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ORIGINAL ARTICLE

Diagnostic and Prognostic Performance of Serum Vascular Endothelial Growth Factor, Vascular Endothelial Growth Factor Receptor 2, and Osteopontin for Gastrointestinal Cancers

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SUMMARY

Background: **Biomarkers for early diagnosis and follow-up of cancers are still underutilized in clinical management. Thus, seeking new biomarkers with better sensitivity and specificity is still a challenge. VEGF, VEGFR2, and OPN are newly emerging biomarkers with clinical potential.**
Methods: **ELISA was used to analyze serum VEGF, VEGFR2, and OPN from 75 gastrointestinal cancer patients and 75 control subjects. The correlation of pre-operative serum VEGF, VEGFR2, and OPN levels with CEA, Ki-67 as well as clinical features (age, gender, tumor size, TNM stage, tumor stage, lymph node involvement, metastasis, and histological grading) in these patients.**
Results: **The pre-operative and post-operative serum VEGF and VEGFR2 levels and the post-operative OPN level in patients were significantly higher than in controls (p = 0.000, for all mentioned). The post-operative VEGF and OPN levels were significantly higher than that of pre-operative (p = 0.000 and 0.007, respectively). There was no correlation between pre-operative serum VEGF, VEGFR2, and OPN levels and serum CEA concentration. The pre-operative serum VEGF level was significantly correlated with the tumor Ki-67 scores; however, there was no correlation between serum VEGFR2 and OPN and Ki-67 scores. Univariate logistic regression analysis revealed that serum VEGF level was significantly higher in patients with advanced TNM (III - IV) stage and with lymph node involvement than in patients with low TNM stage (I - II) and with no lymph node involvement. High OPN level was correlated with metastasis. Multivariate logistic regression analysis results showed that serum VEGF and VEGFR2 were the two most important factors for the diagnosis of gastrointestinal cancers in this study (p = 0.000, for both). Combinatorial analysis of the biomarkers improved the performance of the assays.**
Conclusions: **Serum VEGF and VEGFR2 are potential biomarkers for the diagnosis and prognosis evaluation of gastrointestinal cancers, while serum OPN is a potential biomarker for the prognostication of gastrointestinal cancers.**
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KEY WORDS

vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor 2 (VEGFR2), osteopontin (OPN), biomarker, gastrointestinal cancer

INTRODUCTION

Cancers of the gastrointestinal track are common worldwide [1,2]. Survival of gastrointestinal cancers is directly related to early diagnosis and appropriate clinical management during the course of disease.

Tumor biomarkers, which serve as an index of early detection and prediction of prognosis of gastrointestinal cancers, are pivotal for clinical management. Biomarkers currently used in the diagnostic and prognostic prediction of gastrointestinal cancers such as carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) are rather low in either sensitivity or specificity [3,4]. Thus, seeking more effective biomarkers is still a challenge and a worthwhile task.

Vascular endothelial growth factor (VEGF) and its receptor VEGFR2 play an important role in vasculogenesis, angiogenesis, and tumorigenesis [5,6]. Soluble (serum or pleural effusion) VEGF and VEGFR2 have been implicated to associate with certain types of cancers including oropharyngeal squamous cell carcinoma, ovarian cancer, non-small cell lung cancer, primary liver cancer, metastatic colorectal cancer, and bladder cancer and, thus, serve as potential biomarkers in prognostic prediction [7-18]. In addition, another biomarker, osteopontin (OPN), has been related to certain types of cancers as well and plays a role in predicting prognosis [3, 19-26]. However, the results from previous reports were either inconclusive or contradictory. In this study, we investigated the serum VEGF, VEGFR2, and OPN concentration from 75 gastrointestinal cancers including esophageal, stomach, and colorectal cancers. Among them, 70 cases were paired analyses with both pre-operative and post-operative parameters. We also correlated the serum levels of VEGF, VEGFR2, and OPN with CEA and the cell proliferation index-Ki-67 from clinical data, and compared them with patients' clinical features. The performance of these three biomarkers either alone or in combination has been also analyzed.

