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Prognostic impact of perineural, blood and lymph vessel invasion for esophageal cancer

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Summary. Background: With a median survival of <22 months esophageal cancer is one of the most aggressive tumors, up to 20% of node negative patients develop systemic relapse. Studies investigating the prognostic impact of tumor-micro-invasion in blood (AI) and lymphatic vessels (LVI) as well as perineural invasion (PNI) have shown inconsistent results. The aim of the present study was to investigate the prognostic value of the aforementioned factors in a large homogenously treated cohort of patients with esophageal cancer.

Methods: Data from 695 patients with surgically treated esophageal cancer were analyzed. AI, LVI and PNI were determined and data were statistically correlated with clinico-pathological parameters and survival of the patients.

Results: Thirteen percent of all specimens showed an AI, 35% a LVI and 5% a PNI. The invasion factors were mostly significantly correlated with the established prognostic parameter, including bone marrow micro-metastases. Kaplan-Meier analysis revealed a prognostic impact for LVI in both cancer subtypes, while AI and PNI were significant factors in adenocarcinoma only. In multivariate analysis, none showed statistical significance. However, sub-analysis of completely resected, node negative and non-metastasized patients showed a significant prognostic impact of LVI.

Conclusion: The prognostic significance of AI, LVI and PNI seems to be limited compared to the established prognostic parameters of the UICC staging system. In completely resected, node negative and non-metastasized patients, LVI is an independent prognostic parameter for a worse outcome. Those patients might benfit from additional treatment or more intensive follow up.

Key words: Angiovasion, Vessel Invasion, Lymphangioinvasion, perineural Invasion, Esophageal cancer

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Introduction

Surgery remains the treatment of choice for resectable esophageal cancer (O'Reilly and Forastiere, 1994; Siewert et al., 2001). However, due to its very aggressive tumor biology, a high rate of even low-risk staged (e.g. lymph node negative) patients suffer from relapse after complete surgical treatment (Yekebas et al., 2006; Peyre et al., 2008).

Decision making regarding (neo-) adjuvant therapies requires reliable clinical or histopathological staging systems. The established preoperative staging of the disease, assessed by endoscopy, endosonography and computed tomography, is not precise enough to determine the exact tumor staging. In particular, the accuracy of the endosonographic evaluation is unsatisfactory (Kutup et al., 2007; Shimpi et al., 2007). In addition, with the existing TNM-based staging system, reliable identification of patients with a high risk for relapse is not adequately possible (Khan et al., 2004).

Lymph node metastasis is one of the well-established prognostic factors for esophageal cancer

patients (Paraf et al., 1995; Griffiths et al., 2006; Gertler et al., 2011). A distinct network of longitudinal lymphatic vessels inside the esophageal wall allows an early dissemination of the tumor cells to distant compartments (Nishimaki et al., 1994; Watanabe et al., 2000; Kunisaki et al., 2010). The surgical standard takes this into account by extensive lymph node dissection. Local invasion of vessels and neural structures is an early step in the metastatic cascade, but does not necessarily imply systemic dissemination (Sarbia et al., 1995). Vice versa, up to 20% of patients with small, node-negative tumors develop systemic recurrence (Khan et al., 2004; Kunisaki et al., 2010).

Recently, locally invasive markers, such as tumor micro-invasion in blood (AI) and lymphatic (LVI) vessels, and of perineural invasion (PNI) were included as an optional factor in the Union for International Cancer Control (UICC) classification for esophageal cancer (7^{th} edition) (Sobin et al., 2009). In fact, the so far published studies have shown inconsistent results regarding those factors on the prognostic impact (Theunissen et al., 1991; Paraf et al., 1995; Sarbia et al., 1995; Tanaka et al., 1998; Torres et al., 1999; Watanabe et al., 2000; Brucher et al., 2001; Osugi et al., 2002; Wayman et al., 2002; Zafirellis et al., 2002; Khan et al., 2004; von Rahden et al., 2005; Wijnhoven et al., 2007; Kunisaki et al., 2010; Mirnezami et al., 2010; Reynolds et al., 2010; Kozlowski et al., 2011; Waraich et al., 2011; Imamura et al., 2012).

The objective of the present study was to investigate the prognostic impact of AI, LVI and PNI in a large cohort of surgically resected esophageal cancer patients.

Materials and methods

Patients

The study was approved by the Ethics Committee of the Chamber of Physicians in Hamburg, Germany. Data from 695 patients with esophageal cancer, who underwent esophagectomy at the University Medical Center Hamburg-Eppendorf between May 1995 and November 2011, were collected. Informed consent was obtained from all patients before including them in a prospective database.

