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Association of high expression of non-coding RNA FLJ22447 with HIF-1a in ESCC patients.

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Abstract

Background: This study investigated the prognostic values of hypoxia-regulated markers and their interrelationships in esophageal cancer patients.

Methods: Fresh tumor and margin normal tissue specimens were obtained from 49 patients with esophageal cancer (Age: 48 years; range: 29 to 81 years). Samples collected from the Tumor Bank of Cancer Institute, Imam Khomeini Hospital in 2016-2017. The Quantitative real-time PCR was performed to determine the expression level of HIF-1a, VEGFA, and FLJ22447 and correlated with demographic information of patients, tumor characteristics, and survival.

Results: Significant up-regulation of HIF-1a, VEGFA, and FLJ22447 were observed in ESCC tumor tissues than normal tissues. High expression of VEGFA was associated with advanced stages (III-IV) and metastasis, while HIF-1a correlated with I-II stage. Moreover, high tumor HIF-1a expression was associated with reduced overall survival of ESCC patients.

Conclusions: The evidence from this study suggests that hypoxia-driven angiogenesis by up-regulation of HIF-1a and FLJ22447 in tumor tissues may play a crucial role in ESCC progression. Measuring FLJ22447 expression extends our knowledge to enhance our understanding about its regulatory function on HIF-1a and subsequently angiogenesis by VEGFA.

Keywords: Esophageal cancer, HIF-1a, VEGF, FLJ22447 non-coding RNA.

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Introduction

Esophageal cancer is a common cancer characterized by extremely aggressive nature and poor survival rate in the world [1], and it ranks as 6th fatal cancer among all cancers. This cancer categorizes into squamous cell carcinoma (SCC) and adenocarcinoma, as the epidemiology between the two groups is vastly different [2]. Incidence rates of SCC of the esophagus reported as high as 100 cases per 100000 annually in an area referred to as the "Asian esophageal cancer belt" and this region extends from northeast China to the Middle East.

Hypoxic regions and angiogenesis are common characteristics of solid neoplasms such as esophageal cancer. Hypoxia is a key microenvironment condition that modulates by transcription factors such as hypoxia-inducible factor 1 alpha (HIF-1a). Expression of HIF-1a activates transcription of several genes that regulate cell proliferation, tumor metabolism, and angiogenesis processes [3,4]. Regulation of HIF-1a occurs by PHD and FIH-1 oxygen sensors, that are located upstream of HIF-1a. HIF family members mediate cellular adaptation to low oxygen by activating the transcription of target genes involved in metabolism, angiogenesis, and extracellular matrix (ECM) remodeling [5]. For instance, HIF-1a and HIF2a show overlapping effects on features of angiogenesis and ECM remodeling; however, they also exhibit distinct effects on cell metabolism and proliferation. In cancer biology, the HIF pathway (hsa04066) facilitates tumor growth by regulating cell survival and apoptosis thru TGF-β, bFGF, and p53 [6],

promoting metabolism via GLUT-1 and glycolytic enzymes, angiogenesis via VEGF and SDF-1 [7].

It is well demonstrated the impact of angiogenesis on tumor growth and metastasis through the expression of angiogenic molecules produced by tumor and host cells [8]. This results in formation of blood vessels that nourish the tumor cells with oxygen and nutrients. Research shows HIF-1a not only has a strong impact on the prognosis of pancreatic ductal adenocarcinoma but also it is related to vascular endothelial growth factor (VEGF) expression [9].

VEGF as an angiogenic factor regulates angiogenesis by inducing proliferation, migration, and permeability of endothelial cells. Also, VEGF plays a role in normal physiological functions such as bone formation, hematopoiesis, and wound healing [10]. Deregulated VEGF expression contributes to the development of solid tumors by promoting tumor angiogenesis and to the etiology of several additional diseases that are characterized by abnormal angiogenesis. *In vivo* investigation showed VEGF induces angiogenesis and causes permeabilization of blood vessels [11]. The VEGF family of growth factors play pivotal roles in embryonic development and angiogenesis-dependent disease [12]. Overexpression of VEGF mediates in vascular diseases including retinal, neurodegeneration, and cancer [13].

