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# Expression of Vascular Endothelial Growth Factor (VEGF) in Individuals with Squamous Cell Carcinoma of Esophagus

Aisha Jamil, Maria Tasneem Khattak, Shams ul Hadi, Maria Khan, Sidra Mashal, Iqbal Muhammad Khan

**Abstract**

**Objective:** The aim of the study was find out the vascular endothelial growth factor (VEGF) expression in individuals with esophageal squamous cell carcinoma.

**Study Design and Setting:** The current prospective study was carried out at the department of Histopathology, Rehman College of dentistry / Rehman Medical Institute (RCD/RMI) from July 2024 to December 2024 after taking approval from the ethical board of the institute.

**Methodology:** A total of 53 esophageal squamous cell carcinoma samples were collected and placed at -80ºC. For culturing, humidified five percent carbon dioxide in air was used for cells in monolayer culture at 37°C. Esophageal squamous cell carcinoma tissue samples that were kept at -80°C and EC9706 cells were used to extract total RNA. Gel electrophoresis was used to assess the quality of the extracted RNA. Immunohistochemistry, RT-PCR and in situ hybridization were used to find out the VEGF expression. Data was analyzed using SPSS version 16.

**Results:** Vascular endothelial growth factor was expressed and secreted by EC9706 cells, confirmed by RT-PCR, in situ hybridization and immunohistochemistry staining. Using in situ hybridization, out of 53 cases with esophageal squamous cell carcinoma, 39(73.5%) had positive immunohistochemistry for VEGF. The VEGF positive rate for both metastatic and non-metastatic lymph node patients was 91% (20/22) and 61.2% (19/31) respectively. A significant difference observed in the VEGF expression between the lymph node-positive and node-negative groups ($p<0.05$).

**Conclusion:** This study concluded that in individuals with esophageal squamous cell carcinoma, expression of VEGF is an important and helpful prognostic factor.

**Key words:** Expression, vascular endothelial growth factor, Squamous cell carcinoma, esophagus

**How to cite this Article:**
Jamil A, Khattak MT, Hadi SU, Khan M, Mashal S, Khan IM. Expression of Vascular Endothelial Growth Factor (VEGF) in Individuals with Squamous Cell Carcinoma of Esophagus. J Bahria Uni Med Dental Coll. 2026;16(1):31-36 DOI: https://doi.org/10.51985/JBUMDC2025809



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Received: 04-10-2025
1st Revision: 16-11-25
Accepted: 23-12-2025
2nd Revision: 25-11-25

## INTRODUCTION

In terms of incidence esophageal cancer ranks seventh in the world accounting for 604,000 new cases & 544,000 deaths each year.[1] The two most prevalent histologic subtypes of esophageal cancer, esophageal squamous cell carcinoma (ESCC) & esophageal adenocarcinoma (EAC), have somewhat different incidence rates. The prevalence of these cancer depend on the location and the economic conditions of a country.[2] Although ESCC incidence has lately decreased it still accounts for almost half of all new ESCC cases worldwide[3] According to statistics on cancer epidemiology, dietary carcinogen exposure, alcohol consumption, tobacco use, and micronutrient deficiencies will all contribute to ESCC [4]. Other suspected risk factors include chewing betel quid, eating pickled vegetables, and consuming extremely hot food and drinks[1] Lymphangiogenesis is a critical phase in tumor advancement and metastasis. Prior research has shown that tumors actively stimulate the formation of their own lymphatic networks that interface with adjacent lymphatic veins.[2] The conveyance of tumor cells via lymphatic capillaries is the predominant mechanism for early dissemination, with cancer propagation occurring via

afferent lymphatics along established drainage pathways. [1] Two VEGF family members, VEGF-C and VEGF-D, are recognized as natural ligands for VEGFR-3 and have previously been linked to lymphangiogenesis. [4]

