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Molecular markers predicting lymph node metastasis in early esophageal cancer

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Summary. AIMS: The aim of this study was to identify molecular markers predicting depth of tumor infiltration and presence of lymph node metastasis in early esophageal cancer.

METHODS: Between 1996 and 2004, 67 patients with pT1 esophagus cancer underwent esophagectomy. Resected tumors and lymph nodes were analyzed by immunohistochemistry for tissue infiltration, lymph node metastasis (LNM), micrometastasis and extracapsular lymph node infiltration (ELNI). We focused on MMP-2 (matrix-metalloproteinase-2), TIMP-2 (tissue inhibitor of metalloproteinase-2), PIM-1 and survivin as the most promising marker candidates. The data was correlated with the patients' long term follow-up (median follow-up time 11.4 years).

RESULTS: We found 22 pT1a and 45 pT1b carcinomas. None of the mucosal carcinomas, but 58% (26 patients) of the submucosal carcinomas showed lymph node metastasis or micrometastasis. The rate of LNM positively correlated with the depth of tumor infiltration (23% LNM in sm1 tumors and 82% LNM in sm3 tumors). Low grade PIM-1 expression (<30%) was significantly associated with occurrence of LNM (p=0.034) while high expression TIMP-2 (>70%) were detected in submucosal tumors. Logistic regression analysis revealed PIM-1 and Grading G3 as independent risk factors for LNM (p<0.001). Survival of patients with micrometastasis was comparable to those with LNM (median survival: 5.05 years versus 5.52 years). Patients with ELNI had the worst prognosis (median survival: 1.7 years).

CONCLUSIONS: PIM-1 is a promising marker for prediction of lymph node metastasis in early esophagus cancer. Extracapsular lymph node infiltration has an independent worse prognostic impact.

Key words: Esophageal cancer, MMP-2, TIMP-2, PIM-1, Survivin, Lymph node metastasis, Extracapsular lymph node infiltration, Immunohistochemistry

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Introduction

Esophageal cancer is one of the leading causes of cancer-related death in the western world with rising incidence, especially for adenocarcinoma of the distal esophagus and the cardia (Bollschweiler et al., 2001; Cook et al., 2009; Thrift and Whiteman, 2012; Lepage et al., 2013; Castro et al., 2014). However, the survival rate of patients successfully cured from early esophageal cancer without lymph node infiltration is comparable to the survival of the normal population. Therefore endoscopic interventions or limited surgical procedures for the treatment of superficial esophageal cancer are accelerating. Nevertheless, these procedures should be carried out only in the absence of lymphatic spread of the tumor. The prerequisite for evaluating all lymph nodes infiltrated is radical mediastinal and abdominal lymphadenectomy combined with esophagectomy. Recent literature was able to show that patients with

mucosal carcinoma have low rates of lymph node metastasis: 0-2% for AC and 0-3% for SCC (Bollschweiler et al., 2006). As a consequence, the prognosis of mucosal esophageal cancer is not different comparing endoscopic and surgical procedures (Prasad et al., 2007; Schembre et al., 2008; Pech et al., 2011).

In contrast, lymph node metastases occur in 20% to 30% of submucosal carcinomas (Bollschweiler and Hölscher, 2011; Hölscher et al., 2011). The frequency of lymphatic invasion correlates with the depth of tumor infiltration into the submucosa. One explanation for this fact is the increasing density of lymphatic vessels in the middle and lower third of the submucosa. Figure 1 demonstrates the anatomic and histopathologic view of the esophageal wall in both subtypes of superficial esophageal cancer.

For classification purposes the layers of mucosa and submucosa are currently divided into thirds. We have identified that the probability of lymph node metastases in the upper third of the submucosa (sm1) is about 10%, increases to 20% in the middle third (sm2) and to 40% to 50% in the deepest third (sm3) for adenocarcinoma and squamous cell cancer of the esophagus (Hölscher et al., 2011).

Today, preinterventional diagnostic procedures such as CT-scan or endoscopic ultrasound are not appropriate to predict the depth of infiltration within the mucosa or submucosa. The differentiation between m3 and sm1 tumors is especially difficult (May et al., 2004; Pech et al., 2010). Therefore, there is an urgent need for markers which allow the prediction of lymph node metastases and the depth of infiltration. A huge variety of putative molecular structures were identified within the recent years, but none of them made its way into the daily routine (Plum et al., 2013). Our research group was able to show that matrix-metalloproteinase-2 (MMP-2) and its specific inhibitor tissue of metalloproteinase-2 (TIMP-2) are associated with tumor progression and formation of lymph node metastasis in gastric cancer (Mönig et al., 2001; Alakus et al., 2008). In addition, we proved that up-regulation of the proto-oncogene PIM-1 in gastric glands correlates with formation of lymph node metastases in gastric cancer (Warnecke-Eberz et al., 2009).

