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ORIGINAL ARTICLE

Dysregulation of helper T lymphocytes in *esophageal squamous cell carcinoma* (ESCC) patients is highly associated with aberrant production of miR-21

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Abstract

Dysregulation of helper T (Th) cell subsets has been contributed to the initiation and propagation of *esophageal squamous cell carcinoma* (ESCC). Different microRNAs (miRNAs) have been reported to control the development and functions of tumor-associated immune cells in ESCC. Here, we aimed to assess the IL-10, TGF-β, IFN-γ, and IL-17a-producing CD3+CD8− T cells in association whit miR-21, miR-29b, miR-106a, and miR-155 expression in ESCC patients. A total of 34 ESCC patients including 12 newly diagnosed (ND) and 22 under-treatment (UT) cases and also 34 age-matched healthy donors were enrolled. Flow cytometric characterization of stimulated T cells was performed by staining of the cells with fluorescent conjugated specific anti-human CD3 and CD8 cell surface markers as well as IL-17a, IFN-γ, IL-10, and TGF-β intracytoplasmic cytokines. Circulating RNA was extracted from the plasma, and qRT-PCR was used to evaluate the expression of microRNAs. TGF-β plasma levels were also assessed by ELISA. Results showed that the frequency of Th cells was significantly reduced in patients. A significant increase in Treg as well as Th17 cells population in both patient subgroups was observed. ND patients showed elevated level of Th1 cells and IL-10. However the mean expression of IFN-γ was significantly decreased in Th cells. We also detected higher level of miR-21 in the ESCC patients which was significantly correlated with different subsets of Th cells. Our findings revealed that immune response related to the Th cells is highly impaired in ESCC patients. Association between miR-21 and Th subsets could be correlated with the impairment of anti-tumor immunity and ESCC pathogenesis, which could be potentially used as an important target for immunotherapeutic approaches.

Keywords Esophageal squamous cell carcinoma (ESCC) · Helper T cells · Immunoregulation · miR-21 · miR-29b

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Introduction

Esophageal cancer (EC) still remains a leading cause of cancer-related mortality and morbidity worldwide. The burden of EC is increasing in developing countries including Iran. The highest incidence of *esophageal squamous cell carcinoma* (ESCC) as a major histological type of EC has been reported in an area in the North of Iran which is called *Esocancer belt* [1, 2]. Despite the progresses in the diagnosis and treatment of ESCC, the 5-year survival rate has not markedly improved [3]. Although several studies have proposed mechanisms for ESCC pathogenesis [1, 4, 5], the underlying immunologic causes are not thoroughly elucidated.

Dysregulation of T lymphocytes and related subsets including T helper (Th) cells has been contributed to the initiation and propagation of ESCC [6, 7]. Several studies have demonstrated that interferon γ (IFN-γ)-producing Th1 cells may possess anti-tumor properties and exert direct negative effects on tumors by inhibiting proliferation and stimulating apoptosis [6, 8]. On the other hand, IL-17a-producing $CD4^+$ T (Th17) cells may function as pathogenic Th cells in inflammatory disorders and autoimmunity. However, the role of Th17 cells in cancer pathogenesis including ESCC is still controversial [9, 10]. Previous studies showed the accumulation of Th17 cells in the blood and cancer tissues of ESCC patients and demonstrated that the CCR4-CCL17/22 and CCR6-CCL20 axis might play an important role in Th17 cell infiltration of tumors. Moreover, high levels of Th17 cells have been associated with lymph node metastasis factor and clinical stages, and so Th17 cells were proposed as independent predictors in the assessment of metastasis and prognosis of EC [11, 12]. The increased expression of Th17 cells might also play a proinflammatory role in early ESCC, where the balance of Th17/Treg cells is broken. It might plays an important role in the pathogenesis of ESCC [3]. Regulatory T lymphocytes (Tregs) which are outnumbered in some cancers could be associated with tumor progression and poor prognosis. Transforming growth factor β (TGFβ) and interlukin-10 (IL-10) are major cytokines produced by Tregs with diverse effects on immune response and angiogenesis in various cancers [13]. However, the role of TGFβ pathway and IL-10-producing cells in the development and progression of ESCC is poorly understood [14].

Regarding the immune response in both physiological and pathological conditions, different microRNAs (miRs) have been reported to control the development and functions of tumor-associated immune cells in various cancer types including ESCC [15–17]. According to the role of miR-21 in regulation of cell proliferation and invasion in ESCC, it could be used as a therapeutic marker in this cancer [18]. Moreover, miR-29b which is known as a tumor suppressor miRNA may exert regulatory effects on the differentiation of Th cells [19]. The overexpression of miR-21 in gastric cancer has been associated with the increased percentage of Th17 cells and decreased percentage of Treg cells [20]. Some studies showed that miR-155 has a critical role in both innate and adaptive immune responses [21–23]. Downregulation of miR-155 in EC [24] enhanced Treg and Th17 cells differentiation and IL-17A production by targeting SOCS1 [21]. Expression and functions of miR-106a in esophageal cancer (EC) are not clearly defined, but new studies showed decreased expression and anti-oncogenic roles of miR-106a in human EC [25] and its role in cytokine regulation such as IL-10 and IL-8 [26, 27].

