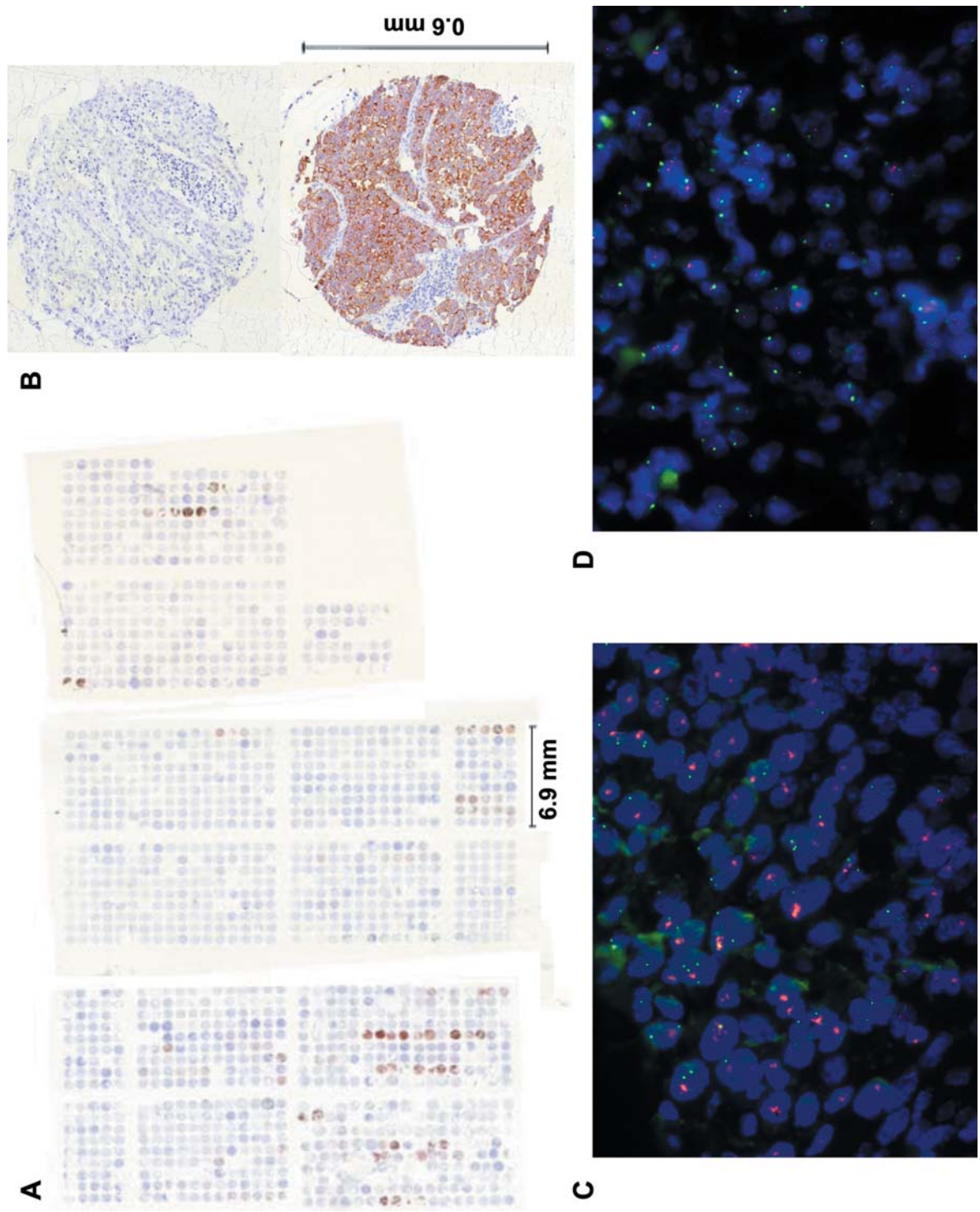


Figure 1. Esophageal cancer tissue microarray (TMA). A: Complete set of TMAs of esophageal cancer samples stained with haematoxylin-eosin. B: Examples of negative (upper panel) and positive (lower panel) human epidermal growth factor receptor-2 (HER2) staining. C: Fluorescence in situ hybridization (FISH) analysis of a tumour cell containing amplified HER2 DNA, with two centromere 17 signals (green) and a tight cluster containing more than five HER2 signals (red). The nuclear DNA is stained blue (x630 magnification). D: FISH analysis of tumour cells containing unamplified HER2 DNA. Each cell has two centromere 17 signals (green) and one or two HER2 signals (red) (x630 magnification).

