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Serum midkine depends on lymph node involvement and correlates with circulating VEGF-C in oesophageal squamous cell carcinoma

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Abstract

Lymph node metastasis (LNM) is a key factor for selection of treatment method and patients' prognosis in oesophageal squamous cell carcinoma (ESCC). However, no biomarkers able to support the clinical detection of LNM have been reported. Recently, vascular endothelial growth factor C (VEGF-C) was found to be a more accurate marker of LNM in lung cancer than computed tomography. Midkine is a multifunctional cytokine involved in cancer development. We investigated circulating midkine levels in ESCC patients ($n=73$) compared with those in healthy subjects ($n=42$) with double-antibody-sandwich indirect enzyme-linked immunosorbent assay (DASI-ELISA). We found that midkine was elevated in ESCC and involved in metastatic disease. Serum midkine (sMK) was a good marker of LNM, evaluated both clinically and pathologically, as revealed by ROC analysis. It also correlated with serum levels of VEGF-C. The increase of sMK was related to cancer cells, although a weak correlation was observed between sMK and platelet and leucocyte counts.

Keywords: *Midkine, vascular endothelial growth factor C (VEGF-C), squamous cell carcinoma, oesophagus, cancer, tumour marker*

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Introduction

Squamous cell carcinoma is one of the deadliest neoplasms. It is too advanced at presentation to be curatively resected, grows faster and spreads to lymph nodes more often and much earlier than other gastrointestinal cancers. None of the standard tumour markers proved to be of use in the early detection of oesophageal squamous cell carcinoma (ESCC), prediction of patients' outcome or treatment response (Enzinger & Mayer 2003). A striking feature of ESCC is the geographic variation in prevalence, with the highest rates occurring in Japan, China and Iran which are called the 'oesophageal cancer belt countries' (Kuwano et al. 2005). Epidemiology is the main reason for endoscopic ESCC screening in some of these countries. However, the

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effectiveness of endoscopic or surgical cancer treatment depends both on early cancer detection and on the involvement of lymph nodes. In about one-third of cancer cases staged as N0, microdeposits of cancer cells are already present in regional lymph nodes (Natsugoe et al. 1998), leading to cancer metastasis and recurrence. The influence of lymph node involvement on surveillance of patients warrants lymph node dissection even in patients with superficial oesophageal cancer (Bollschweiler et al. 2006, Shimada et al. 2006). In this respect, not only is a more optimal screening tool for early cancer detection needed, but also a diagnostic test to help in the evaluation of lymph node involvement.

Midkine, a multifunctional cytokine, has been reported to be overexpressed in many different malignancies. It has been found to possess mitogenic, antiapoptotic and transforming properties towards cancer cells as well as exhibiting proangiogenic potential. Its chemotactic activity towards neutrofils and macrophages contributes to chronic inflammation making midkine responsible for infiltration of transformed tissue with activated inflammatory cells (Muramatsu 2002, Kadomatsu & Muramatsu 2004). Shimada et al. (2003a) explored the potential usefulness of serum midkine (sMK) determination as a disease marker of superficial squamous cell carcinomas in the Japanese population. Comparison of midkine with conventional tumour markers such as carcinoembryonic antigen (CEA), squamous cell carcinoma antigen (SCC-Ag) and CYFRA 21-1 has revealed a much higher positivity rate of cancer detection for midkine, than for SCC-Ag, CEA or CYFRA 21-1. The authors concluded that midkine secretion is associated with early phases of oesophageal squamous carcinogenesis and can serve as an early marker of cancer presence. Additionally, in multivariate analysis, preoperative sMK concentration has been indicated as an independent prognostic marker (Shimada et al. 2003b). However, no correlation between sMK levels and the clinicopathological features of tumours has been observed (Shimada et al. 2003a,b). Taking into account large differences between the aetiopathological factors of ESCC in various regions of the world and the genetic predisposition of the Japanese (Parkin et al. 2005), we were interested in whether overexpression of midkine is a common element of ESCC or rather is a unique feature bound to specific aetiological factors in the Japanese population. Moreover, our preliminary studies suggested that midkine might not only be elevated in ESCC, but its secretion, unlike in studies on the Japanese population, may be related to metastasis and depend on lymph node involvement.