MATERIALS AND METHODS

Patients

A total of 75 cases of gastrointestinal cancer (53 colorectal, 20 stomach, and 2 esophageal) were enrolled consecutively in this study in a period of time from February 2016 to August 2016, of which 49 were males and 26 were female patients with the age from 36 to 83 years old. Patients were diagnosed upon pathological examination. The tumor staging was made according to the TNM staging system from a combination of UICC, AJCC, and WHO. Seventy-five routine healthy individuals without known cancer or a history of cancer were selected as controls, of which the age and gender were matched with that of the patient group (33 - 83 years old, 34 males and 41 females). The study was carried out under the permission of the Institute Review Board of the People's Hospital of Guangxi Zhuang Autonomous Region on the use of human materials. Among 75 patients, 70 had paired pre-operative and post-operative blood samples, the remaining 5 cases did not undergo surgical treatment due to lacking of operative indication. However, the pre-operative serum and clinical features (TNM staging, lymph node involvement, and metastasis) of these 5 cases were used in the analysis when available.

Blood sample collection

Fasting blood samples were obtained either the day before surgical operation or other treatments (baseline) and on day 5 after surgery, using serum separation tubes (BD Healthcare, Franklin Lakes, NJ, USA). The blood samples were transported to laboratory within 30 minutes of collection and centrifuged at 3,000 rpm at 4°C for 10 minutes; the serum was collected into a 2 mL centrifuge tube and stored at -80°C until required.

Enzyme-linked immunosorbent assay (ELISA)

The Quantikine® ELISA human VEGF, VEGFR2/KDR, and OPN immunoassay (catalog number DEV00, DVR-200, and DOST00) kits were purchased from R & D Systems, Inc. (Minneapolis, MN, USA) following the manufacturer's instructions. In brief, all required reagents, working standards, and sample dilutions were prepared and stored properly prior to the assay sampling. A plate layout was prepared and 50 µL or 100 µL of standard, control, or sample was added to corresponding wells. The plate was covered with an adhesive strip and incubated for 2 hours at room temperature (for VEGFR2 and OPN assay, 100 µL of Assay Diluent RD-1W was added to each well prior to this step). After incubation, all liquid in wells were aspirated followed by adding 400 µL of wash buffer into each well and washed 3 times. After the last wash, the plate was inverted and blotted against clean paper towels to drain the remaining wash buffer. This was followed by adding 200 µL of human VEGF, or VEGFR2, or OPN conjugate to each well, covered with a new adhesive strip, and incubated for 2 hours at room temperature. The washing procedures mentioned above were repeated, and then 200 µL of substrate solution was added into each well and incubated for 25 minutes at room temperature while protecting from light. Following this step, 50 µL of stop solution was added to stop the color development, then the plate was gently tapped to ensure thorough mixing and the OD 450 nm was measured immediately on a microplate reader.

Statistical analysis

The statistical analysis was performed using the SPSS 18.0 version of which the independent *t*-test, paired *t*-test, and binary logistic analysis were performed. A p-value less than 0.05 was considered significant. The receiver operating characteristic (ROC) curve was analyzed using the MedCal software and the area under ROC curve (AUC) was obtained.

RESULTS

In this study, the serum levels of VEGF, VEGFR2, and OPN from 75 gastrointestinal cancer patients were analyzed and compared with control subjects as well as correlated with other biomarkers and compared with clinical parameters. The basic characteristics of patients utilized and the summary of the analytical results are shown in Table 1.

The scatter plots and the analytical performance of serum VEGF, VEGFR2, and OPN analysis

Figure 1 shows the scatter plots of serum VEGF, VEGFR2, and OPN from gastrointestinal cancer patients and control subjects.

