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Research Article

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VEGF Expression Evaluation in Patients with Esophageal Cancer by using Reverse Transcriptase Real- Time Polymerase Chain Reaction

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Abstract

Objective: Esophageal cancer is the 8th most important cancer in the world which is responsible for about 1.5 million cases and 1 million morbidity rates per year. One of the most specific regulators of neovascularization is VEGF. VEGF genes are mutated in the different type of esophageal cancers which leads to neovascularization process and tumor metastasis. In this qualitative research work, for the first time, we investigated the VEGF gene expression in esophageal cancer patients in IRAN.

Methods: This study was performed on 30 FFPE (formalin fixed paraffin embedded) (15 specimens were healthy and 15 specimens) were esophageal cancer tissues which were gathered from different hospitals and research centers in Zahedan and Kashan in Iran. We aimed to evaluate the expression of VEGF genes using reverse transcriptase real-time polymerase chain reaction method. PCR reactions for VEGF gene as well as internal control (β-actin) was performed 3 times using 2 -ΔΔCT (Livac) method for all specimens. Then the difference in gene expressions between case and control groups evaluated using the t-test, in which $p \leq 0.05$ was considered to be significant.

Results: There was a meaningful increase in VEGF gene expression in the case group. There was not a meaningful difference in gene expression in male or female in case or control groups (P>0.05).

Conclusion: The results showed that VEGF gene expression in patients was increased compared to control.

Keywords

Esophageal cancer; VEGF; Reverse transcriptase real- time polymerase chain reaction

Introduction

Cancer is a disease in which abnormal cells are divided and multiplied into a malignant tumor and then healthy tissues are destroyed. Cancer cells fall apart from normal mechanisms of cell growth and division. The exact cause of this phenomenon is unknown but probably it is due to genetic factors or external factors such as viruses and carcinogens [1]. Cancer is the third leading cause of death in Iran and 30000 Iranian lose their lives due to cancer every year. Hepatocellular carcinoma is a fatal vascular cancer which is known by activating multiple proliferation paths and different gene mortality [2]. Esophageal carcinoma (cancerous tumor) is one of the common malignant tumors in the world [3]. Different types of esophageal cancer consist of esophageal squamous cell carcinoma (ESCC), esophageal adenocarcinoma (EAC), and Barrett's esophageal (BE), a premalignant disorder that accompanies by chronic acid reflux [4]. EACC is one of the most common malignancies in Iran [5]. Vascular endothelial growth factor (VEGF), which is one of the most important specific regulators of the angiogenesis, is a homodimer glycoprotein with the 34-45 kiloDalton molecular weight [6]. VEGF is a known angiogenic factor that acts in oxygen deficient states in the malignant cells. VEGF has subtypes, VEGF-B, VEGF-C, VEGF-D and VEGF-E [7]. VEGF-A induction is seen in vascular tumors [8]. VEGF gene is contained in chromosome 6p21.3 and has 8 exons with introns. SNP is explained in the VEGF gene which affects induction of gene [9]. SNP causes VEGF resistance to treatment, especially VEGF-A, and It uses for angiogenesis [10,11]. Blood VEGF-A levels are higher in the cancerous patients [12]. At least 12 isoforms of VEGF-A, and VEGF-A_{121}, VEGF-A_{165} are studied [13]. VEGF-A replication is modified by HIF-1, a protein with alpha and beta subunits. However, in oxygen deficient states, gene replications, especially VEGF-A occurs [14]. Angiogenesis response to VEGF and VEGFR-1 activation is adjusted, and in this process, transverse links between VEGFR-1 and VEGFR-2 regulates [15,16]. VEGF-C usually induces in multipotential issues and bonds with VEGFR-3 as well as bonds with VEGFR-2. It is also connected with VEGFR-2 inefficiently [17]. Figure 1 illustrates different VEGFRs and their relations. In this Figure 1, VEGFR-2 is the main receptor and VEGF-A induces angiogenesis [18].

Low flow VEGFR-2 is activated by the Signal pathways including protein kinase C (PCK), related kinase (ERK), phosphatidylinositol 3-kinase (PI3K) and Mediation eNOS pathways [13]. VEGFR-2 inhibition to repress angiogenesis and tumor growth in preclinical different models is expressed and downstream Signal pathways is defined [19,20]. However VEGF Performs phosphorus of phospholipase C (PLC-ϒ), PI3K, PKC, and mitogen-activated protein kinase (MAPK/ ERK) [13]. VEGF-A is connected to VEGFR-1 and VEGFR-2. VEGF-B and PIGF are linked to VEGFR-1 and VEGF-C and VEGF-D both are attached to VEGFR-3 and VEGFR-2 [21].

