Higher T cell immunoglobulin mucin-3 (Tim-3) expression in cervical cancer is associated with a satisfactory prognosis

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Background: Cervical cancer (CC), which has been increasing in incidence in recent years, is the fourth most common gynecological cancer in the world. Therapy targeting T cell immunoglobulin mucin-3 (Tim-3), known as the immune checkpoint, has been rapidly developing as oncotherapy for various carcinomas. However, few studies focus on Tim-3 in CC in terms of patient prognosis. This study demonstrates that higher Tim-3 mRNA levels in CC are associated with a favorable prognosis, which may due to active immune responses in CC.

Methods: First, the clinical and RNA-sequencing (RNA-seq) data of 287 CC patients were downloaded from The Cancer Genome Atlas (TCGA) database and was subsequently analyzed. Then, based on the Tim-3 mRNA levels, the patients were divided into groups categorized by high and low expression, and the overall survival (OS) among the patients was determined. Next, the correlation between the expression of Tim-3 and clinicopathological variables was investigated. Finally, the Tim-3 function was carried out using Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, and a gene set enrichment analysis (GSEA) was performed.

Results: In CC, the median OS of patients with high Tim-3 expression and low Tim-3 expression were 634 and 491 days (P=0.01), respectively. We found that the high expression of Tim-3 was closely associated with smoking history (P=0.012), total number of pregnancies (P=0.002), histological type (P<0.0001), M stage (P=0.036), TNM stage (P<0.0001), papillomavirus (P=0.001), hysterectomy type (P<0.0001) and survival status (P<0.0001). Univariate and multivariate logistic regression tests suggested that the level of Tim-3 was an independent prognostic factor for CC patients. In addition, GSEA further showed that Tim-3 expression was associated with macrophage differentiation, regulation of monocyte chemotaxis, positive regulation of substrate adhesion dependent cell spreading, negative regulation of interleukin 2 production, regulation of NF kappa-B signaling, STAT cascade, erk1 and erk2 cascade and regulation of vascular endothelial growth factor receptor signaling pathway.

Conclusions: A higher expression of Tim-3 was associated with a favorable prognosis, which may due to the activation of immune responses in tumor tissues.

Keywords: Cervical cancer (CC); prognosis; T cell immunoglobulin mucin-3 (Tim-3); immune

Introduction

Cervical cancer (CC) had an estimated 570,000 new cases and 311,000 deaths globally in 2018, and it is the fourth most common gynecological cancer in the world, accounting for over 10% of all female cancers (1,2). A key cause of cervical intraepithelial neoplasia (CIN) and CC is high-risk human papillomavirus (HR-HPV) infection (3). More than 99% of CC are associated with HPV infection, primarily HPV-16 and HPV-18. There are three HPV vaccines that may be used to prevent CC: 2-valent, 4-valent, and 9-valent vaccines. However, the prognosis of patients diagnosed in the advanced stages still remains up for debate in regard to CC (4,5). The prognosis of CC patients is closely related to the patients' FIGO stage, depth of cervical invasion and lymph node metastasis (6). Recently, RPA3, PD-1/PD-L1 and GINS2 were found to be the prognostic biomarkers of CC (7-10). In addition, molecular biomarkers put light on carcinogenic features that can be useful in developing novel anti-CC therapies (11,12). In this study, T cell immunoglobulin mucin-3 (Tim-3) is investigated as a prospective immune-checkpoint target, a topic still under debate.

Tim-3, a cell surface star molecule, consists of immunoglobulin and mucin-like domains and was first discovered in 2002 (13). A previous study reported that Tim-3 was expressed on a large number of immune cells like macrophages, dendritic cells, and T cells, where it was thought to be a novel immune regulator (14). Moreover, Tim-3 may be expressed on tumor cells (15). Another study demonstrated that the expression of Tim-3 is related to the prognosis of gastric cancer (16), hepatocellular carcinoma (17), renal cell carcinoma (18), breast cancer (19), and endometrial carcinoma (20). Recently, a particular study identified that Tim-3 expression in CC patients may promote metastatic potential (21), however, other studies reported that higher Tim-3 mRNA levels found in metastatic prostate cancer tissues served as an indicator of a favorable prognosis (22). Therefore, more comprehensive studies are needed to explore this dynamic.

The prognosis of CC patients in the advanced stages is still unsatisfactory. Targeted therapies confer clinical efficacy than cytokine therapies due to its direct inhibition of the targeted molecules. However, after a certain period of treatment, many patients become intractable to these therapies (23). Checkpoint inhibitors targeting cytotoxic T lymphocyte associated antigen 4 (CTLA-4) or programmed death receptor-1 (PD-1) have been introduced, and clinical trials are ongoing (24-26). To improve survival of patients with advanced CC, ideal therapy targets need to be explored.