In order to evaluate the analytical performance of serum VEGF, VEGFR2, and OPN, the receiver operating characteristic (ROC) curve analysis was performed. The results showed that for VEGF, an area under the curve (AUC) of 0.749 was obtained with the Youden index as 0.440; the specificity and sensitivity under this index were 86.7% and 57.3%, respectively. The cutoff value was 772 pg/mL. While for VEGFR2, an AUC of 0.758 was obtained with the Youden index as 0.427, the specificity and sensitivity under this index were 86.7% and 56.0%, respectively. The cutoff value was 1,379 pg/mL. The AUC for OPN alone was 0.578 with the Youden index of 0.213, and the specificity and sensitivity of 44.0% and 77.3%, respectively. The cutoff value was 1.7 ng/mL. The results of logistic regression analysis on the performances of inter-combination analysis were as followings: AUC: 0.823, Youden index: 0.520, specificity: 88.0%, sensitivity: 64.0%, the cutoff (prediction value): > 0.5201 (VEGF + VEGFR2); AUC: 0.748, Youden index: 0.440, specificity: 88.0%, sensitivity: 56.0%, the cutoff (prediction value): > 0.5471 (VEGF + OPN); AUC: 0.762, Youden index: 0.413, specificity: 85.3%, sensitivity: 56.0%, the cutoff (prediction value): > 0.566 (VEGFR2 + OPN) (Figure 2).

Serum VEGF, VEGFR2, and OPN levels in pre-operative and post-operative gastrointestinal cancers and in control subjects

The serum VEGF and VEGFR2 levels in pre-operative gastrointestinal cancer patients were significantly higher than in control subjects (75 vs. 75, p = 0.000 for both); the levels of VEGF, VEGFR2, and OPN in correlated post-operative patients were significantly higher than in control subjects as well (70 vs. 75, p = 0.000, 0.000, and 0.001, respectively). The post-operative VEGF and OPN levels were significantly higher than those of pre-operative levels (70 vs. 70, p = 0.000 and 0.008, respectively); however, there was no significant difference between the pre-operative and control OPN, and pre-operative and post-operative VEGFR2 levels (p = 0.174, p = 0.292, respectively, Table 2).

Correlation of serum VEGF, VEGFR2, and OPN levels with serum carcinoembryonic antigen (CEA) level in pre-operative gastrointestinal cancers patients

CEA is a common biomarker used in the follow-up of gastrointestinal cancer management. In order to understand whether there was any correlation between serum VEGF, VEGFR2, and OPN levels and serum CEA, the pre-operative VEGF, VEGFR2, and OPN concentrations were correlated with serum CEA (from clinical laboratory data). The results showed that there was no significant correlation among these biomarkers (p = 0.587, 0.547, and 0.381, respectively, Table 3).

Correlation of serum VEGF, VEGFR2, and OPN levels with tumor Ki-67 scores in pre-operative gastrointestinal cancers

Ki-67 is a nuclear protein which is associated with cell proliferation and expresses during all phases of the cell cycle except G0. In addition, Ki-67 is associated with ribosomal RNA transcription. Ki-67 acts as an organizer of chromosome periphery. Inactivation of antigen Ki-67 leads to inhibition of ribosomal RNA synthesis, thus halting the cell cycle progression. In order to determine whether the serum VEGF, VEGFR2, and OPN levels were associated with cell proliferation, the correlation between serum VEGF, VEGFR2, and OPN with Ki-67 scores (from pathology data) in tumor tissues was analyzed. Based on the clinical data on Ki-67 scoring results, a 25% score was set as the cutoff value in this analysis. The results showed that the pre-operative serum VEGF was significantly correlated with the tumor Ki-67 scores (p = 0.027), however, there was no significant correlation between the serum VEGFR2, OPN and tumor Ki-67 scores (p = 0.542 and 0.394, respectively, Table 4).

Evaluation of serum VEGF, VEGFR2, and OPN levels as a potential biomarker for gastrointestinal cancers

In order to evaluate the serum VEGF, VEGFR2, and OPN levels as potential biomarkers for gastrointestinal cancers, univariate logistic regression analysis was performed using experimental data and patients' clinical features including tumor size, TNM stages, histological grading, tumor stage, lymph node involvement, gender, and age. The results show (Table 5) that pre-operative serum VEGF level was significantly higher in high TNM stages (stages III + IV) than in low TNM stages (stage I + II) (p = 0.031). The pre-operative serum VEGF was also higher in patients with lymph node in-

Table 1. Basic characteristics of patient collectives.