According to the important role of Th cell responses in the tumor immunity and the probable role of miR-21, miR-29b, miR-106a, and miR-155 in regulation of T cell responses, we evaluated the IFN-γ, IL-17a, IL-10 and TGF-β producing CD3+CD8− T cells in association with these miRNA levels in ESCC patients.

Materials and methods

Patients and controls

Thirty-four ESCC patients including 12 newly diagnosed (ND) and 22 under-treatment (UT) cases and 34 age-matched healthy donors were enrolled in this study. All of the subjects were recruited from Atrak Clinic, Khatam-al Anbiya Hospital, Gonbad-e Kavus, Golestan Province, Iran. A trained oncologist filled data collection sheets including clinical and laboratory data of all patients at each visit (Table 1). A total volume of 5 mL peripheral whole blood was taken from all participants in a sterile tube and transferred immediately to the laboratory. This study involving human samples was approved by the ethical committee of Golestan University of Medical Sciences and a written informed consent following the declaration of Helsinki was taken from all participants.

Immunophenotyping of T cell subsets by flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque (Baharafshan, Tehran, Iran) density-gradient centrifugation, as previously described [28]. Plasma was also separated and stored at − 80 °C for RNA extraction and ELISA cytokine assay. Viable cells of 3×10^5 were counted and resuspended in RPMI 1640 (Gibco, Life Technologies, USA) supplemented with 2 μL (microliter) of Cell Stimulation Cocktail (eBioscience, San Diego, USA) and incubated for 14–16 h at 37 °C with 5% CO_2, according to the manufacturer's protocols [29]. Flow cytometric characterization of stimulated T cells was

Table 1 Clinical characteristics and laboratory parameters of patients with esophageal squamous cell carcinoma (ESCC) and normal subjects

Characteristics		Normal subjects ($N = 34$)	Newly diagnosed ($N = 12$)	Under-treatment ($N = 22$)
Age		50.44 ± 1.62*	59.92 ± 3.23*	65.22 ± 1.92*
Gender	Male	5 (14.7%)*	3 (25.0%)*	16 (72.7%)*
	Female	29 (85.3%)*	9 (75.0%)*	6 (27.3%)*
Family history		–	1 (8.33%)*	7 (31.81%)*
Positive *H. pylori* test		NA	10 (83.3%)*	18 (81.8%)*
Chemotherapy		–	–	18 (81.8%)*
Surgical resection		–	–	4 (18.2%)*

*Data are presented as means ± SE (standard error) for continuous measures and number (%) for categorical variables

NA not available

performed by staining cells with PerCP-conjugated anti-human CD3 and FITC-conjugated anti-human CD8 (Biolegend, San Diego, USA) cell surface markers. Cells were then incubated and washed with fix/perm buffer (Biolegend) and prepared for intracellular staining according to the manufacturer's instructions. Cells were aliquoted into two separate tubes. Intracellular staining of the cells in the first tube was conducted using PE-conjugated anti-human IL-17a and APC-conjugated anti-human IFN-γ (Biolegend). The second fraction of cells was stained using PE-conjugated anti-human IL-10 (Biolegend) and APC-conjugated anti-human TGF-β (BD PharMingen, San Diego, USA). BD Accuri flow cytometer (BD PharMingen) was used to run the samples and data were analyzed using BD Accuri C6 Flow analysis software to evaluate the distribution of T cell subsets in EC patients and normal subjects.

Circulating RNA extraction and qRT-PCR of mature microRNAs

The preserved plasma samples were analyzed to be free of hemolysis, and cellular debris was removed by centrifugation. Circulating RNA extraction was conducted on 1 ml of fractionated plasma samples using a modified method by TRIzol (Invitrogen, USA) following the manufacturer's instructions. Two microgram of total RNA was then polyadenylated and reverse transcribed to cDNA using the Pars Genome cDNA synthesis kit (Pars Genome, Iran). Real-time q-PCR amplifications were performed using Pars Genome miR-Amp SYBR green kit which was utilized for each miRNA on line Gene Real-time PCR detection system (Bioer Technology, Hangzhou, China). The expression of miRNAs was normalized to 5s rRNA as a suitable internal control. All experiments were tested in triplicates, and $2^{-\Delta Ct}$ method was used to express the expression of miRNAs for each sample.

Assessment of TGF-β plasma level by ELISA

The separated plasma from all patients and controls was used to evaluate the levels of TGF-β using ELISA kit (Biolegend) according to the manufacturer's instruction. Stat Fax 2100 microplate reader (Awareness, USA) was used to obtain the optical density of each sample at the wavelength of 450 nm. All samples were assayed in triplicates and the results were reported as picograms of cytokines per milliliter.

