Evaluation of immune microenvironment in IgG4-related sialadenitis

Qing Hu, Peng Zhou, Yixuan Yan, Qun Huang[*]

Department of Stomatology, Guangdong Women and Children Hospital, Guangzhou, Guangdong, 510010, China

ARTICLE INFO	ABSTRACT
	IgG4-related sialadenitis is a systemic autoimmune disease that can lead to fibro-inflammatory conditions. This study aims to investigate the immune microenvironment and potential signaling pathways associated with IgG4-related sialadenitis. Datasets related to IgG4-related sialadenitis were retrieved from the GEO database. Immune cell infiltration analysis was conducted using the Cell-type Identification by Estimating Relative Subsets of RNA Transcripts (CIBERSORT) method. Differentially immune-related expressed genes (DIEG) and immune-related functional enrichment were identified. Moreover, potential treatment targets for IgG4-related sialadenitis were predicted using The Connectivity Map. Only two datasets from GEO were included for further analysis. The CIBERSORT results indicated dominant immune cell populations in IgG4-related sialadenitis, including CD8+ T cells, resting NK cells, monocytes, and naïve B cells in peripheral blood mononuclear cells. Additionally, high abundance of plasma cells was observed in labial salivary gland tissues. Furthermore, a total of 42 DIEGs were identified, with tumor necrosis factor (TNF) signaling via the NF-Kappa B signaling pathway and the p53 signaling pathway being highly enriched. Autophagy inhibitors and DNA topoisomerase inhibitors were strongly associated with potential targets for the treatment of IgG4-related sialadenitis (P<0.05). This study reveals altered immune microenvironment in peripheral blood mononuclear cells and labial salivary gland tissues in IgG4-related sialadenitis. Autophagy inhibitors and DNA topoisomerase inhibitors show promise as potential targets for treating IgG4-related sialadenitis, providing a novel therapeutic strategy for this disease.

Introduction

IgG4-related disease (IgG4-RD), a systemic autoimmune disease, can cause fibro-inflammatory in nearly any organ system and lead to organ dysfunction and irreversible damage. IgG4-RD was characterized by high concentration of serum IgG4, abundant infiltrated with IgG4-bearing plasma cells and fibrosis in affected organs. The incidence and prevalence of IgG4-RD are poorly described on account of the substantial challenges in its recognition and diagnosis[1]. In the "head and neck-limited" clinical phenotypes of the IgG4-RD subgroup[2], the submandibular, parotid and sublingual glands are frequently involved. Kuttner's tumor, a chronic submandibular gland enlargement with sclerosing sialadenitis, is a typical IgG4-RD manifestation[3].

Despite temporarily spontaneous remissions without treatment reported in IgG4-related sialadenitis[4], this relapsing-remitting pattern leads to progressive organ injury[5]. The treatment for IgG4-related sialadenitis tends to be selected empirically on account of the unclear pathogenesis. At present, glucocorticoids remain the first-line drug for IgG4-related sialadenitis. In addition, immunosuppressive drugs (mainly B-cell depletion therapy-rituximab) were introduced into the refractory and complicated IgG4-RD. Although IgG4-RD patient demonstrates a good response to glucocorticoids and immunosuppressive drugs, the high frequency of relapses existed and limited its radical treatment [1]. Interestingly, the high efficacy of gluco-corticoids and immunosuppressive drugs was noteless in recent double-blind and placebo-control clinical trials (6). Therefore, this study aims to investigate the immunophenotype involved in IgG4-related sialadenitis and explore potential targets for its treatment through bioinformatics analysis.

Materials and Methods

Microarray data collection of IgG4-related sialadenitis

IgG4-related sialadenitis-related transcriptome datasets were retrieved from the GEO database (http://www.ncbi.nih.gov/geo). GSE66465 and GSE40568 were screened and downloaded. Gene expression in peripheral blood mononuclear cells (PBMCs) of IgG4-related sialadenitis (n=2) and health control (n=3) were extracted from GSE66465. Moreover, gene expression in labial salivary glands (LSG) tissue of IgG4-related sialadenitis (n=5) and health control (n=5) were extracted from GSE40568. The extracted data were normalized and processed by log2 transformation. Probes in with more than one gene were eliminated and the average value was calculated for genes corresponding to more than one probe.

