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Differential expression of miRNA-769-5p and Smad2 in patients with or without oral cGVHD

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Conflict of interest
None declared

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Abstract

Background. Oral chronic graft-versus-host disease (cGVHD) impacts quality of life of patients after allogeneic hematopoietic stem cell transplantation (allo-HSCT). However, its precise pathogenesis remains unknown, with potential associations with differential microRNA (miRNA) expression and the TGF-β/Smad signaling pathway.

Objectives. This study aims to explore miRNA expression profiles in the peripheral blood of oral cGVHD patients, focusing on miRNA-769-5p and its relationship with Smad2.

Materials and methods. Peripheral venous blood samples were collected for RNA extraction from 8 patients with oral cGVHD, 8 patients without cGVHD and 8 participants from the healthy control group. The miRNA library was constructed using the Illumina Hiseq 2500 platform. We focused on identifying miRNAs associated with the TGF-β/Smad signaling pathway and subsequently conducted validation experiments. The oral cGVHD and without cGVHD groups were each expanded to include 15 individuals. Peripheral blood samples were subjected to polymerase chain reaction (PCR) analysis to assess miRNA levels and to evaluate Smad2 mRNA levels in peripheral blood mononuclear cells (PBMC). Additionally, enzyme-linked immunosorbent assay (ELISA) was conducted to determine the Smad2 protein levels in peripheral blood.

Results. The most significantly differentially expressed miRNAs among the 3 groups were miRNA-505-5p and miRNA-769-5p. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis indicated an enrichment of the target genes of miRNA-769-5p in the TGF-β signaling pathway. It was observed that miRNA-769-5p expression was higher in patients without oral cGVHD in comparison to those with oral cGVHD. Receiver operating characteristic (ROC) analysis demonstrated that miRNA-769-5p holds diagnostic value for oral cGVHD. As a target of miRNA-769-5p, Smad2 mRNA exhibited a negative correlation with it. Moreover, both Smad2 mRNA and protein levels were higher in patients with oral cGVHD as opposed to those without cGVHD.

Conclusions. Differential expression of miRNAs, particularly the downregulation of miRNA-769-5p, may influence the development of oral cGVHD by diminishing its inhibitory effect on the TGF-β/Smad signaling pathway through its interaction with Smad2.

Key words: oral mucosa, allogeneic hematopoietic stem cell transplantation, miRNAs, chronic graft-versus-host-disease

Background

Chronic graft-versus-host disease (cGVHD) is the leading cause of death following allogeneic hematopoietic stem cell transplantation (allo-HSCT).[1] As an alloimmune and autoimmune disease, it is characterized by lichenoid changes and fibrosis affecting various tissues, thereby compromising organ function.[2] Its clinical signs manifest first in the mucosal tissues.[3] The oral cavity is the primary organ affected by cGVHD, with oral manifestations occurring in 45–83% of cGVHD patients.[4] Encompassing lichen planus-like changes, erythema, ulceration, mucoceles, etc., the clinical presentation of oral cGVHD is diverse and can affect any sites within the oral cavity, including the oral mucosa, salivary glands and periodontium.[5,6] Lichen planus-like changes, marked by hyperkeratotic white lines and lacy-appearing lesions on the oral mucosa, serve as the diagnostic feature of oral cGVHD among its oral manifestations.[7] Our previous study showed that these lichen planus-like changes persist even when the systemic symptoms of cGVHD in patients are well controlled.[8] The repercussions of oral cGVHD significantly impact patient's quality of life, particularly in terms of eating and nutrient absorption.[9] Furthermore, oral cGVHD poses a risk for the development of oral squamous cell carcinomas (OSCCs).[10,11] Given the adverse effects of oral cGVHD on both oral and systemic health, raising awareness is crucial for understanding its occurrence and progression.

The pathogenesis of cGVHD, especially in the oral context, is intricate and remains controversial. Recent studies have shed light on the role of microRNAs (miRNAs) in cGVHD pathogenesis.[12] Reikvam et al. were the first to report miRNA serum profiles in cGVHD,[13] and miRNA profiles from plasma extracellular vesicles (EVs) have been considered markers of cGVHD onset.[14] However, owing to the distinct microenvironments of various organs and tissues, the miRNAs associated with the disease also exhibit variations.[15,16] Consequently, it prompts the question of whether miRNA profiles also change in oral cGVHD patients and how these changes impact the disease.

In our previous research, we uncovered an imbalance of cytokines associated with the TGF-β/Smad pathway in oral cGVHD patients.[17] TGF-β/Smad represents a major subfamily within the TGF-β signaling pathway. Phosphorylation of drosophila mothers against decapentaplegic protein 2 (Smad2) serves to activate this pathway, subsequently exerting its effects.[18] Research supports the involvement of the TGF-β/Smad signaling pathway in the initiation and progression of cGVHD,[19] and it has been shown that therapeutic intervention targeting this pathway can effectively manage cGVHD.[20] Furthermore, it is worth noting that Smads can be subject to regulation by miRNAs.[21,22] This leads us to question whether patients with oral cGVHD may exhibit aberrant miRNA expression profiles that influence the TGF-β/Smad pathway and contribute to the development of oral cGVHD.

Objectives

This study aimed to investigate miRNA expression profiles in the peripheral blood of patients affected by oral cGVHD, with a specific focus on miRNA-769-5p and its relationship with Smad2. We aim to provide an initial exploration of the correlation between miRNA-769-5p and Smad2 and their potential influences on oral cGVHD.

Materials and methods

Study groups and approval

The oral cGVHD group comprised patients whose clinical manifestations of cGVHD exclusively affected the oral cavity. The group without cGVHD comprised patients who underwent transplantation more than a year prior and did not develop cGVHD. The healthy control group comprised healthy volunteers.

All study participants were recruited from Guangxi Medical University (Nanning, China). Informed consent, consistent with the principles of the Declaration of Helsinki, was obtained from all study participants. The research protocol received approval from the Research Ethics Committee of Guangxi Medical University (approval No. IRB: 20170301-4). All procedures adhered to the relevant guidelines and regulations.

Study design

Eight individuals were recruited for sampling in each group for RNA sequencing analysis. To further validate the RNA sequencing results in both the oral cGVHD and without cGVHD groups, an additional 7 individuals were included in each group, resulting in a total of 15 individuals in each group for polymerase chain reaction (PCR)-based testing and analysis.