Materials and Methods

15 esophageal cancer cases and 15 normal samples (control) in paraffin blocks were maintained under -20°C for 24 hours in order to have good cutting sections, then cross section cuts were gathered from samples using microtome. After that, paraffin was separated using xylene [2].

Extraction of RNA

RNA was extracted using RNeasy® FFPE kit which is the product of Qiagen Company then extracted RNA was kept in freezer at -80°C [2].

RNA quality and quantity evaluation

The quality of the extracted RNA was evaluated by uploading

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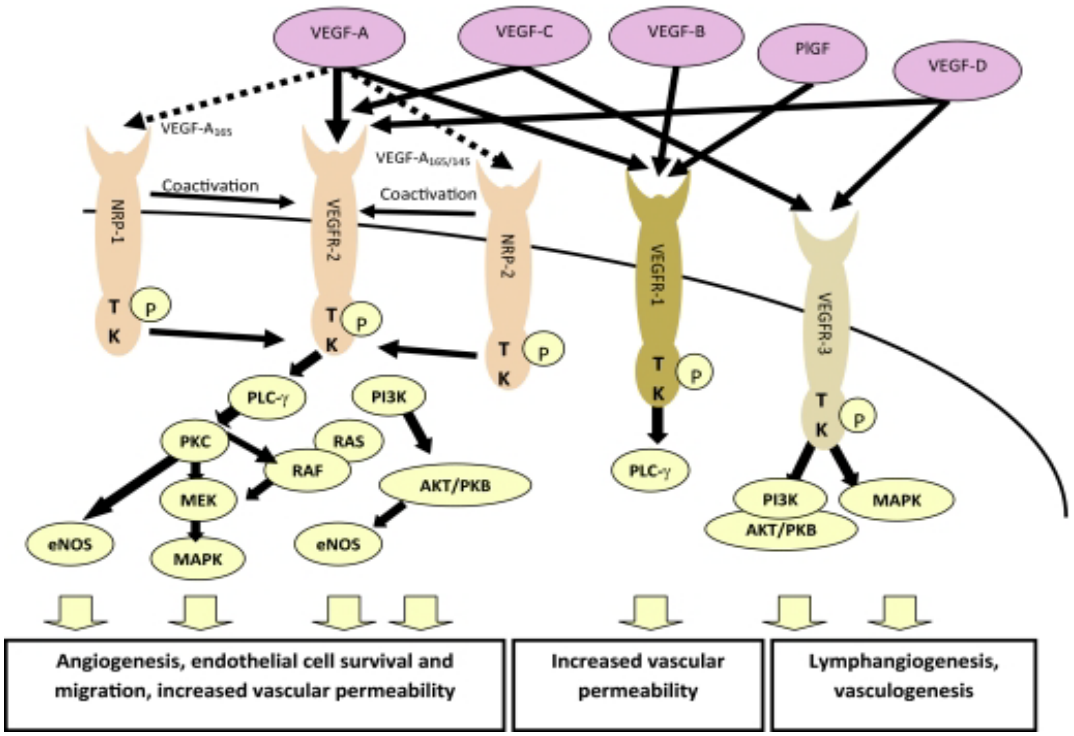

Figure 1: VEGF receptor Here, we would like to appreciate the official journal of the society for translational oncology (The Oncologist) to grant and permission to reproduce figure 1 (The three VEGF receptors, two coreceptors, and downstream signaling pathways) [18].