Statistical analyses

Statistical software SPSS 22.0 and GraphPad Prism 5.04 were used for data analysis and preparation of graphs. One-way ANOVA with Tukey's post hoc test or nonparametric Kruskal-Wallis with Dunn-Bonferroni post hoc test was used to compare the means of multiple samples. Independent samples *t* test or nonparametric Mann-Whitney *U* test was used to compare the means of two groups. Two-tailed Spearman's rank correlation was performed for correlation analyses. Positive *rs* values represented a positive correlation, while negative *rs* values represented a reverse correlation. In order to evaluate the diagnostic value of tested miRNAs, receiver operating characteristic (ROC) curve analysis was conducted and area under the curve (AUC), cut-off value, likelihood ratio (LR), sensitivity and specificity of each variable to be segregated were reported. *p*-values lower than 0.05 were considered as statistically significant.

Results

Evaluation of IFN-γ, IL-17a, IL-10, and TGF-β-producing CD3+CD8– Th cells among ESCC patients in comparison to healthy controls

Intracytoplasmic production of IFN-γ, IL-17a, IL-10, and TGF-β was investigated on CD3+ CD8-gated T cells (Th) in

each group of patients and normal subjects (Fig. 1a). We analyzed both percent and mean fluorescence intensity (MFI) of cytokine expression in Th cell populations. The frequency of Th (CD3+CD8−) cells was significantly reduced in ESCC patients in comparison to normal subjects (p value < 0.001). Our findings demonstrated that the frequencies of IL-10, TGF-β, and IL-17a-producing lymphocytes of patients were significantly higher than the control group (p value < 0.001) (Fig. 1b). Regarding clinical data, under-treatment ESCC patients with negative *Helicobacter pylori* tests expressed elevated numbers of IL-17a-producing lymphocytes (p value < 0.0001). However, the number of Th (CD3+CD8−) cells was not altered in any of the clinical subgroups.

The MFI of IFN-γ-producing lymphocytes in patients was decreased (p value = 0.010). However, no significant difference was observed in the MFI of IL-17a, IL-10, and TGF-β between healthy subjects and patients (Fig. 1c). In order to evaluate the population of regulatory T cells among CD3+ CD8− cells, the frequency of IL-10+TGF-β+-producing cells was evaluated which were significantly increased in ESCC patients (p value = 0.010) and was not different in clinical subgroups.

Comparison of Th1, Th17, and Treg cell populations between newly diagnosed and under-treatment subgroups of patients

Our findings revealed that the population of helper T cells was significantly lower in both newly diagnosed (ND) and under-treatment (UT) patients in comparison to normal individuals (p value < 0.001). We showed that the frequencies of IL-17a-producing Th cells were significantly increased in ND and UT patients (p value < 0.01). The frequency of IFN-γ-producing Th cells was higher in ND patients (p value < 0.05). Analyses of the Th cells in these subgroups showed that the expression of IL-10 and TGF-β was significantly increased in Th cells of ND and UT patients compared with healthy subjects (p value < 0.05) (Fig. 2a).

Regarding the MFI alterations in each subgroup, the expression of IFN-γ was increased in CD3+CD8− T cells of healthy subjects in comparison to ND and UT patients (p value < 0.05). The MFI of IL-10+ Th cells was significantly higher in ND than UT patients (p value < 0.05) and normal individuals (p value < 0.05) (Fig. 2b).

The plasma expression of TGF-β was not changed

We examined the plasma expression of TGF-β between EC patients and normal subjects. Our findings revealed that the plasma concentration of TGF-β was not statistically different in patients and healthy subjects. There was also no significant difference between all subgroups of ND and UT patients and normal subjects (data not shown).

The plasma expression level of miR-21, miR-29b, miR-106a, miR-155, and the diagnostic utility

We evaluated the plasma expression of miR-21, miR-29b, miR-106a and miR-155 in all samples. We showed that miR-21 was significantly overexpressed in patients (p value < 0.01), while no significant difference was observed in the expression of miR-29b (Fig. 3a). The expression of miR-21 was significantly higher in both ND and UT subgroups of patients, in comparison to normal subjects (p value < 0.05). Similarly, no significant difference in the expression of miR-29b (Fig. 3b) was observed between ESCC subgroups and the healthy individuals. Moreover, miR-106a and miR-155 were not detected in ESCC patients and did not significantly change in normal subjects (data not shown).

While miR-21 was significantly higher in ESCC patients, we evaluated the diagnostic utility of miR-21 plasma expression as a potential biomarker. ROC curve analysis was performed, and area under the curve for miR-21 was 0.6987 (95% CI 0.5427 to 0.8547; p = 0.0264). Setting the optimal cut-off value at 0.026 gives a sensitivity of 77.78% and a specificity of 65.38% with the likelihood ratio of 2.247 (Fig. 3c).