The aim of our study was the identification of molecular markers that allow the pretherapeutic detection of lymph node metastasis in early stages of esophageal cancer.

Materials and methods

Patients

We used the database of esophageal cancer patients who underwent surgery at the Department of General, Visceral and Cancer Surgery, University of Cologne between 1996 and 2004 and excluded those patients who received neoadjuvant chemotherapy or radiochemotherapy (ypT1). The histopathologic work-up of the resected tumors identified 67 patients with pT1 esophageal cancer (53 men and 14 women, median age of 61 (range 18-81) years). Recently, we published the data of these patients according to the occurrence of isolated tumor cells within the lymph nodes examined (Prenzel et al., 2012). These findings of lymphatic micrometastasis were included in the present analysis. Written informed consent was obtained by all patients before surgery.

Methods

Surgery

The treatment of choice was a total en bloc esophagectomy using a right transthoracic approach including a so-called 2-field lymphadenectomy of mediastinal and abdominal nodes (Hölscher et al., 2011). The tumor-affected esophagi were removed en bloc, including the lymph nodes. To ensure integrity of the primary tumor, lymph nodes were dissected partially in the operating room and analyzed by the pathologists according to a standardized protocol. On average, 33.7 (min: 14 - max: 73) lymph nodes were removed during resection of 28 SCC; and 30.4 (min: 13 - max: 64) lymph nodes within the 39 AC (not significant). The average number of resected thoracic lymph nodes was 10.5 (min: 5 - max: 50) and of gastric lymph nodes was 18.9 (min: 7 - max: 35). The standard reconstruction for patients receiving transthoracic esophagectomy was done by stomach interposition with high intrathoracic esophagogastrostomy.

Histopathology

Histopathologic examination of all resected esophagi consisted of a thorough evaluation of the tumor stage, residual tumor (R) category, grading and number of resected and involved lymph nodes. All primary carcinomas were completely embedded. The tissue specimens were fixed in 5% formaldehyde and embedded in paraffin. The length and height of the tumors were measured after fixation. The lymph nodes were counted and the maximum diameter of each node was measured with a slide gauge. A series of sections from each node was selected and stained with hematoxylin and eosin as well as with periodic acid-Schiff (PAS). All dissected lymph nodes were microscopically analyzed for metastatic disease. The neoplastic lesions were classified and graded in accordance with WHO recommendations. Using the 7th edition of the TNM-classification of Malignant Tumors, T1 tumors were sub-classified further as T1a (limited to the mucosa or to muscularis mucosae) and T1b (submucosa). The lesions were then subdivided into 3 equal layers of one third of the mucosa (m1, m2, m3) or submucosa: sm1 (invasion of the upper third), sm2 (invasion of the middle third) and sm3 (invasion of the lower third) (see Fig. 1) (Hölscher et al., 2011). The

depth of infiltration was measured at the deepest point of penetration of the cancer cells in the corresponding layer. In addition, invasion of lymphatic and blood vessels was recorded. Lymph node metastasis (LNM) was categorized as pN0=no LNM, pN1=1-2 LNM, pN2=3-6 LNM and pN3=7 or more LNM. Beyond the UICC-pN-category, we defined a new pN-category with extended subdivision of nodal metastasis including pN0, pN0micro+ (=nodal micrometastasis), pN+ELNI- (=regular lymph node metastasis) and pN+ELNI+ (=nodal metastasis with extracapsular lymph node infiltration) for further examinations.

Micrometastasis

The results of the earlier study about micrometastasis were included in this analysis (Prenzel et al., 2012). The micrometastasis was declared as pN0micro+.

Extracapsular lymph node infiltration (ELNI)

The presence of extracapsular lymph node infiltration has been considered as a relevant prognostic factor in advanced esophageal cancer (Metzger et al., 2013). ELNI was defined as metastatic cancer extending through the nodal capsule into the perinodal fatty tissue (pN+ELNI+).