Immune cell infiltration analysis of IgG4-related sialadenitis with CIBERSORT

To investigate the immune signature of IgG4-related sialadenitis, immune cell infiltration analysis was conducted based on Cell-type identification by estimating rela-

* Corresponding author. Email: 2022760254@gzhmu.edu.cn

tive subsets of RNA transcripts (CIBERSORT) (http://cibersort.stanford.edu/) (7).CIBERSORT, based on linear support vector regression (SVR), predicts the fraction of 22 leukocyte subsets in gene expression profiles (GEP) of diseases(8). The 22 leukocyte subsets include seven types of T cells, naïve B cells, memory B cells, plasma cells, monocytes, three types of macrophages, resting NK cells, activated NK cells, resting dendritic cells, activated dendritic cells (DC), resting mast cells, activated mast cells, eosinophils and neutrophils. After normalization and filtration, the IgG4-related sialadenitis-related GEP was analyzed for 100 permutations using a deconvolution algorithm.

Differentially immune-related gene expression in IgG4-related sialadenitis

A total of 1233 immune-related genes list downloaded from the Immunology Database and Analysis Portal (Immport) (https://www.immport.org) (9). GEP of IgG4-related sialadenitis was compared to identify the DEGs by R package DESeq2.(10) Interaction between differentially expressed genes (DEGs) in IgG4-related sialadenitis and immune-related genes was determined. The DEGs and DIEG were filtered when | log2(fold change) | > 1 and false discovery rate (FDR) < 0.05 were considered as the significant cutoff value.

Immune-related functional annotation by GSEA in IgG4-related sialadenitis

To investigate the immune-related functional enrichment in LSGs and PBMCs in IgG4-related sialadenitis, gene set enrichment analysis (GSEA) was performed with the R package (Clusterprofiler) (11) and fgsea. The immunologic signature gene sets were downloaded from mSigDB database which depicts cell states and perturbations within the immune system (http://www.gsea-msigdb.org/gsea/msigdb/collections.jsp#C7). Significant pathway enrichment was identified by the normalized enrichment score (|NES| >1), P value <0.05, and FDR q value <0.05.

CMAP database

To further explore the potential target for the treatment of IgG4-related sialadenitis, DEGs in LSGs of IgG4-related sialadenitis patients were imported into The Connectivity Map (CMAP, https://portals.broadinstitute.org/cmap/) database. CMAP connected drugs, genes and diseases through a collection of gene expression signatures obtained from cultured human cells treated with bioactive small molecules (12, 13). The database contains over 7000 genomic profiles from various cancer cell lines treated with 1309 small bioactive molecules. Small molecule compounds that showed a negative enrichment score were considered as potential target drugs for IgG4-related sialadenitis

Statistical Analysis

Comparisons between IgG4-related sialadenitis and the control group were performed based on a two-sided Wilcoxon test. Pearson correlation coefficient was used to measure the linear correlation among immune cell types fraction. Statistical analyses were conducted with the R software. Results with a P < 0.05 were considered statistically significant.

Results

Composition of infiltrated immune cell subsets in IgG4-related sialadenitis.

To assess the composition of infiltrated immune cell subsets in labial salivary gland (LSG) tissues and peripheral blood mononuclear cells (PBMCs) in IgG4-related sialadenitis, CIBERSORT analysis was conducted. After stringent filtration, only three samples of IgG4-related sialadenitis and three control samples were included for CIBERSORT analysis using the GSE40568 dataset. The CIBERSORT analysis revealed that CD8+ T cells, resting NK cells, monocytes, and naïve B cells were the dominant immune cell populations in PBMCs tissues in IgG4-related sialadenitis. Furthermore, a high abundance of plasma cells was observed in LSG tissues in IgG4-related sialadenitis. Additionally, memory B cells and M2 macrophages were found to be infiltrated in LSGs. However, no statistically significant difference was detected between IgG4-related sialadenitis and the control group upon comparison. (Figure 1).

