Prognostic role of Quantification of Circulating RNA (PDGFRβ) Fragments as Tumor Markers in serum of Patients with Esophageal Cancer

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Abstract

**Background:** Very deadly esophageal cancer (EC) is one of the most common cancers in the world, with a wide distribution in Iran and China and with poor overall survival. Most EC patients are at the metastatic stage of the disease at the time of diagnosis. So, effective non-invasive biomarkers are still needed for the early diagnosis of this malignancy. In this study, platelet-derived growth factor receptor beta (PDGFRB) was selected for further validation as a candidate biomarker for EC diagnosis and prognosis.

**Methods:** Serum from 83 individuals (33 patients with EC and 50 normal volunteer controls) were collected. RNAs of the specimens were extracted and their levels of PDGFRB gene expression were measured by real-time PCR.

**Results.** The results demonstrated that the expression of PDGFRB in the sample of the case group is higher in comparison with the control group (fold change= 1.12 \( p=0.03 \)).

**Conclusions.** According to our results, the evaluation of serum RNAs including beta-platelet-derived growth factor receptor mRNA could help to achieve a non-invasive, rapid diagnostic method and ultimately earlier treatment of EC. Further studies in this field are necessary to find the appropriate diagnostic and differential pattern of esophageal cancer.

1. **Introduction**

Esophageal cancer (EC) is one of the most common malignant tumors in the world, with a wide distribution in Iran and China. It is the 6th leading cause of cancer deaths worldwide (Tan, Qian et al. 2016). It is characterized by a high mortality rate, poor prognosis at time of diagnosis and variability based on geographic location (Uhlenhopp, Then et al. 2020). More than 50% of EC patients presented a distant metastasis when they were first diagnosed (Tan, Qian et al. 2016). Squamous cell carcinoma (ESCC) and adenocarcinoma (EAC) represent the vast majority of ECs. Despite the increasing incidence of EAC in the west, esophageal squamous cell carcinoma (ESCC) continues to be the most prevalent type of EC worldwide accounting for 90% of all ECs each year.

The survival percentage is significantly low, and the average 5-year survival percentage of one-third of patients is 35.45%. These clinical evidences obviously show that early detection is crucial to treatment. However, only 30% of early patients can be examined. As a result, studies on the molecular mechanism of the invasion and metastasis of EC are critical. Biomarkers can be detected using different detection methods, such as blood testing, immunohistochemistry, molecular pathology, gene expression profile and biopsy. The biomarkers of EC are valuable in predicting the outcome and guiding treatments of the disease (Tan, Qian et al. 2016). Cancer is usually related with the unsuitable mRNA expression of nonmutated genes. Lately, numerous tumor-associated RNA species, including tyrosinase mRNA and telomerase RNA, have been confirmed in plasma and serum. The presence of tumor RNA in plasma and serum affords the opportunity to diagnose or stratify patients with cancer when tissue is not readily available (Kopreski, Benko et al. 2001).
Platelet-derived growth factor (PDGFs), a member of a multifunctional polypeptide family, is composed of A, B, C and D polypeptide chains that form homodimeric or heterodimeric molecules. Two receptor isoforms with tyrosine kinase activity have been identified: platelet-derived growth factor-α receptor and platelet-derived growth factor-β receptor (Lin, Sugai et al. 2008). Activation of growth factor receptors generally needs the ligand to promote dimerization or oligomerization of receptor monomers. The extracellular part of the PDGFRs comprises 5 Ig-like domains out of which domain 2–3 are involved in binding to PDGF, Dimerized and activated PDGFRs are autophosphorylated on approximately 10 sites that can interact with SH2-domain-containing signaling proteins (Papadopoulos and Lennartsson 2018).

The PDGFs have critical roles during development, but there is imperfect evidence for normal physiological functions in the adult. Increased PDGF activity has been related with several diseases and pathological conditions. Causal pathogenic roles of the PDGFs have been well-known for some diseases, providing prospects for therapy using PDGF antagonists. PDGF receptor-inhibiting substances are now widely tested in preclinical models as well as in human clinical trials. Furthermore, recombinant human PDGF-BB has been presented in the clinic as a wound-healing therapy (Andrae, Gallini et al. 2008). PDGF functions have been implicated in a wide range of diseases. For a few of them—i.e. some cancers—there is strong evidence for a causative role of PDGF signaling in the human disease process. (Andrae, Gallini et al. 2008).