Immunohistochemistry

We focused on four putative marker proteins for immunohistochemical evaluation of the tumor: Matrix-metalloproteinase-2 (MMP-2), the tissue inhibitor of metalloproteinase-2 (TIMP-2), proto-oncogene PIM-1 and survivin. Analyses were performed with the DAKO EnVisionTM staining kit (DakoCytomation, Hamburg, Germany) as precisely described within our previous studies (Mönig et al., 2001; Alakus et al., 2008; Warnecke-Eberz et al., 2009). Before staining, five μm thick paraffin-embedded tissue sections were deparaffinized according to standard histological techniques.

MMP-2 protein was detected by a polyclonal rabbit anti-MMP-2 antibody (Neomarkers, Fermont, CA, USA), diluted 1:50 (v/v). For staining PIM-1 and survivin within the tumor tissue, we chose rabbit polyclonal anti-PIM-1antibody (Abgent, San Diego, CA, USA), diluted 1:50 (v/v) and rabbit polyclonal anti-survivin antibody (Neomarkers, Fermont, CA, USA), diluted 1:100 (v/v). TIMP-2 protein was marked by mouse monoclonal anti-TIMP-2 antibody, clone 67-4H11, (Diagnostic International, Schriesheim, Germany) used at a dilution of 1:200 (v/v).

The level of expression of MMP-2, TIMP-2, PIM-1 and survivin was estimated by semiquantitative evaluation and divided into four categories according to the number of positively stained tumor cells: score 0 = <5%; score 1 = 5%-30% positive tumor cells; score 2 = 30-70% positive tumor cells and score 3 = >70% positive tumor cells. Sections with score ≥1 were considered positive. The evaluation was performed by two independent staff pathologists who were blinded to all clinical data.

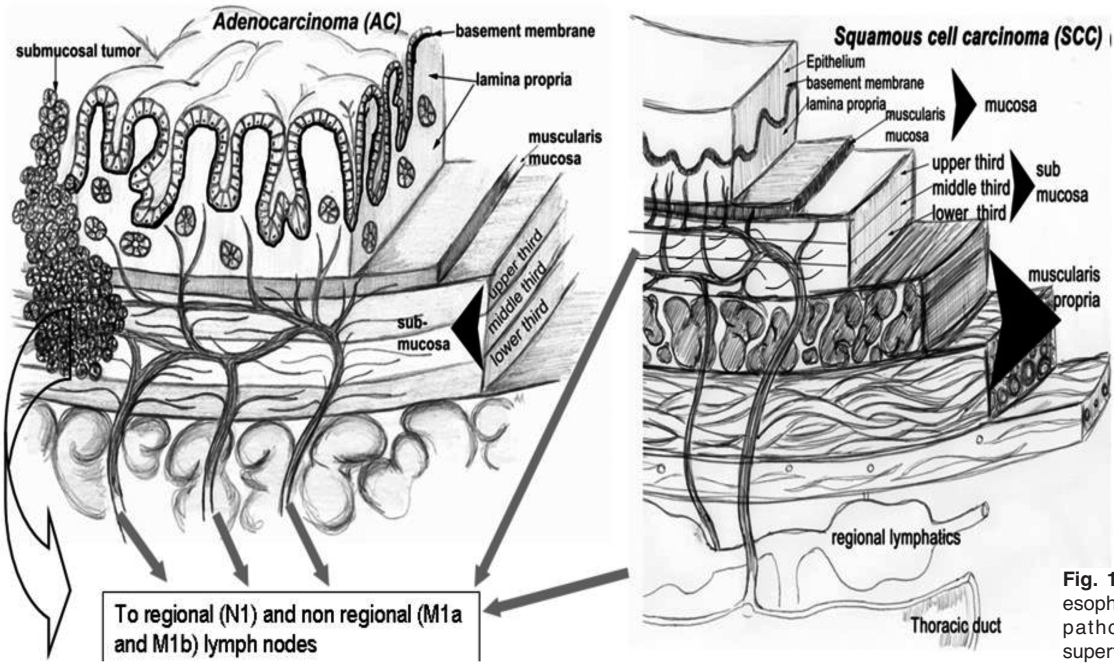

Fig. 1. Schematic view of the esophageal wall in both histo-pathological subtypes of superficial esophageal cancer.

Follow- up

The follow-up of all patients was performed according to a standardized protocol. During the first two years every three months a clinical checkup of the patients was done in the hospital. After this period of time, only one routine examination per year was necessary. This check-up included anamnesis, clinical evaluation, abdominal ultrasound, chest X-ray and if necessary additional diagnostics. All patients were followed up for more than nine years. The median follow-up time was 11.4 years.