In our study, first, we analyzed the medians of PD1, PDL1, CTLA4 and Tim-3 expression with the overall survival (OS) and found that only Tim-3 expression was obviously significant according to the median. We then investigated the relationship among the expression of Tim-3 and the clinicopathological variables. Next, we compared the OS according to Tim-3 expression and investigated the role of Tim-3 expression in tumors in the prognosis of CC based on the clinical parameters and RNA-sequencing (RNA-seq) data from The Cancer Genome Atlas (TCGA) database. Finally, Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) and gene set enrichment analysis (GSEA) was performed on the TCGA data to analyze the biologically meaningful changes in CC and suggest possible treatment strategies in the comprehensive treatment of CC.

Methods

Patients and data

TCGA is a comprehensive publicly available collection of genomic data of more than 30 human tumors that provide researchers with data to improve the treatment of human cancers (27). The clinical characteristics, follow-up information, OS information and mRNA levels in tumors of patients with CC were abstracted from the TCGA dataset (http://cancergenome.nih.gov/). Accordingly, 294 samples of RNA-seq data were collected between 1994 and 2013 and were included in this study. The inclusion criteria were patients suffering from primary CC. Patients who were excluded from the study had incomplete information in regards to their staging or prognosis as well as any undetected genes.

GO annotation analysis

The GO project (http://www.geneontology.org) has been widely used to carry out the biologically meaningful annotation of two groups of Tim-3 mRNA levels.

KEGG pathway enrichment analysis

The enrichment analysis of KEGG pathway was done for the two groups of Tim-3 levels and was performed using

the website (DAVID, http://david-d.ncifcrf.gov/).

GSEA

GSEA was carried out using the GSEA3.0 software (www. broad.mit.edu/gsea) to ascertain the pathways in the low Tim-3 expression group. All phenotype labels (.cls) and dataset (.gct) files were created and loaded into the GSEA software. RNA-seq data were downloaded from TCGA, which contained a total of 20,530 genes. During the GSEA analysis, genes were abstracted from the Molecular Signatures Database (MSigDB). MSigDB collects information on various kinds of genes, including 1,320 canonical pathways from KEGG, BioCarta, PID, Reactome, and other pathway databases (28). Data from TCGA were analyzed by GSEA, and pathways with an FDR <0.05 were considered significant. Herein, the permutations number was 1,000 and the phenotype label was Tim-3-low versus Tim-3-high.

Statistical analysis

All statistical analyses were performed using the SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Patients with CC were separated into two groups based on the median value of Tim-3 expression in tumor cells. Data from different groups were compared using the Student's t-test and chi-square test or Fisher exact test. A Kaplan-Meier curve was conducted to examine whether the Tim-3 expression level in CC has prognostic value for the OS of patients with CC. Then, a univariate and multivariate logistic regression model was done to investigate whether Tim-3 expression was an independent prognostic factor in the OS. Moreover, a Spearman correlation analysis was performed to verify the relevance between immune-related genes and Tim-3 expression levels, and P<0.05 was regarded as statistically significant. Prism 7 (GraphPad Software Inc., La Jolla, USA) was used for all statistics related to clinical samples, while the R software (version 3.4; R Foundation for Statistical Computing, Vienna, Austria) was used for bioinformatics analyses.

Results

Description of the integrated CC data in TCGA

The integrated data of 287 CC patients from TCGA were enrolled for analysis. Of the 287 patients in the cohort, the median age of the CC patients was 46 years, ranging from 20 to 88 years. The clinical characteristics, follow-up information, tumor pathological features and OS information of the CC patients are presented in *Table 1*. The median follow-up time was 549 days among all CC patients, and 90 patients died during follow-up.

The Tim-3 mRNA levels of the CC patients were downloaded from the RNA-seq data, where the median of Tim-3 in CC patients was found to be 7.7168 ng/mL, containing continuous variables in a wide range of 3.823 to 11.1941 ng/mL.

K-M survival curves of Tim-3

The CC patients were further divided into a high Tim-3 group and a low Tim-3 group according to the median Tim-3 mRNA value. A low expression of Tim-3 in tumor tissues was associated with a poor prognosis (P=0.0431), as shown in *Figure 1A*. The 5-year OS rate of patients with early CC was obviously longer than those with advanced CC (P<0.0001), as depicted in *Figure 1B*. Similarly, the 5-year OS rate of patients with a lower tumor size (T), node (N) and metastasis (M) was obviously longer than those with higher T, N and M stage (P<0.0001, P=0.0044 and P=0.0052), illustrated in *Figures 1C,D,E*. The corresponding results imply that Tim-3 may serve as a satisfactory prognostic biomarker in CC.