Identification of differentially immune-related expressed genes (DIEG) in IgG4-related sialadenitis

Only 12 DEGs (MS4A3, CLC, SLC25A37, H2BC3, CPA3, H2AC4, LRRN3, H3C12, S100B, DSC1, SNORA37 and H2BC17) were detected in PBMCs in IgG4-related sialadenitis and S100B was the only differentially immune-related expressed gene. In addition, 423 DEGs (116 down-regulated DEGs and 307 up-regulated DEGs) and 42 DEIGs were screened in LSGs tissues in IgG4-related sialadenitis.
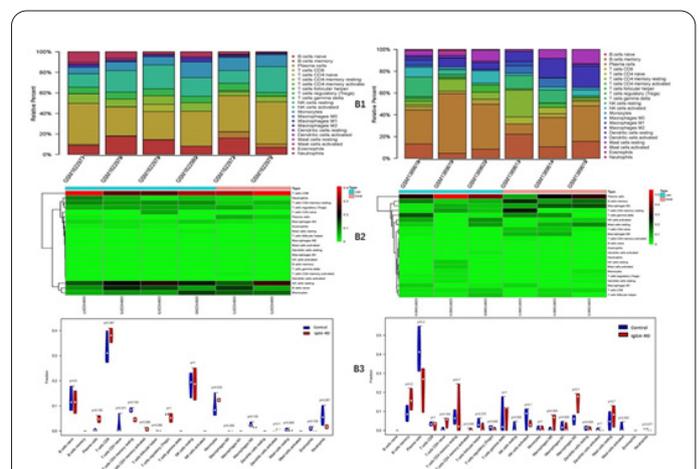

Figure 1. Composition of infiltrated immune cells in IgG4-related sialadenitis. (A1)Bar plot of the abundance of 22 immune cell infiltration in labial salivary glands (LSGs) tissues in IgG4-related sialadenitis determined by CIBERSORT; (B1) Bar plot of the abundance of 22 immune cell infiltration in peripheral blood mononuclear cells (PBMCs) in IgG4-related sialadenitis determined by CIBERSORT; (B1) Heatmap of comparison of infiltrated immune cells subpopulations in LSG tissue in IgG4-related sialadenitis; (B2) Heatmap of comparison of infiltrated immune cells subpopulations in PBMCs of IgG4-related sialadenitis; (A3) Violin plot of the comparison of the composition of 22 immune cells infiltration in LSG tissue between IgG4-related sialadenitis and health control. (B3) Violin plot of the comparison of the composition of 22 immune cells infiltration in PBMCs between IgG4-related sialadenitis and health control.
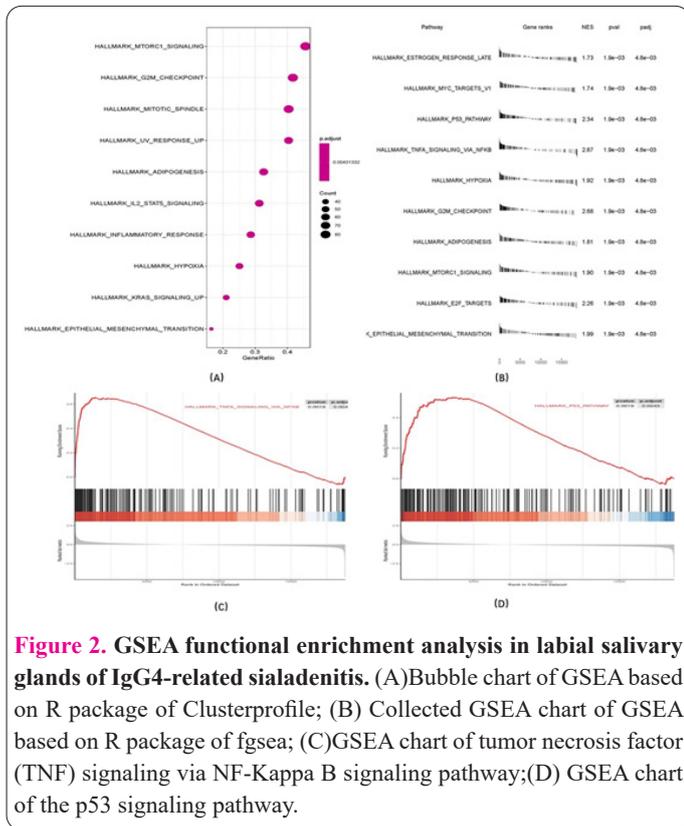
Figure 2. GSEA functional enrichment analysis in labial salivary glands of IgG4-related sialadenitis. (A)Bubble chart of GSEA based on R package of Clusterprofile; (B) Collected GSEA chart of GSEA based on R package of fgsea; (C)GSEA chart of tumor necrosis factor (TNF) signaling via NF-Kappa B signaling pathway;(D) GSEA chart of the p53 signaling pathway.

