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Perturbation of miR-146b and relevant inflammatory elements in esophageal carcinoma patients supports an immune downregulatory mechanism

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ABSTRACT

Background: Esophageal Cancer is known as one of the deadliest cancers worldwide with the squamous cell carcinoma (ESCC) being the predominant subtype. There is a growing body of evidence linking the dysregulated microRNA (miRNA) pathway of immune cells to the progression of several tumors. In a previous study, we investigated molecular alterations pertaining to miR-146a and some components of NF-kB signaling pathway and proposed a possible immune downregulatory mechanism in peripheral blood mononuclear cells (PBMCs) of ESCC patients. Here, we further scrutinized other components of this pathway by evaluating PBMC levels of miR-146b, TLR4, IL10, and TNFA.

Methods: Gene expressions were quantified using RT-qPCR assays. To prevent the vulnerability of results to the expression instability of reference genes, nine additional transcripts were quantified, and stable reference genes for normalizing qPCR data were identified using the NormFinder and the geNorm algorithms. The efficiency-corrected normalized relative quantity values were used to compare gene expressions among study groups.

Results: The PBMC expression of miR-146b and TNFA was downregulated in ESCC patients as compared to healthy subjects. While the level of TLR4 was not different among the study groups, the PBMC level of IL10 was upregulated in ESCC patients. Logistic regression analyses coupled with the ROC curve and cross-validation analysis suggested that PBMC expression may serve as potential candidate biomarker for discriminating ESCC patients from healthy subjects.

Conclusion: The present findings, in line with our previous report, propose a particular gene expression pattern in PBMCs of ESCC patients, providing evidence in support of an immune downregulatory mechanism.

1. Introduction

Esophageal Cancer (EC) is known as one of the deadliest cancers worldwide [49]. Among all cancers, EC is ranked sixth in mortality and eight in incidence, with a great variation by geographical distribution [24]. There are mainly two types of EC, including Esophageal Squamous Cell Carcinoma (ESCC) and Esophageal Adenocarcinoma (EAC), that are originated from the same organ but with a distinct etiology and pathology [18]. In Asia, ESCC remains the predominant subtype and accounts for 90% of EC cases in Asian countries [24]. According to statistics, the highest incidence rate of ESCC has been found in East Asia and Southern and Eastern America. In contrast, it is relatively

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uncommon in Central America and Western Africa [18,34]. Recent studies in Iran have reported that the highest prevalence of ESCC occurs in northern region specifically Golestan province (ASR of 24.3 for men and 19.1 for women) [18]. ESCC affects more men than women and its occurrence peak is observed in the seventh decade of life [53]. In retrospective studies, environmental factors such as high temperature drinks, opium use, smoking, and alcohol consumption have been recognized as strong risk factors [18,53]. In addition, accumulating evidences have suggested that genetic components and various molecular changes are involved in the development of ESCC [9,31,34]. Despite advances in multimodality therapies including surgery, chemotherapy, endoscopy, and radiation, the overall survival rate is 15–25%, and, due to distant metastasis and local invasion, the quality of life still remains poor [21,34,50]. Given the above context, it is essential to characterize biological mechanisms and molecular markers that may help to facilitate early diagnosis [20,24].

There is a growing body of evidence linking inflammation as well as immune deregulation to tumor progression; however the exact underlying molecular mechanisms have been to be elucidated [23,37]. Peripheral blood mononuclear cells (PBMCs) and inflammatory cytokines are the main arms of immune system that participate in important events of several tumor development and exert powerful effects on carcinogenesis processes [6,33]. Moreover, cytokines are capable of providing intercellular communication and creating immune dialogs between PBMCs and cancer cells [12,26]. Recent studies indicated that PBMCs possess the ability to sense tumor-secreted TLR stimulating molecules and to exhibit a unique transcriptional signature including alterations of NF-kB dependent molecules, especially microRNAs, and downstream inflammatory responses in the TLR signaling [8,13,19,40,45]. It is thought that the expression pattern of microRNAs in PBMCs can reflect the cancer status and behave as a novel biomarker for many different tumors [1,5,13,16,28,41,52].

