Bioinformatics analysis identified immune infiltration, risk and drug prediction models of copper-induced death genes involved in salivary glands damage of primary Sjögren’s syndrome

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Abstract
This study aimed to identify copper-induced death genes in primary Sjögren’s syndrome (pSS) and explore immune infiltration, risk and drug prediction models for salivary glands (SGs) damage. The 3 datasets, including GSE40611, GSE23117, and GSE7451 from the Gene Expression Omnibus database were downloaded. The datasets were processed using the affy in R (version 4.0.3). In immune cells, copper-induced death genes were strongly expressed in “activated” dendritic cells (aDcs), macrophages and regulatory T cells (Treg). In immune functions, copper-induced death genes were strongly expressed in major histocompatibility complex (MHC) class I, human leukocyte antigen (HLA) and type I interferon (IFN) response. Correlation analysis showed that 5 genes including SLC31A1, PDHA1, DLD, ATP7B, and ATP7A were significantly correlated with immune infiltration. The nomogram suggested that the low expression of PDHA1 was significant for predicting the risk of pSS and the area under curve was 0.678. Drug model suggested that “Bathocuproine disulfonate CTD 00013590,” “Vitinoin CTD 00007069,” and “Resveratrol CTD 00002483” were the drugs most strongly associated with copper-induced death genes. In summary, copper-induced death genes are associated with SGs injury in pSS, which is worthy of clinicians’ attention.

Abbreviations: aDcs = “activated” dendritic cells, ATP7A = ATPase copper transporting α, ATP7B = ATPase copper transporting β, ATRA = all-trans retinoic acid, CC chemokine receptors, DLD = dihydrolipoamide dehydrogenase, HLA = human leukocyte antigen, IFN = type I interferon, MHC = major histocompatibility complex, PDHA1 = pyruvate dehydrogenase E1 subunit α1, pSS = primary Sjögren’s syndrome, Res = Resveratrol, ROC = receiver operating curve, ROR-γt = receptor-related orphan nuclear receptor γ, SGs = salivary glands, SLC31A1 = solute carrier family 31 member 1, TIL = tumor infiltrating lymphocytes, Treg = regulatory T cells.

Keywords: copper-induced death genes, immune infiltration, prediction models, primary Sjogren’s syndrome, salivary glands damage

1. Introduction
Xerostomia is one of the common clinical manifestations of Sjögren’s syndrome. It is mainly caused by impaired salivary glands (SGs) secretion, resulting in rampant caries, parotid gland enlargement and angular cheilitis. Copper, as one of the essential trace elements, plays an important role in iron transport, expression of vascular endothelial growth factor and angiogenesis. Excessive doses of copper can cause overexpression of genes associated with cell death and damage immune system through the EndoG-Bax-ubiquitin pathway. At present, the pathogenesis of copper-induced death genes in SGs of primary Sjögren’s syndrome (pSS) is not clear. Immune factors and genetic background are considered to be the basis of the occurrence of pSS.

By studying genes and immune cells associated with SGs in pSS, central genes such as CD38, CMPK2, TBC1D9, and PYCR1 were associated with the immune infiltration in SGs, mitochondrial metabolic pathway in gluconeogenesis and tricarboxylic acid cycle. Copper oxide quantum dots significantly reduced the viability of C2C12 cells in a concentration-dependent manner (10–20 μg/mL) and inhibited mitochondrial caspase 3 and 7 by binding to DNA. Further studies showed that copper-dependent apoptosis depended
not only on concentration, but also on type of cell and time of exposure.\textsuperscript{[12]} Studies on apoptosis and pSS suggested that anti-cholinergic autoantibodies mediated apoptosis of the A253 cell line in a dependent manner of inositol phosphate, caspase-3 and matrix metalloproteinase-3.\textsuperscript{[13]} These findings suggested that the expression of copper-induced death genes were related to the disorder of immune microenvironment in SGs in pSS.

As far as we know, studies on copper-induced death genes with immune infiltration in pSS are relatively rare, especially using copper-induced death genes to build risk prediction model and drug model. Therefore, we studied from the perspective of copper-induced death genes in pSS, and may help clinicians to identify potential biomarkers for predicting and diagnosing pSS.