Recently, the regulatory function of non-coding RNAs in the expression of neighboring genes are demonstrated [14-16]. The non-encoding RNA FLJ22447 with 3 transcripts located on chromosome 14q23.1 region in the vicinity of HIF-1a gene. Research on FLJ22447 has been restricted to the RNA-seq data. According to the GEPIA, FLJ22447 ncRNA was highly expressed in testicular, lung, thyroid, skin, and prostate tissues [17]. Although some research has been performed on this lncRNA, only single study reported its remarkable up-regulation in stromal carcinoma-related fibroblasts [18]. What is not yet clear is the impact of FLJ22447 on the expression of HIF-1a in esophageal cancer. The paper explores the significance of FLJ22447 ncRNA in esophageal squamous cell carcinoma (ESCC) and clarifies the correlation of its expression with HIF-1a and VEGF genes.

Materials and Methods

Patients and clinical samples

This cross-sectional study was performed in the Tumor Bank of Cancer Institute, Imam Khomeini Hospital, Tehran, Iran from 2016-2017 among patients who underwent surgery without preoperative chemotherapy or radiotherapy. Eighty fresh tumor and margin normal tissues of esophageal cancer were collected and immediately frozen in liquid nitrogen and storedat -80°C for further experiments.

The association between expression of HIF-1a, VEGF and FLJ22447 as a neighboring ncRNA with clinicopathological features of patients including tumor grade, tumor stage, lymph node, and metastasis were considered. This research was permitted by the Research Ethics committee (IR.GOUMS.REC.1397.006).

Isolation of RNA and cDNA synthesis

Total RNA was isolated from the frozen tissues using TRIZOL (Invitrogen, Life technology, USA) according to the manufacturer's instructions. The RNA quality and quantity was evaluated by PicoDrop (PicoDrop Technologies); the 260/280 ratio of nearby 2.0 was considered as an optimal yield of RNA. The complementary DNA was synthesis by cDNA synthesis kit (Thermo Fisher) with random hexamer primer. The reaction was performed by incubation for 5 min at 25°C followed by 60 min at 42°C and 70°C for 5 min.

Quantitative Real-time PCR

To quantify the expression level of VEGF, HIF1-a, and FLJ22447, the QRT- PCR was performed using SYBER Green PCR master mix kit (TAKARA-Japan). The first strand cDNA synthesis products of VEGF and HIF-1a were subjected to 30 s at 95°C followed by 35 cycles of 5 s at 95°C for denaturing, 20 s of 60°C for annealing/extension steps. Synthesis of FLJ22447, however, was conducted by 40 cycles of 15 s at 95°C and 30 s at 60°C followed by 40 s at 72°C for extension. All experiments were performed in duplicate and the specificity of PCR product was confirmed by melt curve analyses. GAPDH was chosen as an endogenous control to normalize the expression level of candidate genes. The characteristics of primers were indicated in Table 1. Subsequently, the expression level of target genes was calculated using 2–Ct method.

Table 1. *The applied primer for expression analysis in qRT-PCR.*

Genes	Chromosomal location	Primer Sequence 5´-3´	Amplicon length (bp)
HIF-1a	14q23.2	F: TCCAAGAAGCCCTAACGTGT	180
		R: TGATCGTCTGGCTGCTGTAA	
VEGFA (NM_001025366.2)	6p21.1	F: ACTGCCATCCAATCGAGACC	205
		R: TCTCCTATGTGCTGGCCTTG	

FLJ22447 (NR_039985.1)	14q23.1	F:AACCTCCTGAACAGCATCCA	308
		R:ACTGCACTCTGACTCAGCTT	
GAPDH	12p13.31	F: GGTGGTCTCCTCTGACTTCAACA	127
		R: GTTGCTGTAGCCAAATTCGTTGT	

Interaction analysis of FLJ22447

In order to investigate the interaction of FLJ22447 with biological macromolecules, target prediction tools and some bioinformatics resources was used. The LncRNADisease V2 (http://cmbi.bjmu.edu.cn/lncrnadisease) and Lnc2Cancer (http://www.bio-bigdata.net/lnc2cancer) databases was applied to identify the biological role of abnormal expression of FLJ22447 in different cancers. The interaction of FLJ22447 ncRNA with other RNAs and proteins was also identified by the RAID v.2 database (www.rna-society.org/raid).