MicroRNAs (miRNAs) are small non-coding RNA molecules that regulate the translation of numerous different mRNAs and diverse cellular processes [20]. Emerging evidences have demonstrated that miRNAs play a key role in pathways underlying cancer development including ESCC [15,20]. The expression level of miRNA varies depending on the tumor type, and altered miRNA expressions have been associated with progression and tumorigenesis of various types of cancer [10,14,29]. Among the various miRNAs involved in controlling inflammatory challenges, miR-146a and miR-146b have emerged as critical mediators of key events in the inflammatory immune responses through regulating *TLR4* mediated *NF-kB* signaling pathway [46,47]. There is controversy regarding the role and the expression of miR-146a and miR-146b as well as their target genes in cancers, supporting the presence of cancer-specific gene expression patterns [11,35,47]. In a previous study [32], we investigated molecular alterations pertaining to miR-146a and some components of *NF-kB* signaling pathway and proposed a possible immune downregulatory mechanism in PBMCs of ESCC patients. However, the underlying mechanism is not yet clear. Following our previous observations, we set out to further scrutinize other components of this pathway. The present study aimed to investigate the PBMC levels of miR-146b, *TLR4*, and other downstream signaling molecules including *IL10* and *TNFA*. To prevent the vulnerability of qPCR results to the expression instability of a single reference gene, we set out to quantify nine additional transcripts (described in materials and methods) to be used for normalizing PBMC gene expressions.

2. Experimental procedures

2.1. Study population

The study population is described in a previous publication [32]. A total of 40 ESCC patients and 50 cancer-free subjects were recruited in this study. Patients were selected among individuals who referred to the Cancer Institute (Imam Khomeini Hospital, Tehran, Iran). All patients had pathologically confirmed esophageal squamous cell carcinoma (stage T3-4). About five ml blood was obtained from patients before they receive any intervention. The male to female ratio was ~1.22 in the ESCC group (22 out of 40 participants were male). Patients were 60.6 (± 12.8) years old in average, ranging from 38 to 87 years old. Subjects of the control group were selected among individuals who were candidate for a health check-up at the Modarres Hospital (Tehran, Iran). These subjects were required to have no history of any of the following conditions: malignancy, hospitalization during one year before recruitment in the study, inflammatory diseases (e.g. diabetes, ischemic heart disease, and stroke). Participants of this group were 59.4 (±11.2) years old in average, ranging from 36 to 82 years. The male to female ratio was ~1.27 (28 out of 50 participants were male). A written informed consent was obtained from each participant. The Research Ethics Committee of Tabriz University of medical sciences approved the study (IR.TBZMED.REC.1397.488).

2.2. Isolation of mononuclear cells, extraction of RNA, and reverse transcription

We isolated peripheral blood mononuclear cells (PBMCs) from each sample using the standard density-gradient centrifugation (Ficoll-Paque PLUS, Amersham Pharmacia Biotech, Sweden). The phosphate-buffered saline (PBS) was used to wash PBMCs before proceeding into the extraction of total RNA using the miRNeasy Mini kit (Qiagen, Germany). The total RNA obtained with this kit included small RNAs. We leveraged the original stem-loop RT-qPCR strategy [7] to reverse transcribe miR-146b and other small RNAs in 10 μl RT reactions, which contained ~500 ng input RNA. These RT reactions were run using a three-step program: a pre-incubation at 16 °C/30 min, an incubation at 42 °C/30 min, and enzyme inactivation at 82 °C/5 min Table 1 lists all primers that are used in the cDNA synthesis and qPCR. We used separate RT reactions and the protocol provided with the PrimeScript First Strand cDNA Synthesis Kit (Clontech, Takara Bio, Japan) to synthesize the first-strand cDNA of mRNAs and their reference genes.