A variety of cytokines are secreted by carcinoma cells, and these cytokines have an impact on the cells' properties. One of the important cytokines is VEGF.[5] It is believed that VEGF plays significant functions by directly promoting the migration and proliferation of endothelial cells as well as by triggering a number of proteinase activities that break down the surrounding matrix tissues. Recent research on VEGF expression in esophageal carcinoma explains how VEGF influences angiogenesis in esophageal squamous cell carcinoma, which leads to poor prognoses and cancer growth.[6] According to evidence, angiogenesis and lymph angiogenesis are promoted by the high expression of the vascular endothelial growth factor. Angiogenesis is an inherent feature of all cancers, contributes to their aggressiveness and proliferation, and is a prerequisite for metastatic development. [7] Around 50% of esophageal cases of cancer in China are of this subtype, which has a comparatively high prevalence among individuals in South America, South Africa, Iran, and China.[8] Despite significant advancements in diagnostic and treatment methods over the last three decades, the prognosis for esophageal cancer remains dismal, with 5-year rate of survival ranging from 10% to 13%.[2] One of the primary reasons for the poor prognosis is lymph node metastasis. It might be the most significant prognostic variables and is more often seen in ESCC compared to tumors in other areas of digestive tract .[3] The exact processes behind the onset and advancement of LNM in ESCC are unclear, nevertheless. Angiogenesis and lymphangiogenesis are significantly influenced by vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs) .[6] Among them is VEGF-C, which has been identified as an angiogenic and lymphangiogenic growth factor. It starts the related signal transduction pathway by binding to the VEGFR-3 and VEGFR-2 receptors.[9] In a number of tumor forms, including expression of ESCC and VEGF-C has direct association with clinicopathological characteristics.[4-10] The prognosis and clinicopathological characteristics of ESCC have been tightly linked to the VEGF-C expression in tumor tissues.[11] One of the most serious forms of gastrointestinal cancer is esophageal squamous cell carcinoma (ESCC), which has a comparatively high risk of spreading even in its early stages. Specifically, one of the most significant prognostic variables is the metastasis of lymph node.[1] To encourage metastasis to the lymph nodes and beyond, tumor cells exploit the lymphatic vascular system. [1] Tumor-induced lymphangiogenesis often serves as the first stage of tumor spread and encourages metastasis to local lymph nodes. One important predictive factor for the development of human cancer types is lymph node metastasis. According to reports, two components of the vascular endothelial growth factor (VEGF) family, VEGF-C and VEGF-D, stimulate lymphangiogenesis in addition to angiogenesis on lymphatic endothelial cells via the VEGF receptors (VEGFR)-2 and VEGFR-3. [3] These receptors promote lymphatic metastasis in addition to controlling lymphangiogenesis. [4] Furthermore, it was recently found that tumor cells express VEGF-C and VEGFR-3, both of which have been suggested as a marker for lymphatic endothelial cells. These expressions are linked to the invasion, metastasis, and progression of cancer cells. The functions of lymphangiogenesis and the VEGF-C/VEGFR-3 axis have been studied in a number of prior research. A significant contributor to nodal metastasis, lymphangiogenesis is also a predictor of prognosis for a number of carcinomas of the stomach, esophagus, cervix, prostate, colorectum and Lung. [6-12] Few findings exist on the VEGF-C expression in lymph fluid , despite the fact that serum VEGF-C has recently shown significance on both diagnostic and prognostic level.[8-11] Similarly the present study was carried out to determine the VEGF expression in individuals with esophageal Squamous cell carcinoma.

## METHODOLOGY

The current prospective study was carried out at the department of Histopathology, Rehman College of dentistry / Rehman Medical Institute (RCD/RMI) from July 2024 to December 2024 after taking approval from the ethical board of the institute. The ethical approval number for our study was RMI-REC/Ethical Approval/CPSP synopsis/42. The inclusion criteria for our study were all the ESCC patients of both the gender and all ages, without surgical management and distant metastasis. The exclusion criteria were all the patients with other type of carcinoma patients and patients treated with chemotherapy and radiation therapy before surgery. The overall sample size in our study was 53 based on the WHO sample size calculator. All the Individuals who had an incomplete resection were excluded from our study. A total of 53 esophageal squamous cell carcinoma samples were collected from the patients admitted in the Rehman Medical Institute Peshawar and placed at -80$^{o}$C. None of the patients had ever had chemotherapy or radiation treatment. Experienced pathologists clearly categorized each specimen in to grade I, II and grade III. The EC9706 human esophageal cancer cell line was obtained from the institute's research lab. For culturing, humidified five percent carbon dioxide in air was used for cells in monolayer culture at 37°C. RPMI-1640 medium was used to cultivate the cells. Esophageal squamous cell carcinoma tissue samples that were kept at -80°C and EC9706 cells were used to extract total RNA using the TRIzol Reagent. Gel electrophoresis was used to assess the quality of the extracted RNA, and the A260/280 ratio was used to assess the RNA's concentration and purity. The primer pairs used in PCR to amplify vascular endothelial growth factor and internal controls (â-actin) in all reaction were according to the previous study.[8] According to Chen et al., staining was done in tissue sections (6 ìm thick) and