Table I. Patient and tumour characteristics.

	Adenocarcinoma (n=90)	Squamous cell carcinoma (n=68)
Median (range) age, years	62 (34-79)	63 (43-83)
Gender, n (%)		
Male	75 (83.3)	57 (83.8)
Female	15 (16.7)	11 (16.2)
Tumour stage, n (%)		
pT1	3 (3.3)	2 (2.9)
pT2	41 (45.6)	9 (13.2)
pT3	43 (47.3)	43 (63.2)
pT4	3 (3.3)	14 (20.6)
Lymph node status, n (%)		
pN1	20 (22.2)	22 (32.4)
pN2	19 (21.1)	25 (36.8)
pN3	51 (56.7)	21 (30.9)
Distant metastasis, n (%)		
M0	88 (97.8)	65 (95.6)
M1	2 (2.2)	3 (4.4)
Stage groups (UICC 2009), n (%)		
IIB	14 (15.6)	5 (7.4)
IIIA	13 (14.4)	20 (29.4)
IIIB	10 (11.1)	16 (23.5)
IIIC	51 (56.7)	24 (35.3)
IV	2 (2.2)	3 (4.4)
Histological grade, n (%)		
G2	32 (35.6)	37 (54.4)
G3	58 (64.4)	31 (45.6)

pT, Pathological tumour stage; pN, pathological node category; M, metastasis; G, tumour grading; UICC, Union International Against Cancer.

HER2 gene amplification and overexpression in lymph node metastases. Comparison of *HER2* gene amplification and protein overexpression in lymph node metastases revealed correlation in adenocarcinomas (Pearson's $R=0.712$, $p<0.001$) (Table II).

In most cases *HER2* amplification and overexpression in lymph node metastases are in concordance with that of their respective primary tumours. There were, however, discrepant amplification and expression profiles in primary tumour and lymph node metastases. Five patients (four with adenocarcinoma, one with squamous cell carcinoma) with no *HER2* amplification of the primary tumour had *HER2* gene amplification in lymph node metastases. Four patients with squamous cell carcinoma carried *HER2* gene amplification of the primary tumour without amplification in the lymph node metastases (Table III). These results were in concordance with *HER2* overexpression in primary tumours and lymph node metastases (Table III).

Survival analyses according to distinct HER2 gene amplification and overexpression of primary tumours and lymph node metastases. Kaplan-Meier curves for survival

Table II. Human epidermal growth factor receptor-2 (*HER2*) expression and amplification of 158 primary esophageal carcinomas (PT) and their matched lymph node (LN) metastases. AC, Adenocarcinoma; SCC, squamous cell carcinoma; IHC, immunohistochemistry; FISH, fluorescence in situ hybridization

	IHC Score				Total	R	p-Value
	negative		positive				
	0	1+	2+	3+			
PT-All						0.775	<0.001
FISH −	20	118	1	0	139 (88)		
FISH +	0	6	5	8	19 (12)		
Total	144 (91)		14 (9)				
LN-All						0.672	<0.001
FISH −	41	91	6	0	138 (87)		
FISH +	3	4	4	9	20 (13)		
Total	139 (88)		19 (12)				
PT-AC						0.850	<0.001
FISH −	12	66	0	0	78 (87)		
FISH +	0	3	2	7	12 (13)		
Total	81(90)		9 (10)				
LN-AC						0.712	<0.001
FISH −	17	53	4	0	74 (82)		
FISH +	2	2	4	8	16 (18)		
Total	74(82)		16 (18)				
PT-SCC						0.646	<0.001
FISH −	8	52	1	0	61 (90)		
FISH +	0	3	3	1	7 (10)		
Total	63(93)		5 (7)				
LN-SCC						0.469	<0.001
FISH −	24	38	2	0	64 (94)		
FISH +	1	2	0	1	4 (6)		
Total	65 (96)		3 (4)				

Table III. Correlation of human epidermal growth factor receptor-2 (*HER2*) expression by immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) between primary tumours and lymph nodes (LN).