2.3. qPCR

miR-146b and six reference small RNAs (see Section 2.4) was quantified using the Rotor-Gene-Q instrument (Qiagen, Germany) in 10 μl qPCR reactions, which contained RT product (1 μl), 5X qPCR mix (2 μl), forward and reverse primers (1 μl of 5 pmol/μl), and nuclease-free water (5 μl). Quantifications were performed in duplicate reactions using following steps: the incubation: 95 °C/12 min, 40 qPCR cycles: 95 °C/10 s and 60 °C/30 s, and a melting analysis.

mRNAs and their reference genes (i.e. IL10, TLR4, TNFA, GAPDH, ACTB, and 18 S rRNA) were quantified the Light Cycler 96 instrument (Roche Diagnostics, Mannheim, Germany) in 10 μl qPCR reactions, which contained RT product (1 μl), 5X qPCR mix (2 μl), forward and reverse primers (1 μl of 5 pmol/μl), and nuclease-free water (5 μl). Quantifications were performed in duplicate reactions using following steps: the initial incubation: 95 °C/12 min, 40 cycles: 95 °C/10 s and 60 °C/60 s, and a final melting analysis. The mean efficiency value (i.e. E value) for each amplicon was calculated by averaging through efficiency values that were obtained through fitting nonlinear least squares (Levenberg-Marquardt) to the baseline-normalized fluorescence data, as described previously [39,44]. The average efficiency for mRNAs and their corresponding reference genes was obtained as follows: IL10 (1.96), TNFA (1.94), TLR4 (1.95), IL1B (1.97), ACTB (1.94), 18S rRNA (1.95), and GAPDH (1.95).

The relative expression of amplicons was computed according to the following scheme (also presented elsewhere [22]): let $E^{\Delta Ct}$ be the relative quantity (RQ) of each amplicon, with "E" being the mean efficiency value and "ΔCt" being the subtraction of C_t value of the amplicon in a sample from the average C_t of the amplicon considering all samples. Then, the RQ value of an amplicon divided by the geometric mean of RQ

Table 1
Primers that are used in the cDNA synthesis and qPCR. RT: Reverse transcription. F: Forward. R: Reverse.

Gene	Type	Primer sequence
miR-146b-5p (MIMAT0002809)	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGCCTA
	F	CGCTGAGAACTGAATTCCATAGGC
	R	GTGCAGGGTCCGAGGT
SNORD47	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACCTCA
	F	GCGATATCACTGTAAAACCGTTCCA
	R	GTGCAGGGTCCGAGGT
SNORD24	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGCATCAG
	F	CGCTATCTGAGAGATGGTGATGACATT
	R	GTGCAGGGTCCGAGGT
SNORD48	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGTCAGA
	F	AGGTAACTCTTGAGTGTGTCGCT
	R	GTGCAGGGTCCGAGGT
SNORD44	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGTCAGTT
	F	GAATGCTGACTGAACATGAAGGTCT
	R	GTGCAGGGTCCGAGGT
SNORA66	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGACTGTAC
	F	TGATGGAAATGTGTTAGCCTCAGACA
	R	GTGCAGGGTCCGAGGT
RNU6	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAAATAT
	F	GCTTCGGCAGCACATATACTAAAAT
	R	CGCTTCACGAATTTGCGTGTCAT
GAPDH	F	AAGGCTGAGAACGGGAAGCT
	R	CAGCATCGCCCCACTTGATT
18S rRNA	F	CGAACGTCTGCCCTATCAACTT
	R	ACCCGTGGTCACCATGGTA
ACTB	F	ACTTAGTTGCGTTACACCCTT
	R	GTCACCTTCACCGTTCCA
IL10	F	GGCTTGGGGCTTCCTAACTG
	R	GGGAATCCCTCCGAGACACT
TNFA	F	CCCAGGGACCTCTCTCTAATCA
	R	GCTACAGGCTTGTCACTCGG
TLR4	F	AAGCCGAAAGGTGATTGTTG
	R	CTGAGCAGGGTCTTCTCCAC

values of references would yield the normalized relative quantity (NRQ). Statistical analysis was performed using log2(NRQ) values. The geometric mean RQ values of three small RNAs (see Section 2.4) and three RNAs (i.e. ACTB, GAPDH, and 18S rRNA) were used for normalizing miRNA and mRNA quantities, respectively.

