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INCREASE IN SERUM PLATELET-DERIVED GROWTH FACTOR (PDGF)-BB REFLECTS LYMPH NODE INVOLVEMENT IN ESOPHAGEAL CANCER PATIENTS INDEPENDENTLY FROM PLATELET COUNT

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Aim: **To evaluate clinical significance and diagnostic utility of increase in serum PDGF-BB (sPDGF-BB) in esophageal cancer, which have not been addressed yet despite the relevance of PDGF axis in this cancer type.** ***Methods*****: Immunoenzymatically assessed sPDGF-BB was related to clinicopathological features, and inflammatory, angiogenic, and lymphangiogenic indices in 84 patients with esophageal cancer and 47 controls. Its diagnostic utility was evaluated by receiver operating characteristics (ROC) curve analysis.** ***Results:*** **sPDGF-BB was significantly higher in esophageal cancer patients than controls (3.76** ***vs.*** **2.66 μg/l,** $p = 0.0001$**) and corresponded with the disease advancement. Of evaluated clinicopathological features, lymph node metastases and distant metastases were independently associated with an increase in sPDGF-BB; however, only the association with lymph node metastases persist adjustment to platelets. In univariate analysis, sPDGF positively correlated with platelets (r=0.70,** $p < 0.0001$**), vascular endothelial growth factor (VEGF)-A (r=0.50,** $p < 0.0001$**), VEGF-C (r=0.57,** $p < 0.0001$**), white blood cells (r=0.32,** $p = 0.004$**), C-reactive protein (r=0.34,** $p = 0.004$**), IL-6 (r=0.35,** $p = 0.003$**), and IL-8 (r=0.45,** $p < 0.0001$**). In multivariate analysis, VEGF-C and platelets were independently associated with sPDGF-BB explaining 61% in its variability. With >2.845 μg/l cut-off, over 76% of patients had elevated sPDGF-BB. Its accuracy as lymph node metastases marker was 75%, sensitivity and specificity corresponding with >3.029 μg/l cut-off were 84 and 61%, respectively.** ***Conclusions*****: sPDGF-BB owns potential as a possible lymph node metastases marker and might be considered as a diagnostic tool in preliminary evaluation of esophageal cancer patients identifying those likely to be burdened with lymph node metastases, the disease recurrence monitoring, and/or preselecting patients for PDGF-directed cancer therapies.**
Key Words**: esophageal cancer, PDGF-BB, lymph node metastasis, tumor marker, platelets, lymphangiogenesis.**

Esophageal cancer (EC) is the sixth most frequent malignancy worldwide and the fifth cause of cancer-related deaths [1]. The disease is usually recognized late and by the time of its first clinical manifestations, the cancer has already metastasized, primarily to regional then distant lymph nodes. The accurate staging in EC is of primary importance as the involvement of distant lymph nodes determines palliation. Moreover, survival rates following esophagectomy strongly depend on the number of metastatic lymph nodes as well as the number of lymph nodes resected whereas overlooked lymph nodes metastases remain the main culprit of disease recurrence [2, 3]. Currently applied diagnostic procedures either lack accuracy or, like an endoscopic ultrasound (EUS)-guided transesophageal biopsy of lymph nodes, are invasive and not universally available [4]. In this respect, the idea of biomarker evaluation, relatively cheap, non-invasive, and easy to perform procedure, is appealing and lymphangiogenic factors seem to be the best candidates. Moreover, targeting angiogenesis and/or lymphangiogenesis is a promising anti-cancer treatment — relatively non-toxic in comparison with conventional radiochemotherapy and less prone to develop acquired drug resistance, and, yet, capable of inducing tumor shrinking, improving the control of metastasis and the delivery of chemotherapeutic agents into tumor cells [5, 6].

A family of platelet-derived growth factors (PDGFs) has recently emerged as a new potential source of biomarkers and targets for anti-cancer treatment [7]. From among the family members, the isoform consisting of two B chains (PDGF-BB) has been recognized as the most potent lymphangiogenic factor. PDGF-BB is essential for metastatic spread to lymph nodes and acts directly by inducing migration of lymphatic endothelial cells [8]. It also affects cancer-related angiogenesis by up-regulating vascular endothelial growth factor (VEGF)-A expression [9] and by controlling the process of pericyte recruitment during vessel maturation [10].