Statistical analysis

The statistical software, SPSS Statistics version 18, was used for data analysis. Statistical significance between groups was determined by one-way analysis of variance (ANOVA) and Student's t-test. All data were presented as mean ± SD. P values<0.05 was defined to be statistically significant. Kaplan-Meier analysis was performed to calculate overall survival and survival analysis compared by log rank test.

Results

Up-regulation of VEGF in clinical ESCC samples

The VEGF expression level in esophageal cancer tissues were detected by qRT–PCR in 40 paired samples. The expression of VEGF was significantly increased in ESCC samples than adjacent normal tissues ($p<0.05$) (Figure 1). Correlation across VEGF expression and clinicopathological features of ESCC was performed (Table 2), suggesting a remarkable association between increased VEGF expression and advanced stages (III-IV) and metastasis ($p<0.05$).

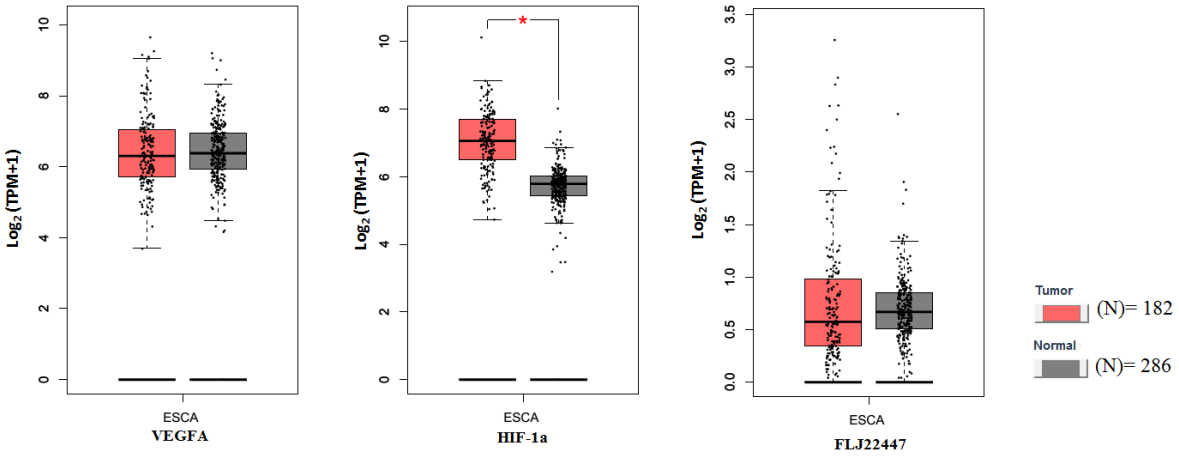

Figure 1. *Comparison expression profile of VEGF, HIF-1a and vicinity ncRNA FLJ22447 in esophageal carcinoma. The expression of HIF-1a are increased in ESCA tissues than normal tissues, respectively. In contrast, the mean expression of VEGFA and FLJ22447 were lower than in ESCA tissues. Log2 (TPM+1) was used for log-scale. ESCA: esophageal carcinoma; TPM: Transcripts per Million.*

Up-regulation of HIF-1a in clinical ESCC samples

The expression level of HIF-1a was significantly increased in clinical ESCC specimens compared to adjacent non-tumor tissues ($p<0.05$) (Figure 2). Furthermore, we identified a significant correlation between HIF-1a expression and tumor stage **(Table 3)**. The high and low expression of HIF-1a was observed in I-II and III-IV stages, respectively.

Up-regulation of FLJ22447 in clinical ESCC samples

The expression of ncRNA FLJ22447 was assessed by qRT–PCR test in 40 paired ESCC. Our data analysis indicated that the expression of FLJ22447 was up-regulated in clinical ESCC tissues than margin tissues, significantly ($p<0.05$).