2.4. The stability of reference small RNAs

The instability of reference genes that are used for normalizing qPCR data may influence the robustness of subsequent analyses. Therefore, it has been suggested to use the geometric mean of multiple genes and evaluate the stability of reference genes [38,48]. Moreover, as the first strand cDNA of miRNAs and mRNAs is synthesized using different reverse transcription procedures, it is advised to use different reference genes to normalize miRNA and mRNA quantifications. Accordingly, six small RNAs, namely RNU6 (U6), RNU47 (U47), RNU48 (U48), RNU66 (U66), RNU24 (U24), and RNU44 (U44), and three RNAs (i.e. ACTB, GAPDH, and 18S rRNA) were quantified in all samples. The stability of small RNAs was evaluated using the NormFinder [2] and the geNorm [48] algorithms, and the geometric mean of most stable genes was used for normalizing miRNA quantities. The NormFinder algorithm takes into account the overall expression variation and the variation between study groups. Specifically, it computes three measures: (i) GroupDif, measures the difference between the study groups; (ii) GroupSD, is the weighted average of intragroup variances and measures the common standard deviation within a group; (iii) Stability, a stability value that is negatively correlated with the stability of the expressed gene. Moreover, it provides a combined stability measure for different pairs of reference genes. The geNorm algorithm calculates the M-value, which is a stability measure. Lower M-values are associated with more stably expressed genes. It uses a procedure in which lease stable reference genes are excluded sequentially and a normalization factor is computed in each step. To determine the optimum number of reference genes, the pairwise variation between two sequential normalization factors (i.e. Vn/Vn+1) is computed. The optimum number of reference genes was obtained when the pairwise variation were close to the cut-off of 0.15.

2.5. Statistical analysis

The Shapiro-Wilk test was implemented to evaluate if quantitative variables came from a normally distributed population. The statistical significance of differences in relative gene expressions (i.e. log2[NRQ]) between patients and controls was assessed using either the student's *t*-test (when expression values were normally distributed) or the Mann-Whitney *U* test (when expression values were not normally distributed). Simple or multiple logistic regression models was fitted to evaluate the potential of PBMC gene expressions in discriminating ESCC patients from subjects of the control group. The disease status was set as the response variable and relative gene expressions (i.e. log2[NRQ]) were set as predictors. Fitted probabilities of the disease status were computed and used in the receiver operating characteristic (ROC) curve analysis. The optimal threshold was obtained by Youden's method. The model performance was evaluated by computing four measures, namely the area under the curve (AUC), specificity, sensitivity, and the prediction error obtained from leave-one-out cross-validation (LOOCV). Statistical analyses were performed in the R environment (version 3.5.1).

3. Results

3.1. The stability of reference genes

The stability of reference genes was evaluated using the NormFinder and the geNorm algorithms. Fig. 1A shows the three measures that were computed by the NormFinder algorithm for the reference small RNAs (see methods for details). Overall, a decrease in the group difference (i.e. GroupDif) and the common group variation (i.e. GroupSD) was