2. Materials And Methods

2.1. Serum Preparation

Serum and information from 83 individuals (33 patients with EC (17 males, 16 females) and 50 normal volunteer controls (26 males, 24 females)) were collected. This study was approved by the Ethics committee of Bojnurd university of Medical sciences. The sera were prepared by centrifugation of peripheral blood of the participants at 830 × g for 10 min at 4°C, followed by careful liquating and freezing of the serum. The sera specimens were frozen at -70°C and stored prior to use.

2.2. RNA extraction

Because the RNA molecule is unstable, RNase-free samplers and microtubes were used in all testing processes. RNA was extracted from 3 ml of serum using a commercial kit (QIAamp Circulating Nucleic Acid, QiaGene), performed according to the manufacturer’s direction. The concentration of RNA was then approximated by Nano drop.

2.3. cDNA synthesis

Comparable quantities of RNA per serum aliquot corresponding to 10–45% of the RNA extracted from 3 ml of serum were reverse transcribed by a commercial cDNA synthesis kit (Yektatajhiz, Cat No: YT4500). A mixture of 6 µl extracted RNA, 1 µl random hexamer and 13.4 µl DEPC-treated water were mixed gently, centrifuged briefly and incubated at 70°C for 5 min and then chilled on ice. After that were added 4 µl buffer, 1 µl dNTP, 0.5 µl RNase inhibitor and 1 µl reverse transcriptase and incubated at 37°C temperature
for 60 min and then maintained in a heat block at 70°C for 5 min. 1 µl of cDNA were then used in the amplification reaction. To verify the presence and integrity of serum RNA, all sera were additionally assayed for human GAPDH mRNA, a mRNA expected to be expressed in all individuals.

2.4. Real-Time PCR

RT-PCR for GAPDH was performed using 1 µl of the cDNA in a reaction mixture with RT-PCR MasterMix (YTA SYBR Green qPCR MasterMix 2X). This mixture was cycled 40 times with an initial denaturing temperature of 95°C for 2 min, followed by a denaturing temperature of 95°C for 20 sec, annealing temperature of 59°C for 30 sec, and extension temperature of 72°C for 20 sec. The primers are as follows: human PDGFRβ sense, 5_ TCCAGCTACAGATCAATGTCCC _3; and antisense, 5_GCAGGCAGACCAGATGATGT _3; and human GAPDH sense, 5_GACAACAGCCTCAAGATCATCAGC_3; and antisense, 5_ATGGCATGGACTGTGGTCATG_3. All RT-PCR amplifications were performed with particular attention paid to prevention of contamination. To correlate the digital results of real-time PCR with conventional gel detection, the PCR products were placed on 2.5% agarose gel and stained with safe stain for visualization. The two methods gave highly comparable results.

Bioinformatic analysis

For investigate genes which collaborate with PDGFRB in specific functions (regulation of phosphatidylinositol 3-kinase signaling, regulation of MAP kinase activity, fibroblast proliferation, growth factor binding, growth factor receptor binding, regulation of ERK1 and ERK2 cascade, vascular endothelial growth factor signaling pathway ) which have critical role in cancer using GeneMANIA database(Warde-Farley, Donaldson et al. 2010).

At the end miRNAs which potentially target PDGFRB identify at miRDB(Chen and Wang 2019) with high score and verified in TargetScan(McGeary, Lin et al. 2019).

3. Results

3.1. Demographic characteristics

A total of 33 patients were included in our study. The mean age of the participants was 63.3 ± 12.4 years in the control group and 65.9 ± 11 years in the case group. Males composed 52.6% of all cancer patients and females 47.4%. Dysphagia was the main complaint of patients. 33.3% of patients consumed hot drinks and 56% of them were smoker.

3.2. Tumor characteristics

Most cases of EC were diagnosed as squamous cell carcinoma (73%), followed by adenocarcinoma (27%). At time of diagnosis most cases of adenocarcinoma were poorly differentiated (42.04%). Squamous cell carcinoma was mostly well differentiated (70.4%) at time of diagnosis. Both Squamous
cell carcinoma and Adenocarcinoma were mainly located in the lower esophagus (54.2% and 77.7%, respectively).

### 3.3. Evaluation of PDGFRβ mRNA expression

Detection of PDGFRβ in RNA extracted from serum of participants demonstrated the ability of the assay to detect PDGFRβ mRNA in all samples. GAPDH mRNA was demonstrated in the serum of all patients and all normal volunteers, confirming both the presence and integrity of RNA in serum. The cycling numbers of real-time PCR reactions in the serially diluted samples were assayed and the correlation coefficients of the RNA concentration and Ct value were very good. Results were 0.99765 and 0.99789 respectively for PDGFRβ and GAPDH, indicating that RNA expression is adequately represented by the Ct value.