Clinical data were obtained from a combination of clinical and pathological record review, reports of outside medical records and communication with patients and with their attending physicians or from the regional Cancer Registry.

Histopathologic examination

All sections were prospectively investigated by experienced pathologists specialized in gastroenterology to determine the histological tumor type, grade, tumor invasion depths, lymph node metastasis and count, and resection margin (classified according to the World Health Organization (WHO) classification).

To determine vascular invasiveness (AI and LVI), representative Hematoxylin and Eosin (H&E)-slides of the interface between the tumor and the surrounding tissue were analyzed. Every vessel outside the tumor circumference is defined as peritumoral without a clearance distance. Only peritumoral vascular invasion was regarded as true invasion; infiltration of intratumoral vessels was disallowed. In cases without direct evidence of vessel wall penetration by tumor extensions, the undermentioned additional criteria were evaluated to determine true vascular invasion and to exclude artificial impaction of tumor material into vessel spaces during processing, as well as misinterpretation of tumor-associated gap-formation as vessel luminae: an endothelial lining of the vessel had to be apparent and an extension of endothelial lining onto the intravascular tumor plug was considered evidentiary. Tumor-associated thrombus-formation in infiltrated vessels was considered equally affirmative. In undetermined cases, immunohistochemical stainings (CD31 and D2-40) were used to identify blood and lymphatic vessels (Tachezy et al., 2010; Imamura et al., 2012).

PNI was defined as tumor cells lying in the perineural space, usually with circumferential or near-circumferential involvement, or intraneural tumor-cell extension.

Statistical analysis

SPSS for Windows (Version 17, SPSS Inc., Chicago, IL USA) was used for statistical analysis. Associations between categorical variables were assessed by Fisher exact test. The Kaplan-Meier method was used to estimate the occurrence probability of an event (death). Overall survival (OS) was calculated from the date of surgery to the date of death or last follow-up. Differences between patient groups with respect to their survival were assessed using log-rank tests. Covariates with a p value <0.05 in univariate survival analysis (log-rank test) were entered into an adjusted multivariate logistic regression test to assess the independent influence of these covariates. Significance statements refer to P values of 2-tailed tests with a P value <0.05.

Results

Patient characteristics

A total of 695 patients aged 32 to 86 years (median 63 years) were included in this study. Of these, 555 men (80%) and 140 women (20%) were surgically treated. Fifty-two patients received neoadjuvant chemotherapy or combined radiochemotherapy. Clinical and histopathologic findings are summarized in Table 1. Median follow-up time of all patients included in survival analysis was 14.5 months (range 1-181 months); the calculated median OS for all patients was 20.3 months. In total, 54 (7.8%) patients died within the first 30 days after surgery.

Correlation of clinical and histopathological parameters with blood vessel-invasion (AI)

Thirteen percent (n=93) of all esophageal cancer specimens showed blood vessel invasion (Fig. 1). No statistical association was found with factors such as sex, age, neoadjuvant therapy, perioperative mortality or tumor cell type. Blood vessel invasion was significantly correlated with advanced cases of tumor size (pT, p<0.001), lymph node metastases (pN, p<0.001), distant metastases (M, p=0.019), tumor grading (G, p<0.001), resection margin (R, p<0.019) and disseminated tumor cells in bone marrow (p=0.027). Data are shown in Table 1.

Correlation of clinical and histopathological parameters with lymph vessel invasion (LVI)

Histopathological examination revealed a LVI rate of 35% (n=241) of the resected esophageal cancer (Fig. 1). In 3% (n=19), lymph vessel (D2-40) and vessel endothelium (CD31) specific immunohistochemical staining was performed. Immunohistochemically stained specimens were classified as LVI positive in 58%, compared to the H&E only stained patients with 42% positivity (p=0.466).

Factors such as sex, age, neoadjuvant therapy or 30-day mortality were statistically not associated with LVI. Lymph vessel invasion was significantly correlated with advanced cases of tumor size (pT, p<0.001), lymph node metastases (pN, p<0.001), tumor grading (G, p<0.001), resection margin (R, p=0.004), tumor cell type (p<0.001) and disseminated tumor cells in bone marrow (p=0.015), while distant metastatis narrowly failed to be associated (M, p=0.064). Results are presented in Table 1.

Immunohistochemistry (CD31 and D2-40) was performed in 23 patients (3.3%, Fig. 1), resulting in a non-significant elevation of the detection rate from 13% to 22% for AI (p=0.207) and from 34% to 42%

Table 1. Correlation between clinical and pathological parameters and angioinvasion (AI), lymph vessel invasion (LVI) and perineural invasion (PNI).

