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EVEN A MILD ANEMIA IS RELATED TO TUMOR AGGRESSIVENESS MEDIATED BY ANGIOGENIC FACTORS

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Esophagogastric cancers have high recurrence rates with lymph nodes being a common pattern. Pre-treatment anemia has been reported an independent prognostic factor of treatment failure regardless of treatment strategy, particularly associated with poor locoregional control. A causative relationship between anemia — tumor hypoxia — tumor aggressiveness mediated by angiogenesis up-regulation is advocated, yet remains controversial. ***Aim:*** **To determine whether and how the pre-treatment anemia is associated with various aspects of disease aggressiveness and to evaluate the possible involvement of angiogenesis mediators.** ***Methods:*** **In 111 esophagogastric cancer patients we investigated the association of pre-treatment hemoglobin concentration and anemia presence with cancer-related, patients-related features and laboratory parameters including angiogenic factors: vascular endothelial growth factors A and C, interleukin-8 and midkine. Serum levels of angiogenic factors were assessed with immunoenzymatic tests.** ***Results:*** **Histology, disease stage, regional metastasis and dissemination in general, malnutrition and angiogenesis represented by midkine were found to correlate with anemia presence and hemoglobin concentration, while tumor extension, patient's age and sex accounted only for anemia presence. A tendency towards hemoglobin correlation with VEGF-A and Il-8 was also observed. Midkine, tumor histology and malnutrition were found to exert an independent effect on pre-treatment hemoglobin concentration and anemia presence in esophagogastric cancer patients. Hemoglobin level of 12 g/dL was found an optimal cut-off value for discrimination between localized and disseminated cancers.** ***Conclusions:*** **Even a mild pre-treatment anemia is associated with cancers metastasizing especially to regional lymph nodes, which seems to be mediated by some of studied angiogenic factors.**
Key Words: **anemia, esophagogastric cancer, metastasis, angiogenesis.**

A negative impact of anemia on the outcome of cancer patients treated with chemoradiotherapy is well known, with a reduction of treatment efficacy by anemia-induced tumor hypoxia being a popular explanation [1, 2]. However, a low concentration of pre-treatment hemoglobin had been related to poor outcome also in patients treated surgically [3–5], even in cases when prognosis had traditionally been favorable [6]. Therefore, it has been hypothesized that the presence of anemia might reflect a more aggressive tumor phenotype [6], related among others to the up-regulation of angiogenic factors due to the anemia-induced tumor hypoxia [7, 8]. Yet, whether or how anemia is associated with tumor hypoxia remains debatable [9]. Moreover, data on anemia impact on up-regulation of angiogenic factors are contradictory as well [10–12]. Furthermore, some of the clinical trials with human recombinant erythropoietin have worsened patients' outcome despite the improvement of hemoglobin concentration [6, 9]. In the light of existing controversies, the detailed studies on mechanisms underlying the advocated association of anemia with tumor aggressiveness mediated by tumor hypoxia and subsequent angiogenesis should precede the implementation of new treatment strategies.

Studies on anemia in esophagogastric cancers have been focused on its adverse impact on a quality of life, duration of survival and treatment response [13–16]. Our objectives were the evaluation of relation between pre-treatment anemia and various manifestations of disease aggressiveness and determination whether the presence of cancer-related anemia is associated with up-regulation of circulating mediators of angiogenesis: vascular endothelial growth factors A and C (VEGF-A and VEGF-C), interleukin 8 (IL-8) and midkine, the latter being a relatively new angiogenic factor [17]. All these cytokines exhibit both angiogenic and proinflammatory properties and have been connected to aggressiveness of esophagogastric cancers [18–21]. Therefore, if causative relationship between anemia-hypoxia-angiogenesis is valid, their adverse relation to hemoglobin concentration might be expected.

METHODS

Patients. The Medical Ethics Committee of our University approved the study protocol.We examined 111 patients of Gastrointestinal and General Surgery Department of our University with histopathologically confirmed esophageal squamous cell carcinomas (SCC; n = 72) or esophagogastric adenocarcinomas (ADC; n = 39), staged clinically according to TNM system [22]. There were: stage II — 20 cases, stage III —

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Abbreviations used: ADC – adenocarcinoma; CI – confidence interval; Hb – hemoglobin; IL-8 – interleukin 8; LR – likelihood ratios; NEU – neutrophil count; PLT – platelet count; ROC – receiver operating characteristics; SCC – squamous cell carcinoma; TLC – total lymphocyte count; VEGF-A – vascular endothelial growth factor A; VEGF-C – vascular endothelial growth factor C; WBC – white blood cell count.