Material and methods

Patients

Levels of sMK were measured in 73 patients with histopathologically confirmed ESCC and 42 apparently healthy blood donors, whose sera were kindly provided by the Regional Center of Blood Donation and Therapeutics in Wroclaw, Poland. Patients enrolled in the studies were treated between 2003 and 2005 in the Gastrointestinal and General Surgery Department of Wroclaw Medical University. There were 60 male and 13 female patients in the cancer group, mean age 60 years (median 59, range 35–86) and 32 men and 10 women, median age 34 years (range 25–56), in the control group. No relation between circulating midkine and age has been reported (Ikematsu et al. 2003).

The Local Medical Ethics Committee approved the project presented in this paper.

Cancers were staged according to the guidelines of the International Union Against Cancer (IUCC) (Sobin & Wittekind 2002) and the American Joint Committee on Cancer (AJCC) (Green et al. 2002) TNM system on the basis of upper digestive tract (udt) endoscopy with biopsy and pathological examination, contrast radiographic studies of the udt with barium or gastrografin, posteroanterior and lateral chest radiography, ultrasound examination of the abdominal cavity and cervical nodes, computed tomography (CT) of thorax and abdominal cavity, and diagnostic laparotomy and thoracotomy.

There were no patients with stage I disease, 16 with stage II, 25 with stage III and 32 with stage IV disease; 34 patients had N1 cancers, 27 N0 and 12 unclassified (Nx); there were 15 cases with T2 stage tumours, 20 with T3, 38 with T4; and 41 cases of M0 and 32 of M1 cancers (26 of one-site metastasis and six of multisite metastasis). We analysed 15 cases of small tumours (<5 cm) and 55 cases of large tumours (≥ 5 cm). After clinical assessment only 22 subjects underwent oesophageal resection with lymph node dissection; some patients refused or postponed treatment; most of them were, however, qualified for palliative treatment.

Determination of midkine and VEGF-C

Blood samples were collected into Sarstedt S-Monovette tubes. Midkine and vascular endothelial growth factor C (VEGF-C) concentrations were determined in serum obtained from blood by clotting for 15 min at room temperature and subsequent centrifugation for 15 min at 3000 rpm. Blood was collected from patients by venous puncture. Patients were not subjected to any treatment including blood transfusions, chemotherapy or radiotherapy prior to study. All samples were run in duplicates.

We assessed sMK levels with double-antibody-sandwich indirect enzyme-linked immunosorbent assay (DASI-ELISA), previously described (Krzystek-Korpacka et al. 2006). Briefly, microtitrate plates were coated with rabbit antihuman midkine polyclonal antibodies (Gentaur, Brussels, Belgium) in a concentration of 4 $\mu g\ ml^{-1}$ in carbonate buffer and subsequently blocked with SuperBlock Protein (Pierce, Rockford, IL, USA). The presence of bound midkine was detected with biotinylated goat antihuman midkine polyclonal antibodies (RnD Systems, Minneapolis, MN, USA) in A concentration of 0.5 $\mu g\ ml^{-1}$ and, subsequently, by streptavidin conjugated with horseradish peroxidase in a concentration of 0.2 $\mu g\ ml^{-1}$ (Jackson Immunoresearch, West Grove, PA, USA). 1-Step Ultra TMB-ELISA (Pierce, Rockford, IL, USA) was used as a peroxidase substrate. Recombinant human midkine (PeproTech, Rocky Hill, NJ, USA) dissolved in diluted human serum depleted of midkine by heparin affinity chromatography served as a standard.

VEGF-C was assayed according to the manufacturer's instructions by commercially available immunoenzymatic tests provided by IBL (Hamburg, Germany).

Statistical analysis

Due to the skewed distribution of midkine and VEGF-C, as analysed with the Shapiro–Wilk normality test, median instead of mean values were calculated for all studied groups and observed differences were examined with the non-parametric Mann–Whitney *U* test, while correlation analysis was conducted with the Spearman rank test; p values below 0.05 were considered significant. For determination of the

best cut-off values of midkine assessment, receiver operating characteristic curves (ROC) were plotted and test specificity, sensitivity, positive and negative predictive values (PPV and NPV), as well as positive and negative likelihood ratios (LR+ and LR-, respectively) were calculated. A ROC analysis with ROCKIT 0.9B software was applied for evaluation of test accuracy by estimating area under the ROC curve (AUC) with 95% confidence interval (CI).