PDGF-BB is overexpressed by many cancer types of epithelial origin as well as by some host cells, that is, macrophages, fibroblasts and vascular smooth muscle and endothelial cells [11]. However, a-granules of platelets remain the main storage site of this growth factor [11], which could be tumor-available following platelet activation and degranulation. Since both these process-

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Abbreviations used: ANCOVA – analysis of covariance; ANOVA – analysis of variance; AUC – area under ROC curve; CRP – C-reactive protein; CT – computed tomography; EC – esophageal cancer; LNM – lymph node metastases; LR – likelihood ratios; PDGF – platelet-derived growth factor; PLT – platelet count; ROC – receiver operating characteristics analysis; sPDGF-BB – serum PDGF-BB isoform; TNF – tumor necrosis factor; VEGF – vascular endothelial growth factor; WBC – white blood cells count.

es occur during clot formation, serum concentrations of PDGF-BB (sPDGF-BB) are believed to better reflect the platelet-stored PDGF-BB than its plasma levels [12].

Data on the possible association between PDGF-BB and esophageal cancer are scanty, suggestive, however, of the relevance of PDGF axis in this cancer type [13, 14]. However, sPDGF-BB levels in EC have not been assessed before. Our objectives were to evaluate clinical significance of increase in sPDGF-BB in EC as well as diagnostic potential of sPDGF-BB as compared to VEGF-A and VEGF–C, the key angiogenic and lymphangiogenic factors.

MATERIALS AND METHODS

Study population. We enrolled 84 EC patients admitted to the Department of Gastrointestinal and General Surgery, Wroclaw Medical University, Wroclaw, Poland for conservative therapy, surgery, or palliative procedure, whose blood samples were collected on admission or soon afterwards, preceding any treatment. Patients were staged clinically according to the guidelines of the UICC TNM [15] system on the basis of upper digestive tract endoscopy with biopsy and pathological examination, contrast radiographic studies with barium or gastrografin, posteroanterior and lateral chest radiography, ultrasound examination of the abdominal cavity and cervical nodes, thorax and abdominal cavity computed tomography (CT), diagnostic laparotomy and thoracotomy, and bronchoscopy, where appropriate. There were 69 men and 15 women, median age 59 (range: 35–84). Number of patients by disease stage and histology is given in Table 1.

Table 1. The association between serum PDGF-BB and clinicopathological features of esophageal cancer

Feature	sPDGF-BB [μg/l]	*p* value	PLT [·10⁹/L]	*p* value
Stage:		0.018		0.041
I/II (n=14)	2.77 (1.8–4.25)		244 (190–298)	
III (n=23)	3.35 (2.62–4.28)		264 (223–305)	
IV (n=47)	4.37 (3.77–5.06)*		310 (281–339)	
Local advancement (T):		0.065		0.046
2 (n=11)	2.76 (1.64–4.63)		250 (183–317)	
3 (n=28)	3.50 (2.92–4.20)*		261 (236–286)	
4 (n=45)	4.25 (3.57–5.05)*		311 (278–345)	
Regional metastases (N):		0.003		0.162
no (n=33)	2.99 (2.37–3.78)		268 (233–302)	
yes (n=51)	4.36 (3.80–5.01)*		299 (271–327)	
Distant metastases (M):		0.008		0.034
no (n=45)	3.22 (2.69–3.86)		265 (236–295)	
yes (n=39)	4.50 (3.81–5.32)*		311 (280–343)	
Histology:		0.958		0.649
scc (n=50)	3.75 (3.24–4.25)*		283 (256–309)	
adc (n=34)	3.78 (2.98–4.79)*		293 (255–331)	

Notes: scc – squamous cell carcinoma; adc – adenocarcinoma; *-significantly different from healthy subjects.