33 cases, stage IV — 58 cases; 20 females, 91 males; median age 59 (range: 35–85).

Data on patients' hemoglobin level, platelet (PLT), leukocyte (WBC), neutrophil (NEU) and total lymphocyte (TLC) counts, were collected retrospectively from case histories on condition that blood samples for hematological analysis and angiogenic factors determination were obtained simultaneously and preceded any treatment. Data on patients' body mass index (BMI) and weight loss due to the disease were also collected. Reported weight loss of > 5% of body weight per three months was considered as substantial.

Anemia was defined in terms of hemoglobin concentration (females: Hb < 12 g/dL; males: Hb < 13 g/dL), verified by hematocrit level.

Analytical methods. Levels of angiogenic factors were measured in sera obtained from blood clotted for 15 min at room temperature and centrifuged (15 min, 3000 rpm). VEGF-A and VEGF-C levels were assayed according to the manufacturer instructions by immunoenzymatic tests (IBL-Hamburg, Germany). Il-8 was determined with PeliKine Compact human Il-8 ELISA kit (Sanquin, Holland). Midkine was determined by double-antibody sandwich indirect ELISA according to the procedure previously described [23]. All measurements were duplicated.

Statistical analysis. Hemoglobin concentrations are presented as mean with 95% confidence interval (CI). Analysis of differences in mean hemoglobin levels was conducted with two-tailed t-test for independent samples or one-way ANOVA. Comparisons of anemia frequency were conducted with Chi-square test. Correlation was analyzed with Pearson moment-product correlation test, Spearman correlation rank test or Kendall tau test with respect to variable distribution and type (categorical/continuous). Distribution normality of raw or log-transformed data was tested with D'Agostino-Pearson test.

Receiver Operating Characteristics (ROC) analysis for determination of optimal hemoglobin cut-off value and likelihood ratios calculation (positive: LR^+; negative: LR^-) was performed.

A stepwise method of multiple and logistic regression was employed (entrance condition: $p < 0.05$, removal condition: $p > 0.1$). Multiple regression was applied for prediction of continuous variables (hemoglobin concentration) and logistic regression for dichotomous variables (anemia and cancer dissemination). Number of entered independent variables was limited to preserve 1 : 10 ratio with sample number. Categorical variables were dichotomized by grouping T2–T3 cancers *vs*. T4 and stages I–III *vs* IV. p values $\leqslant 0.05$ were considered significant.

RESULTS

The prevalence of anemia in our patients was 64%; mean Hb level — 11.9 g/dL (CI: 11.5–12.3). In non-anemic patients mean hemoglobin concentration was 14 g/dL (CI: 13.6–14.3) and 10.8 mg/dL (CI: 10.4–11.1) in anemic patients ($p < 0.0001$). 48 subjects had mild (Hb: 10 to 12–13 g/dL) while 23 moderate/severe anemia (Hb < 10 g/dL).

We analyzed the association of anemia prevalence or hemoglobin concentration with cancer-related and patient-related variables of esophagogastric cancers (Table 1). The strength of the relations with included biochemical parameters (blood cell counts and serum levels of angiogenic factors) was assessed in correlation analysis (Table 2). Additionally, we found that when hemoglobin level dropped < 11 g/dL, a tendency towards a weak negative correlation with VEGF-A occurred (*rho* = –0.178, $p = 0.289$).

Table 1. Relation of anemia and hemoglobin (Hb) concentration to clinico-pathological factors in gastroesophageal cancer patients

