Serum vascular endothelial growth factor receptor-3 levels in patients with esophageal squamous cell cancer

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AIM: *Esophageal cancer is one of the most aggressive tumors of the gastrointestinal tract. In this study, we quantified the serum vascular endothelial growth factor-3 (VEGFR-3) expression in patients with esophageal squamous cell carcinoma (ESCC) to evaluate the role of VEGFR-3 in ESCC.*
MATERIALS AND METHODS: *Ninety five patients with ESCC were studied. Pre-therapy and preoperative samples were stored and ELISA was used to designate the concentrations of VEGFR-3.*
RESULTS: *The serum values of VEGFR-3 were significantly higher in patients with ESCC than in healthy donors ($p<0.0001$).*
CONCLUSIONS: *The results imply a very good sensitivity of VEGFR-3 in ESCC. VEGFR-3 may be a good diagnostic biomarker for ESCC.*

KEY WORDS: Biomarker, ESCC, VEGFR-3

Introduction

Esophageal squamous cell carcinoma (ESCC) is the eighth most common cancer in the world and is the sixth leading cause of cancer mortality in the eastern population of Turkey [1,2].
As the stage of disease has a definite impress on survival, diagnosis at earlier stages is highly critical. Lymphatic spread of ESCC cells to regional lymph nodes is one of the earliest events associated with distant metastasis and poor prognosis [3]. The depth of invasion, lymph node metastasis and the presence of distant metastasis have all been found essential prognostic factors [4-11]. Therefore, it is imperative to find new markers, particularly serum markers, to facilitate the early detection and diagnosis of ESCC.
Vascular endothelial growth factor (VEGF) is a dimeric, heparin-binding glycoprotein that can be expressed in four isoforms, which have 206, 189, 165 and 121 amino acids [12]. These domains have important functional consequences for the VEGF splice variants, as the terminal (exon 8) splice site determines whether the proteins are pro-angiogenic (proximal splice site, expressed during angiogenesis) or anti-angiogenic (distal splice site, expressed in normal tissues). VEGF inspires endothelial

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cells growth and functions as an effective mitogen of vascular endothelial cells, providing a resource for their migration and the neovascularization of micrometastasis [13].

All members of the VEGF family stimulate cellular responses by binding to tyrosine kinase receptors on the cell surface, causing them to dimerize and become activated through transphosphorylation, although to different sites, times and extents. The VEGF receptors have an extracellular portion consisting of 7 immunoglobulin-like domains, a single transmembrane spanning region, and an intracellular portion containing a split tyrosine-kinase domain. Activation of VEGF/VEGF-receptor (VEGFR) axis precipitates multiple signaling pathways that results in endothelial cell survival, mitogenesis, migration and differentiation, and vascular permeability. VEGF-C and VEGF-D are the best defined lymphangiogenic growth factors. These factors cause lymphangiogenesis by activating VEGFR-3, also known as Flt (fms-like tyrosine kinase)-4, a receptor which is expressed in the lymphatic endothelium [14-18].

VEGFR-3 may be defined as a marker of lymphatic endothelial cells because it is certainly expressed in the lymphatic endothelium of adult tissue [19]; at the same time VEGFR-3 has also been designated in the blood vessels within tumors and wounds that are healing [20, 21]. A few studies have featured VEGFR-3 expression in a variety of cancers including gastric [22], small cell lung cancer [23], and in metastatic melanoma patients [24]. Moreover, the high VEGFR-3 levels have been correlated with poor prognosis in breast cancer [25] and non-small cell lung cancer [26,27]. The effect of VEGFR-3 in many cancers and especially esophageal cancer has not been properly explained. So, we conducted this study to investigate the serum VEGFR-3 levels in patients with ESCC. That acted as a trending marker, in that it means levels increased/decreased from healthy controls to high-risk subjects and then ESCC patients.