			Angioinvasion (AI)					Lymphatic vessel invasion (LVI)					Perineural invasion (PNI)				
		Total	negative		positive		p-value	negative		positive		p-value	negative		positive		p-value
			(n)	(%)	(n)	(%)		(n)	(%)	(n)	(%)		(n)	(%)	(n)	(%)	
Age	< 60 years	340	294	86	46	14		228	67	112	33		325	96	15	4	
	≥ 60 years	355	308	87	47	13	0.912	226	64	129	36	0.381	332	94	23	6	0.247
Sex	Female	140	122	87	18	13		93	66	47	34		130	93	10	7	
	Male	555	480	86	75	14	0.890	361	65	194	35	0.843	527	95	28	5	0.306
neoTx	No	643	556	86	87	14		415	65	228	35		606	94	37	6	
	Yes	52	46	88	6	12	0.834	39	75	13	25	0.172	51	98	1	2	0.350
Disease Stage (UICC 7th edition)*																	
pT	1a	41	39	95	2	5		37	90	4	10		41	100	0	0	
	1b	109	104	95	5	5		90	83	19	17		108	99	1	1	
	2	168	149	89	19	11		115	68	53	32		162	96	6	4	
	3	332	278	84	54	16		187	56	145	44		305	92	27	8	
	4	45	32	71	13	29	0.000	25	56	20	44	0.000	41	91	4	9	0.009
pN	0	279	268	96	11	4		247	89	32	11		272	97	7	3	
	1	147	129	88	18	12		95	65	52	35		138	94	9	6	
	2	148	116	78	32	22		65	44	83	56		139	94	9	6	
	3	121	89	74	32	26	0.000	47	39	74	61	0.000	108	89	13	11	0.009
M	0	668	583	87	85	13		441	66	227	34		633	95	35	5	
	1	27	19	70	8	30	0.019	13	48	14	52	0.064	24	89	3	11	0.178
G	1	28	28	100	0	0		24	86	4	14		28	100	0	0	
	2	352	322	91	30	9		258	73	94	27		335	95	17	5	
	3	303	244	81	59	19		165	54	138	46		283	93	20	7	
	4	4	1	25	3	75	0.000	1	25	3	75	0.000	3	75	1	25	0.137
R	0	560	495	88	65	12		382	68	178	32		534	95	26	5	
	1	104	82	79	22	21		57	55	47	45		94	90	10	10	
	2	31	25	81	6	19	0.019	15	48	16	52	0.004	29	94	2	6	0.119
Tumor cell type*	Adenocarcinoma	311	323	104	57	18		223	59	157	41		354	93	26	7	
	Squamous cell carcinoma	380	275	72	36	9	0.218	227	73	84	27	0.000	299	96	12	4	0.095
Bone marrow micrometastasis*	negative	178	168	94	10	6		149	84	29	16		172	97	6	3	
	positive	103	89	86	14	14	0.027	73	71	30	29	0.015	95	92	8	8	0.152

*Numbers do not always add up to 695 in the different categories because of cases with missing data. (tumor size, pT; nodal involvement, pN; distant metastasis, M; tumor cell grading, G; resection margin status, R

(p=0.468).

Correlation of clinical and histopathological parameters with perineural invasion (PNI)

The total rate of esophageal cancer patients with PNI was 5% (n=38) (Fig. 1). Clinical and histopathologic data such as sex, age, neoadjuvant therapy, 30-day mortality, metastasis status, tumor cell grading, resection margin status, tumor cell type and disseminated tumor cells in bone marrow showed no correlation with PNI. It was significantly correlated with advanced cases of tumor size (pT, p=0.009) and lymph node metastases (pN, p=0.009). Table 1 summarizes the data of the analysis.

Survival analysis and multivariate analysis

Survival plots stratified for cancer cell type according Kaplan-Meier plots and results of the log-rank survival tests are shown in Fig. 2. In the case of AI, LVI and PNI OS is significantly reduced in esophageal adenocarcinoma, while survival is significantly affected in esophageal squamous cell carcinoma in case of LVI only, AI narrowly failed to be significant (p=0.066), which might be caused by the small number of positive cases (Fig. 2). Sub-analysis of specimens stained by D2-40 did not show a significant effect in survival analysis, potentially due to the low number of patients. Multivariate Cox regression analysis showed neither in esophageal squamous cell carcinoma, nor in esophageal adenocarcinoma a statistically significant effect of one of the three invasion parameters.