Results

We found statistically higher sMK levels in the cancer patients than in controls (1373 vs. 130 pg ml^{-1}) (Figure 1). The relationship between sMK and the clinical and pathological features of the cancer is presented in Table I. We did not observe a significant relationship between sMK and the patients' sex or age. sMK tended to increase with disease stage and extent of the primary tumour – significant differences were observed between stages II and IV and between T2 and T4 tumours. We also found that patients with stage IIB cancers had significantly higher sMK levels than those with IIA cancers (2073 vs. 775 pg ml^{-1}, $p=0.047$). The sMK level was not significantly elevated in T4 cancers with bronchopulmonary fistulas compared with T4 cancers without fistulas (2080 vs. 1412 pg ml^{-1}, $p=0.418$). sMK levels differed between localised cancers and cancers spreading either to lymph nodes or distant organs and was found to be 775 pg ml^{-1} in N0M0 cancers but 1635 pg ml^{-1} in disseminated cancers ($p<0.001$). We introduced an additional category to N and M and subdivided patients into L0 and L1 groups with respect to the absence/presence of metastasis in lymph nodes, regardless their location (regional or distant). sMK was significantly higher when secondary tumours were present despite the organ type or location. We also observed that tumours with multisite distant metastases tended to be associated with higher sMK levels than single-site tumours: 1430 vs. 1748 pg ml^{-1}, but the increase was not significant ($p=0.770$). There was an insufficient number of cases to allow us to analyse sMK in relation to the type of metastatic organ.

Because the presence of lymph node metastasis (LNM) has been reported to be related to tumour T stage and we observed sMK elevation with increasing T stage and

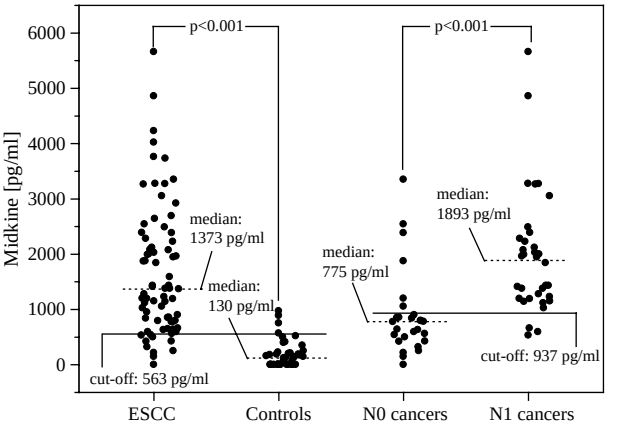

Figure 1. Individual serum midkine levels (sMK) in patients with oesophageal squamous cell carcinoma (ESCC) compared with a control group of healthy blood donors, and comparison of sMK in N0 and N1 cancers.

Table I. Serum midkine level (sMK) with in relation to clinical and pathological characteristics of oesophageal squamous cell carcinomas. Statistical analysis of observed differences between groups with Mann–Whitney *U* test.

	Median sMK (pg ml^{-1})	
Age		
<60 years	2008	$p=0.394$
>60 years	2050	
Sex		
Female	1590	$p=0.713$
Male	1215	
Stage		
II	786	II vs. III: $p=0.199$
III	1329	II vs. IV: $p=0.014$
IV	1430	III vs. IV: $p=0.130$
Primary tumour extent (T)		
T2	780	T2 vs. T3: $p=0.193$
T3	1329	T2 vs. T4: $p=0.028$
T4	1510	T3 vs.T4: $p=0.245$
Regional lymph node metastasis		
N0	775	$p<0.001$
N1	1893	
Regional and/or distant lymph node metastasis (L)		
L0	786	$p<0.001$
L1	1430	
Distant metastasis (M)		
M0	1149	$p=0.019$
M1	1430	
Primary tumour size		
<5 cm	851	$p=0.058$
>5 cm	1378	

the presence of LNM, we additionally investigated sMK in relation to the combined tumour TN status (Table II).

We evaluated the usefulness of sMK determination as a marker of ESCC presence and its correlation with the clinical evaluation of LNM. The best cut-off values calculated from the ROC analysis were 563 pg ml^{-1} (mean sMK in the control group $\pm$1.5 SD) for determination of the presence of disease and 937 pg ml^{-1} (mean $\pm$3

Table II. Relationship of serum midkine (sMK) to TN status of oesophageal squamous cell carcinomas.