The CT of the PDGFRβ gene in the case group was 3.25, while it was 3.53 in the control group. As a result, the case group’s expression of this gene was higher than the control group’s. The case and control groups had significantly different levels of expression of this gene (p = 0.03). Also the expression ratio of the PDGFRβ gene in the case group was 1.12 times that of the control group and Log 2fc for the case group was 0.28 (Fig. 1).

### 3.2. Relationship between PDGFRβ mRNA expression and clinicopathological findings

The independent T-test and one-way ANOVA were used to compare the ΔCT expression of PDGFRβ gene between case groups’ variables including the sex and smoking status of the patients and type, histology and location of tumor. Summarized in the Table 1, the results did not show any significant differences between the corresponding variables.
GeneMANIA database reveal potential collaborate genes such as: PDGFRA, PDGFD, PDGFC in selected functions (Fig. 2). miRNAs which can target PDGFRβ are available in (Fig. 3).

4. Discussion

It has long been identified that RNases are present in blood plasma and serum, and further documented that serum RNase levels augment in patients with cancer. In opinion of the sensitivity of mRNA to degradation by RNase, it was not clear whether RNA could exist in plasma or serum with adequate integrity to permit amplification. Detection of specific tumor mRNA needs an intact and identifiable or amplifiable sequence. (Kopreski, Benko et al. 1999) Serum mRNA levels can be measured for diagnostic purposes, and variations of them in blood may be attributed to altered gene expression in tumor tissues and its reflection in serum. In this study, we used the Real Time PCR technique to assess the expression of the PDGFβ gene in EC by analyzing the circulating mRNA in the serum of two groups: patients with EC (case) and healthy controls. According to the findings of this study, PDGFβ gene expression was higher in the case group than in the control group. This is the first report on alterations in PDGFβ circulating mRNA in EC that we are aware of. Many studies have been performed on the expression of
PDGFRβ in various cancers, including esophageal cancer, but have investigated the expression of this gene in tumor tissue or EC cell lines. (Maderna, Salmaggi et al. 2007, Shinohara, Gonzalez et al. 2007, Gockel, Moehler et al. 2008, Hu and Huang 2021) According to this study about circulating mRNA in the serum, there was a significant difference between the case and control groups \((p = 0.03)\). This could point to the importance of PDGF and PDGFR in tumor tissue formation.

It is well recognized that the development of a fully malignant tumor requires numerous genetic or epigenetic alterations. It is therefore likely that autocrine PDGF stimulation is an initial event in tumor progression, which leads to an expansion of cells that are targets for neoplastic transformation. (Heldin 2012) The platelet derived growth factor ligands (PDGFs) and their receptors (PDGFRs) have emerged as vital regulators of cell growth and division, and mediate significant impact on malignant cells and the tumor microenvironment. (Nordby, Richardsen et al. 2017) In accord with the strong transforming ability of PDGFRβ, most malignant mesothelioma cell lines express PDGFRβ, whereas normal mesothelial cells predominantly express PDGFRα. (Yu, Ustach et al. 2003)

On the other hand, we found that expression of this gene was higher in individuals with less differentiated tumors than individuals with a fully differentiated tumor. Our finding is consistent with a similar study by Sang Yun Ha et al. (Ha, Yeo et al. 2014) So it seems that tumor tissue in the early stages of its formation by expressing the PDGFR gene and its expression on the surface of cells facilitates growth, angiogenesis and eventually metastasis. Therefore, measuring and evaluating PDGFRβ gene expression may be useful in the early stages of tumor formation and the initiation of a rapid treatment process. Interestingly, varied factors such as sex, type of esophageal cancer, tumor grade, and area of esophageal tumor formation had no significant effect on PDGFRβ expression in our study.