Additionally, we performed a Cox regression analysis for completely resected (R0), node negative (pN0) and non-metastasized (M0) patients for esophageal adenocarcinoma and esophageal squamous cell carcinoma separately, and together (n=136, n=115 and n=251, respectively). Survival analysis of esophageal adenocarcinoma showed a significant effect in univariate analysis for patients' age (p=0.001), tumor size (pT; p<0.000) and grading (G; p=0.038) and LVI (p=0.038). Sex (p=0.693), PNI (p=0.788) and AI (p=0.844) failed to be significant. However, age (p=0.001, HR 3.562; 95% CI 1.705/ 7.442), tumor size (p=0.001, HR 1.998; 95% CI 1.348/2.961) and LVI

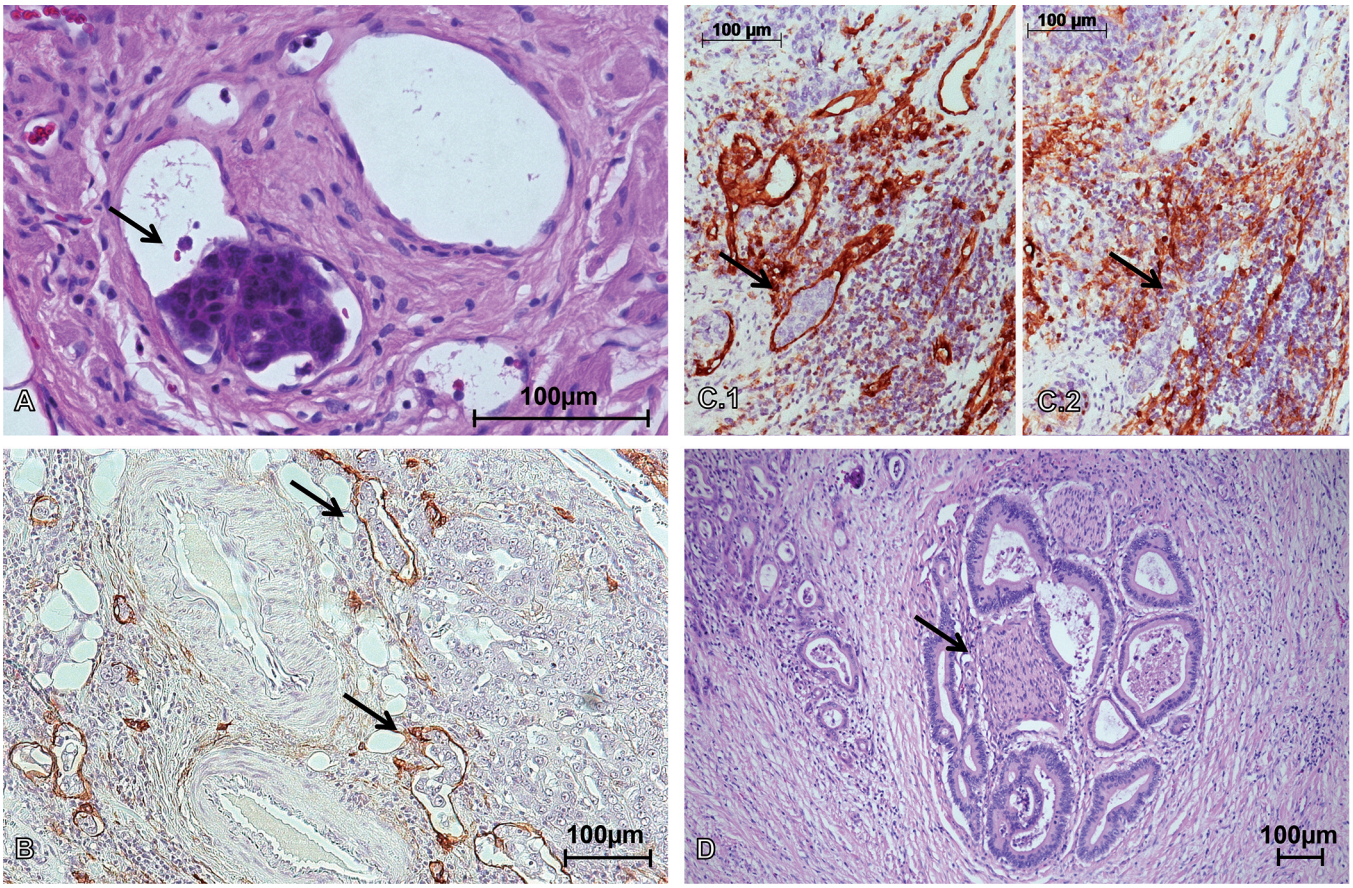

Fig. 1. Representative slides showing lymphangioinvasion in conventional H&E staining **(A)** and with D2-40 immunohistochemistry (**B**, arrows indicate tumor cell infiltration). **C.1.** shows an infiltrated CD31 positive vessel, which is negative in D2-40 staining **(C.2)**. **(D)** Perineural invasion.