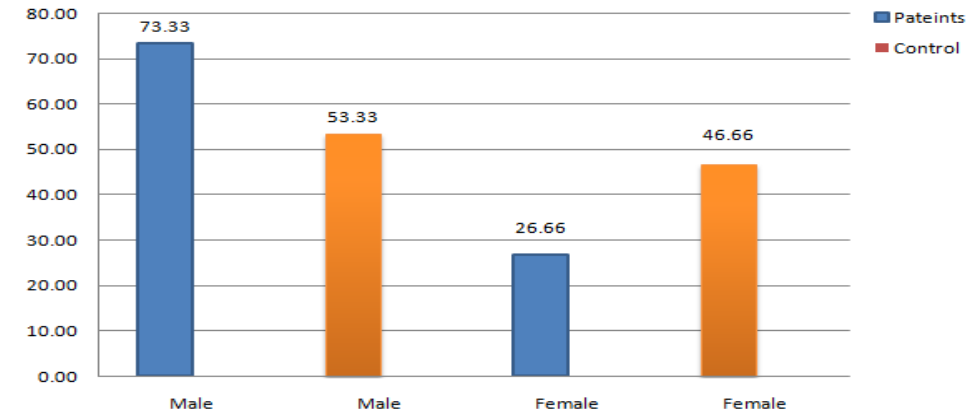

Figure 2: Frequency (%) of men and women were studied in two groups of patients and controls.

the extracted sample on agarose gel 1%; for more reliability, the concentration and OD of samples were measured using optical spectroscopy (ScanDrop – analytic Jena) [2].

The synthesis of cDNA

DNA synthesis is followed by Method of "2–Steps RT–PCR" kit product of Vivantis Company [22].

Designing primers: The design was done using "CLC Main work bench 5" software and Allele ID 7 software [23].

VEGF: FWD: CTTGCCTTGCTGCTCTA, Tm: 53.92

REV: GAACTTCACCACTTCGT, Tm: 51.32

β-actin: FWD: GAAGATCAAGATCATTGCTCC, Tm: 58.4

REV: CTAAGTCATAGTCCGCCTAG, Tm: 57.4

Polymerase chain reaction (PCR)

The reaction is performed using 2–Steps RT–PCR kit, a product of Vivantis Company [2].

Real-Time PCR: In order to perform this reaction, we used "Hot Taq evagreen qPCR master Mix (n.k)", product of cinnagen Company. The Real-Time PCR reactions were performed by "Research RG–3000 Corbett "instrument for evaluation of VEGF gene expression and the internal control gene was the *β-actin* gene [2].

Data and statistical analysis: Data analysis plays an importance role in Real Time–PCR; this can be evaluated by two practical methods as follow [24]:

1. Pffafl method [25],

2.ΔΔCt method [26], In order to decrease mistakes and correcting changes for starting materials in RT-PCR the ΔΔCt method and standard method is applied for RNA proliferation. In this study, two case and control groups were evaluated by t-test using SPSS software (SPSS, Inc, Chicago, IL).

Results

Sampling and patient data

As illustrated in Figure 2, the sample of this study consists of 11 male patients (% 73.3) and 4 female patient (% 26.6) and the control group includes 15 people, 8 of them were male (% 53.3) and 7 (% 46.6) were female.

RNA quality and quantity evaluation

Installing on agarose gel % 1, the quality of extracted RNA was evaluated. It is worth noting that in this study the extraction was performed from paraffinized tissues, the quality of the RNA band wasn't very good and the band appeared on the agarose gel as a smear (Figure 3). After RNA extraction, OD 260.280 nm were measured and the ratio was 1.93 (1.80 to 2.00) for all samples.

The results of thermal slope

The thermal slope was performed in order to obtain the optimum temperature for starters of VEGF gene as well as β-actin gene; after that, every gene was proliferated by its starter gene and the products were installed on agarose gel % 2 and every gene particle was observed in its specified size. The optimum temperature for VEGF gene was 50°C and for *β -actin* gene was 57.1°C accordingly (Figures 4 and 5).

Standard curvature of *B –actin* internal control gene

The reaction efficiency is as follow: for *β -actin* gene was 100%, for R2 was % 99 for VEGF gene was % 94 and for R2 was % 96 accordingly (Figures 6 and 7).

Results of real time-PCR reaction

β -actin gene proliferation were obtained as a progression of reaction curvature in which the x-axis belongs to Cycles number and y-axis belongs to fluorescent light intensity in which the progression of the gene was determined in every cycle (Figure 8). The proliferation of the VEGF gene in every cycle is illustrated in Figure 9.

Results of melting curvature analysis

Figure 10 shows that there are sudden drops of fluorescence in 73°C for *β -actin* gene.

There is a product which is seen under a peak of 77°, this is Tm product of β-actin gene (Figure 11).

The change of melting curve based on gene's temperature

The temperature increases the fluorescent light intensity until the temperature reaches approximately 78° which is related to the PCR reaction product of the VEGF gene and in this point (78°) the fluorescent light intensity suddenly is decreased; then fractures are created in the melting curve (Figure 12). After that, in the melting analysis curve, the PCR product peak is formed (Figure 13).