Association of HIF-1a with overall survival in ESCC patients

In order to demonstrate the correlation of VEGFA and HIF-1a gene with survival rate in ESCC patients, Kaplan-Meier analysis were performed. As can be seen in Figure 3, it is apparent that the overall survival of the patients with the high expression of HIF-1a was markedly reduced compared to those with low expression level ($p=0.019$). However, the high expression of VEGF did not increase the survival rate of patients ($p=0.389$).

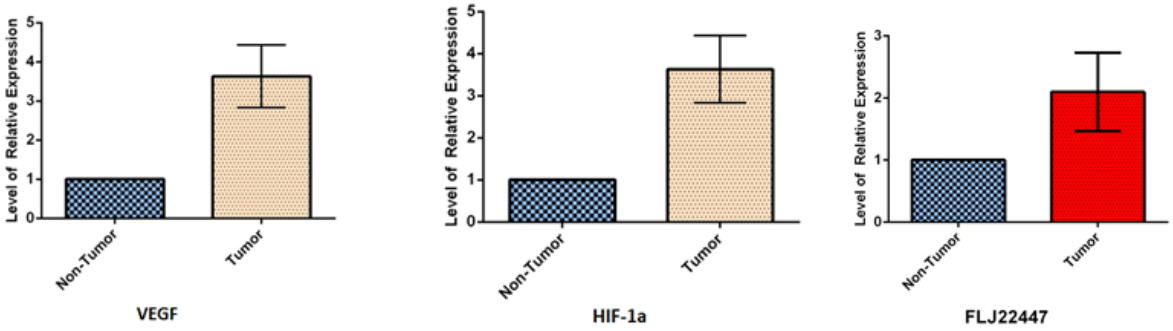

Figure 2. *The gene expression profile of VEGF, HIF-1a and vicinity lncRNA FLJ22447 in esophagus tumor and paired normal tissues. Comparison of VEGF, HIF-1a, and FLJ22447 expression in ESCC tumor tissues normalized to housekeeping gene by 2-ΔCT method. Data are presented as fold change.*

Table 2. *Association of VEGFA expression with clinicopathological factors in ESCC.*

Clinical specify	Samples	VEGFA expression		p-value
		High	Low	
Total	38			
Age				
60	16	8	8	0.344
60	22	13	9	
Gender				
Male	21	11	10	0.416
Female	17	7	10	
Tumor size (cm)				
<5	20	10	10	0.755
5	18	9	9	
Tumor stage				
I-II	11	7	4	0.114
III-IV	27	12	15	
Tumor Grade				
I-II	8	2	6	0.002
III-IV	30	17	13	
Metastasis				
Unknown	9			0.023
Yes	10	8	2	
No	19	10	9	
Lymph node				
Yes	17	9	8	0.732
No	21	9	12	

Table 3. *Association of HIF-1a expression with clinicopathological factors in ESCC.*

Clinical specify	Samples	HIF-1a expression		p-value
Total	38	High	Low	
Age				
60	16	7	9	0.098
60	22	12	10	
Gender				
Male	21	10	11	0.516
Female	17	8	9	
Tumor size (cm)				
<5	20	10	10	0.166
5	18	8	10	
Tumor stage				
I-II	11	7	4	0.043
III-IV	27	12	15	
Tumor Grade				
I-II	8	3	5	0.73
III-IV	30	15	15	
Metastasis				
Unknown	9			0.531
Yes	10	5	5	
No	19	11	8	
Lymph node				
Yes	17	9	8	0.261
No	21	9	12	