2. Materials and methods

2.1. Selection and expression matrix extraction of copper-induced death genes

Study flowchart was showed in Figure 1. Three pSS datasets (GSE7451, GSE23117, GSE40611) were downloaded from the NCBI Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/). All the datasets were pre-processed by affy in R (version 4.0.3), including data merge, normalization, and log2 transformation. In order to explore the interaction between genes, we used weighted gene co-expression network analysis to construct gene co-expression network. To ensure the reliability of network construction results, we removed outlier samples and introduced genes with more than 25% variation in the integrated dataset into weighted gene co-expression network analysis.

In previous studies, genome-wide CRISPR-Cas9 deficiency screening was used to identify 13 genes involved in copper-induced death, including ATPase copper transporting $\alpha$ (ATP7A), ATPase copper transporting $\beta$ (ATP7B), copper importer SLC31A1 (CTR1), dihydrolipoamide dehydrogenase (DLD), dihydrolipoamide S-acetyltransferase, dihydrolipoamide S-succinyltransferase, dihydrolipoamide branched chain transacylase E2, ferredoxin 1, glycine cleavage system protein H, lipoyltransferase 1, lipoyl synthase, pyruvate dehydrogenase E1 subunit $\alpha_1$ (PDHA1), pyruvate dehydrogenase E1 subunit $\beta$ and solute carrier family 31 member 1 (SLC31A1).\textsuperscript{[14]}

2.2. Correlation analysis on copper-induced death genes and immune infiltration

To evaluate the role of copper-induced death genes in the immune microenvironment, we analyzed the correlation of copper-induced death genes and immune microenvironment in SGs of pSS patients by gene expression matrix. The GSVA package was used to calculate the enrichment of immune infiltration including 16 immune cells and 13 immune functions. The pheatmap package was used to show the concentration of copper-induced death genes in immune cells and immune functions.

The corrplot package was used to analyze the correlation of 16 infiltrating immune cells and 13 immune functions, respectively. We used correlation coefficient to evaluate the correlation between immune cells and immune function, respectively. According to the correlation coefficient, we classified the correlation of immune cells and immune functions from strong to weak. We used a plus sign to indicate a positive correlation and a minus sign to indicate a negative correlation.

Figure 1. Study flowchart. Abbreviations: pSS = primary Sjögren’s syndrome; ROC = receiver operating curve.
2.3. Difference in immune score between health controls and pSS

The reshape2 package was used to transform data to calculate the difference of immune score between healthy controls and pSS. The ggpubr package was used to plot the differences between the 2 groups, a P value < .05 was considered statistically significant.

To screen out copper-induced death genes associated with immune infiltration, we used psych package to correlate the immune infiltration expression matrix with the copper-induced death gene expression matrix. The ggcorrplot package was used to calculate correlation coefficients and P values. A P value < .05 was considered statistically significant. If the correlation coefficient |r| was < .5, the correlation was weak, and if the correlation coefficient was ≥ .50, the correlation was medium or strong.

2.4. Construction pSS risk prediction model based on copper-induced death genes

Through the screening of copper-induced death genes related to immune infiltration, we selected the top 4 genes with strong correlation to construct risk prediction model of pSS. The rms package was used to draw nomogram and show the relationship between each variable in the prediction model. Calibration curves were plotted to assess the calibration of the nomogram. The receiver operating curve (ROC) was constructed to predict the risk of pSS. Area under curve was obtained by ROC curve. A P value < .05 was considered statistically significant.

2.5. Enrichment analysis of drug model related to copper-induced death genes

The drug model of copper-induced death genes was constructed with gene enrichment analysis tool (https://maayanlab.cloud/Enrichr/) and the adjust P value < .05 was defined as the cutoff value.[13] We downloaded the therapeutic drug information related to copper-induced death genes from the above website. The clusterProfiler and ggplot2 packages were used for gene and drug enrichment analysis of copper-induced death genes screened in this study. The significant enrichment for analyses threshold was adjust P value < .05. According to the adjust P values, the first 6 drug names were selected and displayed.

3. Results

3.1. Expression of copper death-related genes in immune infiltration

A total of 16 immune cells and 13 immune functions were differentially expressed. Infiltration scores of pSS were obtained by the ssGSEA method. In immune cells, copper-induced death genes were strongly expressed in “activated” dendritic cells (aDCs), macrophages and regulatory T cells (Treg). In immune functions, copper-induced death genes were strongly expressed in major histocompatibility complex (MHC) class I, human leukocyte antigen (HLA) and type I interferon (IFN) response. Among them, copper-induced death genes had the highest expression intensity in immune functions of MHC class I, and were associated with abnormal expression of β-2-Microglobulin, HLA-A and transporter 1. The heatmap of immune cells and immune functions were showed in Figure 2.