We also evaluated the plasma expression of miR-21 in clinically different subgroups of ESCC patients. Although no significant difference was found, miR-21 was elevated in no-metastatic samples (mean rank = 14.93) compared with metastatic ones (mean rank = 3.00). MiR-21 was also overexpressed in well-differentiated ESCC samples compared with moderately differentiated (mean ranks = 14.2 vs 7.33). No other remarkable change was demonstrated in other clinical subgroups.

Correlation analyses between Th cell subsets and plasma expression of circulating microRNAs

The relevance between IL-17a, IFN-γ, IL-10, and TGF-β-producing Th cells and plasma expression of circulating miR-21 and miR29b in each group of patients (ND and UT) and healthy subjects were assessed. As illustrated in Table 2, there was a significant reverse correlation between the frequency of IL-17a-producing cells and miR-21 plasma level ($r_s = -0.711$, p = 0.048) in ND patients. There was also a significant correlation between the frequency of TGF-β-producing Th cells and miR-21 among UT patients (r_s = 0.575, p = 0.040). Moreover, there was a significant reverse correlation between intensity of IFN-γ in Th cells and plasma expression of miR-29b ($r_s = -0.606$, p = 0.005) (Table 3).

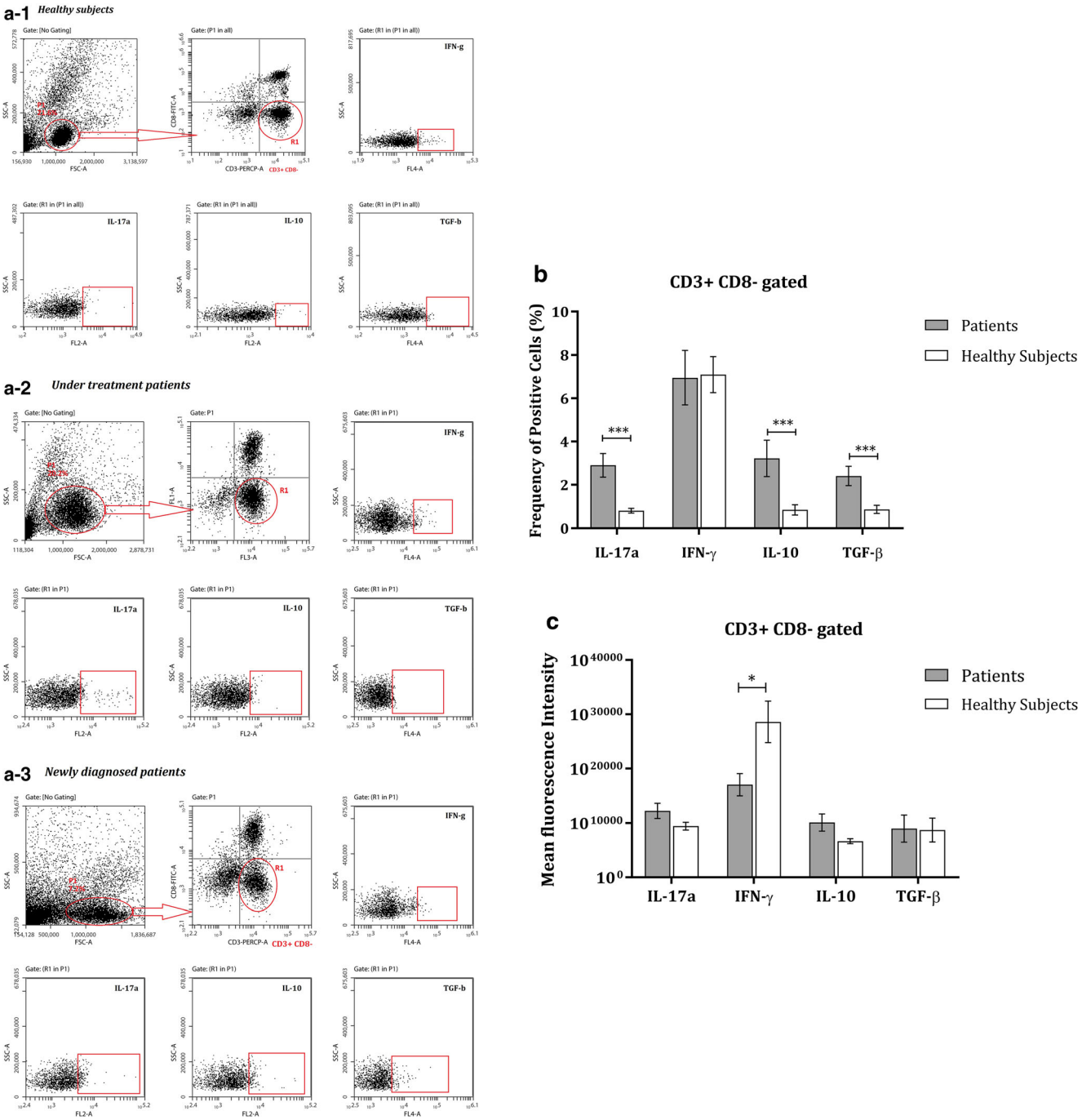


Fig. 1 Immunophenotyping of T cell subsets by flow cytometry; flow cytometric scatter plots and gating for the cell surface expression of CD3 and CD8 markers and intracellular expression of IFN-γ, IL-17a, IL-10, and TGF-β cytokines in a healthy subject (**a-1**), an under-treatment patient (**a-2**), and a newly diagnosed patient (**a-3**). Flow cytometry data are presented as frequency distribution of CD3+CD8− T cells (**b**) and mean fluorescent intensity (MFI) (**C**). Independent samples *t* test or Mann-Whitney *U* test was used to compare the means of two samples. Data of each bar demonstrates mean ± SE. *p* values lower than 0.05 were considered as statistically significant

Discussion

Failure or lack of proper immune response in patients with esophageal cancer could play an important role in the development and progression of this malignancy. Several studies have addressed the alterations of immune cells and cytokines concerning the pathogenesis and survival of patients with ESCC [30]. In this regard, aberrant expression of some