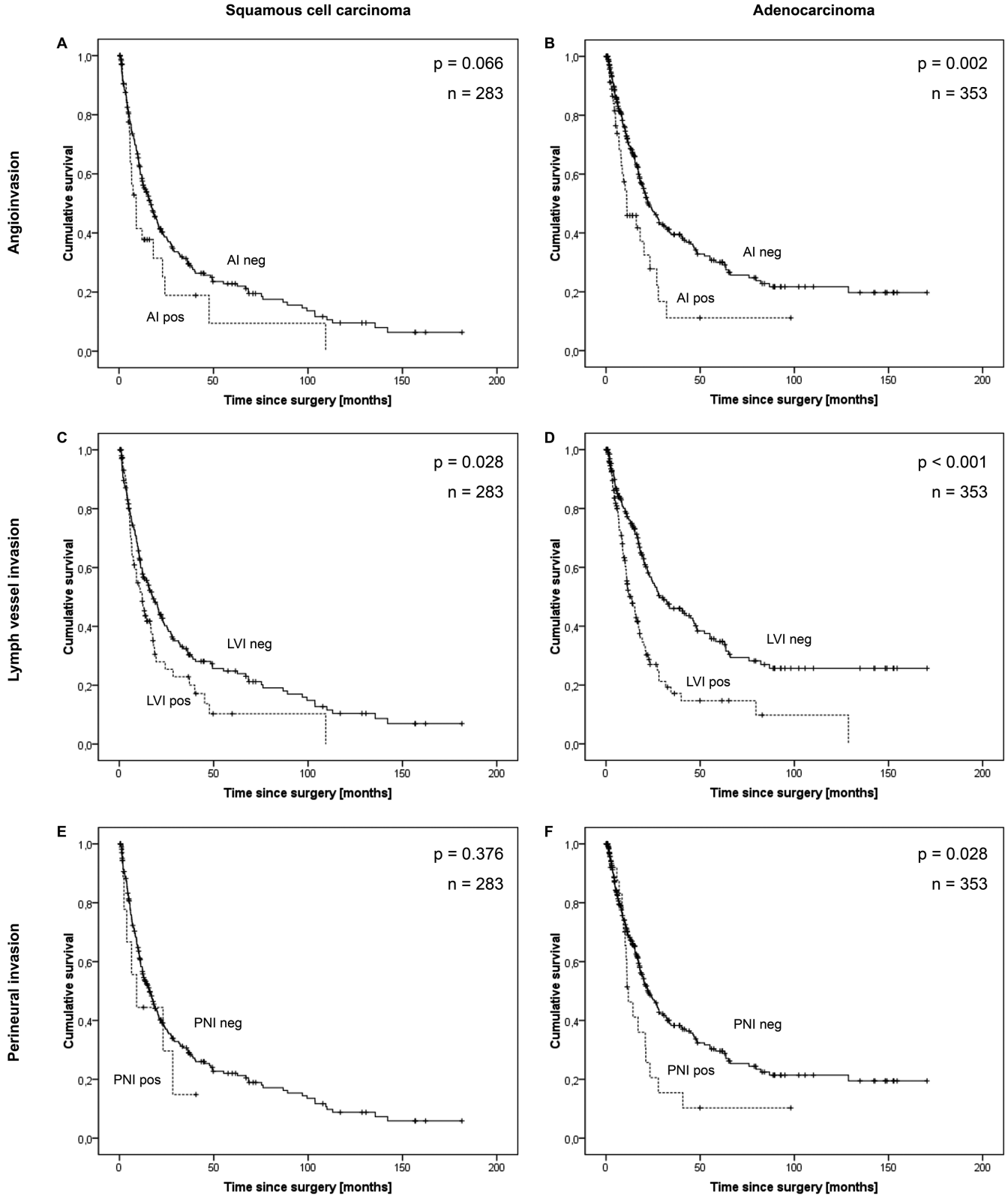

Fig. 2. Kaplan Meier overall survival analysis for angioinvasion (AI), lymph vessel invasion (LVI) and perineural invasion (PNI) stratified for tumor cell type (esophageal adenocarcinoma, EAC and esophageal squamous cell carcinoma, ESCC). Patients who died during the first 30 days after surgery were excluded.