Immune-related functional enrichment based on GSEA

To further explore the function and pathway enrichment of immune-related gene expression, GSEA was performed. GSEA analysis indicated that tumor necrosis factor (TNF) signaling via NF-Kappa B signaling pathway, p53 signaling pathway, epithelial-mesenchymal transition (EMT) signaling pathway, E2F target signaling pathway, G2M checkpoint signaling pathway; apoptosis signaling pathway and oxidative phosphorylation signaling pathway. In particular, both tumor necrosis factor (TNF) signaling via the NF-Kappa B signaling pathway and p53 signaling pathway were highly enriched and associated with alteration of the immune microenvironment in LSGs and PMBC of IgG4-related sialadenitis($p<0.05$)(Figures 2 and 3).

CMAP analysis

The top 15 related small molecules for the potential treatment for IgG4-related sialadenitis were listed in Table 1 ($P<0.05$). Interestingly, the autophagy inhibitors (especially for HSP90 inhibitor) and DNA topoisomerase inhibitors were the most correlated to the target for IgG4-related sialadenitis ($P<0.05$).

Discussion

IgG4-RD is an autoimmune disease characterized by substantial infiltration of plasma cells in multiple organs. The pathogenesis of IgG4-RD involves both innate and adaptive immunity. Recent studies have highlighted that
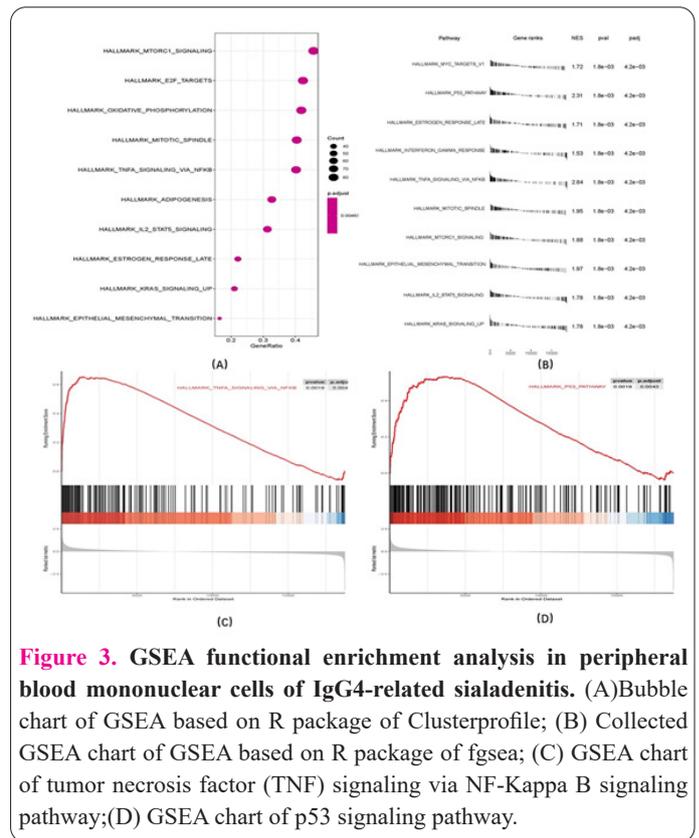

Figure 3. GSEA functional enrichment analysis in peripheral blood mononuclear cells of IgG4-related sialadenitis. (A)Bubble chart of GSEA based on R package of Clusterprofile; (B) Collected GSEA chart of GSEA based on R package of fgsea; (C) GSEA chart of tumor necrosis factor (TNF) signaling via NF-Kappa B signaling pathway;(D) GSEA chart of p53 signaling pathway.

