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RESEARCH ARTICLE

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Simultaneous disruption of circulating miR-21 and cytotoxic T lymphocytes (CTLs): Prospective diagnostic and prognostic markers for *esophageal squamous cell carcinoma* (ESCC)

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Abstract

Background: *Esophageal squamous cell carcinoma* (ESCC) as the most prominent type of esophageal cancer (EC) in developing countries encompasses a substantial contribution of cancer-related mortalities and morbidities. Cytotoxic T lymphocytes (CTLs) are the major subset of effector T cells against cancer. However, the microRNAs involved in the development and regulation of CTLs could be disrupted in cancers such as EC.

Methods: Here, we evaluated the population of IL-10, TGF-β, IFN-γ, and IL-17a-producing CD3+CD8+ T cells, their association with the circulating levels of miR-21 and miR-29b, and their diagnostic and/or prognostic (after 160 weeks of follow-up) utilities in 34 ESCC patients (12 newly diagnosed: ND, 24 under-treatment: UT) and 34 matched healthy donors.

Results: The population of IL-10 and TGF-β-producing CTLs (CD8+ Tregs) were considerably expanded, in addition to the overexpression of miR-21 in both groups (ND and UT) of ESCC patients, while the frequency of Tc17 and CD8+ Treg cells increased only in UT patients. The expression means of TGF-β and IL-10 in CTLs were considered to be excellent biomarkers (1 ≥ area under the curve: AUC ≥0.9) in distinguishing ESCC patients and associated subgroups from healthy subjects. Moreover, the lower

Correction added on 24th November 2021, after first online publication: The author "Ali Memarian" affiliation details have been updated.

expressions of TGF-β, IL-17a, IL-10, and IFN-γ in CTLs were associated with ESCC better prognosis.

Conclusions: The association between the impaired function of CD3+ CD8+ T cell subsets and miR-21 expression could be introduced as novel therapeutic targets and powerful diagnostic and prognostic markers for ESCC.

KEYWORDS
CD3+ CD8+ T cells, cytotoxic T lymphocytes (CTLs), esophageal squamous cell carcinoma (ESCC), Biomarker, miRNA-21

1 | INTRODUCTION

Esophageal cancer (EC) is a solid tumor malignancy with comparatively high mortality and morbidity rates, especially in progressed stages.[1] Adenocarcinoma and squamous cell carcinoma (ESCC) are two main histological types of EC, while ESCC is the most prominent type in developing countries.[2] The incidence of EC is enormously high in the north-eastern regions of Iran, as parts of the area called "*esocancer belt*".[3-5] Despite recent advancements, late and inaccurate diagnosis along with the drug resistance are still the main abiding complexities in ESCC patients. Moreover, the immunopathogenesis of EC related to the aberrant T-cell activation and disproportionate subpopulations is not distinctly identified.[5,6]

The orderly expanded and activated T lymphocytes in tumor milieu and presence of certain T cell subsets is crucial for cancer immunosurveillance.[7-9] CD8+ cytotoxic T lymphocytes (CTLs) are the major subset of T cells in effective compact against tumor, categorized into several subtypes: Tc1, Tc2, Tc17, and CD8+ Tregs.[10] Tc1 cells secrete IFN-γ (Interferon-gamma) and TNF-α (Tumor necrosis factor-α) with strong anti-tumor effect, while Tc2 cells secrete IL-4, IL-5, and IL-10 but not IFN-γ with little or no effect on tumor growth.[11,12] However, the anti-tumor functional and phenotypic features of Tc17 CD8+ T cells (IL-17 secreting) have not been clearly identified, as they do not exhibit any cytotoxicity effects.[13,14] Although accumulation of CD8+ Treg cells has been observed in some tumor environments with anti-tumor suppression,[15-17] there are not well characterized in ESCC patients.

The aberrant expression of several microRNAs has been also described in a variety of cancers including EC with tumor-specific patterns, which may improve tumorigenesis or suppress anti-tumor effects.[2,18-20] MiRNAs modulate mechanisms by which the development and activation of immune cells and responses, including T cells regulation.[21,22] MiR-21, as an oncogene which mainly targets PTEN tumor-suppressor gene, is involved in promoting the proliferation and invasion of ESCC cancer cells.[23] It has been introduced as a useful target for developing treatment strategies and establishing strong prognostic and diagnostic biomarkers with increased expression in ESCC.[24] On the other hand, miR-29b, mainly down-regulated in ESCC, may act as a tumor-suppressor miRNA.[6]

Accordingly, there is an indispensable necessity to track down pertinent molecular mechanisms involved in ESCC immunopathogenesis with regard to the expression of certain miRNAs and the development of specific T-cell subsets to attain timely diagnosis, establish novel therapeutic strategies, and improve the survival of ESCC patients. Regarding the important role of CTLs responses in tumor immunity and the probable role of miR-21 and miR-29b in the regulation of tumor-associated CTLs responses, we evaluated the population of IL-10, TGF-β, IFN-γ, and IL-17a-producing CD3+CD8+ T cells, their association with the plasma level of miR-21 and miR-29b (previously obtained), and their diagnostic and/or prognostic utilities in our cohort of ESCC patients.