(p=0.001; HR 4.456, 95% CI 1.879/10.568) were independent prognosticators for survival in multivariate analysis. Similar to this, esophageal squamous cell carcinoma patient survival is significantly influenced by patient's age (p=0.02), tumor size (pT; p=0.002) and grading (G; p=0.003) and LVI (p=0.043). Again, sex (p=0.601). PNI (p=0.127) and AI (p=0.156) failed to be significant and a significant effect in multivariate analysis was seen for patient's age (p=0.026, HR 1.667; 95% CI 1.062/2.615) and tumor size (p=0.008, HR 1.378; 96% CI 1.088/1.744), but not for LVI (p=0.123, HR 1.788; 95% CI 0.854/3.742). Analyzing the two histological types together, sex (p=0.082), grading (p=0.766), AI (p=0.552) and PNI (p=0.354) failed to be significant in univariate analysis and all other implemented criteria were significant in univariate and also in multivariate analysis: Tumor size (pT), age,

Table 2. Prognostic factors for OS in completely resected, node negative and non metastasized esophageal cancer patients (Multivariate Cox regression analysis).

		Multivariate			
Variable		HR	95%	CI	p-value
Age	≥60y vs <60y	2.362	1.622	3.438	0.000
pT	T2 vs T1	1.561	.986	2.470	0.057
	T3 vs T1	1.981	1.287	3.047	0.002
	T4 vs T1	5.411	1.263	23.192	0.023
Tumor cell type	ESCC vs EAC	.458	.310	.678	0.000
LVI	yes vs no	2.458	1.443	4.186	0.001

HR, hazard ratio with 95% confidence interval (CI); P refers to significance according to Cox regression hazard model comparing specified variables

