Original Article

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Lysine succinylation analysis reveals the effect of *Sirt5* on synovial fibroblasts in rheumatoid arthritis patients

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SUMMARY Rheumatoid arthritis (RA) is an autoimmune disease with complex etiology, and its pathological mechanism remains unclear. Our aim was to explore the effect of protein succinylation on RA by silencing *Sirt5*, sequencing succinylated proteins, and analyzing the sequencing results to identify potential biomarkers. We wanted to gain a clearer understanding of RA pathogenesis, quantitative assessment of succinylated proteins in Fibroblast-like synoviocytes (FLS) from RA patients using liquid chromatography- tandem mass spectrometry and enrichment analysis investigated using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG). A total of 679 proteins and 2,471 lysine succinylation sites were found in RA patients, and 436 differentially expressed proteins and 1,548 differentially expressed succinylation sites were identified. Among them, 48 succinylation sites were upregulated in 38 proteins and 144 succinylation sites were downregulated in 82 proteins. Bioinformatics showed that succinylated proteins were significantly enriched in amino and fatty acid metabolisms. Results indicated that *Sirt5* can affect various biological processes involved in RA FLSs, and succinylation caused by silencing *Sirt5* plays a major role in RA progression. This study provides further understanding of RA pathogenesis and may facilitate searching for potential RA biomarkers.

Keywords rheumatoid arthritis, synovial fibroblasts, *Sirt5*, succinylation

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation in multiple joints. Its pathological features are synovitis and pannus formation, resulting in joint damage, cartilage destruction, bone erosion, and eventually joint deformity and loss of function (*1*). Genetic and environmental factors are involved in the occurrence and development of RA (*2*). Because of the complexity of its pathological factors and pathogenesis, it has not yet been fully clarified. Therefore, understanding the molecular mechanism of RA occurrence and development is very important for RA treatment.

Post-translational modification of proteins can alter the structure and function of proteins and plays a major role in the occurrence and development of diseases (*3*). Lysine is a frequent target of modification among amino acid residues in proteins. In a recent study, lysine succinylation was defined as the transfer of a succinyl group to a lysine residue, which is a newly discovered post-translational modification of proteins (*4*).

Sirt5 is a member of the Sirtuin family and influences the occurrence and development of many diseases. *Sirt5* is involved in the regulation of various cellular processes (*5*), such as reactive oxygen species defense (*5*), fatty acid metabolism (*6*), and apoptosis (*7*). Expression of *Sirt5* is low in RA patients and affects RA development by inhibiting the secretion of proinflammatory factors (*8*). However, the precise mechanism by which *Sirt5* affects RA remains unclear. Therefore, targeting *Sirt5* provides a possibility for treating RA.

In this study, we identified 436 differentially expressed proteins and 1,548 differential succinylation sites after silencing *Sirt5*, followed by protein extraction, affinity enrichment, and liquid chromatography-tandem mass spectrometry (LC-MS/MS). These succinylated proteins were involved in various biological functions and cellular processes. Our study may facilitate understanding the regulatory role of *Sirt5*-mediated lysine succinylation in RA.

2. Materials and Methods

2.1. Synovial tissue collection

Synovial tissue was collected from RA patients during knee replacement surgery. All patients met the RA diagnostic criteria of the American College of Rheumatology. This study was approved by the Medical Ethics Committee of the Institutional Review Board of Shandong Medicinal Biotechnology Center (SMBC-2020-08). Synovial tissue was collected at Shandong Provincial Hospital (Ji'nan, Shandong). Written informed consent was provided by the patients.

2.2. Preparation and culture of synovial fibroblasts from RA patients

The synovial tissue was cut into pieces, digested with type II and III collagenases (Sigma-Aldrich, St. Louis, MO, USA), and cultured at 37°C for 4–5 h. The digestion was terminated by incubation in Dulbecco's modified Eagle's medium DMEM (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA) for 24 h. After washing with phosphate-buffered saline (PBS) (Solabio, Beijing, China), the cells were cultured in 10% fetal bovine serum-containing medium.

2.3. SiRNA-mediated silencing of *Sirt5* expression in synovial fibroblasts

After screening the silencing efficiency of *Sirt5*-targeting siRNAs designed and produced by Gemma Company (Gemma, Shanghai, China), the siRNA with the highest silencing efficiency was selected for the following experiment. The SiSIRT5 sequences were 5′-GGAGAUCCAUGGUAGCUUATT-3′ and 5′-UAAGCUACCAUGGAUCUCCTT-3′. HiPerFect transfection reagent (Qiagen) was used for transfection in accordance with the manufacturer's instructions. SiNC was provided by Qiagen. After incubation for 48 h, cells were collected and stored in liquid nitrogen until analysis.