Clinical features of the integrated CC patients

We investigated the relationship between the expression of Tim-3 and the clinicopathological variables. The results demonstrated a correlation between high expression levels of Tim-3 in patients with CC smoking, BMI, FIGO stage, metastasis and histological type, as seen in *Figures 2A,B,C,D,E,F*. Moreover, we found that the high expression of Tim-3 was strongly association with the smoking history (P=0.012), total number of pregnancies (P=0.002), histological type (P<0.0001), M stage (P=0.036), FIGO stage (P=0.001), papillomavirus (P=0.001), hysterectomy type (P<0.0001) and survival status (P<0.0001), depicted in *Table 2*. Therefore, we identified Tim-3 as a prognostic gene associated with tumor progression in CC patients. Furthermore, logistic regression analysis was done to examine Tim-3's usefulness in the prognosis of CC. Univariate analyses revealed that the T stage (HR =3.80; P=0.001), N stage (HR =2.59; P=0.001), M stage (HR =4.12; P=0.001), TNM stage (HR =1.86;

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Table 1 Characteristics of patients with cervical cancer

Characteristics	No. of cases	%
Age		
≤60	149	51.9
>60	138	48.1
BMI		
<25	91	31.7
≥25	154	53.7
Not recorded	42	14.6
Smoking history		
Non-smoker	139	48.4
≤15 years	105	36.6
>15 years	9	3.1
Not recorded	34	11.9
Total number of pregnancies		
≤5	213	74.2
>5	41	14.3
Not recorded	33	11.5
Number of successful birthed		
≤5	231	80.5
>5	19	6.6
Not recorded	37	12.9
Histological type		
CSCC	238	82.9
Others	49	17.1
M		
M0	105	36.6
M1	10	3.5
Mx	125	43.6
Not recorded	47	16.3
TNM stage		
IA–IIA	171	59.6
IIB–IV	105	36.6
Stage X	5	1.7
Not recorded	6	2.1

Table 1 (*continued*)

Table 1 (*continued*)

Characteristics	No. of cases	%
Histologic grade		
G1/2	147	51.2
G3/4	114	39.7
GX	23	8.0
Not recorded	3	1.1
Papillomavirus		
HPV (−)	25	8.7
HPV (+)	21	7.3
Not recorded	241	84.0
Hysterectomy type		
Simple/radical hysterectomy	161	56.1
Other	126	43.9
Survival status		
Alive	217	75.6
Died	70	24.4

CSCC, cervical squamous cell carcinoma; BMI, body mass index; G, histologic grade.

P=0.001) and high Tim-3 levels (HR =1.62; P=0.04) were associated with the survival of CC patients. Additionally, multivariate analyses showed that the histological type (HR =0.13; P=0.02), TNM stage (HR =3.01; P=0.04) and high Tim-3 levels (HR =3.41; P=0.04) were independent predictors of prognosis, which are detailed in *Table 3*. Collectively, our results suggest that the expression of Tim-3 plays a critical role in the prognosis of CC patients.