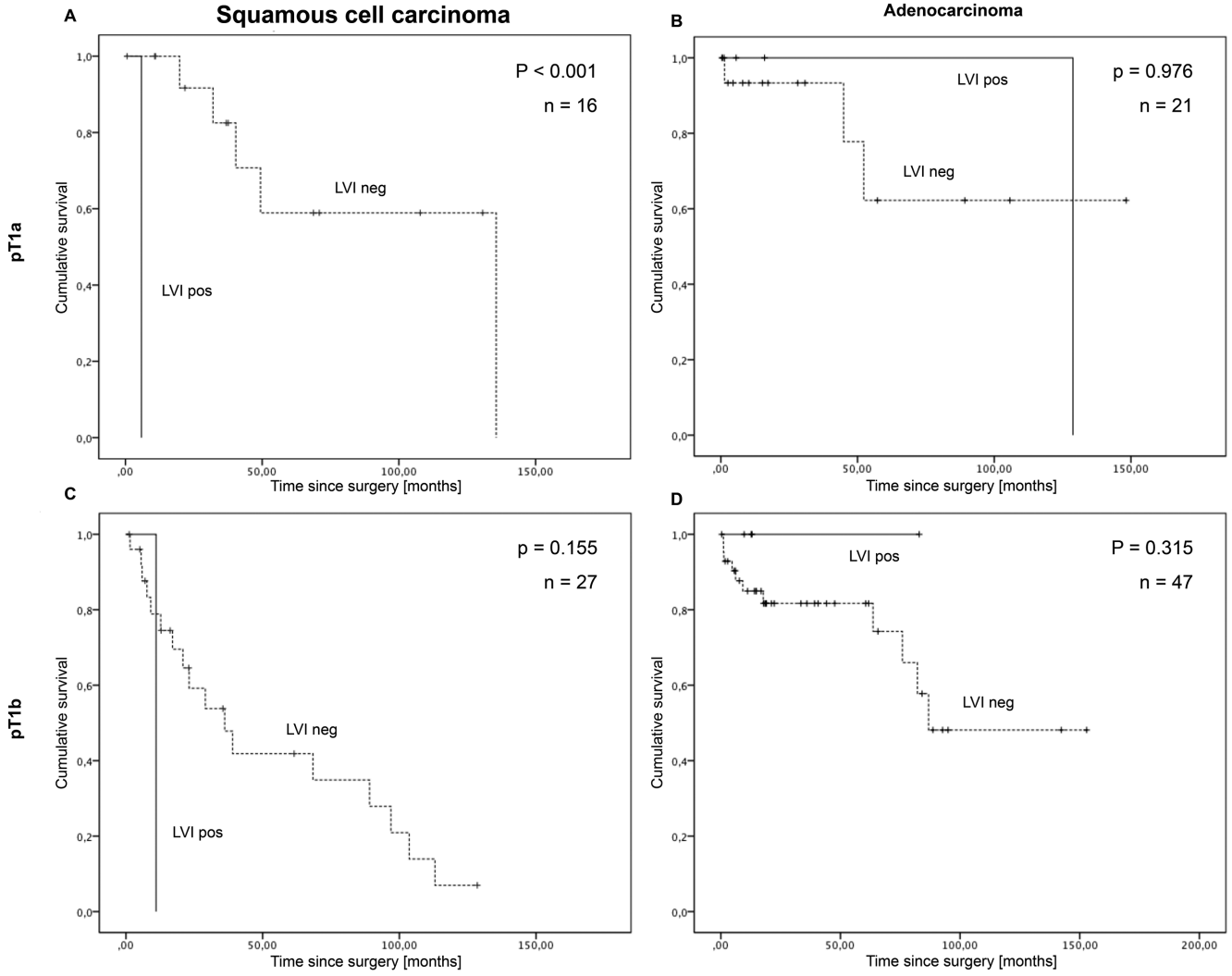

Fig. 3. Kaplan Meier overall survival analysis for lymph vessel invasion of pT1a and pT1b tumors, stratified for tumor cell type (esophageal adenocarcinoma, EAC and esophageal squamous cell carcinoma, ESCC). Patients who died during the first 30 days after surgery were excluded.

tumor cell type and LVI (Table 2).

Analyzing the prognostic effect of LVI on survival of LVI in R0, pN0 and M0 patients in small tumor stadia (pT1a and pT1b), log rank test revealed a significant survival reduction in pT1a esophageal squamous cell carcinoma only (p=<0.001), while pT1a esophageal adenocarcinoma and pT1b failed to be significant (Fig. 3).

Discussion

The present study analyzed the prognostic impact of tumor micro-invasion in blood and lymphatic vessels and of perineural invasion for esophageal cancer patients. These morphological parameters are basically associated with the well-known and established prognostic criteria, which constitute the basis for the TNM staging system of the AJCC and UICC (Sobin et al., 2009).

The multivariate survival analysis revealed no independent effect of LVI, contrary to the vast majority of studies (Sarbia et al., 1995; Watanabe et al., 2000; Brucher et al., 2001; Wayman et al., 2002; von Rahden et al., 2005; Reynolds et al., 2010), while Osugi and colleagues and Tanaka and colleagues also presented no prognostic effect (Tanaka et al., 1998; Osugi et al., 2002). The other established prognosticators of patients' survival seem to prevail over the effect of LVI, but basically two subgroups might profit from the determination of LVI:

First, patients with endoscopical or limited surgical segmental esophageal resections: Ten percent of the patients with tumors limited to the mucosa (pT1a) and 17% to the submucosa (pT1b) show a LVI. Watanabe and colleagues have even found a LVI in 22% of patients with submucosal esophageal squamous cell carcinoma, highlighting the local metastatic potential of the disease. Survival analysis of pT1 tumor stadia showed that only esophageal squamous cell carcinoma patients with a pT1a tumor were significantly influenced by LVI positivity. The results of the small cohorts of patients must be carefully interpreted; In the case of a surgical resection, the treatment might be curative, but potentially to radical. Otherwise, a LVI might indicate a systemic tumor spread and an endoscopical resection might be an under-treatment, as recommended by the National Comprehensive Cancer Network (Sgourakis et al., 2013).

Second, patients after a complete surgical resection, whose tumor has not spread in lymph nodes or distant organs: Twelve percent of completely resected, node-negative and non-metastasized patients had a LVI (in contrast to 49% with lymph node metastases), and in this case, a LVI is an independent significant prognosticator in multivariate analysis (HR 2.458, 95%CI 1.4/4.1; p=0.001). Imamura and colleagues found in their analysis of lymph-node-negative esophageal squamous cell carcinoma LVI rates of 21% with HE staining and even 30% with lymph-vessel-specific immunohistochemistry (D2-40) (Imamura et al., 2012). Our subanalysis, stratified for histologic cell type, has not shown an independent effect of LVI either in esophageal adenocarcinoma or esophageal squamous cell carcinoma, which might be caused by the small number of patients in the subgroups. Nevertheless, patients with LVI might profit from intensified follow up or even additional tumor specific treatment.