Statistics

Beginning in 1996 the data were collected prospectively in accordance with a standardized protocol. Descriptive analysis included the frequency of nominal parameters, the median with the lower (LQ) and upper quartile (UQ) for numeric variables (ordinal or asymmetric distribution) and the mean for numeric variables with normal distribution. Univariate analysis was calculated for tables with the Chi - squared statistics with Yates correction or Fisher's Exact Test if necessary. Mann-Whitney-U-test was used to compare continuous variables.

A multivariate logistic regression analysis was performed to analyze the relevant factors for LNM and depth of tumor infiltration. Significant differences between groups were defined with a $p<0.05$.

The prognosis was analyzed without the postoperative mortality (90-day mortality). The univariate survival analysis was conducted according

Table 1. Association between clinicopathological parameters and survival of the 67 patients with early esophagus cancer.

Factor	Frequency N	Frequency %	5 /10 year SR	Survival Significance
Gender				P=0.072
male	53	79%	69% / 59%	
female	14	21%	61% /27%	
Histology				P=0.250
SCC	28	42%	56% / 39%	
AC	39	58%	68% / 61%	
Grading				P=0.002
G1/G2	51	76%	73% / 65%	
G3	16	24%	42% / 15%	
pT-category				P=0.104
pT1a	22	33%	81% / 62%	
pT1b	45	67%	57% / 45%	
Depth of infiltration				P=0.016
Mucosa	22	33%	81% / 62%	
Sm1	13	19%	67% / 58%	
Sm2	10	15%	70% / 58%	
Sm3	22	33%	40% / 30%	
pN-category				P=0.084
pN0	47	70%	75% / 61%	
pN1	11	16%	45% / 27%	
pN2	8	12%	38% / 38%	
pN3	1	2%	-	
pN-category new				P=0.006
pN0	41	61%	79% / 66%	
pN0micro+	6	9%	50% / 33%	
pN+ELNI-	14	21%	54% / 39%	
pN+ELNI+	6	9%	17% / 17%	

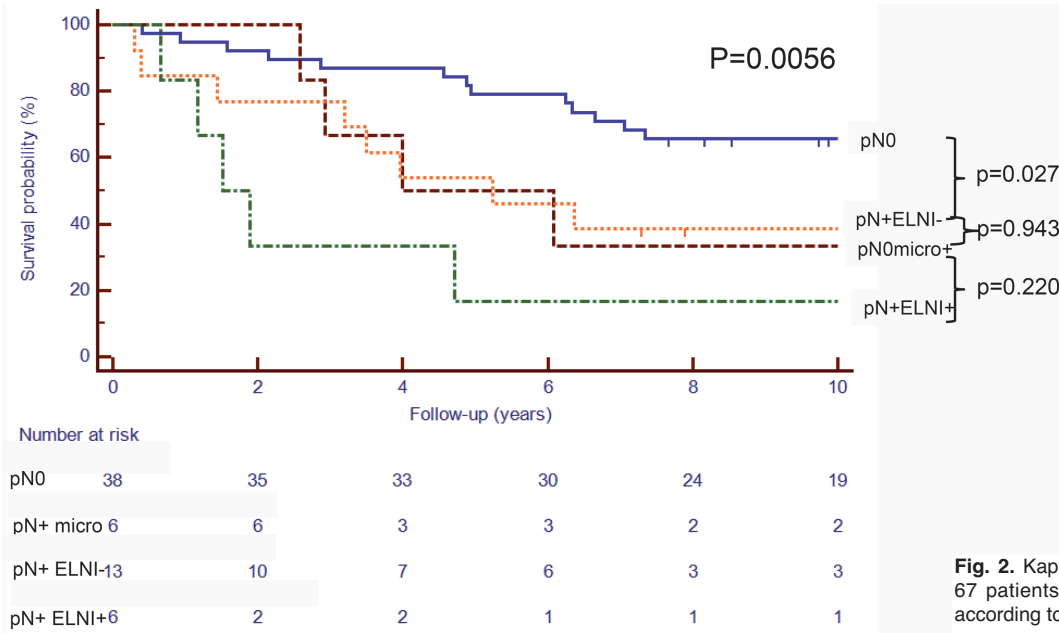

Fig. 2. Kaplan-Meier survival curves of the 67 patients with early esophageal cancer according to the pN-status.

