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SHORT COMMUNICATION

Identification and validation of ferroptosis-related genes for chronic rhinosinusitis with nasal polyps

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Abstract

Purpose Even though the great progress in the field of chronic rhinosinusitis with nasal polyps (CRSwNP) has been achieved, ferroptosis and its molecular mechanism in CRSwNP remain blank. We are the first to study the relationship between CRSwNP and ferroptosis, aiming to identify ferroptosis-related genes in the process of CRSwNP.

Methods Using the GEO database and the FerrDb database, significantly differentially expressed ferroptosis-related genes (DEFGs) were selected between CRSwNP-NP and CRSwNP-IT specimens. Then, the protein–protein interaction (PPI) network of ferroptosis-related genes was constructed. Functional enrichment analyses (GSVA, GO, KEGG, and GeneCodis analyses) were introduced in our study. Besides, based on the GSE136825 data set, DEFGs between CRSwNP-NP and CS-IT specimens were also analyzed. Finally, qRT-PCR was performed to validate the selected ferroptosis-related genes with clinical samples.

Results 31 significantly DEFGs were identified between CRSwNP-NP and CRSwNP-IT specimens. Functional enrichment analyses and the analysis of GeneCodis 4 pointed out that DEFGs may potentially be involved in some related KEGG pathways. 8 DEFGs were selected between CRSwNP-NP and CS-IT specimens. The experimental verification indicated that 4 genes (GPX2, CDO1, CAV1, and TP53) were the important DEFGs of CRSwNP. The Venn diagrams proved that CDO1 and GPX2 were considered as the most important DEFGs genes of CRSwNP, especially GPX2.

Conclusions Though a comprehensive bioinformatics analysis and the experimental verification, CDO1 and GPX2 were considered as the important ferroptosis-related genes of CRSwNP, especially GPX2. However, further molecular biological experiments would be still required to uncover the underlying mechanism between ferroptosis and CRSwNP.

Keywords Chronic sinusitis · Nasal polyps · Ferroptosis · Gene Expression Omnibus (GEO) Database · Experimental verification

Introduction

Chronic rhinosinusitis (CRS), a highly prevalent disease, is a highly heterogeneous chronic mucosal inflammation characterized by chronic inflammation of sinuses and the nasal cavity [1–5]. Nowadays, biologics are available for chronic rhinosinusitis with nasal polyps (CRSwNP) [3], while it would be warranted to understand the pathophysiology of CRSwNP more to adequately match treatment to disease endotype. Thus, the molecular mechanisms' elucidation of CRSwNP still needed to be further revealed [3–5].

In 2012, the Stockwell BR laboratory first proposed the concept of ferroptosis [6]. Ferroptosis is a new mode of programmed cell death, which is distinct from apoptosis, cell necrosis, and autophagy [7]. The toxic accumulation of lipid-reactive oxygen species inside caused the death of cells. The main mechanism of ferroptosis is that ester oxygenase or divalent iron catalyzes the high expression of unsaturated fatty acids on the cell membrane and lipid peroxidation to induce cell death [8]. The ferroptotic process is complicated, consisting of a wide range of biomolecules

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and metabolites [9–12]. In recent years, more and more studies confirmed that ferroptosis is a novel cell death connecting inflammation [13–16]. Thus, the mechanisms between ferroptosis and inflammation may potentially promote the development of therapeutic strategies with ferroptosis inhibitors for CRSwNP [14].

Even though the great progress in the field of CRSwNP has been achieved, ferroptosis and its molecular mechanism in CRSwNP remain blank [3–5]. Therefore, we are the first to study the relationship between CRSwNP and ferroptosis, aiming to further explain the mechanisms and explore more promising targeted treatments.

Methods

Patient dataset

The mRNA expression data (GSE136825) of CRSwNP or non-CRS controls were obtained from the GEO (Gene Expression Omnibus) database (www.ncbi.nlm.nih.gov/geo), containing 42 nasal polyp (CRSwNP-NP) tissues, 33 paired non-polyp inferior turbinate (CRSwNP-IT) tissues from CRSwNP patients, and 28 inferior turbinate samples from non-CRS patients (CS-IT). CRSwNP-IT and CS-IT samples were enrolled as the controls [2].