The control group consisted of 47 blood donors, considered healthy on the basis of physical examination and routine blood tests, whose sera were kindly provided by Regional Center of Blood Donation and Therapeutics in Wroclaw, Poland. There were 41 men and 6 women, median age 50 (range: 42–59). There was no difference in gender distribution ($p = 0.608$) between study and control groups. Although both groups differed with respect to age, sPDGF-BB did not correspond with age in any group: r=0.08, $p = 0.363$ in a whole cohort, r=-0.07, $p = 0.514$ in EC and r=-0.10, $p = 0.510$ in control group.

Ethical considerations. The study protocol was approved by the Medical Ethics Committee of Wroclaw Medical University, Wroclaw, Poland and the study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 1983, and an informed consent has been obtained from all patients.

Analytical methods. sPDGF-BB was measured immunoenzymatically using Human PDGF-BB Stratikine ELISA kits provided by Strathmann Biotec GmbH (Hamburg, Germany) according to the manufacturer's instructions. VEGF-A and -C were assayed using respective ELISA tests provided by IBL (Hamburg, Germany). Serum high-sensitive C-reactive protein (CRP) was determined by immunoturbidimetric method with the Quantia-CRP UV from Tulip Diagnostics Ltd. (Goa, India), adjusted to the micro-manual procedure. Interleukin (IL)-1, 6 and 8, and tumor necrosis factor (TNF)-a were determined in sera by an enzyme double-antibody indirect immunoassays with respective PeliKine Compact human ELISA kits supplied by Sanquin (Amsterdam, the Netherlands). All other indices were measured by routine automatic procedures.

Statistical analysis. Data distribution was tested using the Kolmogorov-Smirnov normality test. If not otherwise stated, data are presented as means together with 95% *CI* and analyzed using the t-test for independent samples, Welch test or one way ANOVA, preceded by the comparison of variances (*F*-test or Levene's test for equality of variances, respectively). Mann-Whitney U test was applied for age comparison. Correlation analysis was conducted with Pearson (r) or Spearman (ρ) correlation tests. For interrelated variables, second-order coefficients of partial correlation (a "net" correlation between two variables of a larger group when the remaining variables are held constant) were calculated. Frequency analysis was carried out using χ^2 test. Multiple regression analysis was conducted with a stepwise model (with $p<0.05$ as an entrance criterion and $p>0.1$ as a removal criterion). Missing data (CRP and interleukins) were handled by restricting the analyses involving CRP and IL to complete cases. Platelet count (PLT) was identified as a potential confounder and adjusted to by analysis of covariance (ANCOVA). All tests were two-sided and p values $\leqslant 0.05$ were considered statistically significant.

sPDGF-BB diagnostic utility was evaluated with reference to VEGF-A and VEGF-C using Receiver Operating Characteristics (ROC) curve analysis. The overall performance was expressed as the area under the ROC curve (AUC) with 95% *CI* and p value for the difference between the calculated AUC and AUC=0.5 (index with no diagnostic power). Additionally, a cut-off value corresponding with the highest accuracy (minimal false positives and false negatives) was determined. The related sensitivities and specificities were calculated and summarized in the Youden index (sensitivity+specificity-1) for comparative purposes. The likelihood ratios for positive (+LR) and negative

(-LR) results were calculated as well. Inter-rater agreement was quantified using K-statistics.

Entire statistical analysis was conducted using MedCalc® version 10.4.0.0 (Mariakerke, Belgium) with the exception of partial correlation coefficients calculated using VassarStats (available from http://faculty.vassar.edu/lowry/VassarStats.html).

RESULTS

sPDGF-BB in esophageal cancer — the association with clinicopathological features. sPDGF-BB in EC patients were significantly higher than in healthy subjects (3.76 μg/l (95%CI: 3.31–4.27) vs. 2.66 μg/l (2.40–2.96), $p = 0.0001$). In EC patients, the increase in sPDGF-BB was associated with the disease advancement, particularly with the presence of regional and distant metastases, whereas sPDGF-BB concentrations in less advanced cancers did not significantly differ from those observed in healthy subjects. There were no differences in factor's concentrations between patients with squamous cell carcinomas and adenocarcinomas. Also PLT significantly differed with respect to the disease advancement (Table 1).