2 | MATERIALS AND METHODS

2.1 | Sample collection, preparation, and T-cell stimulation

A total of 34 confirmed (based on clinical and pathological examinations) ESCC patients and 34 matched healthy controls were enrolled from Atrak Clinic, Khatam-al Anbiya hospital, Gonbad-e Kavus, Golestan province, Iran. Patients were categorized into under-treatment (UT; N = 22), which were receiving treatments including chemotherapy and/or radiotherapy, and newly diagnosed (ND; N = 12) subgroups. Clinical and laboratory information of all patients is shown in our previous publication.[6] Approximately, 5 ml peripheral whole blood was acquired in a sterile tube and immediately transported to the laboratory of the Stem Cells Research Center at Golestan University of Medical Sciences (GoUMS), Gorgan, Iran. This study was approved by the committee of ethics at GoUMS and also Semnan University of Medical Sciences, while a written informed consent according to the ethical principles of Helsinki declaration[25] was signed by all contributors. Peripheral blood mononuclear cells (PBMCs) were separated using Ficoll-Paque (Baharafshan, Tehran, Iran) density-gradient centrifugation, as represented earlier.[26] The viability of isolated PBMCs was determined, and 3×10^5 cells were resuspended in RPMI 1640 (Gibco, Life Technologies) supplemented with 2 μl (microliter) of Cell Stimulation Cocktail (eBioscience). Primed cells were then incubated overnight (14–16 h) at 37°C with 5% CO_2 and 95% humidity, according to the producer's instruction.[27] Plasma was also separated and reserved at −80°C until use. The results of our previous study, using the same samples, were adopted to analyze our current data in which we have revealed that the miR-21 was significantly overexpressed in patients, while no significant difference was observed in the expression of miR-29b between any of the groups. Moreover, the expression of miR-21

was significantly higher in both ND and UT subgroups of ESCC patients. Similarly, no significant difference in the expression of miR-29b was observed between ESCC subgroups and the healthy subjects.[6]

2.2 | Flow cytometric analyses

The cell surface staining on stimulated T cells was performed by PerCP-conjugated anti-human CD3 (Biolegend) and FITC-conjugated anti-human CD8 (Biolegend) markers. Cyto-Fast Fix/Perm buffer (Biolegend) was then used to wash cells according to the provided instructions and prepare them for intracellular staining. Cells were then fractionated into two distinct tubes; the first was stained with PE-conjugated anti-human IL-10 (Biolegend) and APC-conjugated anti-human TGF-β (BD PharMingen), while the next one was stained using PE-conjugated anti-human IL-17a (Biolegend) and APC-conjugated anti-human IFN-γ (Biolegend). BD Accuri C6 flow cytometer (BD PharMingen) and BD Accuri C6 Flow analysis software were utilized to analyze the immunophenotypes of all stained samples and assessing the distribution of T cell subpopulations in ESCC patients and normal subjects. We completely considered any related points and issues for all counterparts including similar cell counts, isotype controls and gating strategies, conjugated antibody concentrations, and compensation of overlapping colors.

2.3 | Statistical analyses

Statistical analyses of the obtained data and preparation of graphs were accomplished by SPSS 22.0 and GraphPad Prism 5.04 statistical software. One-way ANOVA with Tukey's post hoc test or the nonparametric equivalents (Kruskal–Wallis with Dunn–Bonferroni post hoc test) was used to compare the mean(s) differences between multiple samples. Similarly, the independent samples t-test or its nonparametric equivalent (Mann–Whitney U) test was used to compare the mean(s) differences between two groups. Receiver operator characteristic (ROC) curve analysis was conducted to determine the diagnostic utility of significant cytokines within CTLs and miRNAs. Kaplan–Meier survival test was performed following the outcome of ESCC (death or survival) after 160 weeks to examine the prognostic values of the selected variables in ESCC patients. *P*-values (*p*) smaller than 0.05 were regarded as statistically significant.

3 | RESULTS

3.1 | IFN-γ, IL-17a, IL-10, and TGF-β expression in CD3+CD8+ CTLs among ESCC patients and healthy subjects

As demonstrated in Figure 1A, cell surface expression and intracytoplasmic production of IFN-γ, IL-17a, IL-10, and TGF-β markers were studied on CD3+ CD8+ gated T cells (CTLs) in each group of patients and healthy individuals. Both mean fluorescence intensity (MFI; as expression mean) and frequency (percent) of the cells expressing each cytokine were investigated in CTLs. The comparative analyses indicated that the numbers of IL-10, TGF-β, and IL-17a-producing population of lymphocytes was considerably expanded in ESCC patients ($p < 0.001$) (Figure 1B). MFI analysis of CD3+ CD8+ T lymphocytes signified elevated expressions of IL-10 and TGF-β in ESCC patients in comparison with the normal counterparts ($p < 0.001$), while the MFI of IFN-γ ($p = 0.036$) and IL-17a ($p = 0.003$) were decreased in patients (Figure 1C). Besides, ESCC patients indicated significant higher levels of IL-10+ TGF-β+ producing T cells (CD8+ Tregs) ($p = 0.011$).

3.2 | Comparison of CTLs subsets between ND and UT subgroups of patients

Our findings showed that the population of CTLs (CD3+ CD8+ T cells) was remarkably elevated only in UT patients in comparison with both healthy subjects ($p = 0.021$) and ND patients ($p = 0.020$). Frequency of IL-10- and TGF-β-producing cells in both ND ($p = 0.005$ and $p < 0.001$, respectively) and UT ($p < 0.001$ and $p = 0.001$, respectively) patients were significantly increased in comparison with the control group, while the frequency of Tc17 and regulatory CD8+ T cells increased only in UT patients compared with control group ($p < 0.001$ and $p = 0.011$, respectively) (Figure 2A). As expressed in Figure 2B, the MFI of IL-17a in ND patients ($p = 0.015$) and the MFI of IFN-γ in UT patients ($p = 0.007$) were markedly decreased. On the other hand, the MFI of IL-10 and TGF-β was considerably increased in both ND ($p = 0.001$ and $p < 0.001$, respectively) and UT ($p < 0.001$ and $p = 0.002$, respectively) patients (Figure 2B).