A total of 259 ferroptosis-related genes were acquired from the ferroptosis database (FerrDb, http://www.zhounan.org/ferrdb). The FerrDb database is the world's first database about ferroptosis regulators, ferroptosis markers, and ferroptosis-disease associations [17]. The data in the FerrDb database are free to download and utilize.

The data processing and the screening of significantly differentially expressed ferroptosis-related genes (DEFGs)

Utilizing R software (version 4.05, https://www.r-project.org/), the "EdgeR" package was applied to detect the differences between CRSwNP-NP specimens and CRSwNP-IT specimens, selecting significantly DEFGs. $|\log_2 FC| > 1$, p value < 0.05, and false discovery rate < 0.05 were determined as the threshold. The "ggplot2" package was applied to better visualize the differences between CRSwNP-NP specimens and CRSwNP-IT specimens. Moreover, the volcano plot was also drawn to better demonstrate significantly DEFGs.

The protein–protein interaction (PPI) network of ferroptosis-related genes

The PPI network of significantly DEFGs was constructed using the STRING website (https://www.string-db.org/) and a confidence score higher than 0.9 was considered as the threshold. Protein nodes that did not interact with others were excluded. Furthermore, using Cytoscape software (version: 3.9.1, Cytoscape Consortium, USA), the significant modules and hub genes were screened. In detail, based on the criteria (both MCODE score > 10 and number of nodes > 20), the MCODE (version: 2.0.0) plug-in was applied to select significant clustering modules. Besides, the CytoHubba (version: 0.1) plug-in was applied to identify hub genes (degree > 10).

Functional enrichment analyses

As a gene set enrichment method, Gene Set Variation Analysis (GSVA) was applied to estimate the variation of pathway activity in an unsupervised manner [18].

The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis and gene ontology (GO) functional enrichment analysis were introduced to explore the major biological attributes.

GeneCodis

To expand the modular enrichment analysis to regulatory elements, GeneCodis was introduced in our study. GeneCodis, a web-based tool, was designed for the functional analysis of lists of mRNAs, miRNAs, transcription factors (TFs), etc., which can be applied to determine biological annotations according to a reference list [19, 20].

Real-time fluorescence quantitative polymerase chain reaction (qRT-PCR)

According to articles published in authoritative journals, IT was often enrolled as the control [1, 2]. 4 nasal mucosa samples (paired CRSwNP-IT tissues from CRSwNP patients) and 4 CRSwNP samples were obtained to conduct qRT-PCR. This study was approved by the ethics committee of The Second Affiliated Hospital of Nanchang University. Meanwhile, informed consent was obtained from each patient.

Total RNA was isolated from nasal mucosa samples and CRSwNP samples using Trizol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Then, reverse transcription (RT) was performed using The SuperScript III First-Strand Synthesis System (Invitrogen; Thermo Fisher Scientific, Inc.). Subsequently, qPCR was performed using SYBR® Premix Ex Taq™ II (Takara, China) and applied biosystems (USA) according to the manufacturer's protocol.

Gene set enrichment analysis (GSEA)

GSEA was applied to identify the important pathways of the most important ferroptosis-related gene of CRSwNP [21]. *p* values were generated from 1000 random permutations of gene sets. Subsequently, the enrichment score of enrichment results was applied to assess the statistical significance.

Statistical analysis

In this study, Strawberry Perl software for windows (Version 5.32.1.1) was utilized to organize the data. The student's *t* test was utilized for the two-group comparisons. $p < 0.05$ was considered statistically of significance. All experiments were performed in triplicate when indicated. All statistical analyses were performed with R software (Version 4.0.5, The R Foundation).

Result

The identification of significantly DEFGs

A total of 1286 significantly differentially expressed genes (DEGs) were identified, including 669 up-regulated genes and 617 down-regulated genes. The volcano plot (Supplementary Fig. 1A) and the heatmap (Supplementary Fig. 1B) were both drawn to display significantly DEGs between CRSwNP-NP specimens and CRSwNP-IT specimens.

Subsequently, a total of 31 significantly DEFGs were identified, including 22 up-regulated genes and 9 down-regulated genes (Table 1). Up-regulated genes were CDKN2A, SLC2A6, SLC2A1, HSPB1, ATG4D, DUOX1, GPX2, AKR1C2, ALOX15, GDF15, NOX1, RRM2, ALOX5, AKR1C1, NCF2, MT1G, TFR2, NOX4, CYBB, NOS2, HMOX1, and CA9. Down-regulated genes were CDO1, DPP4, IL6, ANGPTL7, CBS, CXCL2, NOX5, PLIN2, and ZFP36. The volcano plot (Supplementary Fig. 1C) and the heatmap (Supplementary Fig. 1D) were both drawn to display significant DEFGs between CRSwNP-NP specimens and CRSwNP-IT specimens.