	Primary AC N=90		Primary SCC N=68	
	Negative	Positive	Negative	Positive
IHC				
LN				
Negative	72	0	61	3
Positive	9	9	2	2
Pearson's R	0.667		0.408	
p-Value		<0.001		<0.001
FISH				
LN				
Negative	74	0	60	4
Positive	4	12	1	3
Pearson's R	0.844		0.532	
p-Value		<0.001		<0.001

AC: Adenocarcinoma; SCC: squamous cell carcinoma.

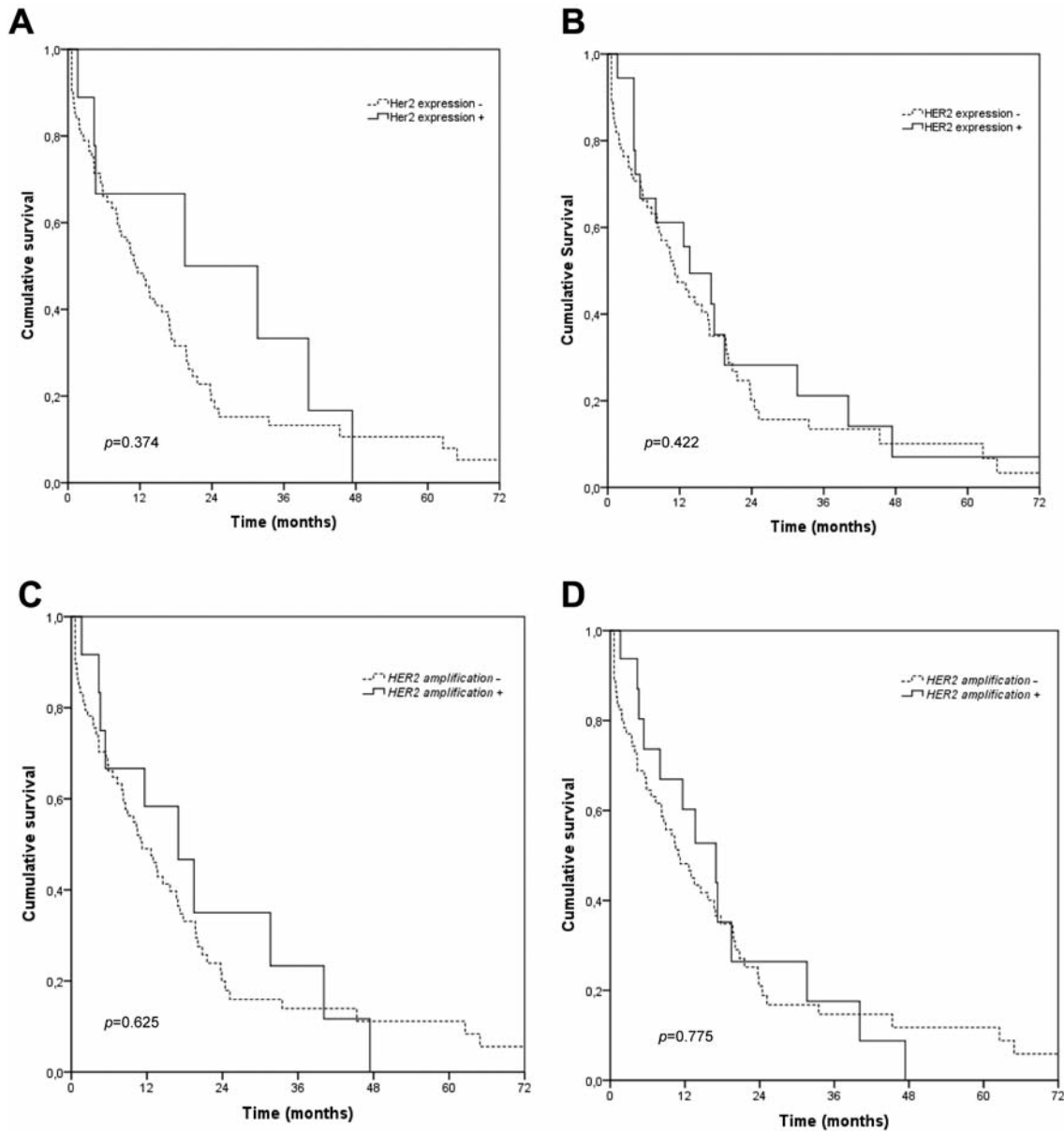


Figure 2. Survival analyses of patients with esophageal adenocarcinomas. Overall survival according to human epidermal growth factor receptor-2 (HER2) expression of the primary tumour (A) and of lymph node metastases (B), and according to HER2 gene amplification of the primary tumour (C) and of lymph node metastases (D) in 90 patients with esophageal adenocarcinoma.

with distinct *HER2* gene amplification and overexpression of primary tumours and lymph node metastases are shown in Figures 2 and 3. *HER2* gene amplification and overexpression of the primary tumour or lymph node metastases showed no correlation to overall survival.