3.3 | Correlation analyses between CD8+ T-cell subsets and plasma expression of circulating microRNAs

The correlation between IL-17a-, IFN-γ-, IL-10-, and TGF-β-producing CD8+ T cells and plasma expression of circulating miR-21 and miR29b in each group of patients (ND and UT) and healthy subjects were analyzed using two-tailed spearman study (Tables 1 and 2). There was a significant reverse correlation between the frequency of IL-17a-producing cells with miR-21 plasma levels in ND ($r_s = -0.874$, $p = 0.005$) and UT ($r_s = -0.615$, $p = 0.025$) patients. There was also a significant reverse correlation between the regulatory CD8+ T cells and miR-29b plasma level ($r_s = -0.698$, $p = 0.025$), and a remarkable reverse correlation between the frequency of Tc1 cells ($r_s = -0.678$, $p = 0.015$), TGF-β MFI ($r_s = -0.664$, $p = 0.002$) and regulatory CD8+ T cells ($r_s = -0.698$, $p = 0.025$) with miR-29b plasma level in ND patients. We also demonstrated a reverse correlation between Tc1 cells and regulatory CD8+ T cells in UT patients ($r_s = -0.490$, $p = 0.028$), while this correlation was direct for ND patients ($r_s = 0.840$, $p = 0.002$).

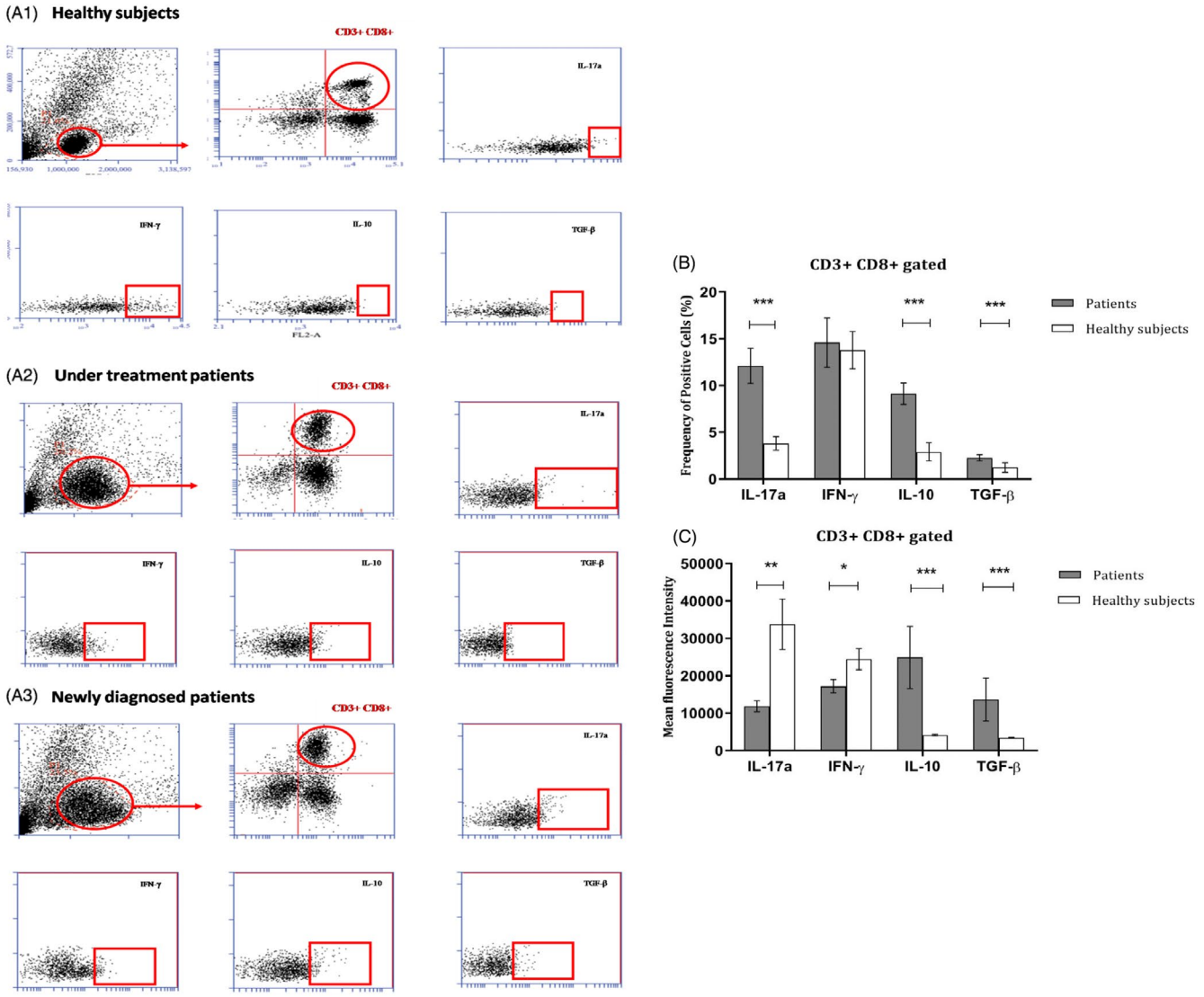

FIGURE 1 Immunophenotyping of T-cell subsets by flow cytometry; flow cytometric scatter plots and gating for the cell surface expression of CD3 and CD8 markers and intracellular expression of IFN-γ, IL-17a, IL-10, and TGF-β cytokines in a healthy subject (A-1), an under-treatment patient (A-2), and a 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 demonstrate means±SEM. *P*-values lower than 0.05 were considered as statistically significant