The PPI network of ferroptosis-related genes and hub genes

The PPI network of ferroptosis-related genes between CRSwNP-NP specimens and CRSwNP-IT specimens was constructed (Supplementary Fig. 2A). Ferroptosis drivers, ferroptosis markers, and ferroptosis suppressors were marked in Supplementary Fig. 2B. 2 significant clustering modules were selected (Supplementary Fig. 2C, D).

Table 1 Significantly differentially expressed ferroptosis-related gene between Control and CRSwNP groups

Symbol	LogFC	*p* value	FDR
CDO1	– 2.47	1.02E–08	5.14E–07
DPP4	– 2.44	7.75E–16	3.44E–13
IL6	– 2.13	7.40E–04	6.09E–03
ANGPTL7	– 2.02	3.06E–04	3.01E–03
CBS	– 1.83	2.29E–08	1.03E–06
CXCL2	– 1.61	3.63E–04	3.44E–03
NOX5	– 1.44	4.24E–03	0.02
PLIN2	– 1.26	1.74E–11	2.18E–09
ZFP36	– 1.04	4.08E–03	0.02
CDKN2A	1.00	3.92E–03	0.02
SLC2A6	1.02	4.61E–03	0.03
SLC2A1	1.03	1.66E–04	1.84E–03
HSPB1	1.06	3.15E–04	3.08E–03
ATG4D	1.13	1.58E–06	3.73E–05
DUOX1	1.15	8.88E–07	2.30E–05
GPX2	1.24	1.81E–03	0.01
AKR1C2	1.25	4.89E–04	4.38E–03
ALOX15	1.32	4.92E–04	4.40E–03
GDF15	1.41	1.60E–03	0.01
NOX1	1.49	1.10E–04	1.31E–03
RRM2	1.50	1.03E–04	1.24E–03
ALOX5	1.55	5.86E–09	3.20E–07
AKR1C1	1.55	9.35E–13	1.65E–10
NCF2	1.63	1.01E–08	5.11E–07
MT1G	1.85	1.07E–04	1.28E–03
TFR2	1.92	1.61E–04	1.79E–03
NOX4	2.05	6.43E–07	1.74E–05
CYBB	2.24	1.15E–12	1.98E–10
NOS2	2.35	3.54E–08	1.50E–06
HMOX1	2.60	3.35E–20	5.25E–17
CA9	3.68	8.68E–07	2.25E–05

Table 2 Top 9 ferroptosis-related genes ranked by degree method

Rank	Name	Score	Class
1	TP53	32	Ferroptosis driver
2	IL6	29	Ferroptosis marker
3	HMOX1	22	Ferroptosis driver
4	GPX2	15	Ferroptosis marker
4	HRAS	15	Ferroptosis driver
6	GPX4	14	Ferroptosis suppressor
6	TFRC	14	Ferroptosis driver
8	CYBB	13	Ferroptosis driver
8	CAV1	13	Ferroptosis suppressor

Subsequently, using the CytoHubba plug-in, 9 hub genes were determined, including TP53, IL6, HMOX1, GPX2, HRAS, GPX4, TFRC, CYBB, and CAV1 (Table 2). Among them, TP53, HMOX1, HRAS, TFRC, and CYBB were ferroptosis drivers. IL6 and GPX2 were ferroptosis markers. Besides, GPX4 and CAV1 were ferroptosis suppressors.

Functional enrichment analyses

The results of GSVA, visualized by the volcano plot (Fig. 1A) and the heatmap (Fig. 1B) showed that significantly differentially expressed genes were enriched in the up-regulated pathway (ferroptosis).

KEGG pathway enrichment analysis is drawn in Fig. 1C, showing that ferroptosis was also one of the important enriched pathways. Enrichment analyses of GO-BP, GO-CC, and GO MF are shown in Fig. 1D–F.