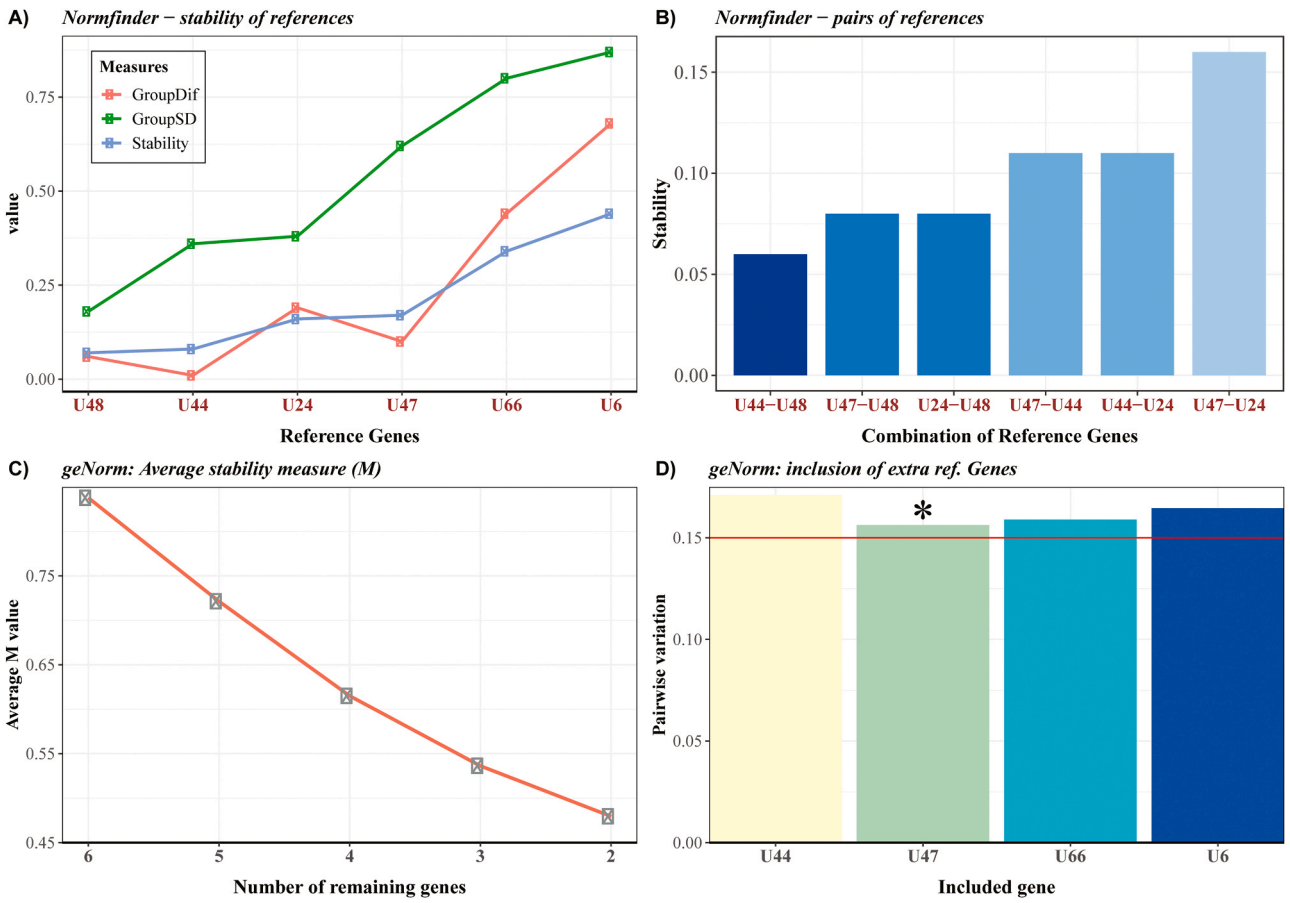

Fig. 1. Results of the Normfinder (A and B) and the geNorm (C and D) stability analyses for reference genes. (A) Plot of the three Normfinder measures for small RNA references. Measures are GroupDif (the group difference), GroupSD (the common group variation), and the stability. (B) The combined stability measure computed by the Normfinder for different pairs of reference genes. (C) The geNorm results for the average M value of the remaining reference genes after each step of excluding the most unstable reference. (D) The geNorm results for the pairwise variation imposed by the stepwise inclusion of more reference genes to the minimum set of two references (i.e. U24 and U48).