3.4 | Evaluation of diagnostic biomarkers in ESCC by ROC curve analyses

We evaluated the diagnostic utility of each variable with significant difference between two groups of patients and healthy subjects. ROC curve analyses were performed, and optimal cutoff points with reliable sensitivity and specificity were identified. The biomarkers were then categorized into 4 groups based on area under the curve (AUC) including excellent biomarkers (1 ≥ AUC ≥0.9), good biomarkers (0.9 > AUC ≥0.8), fair biomarkers (0.8 > AUC ≥0.7), and weak biomarkers (0.7 > AUC ≥0.0) (Figure 3). As illustrated in Figure 3A, the MFI of TGF-β and MFI of IL-10 are considered to be excellent biomarkers in distinguishing ESCC patients (PA) and ND subgroup from healthy subjects (HS). AUC for TGF-β MFI (PA vs HS) was 0.9167 (95% CI, 0.8301 to 1.000; $p = 0.0003$). Setting the optimal cutoff value at 3653 displayed a sensitivity of 100.00% and a specificity of 87.88% with the likelihood ratio (LR) of 8.250. AUC for IL-10 MFI (PA vs HS) was 0.9412 (95% CI, 0.8721 to 1.000; $p = 0.0001$). Setting the favored cut-off point at 4796 gave a sensitivity of 87.50% and a specificity of 88.24% with the likelihood ratio of 7.438. In order to distinguish ND subgroup of ESCC patients from HS, AUC for TGF-β MFI, as an excellent prototypic biomarker, was 1.000 (95% CI, 1.000 to 1.000; $p = 0.0003$). Setting the optimum cutoff value at 3743 resulted in a sensitivity of 100% and a specificity of 100%. Similarly, the AUC for IL-10 MFI was 0.9375 (95% CI, 0.8276 to 1.000; $p = 0.0012$). Setting the favorable cutoff point at 4796 demonstrated a sensitivity of 87.50% and a specificity of 91.67% with the LR of 10.50. IL-10 MFI was also introduced as an excellent biomarker for recognizing UT patients from healthy subjects. AUC for IL-10 MFI was 0.9432 (95% CI, 0.8644 to 1.000; $p = 0.0003$). Setting the optimal cutoff

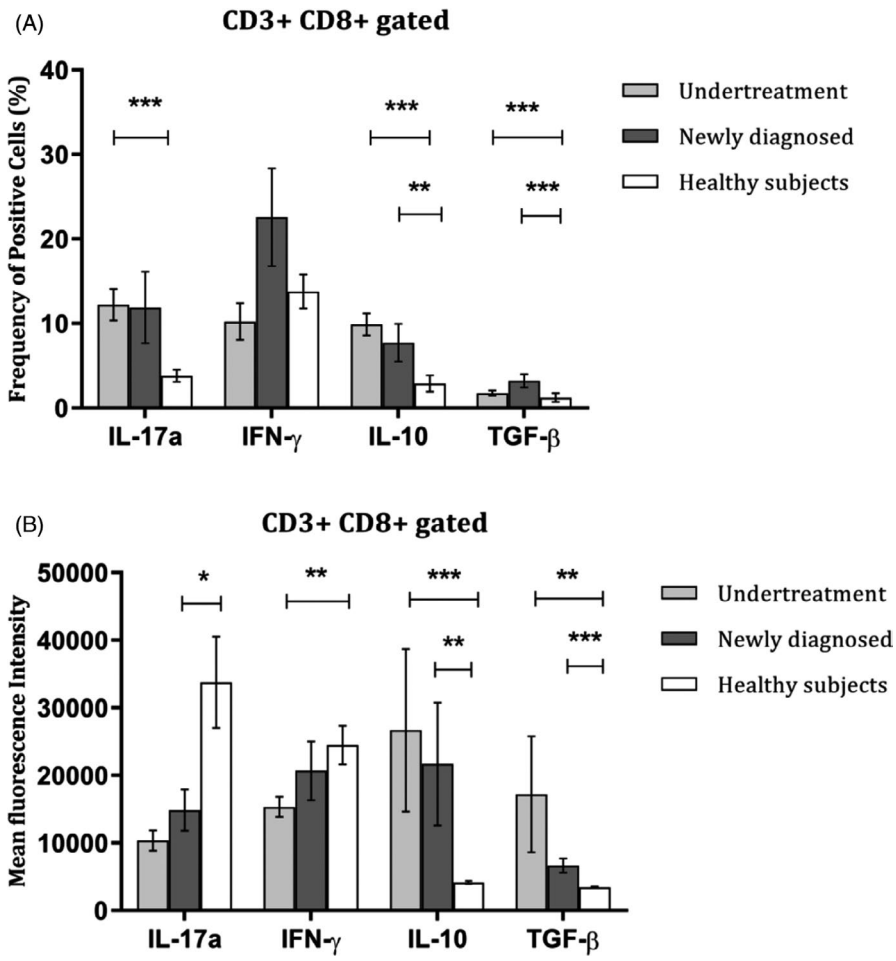

FIGURE 2 Immunophenotyping of T-cell subsets in ESCC subgroups and healthy subjects; flow cytometry data are presented as frequency distribution of CD3+CD8- T cells (A) and mean fluorescent intensity (MFI) (B). One-way ANOVA with Tukey's *post hoc* test or Kruskal–Wallis with Dunn–Bonferroni *post hoc*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

TABLE 1 Correlation analyses of circulating expression of miR-21and miR29-b with the frequencies of CD3+ CD8+ T cells, which produce IL-17α, IFN-γ, IL-10, and TGF-β in ESSC subgroups and healthy subjects

		IL–17α		IFN-γ		IL–10		TGF-β	
Groups		r_s	*p*-value	r_s	*p*-value	r_s	*p*-value	r_s	*p*-value
Healthy subjects	miR–21	0.300	0.624	0.200	0.747	0.400	0.600	0.000	1.000
	miR–29b	−0.326	0.391	−0.226	0.559	0.167	0.693	0.405	0.320
Newly diagnosed	miR–21	**−0.874**	**0.005**	−0.643	0.086	−0.120	0.778	0.012	0.978
	miR–29b	−0.319	0.313	**−0.678**	**0.015**	−0.319	0.312	−0.298	0.346
Under-treatment	miR–21	**−0.615**	**0.025**	0.094	0.761	0.047	0.879	0.488	0.091
	miR–29b	−0.284	0.224	−0.144	0.546	0.057	0.811	0.252	0.283

P-values smaller than 0.05 were assumed as statistically significant. ***Significant*** or ***strong*** correlations (r_s ≥0.6) are expressed in bold; r_s: Spearman correlation coefficient.

value at 4962 showed a sensitivity of 100.00% and a specificity of 81.82% with the LR of 5.500 (Figure 3A and Table 3).