Significant GOs and pathways

To predict the biological function of Tim-3 in the progression of CC, a GO enrichment analysis and a KEGG analysis were performed. The results demonstrated Tim-3's involvement in various biological processes. *Figure 3A,B* depict the associated functional pathways, where it is observed that Tim-3 closely relates to macrophage differentiation, monocyte chemotaxis, macrophage activation, lymphocyte mediated immunity, leukocyte differentiation and interleukin 2 production. In order to identify the key
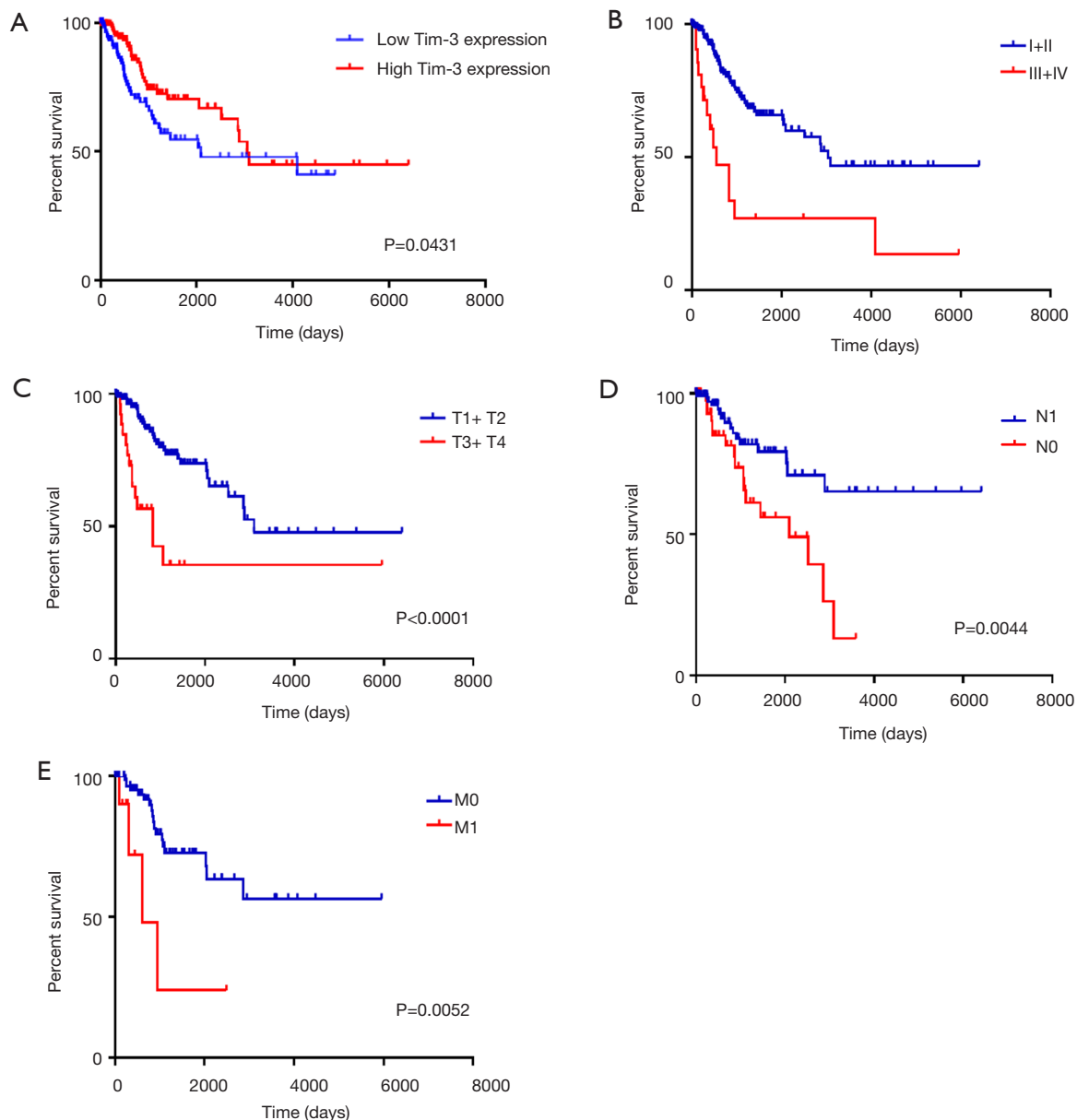
Figure 1 KM analysis of Tim-3 and other clinicopathological features. (A) The Kaplan-Meier (KM) curve illustrates the association between Tim-3 expression and OS of cervical cancer patients. (B) The KM curve shows the association between TNM stage and OS of cervical cancer patients. (C) The KM curve depicts the association between the T stage and OS of cervical cancer patients. (D) The KM curve demonstrates the association between the N stage and OS of cervical cancer patients. (E) The KM curve illustrates the association between M stage and OS of cervical cancer patients. OS, overall survival.

pathways, a pathway analysis was performed, where it was found that the major regulated biological pathways are B cell receptor signaling, ECM receptor interaction, T cell receptor signaling, cell cycle, apoptosis, cell adhesion molecules and JAK-STAT signaling, implying that Tim-3 potentially regulates the immune system.