Median sMK (pg ml^{-1})	p Value of TN groups comparison
T2N0: 542	T2N0 vs. T2N1: $p=0.025$
T3N0: 630	T3N0 vs. T3N1: $p=0.077$
T4N0: 839	T4N0 vs. T4N1: $p=0.003$
T2N1: 2052	T2N0 vs. T3N0: $p=0.223$
T3N1: 1378	T2N0 vs. T4N0: $p=0.119$
T4N1: 1999	T3N0 vs. T4N0: $p=0.892$
	T2N1 vs. T3N1: $p=0.314$
	T2N1 vs. T4N1: $p=0.762$
	T3N1 vs. T4N1: $p=0.204$

Table III. Attributes of serum midkine (sMK) determination as a marker for oesophageal squamous cell carcinoma (ESCC) and lymph node metastasis (LNM).

	sMK as ESCC marker	sMK as LNM marker
Sensitivity	85%	91.2%
Specificity	90.5%	77.8%
Positive predictive value	94%	84%
Negative predictive value	77.5%	87.5%
Positive likelihood ratio	8.9	4.1
Negative likelihood ratio	0.16	0.11

SD) for evaluation of lymph node involvement. Test sensitivity, specificity, PPV and NPV, LR+ and LR- are presented in Table III. Midkine determination as an ESCC marker was characterised by 95% accuracy, as calculated from the AUC (0.9544, 95% CI 0.9083–0.9797). The sensitivities of midkine at particular stages of disease were 62.5% in stage II, 80% in stage III and 81% in stage IV. Accuracy of midkine determination with clinical evaluation of LNM was 83% (AUC 0.8311, 95% CI 0.7040–0.9164). The comparison of midkine and VEGF-C as LNM markers is presented in Figure 2. We additionally conducted a ROC analysis on the results obtained for patients with resected tumours, where presence or absence of metastatic lymph nodes was confirmed by pathological examination. In spite of the relatively small number of cases, we obtained 87% accuracy of midkine determination as a marker of lymph node involvement.

Pathological examination of resected tumours was available in 22 cases. The median sMK level in T2 vs. T3/T4 tumours was 971 vs. 1378 pg ml^{-1}, respectively ($p = 0.622$), and in N0 vs. N1 tumours 497 vs. 1635 pg ml^{-1}, respectively ($p = 0.007$). The median sMK level in patients with IIA tumours was 780 pg ml^{-1} but 2073 pg ml^{-1} in patients with IIB tumours ($p = 0.088$).

Analysis correlation between sMK and potential sources of its elevation in serum revealed that, in ESCC, sMK is related to leucocyte (WBC) and platelet (PLT) counts (Table IV). However, correlation analysis with the Spearman rank test showed a rather

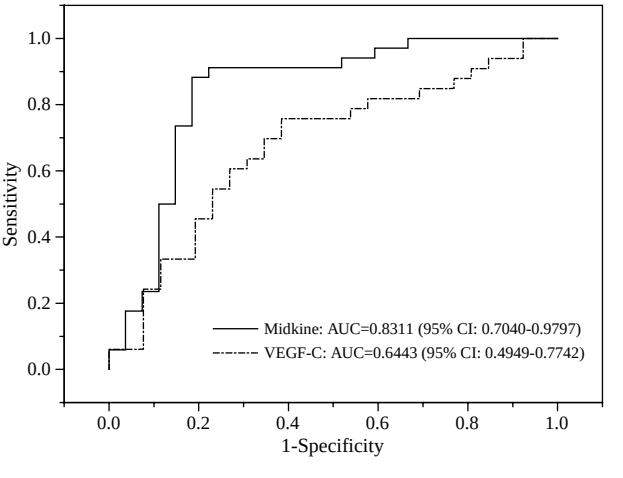

Figure 2. Comparison by ROC analysis of serum midkine and vascular endothelial growth factor-C (VEGF-C) levels as markers of lymph node metastasis.

Table IV. Serum midkine levels (sMK) in oesophageal squamous cell carcinoma in relation to counts of platelet (PLT) and leucocytes (WBC) in patients.

Low PLT (l-PLT): ($<150\times10^9\,l^{-1}$) Median sMK: 930 pg ml^{-1} l-PLT vs. n-PLT: $p=0.18$	Normal PLT (n-PLT) ($150-340\times10^9\,l^{-1}$ Median sMK: 1375 pg ml^{-1} l-PLT vs. h-PLT: $p=0.02$	High PLT (h-PLT) ($\geq340\times10^9\,l^{-1}$) Median sMK: 2250 pg ml^{-1} n-PLT vs. h-PLT: $p=0.01$
Low WBC (l-WBC) ($<4800\times10^6\,l^{-1}$) Median sMK: 775 pg ml^{-1} l-WBC vs. n-WBC: $p=0.07$	Normal WBC (n-WBC) ($4800-10800\times10^6\,l^{-1}$) Median sMK: 1378 pg ml^{-1} l-WBC vs. h-WBC: $p=0.01$	High WBC (h-WBC) ($\geq10800\times10^6\,l^{-1}$) Median sMK: 2117 pg ml^{-1} n-WBC vs. h-WBC: $p=0.02$