Characteristic	n (%)	Median (range)
Gender		
Male	49 (65.3%)	
Female	26 (34.7%)	
Age		60 (36 - 83)
< 50 years	16	
≥ 50 years	59	
No. of patients who had surgery	70	
No. of patients who had no surgery	5	
Histological type		
Adenocarcinoma	73	
Squamous cell carcinoma	2	
Gastrointestinal cancer types		
Colorectal cancer	53	
Esophagus cancer	2	
Gastric cancer	20	

Table 2. Comparison of serum VEGF, VEGFR2, and OPN concentrations in pre- and post-operative gastrointestinal cancers and normal controls.

Category	n	VEGF ($\bar{x} \pm s$, pg/mL)	p	VEGFR2 ($\bar{x} \pm s$, pg/mL)	p	OPN ($\bar{x} \pm s$, ng/mL)	p
* Pre-op vs. controls	75/75	1,063 ± 901 vs. 507 ± 315	0.000	1,478 ± 551 vs. 1,024 ± 340	0.000	3.06 ± 2.29 vs. 2.59 ± 1.91	0.174
* Post-op vs. controls	70/75	1,398 ± 922 vs. 507 ± 315	0.000	1,447 ± 476 vs. 1,024 ± 340	0.000	3.94 ± 2.58 vs. 2.59 ± 1.91	0.001
Δ Pre-op vs. post-op	70/70	1,070 ± 916 vs. 1,398 ± 922	0.000	1,520 ± 539 vs. 1,447 ± 476	0.292	3.05 ± 2.34 vs. 3.94 ± 2.58	0.008

*** - independent-samples *t*-test, Δ - paired-samples *t*-test, pre-op - pre-operative, post-op - post-operative.**

Table 3. Correlation of pre-operative serum VEGF, VEGFR 2, and OPN concentrations with carcinoembryonic antigen values.

	n	Pre-op VEGF ($\bar{x} \pm s$, pg/mL)	p	Pre-op VEGFR2 ($\bar{x} \pm s$, pg/mL)	p	Pre-op OPN ($\bar{x} \pm s$, ng/mL)	p
CEA (µg/L)			0.587		0.547		0.381
≥ 5	20	1,175 ± 1,414		1,402 ± 525		3.06 ± 2.11	
< 5	47	1,038 ± 654		1,488 ± 536		2.67 ± 1.40	

CEA - carcinoembryonic antigen.

volvement (N1 + N2 + N3) than in patients without lymph node involvement (N0) (p = 0.028). The pre-operative serum OPN level was significantly higher in patients with metastasis (M1) than patients without metastasis (M0) (p = 0.042).

Multivariate logistic regression analysis results showed

Table 4. Correlation of pre-operative serum VEGF, VEGFR2, and OPN concentrations with Ki-67 scores.

	n	Pre-op VEGF ($\bar{x} \pm s$, pg/mL)	p	Pre-op VEGFR2 ($\bar{x} \pm s$, pg/mL)	p	Pre-op OPN ($\bar{x} \pm s$, ng/mL)	p
Ki-67			**0.027**		**0.542**		**0.394**
≥ 25%	**59**	**1,193 ± 962**		**1,530 ± 519**		**3.16 ± 2.50**	
< 25%	**10**	**499 ± 247**		**1,415 ± 712**		**2.46 ± 1.45**	

Table 5. Evaluation of serum VEGF, VEGFR2, and OPN as a potential biomarker for gastrointestinal cancers (Univariate analysis).