associated with a decrease in the stability measure and an increase the expression stability of the reference gene (Fig. 1A). The NormFinder algorithm identified U48 as the most stable reference gene with the lowest stability value followed by U44, U24, U47, and U66 (Fig. 1A). U6 was identified as the least stable gene by NormFinder. Moreover, a combined stability measure was computed for different pairs of reference genes (Fig. 1B). The NormFinder analysis identified that the combination of U48 and U44 was the most stable pair of reference genes. Moreover, the combination of U48 with either U47 or U24 was the second most stable pair of reference genes (Fig. 1B).

The geNorm analysis identified U48 and U24 as the most stable reference genes. The ranking of reference genes was as follow (from the least stable to the most stable): U6, U66, U47, U44, U24-U48. This algorithm started with all six reference genes and sequentially excluded the least stable reference gene monitors while monitoring changes in the average stability measure (i.e. M value) of remaining reference genes. Fig. 1C shows the average M value of the remaining reference genes after each step of excluding the most instable reference. As expected, excluding the least stable references led to a decrease in M value (i.e. an increased stability). Fig. 1D shows the pairwise variation imposed by the stepwise inclusion of more reference genes to the minimum set of two references (i.e. U24 and U48). The significant effect of including U44 is evident from the relatively high pairwise variation observed after its inclusion (Fig. 1D). However, the inclusion of the next gene (i.e. U47) may not be required for a reliable normalization as it is evident by a low pairwise variation of close to 0.15. Therefore, the geNorm analysis suggested that the geometric average of U48, U24, and U44 may serve as a proper value for reliable normalization of PBMC miRNA quantities.

3.2. PBMC gene expressions in ESCC patients

Fig. 2A shows the PBMC expression of the studied genes in ESCC patients and the control group. As suggested by the geNorm analysis, the expression of miR-146b was normalized using the geometric average of U48, U24, and U44. The mean expression level of miR-146b was significantly lower in PBMCs of ESCC patients than that in PBMCs of healthy subjects (Fig. 2A, *t*-test *P*-value: 3.17E-05). To evaluate whether this finding was sensitive to the selection of proper reference genes, the expression of miR-146b was also normalized relative to the geometric mean of U48 and U44 (as suggested by NormFinder), U48 and U24 (as suggested by NormFinder) or all six reference genes. The down-regulation of miR-146b in ESCC patients was remained statistically significant after normalizing expressions relative to the geometric mean of U48 and U44 (*t*-test *P*-value: 1.8E-03), U48 and U24 (*t*-test *Pvalue*: 8.3E-05) or all six reference genes (*t*-test *P*-value: 2.14E-04).

The Mann-Whitney *U* test revealed no statistically significant difference between ESCC patients and healthy subjects regarding PBMC levels of TLR4 (Fig. 2A, *P*-value: 0.13). However, TNFA was down-regulated in PBMCs of ESCC patients as compared to healthy subjects (Fig. 2A, Mann-Whitney *U* test *P*-value: 0.014). Moreover, an increased level of IL10 was observed in PBMCs of ESCC patients compared to healthy subjects (Fig. 2A, *t*-test *P*-value: 2.14E-05).