Clinical examination

The oral cGVHD diagnosis and grading were determined based on established diagnostic and scoring criteria.[7] Oral discomfort was assessed using a Visual Analogue Scale (VAS). Demographic information was collected through a structured questionnaire, and clinical evaluations and examinations were recorded.

RNA sequencing

Peripheral venous blood was collected from 8 participants in each of the 3 groups. Total RNA was isolated from the whole blood using TRIZOL (Invitrogen, Waltham, USA) to create the miRNA library. Sequencing and the subsequent analysis of miRNA differential expression were performed on the Illumina Hiseq 2500 platform by LC-Bio (Hangzhou, China).

qPCR

Peripheral venous blood samples were collected, and total RNA was extracted using TRIzol (Invitrogen) to measure miRNA levels. Concurrently, peripheral blood mononuclear cells (PBMCs) were isolated and subjected to RNA extraction to determine the Smad2 mRNA levels. The RNA was subsequently reverse-transcribed into cDNA utilizing TruScript™ First Strand cDNA Synthesis Kit. Quantitative real-time polymerase chain reaction (qPCR) was conducted using qTOWER 2.2 system (Analytik Jena AG, Jena, Germany). Cel-miR-39-5p served as the control for miRNA detection, while GAPDH acted as the control for Smads mRNA. Primer sequences are provided in Table 1. Data analysis was performed using the following formula: $R = 2^{-(\Delta Ct\ sample\ -\ \Delta Ct\ control)}$.

ELISA

Plasma samples from participants in the oral cGVHD and without cGVHD groups were collected and subjected to analysis using a human Smad2 immunoblot assay enzyme-linked immunosorbent assay (ELISA) kit (Cusabio, Wuhan, China). Spectrophotometric measurements were taken at 450 nm using the Wellscan Mk3 instrument (Thermo Fisher Scientific, Waltham, USA).

Luciferase reporter assay

The 3'-UTR of Smad2, containing either wild-type (WT) or mutated (MUT) binding sites for miRNA-769-5p, was amplified and inserted into the pGL3 vector to generate the plasmids pGL3-WT-Smad2-3'-UTR or pGL3-MUT-Smad2-3'-UTR, respectively. In the luciferase reporter assay, HEK-293 cells were co-transfected with the luciferase reporter vectors and miRNA-769-5p mimics, or their respective negative controls, using Lipofectamine LTX (Invitrogen). The pRL-TK plasmid (Promega, Madison, USA) served as a normalization control. After 24 h of incubation, luciferase activity was measured using a Luciferase Reporter Assay kit (Promega) following the manufacturer's instructions.

Statistical analyses

RNA sequencing results were expressed as the normalized fragments per kilo base per million mapped reads (FPKM) (log base per million reads) and subjected to analysis of variance (ANOVA) testing. The miRNAs with differential expression were defined based on a Benjamini–Hochberg adjusted p-value <5% and |log2 (fold-change FC) | ≥1. Significant differential miRNAs were then analyzed for target gene prediction using TargetScan (https://www.targetscan.org/vert_80/)[23] and miRWalk (http://mirwalk.umm.uni-heidelberg.de).[24] The resulting overlapping datasets were subsequently utilized for the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis with the aid of Omic-Studio (LC-Bio). In the following experiments, we employed the Shapiro–Wilk normality test to evaluate the distribution of the data (Supplementary Table 1). The miRNA expression displayed skewness and underwent transformation using the Box–Cox method to conform to a normal distribution. Differences in miRNA and Smad2 expression between groups were assessed using t-test. Diagnostic analysis was conducted through receiver operating characteristic (ROC) curve analysis. Furthermore, after assigning ranks to miRNA-769 based on its expression values, Spearman's correlation method was utilized to assess its association with Smad2. A p-value of less than 0.05 was considered statistically significant. The statistical software IBM SPSS v. 22.0 (IBM Corp., Armonk, USA) was utilized for data analysis.

Results

miRNAs differential expression among allo-HSCT patients and healthy controls

The demographic and clinical characteristics of the study participants are presented in Table 2. Among all 3 groups, a total of 518 miRNAs were expressed. After conducting an ANOVA analysis (details not presented), it was found that 191 of these miRNAs exhibited differential expression (Fig. 1). The most significantly differentially expressed miRNAs were identified as miRNA-505-5p and miRNA-769-5p (Table 3), sorted in descending order of significance based on p-values. Enrichment analysis of these 2 miRNAs was conducted using KEGG pathway functional annotation (Fig. 2,3).

Table 1. Primer information

Name	Sequence (5'to3')
has-miR-769-5p	F: TCGCGTGAGACCTCTGGG R: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGCTCA
cel-miR-39-5p	F: TGGGAGCTGATTTCGTCTTG R: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTATTAC
Smad2	F: TGTTAACCGAAATGCCACGGTA R: GGCTCTGCACAAAGATTGCACTA
GAPDH	F: GCACCGTCAAGGCTGAGAAC R: TGGTGAAGACGCCAGTGGA

Table 2. Demographic and clinical characteristics of study participants

Demographic and clinical information	Oral cGVHD (n = 8)	Without cGVHD (n = 8)	Healthy control (n = 8)
Demographic information			
Age (years, M ±SD)	35.12 ±14.38	34.37 ±15.31	32.12 ±4.79
Gender (male/female)	6/2	6/2	6/2
Donor			
Donor relationship (unrelated/related)	1/6	3/5	/
HLA match (non-identical/identical)	5/3	4/4	/
Blood type (non-identical/identical)	5/3	4/4	/
Cell source			
BMT/PBSCT/BMT+PBSCT/BMT+CBT	0/5/3/0	0/4/3/1	/
Clinical sign			
Severity score of oral manifestation	4.25 ±2.19	0	0
Oral pain VAS scores	4.5 ±2.39	0	0

M ±SD – mean ± standard deviation; cGVHD – chronic graft-versus-host disease; HLA – human leukocyte antigen; BMT – bone marrow transplantation; PBSCT – peripheral blood stem cell transplantation; CBT – cord blood transplantation.