Clinico-pathological variables	Frequency of anemia in respect to cancer clinico-pathological variables			Relation between Hb level and cancer clinico-pathological variables	
	Normal	Anemia	*p* value	Mean Hb level [g/dL] (with 95% CI)	*p* value
Gender:					
Female	10	10	0.238[Ch]	11.5 (10.9–12.2)	0.214[t]
Male	30	61		12.0 (11.5–12.4)	
Tumor histology:					
squamous cell	32	40	0.024[Ch]	12.5 (12.1–12.8)	<0.001[t]
carcinoma					
adenocarcinoma	8	31		10.9 (10.2–11.7)	
Regional					
metastasis:					
N0	19	15	< 0.001[Ch]	12.6 (11.9–13.3)	0.003[t]
N1	13	52		11.3 (10.8–11.8)	
Distant					
metastasis:					
M0	25	39	0.565[Ch]	12.0 (11.5–12.5)	0.544[t]
M1	15	32		11.8 (11.2–12.4)	
Cancer					
dissemination:					
localized	14	9	0.006[Ch]	13.0 (12.2–13.9)	0.001[t]
disseminated	23	61		11.6 (11.1–12.0)	
BMI:					
> 18.5	21	41	0.477[Ch]	12.2 (11.6–12.7)	0.382[t]
< 18.5	12	15		11.8 (11.2–12.4)	
Weight loss:					
unsubstantial	17	33	0.679[Ch]	11.9 (11.3–12.5)	0.756[t]
substantial	20	30		12.0 (11.4–12.6)	
Tumor size:					
< 5 cm	9	10	0.428[Ch]	12.4 (11.3–13.5)	0.253[t]
> 5 cm	30	57		11.8 (11.4–12.2)	
Primary tumor					
extension:					
T2	8	9	0.192[Ch]	12.4 (11.2–13.5)	0.549[A]
T3	16	21	(0.087)[ChT]	12.0 (11.3–12.6)	
T4	16	41		11.8 (11.2–12.3)	
Disease stage:					
I/II	11	9	0.119[Ch]	12.6 (11.6–13.7)	0.041[A]
III	12	21	(0.047)[ChT]	12.3 (11.6–12.9)	
IV	17	41		11.5 (10.9–12.0)	

Notes: Hb – hemoglobin concentration; CI – confidence intervals; [Ch]Chi-square test; [ChT]Chi-square test for trend; [t]two-sided *t*-test for independent samples; [A]one way ANOVA.

Since our results revealed hemoglobin association with cancer metastasis, ROC analysis was performed to determine the optimal hemoglobin levels discriminating between non-metastasizing and metastasizing cancers. The optimal hemoglobin threshold value was 12.1 g/dL for disseminated cancers and 12.5 g/dL for cancers metastasizing to lymph nodes. Likelihood ratios were $LR^+ = 2.85$, $LR^- = 0.49$ and $LR^+ = 1.91$, $LR^- = 0.37$, respectively.

We conducted multiple regression analysis in order to establish which of the cancer or patient-related variables and/or biochemical parameters account for changes in blood hemoglobin concentration. Similarly, logistic regression was applied to determine predictors of anemia. Only those variables, which were found

significant in correlation analysis (marked with asterisk see in Table 2), were included.

Table 2. Correlation analysis between the presence of anemia or hemoglobin concentration and patient-related, cancer-related and biochemical variables in esophagogastric cancer patients

Variable	Presence of anemia	Hemoglobin level
	Correlation coefficient; *p* value	Correlation coefficient; *p* value
Age	rho = 0.194, $p = 0.044$*	rho = –0.100, $p = 0.293$
Sex	tau = 0.136, $p = 0.034$*	rho = 0.085, $p = 0.373$
BMI	tau = 0.101, $p = 0.160$	rho = –0.079, $p = 0.461$
Weight loss	tau = –0.062, $p = 0.356$	rho = 0.042, $p = 0.679$
Histology	tau = 0.238, $p < 0.001$*	rho = –0.348, $p < 0.001$*
Disease stage	tau = 0.166, $p = 0.010$*	rho = –0.218, $p = 0.022$*
Tumor extension (T)	tau = 0.163, $p = 0.011$*	rho = –0.102, $p = 0.287$
Tumor size	tau = 0.102, $p = 0.121$	rho = –0.095, $p = 0.329$
Dissemination	tau = 0.289, $p < 0.0001$*	rho = –0.300, $p = 0.002$*
Regional metastasis	tau = 0.364, $p < 0.0001$*	rho = –0.296, $p = 0.003$*
Distant metastasis	tau = 0.074, $p = 0.255$	rho = –0.052, $p = 0.585$
WBC	rho = –0.007, $p = 0.939$	rho = 0.063, $p = 0.511$
TLC	rho = –0.411, $p < 0.001$*	rho = 0.419, $p < 0.001$*
NEU	rho = 0.108, $p = 0.305$	rho = –0.095, $p = 0.368$
PLT	rho = 0,043, $p = 0.650$	rho = –0.089, $p = 0.351$
VEGF-A	rho = 0.047, $p = 0.628$	r = 0.052, $p = 0.592$
VEGF-C	rho = –0.022, $p = 0.821$	rho = 0.096, $p = 0.331$
Il-8	rho = 0.076, $p = 0.437$	r = –0.155, $p = 0.113$
Midkine	rho = 0.266, $p = 0.005$*	rho = –0.286, $p = 0.003$*

Notes: Test application depended on variable distribution and type (*rho*-Spearman, tau-Kendall, r-Pearson); *significant relations.