The results of ROC curve analyses (AUC, cutoff values, sensitivity, specificity, and LR) for the variables categorized as good biomarkers including the intensities of IL-17a (PA vs. HS) and IL-10 (PA vs. HS), TGF-β % (ND vs. HS) and IL-17a (UT vs. HS), and IL-10% (UT vs. HS) and TGF-β MFI (UT vs HS) are illustrated in Figure 3B, while complementary results are listed in Table 3. Additionally, the diagnostic value results for the combinations of variables classified as fair biomarkers including miR-21 (PA vs. HS) and (ND vs. HS), TGF-β% (PA vs. HS) and (UT vs. HS), and IL-10% (ND vs. HS) are represented in Figure 3C and Table 3.

3.5 | Evaluating the prognostic values of biomarkers to predict ESCC outcome

In order to evaluate the prognostic utility of significant variables, we fulfilled a log-rank Kaplan–Meier survival test after 160 weeks of

TABLE 2 Correlation analyses of circulating expression of miR-21and miR29-b with the intensities of IL-17α, IFN-γ, IL-10, and TGF-β in CD8+ T cells among ESSC subgroups and healthy subjects

		IL–17α		IFN-γ		IL–10		TGF-β	
Groups		r_s	*p*-value	r_s	*p*-value	r_s	*p*-value	r_s	*p*-value
Healthy subjects	miR–21	0.100	0.873	0.400	0.600	−0.200	0.800	0.400	0.600
	miR–29b	0.167	0.667	**−0.766**	**0.027**	0.381	0.352	0.238	0.570
Newly diagnosed	miR–21	0.000	1.000	−0.333	0.420	−0.167	0.693	−0.071	0.879
	miR–29b	−0.409	0.212	−0.448	0.145	−0.301	0.342	**−0.664**	**0.026**
Under-treatment	miR–21	−0.088	0.755	−0.170	0.578	−0.017	0.957	0.176	0.566
	miR–29b	−0.214	0.366	−0.386	0.092	−0.249	0.290	0.290	0.214

P-values smaller than 0.05 were assumed as statistically significant. ***Significant*** or ***strong*** correlations ($r_s \geq 0.6$) are expressed in bold; r_s: Spearman correlation coefficient.

follow-up and monitoring the disease outcome. Based on the cutoff point obtained from ROC curve results, patients were divided into two groups of high and low expression. Subsequently, the outcome (death or death-free) for each patient was registered and adopted the relevant expression group. As demonstrated in Figure 4, the lower expressions of TGF-β %, TGF-β MFI, IL-17a %, IL-17a MFI ($p = 0.0001$), IL-10 MFI, and IFN-γ MFI in CD3+ CD8+ CTLs were associated with better prognosis in ESCC patients, while the lower expressions of circulating miR-21 and IL-10% in CTLs were related to a poor prognosis among patients.

4 | DISCUSSION

The microenvironment in which cancer cells thrive is comprised of epithelial cells, vascular and lymphatic vessels, infiltrating immune cells, cytokines, and chemokines. The interaction between these components, especially an efficacious immune response, is influential in determining the clinical outcome.[7] Since there is lack of reliable diagnostic and prognostic markers in ESCC, the immune cell populations and their associated cytokines, and also the factors regulating their function (such as microRNAs), could be employed to diagnose and predict the disease outcome.[28,29] In addition to the factors such as surgery, radiotherapy, and chemotherapy that weaken the effective immune response in most of the cancer types, the EC patients suffer from poor nutritional status due to dysphagia and subsequently a weaker immunological condition.[30,31] In order to increase the life-expectancy of ESCC patients, we need to have detailed information about the molecular mechanisms controlling the immune cell populations and responses. Among all infiltrating T-cell subtypes, particularly CD3+CD8+ CTLs have a major effect on the clinical attributes of human ESCC.[7,32]

Accordingly, we evaluated the population of IFN-γ-, IL-17a-, TGF-β-, and IL-10-producing CD3+ CD8+ T cells (CTLs) in ESCC patients and normal subjects. Since we have quantified the plasma levels of miR-21 and miR-29b in our previous work, we assessed their association with CTLs. Finally, we analyzed the diagnostic/prognostic utility of significant variables after 160 weeks of follow-up. The frequencies of Tc1 cells between the normal and patient groups were not significantly different. However, the IFN-γ fluorescent intensity was lower among the patients (Figure 1). IFN-γ is one of the major cytokines involved in the activation of innate and adaptive immune responses against tumor[33] and has direct anti-proliferative effects on some tumor cell lines including ESCC and immunomodulatory function.[34-36] Reduction of IFN-γ in Tc1 lymphocytes could be related to the dysregulation of anti-tumor immune responses in ESCC patients.[35,37,38] Similarly, Saha et al reported that decreased levels of IFN-γ in breast cancer patients were associated with the immune escape and tumor progression.[35]

The frequency Tc17 cells was significantly increased among patients (Figure 1A). IL-17a plays an essential but controversial role in promoting and/or inhibiting tumor.[39] IL-17a is capable of both enhancing natural killer (NK) cells and CTLs activity to generate anti-tumor effects and promoting tumor growth by inducing tumor angiogenesis.[40] Here, we showed that IL-17a MFI was lower among ESCC patients (Figure 1B). Although the precise role of Tc17 cells in tumor is not well discovered, some studies reported that IL-17a may enhance the antitumoral effects of CTLs.[41] However in gastric cancer, Tc17 cells may be involved in tumor progression via inflammation.[42] Recent studies mostly revealed the protective roles of IL-17a in the tumor microenvironment (TME) of esophageal cancer.[43,44] IL-17a-producing cells in ESCC cancer cells induced tumor protective immunity by recruiting CD8+T lymphocytes, NK cells, and B lymphocytes into the TME.[44] Thus, the lower expression of IL-17a in spite of the high frequency of Tc17 cells may define that the immune system is attempting to regulate its effects on TME.