weak overall relationship: $r=0.23$, $p=0.048$ and $r=0.25$, $p=0.001$, respectively. sMK levels tended to correlate with the number of neutrophils rather than lymphocytes ($r=0.20$, $p=0.065$ and $r=0.01$, $p=0.968$, respectively).

Due to the strong relationship of sMK levels to lymph node involvement, we investigated whether there is a correlation between sMK and sVEGF-C levels in ESCC patients. We found a significant overall correlation with $r=0.36$ ($p=0.002$), which depended on cancer TNM stage (Table V).

Discussion

We found significantly elevated midkine levels in cancer patients, which may indicate that midkine overexpression is a feature shared by SCC in spite of very dissimilar aetiopathogenesis (see Figure 1). In our studies conducted on a Polish population, a correlation with clinicopathological features was observed. We found that the circulating midkine level tended to increase with cancer stage. Similarly, Ren et al. (2006) reported a relationship between midkine cancer tissue expression and disease

Table V. Correlation of serum midkine with serum vascular endothelial growth factor-C (VEGF-C) level in relation to TNM stage of oesophageal squamous cell carcinomas.

TNM stage	Correlation coefficient
Disease stage	
II	$r=0.36$, $p=0.172$
IIA	$r=0.10$, $p=0.770$
IIB	$r=0.90$, $p=0.047$
III	$r=0.13$, $p=0.535$
IV	$r=0.49$, $p=0.005$
Primary tumour extent	
T2	$r=0.35$, $p=0.196$
T3	$r=0.04$, $p=0.865$
T4	$r=0.43$, $p=0.010$
Lymph node involvement	
N0	$r=0.00$, $p=0.990$
N1	$r=0.45$, $p=0.008$
L0	$r=0.01$, $p=0.964$
L1	$r=0.49$, $p=0.001$
Distant metastasis	
M0	$r=0.22$, $p=0.180$
M1	$r=0.49$, $p=0.005$

stage, while Shimada et al. (2003a,b) found increasing sMK levels with cancer stage. Yet, in contrast to data reported by Shimada et al. (2003a,b), in our studies midkine level was also related to the cancer T category. However, this discrepancy seems to be caused by different clinical and pathological interpretation of cancer T features. In our studies, as well as those conducted by Shimada (2003b), the midkine level was related to tumour size and this parameter is included in the clinical T category, while pathological T category is limited to evaluation of cancer transmural penetration (Sobin & Wittekind 2002). Such explanation appears to be validated by the lack of significant correlation between midkine and the depth of tumour invasion in resected tumours. Either due to population differences or to the lack of non-invasive early cancers and a strong representation of advanced cancers in the examined group, we observed that sMK concentration was related to the ability of cancer cells to spread. Midkine involvement in metastasis to lymph nodes, regardless of their location in respect to the primary tumour, seems to be especially promising. Lymph node involvement is the most important prognostic factor in oesophageal cancers (Mukai et al. 2004), as early lymphatic invasion is characteristic for ESCC – in up to 60% of cancers invading muscle (T2), involvement of lymph nodes is already observed (Enzinger & Mayer 2003). Moreover, the process of lymph node metastasis is chaotic and difficult to predict, which complicates the detection of metastatic lymph nodes by standard imaging techniques (Mitsuhashi et al. 2005). In this respect, a non-invasive biomarker predicting lymph node involvement would be of a great value.

Recently, the dependence of LNM-related genes expression on tumour T stage has been reported (Sato et al. 2006). It has been observed that different genes are expressed in T1 cancers metastasizing to lymph nodes as compared with T2–T4 cancers. In our studies, the sMK level was significantly higher in cancer metastasizing to lymph nodes, both regional and distant. This correlation was sustained when the presence of cancer in lymph nodes was confirmed histopathologically. Moreover, the observed increase in sMK level in stage II cancers with metastatic lymph nodes (IIB), as compared with cancers free from metastasis (IIA), was significant in cancers staged clinically and close to significance in cancers staged pathologically. However, we also observed that sMK level was related to the extent of the primary tumour as well, and the T parameter was found to be an independent prognostic factor of LNM (Shimada et al. 2006). In respect to this interrelationship between T and N cancer features, we examined midkine level in cancers stratified according to their TN status and found that the presence of metastasis in lymph nodes and not tumour extent significantly contributed to the observed rise in sMK level in ESCC patients.