3.2. Correlation analysis of immune infiltration

Correlation analysis showed the top 3 correlation in immune cells were tumor infiltrating lymphocytes (TIL) and B cells, TIL and follicular helper T, B cells and follicular helper T. The top 3 correlation in immune functions were T cell co-stimulation and check-point, CC chemokine receptors (CCR) and check-point, T cell co-inhibition and check-point, as showed in Figure 3A and B.

The boxplots of differences between immune score in health controls and pSS were illustrated in Figure 4. In immune cells, aDCs (.657 vs 594, P < .001), TIL (.549 vs 492, P < .001), Treg (.621 vs 598, P < .001), neutrophils (.550 vs 489, P < .001), pDCs (.478 vs 445, P = .001), B cells (.492 vs 434, P = .004), Th2 cells (.500 vs 470, P < .004), T helper cells (.399 vs 327, P = .006) and macrophages (.631 vs 601, P = .010) were significantly increased in pSS. In immune functions, HLA (.793 vs 743, P < .001), MHC class I (.916 vs 873, P < .001), inflammation-promoting (.545 vs 478, P < .001), parainflammation (.645 vs 601, P < .001) and type I IFN response (.728 vs 625, P < .001) were significantly higher in pSS than controls. Antigen presenting cell (APC) co inhibition (.657 vs 466, P = .009), T cell co-inhibition (.466 vs 439, P = .032) and cytolytic activity (.614 vs 559, P = .014) were also higher in pSS, as shown in Figure 3C and D.

3.3. Correlation between copper-induced death genes and immune infiltration

Correlation analysis of copper-induced death genes and immune infiltration in pSS patients showed that among the 12 copper-induced death genes, 5 genes were significantly correlated with immune infiltration, including SLC31A1, PDHA1, DLD, ATP7B, and ATP7A. The DLD and ATP7A were positively correlated with immune infiltration while SLC31A1, PDHA1, and ATP7B were negatively correlated with immune infiltration (P < .05). SLC31A1 and PDHA1 were associated with the abnormality of various immune cells and immune functions in pSS, especially Th1 cells, CCR and T cell co-inhibition, as shown in Figure 4A.

3.4. Copper-induced death genes construct ROC curves

According to the correlation between copper-induced death genes and immune infiltration, the top 4 genes were selected to construct ROC curve and the risk of pSS were predicted. The copper-induced death genes were divided into high and low expression by the median expression value. According to the nomogram, the results suggested that the expression of PDHA1 was significant for predicting the risk of pSS, as shown in Table 1. When expression of PDHA1 was low, the total score was near 180 and the risk of pSS was greater than 80%, as shown in Figure 4B. ROC curve indicated that the area under curve was 0.654, indicating that this model had a good predictive ability for pSS, as shown in Figure 4C.

3.5. Enrichment analysis of copper-induced death gene related drug model

A total of 175 drug-related messages of copper-induced death gene (SLC31A1, PDHA1, DLD, and ATP7B) were downloaded from gene enrichment analysis tool. After screening through adjust P value < .05, a total of 71 drug information were included. Gene enrichment analysis showed that the drugs were mainly involved in SLC31A1 term, including “CHEMBL1182312 CTD 00004324,” “cytochalasin D CTD 00007076,” and “Trimethyl - beta - cyclodextrin CTD 00003512.” In PDHA1 term, the drugs were significantly enriched in “Vitinin CTD 00007069,” “2, 6 – DICHLORO - 4 – NITROPHENOL CTD 00000815,” and “resveratrol CTD 00002483.” DLD term showed that the drugs were mainly enriched in “Isoquercitrin TTD 00008703,” “RUTIN TTD
00010730,” “Indatraline hydrochloride TTD 00008587,” “5, 7 – Dimethoxyflavone CTD 00002502,” “brimonidine CTD 00000810,” and “flavin adenine dinucleotide TTD 00008045.” Additionally, ATP7B term was found to be enriched in “Bathocuproine disulfonate CTD 00001350,” “MG – 132 CTD 00002789,” and “CID755673 CTD 00004896,” as shown in Figure 5A.

We downloaded the figure of copper-induced death gene related drug and verified the drug use frequency chart, as shown in Figure 5B. The results suggested that “Bathocuproine disulfonate CTD 00001350”, “Vitinoin CTD 00007069” and “Resveratrol CTD 00002483” were the drugs most strongly associated with copper-induced death genes. It was consistent with the results of this study.