Table 1. CMAP and Mechanism of action analysis of IgG4-related sialadenitis.

Cmap name	mean	enrichment	p	Mechanism of actions
irinotecan	-0.916	-0.997	0	DNA topoisomerase inhibitors
LY-294002	0.279	0.347	0	PI3K inhibitors
tanespimycin	0.346	0.374	0	HSP90 inhibitor; autophagy inhibitors
sirolimus	0.271	0.404	0	mTOP inhibitors
alvespimycin	0.528	0.718	0	HSP90 inhibitors
diloxanide	-0.587	-0.934	0.00002	Anti-protozoal agents
camptothecin	-0.729	-0.973	0.00006	DNA topoisomerase inhibitors
amantadine	-0.702	-0.911	0.0001	Antiviral; antiparkinsonian agent; adamantane derivative.
6-bromoindirubin-3'-oxime	-0.406	-0.75	0.00012	GSK 3 inhibitors
doxylamine	0.622	0.846	0.00018	H1 receptor antagonists
galantamine	0.581	0.866	0.00044	Acetylcholinesterase inhibitors
penbutolol	-0.598	-0.92	0.00092	Beta-blocker
benzocaine	-0.547	13.148	0.00092	Anesthetic agent
meteneprost	-0.626	-0.849	0.00095	PGE2 analogue
monorden	0.358	0.396	0.00114	HSP90 inhibitors

lymphocytes (14), including plasmablast, T follicular helper (Tfh) cells, T type 2 helper (Th2) cells, T regulatory (Treg) cells, and cytotoxic T lymphocytes (CTL)(15), NK cells and macrophages participated in the local inflammatory infiltration in IgG-RD. An increased number of circulating plasmablasts, largely IgG4+, were detected in patients with active and relapsing IgG4-RD(16, 17). In addition, a declined abundance of plasmablasts was observed in IgG-RD patients with remission after B cell depletion therapy with rituximab (RTX)(17). Plasmablast function has an important role in the biomarker for diagnosis and response evaluation.

Both effector CD8+ cytotoxic T lymphocytes (CTLs) and CD4+ CTLs induced apoptotic cell death in blood and lesions of IgG4-RD(18). Recent research has discovered a positive correlation between the abundance of CD8+ CTLs and serum IgG4 levels in IgG4-RD patients(18). Clonal expansion of CD4+ effector/memory T cells with CTLs cells has been detected in IgG4-RD patients, while the number of Th1 and Th2 cells is low (18-21). Furthermore, the abundance of CD4+ CTL cells decreases in IgG4-RD patients in clinical remission following B cell depletion therapy. These findings suggest a close interplay between activated B cells and CD4+ CTL cells. CD4+ CTLs are capable of secreting pro-inflammatory and pro-fibrotic cytokines (such as IL-1beta, TGF-beta1, and IFN-gamma), as well as cytolytic molecules such as perforin and granzymes A and B, leading to inflammation and fibrosis in affected tissues. In addition, extensively infiltrated CD4+ CTLs in skin lesions contribute to endothelial cell apoptosis in systemic sclerosis(22). However, our study failed to observe statistically significant differences in immune cell populations between IgG4-RD and healthy controls, likely due to the limited sample size.