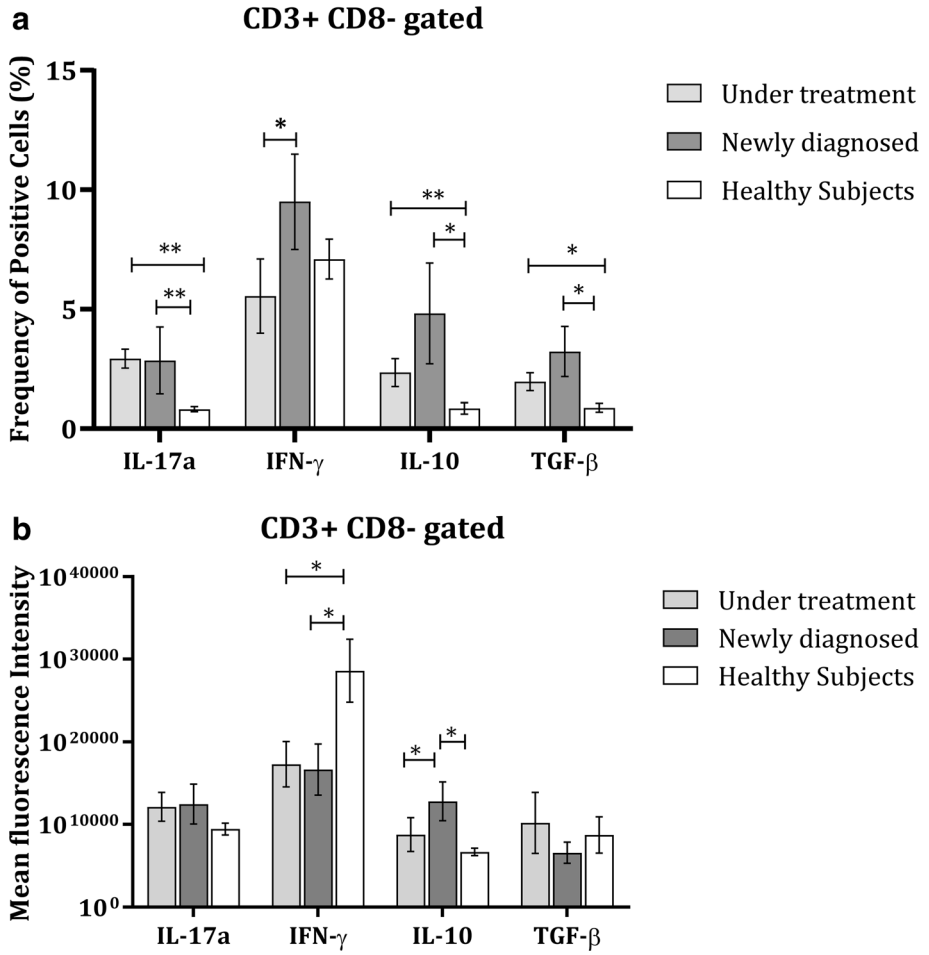


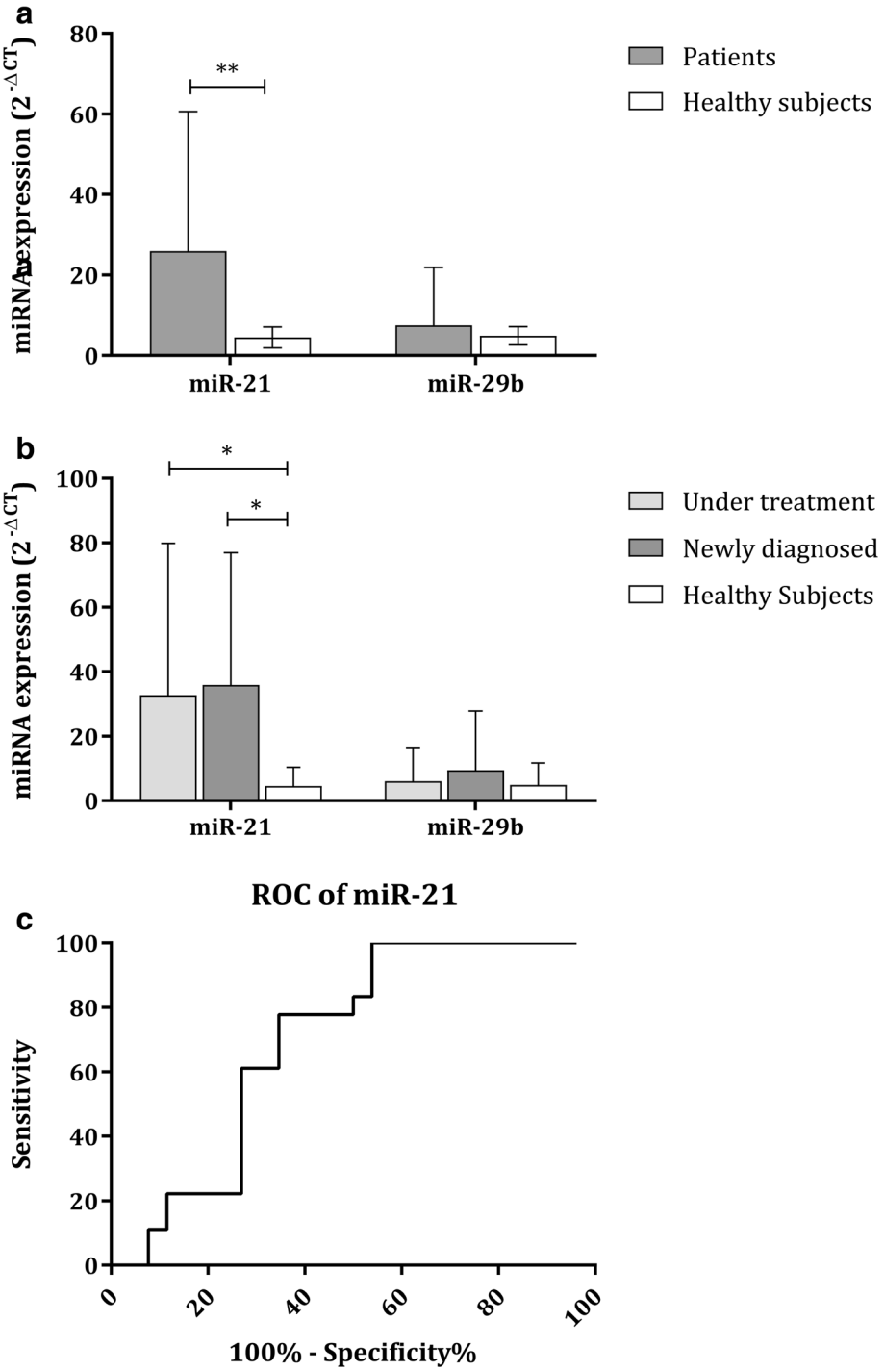
Fig. 2 Immunophenotyping of T cell subsets in ESCC subgroups and healthy subjects; flow cytometry data are presented as frequency distribution of CD3+CD8− T cells (**a**) and mean fluorescent intensity (MFI) (**b**). One-way ANOVA with Tukey's post hoc test or Kruskal-Wallis with Dunn-Bonferroni post hoc test was used to compare the means of multiple samples. Data of each bar demonstrates mean ± SE. *p* values lower than 0.05 were considered as statistically significant

microRNAs in various tumors could be associated with abnormal immune response [31]. Moreover, microRNAs play an important role in regulating proliferation, survival, differentiation, and production of cytokines by T cells and they could be signified as biomarkers in the diagnosis and prognosis of cancers [32]. However, the exact role and interaction of microRNAs and helper T cell subtypes in various cancer types such as ESCC are still under investigation. In the present study, IFN-γ, IL-17a, TGF-β, and IL-10-producing Th cells were evaluated in EC patients and normal subjects. We also quantified the plasma expression levels of miR-21, miR-29b, miR-106a, miR-155, and TGF-β cytokine and their association with different Th lymphocytes.