Tumor histology, TLC and midkine were found to exert independent effect on hemoglobin concentration in esophagogastric cancer patients. Resulting coefficient of determination (R^2) of the model was 0.338 and multiple correlation coefficient (R) was 0.581 ($p < 0.001$). Regression coefficients of individual variables are presented in Table 3. We excluded midkine from the model to evaluate the usefulness of this parameter. The strength of a new model was similar ($R^2 = 0.321$, $R = 0.566$, $p < 0.001$), yet midkine was replaced by dissemination variable (Table 3).

Table 3. Results of multiple regression analysis (stepwise method) with hemoglobin concentration in esophagogastric cancer patients as a dependent variable

Entered variables: histology, midkine, TLC, dissemination, stage, regional metastasis			
Independent variables retained in the model	Regression Coefficient	Standard error	*p* value
Histology	–1.21333	0.36857	0.002
Midkine	–0.00045	0.00016	0.007
TLC	1.07550	0.27780	< 0.001
Entered variables: histology, TLC, dissemination, stage, regional metastasis			
Dissemination	–1.02697	0.43865	0.022
Histology	–1.20672	0.37777	0.002
TLC	0.97746	0.28143	< 0.001

Note. TLC – total lymphocyte count.

In turn, variables that exert an independent effect on anemia presence were midkine, TLC, age and regional metastasis. The significance of overall model fit was $p < 0.0001$ and its accuracy 86%. Odds' ratios for individual variables are presented in Table 4.

In multiple regression model midkine was replaced by cancer dissemination without substantial loss of model's strength, suggesting that hemoglobin association with cancer dissemination can be mediated by midkine. Hence, we performed logistic regression analysis in search for variables, which exert an independent effect on cancer dissemination and found that midkine alone was an independent variable (Table 5).

Table 4. Results of logistic regression analysis (stepwise method) with the presence of anemia in esophagogastric cancer patients as a dependent variable

Entered variables: midkine, TLC, age, regional metastasis, dissemination, histology, sex, stage, tumor extension				
Retained variable	Regression coefficient	Standard error	*p* value	ODDs ratios (95% CI)
Midkine	0.0011	0.0004	0.0097	1.0011 (1.0003–1.0019)
TLC	-1.4059	0.5265	0.0076	0.2452 (0.0874–0.6880)
Age	0.0770	0.0347	0.0264	1.0800 (1.0091–1.1560)
Regional metastasis	1.4746	0.6829	0.0308	4.3692 (1.1459–16.6595)

Notes: TLC – total lymphocyte count; CI – confidence interval.

Table 5. Results of logistic regression analysis (stepwise method) with the presence of cancer dissemination in esophagogastric cancer patients as a dependent variable

Entered variables: stage, tumor extension, histology, hemoglobin, PLT, WBC, TLC, VEGF-A, Il-8, midkine				
Retained variable	Regression coefficient	Standard error	*p* value	ODDs ratios (95% CI)
Midkine	0.0015	0.0005	$p < 0.001$	1.001 (1.000–1.002)

Notes: PLT – platelet count, WBC – leukocyte count; TLC – total lymphocyte count.

DISCUSSION

We found that anemia prevalence and hemoglobin concentration were related to cancer dissemination in esophagogastric cancers. Similarly, Knight *et al.* [24] summarized that the prevalence of anemia associated with other cancer types varied not only with tumor type but also with metastasis presence. Our finding that stronger association can be found with regional rather than distant metastasis corroborates well the results of Rades *et al.* [14, 16], who reported that anemia in patients with esophageal cancer was one of the strongest independent predictors of locoregional recurrence after radiotherapy but not of shortened metastasis free survival. The same authors demonstrated that locoregional recurrence rates were three times higher in anemic than non-anemic patients and that locoregional control of esophageal cancer could be improved by the administration of epoetin-alpha [15, 16].

Some investigators argue that the impact of anemia on prognosis results from anemia relation to tumor volume [25]. Indeed, anemia prevalence has been higher in T4 prostate cancer than T0–T3 cancers [24]. But Rades *et al.* [13, 14] showed that in esophageal cancer both hemoglobin concentration and tumor length were independent prognostic variables. Hemoglobin has been associated with locoregional control while tumor length with metastasis free survival [13, 16]. Other authors [26] reported that in multivariate analysis only hemoglobin concentration was an independent survival predictor in esophageal carcinoma. We, in turn, observed that hemoglobin concentration was related neither to tumor size nor to the extension of primary tumor, although frequency of anemia tended to depend on tumor extension with a weak, yet significant, correlation between variables. However, tumor extension was not selected as variable exerting independent effect on hemoglobin level or anemia presence.