Several immune checkpoint molecules have been identified as an immune regulator in immune-mediated processes in autoimmune diseases(23). Recent evidence suggests a close interaction between IgG4-RD and immune checkpoint molecules. Not only high serum levels of programmed cell death (PD)-1, PD-ligand 1(PD-L1) and PD-L2 but also increased expression of of PD-1 and PD-L2 on Treg cells were detected in patients with IgG4-RD(24). Interestingly, a new type of immune-related adverse event, IgG4-related pleural disease, has emerged after treatment with PD-L1 inhibitors for non-small cell lung cancer(25). In addition, compared with rheumatoid arthritis (RA), compared to rheumatoid arthritis (RA), IgG4-RD patients with visceral involvement show higher serum levels of immune checkpoint molecules including T cell immunoglobulin and mucin-containing molecule-3 (TIM-3) and its ligand, galectin-9 (Gal-9)(23). In IgG4-related disease, the PD-1/PD-L1 pathway can enhance the differentiation of Treg cells (24).

The results of functional enrichment analysis and cMAP analysis suggest that autophagy, particularly the mammalian target of the rapamycin (mTOR) signaling pathway, is closely linked to the occurrence of IgG4-related disease (IgG4-RD). Excessive autophagy has been shown to contribute to acinar cell injury and salivary gland disorders(26, 27). Similarly, the mTOR signaling pathway was involved in salivary gland injury by phosphorylation of 4E-BP1(27). In addition, recent research indicated that a high level of TNF-α IgG4-related sialadenitis reduced autophagic flux through phosphorylation of extracellular signal-regulated kinase (ERK) 1/2(28).

Epithelial-mesenchymal transition (EMT), a cellular process involved in embryonic development, tissue repair and tumor migration, is characterized by the alteration of morphology and behavior in epithelial cells with changes in specific proteins (E-cadherin, zonula occludens-1, α-smooth muscle actin, vimentin and laminin)(29). Accumulated evidences have confirmed that EMT plays an important role in inflammation with fibrosis in autoimmune diseases. Sjögren's Syndrome, in particular, features lymphocytic infiltration of the salivary and lachrymal glands with advanced fibrosis, which is ascribed to tissue damage and recurrent episodes of inflammation. Recent studies have found EMT program was involved in the fibrosis of Sjögren's Syndrome. Salivary gland epithelial cells transition to the mesenchymal cell with myofibroblast type was activated in inflamed salivary glands through TGF-β1/SMAD/Snail signalling pathway(29). Interestingly, increased expression of α-smooth muscle actin, snail, and heat-shock protein 47 level and decreased E-cadherin expression, were observed in the lacrimal glands in IgG4-related Mikulicz's disease, which suggests an EMT process(30). Additionally, several studies have demonstrated that heat shock protein 90 (HSP90) can initiate immune regulation in vitro and in vivo (31). Autoantibodies against HSP90 are exclusive of the IgG class, and approximately 25%-50% of systemic lupus erythematosus (SLE) patients exhibit elevated levels of heat shock protein 90 in PBMC (32-34). HSP90 inhibition can suppress inflammation through binding to HSP90-dependent substrate proteins (e.g., nuclear factor-kappa B (NF-κB))(31, 35). HSP90 inhibitors have become a focus of research in autoimmune diseases. Tumor Necrosis Factor (TNF) plays a crucial role in inflammation irritation and immune regulation. Dysfunction of TNF receptor 1 (TNFR1)-induced NF-κB signaling leads to chronic inflammation and is associated with autoimmune diseases(36-38). The NF-κB signaling pathway in response to TNF-α is involved in the regulation of CD4+ T cell differentiation toward Th2 cell immune responses (39).

Altered immune microenvironments have been observed in PBMC and LSG in IgG4-related sialadenitis. Autophagy inhibitors and DNA topoisomerase inhibitors may represent potential targets for IgG-RD and offer new perspectives for diagnosis and treatment.

Author Contributions
The authors confirm their contribution to the paper as follows: study conception and design: Hu Q and Huang Q; data collection: Zhou P and Hu Q; analysis and interpretation of results: Yan YX; draft manuscript preparation: Hu Q and Zhou P. All authors reviewed the results and approved the final version of the manuscript.

Availability of data and materials
The datasets involved can be accessed from the GEO database(http://www.ncbi.nih.gov/geo)

Ethics approval

This study does not involve ethical issues.

Conflict of interest

The authors declare that they have no conflicts of interest to report regarding the present study.

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