Patients and Methods

The patient group comprised ninety five (40 females and 55 males) patients with esophageal cancer and the control group comprised forty healthy (18 females and 22 males) subjects. The age range was 40-65 years (mean 53.7 ± 9.5 years) for the esophageal cancer group and 39-63 years (mean 52.9 ± 7.8 years) for the control group.

None of the cases involved in our study had undergone chemotherapy and radiotherapy prior to sampling. Informed consent was obtained from all participants for the use of their blood samples in the study. This project was approved by the Ethics Committee of the Yuzuncu Yıl University Medical Faculty, Van, Turkey. Written informed consent was obtained from each subject following a detailed explanation of the objectives and protocol of the study which was conducted in accordance with the ethical principles stated in the "Declaration of Helsinki" and approved by the institutional ethics committee. CMJE principles were totally obeyed. The clinical diagnosis of ESCC was confirmed by a pathologist who examined the biopsy samples obtained during gastroscopy. Healthy volunteers were recruited as normal controls.

Blood samples were collected from each patient before surgery. Venous blood samples were centrifuged at 3000 x g to obtain serum samples and stored at -80º C until assayed. Serum levels of VEGFR-3 were analyzed with enzyme-linked immune-sorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA). An ELISA component kit that measures the extracellular (soluble) domain of VEGFR-3 was employed. Serum VEGFR-3 assays were calibrated against recombinant proteins that consisted of the full-length extracellular domain of the respective receptors. A 96-well micro-plate was shielded with diluted hold antibody, and incubated overnight.

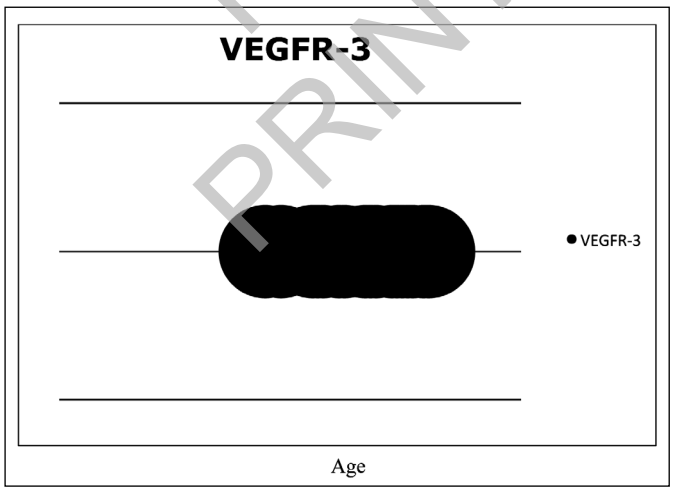

Fig. 1: Graphic of the age distribution of the control group.

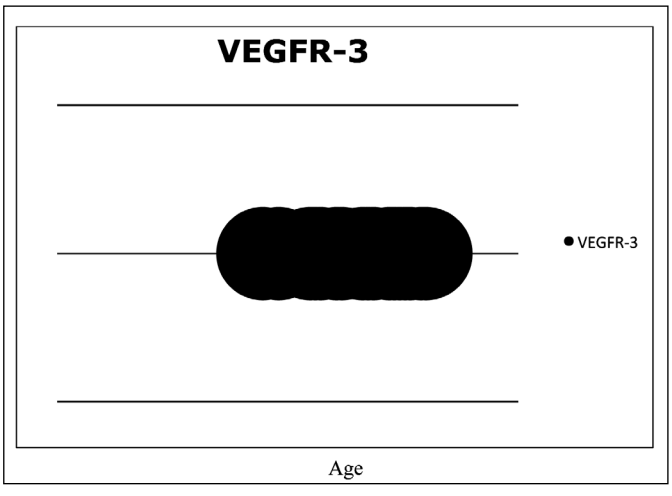

Fig. 2: Graphic of the age distribution of the patients.

After washing, the plate was blocked by adding diluent reagent. Plate preparation was finished. Samples or standards were added, then the plates were washed, detection antibody was added, and washing was repeated. Streptavidin-horseradish peroxidase was added to each well. After washing, substrate solution was added to each well. Finally, stop solution was added to each well. The plate was tapped gently. The optical densities of each well were quantified within 30 min at dual wave-lengths of 450 nm corrected to 540 nm using a micro-plate reader.