the cell slides ready for hybridization.[9] Immunohistochemistry was also done according to the previous study.[9] After being stored in 10% neutral buffered formalin for 8 to 48 hours, each sample was dehydrated using xylene and alcohol. Paraffin was used to encase the dried samples. The MaxVision two-step method was used for immunohistochemical staining. Slices of tissues measuring 4 µm were cut out. Following an hour of baking at 65 °C, the slices were deparaffinized by using xylene and then ethanol gradient was used for rehydration. To inhibit the activity of endogenous peroxidases, the slices were exposed to 0.3% H2O2 for 10 minutes. Then, for VEGF-C, survivin, and Ki-67, antigen retrieval was performed under high-pressure circumstances utilizing pH 6.0, 0.01 M citrate buffer. For VEGFR-3, the retrieval was performed under conditions of high temperature employing pH 9.0 EDTA. The samples were washed three times with phosphate-buffered saline for three minutes after being allowed to settle at room temperature. The sections were incubated with the secondary antibody at 37 °C for 15 minutes after being treated with the primary antibody for an hour. After five minutes of DAB staining, the slices were counterstained for one minute with hematoxylin. Neutral gum was used to secure the slides. Instead of using the primary antibodies, PBS buffer was used to incubate the negative controls. A blinded study of the stained slides was performed by two pathologists. The color of the brownish-yellow cellular staining was positive. The lack of significant color intensity fluctuation relative to the backdrop was a defining feature of negative staining. Light microscopy was used to observe staining. After identifying the area with the highest staining intensity at low magnification (50×), 10 visual fields were examined at high magnification (400×). Each patient sample's staining of 100 cells was assessed. As previously mentioned, a semi-quantitative scoring system was used to assess staining for VEGF-C, VEGFR-3, and survivin.[9] The intensity of certain stains was used as the basis for scoring. 0 represents no staining; 1 represents light staining; 2 represents moderate staining; and 3 represents severe staining. The following semi-quantitative method was used to determine the percentage of positive cells amongst all the cells counted: Zero is negative; one is 1% to 10% positive; eleven is to 50% positive; and three is above 50 percent positive. The immunohistochemistry score was finally calculated by combining the intensity and percentage evaluations. A score of =3 on immunohistochemistry was considered positive. The following is the breakdown of the final immunohisto-chemistry scores: 5 to 7 are fairly positive (++); 8 to 9 are highly positive (+++); and 3 to 4 are mildly positive (+). The percentage of Ki-67-positive cells amongst all tumor cells was used to assess Ki-67 staining. Data was analyzed using SPSS version 16. Data analysis was done using the Student's t-test or the Chi-square test. Statistical significance was defined as values having a $P < 0.05$.

## RESULTS

A total of 53 patients were included in this study out of which 27 patients were male and female patients 26. The mean age was 59.4 (44-76 years). Vascular endothelial growth factor was expressed and secreted by EC9706 cells, confirmed by RT-PCR, in situ hybridization and immunohistochemistry staining. For VEGF, in cytoplasm of EC9706, the brown positive stained granules were found**.** figure 1. Using in situ hybridization. It was discovered that the cytoplasm of the EC9706 cells contained VEGF mRNA (blue-purple granules) as shown in figure 2. RTPCR revealed positive bands of VEGF mRNA in EC9706 cells. When at least thirty percent of the tumor cells exhibited vascular endothelial growth factor immunoreactivity, this was referred to as positive staining.[11] Out of 53 cases with esophageal squamous cell carcinoma, 39(73.5%) had positive immunohistochemistry for VEGF. The VEG positive rate for both metastatic and non-metastatic lymph node patients was 91% (20/22) and 61.2% (19/31) respectively. A significant difference observed in the VEGF expression between the lymph node-positive and node-negative groups (p?0.05). In esophageal squamous cell carcinoma participants, immunohistochemistry revealed that the lymph node-involved group had significantly greater VEGF expression than the node-negative group. Reverse transcriptase -PCR identified VEGF-C mRNA in tumor tissues in 31 out of 53 esophageal squamous cell carcinoma patients. The lymph node-involved group's expression of VEGF differed significantly from that of the involved group ($P<0.01$). Using RT-PCR, the lymph node-involved group's expression of VEGF- mRNA was noticeably greater than that of the lymph node-noninvolved group. In situ hybridization for VEGF mRNA was found to be positive in 25 out of 53 cases of esophageal cancer. In contrast to the non-metastatic lymph node group (4 of 31; 12.9%), VEGF positive staining was observed in majority of patients with metastatic lymph nodes (19 of 22; 95.40%). When comparing the carcinomas with lymph node metastases to those without, the staining was much greater in the former group ($P<0.01$). The group with lymph node involvement had substantially greater levels of VEGF-C mRNA expression than the group with and without involvement of lymph node, according to ISH technique. VEGF levels were not substantially linked with age, sex, or pathological grade as described in table 1.