IL-10 could inhibit CD4+ T-cell proliferation and cytokine production,[45] while TGF-β is a key factor in cancer development and progression.[46] Interestingly, TGF-β signaling appears to have a dual role in regulating tumorigenesis; in early stages, it is a growth suppressor, but later it promotes EMT and metastasis.[47,48] The frequencies of TGF-β- and IL-10-producing CD8+ T cells in both groups of patients were increased in comparison with the normal subjects (Figure 2A). Moreover, there was a positive correlation between TGF-β and IL-10 production in CD3+ CD8+ T cells (Table 1). CD8+

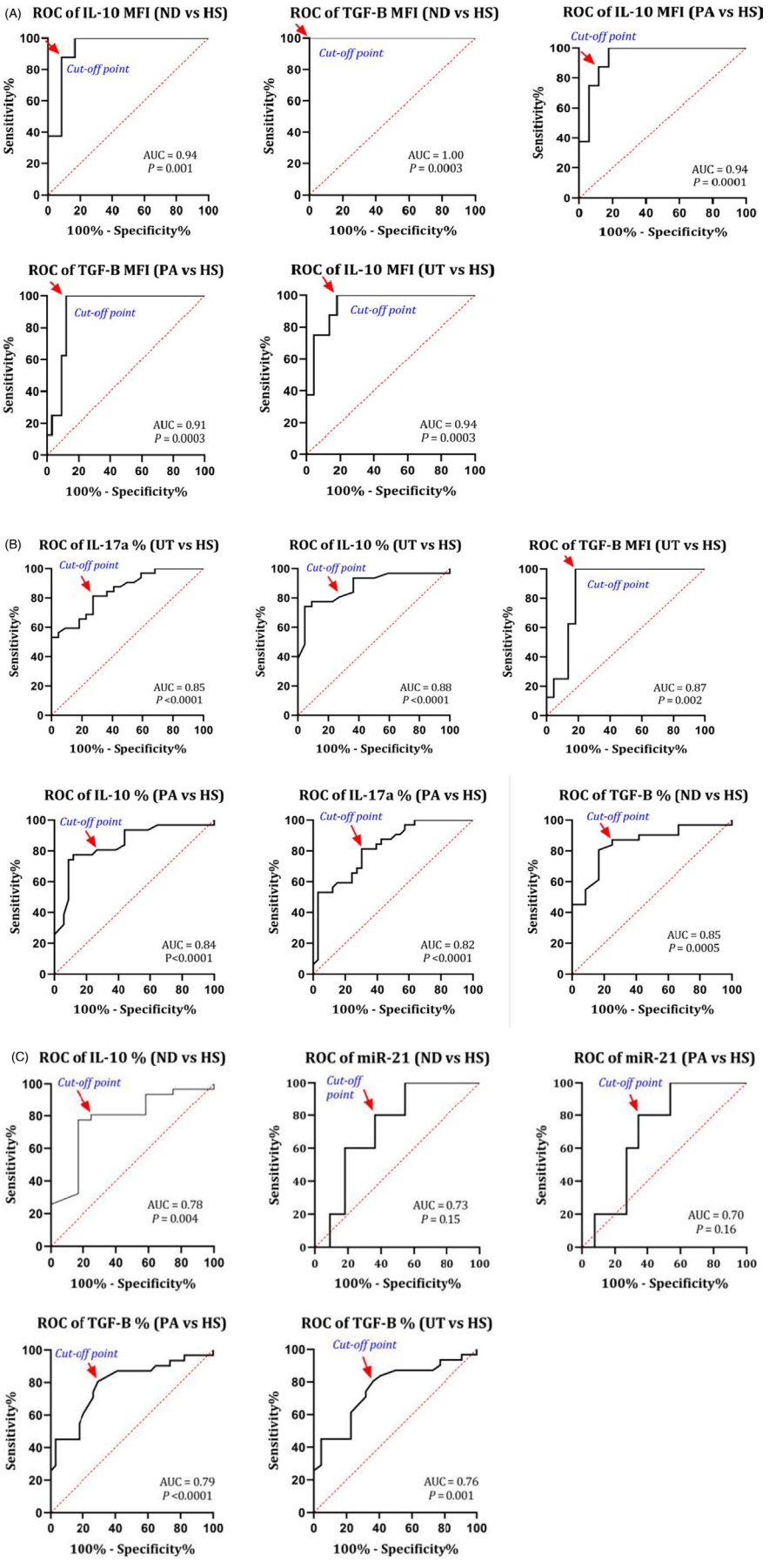

FIGURE 3 Assessment of diagnostic biomarkers by ROC curve analyses; biomarkers were categorized into groups including of excellent biomarkers (AUC: 0.9–1) (A), good biomarkers (AUC: 0.8–0.9) (B), and fair biomarkers (AUC: 0.7–0.8) (C). AUC, p-value, cutoff value, specificity, sensitivity, and likelihood ratio determined for each variable. HS, Healthy Subjects; ND, Newly diagnosed; PA, Patients; UT, Under-treatment

TABLE 3 Diagnostic values (ROC curve results) of each variable with significant difference between two groups of patients and healthy subjects