	n	Pre-op VEGF ($\bar{x} \pm s$, pg/mL)	p	Pre-op VEGFR2 ($\bar{x} \pm s$, pg/mL)	p	Pre-op OPN ($\bar{x} \pm s$, ng/mL)	p
Age			**0.255**		**0.598**		
≥ 50	**59**	**1,030 ± 958**		**1,460 ± 511**		**3.21 ± 2.49**	**0.255**
< 50	**16**	**1,090 ± 700**		**1,543 ± 596**		**2.48 ± 1.18**	
Gender			**0.465**		**0.933**		**0.056**
Male	**49**	**1,119 ± 1,045**		**1,481 ± 546**		**3.42 ± 2.59**	
Female	**26**	**958 ± 534**		**1,470 ± 527**		**2.37 ± 1.38**	
Tumor size (cm)			**0.658**		**0.180**		**0.568**
≥ 5	**26**	**1,007 ± 688**		**1,408 ± 584**		**3.26 ± 1.60**	
< 5	**44**	**1,108 ± 1,033**		**1,587 ± 505**		**2.93 ± 2.69**	
TNM stage			**0.031**		**0.267**		**0.237**
III + IV	**32**	**1,321 ± 1,157**		**1,395 ± 552**		**3.42 ± 1.94**	
I + II	**43**	**871 ± 593**		**1,539 ± 549**		**2.79 ± 2.51**	
Tumor stage			**0.160**		**0.365**		**0.640**
T3 + T4	**51**	**1,165 ± 1,017**		**1,485 ± 546**		**2.97 ± 1.78**	
T1 + T2	**19**	**817 ± 506**		**1,617 ± 519**		**3.27 ± 3.48**	
Lymph node involvement			**0.028**		**0.672**		**0.335**
N1 + N2 + N3	**31**	**1,345 ± 1,157**		**1,474 ± 534**		**3.41 ± 1.90**	
N0	**40**	**868 ± 598**		**1,530 ± 565**		**2.80 ± 2.60**	
Metastasis			**0.614**		**0.501**		**0.042**
M1	**8**	**1,216 ± 444**		**1,353 ± 616**		**4.60 ± 1.45**	
M0	**67**	**1,045± 941**		**1,492 ± 546**		**2.87 ± 2.31**	
Histological grading			**0.812**		**0.215**		**0.939**
Low grade	**21**	**1,103 ± 664**		**1,350 ± 566**		**3.02 ± 1.69**	
Medium and High grade	**54**	**1,048 ± 983**		**1,527 ± 543**		**3.07 ± 2.50**	

Table 6. Multivariate analysis of experimental data and clinical features of gastrointestinal cancers (Binary logistic regression).

	B	S.E	Wals	df	Sig.	Exp (B)
Gender	**0.522**	**0.415**	**1.580**	**1**	**0.209**	**1.666**
Age	**-0.032**	**0.021**	**2.236**	**1**	**0.135**	**0.969**
VEGF	**0.002**	**0.001**	**12.251**	**1**	**0.000**	**1.002**
VEGFR2	**0.002**	**0.001**	**13.993**	**1**	**0.000**	**1.002**
OPN	**0.068**	**0.108**	**0.396**	**1**	**0.529**	**1.070**
Constant	**-2.579**	**1.476**	**3.055**	**1**	**0.080**	**0.076**

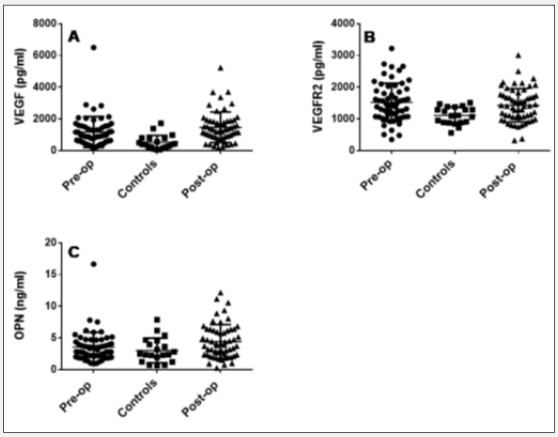

Figure 1. Scatter plots of serum VEGF, VEGFR2, and OPN concentrations in gastrointestinal cancer patients and in control subjects.

The scatter plot analysis shows that serum VEGF, VEGFR2, and OPN from gastrointestinal cancer patients and control subjects, showing the distribution of data.