Table 3. The information of top 2 different expression miRNAs

Statistical information	Normalized FPKM of miR-505-5p (miR sequence: GGGAGCCAGGAAGTATTGATGT)			Normalized FPKM of miR-769-5p (miR sequence: TGAGACCTCTGGGTTCTGAGCT)		
	oral cGVHD (n = 8)	without cGVHD (n = 8)	healthy control (n = 8)	oral cGVHD (n = 8)	without cGVHD (n = 8)	healthy control (n = 8)
M ±SD	202.625 ±48.928	301.000 ±48.196	169.500 ±36.493	1145.250 ±196.738	1178.125 ±223.850	752.250 ±161.667
Levene's test for variance homogeneity H_0: Equal variances H_1: Unequal variance	0.337			0.482		
p-value for Levene's test	0.718			0.624		
ANOVA for difference among groups H_0: equal means among groups H_1: unequal means among groups	18.561			11.723		
p-value for ANOVA	<0.001 (2.26E-05)			<0.001 (3.83E-04)		
df	23			23		

M ±SD – mean ± standard deviation; ANOVA – analysis of variance; cGVHD – chronic graft-versus-host disease; H_0 – null hypothesis; H_1 – alternative hypothesis; df – degrees of freedom.

The KEGG results indicated that target genes of miRNA-769-5p were enriched in the TGF-β signaling pathway (Fig. 3). While high-throughput sequencing indicated a modest reduction in miRNA-769-5p abundance in the oral cGVHD group compared to the without cGVHD group, the observed difference was not statistically significant.

Lower expressions of miRNA-769-5p in patients with oral cGVHD

To further validate the differential expression of miRNA-769-5p, we expanded the sample size to include 15 patients in both the oral cGVHD and without cGVHD groups (Table 4), and conducted verification through PCR analysis. It was observed that the expression of miRNA-769-5p was higher in patients without oral cGVHD compared to those with oral cGVHD (Table 5, Fig. 4). Among 30 patients after allo-HSCT (both the oral cGVHD and without cGVHD groups), ROC analysis indicated that miRNA-769-5p holds a certain diagnostic value for oral cGVHD, with an area under the curve (AUC) of 0.809, signifying a moderate diagnostic efficacy (95% confidence interval (95% CI): 0.657, 0.961; p = 0.004, Fig. 5).

Higher expressions of Smad2 in patients with oral cGVHD

Following predictions made by TargetScan and miRWalk, Smad2 was validated as a potential target gene of miRNA-769-5p through a dual-luciferase test (Fig. 6).

Table 4. Demographic and clinical characteristics of study participants

Demographic and clinical information	Oral cGVHD (n = 15)	Without cGVHD (n = 15)
Demographic information		
Age (years, M ±SD)	33.33 ±16.70	33.40 ±12.61
Gender (male/female)	12/3	11/4
Donor		
Donor relationship (unrelated/related)	4/11	5/10
HLA match (non-identical/identical)	8/7	7/8
Blood type (non-identical/identical)	7/8	8/7
Cell source		
BMT/PBSCT/BMT+PBSCT/BMT+CBT	0/5/10/0	0/8/7/0
Clinical sign		
Severity score of oral manifestation	4.27 ±2.22	0
Oral pain VAS scores	3.40 ±2.72	0

M ±SD – mean ± standard deviation; VAS – Visual Analogue Scale; cGVHD – chronic graft-versus-host disease; HLA – human leukocyte antigen; BMT – bone marrow transplantation; PBSCT – peripheral blood stem cell transplantation; CBT – cord blood transplantation.

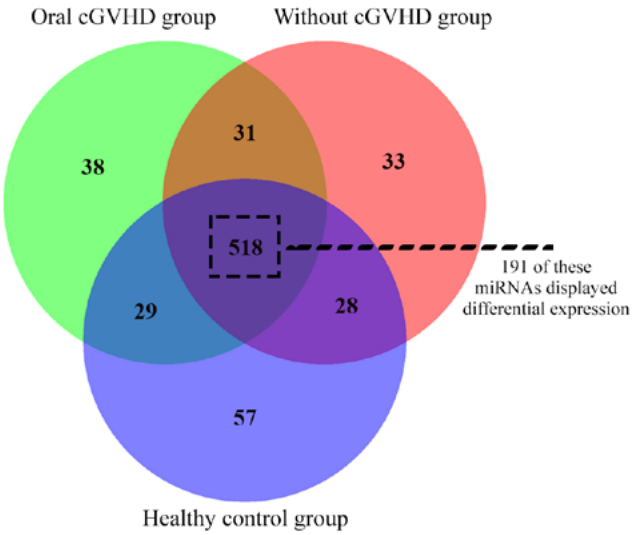

Fig. 1. Venn diagrams of miRNA profiles of the 3 groups. Among the 3 groups (oral chronic graft-versus-host disease (cGVHD) group, without cGVHD group and the healthy control group), a total of 518 miRNAs were expressed. After conducting an analysis of variance (ANOVA) analysis (details not presented), it was found that 191 of these miRNAs exhibited differential expression