In this study, Squamous Cell Carcinoma (SCC) was the most common esophageal cancer \((ESCC = 73\%\) percent EAC = 27 percent), however, in Western nations such as Northern and Western Europe, Northern America, adenocarcinoma is the most common type of EC. In most of Asia and Sub-Saharan Africa, esophageal carcinomas happen nearly entirely as ESCCs. Within countries, the proportion of ESCC and EAC can vary greatly among population subgroups. For instance, in the US, African-Americans are 7-fold more probable to be diagnosed with ESCC than EAC, whereas US whites are about 4-fold more probable to develop EAC than ESCC. The reasons for this large difference are not clear and can’t be fully explained by known risk factors. (Abnet, Arnold et al. 2018, Then, Michell Lopez et al. 2020) Also, (66.1%) of all the patients had tumors in the lower esophagus, \((ESCC 54.2\% \text{ and EAC 77.7}\%)\) whereas this finding is in contrast to some other researches. For example,

Eric Omar Then et al in their research found that Adenocarcinoma was mainly located in the lower esophagus (78.9%), whereas the middle esophagus was the most common location for squamous cell carcinoma. (Maley and Rustgi 2006, Then, Michell Lopez et al. 2020) So it seems that the location of
tumor in the esophagus varies among different countries and races. Although PDGFRβ gene expression did not differ significantly between men and women (p = 0.790) however it was higher in men.

Because of the high frequency and high mortality rate, poor prognosis at the time of diagnosis of esophageal cancer, it is critical to develop adequate diagnostic tools and tumor markers to diagnose the disease as soon as possible and initiate treatment. Given that circulating RNAs are more stable than previously assumed when combined with lipids, proteins, and vesicles,(El-Hefnawy, Raja et al. 2004) it appears that these nucleic acids can be employed to improve/replace the routine diagnostic methods because blood collection is easier, less invasive, and more repeatable than biopsy and other methods. The current study found that those with a family history of cancer were more likely to get esophageal cancer. In the cancer group, 37.1 percent had a family history of cancer, compared to 18 percent in the control group. Our finding is consistent with similar studies but some studies have different findings.(Hu, Dawsey et al. 1992, Wang, Han et al. 1992, Slattery and Kerber 1994, Winawer, Zauber et al. 1996, Chang-Claude, Becher et al. 1997, Keku, Millikan et al. 2003)

5. Conclusion

In conclusion, we found a positivity for PDGFRβ in patients with esophageal cancer. These finding shows that PDGFRβ could be a useful biomarker for EC prognosis. As a result of the findings of real-time PCR and other statistical analysis, it is believed that further research into serum RNAs, including beta-platelet-derived growth factor receptor mRNA, could lead to a non-invasive, rapid diagnostic method and, ultimately, earlier treatment of EC and more prospective studies on individual gene expression profiles are necessary to clarify their influence on prognosis in this cancer. On the other hand, because this study found a significant likelihood of hereditary impacts on the prevalence of esophageal cancer, family members of patients with EC can be monitored for medical examinations. However, further studies and researches in these areas are needed.

Declarations

Aknowledgements

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Ethical Approval

The authors declare that the protocol herein described complies with the Bojnurd University of Medical Sciences and that they obtained institutional review board approval and have been performed in accordance with the ethical standards (IR.nkums.REC.1396.29).

Conflicts of Interests

The authors declared no conflict of interests.
Authors’ Contribution

Ali Babaei: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Visualization, Writing - original draft. Hadi Mohammad doost: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Validation, Visualization, Writing - original draft. Vahid Dashti: Investigation, Supervision, Writing - original draft, Writing - review & editing. Taha Mohammadreza poor: Investigation, Visualization, Writing - original draft, Writing - review & editing. Seyyedehsara Raeisalsadati, Visualization Fatemeh Nour Mohammadi1: Conceptualization, Funding acquisition, Writing - review & editing. Alireza Salimi: Data curation analysis, Funding acquisition, Investigation, Methodology, Resources, Visualization, Writing. Alireza Abbaspoor: Data curation, Funding acquisition, Supervision, Validation, Writing - review & editing, Project administration. Reza Salarinia: Data curation, Funding acquisition, Supervision, Validation, Writing - review & editing, Project administration.

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References


Figures

**Figure 1**

(A) The rate of ΔCT expression of PDGFRβ gene in both case and control groups, (B) The rate of PDGFRβ gene expression (-ΔΔCT) in the case group compared to the control group, (C) Results of PDGFRβ gene expression analysis (fold change _2 ^ΔΔCT_) in case group compared to control group (* p < 0.05).
Figure 2

Analyzing in GeneMANIA database shows that genes collaborate with each other in selected functions.

Figure 3

hsa-miR-30a-5p, hsa-miR-24-3p, and hsa-miR-9-5p could potentially target PDGFRB according to TargetScan and miRDB databases.