Kaplan-Meier and survival curves were compared with log rank test. The multivariate analysis was performed with the Cox-regression method, using the backward option. Statistical significance was set at $p<0.05$. For statistical analysis the SPSS for Windows (version 20.0) application (SPSS Inc., Chicago IL, USA) was used. The survival curves were generated by the statistic software MedCalc (version 14.12.0) (MedCalc Software bvba, Ostend, Belgium).

Results

Depth of infiltration and lymph node metastasis (LNM)

Table 1 presents the clinicopathological parameters of the patients analyzed. There were 22 mucosal carcinomas (6 SCC, 16 AC) and 45 submucosal carcinomas (22 SCC; 23 AC). None of the pT1a carcinoma, but 26 (58%) of the pT1b carcinoma showed lymph node metastases or micrometastasis. The frequency of lymph node metastasis increased with the depth of tumor infiltration into the esophageal submucosa: carcinomas in the upper third of the submucosa (sm1) had 23% LNM, in the middle third (sm2) 40% LNM and in the deepest third (sm3) 82% LNM. Micrometastases were found in 20% of the sm2 as well as in the sm3 tumors. The rate of pN+ELNI+ was 15% for sm1 tumors and 18% for sm3 tumors.

Molecular markers and lymph node metastasis

MMP-2, TIMP-2, PIM-1 and survivin were not significantly different between adenocarcinomas and squamous cell carcinomas (data not shown). Table 2 visualizes the correlation between the expression of the four putative molecular markers and the occurrence of lymph node metastasis. Low grade PIM-1 expression (<30%) showed in 62% (10/16) of the cases LNM compared to 31% (16/51) in tumors with higher expression (p=0.034). The other markers showed no correlation to the existence of LNM. The detailed results of univariate and multivariate analysis are shown in Table 2.

Grading of the primary tumor significantly influenced the status of LNM. Only 25% of the 16 G3-tumors had no LNM in contrast to G2-tumors with 73% (p=0.002). There was no correlation between Grading and level of PIM-1.

We performed a logistic regression analysis with backward elimination of non-significant factors to predict lymph node metastases including the variable depth of tumor infiltration (pT1a-pT1b), type of histology (AC versus SCC), grading (G1/2-G3) and PIM-1 (<30% - >30). Grading G3 and low PIM-1 expression (<30% expression) were independent predictive factors for the presence of lymph node metastasis. For PIM-1, the odds-ratio was 7.6 (95%

Table 2. Correlation between lymph node status, histopathological grading and molecular markers (MMP2, PIM-1, TIMP-2 and survivin) from 67 patients with early esophageal cancer.

Factor	pN0 N - %	pN+ including pN0micro+ N - %	P Chi^{2-} (Trend)	Odds-Ratio for pN+	95%-Confidence intervall	Logistic Regression Significance
Total	N=41	N=26	--			P=0.007
MMP-2			P=0.429			
<30%	4 11%	3 12%		1		
30%-70%	35 85%	19 72%		0.81	0.07-9.42	P=0.307
71%-100%	2 4%	4 6%		0.16	0.01-5.57	
PIM-1			P=0.034			
<30%	6 15%	10 38%		1		
30%-100%	35 85%	16 62%		0.06	0.01-0.45	P=0.004
TIMP-2			P=0.354			
<5 %	6 4%	1 4%		1		
5-30%	13 33%	6 27%		12.3	0.59-25.3	P=0.105
30%-100%	28 63%	19 65%		14.4	0.59-21.8	p=0.105
Survivin			P=0.857			
<30%	7 17%	7 27%		1		
30%-70%	29 71%	14 54%		3.34	0.45-24.2	P=0.234
>70%	5 12%	5 19%		12.25	0.80-187	P=0.072
Survivin Ncl			P=0.079			
<5%	9 22%	10 38%		1		
5%-30%	24 59%	14 54%		0.31	0.06-1.44	P=0.078
>30%	8 19%	2 8%		0.15	0.01-1.54	P=0.502
Grading			P=0.002			
G1-G2	37 90%	14 54%		1		
G3	4 10%	12 46%		17.41	2.68-113	P=0.004

CI=1.3-45.2) and the p-value was 0.025. For Grading G3, the odds-ratio was 9.3 (95% CI=2.4-36.4) and the p-value was 0.001. The area under the receiver operator curve (ROC) was 0.75 (95% CI=0.63-0.85), with a sensitivity of 70% and specificity of 76%.