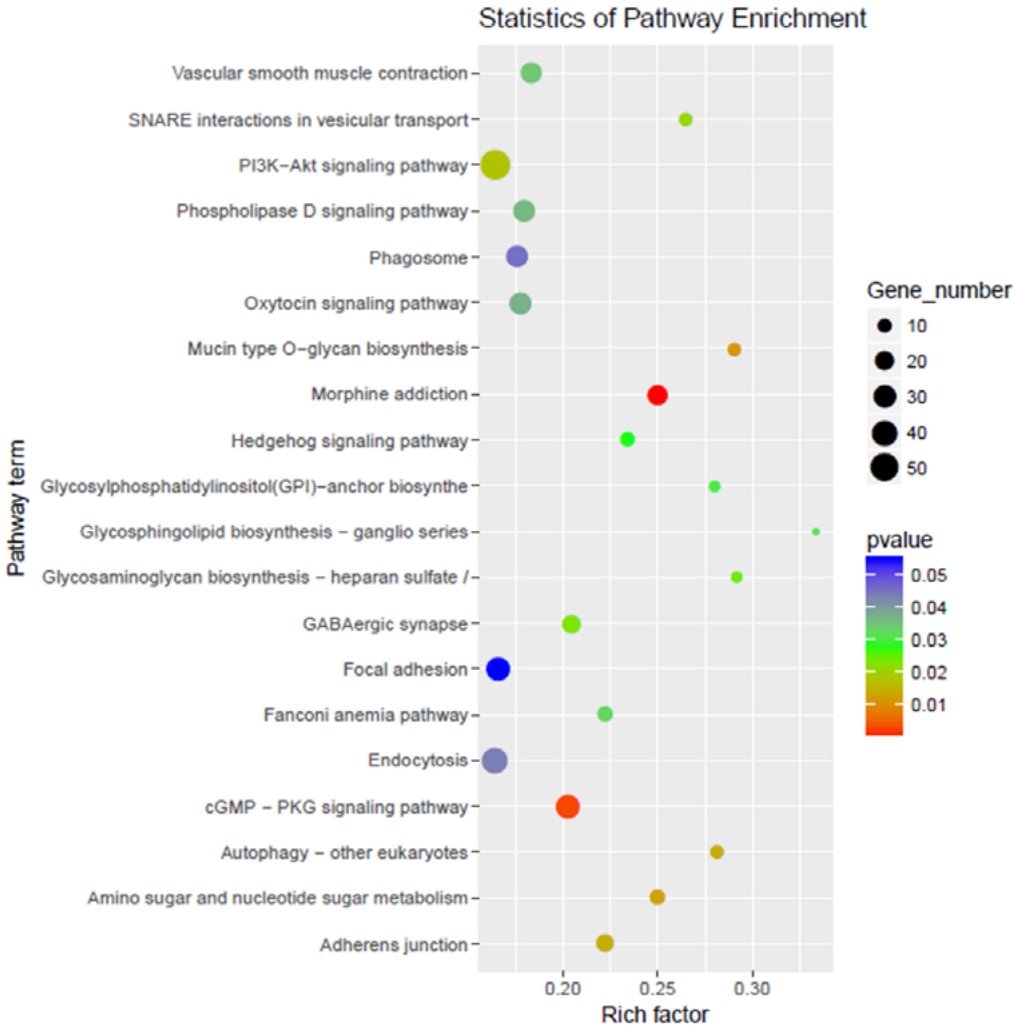

Fig. 2. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment of the target genes for miRNA-505-5p. Y-axis: pathway names, x-axis: rich factor. Bubble colors represent significance, with deeper red indicating smaller p-values and more prominent statistical significance. The size of the bubbles reflects the number of genes associated with the pathway

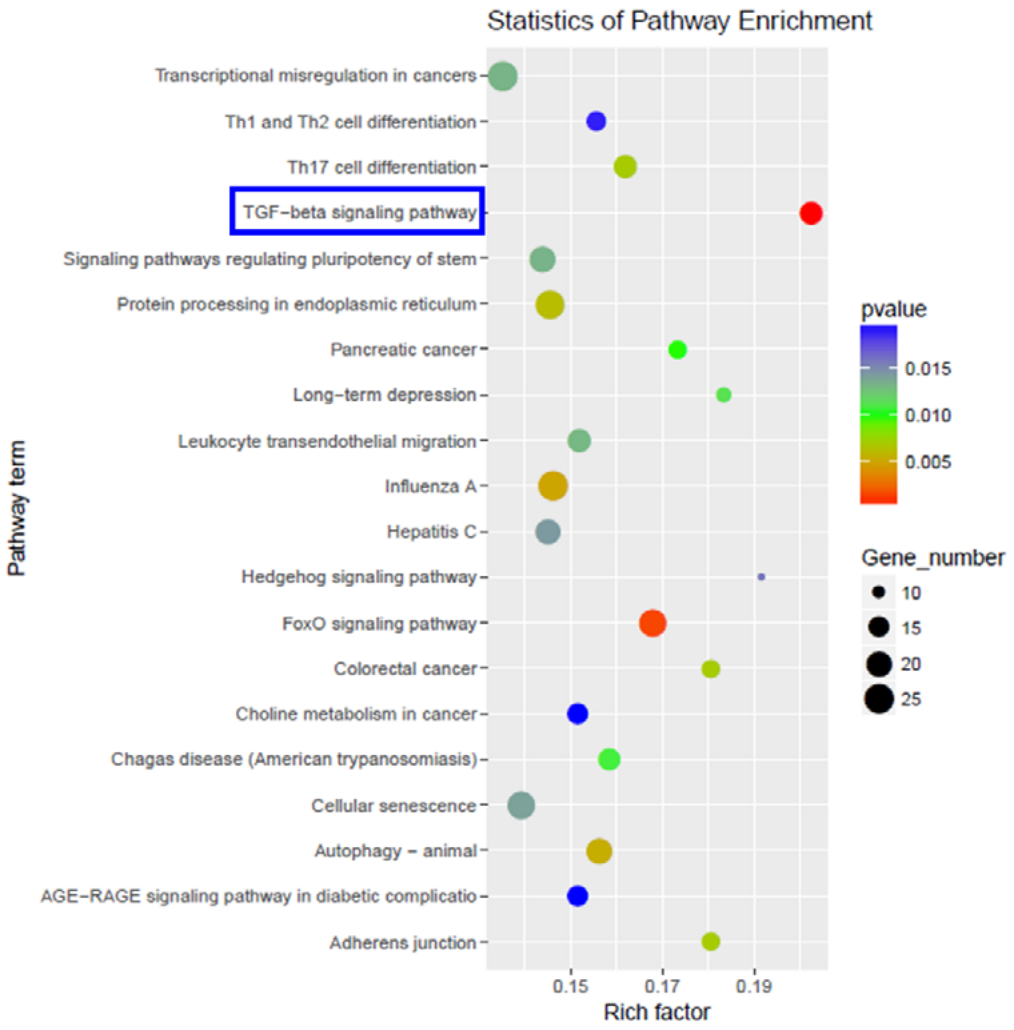

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Table 5. Differences in miRNA and Smad2 expression between groups

Statistical information		miR-769-5p*		Smad2 mRNA		Smad	
		oral cGVHD (n = 15)	without cGVHD (n = 15)	oral cGVHD (n = 15)	without cGVHD (n-15)	oral cGVHD (n = 15)	without cGVHD (n = 15)
M ±SD		−4.342 ±2.726	−0.447 ±2.004	1.455 ±0.432	1.019 ±0.361	77.238 ±13.318	62.799 ±15.238
Levene's test for variance homogeneity H_0: equal variances H_1: unequal variance		2.326		<0.001		0.002	
p-value for Levene's test		0.138		0.993		0.963	
t-test for difference between groups H_0: equal means for 2 groups H_1: unequal means for 2 groups		−4.459		3.000		2.763	
p-value for t-test		<0.001		0.006		0.010	
df		28		28		28	
95% CI	upper	−2.106		0.733		25.142	
	lower	−5.686		0.138		3.734	

cGVHD – chronic graft-versus-host disease; H_0 – null hypothesis; H_1 – alternative hypothesis; df – degrees of freedom; 95% CI – 95% confidence interval. M ±SD – mean ± standard deviation.