The analysis of GeneCodis 4

The results showed the enriched KEGG pathways analysis (Supplementary Fig. 3A), panther pathway analysis (Supplementary Fig. 3B), wikipathways analysis (Supplementary Fig. 3C), and GO-BP analysis (Supplementary Fig. 3D). These analyses also confirmed that ferroptosis played an important role in CRSwNP.

The validation of expression levels of significantly DEFGs between CRSwNP-NP specimens and CS-IT specimens

Based on the expression data between 42 CRSwNP-NP specimens and 28 CS-IT specimens, we detected differences in significant DEFGs. Then, the significant differences on expression levels were observed in CAV1 (Fig. 2A, $p < 0.001$), CDO1 (Fig. 2B, $p < 0.001$), G6PD (Fig. 2C, $p < 0.001$), GPX2 (Fig. 2D, $p = 0.01$), GPX4 (Fig. 2E, $p < 0.001$), HMOX1 (Fig. 2F, $p < 0.001$), HRAS (Fig. 2G, $p = 0.01$), and TP53 (Fig. 2H, $p < 0.001$).

The experimental verification

The experiment on relative iron concentration indicated a significant difference between nasal mucosa samples and CRSwNP samples (Fig. 3A, $p < 0.001$). Furthermore, significant differences in relative expression levels between nasal mucosa samples and CRSwNP samples were observed in TP53 ($p < 0.05$), GPX2 ($p < 0.05$), CDO1 ($p < 0.05$), and CAV1 ($p < 0.01$) (Fig. 3B). qRT-PCR primer sequences are described in Table 3.

The Venn diagrams

In Fig. 4A, 2genes (CDO1 and GPX2) were overlapped, which were considered as the important ferroptosis-related genes of CRSwNP. Adding the top 9 hub ferroptosis-related genes, only GPX2 was all overlapped, potentially being considered as the most important ferroptosis-related genes of CRSwNP (Fig. 4B).

GSEA

The results of GSEA showed that GPX2 may probably affect many KEGG pathways, such as glycolysis gluconeogenesis, glutathione metabolism, etc. (Fig. 4C).

Discussion

Ferroptosis, a newly discovered type of cell death, is triggered by intracellular phospholipid peroxidation [13, 15, 16]. Classified as regulated necrosis, ferroptosis would be more immunogenic than apoptosis. Inspiring evidence indicates that ferroptosis plays a vital role in inflammation [13–16]. Furthermore, functioning as ferroptosis inhibitors, some antioxidants have been proven to exert anti-inflammatory effects in several experimental models with certain diseases [14, 16]. Thus, it would be more promising to study the relationship between CRSwNP and ferroptosis, aiming to further explain the mechanism and explore more accurate targeted treatment involving ferroptosis inhibitors.

In this study, we conducted a comprehensive bioinformatics analysis and experimental verification. First, 31 significant DEFGs were identified between CRSwNP-NP specimens and CRSwNP-IT specimens. Second, TP53, IL6, HMOX1, GPX2, HRAS, GPX4, TFRC, CYBB, and CAV1 were considered as top 9 ferroptosis-related genes between CRSwNP-NP specimens and CRSwNP-IT specimens. Third, functional enrichment analyses and the analysis of GeneCodis 4 pointed out that significantly DEFGs may potentially be involved in some related KEGG pathways, especially ferroptosis. Fourth, CAV1, CDO1, G6PD, GPX2, GPX4, HMOX1, HRAS, and TP53 were considered as significant DEFGs between CRSwNP-NP specimens and CS-IT specimens. Fifth, the experimental verification indicated that 4 genes (GPX2, CDO1, CAV1, and TP53) were the important DEFGs of CRSwNP. Sixth, the Venn diagrams proved that CDO1 and GPX2 were considered as the most important DEFGs of CRSwNP, especially GPX2.