Molecular markers and depth of tumor infiltration

We analyzed the scores of protein expression of the four molecular markers MMP-2, TIMP-2, PIM-1 and survivin according to the depth of tumor infiltration into

Table 3. Correlation between depth of tumor infiltration (pT1a vs. pT1b), histopathological grading and molecular markers (MMP2, PIM-1, TIMP-2 and survivin) from 67 patients with early esophageal cancer.

Factor	Mucosal tumor pT1a n	Submucosal tumor pT1b n	P Chi^{2}- (Trend)	Multivariate Odds-Ratio	95% CI- Interval	Logistic Regression Significance
Total	22	45	-			P=0.016
MMP-2			0.323			
<30%	2 9%	5 11%		1	-	
30%-70%	20 91%	34 76%		0.24	0.02-3.68	P=0.307
71%-100%	0	6 13%		3.6x10^{6}	-	P=0.994
PIM-1			0.645			
<30%	4 18%	12 27%		1	-	-
30%-100%	18 72%	33 73%		0.25	0.04-1.78	P=0.168
TIMP-2			0.046			
<30%	5 23%	2 4%		1	-	
30%-70%	4 18%	9 20%		11.98	0.85-167	P=0.065
71%-100%	13 59%	34 76%		19.47	1.44-262	P=0.025
Survivin			0.240			
<30%	6 27%	8 18%		1	-	-
30%-70%	14 64%	29 64%		4.76	0.68- 33	P=0.115
>70%	2 9%	8 18%		8.61	0.53-139	P=0.130
Survivin Ncl			0.232			
<5%	6 27%	13 29%		1	-	
5%-30%	10 46%	28 62%		1.00	0.21- 4.73	P=0.991
>30%	6 27%	4 9%		0.14	0.02- 1.09	P=0.061
Grading			0.201			
G1-G2	20 91%	31 69%		1	-	
G3	2 9%	14 31%		4.01	0.52- 31	P=0.180

Table 4. Cox-regression analysis of independent prognostic factors for 67 patients with early esophageal cancer.

Factor	Univariate Hazard-Ratio (95% CI)		Sign	Cox-regression Hazard-ratio	95%-Confidence interval	Significance
Total n=67						P=0.003
Age (each year)				1.06	1.01-1.11	P=0.016
Histology			P=0.249			
SCC	1			1		
AC	0.67	0.33-1.37		0.82	0.28-2.38	P=0.784
pT-category			P=0.104			
pTa	1			1		
pTb	1.83	0.92-3.68		1.33	0.46-3.76	P=0.598
pN-category new			P=0.001			
pN0	1			1		
pN+ELNI-	2.16	0.84-5.50		1.31	0.55-3.09	p=0.545
pN+ELNI+	4.78	0.93-24.58		3.92	1.45-12.24	P=0.008
Grading			P=0.008			
G1/G2	1			1		
G3	3,11	1.21-7.99		2.64	1.02-6.90	P=0.045

the mucosa and submucosa: High scores of MMP-2, TIMP-2 and survivin could only be found in submucosal tumors. Furthermore, we demonstrated that lesions infiltrating the deepest third of the submucosa (sm3) had more often higher values of summated scores of these three markers than other more superficial esophageal tumors.

The logistic regression analysis identifying risk factors for submucosal carcinoma compared to mucosal infiltration (including grading, MMP-2, TIMP-2 and survivin) revealed TIMP-2 as an independent risk factor for submucosal infiltration (p=0.025). Table 3 shows the detailed results of univariate and multivariate analysis. 91% of the patients with pT1b tumor were correctly classified. The area under ROC was 0.809 (95% CI=0.69-0.89).

Prognosis

The univariate analysis of the most important prognostic factors is illustrated in table 1. Patients with micrometastasis (pN0micro+) had a median survival of 5.05 years (95% CI=2.9-6.1) which is comparable to those patients with lymph node metastasis (pN+ELNI-) who had a median survival rate of 5.2 years (95% CI=3.2-6.2). The greatest difference was discovered between patients without LNM (pN0) (median survival rate of 16.6 years (95% CI=10.0)) and patients with extracapsular lymph node metastasis infiltration (pN+ELNI+) (median survival rate of 1.7 years (95% CI=1.2-4.7)) who died significantly earlier (p=0.0056) (see also Fig. 2). A new pN-category (pN-new) for patients with early esophageal cancer was defined: pN0, pN+ELNI- including pN0micro+, and pN+ELNI+.