GSEA analysis of Tim-3

Based on the above results, we discovered that Tim-3 plays a crucial role in the prognosis of CC. Consequently, it was necessary to explore the biological functions of Tim-3. GSEA, a powerful tool used to estimate biological
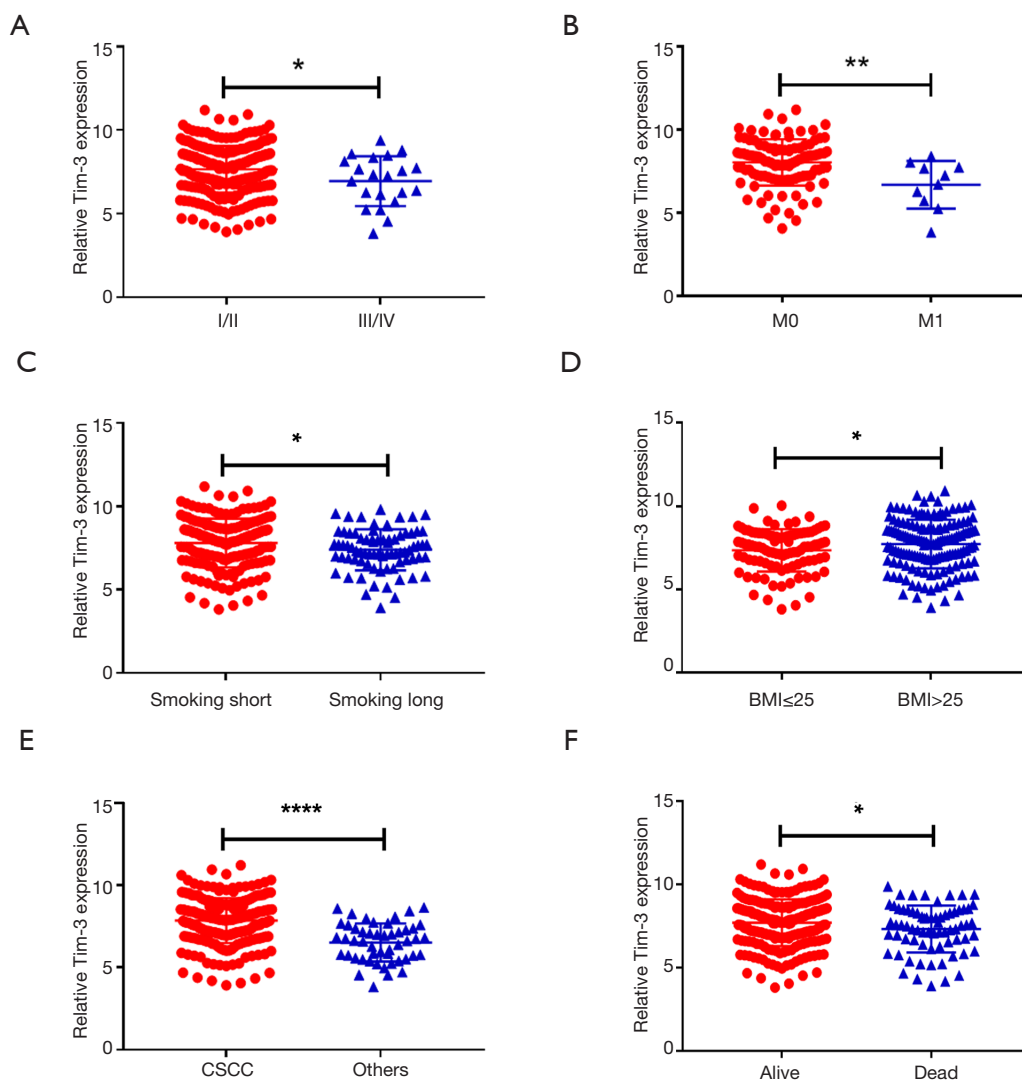
Figure 2 Tim-3 is upregulated in The Cancer Genome Atlas (TCGA) database and is attributed with a good prognosis. (A) Tim-3 expression levels in different groups of TNM stages are displayed. (B) Tim-3 mRNA levels were compared with respect to their M stage. (C) Effect of Tim-3 expression on smoking in cervical cancer patients. (D) Effect of Tim-3 expression on BMI in cervical cancer patients. (E) Effect of Tim-3 expression on histological type in cervical cancer patients. (F) Effect of Tim-3 expression on survival status in cervical cancer patients. *, P<0.05; **, P<0.01; ****, P<0.0001. CSCC, cervical squamous cell carcinoma; BMI, body mass index.

functions, was utilized in this regard. The results showed that Tim-3 expression was associated with macrophage differentiation, regulation of monocyte chemotaxis, negative regulation of interleukin 2 production, positive regulation of substrate adhesion dependent cell spreading, regulation of NF kappa-B signaling, STAT cascade, erk1 and erk2 cascade and regulation of vascular endothelial growth factor receptor signaling pathway. These functions were significantly enriched at low levels of Tim-3 expression in CC, as seen in *Figure 4*. This demonstrated that Tim-3

expression levels may serve as a prognostic indicator in CC patients with favorable outcomes. Then, a database search was done for the expression profiles of Tim-3 as well as any immune-related genes, where a positive correlation was observed between Tim-3 expression and the expression of immune markers, such as CD4 (P<0.0001, r=0.9114) and CD163 (P<0.0001, r=0.6904), as illustrated in *Figures 5A,B*. Seemingly, a high level of Tim-3 confers an immunological tumor-promoting role. Essentially, our study infers that a high level of Tim-3 is closely associated with a favorable

Table 2 Association between Tim-3 expression and clinicopathological characteristics in patients with cervical cancer

Characteristics	Total, N (%)	Tim-3 expression		P value
		High	Low	
Age				0.955
≤46	149	74	75	
>46	138	69	69	
BMI				0.082
<25	91	38	53	
≥25	154	82	72	
Not recorded	42	23	19	
Smoking history				0.012*
Non-smoker	139	82	57	
≤15 years	105	45	60	
>15 years	9	4	5	
Not recorded	34	12	22	
Total number of pregnancies				0.002**
≤5	213	104	109	
>5	41	25	16	
Not recorded	33	13	20	
Number of successful birthed				0.756
≤5	231	118	113	
>5	19	9	10	
Not recorded	37	15	22	
Histological type				<0.0001****
CSCC	238	136	102	
Others	49	7	42	
M				0.036*,b
M0	105	67	38	
M1	10	3	7	
Mx	125	59	66	
Not recorded	47	14	33	
FIGO stage				0.001*
IA-IIA	171	101	70	
IIB-IV	105	40	65	
Stage X	5	0	5	
Not recorded	6	2	4	
Histologic grade				0.067
G1/2	147	67	80	
G3/4	114	65	49	
GX	23	9	14	

Table 2 (*continued*)

Table 2 (*continued*)

Characteristics	Total, N (%)	Tim-3 expression		P value
		High	Low	
Not recorded	3	2	1	
Papillomavirus				0.001**
HPV (−)	25	12	13	
HPV (+)	41	35	6	
Not recorded	221	96	125	
Hysterectomy type				<0.0001****
Simple/radical hysterectomy	161	90	71	
Other	126	53	73	
Survival status				<0.0001****
Alive	197	114	83	
Died	90	29	61	

[b], indicates the Fisher test, and the other test was the Chi-square test. *, P<0.05; **, P<0.01; ***, P<0.001****, P<0.0001. CSCC, cervical squamous cell carcinoma; BMI, body mass index; G, histologic grade.