## DISCUSSION

In light of the expanding understanding of the molecular pathways that govern tumor life, the pursuit of prognostic indicators has become the most dynamic areas in studying cancer. In order to determine the prognosis of patients with solid cancers, efforts are now being made to find molecular biological markers. To link markers to survival, for example, parameters related to cell cycle, development, or apoptosis have been studied.[1-3] It has been shown that the production

Table 1 Correlation between the clinical factors and the VEGF expression by 3 methods in squamous cell carcinoma of esophagus

| Features | N | mmunohistochemistry | | | in situ hybridization | | | RT-PCR | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | (+) | (-) | P Value | (+) | (-) | P Value | (+) | (-) | P Value |
| Gender | | | | | | | | | | |
| Male | 27 | 21 | 6 | > 0.05 | 17 | 10 | > 0.05 | 14 | 13 | > 0.05 |
| Female | 26 | 17 | 9 | | 14 | 12 | | 11 | 15 | |
| **Lymph node metastasis** | | | | | | | | | | |
| Positive | 22 | 20 | 2 | > 0.05 | 20 | 2 | < 0.01 | 22 | 0 | < 0.01 |
| Negative | 31 | 19 | 11 | | 4 | 27 | | 11 | 21 | |

Figure 1.In the cytoplasm of EC9706, the brown positive staining granules for VEGF

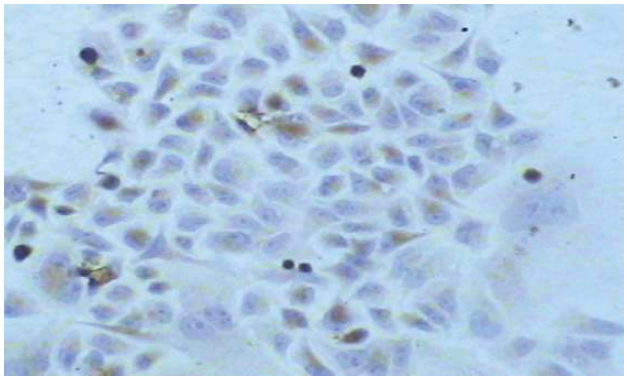
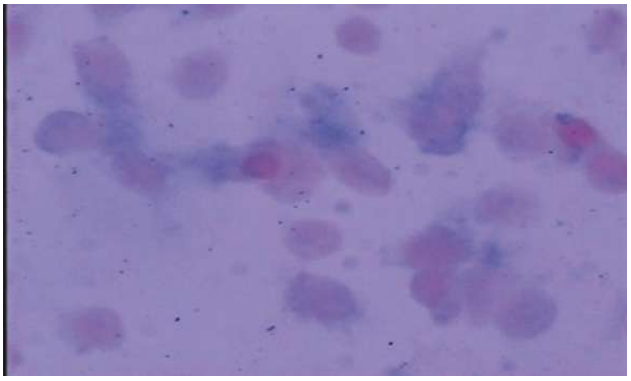


Figure 2. Cytoplasm of the EC9706 cells contained VEGF mRNA (blue-purple granules)



of VEGF, a key angiogenic factor, promotes the development and maintenance of a vascular network that promotes tumor growth and metastasis in a variety of human cancers and cell lines.[9] VEGF expression is highly associated with poorer outcomes in cancer patients, according to a large and growing body of research.[9]