Among 30 patients after allo-HSCT, a negative correlation was observed between miRNA-769-5p and Smad2 mRNA ($r = -0.635$, 95% CI: −0.8136, −0.3460; $p < 0.001$, Fig. 7). Additionally, both Smad2 mRNA and protein levels were higher in the patients with oral cGVHD compared to those without cGVHD (Table 5, Fig. 8).

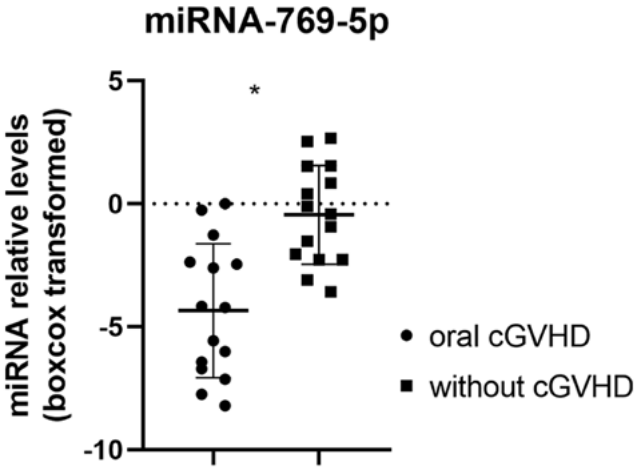

Fig. 4. Different expression of miRNA-769-5p between the oral chronic graft-versus-host disease (cGVHD) and without cGVHD groups. The expression data of miRNA-769-5p were transformed using the Box–Cox method. The differences were measured with t-test. The bars represent the mean and standard deviation (M ±SD). The expression of miRNA-769-5p was higher in patients without oral cGVHD compared to those with oral cGVHD

*oral cGVHD compared to without cGVHD, t = –4.459, degrees of freedom (df) = 28, 95% confidence interval (95% CI): –5.686, –2.106; p < 0.001, tested using t-test.

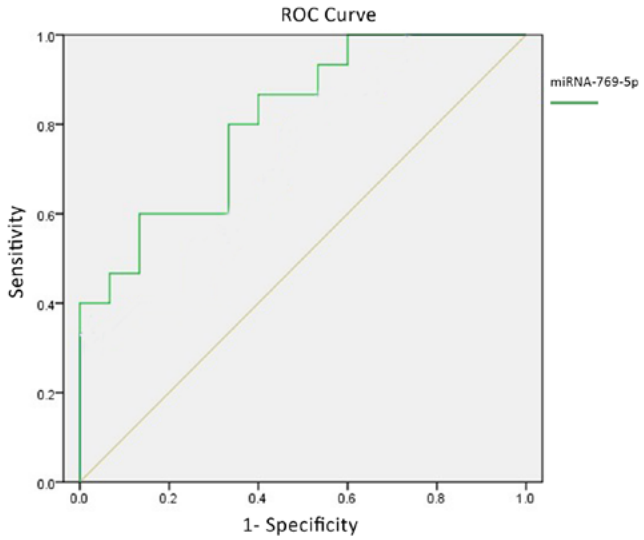

Fig. 5. Receiver operating characteristic (ROC) curve of circulating miRNA-769-5p in the peripheral blood. Among the 30 patients after allogeneic hematopoietic stem cell transplantation (allo-HSCT) (both the oral chronic graft-versus-host disease (cGVHD) and without cGVHD groups), ROC analysis indicated that miRNA-769-5p holds a certain diagnostic value for oral cGVHD, with an area under the curve (AUC) of 0.809, signifying a moderate diagnostic efficacy (95% confidence interval (95% CI): 0.657, 0.961; p = 0.004)

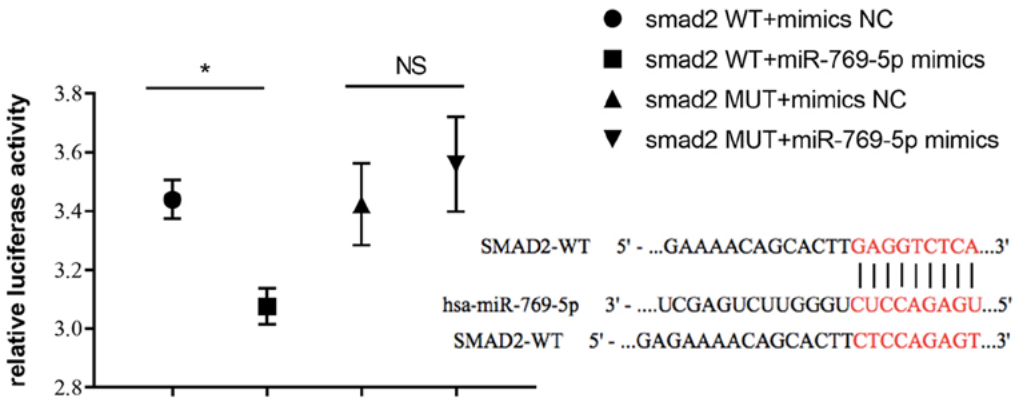

Fig. 6. Dual-luciferase detection of the interaction between hsa-miR-769-5p and *Smad2*. *Smad2* was validated as a potential target gene of miRNA-769-5p. The difference was measured with t-test. The bars represented mean and standard deviation (M ±SD)

*Smad2 wild-type (WT)+mimics negative control (NC) vs Smad2 WT+miR-769-5p mimics, t = 7.021, degrees of freedom (df) = 4, 95% confidence interval (95% CI): –0.507, –0.220, p = 0.002; NS Smad2 mutant-type (MUT)+mimics, NC+Smad2 MUT+miR-769-5p mimics, t = 1.114, df = 4, 95% CI: –0.204, 0.477; p = 0.328.

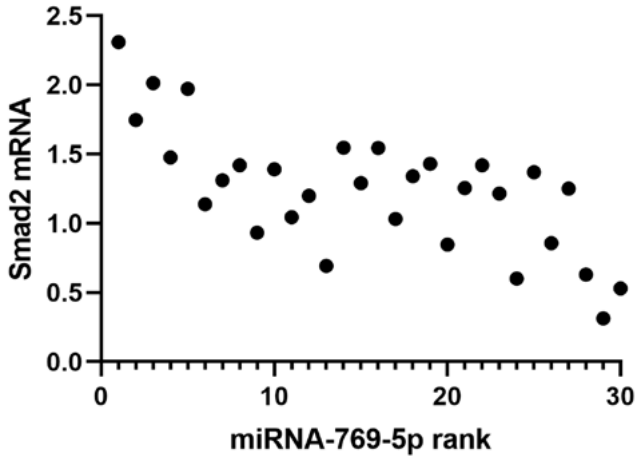

Fig. 7. The relationship between miRNA-769-5p and Smad2 mRNA in peripheral blood mononuclear cells (PBMCs). Among 30 patients after allogeneic hematopoietic stem cell transplantation (allo-HSCT), a negative correlation was observed between miRNA-769-5p and Smad2 mRNA (r = –0.635, 95% confidence interval (95% CI): –0.814, –0.346; p < 0.001)

Discussion

The study successfully established an expression profile of circulating miRNAs in oral cGVHD and made initial observations indicating a phenomenon of a low expression of miRNA-769-5p and high expression of Smad2 in oral cGVHD patients.