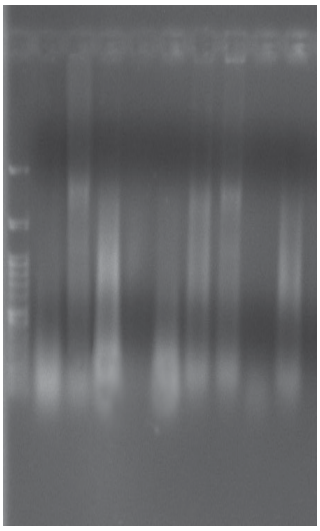

Figure 3: RNA extraction results in a 1% agarose gel.

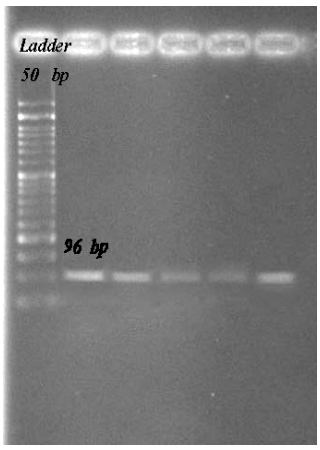

Figure 4: VEGF gene PCR thermal gradient.

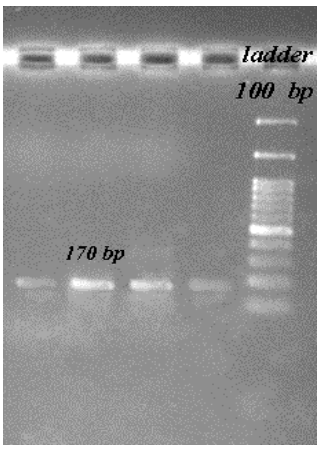

Figure 5: *β -actin* gene PCR thermal gradient.

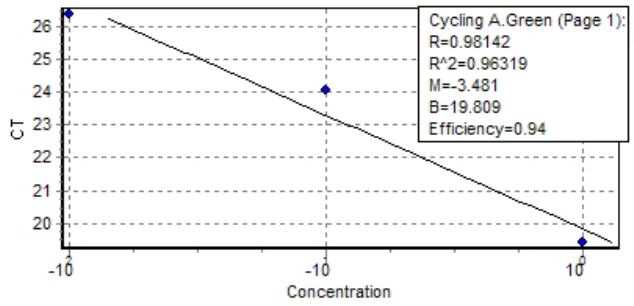

Figure 6: *β -actin* gene Standard curve.

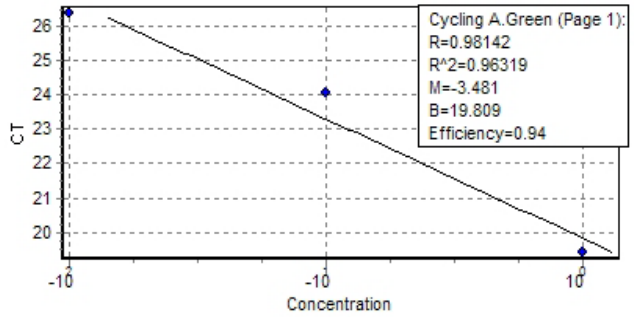

Figure 7: Standard curves of VEGF gene.

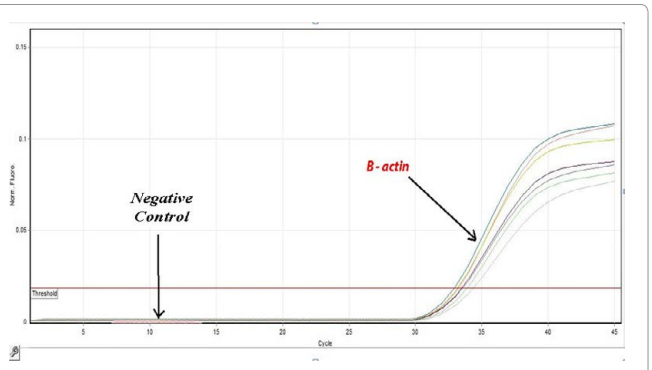

Figure 8: *β -actin* gene amplification curve.