GPX2 (Glutathione peroxidase 2), a protein-coding gene, can catalyze the reduction of organic hydroperoxides and hydrogen peroxide by glutathione, which can protect cells against oxidative damage. Pathways associated with GPX2

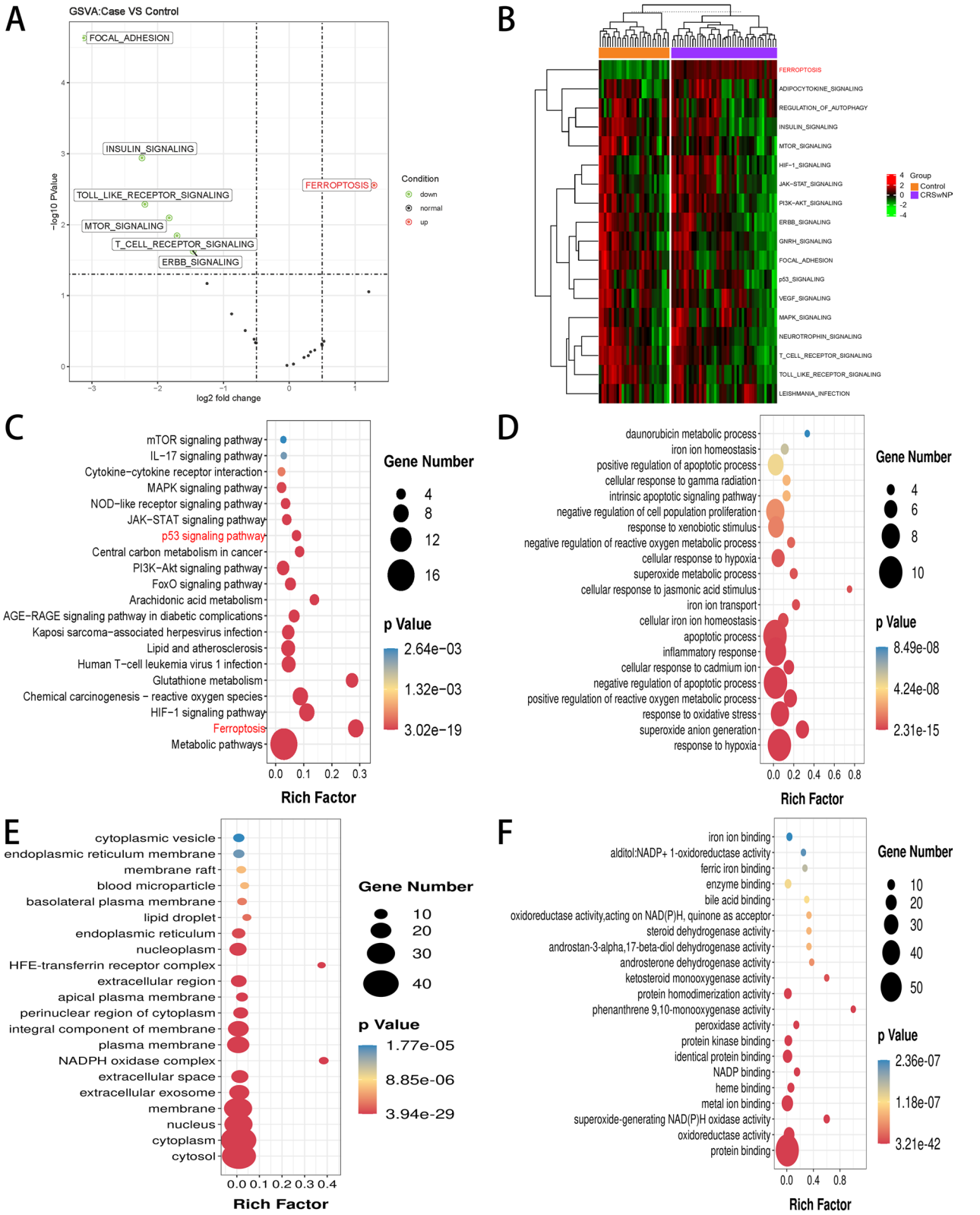

Fig. 1 Functional enrichment analyses. **A** Volcano plot showing the results of GSVA. **B** Heatmap showing the results of GSVA. **C** Visualization of KEGG pathway enrichment analysis. **D** Visualization of GO-BP enrichment analysis. **E** Visualization of GO-CC enrichment analysis. **F** Visualization of GO-MF enrichment analysis. GSVA, gene Set Variation Analysis. *KEGG* Kyoto Encyclopedia of Genes and Genomes, *GO* gene ontology, *BP* biological process, *CC* cellular component, *MF* molecular function