Table 3 Logistic regression model of prognostic factors in patients with cervical cancer

Characteristics	Univariate			Multivariate		
	HR	95% CI	P value	HR	95% CI	P value
Age (>46 *vs.* ≤46)	1.35	0.59–3.17	0.41	1.39	0.87–3.45	0.67
BMI (<25 *vs.* ≥25)	1.72	0.97–3.04	0.05	0.48	0.14–1.61	0.23
Smoking years (>15 to ≤15)	0.95	0.31–2.95	0.93	1.30	0.01–119.00	0.91
Total number of pregnancies (≥5 *vs.* <5)	1.23	0.63–2.41	0.51	2.95	0.52–16.71	0.22
Number of successful birthed (≥5 *vs.* <5)	1.24	0.49–3.11	0.62	0.02	0.01–0.03	0.99
Histological type (CSCC *vs.* others)	1.03	0.55–1.96	0.91	0.13	0.02–0.74	0.02*
T (T3/4 *vs.* T1/2)	3.80	1.47–9.86	0.001***	0.13	0.04–4.04	0.25
N (N1 *vs.* N0)	2.59	1.22–5.49	0.001**	1.49	0.30–7.34	0.63
M (M1 *vs.* M0)	4.12	1.712–8.38	0.001**	3.44	1.68–6.13	0.001**
TNM stage (IIB-IV *vs.* IA-IIA)	1.86	1.13–3.08	0.001**	3.01	1.03–8.82	0.04*
G (G1/2 *vs.* G3/4)	1.16	0.70–1.95	0.57	0.73	0.23–2.37	0.60
Papillomavirus (+ *vs.* −)	1.11	0.33–3.72	0.87	0.89	0.16–3.28	0.07
Hysterectomy type (simple/radical hysterectomy *vs.* others)	0.70	0.13–3.76	0.62	0.89	0.45–3.32	0.86
Radiotherapy (yes *vs.* no)	1.06	0.60–1.87	0.85	0.63	0.13–5.16	0.63
Chemotherapy (yes *vs.* no)	0.94	0.12–7.27	0.95	3.12	0.39–4.26	0.76
Targeted molecular therapy (yes *vs.* no)	0.94	0.54–1.65	0.83	2.22	0.68–3.21	0.65
Tim-3 (low *vs.* high)	1.62	1.43–3.67	0.04*	3.41	1.56–12.33	0.04*

CSCC, cervical squamous cell carcinoma; BMI, body mass index; G, histologic grade. *, P<0.05; **, P<0.01; ***, P<0.001.
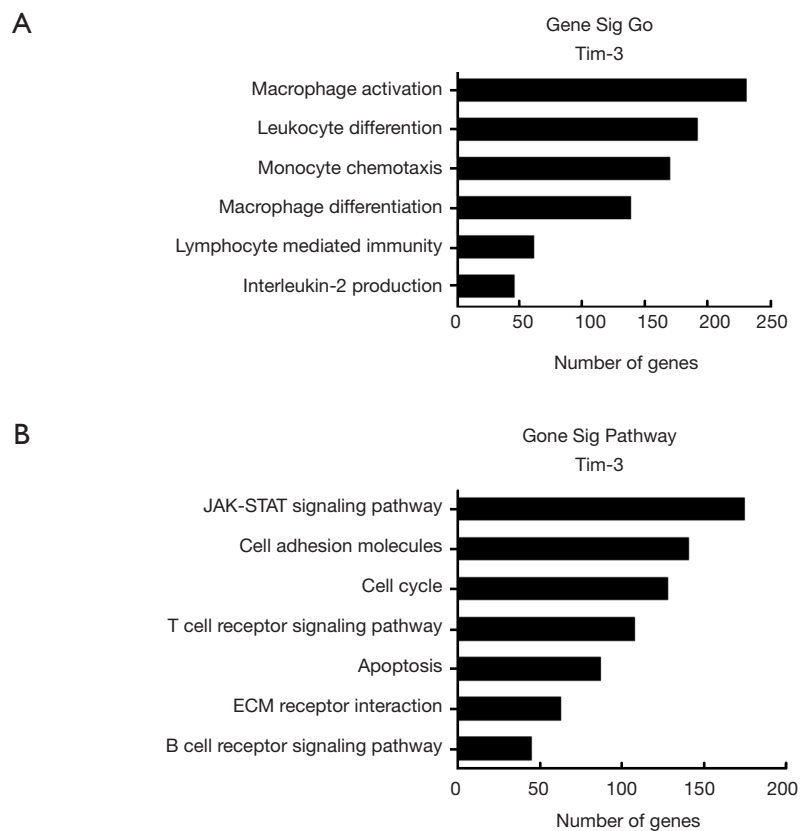
Figure 3 Functional associations of Tim-3. (A) Gene ontology analysis revealed that Tim-3 is involved in lymphocyte mediated immunity, macrophage differentiation, monocyte chemotaxis, leukocyte differentiation, macrophage activation and interleukin-2 production. (B) KEGG showed that Tim-3 was associated with the B cell receptor signaling pathway, ECM receptor interactions, apoptosis, cell cycle, T cell receptor signaling pathway, cell adhesion molecules and JAK-STAT signaling pathway. KEGG, Kyoto Encyclopedia of Genes and Genomes.