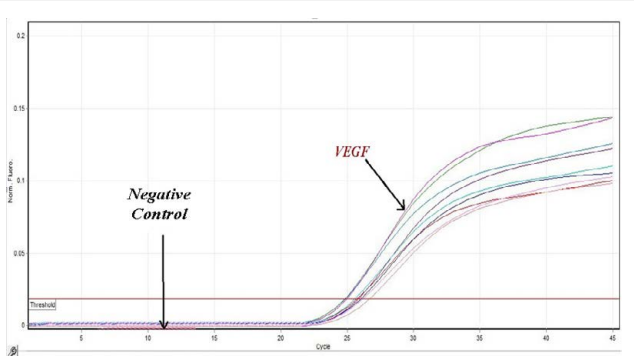

Figure 9: VEGF gene amplification curve.

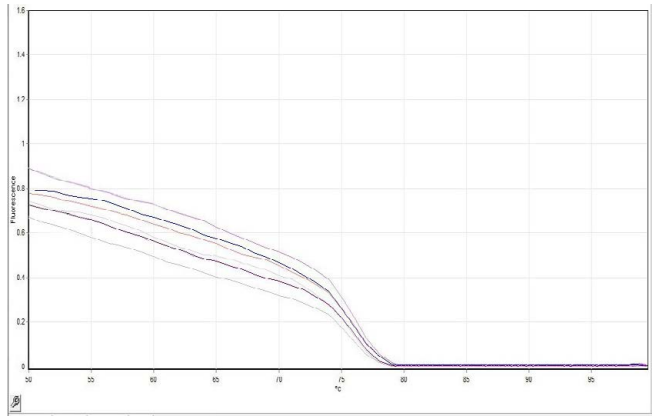

Figure 10: Fluorescence changes in temperature for *β -actin* gene.

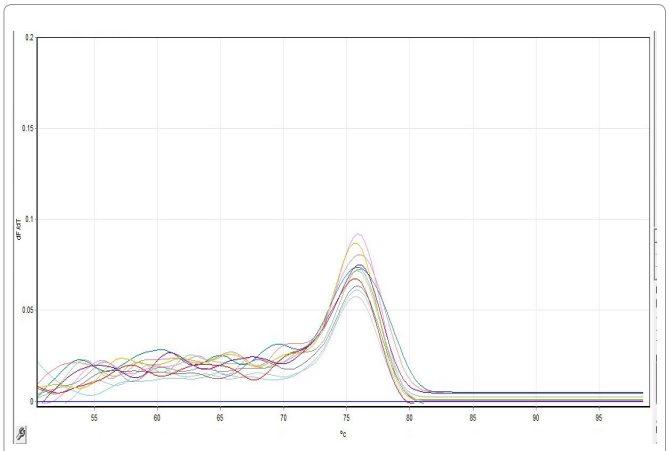

Figure 11: The melting curve of *β -actin* gene.

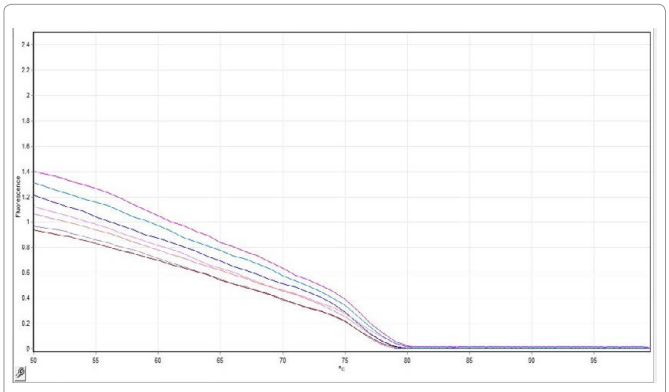

Figure 12: Fluorescent light changes depending on the temperature of the incoming samples for VEGF gene.

Checking the uniqueness of the real time PCR

To ensure the amplification of the gene, the samples which resulted from PCR amplification and two amplified samples of each gene were randomly chosen and were put on 2% agarose gel for 60 minutes with the voltage of 100. The results obtained from agarose gel image confirmed the correct amplification of VEGF and *β –actin* (Figure 14).