Discussion

HER2 amplification was found in 12% of primary tumours, with amplification in corresponding lymph node metastases

in 78.9% (15/19 patients). *HER2* gene amplification was discordant between primary tumour and lymph node metastases in 5.7% of the 158 patients. Four patients with *HER2* gene amplification in the primary tumour had no amplification in lymph node metastases, while in five patients without *HER2* amplification of the primary tumour, *HER2* was amplified in the corresponding lymph node metastases. There was a positive correlation between *HER2* amplification status and overexpression. Although the *HER2* status, overall, remains highly preserved in the lymph node metastases, our

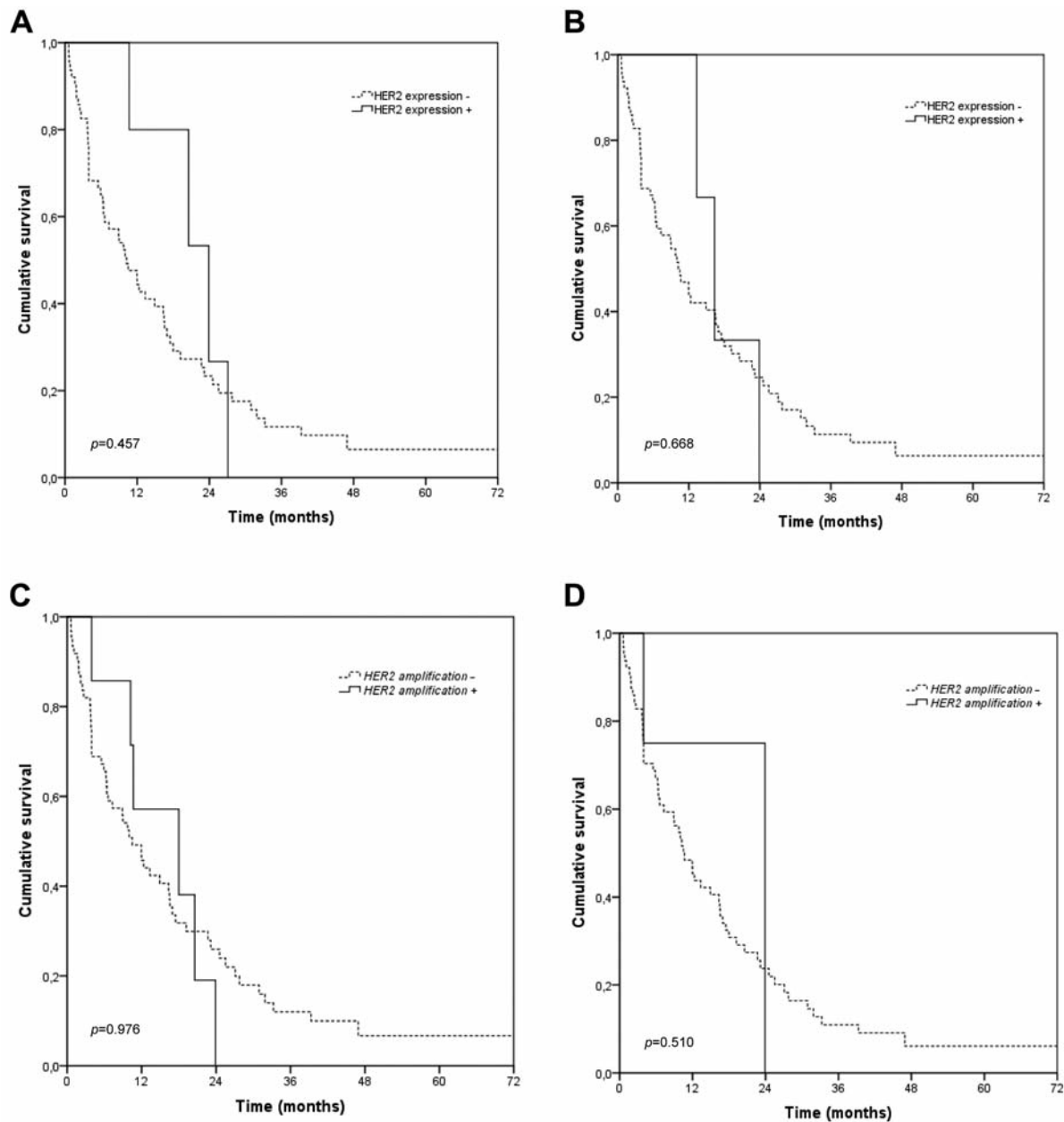


Figure 3. Survival analyses of patients with esophageal squamous cell carcinoma. Overall survival according to human epidermal growth factor receptor 2 (HER2) expression of the primary tumour (A) and of lymph node metastases (B), and according to HER2 gene amplification of the primary tumour (C) and of lymph node metastases (D) in 68 patients with esophageal squamous cell carcinoma.

results suggest differences in the metastatic pathway, which might be due to heterogeneity of the primary tumour.

In a previous study by Tapia *et al.* only 4% of esophageal SCCs showed *HER2* amplification (33). In contrast, our dataset of squamous cell carcinomas showed an *HER2* amplification rate of 10.3% (7/68) with three cases of high-level amplification, which is relatively high compared to the results of Tapia *et al.* and Reichelt *et al.* (5%, 7/145) (23). Other studies found *HER2*

amplification in esophageal squamous cell carcinoma in 19.1% (9/47) (26) and 11% (7/69) (22). This is not only a demonstration of the variation in results from FISH analysis, but also re-affirms the tendency towards a higher amplification rate in squamous cell carcinomas than initially expected. In the diagnosis of esophageal squamous cell carcinomas *HER2* status is not routinely tested. The guidelines of diagnosis for SCCs should therefore perhaps be reconsidered (2, 24, 25, 35).

We did not find any prognostic value of *HER2* amplification or expression for esophageal carcinoma, as was described by previous studies (23, 34). However, there is evidence that *HER2* amplification in disseminated tumour cells (DTCs) is a prognostic factor for esophageal carcinoma, although the mechanisms of tumour cell dissemination and reactivation of latent tumour cells remain largely unknown (31). It was also shown in an orthotopic animal model of metastatic esophageal carcinoma that *HER2*-targeted therapy with trastuzumab resulted in significant reduction of primary tumour growth and lymph node metastases (11). Even adjuvant therapy of this highly treatment-resistant disease with trastuzumab to prevent from lymph node metastasis after primary tumour resection has been suggested (14, 30).