As could be expected, a large number of our patients was anemic and anemia presence was related

to age. The prevalence of anemia was in agreement with the results summarized by Varlotto *et al.* [9] and with the data from The European Cancer Anemia Survey (ECAS) [27]. Although only 31% of patients with gastrointestinal cancers were found to be anemic at the beginning of ECAS, the number was doubled during the survey. High anemia prevalence can be explained by its tendency to depend on disease stage. On one hand we found a positive correlation between disease stage and both anemia presence and hemoglobin level. On the other hand, which is characteristic for esophagogastric cancers, most of our patients were diagnosed with advanced, non-operable tumors and submitted to palliative treatment.

Results of our studies support the hypothesis that anemia is related to tumor aggressiveness, especially to lymph node involvement. Assuming that a link between hemoglobin level and tumor oxygenation is valid, this result corroborates the finding that poorly oxygenated tumors had larger lymphatic-vascular space invasion as compared to better oxygenated ones [9]. Angiogenesis is a condition of successful dissemination of transformed cells. Since many angiogenic factors have been implicated in regional metastasis [28], we evaluated whether they may mediate the anemia association with cancer dissemination. And indeed, a significant relation to hemoglobin concentration and anemia presence was observed in case of midkine, while a tendency towards weak adverse association of hemoglobin level with Il-8 and VEGF-A was also found. Yet, the latter relation manifested itself when the hemoglobin dropped below 11 g/dL. Moreover, midkine was found to exert an independent effect on both hemoglobin concentration and anemia presence. In our previous studies we observed that midkine levels were elevated in cachectic patients with esophagogastric cancer [29] and mechanisms of both cancer-related cachexia and anemia seem to share a lot of similarities [30]. Association of anemia with VEGF-A, a key regulatory factor of angiogenesis, has already been studied in selected cancers but yielded contradictory results. Dunst *et al.* [10, 31, 32] consequently reported a significant relation between hemoglobin concentration and circulating VEGF-A, but has not been confirmed by other authors [11, 12]. We also failed to corroborate such correlation.

The cause-effect relationship between anemia, tumor hypoxia, angiogenic factors' up-regulation, promotion and facilitation of cancer cells dissemination appears to be an attractive explanation of anemia association with tumor aggressiveness. Our results seem to support individual elements of this sequence since at least some of the angiogenic factors are related to anemia presence and to cancer metastasis. Moreover, midkine was directly replaced by cancer dissemination in multiple regression analysis without significant lost to the model's strength. At the same time, however, our results may be interpreted as indirect evidence supporting the hypothesis that other factors, additional to tumor hypoxia, mediate anemia impact on tumor aggressive behavior. We found that even a mild anemia was related to tumor aggressiveness, which is in agreement with recent understanding of anemia impact on cancer patients' outcome [33]. ROC analysis revealed that hemoglobin concentration below 12.1 g/dL was almost three times more likely to be seen in patients with disseminated than localized cancers, while it was almost twice more probable to find hemoglobin < 12.5 g/dL in patients with regional metastasis than in those without. In the meanwhile, markedly lower hemoglobin levels were reported to impair significantly tumor oxygenation [6, 25, 34, 35]. In accordance with it, we observed that only in patients with moderate/severe anemia VEGF-A tended to adversely correlate with hemoglobin concentration. Therefore, it can be speculated that apart from tumor oxygenation status there are other mechanisms that can trigger oversecretion of proinflammatory and angiogenic cytokines, which may account for their up-regulation in mild anemia. The existence of more complex mechanisms behind anemia association with tumor aggressiveness seems to be advocated by the results of most of studies comparing anemia and tumor hypoxia as predictors of cancer patients' outcome, where the level of pre-treatment hemoglobin was more important prognostic determinant than oxygenation status of tumor tissue [9].

Nutrient deprivation might be a good candidate and oversecretion of some angiogenesis mediators due to nutrient deprivation has been found [36]. We, in turn, observed that both hemoglobin concentration and anemia presence were strongly associated with total lymphocyte count, a surrogate marker of nutritional status. Moreover, other markers of malnutrition have been reported to correlate with hemoglobin on one hand [30] and patients' survival on the other [3].

Additionally, contrary to lung cancer [37], we observed that tumor histology was related to hemoglobin concentration and anemia presence. However, the proportion of metastasizing cancers was higher in adenocarcinoma than squamous cell carcinoma group, what may account for the higher frequency of anemia in the former. Yet, since histology was found to exert an independent effect on hemoglobin concentration, further elucidation of this issue is required.

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