None of the molecular markers - MMP-2, TIMP-2, PIM-1 and survivin - were of prognostic relevance (data not shown). Further, Table 4 summarizes the results of the univariate and multivariate analysis concerning the independent prognostic factors for the patients with early esophageal cancer analyzed.

Discussion

Lymph node status and depth of tumor infiltration in patients with early esophageal cancer are requisite parameters for planning an individual therapeutic concept and for estimating the patient's long-term outcome. Unfortunately, we are not yet able to predict putative affected lymph nodes with high accuracy. Today diagnostic procedures like endoscopic ultrasound or computer tomography only hint at the presence of local nodal metastasis. But parameters like lymph node size are no irrevocable proof (Mönig et al., 2002; May et al., 2004) since lymphatic metastasis in normal-size lymph nodes and the phenomenon of lymph node micrometastasis are not detectable by these techniques. Recently, Bergeron reported that tumor depth was only correctly staged in 39% of pT1a tumors and in 51% of pT1b-tumors by using endoscopy ultrasound (Bergeron et al., 2014). Furthermore, there were 15% positive lymph nodes in the endoscopic ultrasound-staged cN0 pT1a-tumors while 17% of the cN0 staged lymph nodes in pT1b-tumors showed lymphatic metastasis within the pathologic specimens. As a consequence of limited sensitivity and specificity, new diagnostic methods are required in order to optimize the diagnosis of early esophageal cancer. Improved knowledge of the local tumor progression forms the basis of a more personalized treatment with better differentiation of which patients benefit the most from either a local endoscopic resection or an esophagectomy with lymphadenectomy.

Our study focused on the aspects of tumor infiltration and lymph node metastasis in pT1-esophageal cancer. We have characterized novel promising marker candidates and compared the histopathological results with the clinicopathological information of the patients.

Several studies have already shown the positive correlation between depth of tumor infiltration and lymph node metastasis (LNM) (Liu et al., 2005; Ancona et al., 2008; Bollschweiler and Hölscher, 2011; Hölscher et al., 2011). However, only few data exist concerning the impact of so-called lymphatic micrometastasis on the prognosis of patients with mucosal or submucosal esophageal tumors. The term "lymphatic micrometastasis" describes the occurrence of small clusters of tumor cells within a lymph node which are difficult to identify during the conventional histopathological examination in HE staining. In this current study, we found micrometastasis in sm2 and sm3 tumors of both entities, esophageal adenocarcinomas and squamous cell carcinomas, associated with worse survival. But there were no micrometastasis in mucosal tumors. In contrast to our results, Grotenhuis also detected isolated tumor cells in about 8% of the examined mucosal (m3) adenocarcinomas (Grotenhuis et al., 2010). Explicit analyses of a putative prognostic impact of lymphatic micrometastasis in mucosal or submucosal esophageal squamous cell carcinoma do not exist so far. However, results described by Natsugoe, Matsumoto and Koenig indicate a worse long-term survival in the presence of nodal micrometastasis, though their studies did not focus on pT1 tumors, but also included pT2 and pT3 squamous cell carcinomas and had shorter follow-up times (Natsugoe et al., 1998; Matsumoto et al., 2000; Koenig et al., 2009). We could show that the prognosis of patients with micrometastasis was comparable to those with lymph node metastasis but without extracapsular lymph node infiltration.

Extracapsular lymph node infiltration (ELNI) within the perinodal (fatty) tissue is another aspect of lymph node metastasis which was further examined here. In recent years it has become more and more clear that this phenomenon reflects a more aggressive tumor growth and therefore seems to have negative prognostic potential. In Caucasian and Asian cohorts such correlations have been described so far: Considering