Although a positive correlation of the *HER2* status between FISH and immunostaining was observed for the primary tumor tissue, and *HER2* status was highly conserved in the matched lymph node samples, divergent results from the primary site were also found in lymph node metastases. Neither in breast cancer nor gastric cancer, which are well-researched, are molecular mechanisms explaining these divergences (27).

Four cases with *HER2*-amplified lymph nodes were found without *HER2*-amplified primary tumours. Considering these cases individually, the divergence in status between *HER2*-negative primary tumours and -positive corresponding lymph nodes might occur on account of spots of low-level amplification (ratio 2-3). These effects might be related to borderline amplification in these individual tissue spots. Cases with *HER2*-positive primary tumour and -negative lymph node metastases can be explained by heterogeneity of the primary tumour. However, the important question remains: What makes cells metastasize? It is as yet unknown at what stage and which tumour cells acquire the ability to metastasize. Previous studies have suggested that tumour cells can begin to disseminate from the primary site at very early stages of transformation (15, 31, 32). It is possible that only a subclone of tumour cells disseminate, migrate, invade and form metastases in distant organs and lymph nodes. Immunophenotyping and molecular studies show that the DTC and circulating tumour cell (CTC) population is highly heterogeneous. It can be assumed that only a small subset of these cells might be the founders of overt metastases (32).

Conclusion

Although the *HER2* status overall remains highly conserved in lymph node-metastatic esophageal carcinoma, discrepancies occur between primary tumour and lymph node metastases. Our results suggest that *HER2* amplification varies in a subset of cells, and that changes in

the *HER2* status of primary tumour and lymph node or distant metastases are essential for the prediction of the individual disease course. Further studies investigating the heterogeneity of metastatic cells and the dissemination process are needed for esophageal carcinoma.

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