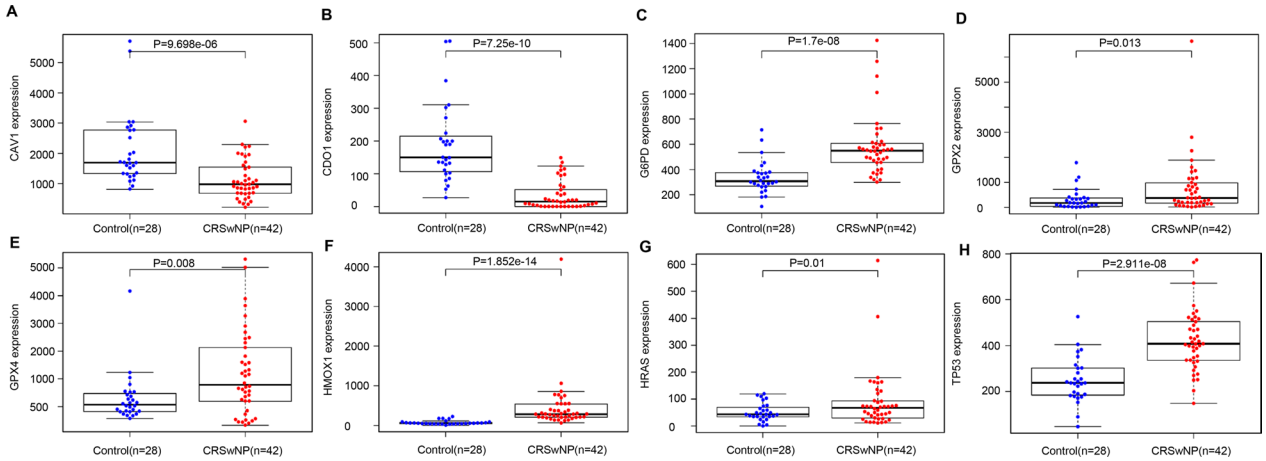

Fig. 2 Validation of expression levels of significantly DEFGs between CRSwNP-NP specimens and CS-IT specimens. **A** CAV1. **B** CDO1. **C** G6PD. **D** GPX2. **E** GPX4. **F** HMOX1. **G** HRAS. **H** TP53. *DEFG* differentially expressed ferroptosis-related genes, *CRSwNP* chronic rhinosinusitis with nasal polyps, *NP* nasal polyps, *CS-IT* inferior turbinate samples from non-CRS controls

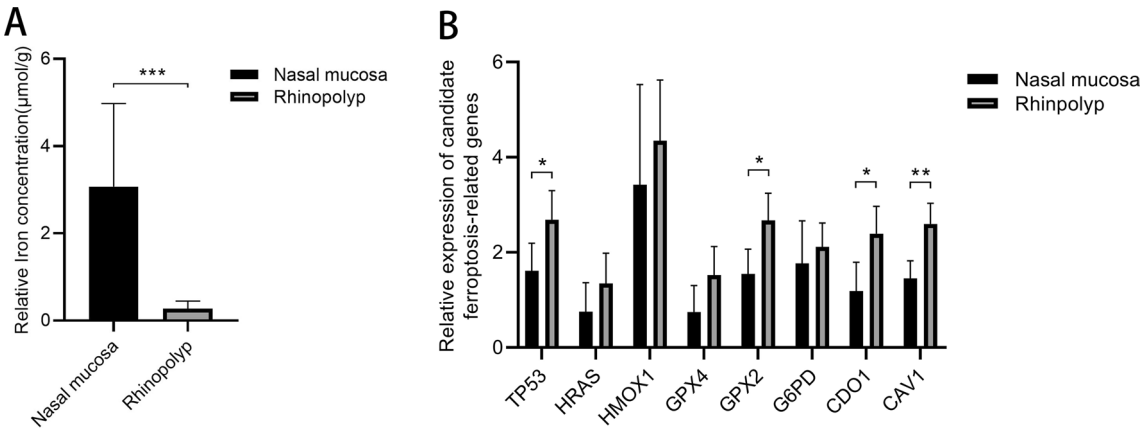

Fig. 3 Experimental verification. **A** Experiment on relative iron concentration. **B** Relative expression levels of candidate ferroptosis-related genes between nasal mucosa samples and CRSwNP samples using qRT-PCR. *CRSwNP* chronic rhinosinusitis with nasal polyps, *qRT-PCR* real-time fluorescence quantitative polymerase chain reaction