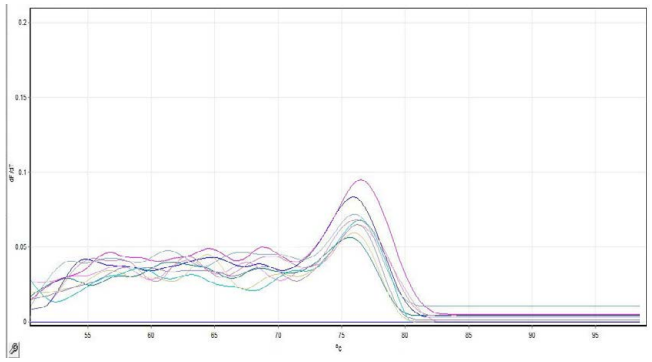

Figure 13: The melting curve of VEGF gene.

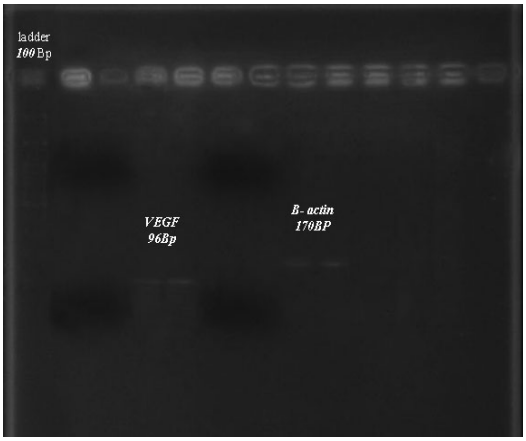

Figure 14: Amplified fragments in the reaction Real Time – PCR.

The statistical results obtained from the analysis

The results showed that the VEGF gene expression in patients was 4.369 times higher compared with non-patients as shown in Figure 15.

Discussion

Esophageal cancer is considered to be one of the most important malignant cancers in terms of prognosis and outcome [28]. Esophageal cancer is the result of a series of environmental factors such as tobacco smoke, gastroesophageal reflux, and genetic changes. Molecular studies have shown there may be genetic abnormalities such as changes in the expression of p16, p53, VEGF genes and several other genes in esophageal squamous cell carcinoma and adenocarcinoma of the esophagus [29]. In another study, Noguchi et al., examined 71 patients with esophageal squamous cell carcinoma and estimated the expression intensity of VEGF-C gene. They reported that they hadn't seen any significant effect on the VEGF-C gene expression and they studied clinic pathological parameters [30]. In another study, the participation of VEGF gene in the lung organ's response to acute and chronic hypoxia conditions was reported [31]. The stimulation of VEGF gene under oxygen deficiency conditions plays the vital role in the development of the malignant cells survival in local tumor growth and invasion and the extension of invasion [31]. The purpose of this study was to estimate the VEGF gene expression in patients suffering from esophageal cancer in order to advance therapy goals. The difference in the intensity of reported mutations in VEGF gene in different regions confirms the fact that the mutation occurrence in this gene is highly influenced by environmental, geographical and ethnic factors. However, the test method and the mutation detection technique are highly important. Since there has never been a research on VEGF gene in patients suffering from esophageal cancer using Real Time PCR, the necessity to do this research, using the mentioned method, is strongly felt. In contrast, Hongxin et al. [32], investigated the VEGF gene expression in patients suffering from ESCC cancer using immunohistochemistry (IHS), RT-PCR and in situ hybridization(ISH) methods. Technological advances, such as the development of the sensitive Real Time PCR system, have made the fast and accurate quantitative measurement of small quantities of mRNA possible [33]. In Real Time PCR method, the gene copy number is not important and only the decrease or increase of the gene expression is important. This decrease or increase is compared to a standard or a reference gene which is considered as an internal control. This method is both time consuming and economically affordable [34]. The VEGF gene expression analysis by Real Time PCR method can be used as a tool to measure this phenotypic biomarker in the development or nondevelopment of Coronary artery disease (CAD). The VEGF gene is on chromosome 6 and 14 kb in length and includes 8 exons and 7 introns [35]. In our study, we used the β-actin gene to normalize the data obtained from the Real Time PCR. Considering the importance and place of the standard gene in the statistical analysis of this method, using and choosing an appropriate standard gene is of significant importance. Because stability in esophageal is approved, it is more stable compared to *18s rRNA* and *GAPDH* genes is more reliable for the normalization of the data and it is suggested for this subject [36]. As mentioned before, VEGF gene is located in the 6p21.3 chromosome and it includes 8 separated exons with introns. It has been reported that single-nucleotide changes in VEGF gene affect the induction of gene function [9]. As the results of the study shows, the VEGF gene expression indicates an increase in the patient group compared to the control group. In this respect, the results obtained from our research are the same as those of the following researchers. Hata et al. [37], the relationship of VEGF gene in the stages III and IV of cancer were found. The present study indicated that the expression intensity of VEGF gene was increased, so the increase in the expression intensity of this gene is related to the patients being in stages III and IV of the cancer. Baatar et al. [38], reported that ulceration of the esophagus by acid can increase the HIF-1a expression and consequently the VEGF expression. Nagata et al. [39], described a point based on the interleukin 10 and concluded that gene expression is significantly related to the VEGF gene expression and the increase in interleukin 10 stimulates the gene expression of angiogenesis factor. As the results of the study indicates the VEGF gene expression increase