prognosis in CC patients in regard to their immunity.

Discussion

Thanks to the increasing availability of high-throughput technologies, many novel therapeutic targets and biomarkers have been identified through the transcriptomic analysis of various tumors. In this paper, our results imply that high Tim-3 mRNA levels in CC are associated with a satisfactory prognosis via activation of immune responses in cervical tumor tissues. The identification of CC biomarkers may help predict and prolong the survival of CC patients and may serve as an ideal therapeutic target.

Tim-3 is known to be a novel immune regulator that is expressed in a variety of tumor tissues and is closely related with tumor development (17,29). High levels of Tim-3 expression in peripheral NK cells (30), tumor infiltrating T cells (31,32), macrophages/monocytes (33), contributed to

an unfavorable prognosis in a variety of cancers. However, in this study, we found that downregulation of Tim-3 indicates a worse outcome. The underlying mechanism explaining the according results lies in that the Tim-3 expressed in tumor cells and tumor-associated immune cells exhibit different tumorigenic patterns (34). Therefore, conflicting results pertaining to the relationship between Tim-3 expression in tumor cells and the survival data in different cancers were obtained (21,22). The upregulation of Tim-3 in esophageal cancer indicated a shorter survival of patients (35), while a higher expression of Tim-3 may be a protective factor in patients with colon cancer (36). This complicated phenomenon may demonstrate that the localization of varying levels of Tim-3 expression encompass different functions in different tumor tissues.

Our study provides new insight into the prognostic value of Tim-3 in patients with CC. Accordingly, we obtained CC data from TCGA and evaluated the different