Statistical Analysis

Shapiro Wilk normality and Student *t*-tests, assessment and histogram charts were drawn. Defining the average values, standard deviation, median, minimum and maximum given in the form. Analysis, independent samples t-test (normally distributed variables), Mann-Whitney U test, Kruskal-Wallis one-way analysis of variance and Bonferroni correction after the Mann-Whitney U test were used for binary comparisons. The limit of significance was set at $P<0.05$. Analyses were performed using an SPSS 17.0 program.

Results

Serum VEGFR-3 levels were detected in all the patients and healthy controls. Serum VEGFR-*3* levels varied in healthy donors, and the median level [range: 9.5, 95% confidence interval (CI):]. In esophageal cancer, the median VEGFR-3 level was (range: 78.4, 95% CI). A highly significant difference was found in the median VEGFR-3 level between esophageal cancer patients ($p< 0.0001$) and healthy donors (Table I).

We used receiver operating characteristics (ROC) curves to determine the cut-off values and sensitivity and specificity of serum VEGFR-3 test in the patients. The cut-off value was chosen according to the ROC curve coordinate points and the cut-off point for serum VEGFR-3 was equal to its mean value. The sensitivities and specificities determined from the ROC curves at a cut-off level of 62 were 93% and 87% for serum VEGFR-3 respectively.

Table I - *The expression of VEGFR-3 in the normal controls and in patients with ESCC.*

	Controls (n=40)	Patients (n=95)	p
Age (year)	52.9 ± 7.8	53.7 ± 9.5	p>0.05
VEGFR-3	9.5 ± 1.5	78.4 ± 29.6	p<0.0001

Discussion

In this study, serum VEGFR-3 levels in esophageal cancer were independent marker for the patients. The biological implications of VEGF for tumor growth are not fully understood yet. Additionally, the biological effects of VEGFR and VEGFR-3 in particular, are not known. Increased VEGFR-3 levels are related to regional lymph node metastasis in colorectal cancers (28-30) and in gastric cancers [31]. Also, VEGFR-3 levels are related to lymphatic metastasis, generally via tumor lymphangiogenesis in animal models and human tumors [32,33].

VEGFR-3 belongs to the family of tyrosine kinase receptors and is able to activate the FAK/Rac-1 pathway [34-36]. Garces et al showed that the NH2-terminus of VEGFR-3 was related to the COOH terminus of FAK, and corruption of VEGF/FAK interactivity results in the dispersion of FAK from the focal adhesions [35].

Expressions of VEGFR-3 are associated with portal vein invasion, tumor recurrence, lymphangiogenesis, and shorter disease-free survival in hepatocellular carcinoma [37,38]. Also, a few studies have searched the association between the expression of VEGFR-3 and tumor stage [39,40]. It has been indicated that the activation of VEGFR-3 contributes in cell proliferation, migration, and survival [41].

The invasion of cancer cells into contiguous tissues is a crucial step in the process of tumor metastasis [42]. Development of the migration capacity plays an important role in cancer cell invasion [43]. In this study, we demonstrated the stimulatory effects of VEGR-3 expression on the migration and invasion and metastasis of ESCC.

Another effect of the expression of the VEGFR-3 is the stimulation of lymphatic vessel permeation, lymph node metastasis and tumor differentiation [28-33].

The process of tumor cells follows a series of complex biologic reactions. In the initial phase, tumor cells first invade the stroma. The expression of growth factors such as VEGF-C, platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) from tumor cells increases the expression VEGFR-3, which initiates lymphangiogenesis followed by the development of new lymphatic capillaries and increases the number of LYVE-1-secreting cells [44]. The elevated VEGFR-3 and LYVE-1 expressions are highly important because they reflect lymphangiogenesis, indicating how tumor cells might be lightly attained the lymphatic system [45].