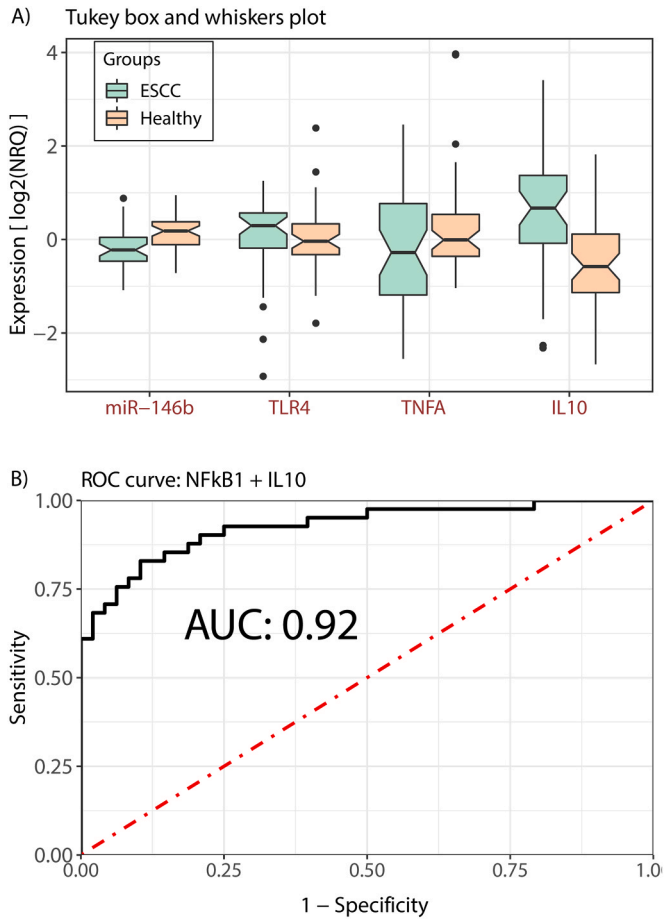

Fig. 2. PBMC gene expressions and ROC curve analysis A) Box and whisker plots of PBMC gene expressions in ESCC patients and control subjects. The vertical axis represents base two logarithm of normalized relative quantity (NRQ) values. *: *P*-value < 0.02, ***: *P*-value < 0.0001, ns: *P*-value > 0.05. Whiskers represent Tukey's IQR. B) The ROC curve showing the discriminatory power of a model including NF-κB1 and IL10 with an area under the curve (AUC) of 0.92.

3.3. Potentials of PBMC expressions in discriminating patients from healthy subjects

Logistic regression followed by ROC curve analysis was performed to evaluate the potential of PBMC expressions in discriminating ESCC patients from healthy subjects (Table 2 and Fig. 2B). PBMC levels of miR-146b and IL10 had moderate potentials in discriminating ESCC patients from healthy subjects (Table 2, AUC of 0.76 and 0.73, respectively). In our previous publication on the same cohort [32], we demonstrated that the combination of NF-κB1 and IL6 in a multivariate analysis had better performance in discriminating ESCC patients from healthy subjects in terms of both AUC and estimated prediction error (Table 2, AUC: 0.87, estimated error: 0.18). Here, we showed that the combination of NF-κB1 and IL10 may provide even better performance in terms of AUC and a comparable error rate (Table 2 and Fig. 2B, AUC: 0.92, estimated error: 0.19).

Table 2
Discriminatory potentials of PBMC gene expressions. ROC curve analyses and leave-one-out cross-validation of various models are shown.

Predictor(s)	AUC[a] (95%CI)	Specificity	Sensitivity	CV prediction error rate[b]
miR-146b	0.76 (0.65–0.87)	0.66	0.86	0.32
TNFA	0.63 (0.50–0.76)	0.92	0.43	0.47
IL10	0.73 (0.61–0.84)	0.76	0.66	0.33
NF-κB1 + IL6	0.87 (0.79–0.95)	0.89	0.76	0.18
NF-κB1 + IL10	0.91 (0.85–0.98)	0.87	0.84	0.19

[a] AUC: area under the curve.
[b] The leave-one-out cross-validation estimate of the prediction error.