Alterations in the production of IFN-γ from Th cells are variable and dependent on disease conditions in patients with ESCC

As shown in Fig. 1, the percentage of IFN-γ-producing Th cells (Th1 cells) in the normal group is not significantly different from the patients. However, the IFN-γ fluorescent intensity (MFI) was lower in Th cells of ESCC patients. Since IFN-γ is involved in the activation of the immune system against tumors, several studies showed that the neutralization or inhibition of the IFN-γ-dependent pathway may contribute to tumor formation and its reduction in Th lymphocytes may indicate a failure of the anti-tumor immune in these patients [33–35]. IFN-γ could directly inhibit tumor cell proliferation and induce apoptosis [36]. On the other hand, Wang et al. showed that increased expression of IFN-γ and

Fig. 3 The plasma expression of miR-21 and miR-29b in ESCC patients and healthy subjects; miR-21 is significantly increased in ESCC patients, while miR-29b is not changed (**a**). MiR-21 is significantly higher in newly diagnosed and under-treatment ESCC patients (**b**). ROC curve analysis of miR-21 plasma expression to distinguish ESCC patients from normal subjects (**c**). $2^{-\Delta Ct}$ method was used to calculate the microRNA expression level for each group. The nonparametric Mann-Whitney *U* or Kruskal-Wallis with Dunn-Bonferroni post hoc test was used to compare the means of samples. All of the experiments were repeated in triplicates. Data of each bar demonstrates mean ± SE. *p* values lower than 0.05 were considered as statistically significant



downregulation of IFNGR1 (IFN-γ receptor 1) may play a crucial role in the development of ESCC and oncogenic properties by inducing the overexpression of IRF-2 (interferon regulatory factor 2) [37, 38]. Accordingly, the decrease in the amount of IFN-γ-producing T cells among our UT patients (Fig. 2b) can be associated with the reduced cell growth during the treatment.

High levels of IL-17a production by T lymphocytes could play a role in the pathogenesis of ESCC

As shown in Fig. 1, the percentage of IL-17a-producing Th cells has significantly increased among patients versus normal group. Chen et al. reported that Th17 cells were increased in the peripheral blood and tumor tissue of EC

Table 2 Correlation of miR-21and miR29-b expression with the frequencies of Th cells which produce IL-17a, IFN-γ, IL-10, and TGF-β in ESSC patients and normal subjects

Groups		IL-17a		IFN-γ		IL-10		TGF-β	
		r_s	*p* value	r_s	*p* value	r_s	*p* value	r_s	*p* value
Healthy subjects	*miR-21*	0.205	0.741	0.462	0.434	0.200	0.800	*0.800*	0.200
	miR-29b	*0.646*	0.060	− 0.416	0.265	*0.614*	0.106	0.132	0.756
Newly diagnosed	*miR-21*	*− 0.711*	*0.048*	− 0.263	0.528	− 0.132	0.756	0.072	0.865
	miR-29b	− 0.475	0.135	− 0.175	0.586	− 0.480	0.114	− 0.021	0.948
Under-treatment	*miR-21*	− 0.116	0.706	0.202	0.508	0.452	0.121	*0.575*	*0.040*
	miR-29b	− 0.129	0.588	− 0.085	0.721	0.407	0.075	0.357	0.122

Two-tailed spearman correlation study was performed to evaluate the correlation between parameters. *p* values lower than 0.05 were considered as statistically significant. Significant or strong correlations ($r_s > 0.5$) are shown in italics

r_s Spearman correlation coefficient

which was markedly higher in patients with advanced stages [12]. The role of IL-17a-producing cells in tumor initiation and progression is controversial. Th17 cells can promote tumor growth by enhancing angiogenesis [39], while they may play a regulatory role by enhancing the anti-tumor immune response [40]. Furthermore, Th17 cells accumulate specifically in esophageal carcinomas which explain they distinctively recruit and infiltrate to tumor sites and may be related to good or bad prognosis. Since the chronic inflammation is strongly associated with invasion, migration, and tumor metastasis [39], and Th17 cells play major roles in inflammation [41], we need to elucidate the precise physiological role of these cells in the tumor. Although *H. pylori* infection is known to induce Th17 response [42], we realized that the population of IL-17-producing Th cells was increased among infected patients who were receiving treatment. This could be justified by the effectiveness of therapeutic approaches, and further studies with bigger sample sizes are suggested for illumination. Here, we also observed a positive correlation between TGF-β and IL-17a production in Th lymphocytes (Table 2). This association could indicate the role of TGF-β in induction and differentiation of Th17 cells [43]. T cell Ig and ITIM domain (TIGIT) as an immune receptor involved in inhibiting the production of IL-17a [44] is decreased among both CD4+ and CD8+ T cells in esophagus cancer [45]. So reduction its inhibitory effect in EC patients may lead to increase the number of Th17 cells.

Increased activity of regulatory T lymphocytes and immunosuppressive responses, especially among ND patients

As depicted in Fig. 2, the percentage of TGF-β and IL-10-producing Th cells in both groups of patients is increased in comparison to normal subjects. Moreover, there was a significant positive correlation between TGF-β and IL-10 production in Th cells. Increasing the expression of IL-10 and TGF-β from Th lymphocytes could play an

Table 3 Correlation of miR21and miR29b expression with the intensity of IL-17a, IFN-γ, IL-10, and TGF-β expression in Th cells of ESSC patients and normal subjects