To identify factors independently associated with and increase in sPDGF-BB in a whole cohort, we performed multiple regression analysis with sPDGF-BB as response variable and cancer presence (0/1), disease stage, lymph node (0/1) and distant (0/1) metastases as explanatory variables. Of these, lymph node metastases (LNM) ($p<0.001$) and distant metastases ($p=0.002$) were independently associated with the increase in sPDGF-BB. Also when the analysis was restricted to EC patients, LNM and distant metastases but not the disease stage were predictors of sPDGF-BB. However, only the association with LNM was pertinent following PLT adjustment ($p=0.033$) by ANCOVA (sPDGF-BB as dependent variable, LNM and distant metastases as factors, and PLT as covariate).

Univariate and multivariate correlation analyses. sPDGF-BB in EC patients correlated positively with VEGF-A ($r=0.50$, $p < 0.0001$), VEGF-C ($r=0.57$, $p<0.0001$), and platelets ($r=0.70$, $p < 0.0001$). Since all these variables were interrelated, we calculated second order partial correlation coefficients to evaluate the "net"correlations. sPDGF-BB remained correlated with VEGF-C and PLT ($r=0.33$, $p=0.003$ and $r=0.48$, $p<0.0001$, respectively) but not with VEGF-A ($r=0.13$, $p=0.262$).

sPDGF-BB correlated also with white blood cells (WBC) count ($r=0.32$, $p=0.004$) and other inflammatory indices, that is, CRP ($r=0.34$, $p=0.004$), IL-6 ($r=0.35$, $p=0.003$), and IL-8 ($r=0.45$, $p<0.0001$) but not with IL-1 ($\rho=-0.05$, $p=0.711$) or TNF-α ($\rho=-0.04$, $p=0.768$).

Of the factors associated with sPDGF-BB in univariate analysis (PLT, WBC, CRP, IL-6, IL-8, VEGF-A, and VEGF-C), PLT ($b=0.016$, $p<0.0001$) and VEGF-C ($b=3.27$, $p=0.005$) were independently associated with sPDGF-BB in multiple regression analysis, explaining 61% in data variability ($R^2=0.61$; const=-4.16; $F=46.95$, $p<0.001$).

Diagnostic potential of sPDGF-BB. Using the cut-off values established in ROC analysis to optimally differentiate diseased and healthy subjects (>2.845 μg/l for sPDGF-BB, >13.14 μg/l for VEGF-C and >222 ng/l for VEGF-A), 76.2%, 72.7%, and 63.4% of EC patients had elevated sPDGF-BB, VEGF-C, and VEGF-A, respectively.

The overall diagnostic accuracy of all growth factors was comparable. The sensitivity of sPDGF-BB using optimal cut-off values was higher than these of VEGF-C and VEGF-A but the corresponding specificity was lower than calculated for VEGF-C (Table 2). The optimal cut-off values are chosen assuming that sensitivity and specificity are equally important. However, in case of LNM detection, falsely negative predictions should be minimized thereby favoring high test sensitivity. Using optimal cut-offs, sPDGF-BB had the highest sensitivity. For comparative purposes, we chose cut-off values for VEGF-A and VEGF-C to approach sensitivity calculated for sPDGF-BB. Using a new cut-offs with fixed high sensitivity, sPDGF-BB was the marker with the highest specificity and Youden index and favorable likelihood ratios (Table 2).

Table 2. Diagnostic potential of sPDGF-BB, VEGF-C and VEGF-A as indicators of lymph node metastases

	sPDGF-BB	VEGF-C	VEGF-A
AUC (95%CI),	0.75 (0.66–0.82),	0.78 (0.70–0.85),	0.71 (0.63–0.79),
$p_{AUC}=0.5$	p<0.001	p < 0.001	$p < 0.001$
Optimal cut-off	>3.029 μg/l	>15.96 μg/l	>173.8 ng/l
Sens. and Spec.	84.3 and 61.3%	69.6 and 80.8%	81.6 and 53.8%
Youden index	0.456	0.504	0.354
LR+ and LR-	2.18 and 0.26	3.62 and 0.48	1.77 and 0.34
Adjusted cut-off*		>12.4 μg/l	>142 ng/l
Sens. and Spec.		84.8 and 52.6%	83.7 and 50%
Youden index		0.374	0.337
LR+ and LR-		1.79 and 0.29	1.67 and 0.33

Notes: AUC – area under ROC curve; CI – confidence interval; Sens – sensitivity; Spec – specificity; LR – likelihood ratios. *Cut-off values for VEGF-C and VEGF–A have been changed to reach sensitivities comparable to that of sPDGF-BB.