2.4. Protein extraction and pancreatic enzymolysis

Samples were mixed with a four-fold volume of cracking buffer (8 mol/L urea, 1% protease inhibitor, 3 μmol/L TSA, and 50 mmol/L NAM) for ultrasonication. After centrifuging at 4°C for 10 min at 12,000 × g, the supernatant was transferred to a new centrifuge tube, and the protein concentration was determined using a BCA protein concentration determination kit. The proteins in each sample were enzymolized in the same amount, and then the volume was adjusted to the same volume as the lysate. Trichloroacetic acid (TCA) (Sigma-Aldrich, St. Louis, MO, USA) was slowly added to a final concentration of 20%, and the sample was precipitated at 4°C for 2 h. After centrifugation at 4,500 × g for 5 min, the precipitate was washed with pre-cooled acetone two to three times. After drying and precipitation, tetraethylammonium bromide (TEAB) (Sigma-Aldrich, St. Louis, MO, USA) was added to a final concentration of 200 mmol/L. The precipitate was dispersed by ultrasonication, and trypsin (Promega Corporation, Fitchburg, Wisconsin, United States) was added at a ratio of 1:50 (protease:protein, m/m), and enzymatic hydrolysis was carried out overnight. Dithiothreitol (DTT) (Sigma-Aldrich, St. Louis, MO, USA) was added to a final concentration of 5 mmol/L and reduced at 56°C for 30 min. Then, iodoacetamide (IAM) (Sigma-Aldrich, St. Louis, MO, USA) was added to a final concentration of 11 mmol/L, followed by incubation at room temperature for 15 min while protected from light.

2.5. Enrichment by Ksu modification

Proteins were dissolved in IP buffer (100 mmol/L NaCl, 1 mmol/L EDTA, 50 mmol/L Tris-HCl, and 0.5% NP-40, pH 8.0). The supernatant was transferred to pre-washed resin (antibody resin catalog number PTM402, PTM Bio, Hangzhou, China) and placed on a rotating mixer at 4°C overnight. Then, the resin was washed with IP buffer four times and then with deionized water twice. Finally, the resin-bound peptides were eluted using 0.1% trifluoroacetic acid eluent (TFA)(Sigma-Aldrich, St. Louis, MO, USA), the eluate was eluted three times, and the eluent was collected and dried using vacuum freezing. After desalting using C18 ZipTips (Millipore) in accordance with the manufacturer's instructions, the sample was dried again using vacuum freezing for LC-MS analysis.

2.6. Liquid chromatography-mass spectrometry

Peptides were dissolved in mobile phase A and separated in a NanoElute (Bruker, Germany) ultra-high performance liquid phase system. Mobile phase A was an aqueous solution containing 0.1% formic acid and 2% acetonitrile. Mobile phase B was an acetonitrile-aqueous solution containing 0.1% formic acid. The liquid phase gradient settings were: 0–40 min, 7–24% B; 40–52 min, 24–32% B; 52–56 min, 32–80% B; 56–60 min, 80% B. The flow rate was maintained at 450 nL/min. Peptides were separated in an ultra-high performance liquid phase system, injected into the capillary ion source for ionization, and then analyzed by timsTOF Pro 2 mass spectrometry. The ion source voltage was set to 1.5 kV. The parent ion of the peptide segment and its secondary fragments were detected and analyzed using high-resolution TOF. The secondary mass spectrometry scan range was set to 100–1700. The data acquisition mode used the parallel cumulative serial fragmentation

(PASEF) mode. A secondary spectrum with a charge number of parent ions in the range of 0–5 was collected in PASEF mode 10 times after primary mass spectrum collection. The dynamic exclusion time of the series mass spectrometry scanning was set to 24 s to avoid repeated scanning of parent ions.

2.7. Database searching

Secondary mass spectrum data were retrieved using Maxquant (v1.6.15.0), and the enzyme digestion method was set to Trypsin/P. The number of missing cuts was set to 4. The minimum length of the peptide was set to 7 amino acid residues. The maximum number of peptide modifications was set to 5. The mass error tolerance for primary parent ions was set to 20 ppm for first and main searches, and secondary fragment ions. The false discovery rate (FDR) for protein, peptide and modification site identification was set to 1%.

2.8. Bioinformatics data analysis

Gene Ontology (GO) was performed for functional analysis of differentially expressed proteins, including biological processes, cell localization and molecular function. Pathway enrichment analysis of differential genes was performed by Kyoto Encyclopedia of Genes and Genomes (KEGG). Motif analysis of modification sites employed the MoMo analysis tool based on the MotiF-X algorithm. After database comparison with the STRING protein interaction network, the R package visNetwork tool was used for visual analysis of differentially expressed proteins. The interaction relationship of differentially modified proteins was extracted using a confidence score of > 0.7 (high confidence).

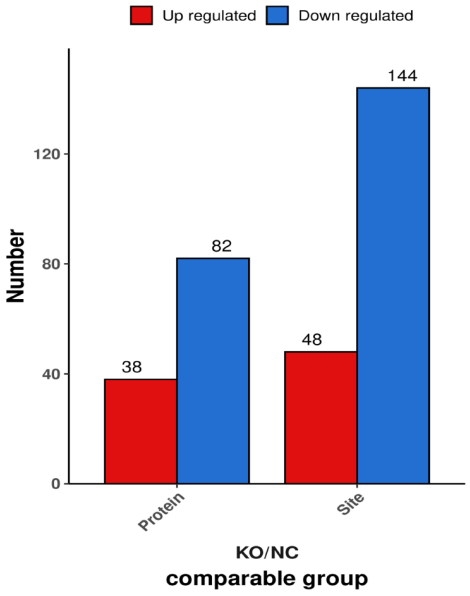


Figure 1. Expression of differentially modified proteins and modification sites.