4. Discussion

MiR-146a and miR-146b are members of the miR-146 family of miRNAs that share the same 'seed' sequence, suggesting the same repertoire of transcript targets [46]. However, the fact that they are located on separate chromosomes (chromosomes 5 and 10, respectively) raises the possibility of distinct posttranscriptional processing mechanisms and functions [11,27]. This possibility is reinforced by studies reporting divergent and asynchronous transcriptional regulation for these miRNAs [11,35].

MiR-146b serves as an IL-10 dependent miRNA with an anti-inflammatory function [11]. Similar to miR-146a, miR-146b seems to modulate the TLR4 signaling pathway as well as proinflammatory cytokine production through negative feedback regulation of *NF-κB* pathway key adaptors including IRAK1 and TRAF6 [46,47], which has been reported to be downregulated in PBMCs obtained from ESCC patients compared to healthy controls. We showed that miR-146b was downregulated in PBMCs of ESCC, which is consistent with reports in other diseases [3,11,25]. Downregulation of miR-146b, along with our previous observation regarding up-regulation of miR146a, reinforces divergent regulatory roles for PBMC miR-146b in NF-κB mediated inflammatory processes in ESCC.

IL-10, which is primarily produced by T helper 2 cells, has been known as a strong anti-inflammatory cytokine with a broad range of immunosuppressive effects [4]. It participates in the development of M2 macrophage phenotype polarization, which resemble tumor-associated macrophages (TAMs), and in preventing certain cytokine secretion by Th1 cells [4,36]. IL-10 also has the ability to stimulate and promote regulatory T cells (Tregs) function which, together with TAMs, are the important components of solid tumor microenvironment [17,54]. Moreover, IL-10 may be involved in deactivation or unresponsiveness of monocytes via repression of proinflammatory cytokines production, such as TNF, resulting in non-responsiveness to tumor cells [30,42,43]. TNFα is involved in inflammation-associated carcinogenesis as a multifunctional and double-dealer cytokine that could have either pro- or anti-tumorigenic effects [51]. In a word, upregulation of IL10 and downregulation of TNFα are conceptually consistent with our previously proposed immune suppressive effect in PBMCs of ESCC patients [32]. This IL-10-TNF phenotype has also been proposed for other tumors especially advanced cancers [4,33], implying that the same phenomenon may also occur in PBMCs of ESCC patients. Specifically, the elevated level of immunosuppressive cytokine (i.e., IL-10) and suppressed production of proinflammatory cytokine (i.e., TNFα) have been reported as a plausible phenotype of the impaired host immune response originating from a complex process, which is induced by tumor cells in PBMCs of advanced ovarian cancer and head and neck squamous cell carcinoma [4,33]. This hypothesis is further supported by observations in PBMCs of other cancers stating that a reduced TNF level is correlated with a diminished IRAK1 after pre-exposure to tumor cells, paving the way for the impairment of immune response in advanced cancers [30,43]. Interestingly, a reduced expression of PBMC IRAK1 has been reported in the same cohort that was used in this study [32].

Taken together, the present findings, in line with our previous report, propose a particular gene expression pattern in PBMCs of ESCC patients, providing evidence in support of an immune downregulatory mechanism. Further researches are necessary to identify downstream changes

induced by the perturbation of miR-146b/a and whether such changes contribute to the reduced capability of PBMCs to adequately respond to ESCC tumor cells and tumor development.

CRediT authorship contribution Statement

Milad Bastami: Methodology, Investigation, Writing – original draft, Visualization, Formal analysis. **Habibollah Mahmoodzadeh:** Methodology, Resources. **Zahra Saadatian:** Resources, Data curation. **Abdolreza Daraei:** Data curation, Validation. **Sepideh Zununi Vahed:** Resources, Data curation. **Yaser Mansoori:** Conceptualization, Supervision, Writing – review & editing. **Ziba Nariman-Saleh-Fam:** Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing.

Declarations of interest

The authors declare that they have no conflicting interest.

Data availability

Data available on request due to privacy/ethical restrictions.

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