Diagnostic potential of simultaneous evaluation of sPDGF-BB and VEGF-C. To evaluate the agreement between LNM predictions conducted on the basis of increase in sPDGF-BB and VEGF-C concentrations (using optimal cut-offs), we applied K-statistics. Inter-rater agreement was fair with K=0.359. Therefore, we examined whether substantial improvement can be achieved with their simultaneous measurement. Optimal cut-off values determined previously in ROC analysis were used and a rule minimizing false negative results was applied: a test was considered positive when at least one marker was elevated above established cut-off values, whereas a test was considered negative only when both markers were below their thresholds. The diagnostic accuracy expressed in terms of AUC was 0.730 (0.645–0.803), sensitivity and specificity were 92.16 and 53.75%, Youden index was 0.459, LR+ and LR- were 1.99 and 0.15, respectively.

DISCUSSION

To the best of our knowledge, clinical and diagnostic significance of the increase in sPDGF-BB in EC has not been addressed yet. Matsumoto et al. [14] showed up-regulated expression of PDGF-BB in EC tumors, which corresponded with the disease progression. Their results added to the *in vitro* findings on the fac-

tor's relevance for the growth promotion of EC. Liu et al. [13] demonstrated the presence of binding sites for PDGF-BB on tumor cells, which implies autocrine mode of action and provides the growth advantage and prevents apoptosis in esophageal carcinoma cell line. Correspondingly, we found increased sPDGF-BB in EC patients as compared to healthy individuals. sPDGF-BB elevation was associated exclusively with advanced disease, particularly with the presence of regional and distant metastases.

Advanced EC in our patients was also associated with increased platelet count, corroborating previous findings showing high PLT to correspond with EC progression and poor outcome [16]. Since platelets greatly contribute to serum PDGF-BB, reflected by high correlation coefficient for sPDGF-BB and PLT in our patients, the associations we observed might be mediated by increase in platelets. Indeed, following adjustment to PLT, sPDGF-BB association with distant metastases lost its relevance. However, sPDGF-BB association with lymph node metastases remained significant and was further supported by a positive and independent from PLT correlation between sPDGF-BB and VEGF-C, a key lymphangiogenic factor [17]. Correspondingly, Onimaru et al. [18] demonstrated that both these growth factors are tightly associated, with VEGF-C regulating PDGF-BB expression during capillary formation.

sPDGF-BB in our patients was also correlated with VEGF-A, IL-6, IL-8, CRP, and WBC, but, otherwise than in case of VEGF-C, all these correlations did not persist PLT adjustment. PDGF-BB has been shown to trigger VEGF-A expression in malignant and non-malignant settings [9, 19]. However, in our study the relationship reflected the close association of both growth factors with platelet count. Similarly to PDGF-BB, circulating VEGF-A is actively scavenged and stored in platelets, and released *ex vivo* during blood clotting [20, 21]. Also IL-8 is associated with angiogenic and lymphangiogenic potential of EC [22] and as such may be sequestered by platelets from circulation. Concerning IL-6, platelet mediation is consistent with the findings showing IL-6 to be a potent inductor of platelet production [23] and PDGF-BB to be preferably expressed isoform by megakaryocytes, platelets precursors [24]. IL-6 also induces CRP synthesis [25] and correlates with WBC and might mediate the association between sPDGF-BB and these inflammatory indices. Accordingly, the correlation between IL-6 and CRP or WBC in our patients was stronger than found for sPDGF-BB, while calculated partial correlations were significant for IL-6 and CRP or WBC but not for sPDGF-BB (data not shown).