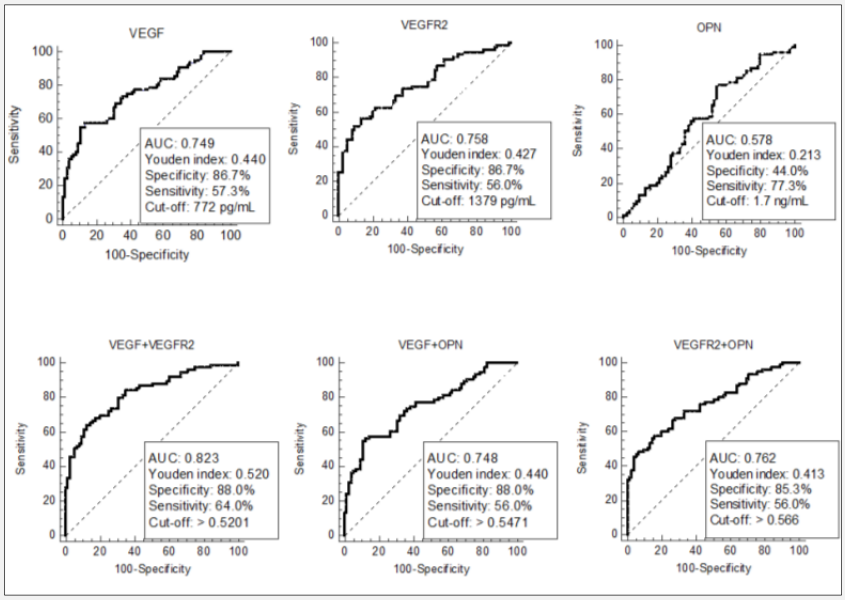

Figure 2. The ROC curve and the performance of serum VEGF or VEGFR2, or OPN alone, and the inter-combinatorial analysis of three biomarkers.

The ROC curve analysis shows that for VEGF, an area under curve (AUC) of 0.749 was obtained with the Youden index as 0.440, the specificity and sensitivity under this index are 86.7.0% and 57.3%, respectively. The cutoff value for the assay was 772 pg/mL (A). While for VEGFR2, an AUC of 0.758 was obtained with the Youden index as 0.427, the specificity and sensitivity under this index are 86.7% and 56.0%, respectively. The cutoff value for the assay was 1,379 pg/mL (B). For OPN, an AUC of 0.578 was obtained with the Youden index as 0.213, the specificity and sensitivity under this index are 44.0% and 77.3%, respectively. The cutoff value for the assay was 1.7 ng/mL (C). D to F shows the performance of inter-combinatorial analysis of three biomarkers: AUC: 0.823, Youden index: 0.520, specificity: 88.0 %, sensitivity: 64.0%, the cutoff (prediction value of performance for combinatory analysis, same below): > 0.5201 (VEGF + VEGFR2); AUC: 0.748, Youden index: 0.440, specificity: 88.0%, sensitivity: 56.0%, the cutoff: > 0.5471 (VEGF + OPN); AUC: 0.762, Youden index: 0.413, specificity: 85.3%, sensitivity: 56.0%, the cutoff: > 0.566 (VEGFR2 + OPN).

that serum VEGF and VEGFR2 were the two most important factors for the diagnosis of gastrointestinal cancers in this study ($p = 0.000$, for both, Table 6).

DISCUSSION

Tumor biomarkers play an important role as indicators of cancer diagnosis, progression, and prognosis. The soluble tumor biomarkers are especially useful in clinical practice because of the noninvasive properties associated with their measurement, their ease of handling, and the advantage of being low cost. However, none of the tumor biomarkers used today possess both high specificity and sensitivity. Thus, they are insufficient as an early screening tool of cancer. Furthermore, results from tumor biomarker measurement during disease progression cause confusion in clinical interpretation and treatment decisions.