included metabolism and glutathione metabolism. As one of the ferroptosis-related markers, its expression was upregulated during ferroptosis, which was induced by erastin or RSL3 [12]. Integrating the bioinformatics analysis and the Vitro experiments, a recent study confirmed that GPX2 was the ferroptosis-related gene, influencing the overall survival of patients with lung adenocarcinoma [22]. In terms of the associated pathways of GPX2, Ren Z et al. stated that GPX2 loss can promote phosphorylation and glycolysis in breast cancer [23]. Besides, Du H et al. concluded that high expression of GPX2 is correlated with a glycolysis and gluconeogenesis gene set, a hallmark glycolysis gene set, an oxidative phosphorylation gene set, etc.[24]. However, still being not explored in published studies of CRS or CRSwNP, GPX2 may probably play a vital role in the ferroptosis of CRSwNP.

CDO1 (Cysteine dioxygenase type 1), a protein-coding gene, was associated with metabolism and sulfur amino acid metabolism. As one of the ferroptosis-related drivers, its expression contributed to ferroptosis resistance [25]. Integrating the Vitro and Vivo experiments, Hao S et al. demonstrated that CDO1 plays an important role in this process of ferroptosis in human gastric cancer cells. In detail, silencing CDO1 can inhibit ferroptosis in gastric cancer cells in vitro or in vivo [25]. However, still being not explored in published studies of CRS or CRSwNP, CDO1 may probably play a vital role in the ferroptosis of CRSwNP.