VEGFR-3 provides information on the behavior of the tumor, the expression and over-expression of the VEGFR-3 were increased by the existence of neural or perineural invasion as well as vascular invasion; in our study, we also investigated the correlation between elevated VEGFR-3 values. Although we could not find the reason for these results, we thought that these conflicting findings might be unexplainable by the low binding affinity of the antibody in ESCC via an unexplained

mechanism or by the existence of other molecules that could promote lymphangiogenesis. Further studies are needed to elucidate the cause of this apparent conflict. The development of tumor cells is one of the important mechanism which contribute to the progression of cancer. Because inhibitors that block the VEGF-C/VEGF-D/VEGFR-3 signaling pathway might potentially block the process of the tumor cells, the VEGF-C/VEGF-D/VEGFR-3 interference has been largely searched as a feasible target for cancer treatment. Probable therapeutics comprise soluble versions of VEGFR-3 that bind VEGF-C and VEGF-D, thereby inhibiting activation of endogenous VEGFR-3, neutralizing monoclonal antibodies to VEGF-C and VEGF-D that inhibit binding to both VEGFR-2 and VEGFR-3, monoclonal antibodies to VEGFR-3, and small molecules that inhibit VEGFR-3 tyrosine kinase or downstream signaling molecules [46].

Downstream of VEGFR-3, the nuclear factor of activated T cells and FoxC2 cooperatively control the expression of the set genes required for the differentiation of lymphatic capillaries and valves [47]. The collecting of the tumor cells down-regulates FoxC2, which leads to decreased expression of VEGFR-3 [47]. Also, promotes circumferential enlargement of the collecting vessels, transport of tumor cells, as well as accommodation of larger tumor cell clusters [48].

Furthermore, growth factors produced by the primary tumor appear to act at a distance by inducing lymphangiogenesis in the sentinel lymph node even before the arrival of the first metastatic cells [49-51].

In our priory study, we analyzed the utility of serum VEGFA, VEGFC, and VEGFRI levels as an importance marker in cachectic patients with esophageal carcinoma [52]. ESCC have a high morbidity and mortality rate mainly because of their high invasive and metastatic potential and high recurrence rates. The often late diagnosis of this cancer is one of the main problems.

We propose that VEGFR-3 may also be a more valuable marker for ESCC has been appreciated. VEGFR-3 may therefore not only have value as a prognostic marker but possibly also as a marker for predicting response to adjuvant treatment in advance ESCC. Whether VEGFR-3 itself could be a drug target depends on whether it is causally involved in facilitating the metastatic process.

Conclusion

Our results clearly suggested that serum VEGFR-3 levels have the potential to be a marker for ESCC. VEGFR-3 is over-expressed in many cancers; therefore, elevated VEGFR-3 may also be present in other cancers as a non-specific cancer biomarker which can deduce that the levels of VEGFR-3 detected and TNM classification of cases can mostly be used for screening the prognosis. The samples in our study were aggressive ESCC serum samples; therefore it is necessary to further validate these results using a large cohort of well-characterized patient samples, especially with clinically stage patient sample. It might be also a good approach to further analyze VEGFR-3 values with the current tumor marker in ESCC.

Riassunto

Il cancro dell'esofago è uno dei tumori più agressivi del tratto gastrointestinale. In questo studio abbiamo quantificato nel siero il VEGFR-3, espressione fattore di accrescimento dell'endotelio vascolare in pazienti portatori di tumore squamocellulare esofageo (ESCC) per valutare il ruolo del VEGFR-3 in questo tipo di cancro.

Lo studio è stato eseguito su 95 pazienti con ESCC, raccogliendo campioni sia in pre-trattamento che preoperatori usando ELISA per definire la concentrazione del VEGFR-3.

Questi valori sono risultati significativamente più elevati nei pazienti con ESCC che non in soggetti sani ($p < 0.0001$), portando a concludere per un'elevata sensibilità del VEGFR-3 nel carcinoma squamodo dell'esofago, che quindi può essere un valido marker biologico per questo tumore.

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