Through the sequencing of miRNAs in peripheral blood samples of patients, we identified a total of 518 miRNAs expressed across all 3 groups. Subsequent ANOVA analysis revealed differential expression in 191 of these miRNAs. Guided by the ranking of p-values, we selected the top 2 miRNAs for KEGG pathway analysis, which unveiled an enrichment of target genes for miRNA-769-5p within the TGF-β signaling pathway. Based on the sequencing

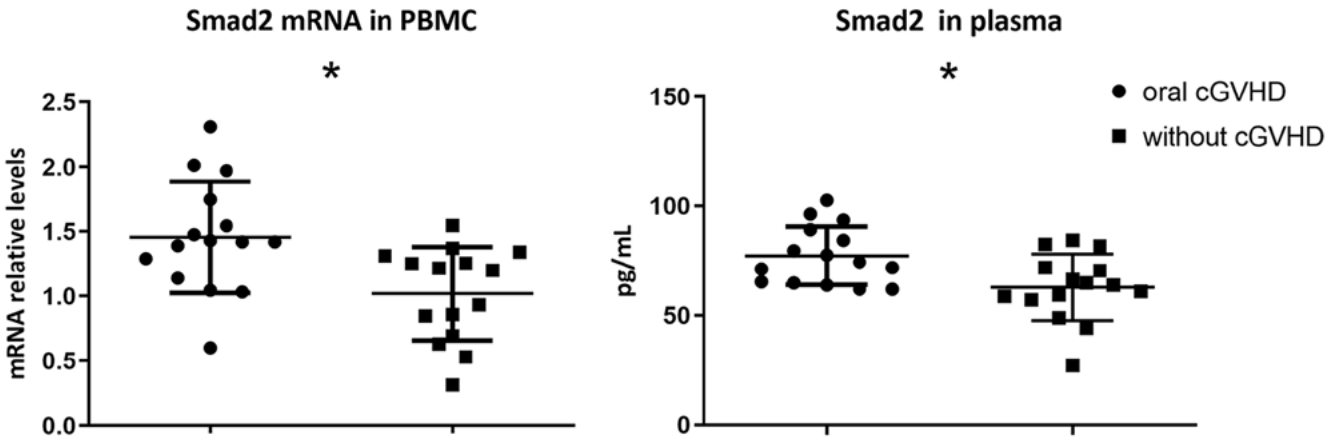

Fig. 8. Different expressions in Smad2 between the oral chronic graft-versus-host disease (cGVHD) and without cGVHD groups. The differences were measured with t-test. The bars represented mean and standard deviation (M ±SD). Both Smad2 mRNA and protein levels were higher in patients with oral cGVHD compared to those without cGVHD

*oral cGVHD compared to without cGVHD, $t_{smad2\ mRNA} = 3.000$, degrees of freedom (df) = 28, 95% confidence interval (95% CI): 0.138, 0.733; p = 0.006; $t_{smad2} = 2.763$, df = 28, 95% CI: 3.734, 25.142; p = 0.010, tested with t-test.

results, we expanded the sample size and conducted PCR validation, which revealed the lower expression of miRNA-769-5p in oral cGVHD. Importantly, it was ascertained that miRNA-769-5p holds diagnostic significance in the context of oral cGVHD.

Research on miRNA-769-5p has primarily centered on its involvement in cancer. Studies have revealed its function as an oncogene in gastric cancer,[25] hepatocellular carcinoma[26] and prostate cancer.[27] Conversely, in oral squamous cell carcinoma (OSCC), elevated miRNA-769-5p expression has been found to inhibit tumor cell proliferation, migration and invasion while promoting apoptosis.[28] Studies have shown that patients after allo-HSCT are 2–3 times more likely to develop solid tumors than healthy individuals.[29] About 1/3 of solid tumors are secondary tumors in the skin and mucosa of recipients of transplants, with OSCC accounting for 50% of these patients.[30,31] Consequently, the question arises as to whether the low expression of miRNA-769-5p in oral cGVHD patients is one of the factors predisposing them to the development of oral cancer, a matter that warrants further consideration.

Furthermore, the study unveiled that the overexpression of miR-769-5p has the potential to alleviate tissue fibrosis by modulating the TGF-β/Smad signaling pathway.[32] It is well-established that the TGF-β/Smad signaling pathway can stimulate tissue fibrosis.[33] Both mRNA and protein levels were higher in the oral cGVHD group than those in patients without cGVHD. The high expression of Smad2 hinted at the activation of TGF-β/Smads, which leads to oral cGVHD. In this study, after being predicted using TargetScan and miRWalk, we verified that Smad2 was the target gene of miRNA-769-5p using the dual-luciferase test. A negative relationship between miRNA-769-5p and Smad2 mRNA was also observed. Fibrosis is a feature of oral cGVHD, and the effective treatment of cGVHD often involves inhibiting the TGF-β/Smad signaling pathway.[34] Consequently, the high expression of miRNA-769-5p in patients without oral cGVHD may also act to suppress the expression of SMADs, subsequently inhibiting the activation of the TGF-β/Smad signaling pathway, ultimately leading to a reduction in fibrosis and symptom relief.

Additionally, it is noteworthy that the TGF-β/Smad signaling pathway is involved in Th17/Treg immunoreactions.[35] In patients suffering from skin cGVHD, the study identified a dysregulation in cytokine secretion, such as TGF-β, alongside an imbalance in Th17/Treg cell ratios.[36] In our earlier research, we also observed imbalances in cytokines related to Th17 and Treg cells in oral cGVHD.[17] Previous studies have demonstrated that downregulating Smad2 can mitigate the severity of cGVHD[20] by not only reducing fibrosis[37] but also by inhibiting the generation of Th17.[38] The investigations further emphasized that by inhibiting the TGF-β/Smad pathway, Th17-mediated immune responses could be effectively controlled, thus ameliorating murine cardiac transplant rejection.[39] Therefore, the high expression of miRNA-769-5p in patients without oral cGVHD may serve to modulate the immune response by suppressing the TGF-β/Smad pathway, ultimately reducing the manifestation of oral cGVHD symptoms.