Our finding on sPDGF-BB being associated with lymph node metastases is of clinical relevance. First, its assessment might be considered in preliminary evaluation of EC patients as an inexpensive, available, and non-invasive diagnostic tool for selecting those who are likely to be LNM positive. We found sPDGF-BB specificity and sensitivity to be comparable to these reported for CT (sensitivity 84% and specificity 67% [4]) while its sensitivity was better than that reported for positron emission tomography with fluorine-18–2-fluoro-2-deoxy-D-glucose (FDG-PET; sensitivity 51% and specificity 84% [4]). Previously, Tamura et al. [26] demonstrated circulating lymphangiogenic growth factor, VEGF-C, to be a more accurate indicator of lymph node metastases in lung cancer than chest CT. Correspondingly, we demonstrated VEGF-C to be equally accurate in LNM detection in esophageal squamous cell carcinoma [27]. In our present study, VEGF-C as LNM marker had the best Youden index and the overall accuracy of all evaluated growth factors but, at its optimal cut-off, specificity was favored over sensitivity. Taking into account the results of clinical studies demonstrating prolonged survival for patients with extended lymphadenectomy [3], from clinical point of view a false negative prediction is more critical for patient's survival than a false positive one. Therefore, sensitivity is favored over specificity and LR- over LR+. sPDGF-BB had the highest sensitivity and, when cut-offs of other markers were adjusted to approach 85% sensitivity, it had also the highest specificity. Second, measuring sPDGF-BB might be considered in monitoring of patients following esophagectomy as overlooked metastatic lymph nodes remain the main culprit of disease recurrence [3]. Further studies are needed to evaluate sPDGF-BB as a possible indicator of micrometastases, which are easy to be missed during tissue sampling. The potential of lymphangiogenic factors in detecting micrometastases is supported by the finding that the arrival of tumor cells into the nodes is preceded by lymph node lymphangiogenesis associated with overexpression of lymphangiogenic growth factors [17]. Fair inter-rater agreement between sPDGF-BB and VEGF-C as predictors of LNM inn our patients raised the possibility that their simultaneous evaluation may improve the diagnostic power of a single evaluation. Indeed, the sensitivity was slightly improved; however, specificity was reduced and the overall accuracy as well as Youden index for simultaneous evaluation of sPDGF-BB and VEGF-C remained almost unaltered.

Another possible clinically relevant application of sPDGF-BB evaluation is identifying patients with high angiogenic/lymphangiogenic potential who are likely to benefit from angiogenesis and/or lymphangiogenesis directed therapies. It may also guide the selection of treatment that would suit the actual angiogenic/lymphangiogenic phenotype of a specific EC patient since pattern of growth factor expression varies by tumor type and changes during the disease progression. Moreover, there are inter-individual molecular differences that have to be taken into account while designing a treatment [5].

We evaluated PDGF-BB concentration in serum, although plasma is believed to better reflect circulating pool of this growth factor. However, serum concentrations represent also PDGF-BB pool stored in platelets [12]. Taking into account that platelet-stored PDGF-BB is easily available for the tumor and surrounding stroma due to complex action of thrombin [24, 28], sPDGF-BB better correspond with the true angio-

genic/lymphangiogenic potential related to this growth factor. Moreover, Klement et al. [20] not only demonstrated that platelets actively sequester angiogenic growth factors but, even more importantly, that during cancer development the enlargement of platelet pool of angiogenic growth factors precedes their rise in plasma. Since sPDGF-BB is not an exact representation of PDGF-BB content in platelets as it might be secreted by several other cells [11], it would be of interest to evaluate in future studies whether EC is associated with increased load of PDGF-BB in individual platelets.

Summarizing, we demonstrated the increase in serum concentrations of PDGF-BB in esophageal cancer patients, exclusively associated with the presence of lymph node metastases. PDGF-BB association with lymphangiogenesis was supported by its correlation with VEGF-C, independent from platelets, with the number of which sPDGF-BB was closely correlated. If confirmed in a larger study, sensitivity of sPDGF-BB as an indicator of lymph node metastases, comparable to that of CT, suggest its clinical relevance as potential LNM biomarker in preliminary evaluation of EC patients, the disease recurrence monitoring, and/or preselecting patients for directed cancer therapies.

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