esophageal squamous cell carcinoma, Baba, Tanabe and Sakai were able to present an association between ELNI and a significant higher recurrent risk as well as a shorter overall survival of patients with ELNI (Baba et al., 1997; Tanabe et al., 2007; Sakai et al., 2012). Zhang and coworkers recently described similar circumstances when comparing clinicopathological data from 5,467 lymph nodes of 284 patients with adenocarcinoma of the esophagogastric junction after curative resection and their status of extracapsular lymph node infiltration (Zhang et al., 2013). In 2007, Wind published a systematic review including seven studies with squamous cell carcinomas and adenocarcinomas of the esophagus where they identified a significantly worse long-term survival among patients with ELNI (Wind et al., 2007). Further, Lagarde and coworkers analyzed 1562 positive lymph nodes in 251 patients with lymph node dissemination of adenocarcinomas of the distal esophagus or the gastroesophageal junction. They found ELNI in 38% of the patients examined significantly associated with increased depth of tumor infiltration, presence of positive resectable truncal nodes as well as number of tumor positive lymph nodes (Lagarde et al., 2006). In an earlier study our group demonstrated a significant difference between the occurrences of ELNI in patients with pN1 esophageal adenocarcinomas compared to those with squamous cell carcinomas (Metzger et al., 2009). All these studies have in common that they are based on tumors staged from pT1 until pT4. Therefore, this was the first published study which performed these examinations explicitly focused on extracapsular lymph node infiltration in early esophageal carcinomas of both histopathological subtypes.

We further screened for putative molecular markers which could be able to detect the existence of lymph node metastasis or depth of tumor infiltration before any therapeutic interventions. Matrix-Metalloproteinase-2 (MMP-2), tissue inhibitor of metalloproteinase-2 (TIMP-2), proto-oncogene PIM-1 and survivin were chosen as promising candidates for the examinations. MMP-2 and its inhibitor TIMP-2 are considered to be involved within the progression as well as in the development of local nodal metastasis in gastric cancer (Mönig et al., 2001; Alakus et al., 2008), since a degradation of the extracellular matrix simplifies the expansion of the tumor. In esophageal cancer, Groblewska reported an association between serum expression levels of MMP-2 and TIMP-2 and clinicopathological tumor characteristics (Groblewska et al., 2012). PIM-1 overexpression in gastric glands correlates with lymph node metastases in gastric cancer (Warnecke-Eberz et al., 2009). In addition, analyses from tissue samples of esophageal squamous cell cancer (ESCC) patients and ESCC cell lines supported these findings (Li et al., 2010; Liu et al., 2010). High expression of PIM-1 detected by immunohistochemistry and in-situ-hybridization were found in samples of patients with poor prognosis and lymph node metastasis (Liu et al., 2010) while treatment of ESCC cells with PIM-1 siRNA resulted in inhibited cell proliferation and induced apoptosis (Li et al., 2010). Survivin is an inhibitor of apoptosis and there is evidence concerning its impact on the patients' prognosis (Hoffmann et al., 2010).

Regarding the results from our study, PIM-1 was the only marker with significant influence on the existence of lymph node metastasis ($p=0.034$). Immunohistochemical low-grade expression of PIM-1 (<30%) correlated with LNM. On the other hand, high grade expression (>70%) of MMP-2, TIMP-2 and survivin were only found in submucosal tumors and therefore mark the tumor's progression. These findings are partially in contrast to the results shown by Groblewska (Groblewska et al., 2012). Within their work they postulated that serum levels of MMP-2 and TIMP-2 do not correlate with the stage of disease and that lowest levels were detected in stage III cancer patients. Nevertheless, their study was not focused on early esophagus cancer, but included all stages of the malignancy. That is why the number of pT1 patients was considerably lower compared to our study. Additionally, the analyzing methods differed: We detected tissue level of the markers using immunohistochemistry while Groblewska and coworkers examined serum protein levels. Performing logistic regression analysis also revealed TIMP-2 to be an independent risk factor for submucosal tumor growth ($p=0.025$).

In summary, we have identified PIM-1 together with Grade 3 in Grading as promising molecular markers for the prediction of lymph node metastasis. Using these two histopathologic markers it was possible to predict more than 70% of the patients with LNM correctly. This is of great importance because we had included patients with micrometastasis, which are not detectable by routine histopathology. In addition, another molecular marker TIMP2- was relevant for the prediction of depth of tumor infiltration in early esophagus cancer.

Nevertheless, the current study has its limitations being planned as retrospective analysis based only on histopathological analyses such as immunohistochemistry. Therefore, it will be necessary to perform larger prospective studies using additional analyzing methods (such as non-invasive serum protein detection) to evaluate and verify the current results in the future. Someday, these findings could result in novel diagnostic approaches for risk estimation of patients with mucosal or submucosal esophageal cancer improving the personalized process of treatment. In addition, it would be extremely useful to include micrometastasis and ELNI within the pN-category since we have demonstrated that these factors have negative impact on the patients' prognosis indicating a more aggressive tumor biology.

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