Limitations

In this study, we have examined the distinctive expression patterns of miRNAs in patients affected by oral cGVHD. We have also undertaken an initial exploration of the potential relationship between miRNA-769-5p and Smad2 differential expression and oral cGVHD. Nevertheless, the precise mechanistic interactions of miRNAs influencing the development of diseases require further

in-depth exploration and validation through in vitro experiments. Additionally, during the sequencing phase, we observed lower expression levels of miRNA-769-5p in the peripheral blood of healthy volunteers compared to individuals with oral cGVHD and those without it. However, this study did not proceed to validate these findings using PCR. Subsequent research could involve further validation and exploration of the implications of elevated miRNA-769-5p expression in post-transplant patients.

Conclusions

Differential expression of miRNAs, particularly the downregulation of miRNA-769-5p, may influence the development of oral cGVHD by diminishing its inhibitory effect on the TGF-β/Smad signaling pathway through its interaction with Smad2. This interaction potentially plays a significant role in the pathogenesis of oral cGVHD and offers a promising avenue for further research and therapeutic exploration.

Supplementary data

The Supplementary materials are available at https://doi.org/10.5281/zenodo.10551750. The package includes the following file:

Supplementary Table 1. Normality test for the data distribution.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Consent for publication

Not applicable.

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References

1. Kröger N, Solano C, Wolschke C, et al. Antilymphocyte globulin for prevention of chronic graft-versus-host disease. *N Engl J Med*. 2016;374(1):43–53. doi:10.1056/NEJMoa1506002
2. Tollemar V, Garming Legert K, Sugars RV. Perspectives on oral chronic graft-versus-host disease from immunobiology to morbid diagnoses. *Front Immunol*. 2023;14:1151493. doi:10.3389/fimmu.2023.1151493
3. MacDonald KPA, Hill GR, Blazar BR. Chronic graft-versus-host disease: Biological insights from preclinical and clinical studies. *Blood*. 2017;129(1):13–21. doi:10.1182/blood-2016-06-686618
4. Fall-Dickson JM, Pavletic SZ, Mays JW, Schubert MM. Oral complications of chronic graft-versus-host disease. *J Natl Cancer Inst Monogr*. 2019;2019(53):lgz007. doi:10.1093/jncimonographs/lgz007
5. Bassim CW, Fassil H, Mays JW, et al. Oral disease profiles in chronic graft versus host disease. *J Dent Res*. 2015;94(4):547–554. doi:10.1177/0022034515570942
6. Haverman TM, Raber-Durlacher JE, Raghoebar II, et al. Oral chronic graft-versus-host disease. *J Am Dent Assoc*. 2020;151(11):846–856. doi:10.1016/j.adaj.2020.08.001
7. Jagasia MH, Greinix HT, Arora M, et al. National Institutes of Health Consensus Development Project on Criteria for Clinical Trials in Chronic Graft-versus-Host Disease: I. The 2014 Diagnosis and Staging Working Group Report. *Biol Blood Marrow Transplant*. 2015;21(3):389–401.e1. doi:10.1016/j.bbmt.2014.12.001
8. Xiao LT, Peng YT, Tao RC, et al. Oral mucosa health status among patients after allogeneic hematopoietic stem cell transplantation in Guangxi district [in Chinese]. *J Oral Sci Res*. 2011;27(10):881–885. https://www.cnki.net/KCMS/detail/detail.aspx?dbcode=CJFD&dbname=CJFD2011&filename=KQYZ201110015&uniplatform=OVERSEA&v=dTrPAV-h1ZE9xeOJrTgb-L7gcsaevGIb4eqTX0SyjoTQB43bRJHQ3csVBT0_2-jQ. Accessed July 12, 2023.
9. Bassim CW, Fassil H, Dobbin M, et al. Malnutrition in patients with chronic GVHD. *Bone Marrow Transplant*. 2014;49(10):1300–1306. doi:10.1038/bmt.2014.145
10. Wu T, Liu Z, Yong X, et al. P16-positive secondary tongue squamous cell carcinoma following allogeneic hematopoietic stem cell transplantation: A case report and literature review. *Oral Oncol*. 2021;121:105399. doi:10.1016/j.oraloncology.2021.105399
11. Liu Z, Peng Y, Yong X, et al. A case report of secondary oral squamous cell carcinoma after allogeneic hematopoietic stem cell transplantation. *Oral Oncol*. 2020;109:104629. doi:10.1016/j.oraloncology.2020.104629
12. Salvi V, Gianello V, Tiberio L, Sozzani S, Bosisio D. Cytokine targeting by miRNAs in autoimmune diseases. *Front Immunol*. 2019;10:15. doi:10.3389/fimmu.2019.00015
13. Reikvam H, Vo AK, Johansen S, et al. MicroRNA serum profiles and chronic graft-versus-host disease. *Blood Adv*. 2022;6(18):5295–5306. doi:10.1182/bloodadvances.2021005930
14. Łacina P, Crossland RE, Wielińska J, et al. Differential expression of miRNAs from extracellular vesicles in chronic graft-versus-host disease: A preliminary study. *Adv Clin Exp Med*. 2022;32(5):539–544. doi:10.17219/acem/155373
15. Vajari MK, Moradinasab S, Yousefi A, Bashash D. Noncoding RNAs in diagnosis and prognosis of graft-versus-host disease (GVHD). *J Cell Physiol*. 2022;237(9):3480–3495. doi:10.1002/jcp.30830
16. Lv Q, Tian Z, Dong E, Cao X. Tissue/organ-specialized immunologic features and disease mechanisms [in Chinese]. *Chin Sci Bull*. 2012;36:3450–3458. doi:10.1360/972012-1602
17. Yong X, Peng Y, Liu Z, et al. Analysis of serum and salivary cytokines among patients with oral cGVHD after Allo-HSCT. *Oral Dis*. 2021;27(5):1320–1324. doi:10.1111/odi.13658
18. Wang H, Li M, Fei L, et al. Bone marrow-derived mesenchymal stem cells transplantation attenuates renal fibrosis following acute kidney injury in rats by diminishing pericyte-myofibroblast transition and extracellular matrix augment. *Transplant Proc*. 2023;55(1):225–234. doi:10.1016/j.transproceed.2022.12.002
19. Zhang H, Liu J, Sun Y, et al. Nestin$^+$ mesenchymal stromal cells fibrotic transition mediated by CD169$^+$ macrophages in bone marrow chronic graft-versus-host disease. *J Immunol*. 2023;211(7):1154–1166. doi:10.4049/jimmunol.2200558
20. Marinelli Busilacchi E, Costantini A, Mancini G, et al. Nilotinib treatment of patients affected by chronic graft-versus-host disease reduces collagen production and skin fibrosis by downmodulating the TGF-β and p-SMAD pathway. *Biol Blood Marrow Transplant*. 2020;26(5):823–834. doi:10.1016/j.bbmt.2020.01.014
21. Li S, Fan Q, He S, Tang T, Liao Y, Xie J. MicroRNA-21 negatively regulates Treg cells through a TGF-β1/Smad-independent pathway in patients with coronary heart disease. *Cell Physiol Biochem*. 2015;37(3):866–878. doi:10.1159/000430214