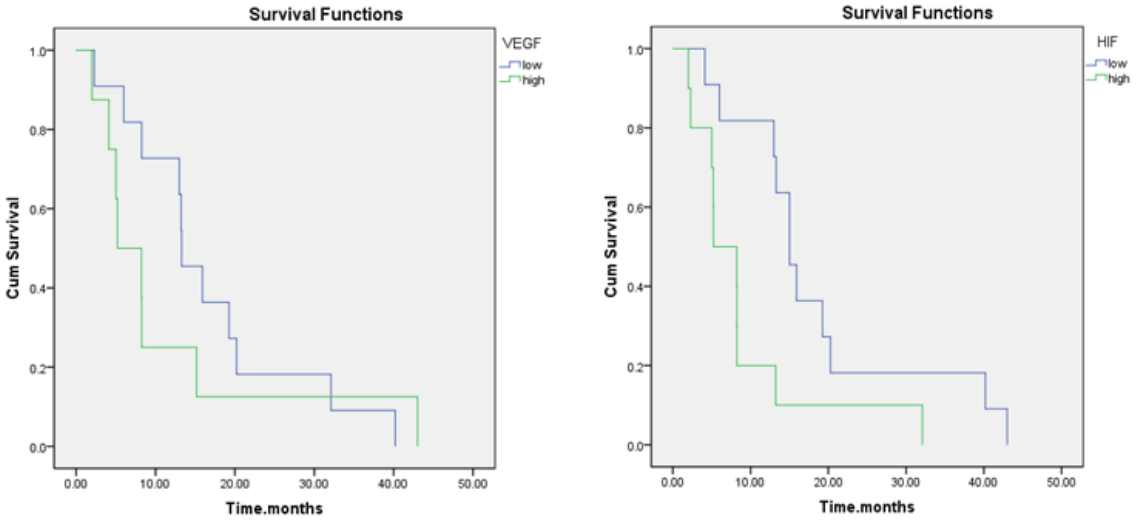

Figure 3. Association of VEGF and HIF-1a with survival rate in ESCC patients. Survival rate of patients with high vs. low expression levels of VEGF and HIF-1a are illustrated. Patients with high HIF-1a expression have poorer survival.

Interaction analysis of FLJ22447 in human tumors

According to the LNCipedia database, the ncRNA FLJ22447 contains three lncRNA transcripts with the length of 1368, 1312, and 577 nucleotides. The term of its sequence ontology is sense_intronic_ncRNA. The LncRNADisease predicts the involvement of FLJ22447 in cervical, lymphoma, glioma, stomach, thyroid, and bladder cancers by the TAM method. The retrieved data from the RAID v.2 resource show 67 protein coding-genes and RNAs that interact with FLJ22447 (Table 4). Overall, these predicted results provide important insights into FLJ22447 function in biological processes. However, further experimental research is demanded.

Table 4. The predicted interaction of ncRNA FLJ22447 with Proteins and RNAs. Note: Data retrieved from RAID v2.0 resource.

Protein interaction	RNA interaction
--	FLJ22447-let-7e-5p
--	FLJ22447-let-7f-5p
--	FLJ22447-miR-302d-3p
FLJ22447-FOXH1	FLJ22447-miR-302c-3p
FLJ22447-BATF	FLJ22447-miR-200b-3p
FLJ22447-AR	FLJ22447-miR-204-5p
FLJ22447-SPI1	FLJ22447-miR-15a-5p
FLJ22447-FOS	FLJ22447-miR-302a-3p
FLJ22447-BRF1	FLJ22447-miR-15b-5p
FLJ22447-SMARCC2	FLJ22447-miR-144-3p
FLJ22447-JUN	FLJ22447-miR-421
FLJ22447-POU2F2	FLJ22447-miR-216a-5p
FLJ22447-JUND	FLJ22447-miR-302b-3p
FLJ22447-SP1	FLJ22447-miR-455-5p
FLJ22447-CEBPB	FLJ22447-miR-135b-5p
FLJ22447-E2F1	FLJ22447-miR-410-3p
FLJ22447-TCF12	FLJ22447-let-7g-5p
FLJ22447-TAF1	FLJ22447-miR-155-5p
FLJ22447-MAX	FLJ22447-miR-16-5p
FLJ22447-NANOG	FLJ22447-miR-216b-5p
FLJ22447-NRF1	FLJ22447-miR-107
FLJ22447-SMARCA4	FLJ22447-miR-205-5p
FLJ22447-TCF7L2	FLJ22447-miR-424-5p
FLJ22447-E2F4	FLJ22447-miR-433-3p
FLJ22447-PPARG	FLJ22447-miR-103a-3p
FLJ22447-SMARCB1	FLJ22447-miR-135a-5p
FLJ22447-CEBPA	FLJ22447-miR-154-5p
FLJ22447-MYC	FLJ22447-miR-539-5p
FLJ22447-SMARCC1	FLJ22447-miR-497-5p
FLJ22447-RAD21	FLJ22447-miR-195-5p
FLJ22447-HNF4A	FLJ22447-let-7i-5p
FLJ22447-BCL11A	FLJ22447-let-7b-5p
FLJ22447-EBF1	FLJ22447-miR-98-5p
FLJ22447-STAT1	FLJ22447-miR-153-3p
--	FLJ22447-miR-211-5p
--	FLJ22447-miR-544a