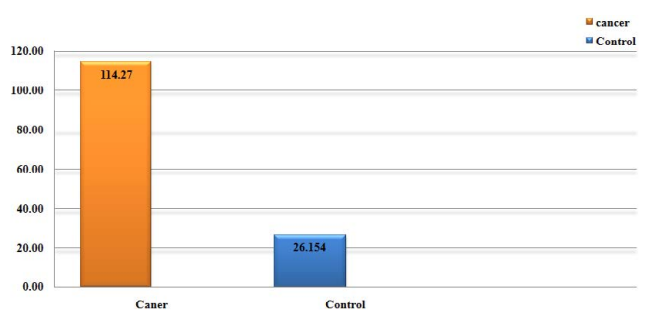

Figure 15: The Average ΔCt VEGF genes, the two groups.

pathway, and the VEGF gene expression increase through P13K/AKT pathway in the way that the phosphoinositide 3-kinase (PI3K/AKT) pathway regulates the activity of hypoxia-inducible factor (HIF). The hypoxia-inducible factor (HIF) is a key transcriptional regulator in response to reduced oxygen levels (hypoxia) [40]. Heterodimer HIF includes β manufacturer (HIF-1β) and one of α manufacturer (HIF-1α, HIF-2α, and HIF-3α) [41]. HIF is regulated through two types of oxygen sense control. The first witness of regulating the stabilization of HIF-α is done by hydroxylation in proline 462 and 564 (HIF-α) in the oxygen-dependent degradation domain (ODD) [42]. In mixed lighting conditions, hydroxylation from proline by a family of proline 4-hydroxylase. O_2, iron (II), and 2-oxoglutarate lets the special recognition of HIF-α with Von Hippel–Lindau (VHL) protein [43,44]. In addition to ODD, HIF-α1 also includes two transcriptional activation domains (AD), N-terminus NAD and C-terminus CAD. The second oxygen sensor control is the transcriptional activity of CAD whose regulation happens through aspergillus of hydroxylase factor depending on inhibition of oxygen FIH-1 (HIF-1) [45]. The HIF collection, then, in response to hypoxia ingredients (HRE) joins in the target gene and activates its own transcription. In addition to hydroxyproline, other legal pathways, such as phosphatidylinositol 3-kinase (PI3K)/AKT, have been implicated in controlling the expression of HIF-α protein. It's the (PI3K)/AKT pathway that is usually activated in human cancers through losing tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) or through the signaling growth factor [40]. High PI3K activity can also affect the participating interaction of hypothetical repressor with HIF-1. The more activity is shown by the mechanisms of this physiological inhibitory effect, the more the literature supports the relationship between activating HIF-1a through PI3K/AKT pathway and the VEGF expression which is most likely important in the development of the tumor [40]. Our results, did not show any significant difference between the two men and women sexes; this is in accordance with the researchers of Shimada et al. [46], Shimada et al. [47] and Shimada et al. [48], in the way that serum VEGF is greatly related to the tumor size, stage of tumor growth, nodal status, and distant metastasis but is irrelevant to the patient's sex or age.

Conclusion

There was a meaningful increase in VEGF gene expression in the case group. There was not a meaningful difference in gene expression in male or female groups in case or control groups (P>0.05).

Compliance with Ethical Standards

i. Conflict of Interest: None.

ii. Funding: This work was a self-funded M.Sc. student project (Mrs. Hadiseh Farzanfar) that was funded, in part, by University of Zabol, and supported by Payam Noor University (PNU), Tehran, Islamic Republic of Iran.

iii. Ethical approval: This research was approved by Payam Noor University (PNU), Tehran, Islamic Republic of Iran (ethical committee approval No. #3871/1938).

iv. Informed consent: The informed consent from all individuals participated was obtained in this study.

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