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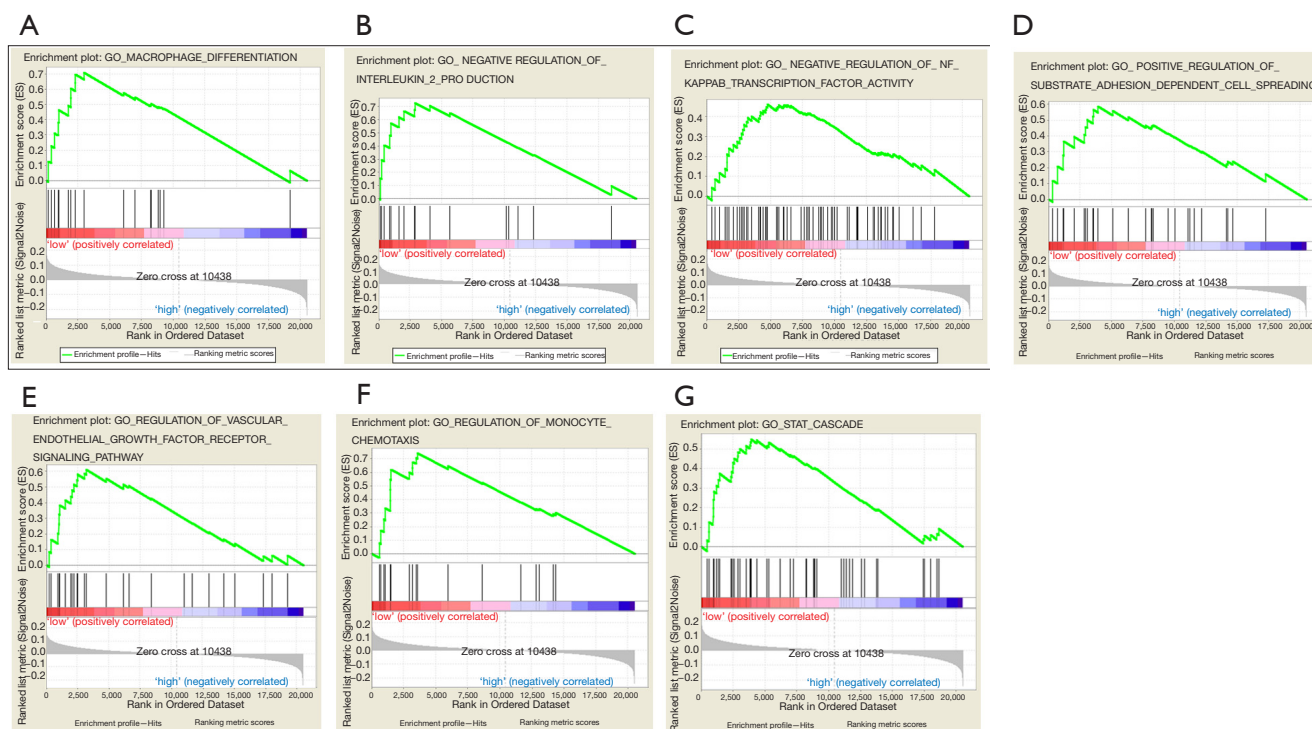
Figure 4 GSEA analysis of Tim-3. (A,B,C,D,E,F,G,H) Gene set enrichment analysis (GSEA) showed that Tim-3 was associated with macrophage differentiation, regulation of monocyte chemotaxis, positive regulation of substrate adhesion dependent cell spreading, negative regulation of interleukin 2 production, regulation of NF kappa-B signaling, stat cascade, erk1 and erk2 cascade and regulation of vascular endothelial growth factor receptor signaling pathway.
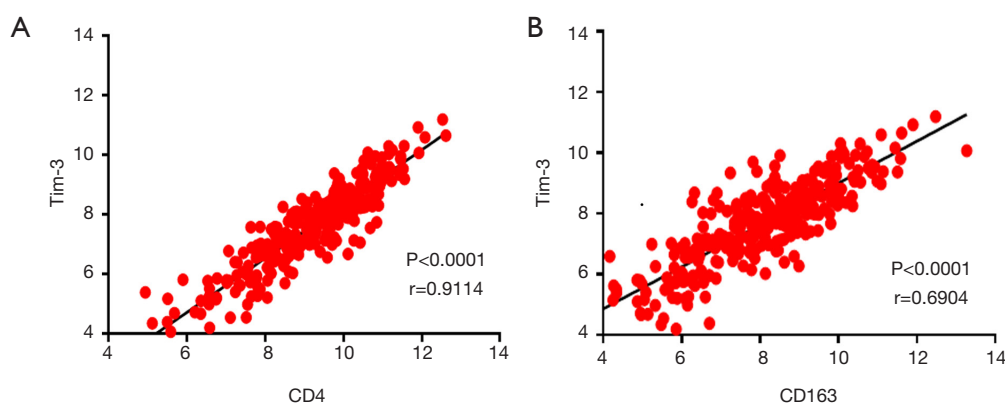
Figure 5 Tim-3 is positively correlated with the promotion of immune cells. (A) The correlation between Tim-3 and CD4. (B) The correlation between Tim-3 and CD163. Pearson's correlation coefficients (r) and P values were determined.

expression levels of Tim-3 in CC tissues as well as the clinicopathological variables. We found that Tim-3 is a favorable prognostic biomarker for patients with CC, and a logistic regression analysis suggests that the expression level of Tim-3 plays an important role in

predicting the prognosis of CC patients. According to the pathway analysis, the underlying mechanism may deal with macrophage differentiation, macrophage activation, lymphocyte mediated immunity, regulation of monocyte chemotaxis, negative regulation of interleukin 2 production,

positive regulation of substrate adhesion dependent cell spread and other mechanisms.

However, the results obtained in this study contrasts with a previous study suggesting that Tim-3 expression in patients with CC may be attributed with increased metastatic potential (21). Hence, controversial results in regard to the association between Tim-3 expression levels in CC cells and CC patient prognosis were obtained. This phenomenon suggests that the role of Tim-3 in CC remains ambiguous. Moreover, the underlying reason for these results has not been fully clarified. Therefore, further investigation and experimental validation are needed in order to elucidate the exact role of Tim-3 in patients with CC in order to develop a more accurate treatment plan.

In summary, this report first indicated that Tim-3 expression was downregulated in CC tissues in advanced stages compared to those in early stages. The absence of Tim-3 contributed to tumor progression, resulting in poor patient outcomes. This is the first study showing that Tim-3 exerts a detrimental effect on the progression of CC via bioinformatic screening. The expression levels of Tim-3 may be a valuable adjuvant parameter to predict the prognosis of patients with CC and provides a potential therapeutic foundation.

This study has the following limitations: no clinical sample was verified, and different types of CC were included. Therefore, further investigations should focus on the observation of the differences between expression levels and prognosis in CC.

Conclusions

To conclude, a higher level of expression of Tim-3 serves as a biomarker in the favorable prognosis of CC. Hence, Tim-3 has the potential to shape the landscape of immunotherapy for CC.

Acknowledgments

Footnote

Conflicts of Interest: The authors have completed the ICMJE uniform disclosure from (available at http://dx.doi.org/10.21037/tcr.2020.02.41). The authors have no conflicts of interests to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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