4. Discussion

pSS is a chronic inflammatory autoimmune disease characterized by specific pathological changes. Previous studies on pSS and genes mainly focused on screening differentially expressed genes, correlation analysis between signaling pathways, genes and protein interaction network. There are few studies on copper-induced death genes, immune infiltration score and prediction model construction. In this study, we found that copper-induced death genes were associated with a variety of immune cells and immune functions. Copper-induced death genes were significantly expressed in aDCs, macrophages, and Treg in immune cells. They were strongly expressed in immune functions such as MHC class I, HLA and type I IFN response. In early studies, CD4 + cytotoxic T lymphocytes (CTL) and DCs may be involved in the proliferation of activated B lymphocytes in SGs of pSS. Further studies showed that RNA interacting with Fcγ receptor IIa and triggered the activation of RNA-containing immune complexes in plasmacytoid dendritic cells (pDCs).

Expression of C-C chemokine receptor 5 (CCR5) and its ligands C-C chemokine ligand type 3 and CCL4 in SGs played an important role in effective migration of DCs. DCs recognized NK cells that bound to B7-H6 in SG epithelial cells and secreted Th1 cytokines such as IFN-γ and interleukin (IL)-12. In this study, it was found that aDCs and pDCs of pSS were significantly increased, while DCs and iDCs were not significantly different. This was an important complement to DCs in the pathogenesis of pSS. In this study, copper-induced death genes were also highly expressed in MHC I, HLA and type I IFN reactions. Copper-induced death genes may also be related to abnormal activation of DCs in SGs, and the specific subtypes of activated dendritic cells need to be verified in further experiments.

Macrophage was the main leukocytes in tissues and the phenotypic characteristics were closely related to the immune microenvironment. Macrophages were activated by IFN-γ and IL-17 from Th1 and Th17 cells in SGs of pSS. Activated macrophages were mainly involved in the pathogenesis of pSS in the following 2 ways: one is to produce inflammatory cytokines such as IL-1, tumor necrosis factor α, IL-18 and metalloproteinases, which led to epithelial cell damage. Another is...
to activate CD4+ T cells through MHC-II as antigenic peptide presenting cells. The 2 interacted to maintain the pro-inflammatory automatic maintenance cycle.\[28\]

Early studies showed that Treg could effectively inhibit the activation of self-reactive T cells, further avoiding the accelerated maturation of DCs.\[29\] Another study showed that labial gland-derived mesenchymal stem cells effectively inhibited the differentiation of Th17 cells and induced the differentiation into Treg cells. Labial gland-derived mesenchymal stem cells educated the secretion of IL-17, IFN-γ and IL-6, and restored SG secretion function.\[30\] Our study showed that macrophages, TIL, Th2 and Treg in pSS were significantly increased. TIL and Treg were associated with abnormal activation of various immune cells. These results suggested that DCs and macrophages were closely associated with abnormal activation of T cells in SGs of pSS. Although the role of Treg in pSS is still controversial.\[31,32\] It is worth further studies in the activation of macrophages and DCs and relationship with pSS.

In this study, immune functions of pSS were characterized by MHC class I, HLA, and type I IFN responses. This result was also reported in the previous studies. Levels of HLA-DR were elevated in exocrine epithelial cells of pSS, inducing activation and infiltration of CD4+ T cells.\[33\] Recent studies using microarrays to analyze the expression of SGs also found that gene expression patterns in pSS involve multiple chronic inflammatory pathways, such as chemokines, cytokines, MHC, and IFN.\[34–36\] This study showed that copper-induced death genes were mainly related to the inflammatory response of pSS. We speculated that copper-induced death genes were associated with immune infiltration in SGs of pSS patients. It may provide important clues for the treatment of pSS.

We also explored the relationship between copper-induced death genes and the risk of pSS, which was an innovative attempt. In this study, we found that SLC31A1 and PDHA1 were associated with various immune cells and immune functions in pSS. It was suggested that excessive copper could target...
to induce tumor cell death in tumor therapy. It was suggested that the role of copper-induced death genes in pSS may be inconsistent. Low expression of PDHA1 gene was closely associated with risk of pSS, while high expression of DLD gene was associated with risk of pSS. PDHA1 gene encoded the E1 subunit α 1 of pyruvate dehydrogenase and played a key role in pyruvate dehydrogenase complex. Inhibition of PDHA1 expression in human esophageal squamous cell cancer significantly reduced oxidative phosphorylation, leading to increased angiogenesis and malignancy. In this study, we found that the low expression of PDHA1 in pSS had biological significance and was associated with the risk of pSS. It is an extension of the research field of PDHA1 gene. More studies on the metabolic reprogramming of PDHA1 and its protein expression products may provide potential therapeutic targets for pSS.