On the basis of literature study, there is no published data about VEGF expression in esophageal cancer in the literature. The authors thus investigated microvessel density and VEGF expression in 53 primary esophageal squamous cell carcinomas in order to elucidate the relationship between angiogenesis and disease clinical characteristics. The prognosis and clinicopathological characteristics of ESCC have been tightly linked to the VEGF-C expression in tumor tissues.[11] Few findings exist on the VEGF-C expression in lymph fluid , despite the fact that serum VEGF-C has recently shown significance on both diagnostic and prognostic level.[8-11] Only the members of the VEGF family ("PlGF, VEGF-A, VEGF-B, VEGF-C, VEGF-D, and VEGF-E") are distinct growth factors for vascular endothelial cells. Lymphangiogenesis is induced due to VEGF-C.[12] Transgenic mice's skin has been demonstrated to show lymphatic endothelial proliferation due to its overexpression, suggesting that VEGF contributes to the maintaining of the lymphatic endothelium.[13] In experimental tumors, overexpression of these factors transgenes demonstrated a clear link between lymphangiogenesis and lymph node metastases.[14] VEGF-C expression and lymph node metastases are strongly positively correlated, according to the majority of clinical research.[6-9] VEGF-C stimulates the proliferation of tumor cells, which is associated with the expansion of lymphatic capillaries around tumors and the spread of cancer inside the lymphatic system. The metastasis of lymph nodes is intimately linked to the production of VEGF-C in tumor cells.[15] Using reverse ploymerase chain reaction, in situ hybridization, and immunohistochemistry, all of the EC9706 cells in the current investigation showed positive expression of Vascular endothelial factors. The outcomes were consistent with the invasive nature of the highly metastatic EC9706 cell line. Furthermore, the results of this investigation showed that in squamous cell carcinoma of esophagus patients, VEGF expression was positively correlated with lymph node metastasis. VEGF-C protein and mRNA expression and metastasis in squamous cell carcinoma of esophagus were revealed to be strongly correlated by immunohisto-chemistry, in situ hybridization, and Reverse transcriptase polymerase chain reaction. The findings aligned with those of earlier publications. [8-12] There was no significant correlation found between age, gender, or disease grade and any of the VEGF-C tissue expressions. These findings contrast from those of Onogawa et al.[16] This causes the growth and proliferation of new lymphatic capillaries, which in turn increases the likelihood of lymph node metastases in animal models. As per specific investigations,[17-21] lymph node metastasis and levels of VEGF-C in primary tumors are correlated. Additionally, it has been found that

individuals with some malignancies that express high amounts of VEGF-C have worse prognoses in comparison to tumors that express decrease levels of VEGF-C. Expressions of VEGF-C are not always associated with lymphatic involvement or the advancement of cancer, according to certain research[17-21]Anyhow these doubts, a treasure of data from clinicopathologic and experimental research supports the clinical targeting of the VEGF-C/VEGFR-3 lymphangiogenic signaling system. This will take precedence in the future.[22]. RT-PCR techniques may amplify the RNA from a small number of contaminating cells, masking tumor-specific changes.[23] There is no site-dependent differential expression of VEGF-C detected by RT-PCR analysis. However, ISH and IHC can detect intra-tumor heterogeneity in expression and pinpoint the cellular source.

Small sample size and single centre nature of the study were the main limitations of the study. To fully understand the relevance of these two biomarkers in ESCC, more research into the molecular mechanisms linking them and their roles in angiogenesis and metastasis is required.

## CONCLUSION

In human squamous cell carcinoma of esophagus, vascular endothelial growth factor expression may trigger lymphangiogenesis. The expression of VEGF and lymph node metastases were closely related. This study concluded that in individuals with esophageal squamous cell carcinoma, expression of VEGF is an important and helpful prognostic factor.

**Conflicts of interest:** Nil

**Source of Funding:** Nil

**Acknowledgement:** Nil

**Authors Contribution:**
**Aisha Jamil:** Data collection, article writing
**Maria Tasneem Khattak:** Literature review
**Shams ul Hadi:** Statistical analysis
**Maria Khan:** References article search
**Sidra Mashal:** Help in data collection
**Iqbal Muhammad Khan:** supervision

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