Lymph vessel invasion in esophageal cancer has been analyzed in several studies, and the published LVI rates show a wide range from 18% to 72%, which is basically caused by the lack of an accepted definition for LVI: There is a need for a generally accepted definition, making the results of the pathological examinations more comparable. For colorectal cancer, such initiatives are made (Kojima et al., 2013). The identification of lymph vessels in histopathologic sections with conventional H&E staining is sometimes difficult and the detection sensitivity can be elevated for 15-45% by specific staining of lymph vessels (Buskens et al., 2008; Saad et al., 2009; Kozlowski et al., 2011; Imamura et al., 2012). In the current study, LVI was analyzed by our standard procedure; by microscopic examination of H&E stained samples. Just in non-ambiguous cases, immunohistochemical methods (D2-40 and CD31 staining) were used and increased the detection rate by 16 percent, underlining the known effect of LVI underreporting by H&E staining. However, our results show similar data compared to other studies using morphological criteria only (35% of all patients) (Brucher et al., 2001; von Rahden et al., 2005). In our opinion, a stepwise scheme as performed in the present study should be appropriate in the clinical cost-conscious routine. Apart from this general conclusion, subgroups such as the aforementioned might profit from routine staining, as already discussed and investigated by several authors (Gockel et al., 2009; Moriya et al., 2011; Imamura et al., 2012). Patients with a higher risk of recurrence might be identified by additional lymph vessel specific staining, stratifying patients, which benefit from additional surgical or adjuvant treatment.

In contrast to LVI, no significant difference was found regarding the presence of AI between the two main types of esophageal cancer (esophageal adenocarcinoma 18% and esophageal squamous cell carcinoma 9%, respectively). Other authors described an AI rate in 11-67% of the patients (Theunissen et al., 1991; Paraf et al., 1995; Sarbia et al., 1995; Tanaka et al., 1998; Torres et al., 1999; Watanabe et al., 2000; Osugi et al., 2002; Wayman et al., 2002; Zafirellis et al., 2002; Khan et al., 2004; Wijnhoven et al., 2007; Kunisaki et al., 2010; Waraich et al., 2011; Imamura et al., 2012). Angioinvasion is significantly associated with the established prognosticators of the TNM classification and also bone marrow micrometastasis (Table 1). Although not an independent prognosticator for survival, AI turned out to be significant in univariate analysis of esophageal adenocarcinoma and both cell types combined, but not esophageal squamous cell carcinoma.

The published data regarding the prognostic significance of AI are inconsistent; some authors have found an independent significance in one or both histologic subtupes and others have not (Theunissen et al., 1991; Paraf et al., 1995; Sarbia et al., 1995; Tanaka et al., 1998; Torres et al., 1999; Watanabe et al., 2000; Osugi et al., 2002; Wayman et al., 2002; Zafirellis et al., 2002; Khan et al., 2004; Waraich et al., 2011). Notwithstanding, Kunisaki and colleagues described an independent prognostic value of AI even in pN0-staged esophageal squamous cell carcinoma patients (Kunisaki et al., 2010). Sub-analysis of our data did not show an impact on the survival of pN0, M0 and R0-staged cancer (p=0.552). In conclusion, the prognostic role of AI in esophageal cancer remains unclear and needs further clarification.

In our study, PNI was present in a small subset of patients (5.8% of all esophageal cancer patients) and was significantly associated with tumor size (pT) and lymph node metastasis (pN). Published data range from 5% to 54% (Paraf et al., 1995; Sarbia et al., 1995; Tanaka et al., 1998; Torres et al., 1999; Wayman et al., 2002; Khan et al., 2004; Wijnhoven et al., 2007; Reynolds et al., 2010). Perineural invasion was of prognostic significance regarding overall survival in esophageal adenocarcinoma only according to univariate, but not to multivariate analysis. This is in concordance to most of the other studies of esophageal adenocarcinoma (Paraf et al., 1995; Torres et al., 1999; Wayman et al., 2002; Reynolds et al., 2010). Findings for esophageal squamous cell carcinoma are inconsistent, as studies investigating prognosticators for esophageal cancer without cell type differentiation are (Sarbia et al., 1995; Tanaka et al., 1998; Khan et al., 2004; Wijnhoven et al., 2007). Further studies should be performed in larger patient groups to evaluate the independent prognostic value of PNI in esophageal carcinoma.

In conclusion, the investigated invasion-parameter may provide additional prognostic information for a more precise identification of patients with a high risk of relapse, although significant in univariate analysis only. Particularly, LVI might be included in the prognostic UICC Stage group system, helping to identify those patients who might profit from additional treatment or intensified follow-up. Further efforts must be made investigating predictive parameters, to optimize the treatment for each individual patient.

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