Groups		IL-17a		IFN-γ		IL-10		TGF-β	
		r_s	*p* value	r_s	*p* value	r_s	*p* value	r_s	*p* value
Healthy subjects	*miR-21*	*0.700*	0.188	− 0.400	0.505	*0.800*	0.200	0.400	0.600
	miR-29b	− 0.418	0.262	0.218	0.574	− 0.095	0.823	0.286	0.493
Newly diagnosed	*miR-21*	0.286	0.535	− 0.119	0.779	− 0.167	0.693	*0.536*	0.215
	miR-29b	− 0.136	0.689	*− 0.510*	0.090	− 0.209	0.537	− 0.336	0.312
Under treatment	*miR-21*	0.082	0.789	− 0.192	0.529	0.225	0.459	0.308	0.306
	miR-29b	− 0.053	0.826	*− 0.606*	*0.005*	− 0.113	0.636	− 0.033	0.890

Two-tailed spearman correlation study was performed to evaluate the correlation between parameters. *p* values lower than 0.05 were considered as statistically significant. Significant or strong correlations ($r_s > 0.6$) are shown in italics

r_s Spearman correlation coefficient

important role in the pathogenesis of EC and may lead to tumor escape from the immune system. Other study also showed that TGF-β levels increased along with the IL-10 levels in EC [46]. In the present study, we also showed a significant negative correlation between the frequency of TGF-β-producing cells and Th lymphocytes in ND patients. Since ND patients showed higher fequency of TGF-β producing cells and lower frequency of Th cells, than the normal group, it could be indicated that Tregs may help tumor initiation and support [47] by suppressing T cells in ND patients.

Here, there was no correlation between the percentage of TGF-β-producing cells and the plasma concentration of this cytokine. It could be due to the different origins of TGF-β produced from somatic and phagocytic cells [48]. Similarly, another study demonstrated no association between serum levels of TGF-β and TGF-β-producing cells in patients with ovarian cancer [49]. It was found that breast cancer patients with a more advanced TNM stage had higher serum levels of TGF-β1 which may be and indicator of the invasion rate [50]. Although the level of TGF-β was not significantly altered in our patients, it may play a role in inhibiting antitumor immune responses locally [51].

IL-10 inhibits the proliferation of CD4 + T cells and its cytokine production [52]. IL-10 could lead to the development of regulatory phenotypes during the activation of CD4 + T cells [53]. The expression of IL-10 in various tumors including in EC (our study) may be associated with the tumor development by suppressing the immune response [54].

microRNA-21 play a more significant role in the pathogenesis and diagnosis of ESCC

The plasma expression of miR-21 was significantly higher in ESCC patients (Fig. 3a), while the expression of miR-29b was not different in patients and normal individuals. miR-21 was also introduced as a potential marker to discriminate ESCC patients and normal subjects. Moreover, there was a significant positive correlation between the expression of miR-21 and expression of IL-17a and TGF-β production in Th lymphocytes among ND patients (Table 3). According to the fact that this relationship is observed merely among ND and not UT patients, miR-21 could probably play a crucial role in direct induction of Th17 cells [55] and consequently the pathogenesis of the ESCC or indirect effect on Th17 by inducing TGF-β production [12]. Due to the oncogenic role of miR-21 by inhibiting tumor suppressor genes such as phosphatase and tensin homolog (PTEN) and programmed cell death-4 (PDCD4) [56, 57], the amplification of TGF-β production might be introduced as one of the other miR-21-associated tumorigenesis mechanisms.

On the other hand, miR-21 directly increases the expression of BCL-2 which results in inducing resistance to chemotherapy and proliferation of tumor cells [58]. While miR-29b makes cells susceptible to apoptosis by targeting BCL-2 [59] and regarding the elevation of BCL-2 in EC [60], the remarkable increase of miR-21 in comparison to miR-29b could be of great importance in EC pathogenesis.

However, miR-29b has been also reported to be reduced in a few research studies among EC patients [61] with diverse and contradictory roles [62]. We showed that there were reverse correlations between the expression of miR-29b and the production of IFN-γ and IL-17a from Th cells in our patients. This reverse relationship was also previously reported between miR-29b, IFN-γ, and TNF-α production in Th cells [53]. miR-29b may inhibit the production of IFN-γ by inhibiting *T-bet* and *Eomes*, as two major regulators of IFN-γ transcription [19].

The results of the present study revealed that immune response is highly disturbed in the ESCC patients. Our findings indicate that association between some small molecules such as miR-21, miR-29b, and impaired function of effector lymphocytes in ESCC could introduce a novel and useful approach for the targeted immunotherapy. We also suggest fulfilling functional and in vivo studies to investigate the direct interaction between miRNAs and T lymphocytes.

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Compliance with ethical standards

Research involving human participants The present study which involved human participants was approved by the ethical committee of Semnan University of Medical Sciences (Code of Ethics: IR.SEMUMS.REC.1394.215) and also Golestan University of Medical sciences (Code of Ethics: IR.GOUMS.REC.1395.28). A written informed consent following the declaration of Helsinki was taken from all participants.

Conflict of interest The authors declare that they have no conflict of interest.

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