We also attempted to conduct enrichment analysis of copper-induced death genes in therapeutic models, and verified by relevant websites. Results showed that “bathocuproine disulfonate,” “vitinoin,” and “resveratrol” were the drugs most strongly associated with copper-induced death genes. Retinoic acid was the active metabolite of vitamin A, and its main active ingredient was all-trans retinoic acid (ATRA). ATRA produced by DCs promoted differentiation of T cells into FoxP3 + T cells and inhibit differentiation of Th17. The combination of ATRA and TGF-β transferred the balance of Treg/Th17 and reduced the immune inflammatory response. ATRA regulated the expression of FoxP3 gene and induced the proliferation and differentiation of Treg in CD4 + T cells. Retinoic acid receptor α and γ were

![Figure 4. Diagnostic efficacy of copper death-related genes. (A) Correlation of copper death-related genes and immune infiltration, horizontal axes demonstrate copper death-related genes. (B) Column map of top 4 genes, horizontal axes demonstrate risk of pSS, vertical axes demonstrate genes. (C) Diagnostic efficacy of combined prediction model. Abbreviations: AUC = area under curve, CI = confidence Interval, FPR = false positives ratio, pSS = primary Sjogren’s syndrome, TPR = true positives ratio.](image)

| Predictor variable | AUC    | Sensitivity | Specificity | 95% CI       | Standard Error | P    |
|-------------------|--------|-------------|-------------|--------------|----------------|------|
| ATP7B             | .538   | .740        | .500        | .402-.674    | .468           | .851 |
| DLD               | .598   | .420        | .794        | .473-.722    | .477           | .356 |
| PDHA1             | .655   | .960        | .441        | .519-.791    | .519           | .038*|
| SLC31A1           | .464   | .588        | .632        | .336-.591    | .516           | .786 |
| Combined 4 genes  | .678   | .880        | .441        | .558-.799    | .453           | .118 |

AUC = area under the curve, ROC = receiver operating characteristic.
*P < .05.
expressed on the surface of T cells. ATRA also promoted FoxP3+ T cell expression by activating CD4+ T cells with Retinoic acid receptor α.[41,42] ATRA inhibited Th17 mainly by binding receptor-related orphan nuclear receptor γ (ROR-γ) to exert anti-inflammatory effect.[43] In mouse model, overexpression of ROR-α not only attenuated ROR-γ and IL-17, but also regulated production of tumor necrosis factor-α, improving the inflammatory environment.[44] IL-6 can induce high levels of ROR-γ and up-regulate IL-23R, finally promoted the production of IL-17 through the signal transducers and activators of transcription 3 pathway.[45,46] ATRA down-regulated IL-6Rα and IL-23R and played an important role in differentiation of Th17.[47,48]

Resveratrol (Res) is a natural non-flavonoid polyphenol compound, which has strong immunomodulatory and anti-inflammatory effects.[49] Res increased IL-10 and deacetylase 1, and effectively improved the salivation dysfunction in non-obese diabetic mice.[50] In addition, Res reduced dextran sodium sulfate-induced IBD by regulating small ubiquitin-like modifier protein 1 through the Wnt/β-catenin pathway. The expression of anti-inflammatory cytokines increased and pro-inflammatory cytokines decreased in colon and spleen tissues of mice.[51] Since there are few studies on Res and pSS, we speculate that whether drugs based on copper-induced death gene needs to be verified by further models.

Our study has some limitations. First, there are only a few genes related to copper-induced death that can be found at present, and the potential genes related to pSS may be neglected. Second, further studies on immune infiltration are needed to screen potential risks and drug treatment models of pSS.

5. Conclusion
We hypothesized that copper-induced death genes may lead to SGs damage in pSS by influencing immune infiltration disorder. SLC31A1, PDHA1, DLD, ATP7B, and ATP7A were significantly associated with abnormal expression of immune infiltration and may be important genes in the regulation of pSS. PDHA1 gene has a high predictive value for the risk of pSS. In addition, ATRA and Res may have certain reference value for drug treatment of pSS.

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Zhang et al. • Medicine (2022) 101:41

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