Tamura et al. (2004) found that the serum level of the main regulatory factor of lymphangiogenesis, VEGF-C, was a more accurate marker of LNM in lung cancer than chest CT. We evaluated the VEGF-C serum level as a marker for LNM in ESCC (Krzystek-Korpacka et al. 2007) and reported similar accuracy in LNM detection to that described for lung cancer by Tamura et al. (2004). Here, we evaluated attributes of midkine as a possible marker of LNM in ESCC and on the basis of ROC analysis we found a high correlation between the ability of midkine to predict LNM and the clinical evaluation of the presence of metastasis. As already mentioned, midkine involvement in LNM was also observed in cancers with histopathologically confirmed metastasis in lymph nodes, and midkine correlation with the results of pathological examination was also high. However, due to the limited number of resected cancers, this result needs to be verified in a larger group. Comparison of midkine and VEGF-C

as LNM markers by ROC analysis revealed that sMK determination is a more accurate indicator of metastasis presence than VEGF-C.

A strong relationship between midkine level and the presence of metastasis in lymph nodes, as well as its relationship to prolymphangiogenic VEGF-C level, especially in metastasizing cancers may suggest midkine involvement in tumour lymphangiogenesis.

Midkine has already been reported to be a prognostic factor in ESCC and an early marker of disease presence, characterised by a higher sensitivity than SCC-Ag, CYFRA 21-1 and CEA (Shimada et al. 2003a). Midkine elevation, however, has been reported to be involved only in early stages of ESCC, whereas our studies indicate midkine involvement in metastatic disease as well. Midkine, besides its involvement in vascular endothelial cell mitosis, differentiation and migration, is a factor stimulating the expression of plasminogen activator (uPA) while suppressing expression of its inhibitor PAI-1 (Muramatsu 2002, Kadomatsu & Muramatsu 2004). The uPA to PAI-1 ratio has been found to be one of the most important factors determining the invasiveness of oesophageal cancers (Nomiya et al. 2002). The elevation of plasma midkine, although not significant, has already been described by Soulie et al. (2004) in non-localised tumours of various types, while Konishi et al. (1999) found the midkine gene to be one of the 14 genes, whose overexpression was responsible for the aggressive behaviour of prostate cancer. The ability of midkine to enhance metastatic potential has also been reported in Lewis lung carcinoma (Salama et al. 2006). We found midkine to be a good marker of T2–T4 cancers with high positive likelihood ratios close to values that occur in the presence of the disease. The comparison of midkine sensitivity as a marker of ESCC existence for each disease stage revealed that there are more 'midkine-positive' cases in all analysed stages in the Polish population compared with the Japanese population (54%, 60% and 76%, respectively) (Shimada et al. 2003). This observation together with generally higher midkine levels in cancer patients in the studied population may suggest population-based differences in midkine expression and secretion during ESCC cancer development.

We analysed the relationship between circulating midkine and WBCs as well as PLTs in order to establish the main source of its elevation in ESCC patients. Not only cancer cells but also host cells, such as macrophages, neutrophils and lymphocytes are able to express and secrete this growth factor (Salama et al. 2006), while platelets are known to scavenge various biologically active factors (Klinger & Jelkmann 2002). To date, no relationship of midkine to WBC or PLT count has been described, although a correlation between PLT and the level of pleiotrophin, a midkine homologous protein, has been found (Soulie et al. 2004). We observed that the midkine level was significantly higher in cancer patients with leucocytosis and thrombocytosis, while the lowest midkine values were observed in cancer patients with leucocytopenia and thrombocytopenia (see Table IV). We also found that the sMK level directly correlated with leucocyte and platelet number. However, the relationship examined by Spearman's test was rather weak and did not account for the midkine rise observed in LNM. This fact, together with a notably strong dependence of midkine level on the number of cancer cells as indicated by its relationship to tumour size and the presence of secondary tumours in lymph nodes and other organs as well as the tendency to increase with the number of metastases, suggests that cancer cells, rather than host cells, remain the main source of midkine in the circulation. This is, however, the first

report that may indicate the capability of platelets to store midkine among other biologically active factors.

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