Biomarkers		Group distinguish	*p*-value	Cutoff value	Specificity	Sensitivity	Likelihood ratio
Excellent	TGF-β MFI	PA vs. HS	0.0003	3653	87.88%	100.00%	8.250
		ND vs. HS	0.0003	3743	100%	100%	8.450
	IL-10 MFI	PA vs. HS	0.0001	4796	88.24%	87.50%	7.438
		ND vs. HS	0.0012	4796	91.67%	87.50%	10.50
		UT vs. HS	0.0003	4962	81.82%	100.00%	5.500
Good	IL-17a %	PA vs. HS	<0.0001	5.650%	69.70%	81.25%	2.681
		UT vs. HS	<0.0001	5.650%	72.73%	81.25%	2.979
	TGF-β%	ND vs. HS	0.0005	3.950%	75.00%	87.10%	3.484
	TGF-β MFI	UT vs. HS	0.0020	3843	81.82%	100.00%	5.500
	IL-10%	PA vs. HS	<0.0001	3.950%	73.53%	80.65%	3.047
		UT vs. HS	<0.0001	4.450%	72.73%	80.65%	2.957
Fair	TGF-β%	PA vs. HS	0.0003	1.050%	70.59%	80.65%	2.742
		UT vs. HS	0.0014	1.050%	63.64%	80.65%	2.218
	miR-21	PA vs. HS	0.1626	0.02058	65.38%	80.00%	2.311
		ND vs. HS	0.1567	0.01387	63.64%	60.00%	1.650
	IL-10%	ND vs. HS	0.0049	3.950%	75.00%	80.65%	3.226
Poor	IFNγ-γ MFI	PA vs. HS	0.1561	16491	64.71%	61.29%	1.737
		UT vs. HS	0.1042	16491	68.18%	61.29%	1.926
	IL-17a MFI	PA vs. HS	0.1313	9845	60.61%	62.50%	1.587
		ND vs. HS	0.3031	16149	63.64%	56.25%	1.547
	miR-21	UT vs. HS	0.2386	0.02058	66.67%	80.00%	2.400

Patients, ND: Newly diagnosed, UT: Under-treatment, HS: Healthy Subjects.

Treg cells might be involved in inhibiting the effective immune responses to tumors. Zhang et al suggested that CD8+ Treg cells were increased in ovarian cancer patients and could be induced in vitro, which may be the way tumors limit the antitumor immunity and evade immune surveillance.[49] CD8+ T cells could mediate antigen-specific immunosuppression by polishing off T helper cells, antigen presenting cells or by noncytolytic pathways, which are not clearly defined.[50] IL-10- and TGF-β-expressing CD8+ regulatory T cells are also recruited or activated by the immunosuppressive environment of the lung, where they may suppress the induction of antitumor immunity.[51] We also represented a direct correlation between Tc1 and CD8+ Treg cells in ND patients, whereas the reverse correlation was observed in UT patients. On the other hand, we also demonstrated a reverse correlation between Tc17 cells with IL-10 and IFN-γ MFI in normal subjects, while the correlation was direct in ND patients. All these findings are mainly in favor of the dysregulation of immune responses in the onset of ESCC.[6,52]

Several researchers have revealed that the expression of miR-21 is elevated in various malignancies including brain, breast, cervix, lung, liver, prostate, pancreas, colon, and esophageal cancer.[18,53-59] Accordingly, the elevated serum levels of miR-21 were suggested as a potential prognostic marker and/or diagnostic indicator in some cancers.[53,60-62] In our previous publication, we showed that the plasma expression of miR-21 was significantly higher in ESCC patients, while the expression of miR-29b was not significantly changed.[6] Here, we demonstrated a significant reverse correlation between the expression of miR-21 and Tc17 frequency among patients (not normal subjects). The reverse correlation between the expression of miR-21 and frequency of Tc17 lymphocytes among ESCC patients denotes the regulatory role of miR-21 in immune responses of ESCC patients and could be related to the reduction of IL-17a production from Tc17 cells.

We also showed that there were reverse correlations between the expression of miR-29b and the production of IFN-γ and IL-17a from CD8+ T lymphocytes (Table 1). There were also reverse correlations between the expressions of miR-29b with the CD8+ Treg cell frequency (Table 2). MiR-29b targets the expression of diverse proteins such as collagens, transcription factors, and methyltransferases, which may contribute in abnormal migration, invasion, or proliferation of the cells. MiR-29 family could also be activated by interferon signaling, which suggests a crucial role in the immune system. MiR-29b could limit TH1 cell differentiation and IFN-γ production by targeting the mRNAs encoding T-bet, eomesodermin (Eomes), and IFN-γ itself.[63,64] These findings in addition to the reverse correlations between the expression of miR-29b and the production of IFN-γ and IL-17a from CD8+ T lymphocytes in our study could highlight the significant role of mir-29b in immunopathogenesis of ESCC. Altogether, mir-29b may possess a dual role