Vascular endothelial growth factor (VEGF) is a secreted cytokine implicated in angiogenesis, tumorigenesis, and vascular permeability [14,27]. Soluble VEGF is an autocrine cytokine of different types of cells [7,28,29]. VEGF binds to its receptor VEGFR2 (KDR/Flk-1), which then is activated and triggers cellular signaling cascades [30]. Therefore, VEGF and VEGFR2 have been the targets of cancer chemotherapy [31-33].

Studies have found that serum or pleural effusion VEGF and VEGFR2 levels were altered in multiple cancers and might be potential cancer biomarkers in either diagnosis or evaluation of prognosis [7-18,34].

In this study, we found that the pre-operative and post-operative serum VEGF and VEGFR2 and post-operative serum OPN were significantly higher than in control subjects. Furthermore, post-operative VEGF and OPN levels were significantly higher than that of pre-operative levels (Table 2). Chen et al. observed serum VEGF levels prior to treatment, and 1 day, 7 days, and 30 days after percutaneous microwave coagulation treatment in primary hepatocellular carcinoma patients. They reported that the serum VEGF had no significant difference between day 1 post treatment and baseline (pretreatment), however, it was reduced significantly 7 days post treatment, while it slightly increased on day 30 post treatment in primary hepatocellular carcinoma patients. Thus, serum VEGF level was an effective hematologic evaluation index of percutaneous microwave coagulation treatment for primary hepatocellular carcinoma [35]. In another report, the serum VEGF-A level was decreased in metastatic colorectal cancers on day 21 after regorafenib salvage therapy, and this phenomenon was significantly associated with a better progression-free survival. Thus, serum VEGF-A level may serve as a potential predictive marker for survival in metastatic colorectal cancer patients [11]. In bladder cancer, the baseline serum VEGF level was associated with patients' overall survival (OS), cancer survival (CS), and bladder cancer survival (BCS), while the serum VEGF levels 12 months after treatment did not show significant correlations with the assessment of disease progression. Patients with high baseline serum VEGF levels had significant shorter OS, CS, and BCS. Therefore, serum VEGF was a significant predictor of overall and cancer death [12]. In gastric cancer patients, serum VEGF levels were significantly higher in patients than in healthy controls and were reduced significantly on day 7 and day 30 after radical resection [36,37]. Blank et al. also reported that high serum VEGF level in gastric cancers was associated with poor prognosis [38]. Our results showed that serum VEGF and OPN levels on day 5 after operation were significantly higher than levels before operation (baseline). The principle and mechanism of this phenomenon is unknown, however, this could be interpreted as traumatic response to the surgery since the secretion of VEGF is affected by stress response [39-43]. Further study is needed to interpret this circumstance and mechanism. In addition, Loosen et al. found that pre- and post-operative elevated serum OPN level was significantly associated with poor post-operative survival of cholangiocarcinoma. Thus, serum OPN concentrations represent a promising prognostic biomarker in patients with resectable CCA which could help to guide pre-operative treatment decisions and to identify patients that will particularly benefit from extended liver surgery [44].

While serum VEGFR2 is a naturally occurring soluble form of the receptor caused by proteolytic hydrolysis of the membrane form of VEGFR2 [45]. The biological significance of the soluble form of VEGFR2 (sVEGFR2) has been suggested in several studies [46-49]. The sVEGFR2 could inhibit angiogenesis by binding to its ligand, VEGF, which blocks the binding of VEGF to VEGFR2 [45,46]. Our study results indicated that pre-operative and post-operative VEGFR2 levels were elevated alongside VEGF compared with control subjects (Table 2), which indicates that there is a need in the balance of the receptor to its ligand, hence, more proteolytic hydrolysis of the receptor occurred during the course of the cancer. Zheng et al. analyzed the serum VEGFR2 in 169 patients with unresectable hepatocellular carcinoma before and after undergoing transarterial chemoembolization. Serum VEGFR2 concentrations decreased in 44 (26.0%) patients at week 4. Patients who had a VEGFR2 response at week 4 had a longer median survival than those who did not have a VEGFR2 decrease. Thus, the reduction of serum VEGFR2 after transarterial chemoembolization may predict favorable overall survival in patients with advanced hepatocellular carcinoma. In this context, serum VEGFR2 may serve as an index of prognosis for these patients. When correlating the pre-operative serum VEGF, VEGFR2, and OPN levels with serum CEA levels (based on a cutoff of 5 µg/L), we found that there was no significant correlation among these biomarkers ($p = 0.587$, $p = 0.547$, and $p = 0.381$, respectively, Table 3).