Discussion

Angiogenesis and adaptation to hypoxia are common features of solid cancers [3]. Hypoxia modulates the expression of genes that stimulate several pathway including angiogenesis

which results in many pathologic processes [19]. It seems that, supplying food and oxygen to grow cancer cells and metastatic of several tumors depend on angiogenesis. There are numerous factors that have a critical role during induced angiogenesis by tumor [20]. It was suggested that HIF-1a is an essential factor to induce angiogenic factors such as VEGF in hypoxia microenvironment condition [4,21]. The presence of correlation between Hif-1a and VEGF expression with clinical parameters have been reported in ESCC [19], pancreatic ductal adenocarcinoma [9], and breast cancer [12].

The present study was designed with the aim of assessing the importance of VEGF and HIF-1a expression in ESCC patients and a possible relation between them and clinicopathologic outcomes. A positive association between the expression of HIF-1a and VEGF in ESCC tissues than adjacent non-tumor tissues in this study corroborates the findings of previous research [22]. The clinically relevant finding indicted a significant correlation between HIF-1a expression and advanced tumor stage. This result, however, differs from earlier finding, who reported that higher expression of HIF-1a is not related to tumor stage but it had positive correlated with vascular invasion in ESCC tumor specimens [22]. Also, our results showed up-regulated of VEGF and HIF-1a expression correlated inversely with patients' survival. Recent studies have demonstrated that high expression of VEGF and HIF-1a are usually proposed as poor prognostic predictors in several cancers including ESCC [23]. In consistent with this result, other study have demonstrated that up-regulated of HIF-1a expression is related to poor survival and high risk of metastasis but in breast cancer [24].

The up-regulation of ncRNA FLJ22447 was firstly reported in stromal carcinoma-related fibroblasts [18]. Contrary to our expectation, based on GEPIA resource, this study did not find the reduced expression of FLJ22447 in ESCC tissue. This is the first study reporting a notable expression of FLJ22447 in ESCC tumor tissues than adjacent normal tissues that concomitant with high expression of HIF-1a. The exact biological function and signaling pathways related to FLJ22447 have not yet been investigated. Since the non-coding RNAs, lncRNA and/or miRNAs, regulate the expression of their nearby genes [25]. It is possible, therefore, that the vicinity of HIF-1a with FLJ22447 effect on the gene expression. Further research is required to focus on this interaction is suggested.

In conclusion, HIF-1a regulated the expression of VEGF and outcomes of our study reported a role of HIF-1a expression as survival predictor in ESCC. Also, these factors alongside with FLJ22447 could be useful as new therapeutic targets for ESCC in the future. However, this should be assessed in experimental research.

Declaration

Ethical Approval and Consent to participate: This research was approved in Ethic Committee of by IR.GOUMS.REC. 1397.006.

Consent for Publication

Not Applicable.

Availability of Supporting Data

Data will be available while editor requested.

Competing Interests

No potential conflicts of interest were disclosed.

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Authors' Contributions

Bahramian, sahebi, and roohinejad performed all experiments of the study. Razavi provided tumor samples. Javid and Shamsabadi analyzed the experiments and wrote the manuscript. Delshad analyzed the bioinformatic data. Shamsabadi and Shafiee conceived and coordinated the study. All authors have seen and approved the final manuscript.

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