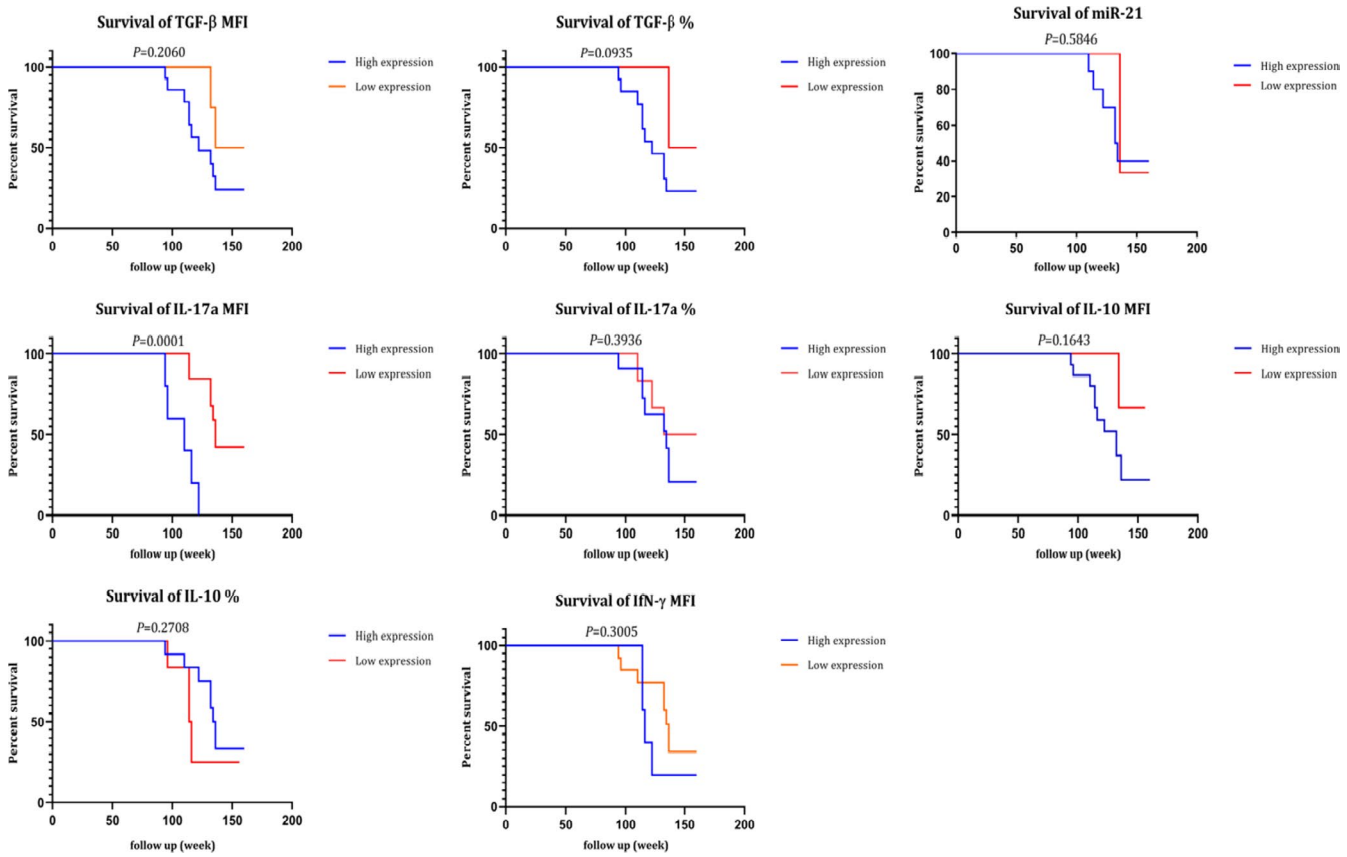

FIGURE 4 Evaluating the prognostic values of biomarkers to predict ESCC outcome. Log-rank (Mantel–Cox) test analyses showed that IL-17a MFI ($p = 0.0001$) is a good biomarker for prediction of survival. Patients with IL-17a high expression had shorter life span compared to patients with IL-17a low expression. Life span defined from time when samples were acquired until the date of death or date of last follow-up

in ESCCimmunopathogenesis. According to the fact that miR-29b is under negative regulation of TGF-β–Smad3 signaling,[65] its reverse association with the regulatory CD8+ T cells could be introduced as one of the other miR-29b-related tumorigenesis mechanisms.

As shown in Figure 3 and Table 3, we found that the MFI of both TGF-β and IL-10 could be considered as excellent biomarkers in distinguishing ESCC patients and ND subgroup from healthy subjects. Application of effective treatment modalities and increasing the life expectancy of ESCC patients depends on introducing powerful prognostic and diagnostic tools. The current low-sensitive, expensive, and mostly invasive current diagnostic markers including CT, MRI, positron emission tomography (PET), and endoscopic ultrasound (EUS) should be replaced with more sensitive options.[66] These biomarkers mainly include liquid biopsy, genomics biomarkers, epigenetic markers (such as miRNAs), immunologic tests, inflammatory chemokines and cytokines, and proteomic molecules (72–76).

Among immunologic mediators which have been introduced as ESCC biomarkers, serum IL-6,[67] circulating antibodies against tumor-associated antigens,[68,69] tumor-infiltrating lymphocytes,[70] intratumoral dendritic cell infiltration,[71] CXCL-8 and CXCR-2,[72] and CXCR4[73] have drawn notable attention. The intratumoral CD8+ T-cell infiltration have been also regarded as a good prognosis in both squamous cell and adenocarcinomas.[74] Although our research work was accompanied by limitations including the small sample size in studied groups, and lack of functional analyses to evaluate the role of miR-21 and miR-29b on altering the frequencies of CD8+ CTLs, to the best of our knowledge, no study has investigated the immunophenotype and cytokine-specific patterns of CD8+ CTLs in circulation as potential diagnostic/prognostic markers, and the current study highlights the significance of various CTLs in association with the miRNAs in improving ESCC diagnosis and enhancing the prediction of disease outcome.

5 | CONCLUSION

Our findings represented that the effector immune responses especially related to the CD3+CD8+ T lymphocytes are disrupted in ESCC patients. According to the correlation of CD8+ T lymphocyte impaired function with the aberrant miR-21 production in ESCC patients, the association could somehow be involved in a novel approach for the targeted immunotherapy. These alterations are so intense and would also be introduced as useful and powerful diagnostic and prognostic markers for ESCC.

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CONFLICT OF INTEREST

None.

AUTHORS CONTRIBUTION

Hadiseh Samiei involved in participation in literature bibliography, acquisition of data, analyses and interpretations of data, manuscript drafting, and revision of the manuscript. Abdolsamad Gharavi involved in participation in data acquisition and participation in manuscript drafting. Sara Abdolmaleki involved in participation in data acquisition and manuscript drafting. Parviz Kokhaei involved in participation in data acquisition and manuscript drafting. Saeed Mohammadi involved in participation in literature bibliography, acquisition of data, analyses and interpretations of data, manuscript drafting, and revision of the manuscript. Ali Memarian involved in study design and concept, participation in literature bibliography, acquisition of data, analyses and interpretations of data, manuscript drafting, and revision of the manuscript. All authors read and approved the final version of the manuscript.

ETHICAL STATEMENT

The present study that involved human participants was approved by the Ethics Committee of Golestan and Semnan Universities of Medical Sciences (Code of Ethics: IR.GOUMS.REC.1395.28 and IR.SEMUMS.REC.1394.215, respectively). A written informed consent following the declaration of Helsinki was signed by all participators.

DATA AVAILABILITY STATEMENT

Data are available on request from the authors.

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