The antigen Ki-67 is a nuclear protein that is associated with and may be necessary for cellular proliferation and is widely used in clinical practice. Furthermore, it is as-

sociated with ribosomal RNA transcription [50]. In this study, we correlated the serum VEGF, VEGFR2, and OPN levels with Ki-67 scores in tumor tissues. The results showed that the pre-operative serum VEGF was significantly correlated with the tumor Ki-67 scores based on a cutoff of 25% ($p = 0.027$); however, there was no significant correlation between the serum VEGFR2, OPN, and tumor Ki-67 scores ($p = 0.542$ and $p = 0.394$, respectively, Table 4). To the best of our knowledge, this is the first report on the association of the proliferation marker Ki-67 with serum VEGF levels which may be useful as combination testing in gastrointestinal cancers for prediction of prognosis.

Guo et al. reported that serum VEGF levels were significantly related to tumor stage and lymph node and lung metastasis in liver cancer patients [10]. Another report indicated that serum VEGF level in gastric cancers was significantly correlated with degree of tumor cell differentiation, clinical stages, tumor infiltration depth, lymph node metastasis, and tumor size [51]. Our study results (Table 5) showed that pre-operative serum VEGF level was significantly higher in high TNM stages (stages III + IV) than in low TNM stages (stage I + II) ($p = 0.031$). The pre-operative serum VEGF was also higher in patients with lymph node involvement (N1 + N2 + N3) than in patients without lymph node involvement (N0) ($p = 0.028$). The pre-operative serum OPN level was significantly higher in patients with metastasis (M1) than patients without metastasis (M0) ($p = 0.042$). Ock et al. reported that in gastric cancer patients, high-serum VEGF was correlated with poor overall survival; however, the VEGFR2 level had no association with survival. The authors suggested that VEGF and VEGFR2 have distinct cytokine angiogenic factor signatures. High VEGF/VEGFR2 ratio was significantly correlated with worse overall survival. Combination analysis of serum VEGF and VEGFR2 confers more accurate prognostic implications [14].

Multivariate logistic regression analysis results showed that serum VEGF and VEGFR2 were the two most important factors for the diagnosis of gastrointestinal cancers in the analysis ($p = 0.000$, for both, Table 6). This may be a new set of tumor markers with clinical potential.

Our results indicate that VEGF analysis alone had a moderate performance (AUC: 0.749, Youden index: 0.440, specificity: 86.7%, and sensitivity: 57.3%), while VEGFR2 analysis alone had a similar performance (AUC: 0.758, Youden index: 0.427, specificity: 86.7%, and sensitivity: 56.0%), Figure 2. Inter-combinatorial analysis improved the performance assays and the specificity and sensitivity were both improved, Figure 2. Previous studies have shown that OPN displayed a low sensitivity of 30.2% when the specificity was fixed at 90% [7]. In our study, the OPN analysis alone had a relatively poor performance with an AUC of 0.578, a specificity of 44.0% and sensitivity of 77.3%; however, combination analysis of VEGF with OPN increased the assay specificity to 88.0% with an AUC of 0.748; combination analysis of VEGFR2 with OPN also increased the specificity of OPN alone from 44.0% to 85.3%. Altogether, the results indicate that the serum VEGF and VEGFR2 are potential biomarkers for the diagnosis and prognosis of gastrointestinal cancers, while serum OPN is a biomarker correlated with metastasis of gastrointestinal cancers. Inter-combinational analysis of serum VEGF, VEGFR2, and OPN further improved the assay performance, and thus, may be useful in the diagnosis and prognostic evaluation of gastrointestinal cancers.

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Declaration of Interest:
There is no conflict of interest to this work.

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