22. Liang J, Zhu W, Zhang Z, et al. MicroRNA-199a-3p enhances expressions of fibrosis-associated genes through targeting Smad1 in mouse cardiac fibroblasts [in Chinese]. *J Southern Med Univ*. 2018;38(10): 1203–1208. https://kns.cnki.net/kcms/detail/44.1627.R.20181026.2108.040.html. Accessed July 12, 2023.
23. Agarwal V, Bell GW, Nam JW, Bartel DP. Predicting effective microRNA target sites in mammalian mRNAs. *eLife*. 2015;4:e05005. doi:10.7554/eLife.05005
24. Sticht C, De La Torre C, Parveen A, Gretz N. miRWalk: An online resource for prediction of microRNA binding sites. *PLoS One*. 2018;13(10): e0206239. doi:10.1371/journal.pone.0206239
25. Luan PB, Jia XZ, Yao J. MiR-769-5p functions as an oncogene by down-regulating RYBP expression in gastric cancer. *Eur Rev Med Pharmacol Sci*. 2020;24(12):6699–6706. doi:10.26355/eurrev_202006_21657
26. Xian Y, Wang L, Yao B, et al. MicroRNA-769-5p contributes to the proliferation, migration and invasion of hepatocellular carcinoma cells by attenuating RYBP. *Biomed Pharmacother*. 2019;118:109343. doi:10.1016/j.biopha.2019.109343
27. Lee D. miR-769-5p is associated with prostate cancer recurrence and modulates proliferation and apoptosis of cancer cells. *Exp Ther Med*. 2021;21(4):335. doi:10.3892/etm.2021.9766
28. Zhou Y, Xu XM, Feng Y. MiR-769-5p inhibits cancer progression in oral squamous cell carcinoma by directly targeting JAK1/STAT3 pathway. *Neoplasma*. 2020;67(3):528–536. doi:10.4149/neo_2020_190703N582
29. Shimada K, Yokozawa T, Atsuta Y, et al. Solid tumors after hematopoietic stem cell transplantation in Japan: Incidence, risk factors and prognosis. *Bone Marrow Transplant*. 2005;36(2):115–121. doi:10.1038/sj.bmt.1705020
30. Mawardi H, Elad S, Correa ME, et al. Oral epithelial dysplasia and squamous cell carcinoma following allogeneic hematopoietic stem cell transplantation: Clinical presentation and treatment outcomes. *Bone Marrow Transplant*. 2011;46(6):884–891. doi:10.1038/bmt.2011.77
31. Santarone S, Natale A, Angelini S, et al. Secondary oral cancer following hematopoietic cell transplantation. *Bone Marrow Transplant*. 2021;56(5):1038–1046. doi:10.1038/s41409-020-01147-z
32. Bontempi G, Terri M, Garbo S, et al. Restoration of WT1/miR-769-5p axis by HDAC1 inhibition promotes MMT reversal in mesenchymal-like mesothelial cells. *Cell Death Dis*. 2022;13(11):965. doi:10.1038/s41419-022-05398-0
33. Chen C, Chen J, Wang Y, et al. Ganoderma lucidum polysaccharide inhibits HSC activation and liver fibrosis via targeting inflammation, apoptosis, cell cycle, and ECM-receptor interaction mediated by TGF-β/Smad signaling. *Phytomedicine*. 2023;110:154626. doi:10.1016/j.phymed.2022.154626
34. Mays J, Fassil H, Edwards D, Pavletic S, Bassim C. Oral chronic graft-versus-host disease: Current pathogenesis, therapy, and research. *Oral Dis*. 2013;19(4):327–346. doi:10.1111/odi.12028
35. Wang F, Yang Y, Li Z, et al. Mannan-binding lectin regulates the Th17/Treg axis through JAK/STAT and TGF-β/SMAD signaling against *Candida albicans* infection . *J Inflamm Res*. 2022;15:1797–1810. doi:10.2147/JIR.S344489
36. Yang H. Clinical observation and pathogenetic study on the sclerodermatous chronic graft-versus-host disease after hematopoietic stem cell transplantation [in Chinese] [doctoral thesis]. Guangzhou, China: Southern Medical University; 2016. https://www.cnki.net/KCMS/detail/detail.aspx?dbcode=CMFD&dbname=CMFD201701&filename=1016269721.nh&uniplatform=OVERSEA&v=89vORq70QkQPcXUbbRKeCe9ngGsu99_fKuM-4NUeQ3--TCIaBX21cNuVLWHQEszv. Accessed July 12, 2023.
37. Chen H, Moreno-Moral A, Pesce F, et al. WWP2 regulates pathological cardiac fibrosis by modulating SMAD2 signaling. *Nat Commun*. 2019;10(1):3616. doi:10.1038/s41467-019-11551-9
38. Martinez GJ, Zhang Z, Reynolds JM, et al. Smad2 positively regulates the generation of Th17 Cells. *J Biol Chem*. 2010;285(38):29039–29043. doi:10.1074/jbc.C110.155820
39. Wang YY. Deletion of Smad3 improves cardiac allograft rejection in mice [in Chinese] [doctoral thesis]. Hangzhou, China: Zhejiang University; 2016. https://www.cnki.net/KCMS/detail/detail.aspx?dbcode=CDFD&dbname=CDFDLAST2017&filename=1016282995.nh&uniplatform=OVERSEA&v=hth6zz6Eq-DdKsHO0-j8PxdBDGnbyCr531HZQMkrQMHkAFR6V1yEve9t5b_S_sHQ. Accessed July 12, 2023.