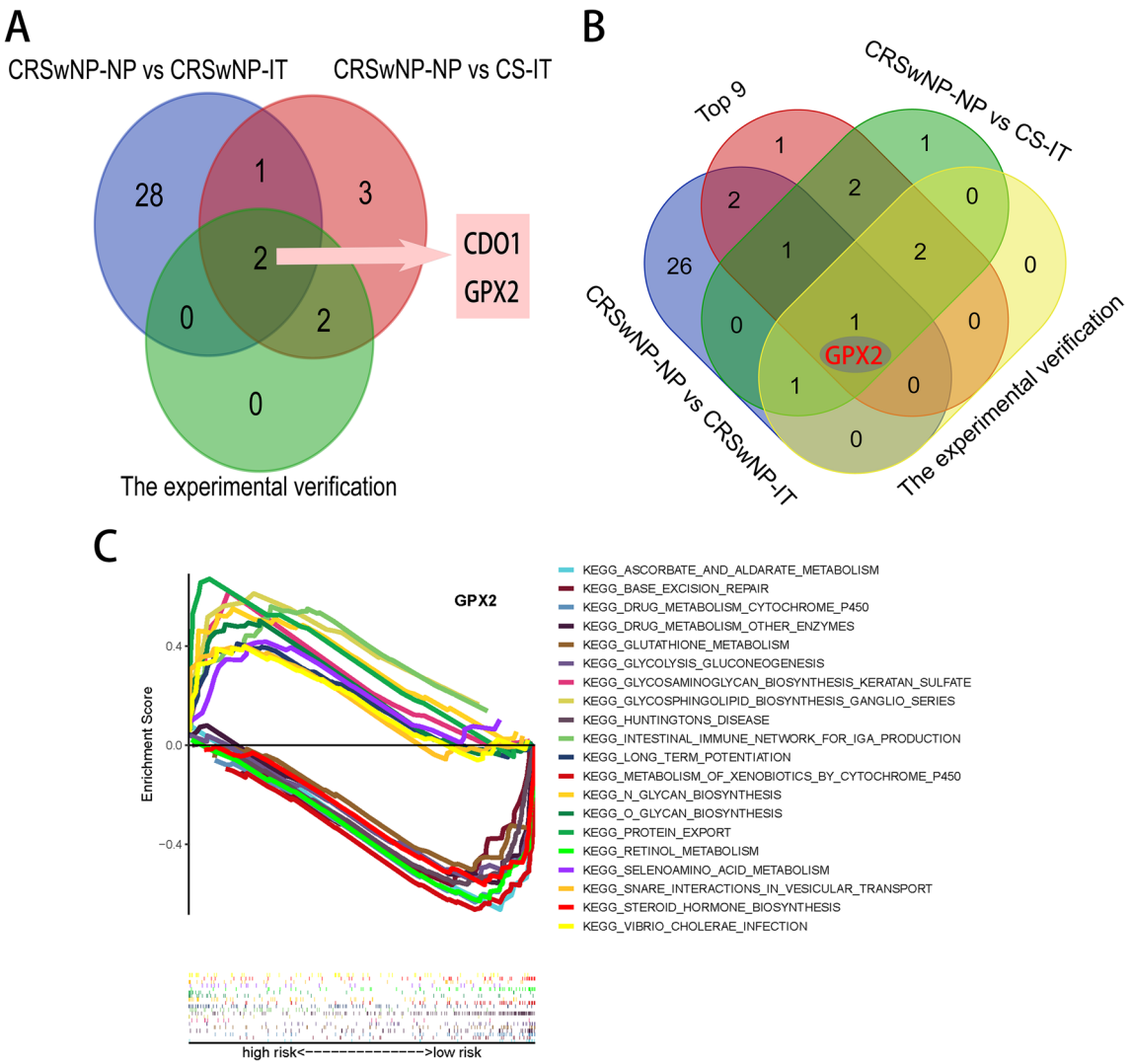

Fig. 4 Venn diagrams and the GSEA analysis of GPX2. **A** Venn diagram showed that CDO1 and GPX2 were overlapped. The blue area represents significantly DEFGs between CRSwNP-NP and CRSwNP-IT specimens; the red area represents significantly DEFGs between CRSwNP-NP and CS-IT specimens; the green area represents significantly DEFGs in the experimental verification. **B** Venn diagram showed that GPX2 were all overlapped. The blue area represents significantly DEFGs between CRSwNP-NP and CRSwNP-IT specimens; the green area represents significantly DEFGs between CRSwNP-NP and CS-IT specimens; the yellow area represents significantly DEFGs in the experimental verification; the red area represents 9 hub DEFGs after using the CytoHubba plug-in. **C** GSEA analysis of GPX2. *CRSwNP* chronic rhinosinusitis with nasal polyps, *NP* nasal polyps, *IT* inferior turbinate, *DEFG* differentially expressed ferroptosis-related gene, *GSEA* gene set enrichment analysis

Therefore, we are the first to explore ferroptosis and its molecular mechanism in CRSwNP, with a comprehensive bioinformatics analysis and experimental verification. However, several limitations still existed in this study. First, ferroptosis-related genes in this study were solely based on the FerrDb database with the current evidence based on previously published studies, which probably omitted many unreported ferroptosis-related genes. Second, the underlying mechanism between the important ferroptosis genes (GPX2 and CDO1) and CRSwNP needs to be further experimentally addressed.

Conclusions

Our work identified and validated ferroptosis-related genes for CRSwNP, which may reveal the relationship between ferroptosis and CRSwNP. CDO1 and GPX2 were considered as important ferroptosis-related genes of CRSwNP, especially GPX2. However, further molecular biological experiments are still required to uncover the further mechanism.

Table 3 Primer sequences

Target name	Primer	
Actin	Actin-F	TCCTCCTGAGCGCAAGTACTCC
	Actin-R	CATACTCCTGCTTGCTGATCCAC
TP53	TP53-F	GGCCCATCCTCACCATCATCACA
	TP53-R	GCTCCCCTTTCTTGCGGAGA
HRAS	HRAS-F	GCTGCACGCACTGTGGAATCTCG
	HRAS-R	CGCACCAACGTGTAGAAGGCATC
HMOX1	HMOX1-F	CCTTCCCCAACATTGCCAGT
	HMOX1-R	CTTGGCCTCTTCTATCACCCTC
GPX4	GPX4-F	ACACCGTCTCTCCACAGTTCC
	GPX4-R	GCTCCTCCATGGGTCCGTAGC
GPX2	GPX2-F	TCCTTGGCTTCCCTTGCAAC
	GPX2-R	CTCGTTCTGCCCATTCACCT
G6PD	G6PD-F	AGATTTGCCAACAGGATCTTCGG
	G6PD-R	TCACGTCCCGGATGATCCCAA
CDO1	CDO1-F	GCCATCATGGAAGCCTACGA
	CDO1-R	ACAAACTGCAAACCACGAC
CAV1	CAV1-F	TCTCTCTTTCCTGCACATCTGG
	CAV1-R	AACAGCTTCAAAGAGTGGGTCA

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00405-022-07696-x.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was approved by the ethics committee of The Second Affiliated Hospital of Nanchang University.

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