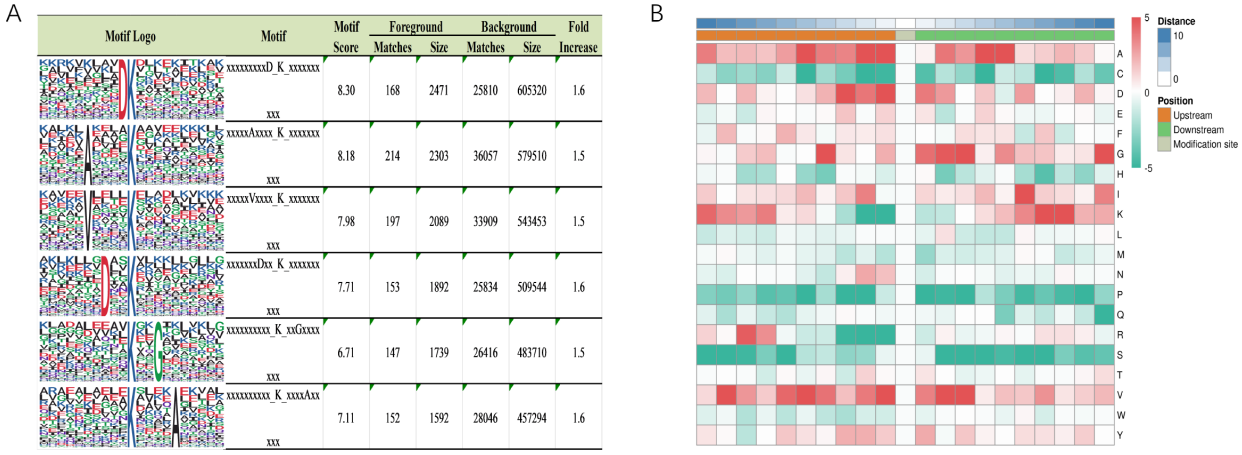
3. Results

3.1. Quantitative modification of Ksu in siSIRT5 and siNC groups

A total of 436 differentially expressed proteins were identified by LC-MS/MS in siSIRT5 and siNC groups with 1,548 lysine succinylation sites. Among them, 38 upregulated proteins had 48 succinylation sites and 82 downregulated proteins had 144 succinylation sites(Figure 1). The samples were set as three group repeats. At $p < 0.05$, the differential expression level was > 1.5 as the significantly upregulated change threshold and < 0.67 as the significantly downregulated change threshold.

3.2. Ksu motif analysis

To clarify the specific lysine succinylation sequence of



Motif Logo	Motif	Motif Score	Foreground Matches	Foreground Size	Background Matches	Background Size	Fold Increase
	xxxxxxxxxD_K_xxxxxx	8.30	168	2471	25810	605320	1.6
	xxxxxAxxxx_K_xxxxxx	8.18	214	2303	36057	579510	1.5
	xxxxxVxxxx_K_xxxxxx	7.98	197	2089	33909	543453	1.5
	xxxxxxxDxx_K_xxxxxx	7.71	153	1892	25834	509544	1.6
	xxxxxxxxx_K_xxGxxxx	6.71	147	1739	26416	483710	1.5
	xxxxxxxxx_K_xxxxAxx	7.11	152	1592	28046	457294	1.6

Figure 2. Motif analysis of succinylated peptides. (A) Sequence markers of succinylation motifs; (B) Heat map of amino acid frequency near the succinylation site.

SIRT5 in rheumatoid joints, we conducted motif analysis of amino acids at succinylation sites around lysine. The lysine succinylation sites were mainly enriched in DK, A****K, V****K, D**K, K**G, and K****A (Figure 2A). By evaluating the frequency of amino acids at the succinylation sites, we found highly site-specific alanine (A), aspartic acid (D), and valine (V) at multiple sites (-1, -2, -3, -4, and -5), but different degrees of deletion at corresponding sites upstream (Figure 2B). Cysteine (C), proline (P), and serine (S) were rarely present, and glycine (G) and leucine (L) were highly enriched at +2, but absent at -2, whereas aspartate (N) was only highly present at -1 and -2, and lysine was highly present at ±7, ±8, ±9, and ±10.

3.3. Enrichment analysis of differentially expressed succinylated proteins

To elucidate the function of differentially regulated lysine succinylation genes after *Sirt5* silencing, the GO database was used for functional analysis and the KEGG database was used for pathway analysis. The GO database includes three major components: cells, biological processes, and molecular functions. For cell components, expression was mainly upregulated in mitochondria and downregulated in the cytoplasm, nucleus, and chromosomes (Figure 3A,3B). For molecular functions, enzyme binding, unfolded protein binding, and cytoskeletal protein binding were downregulated

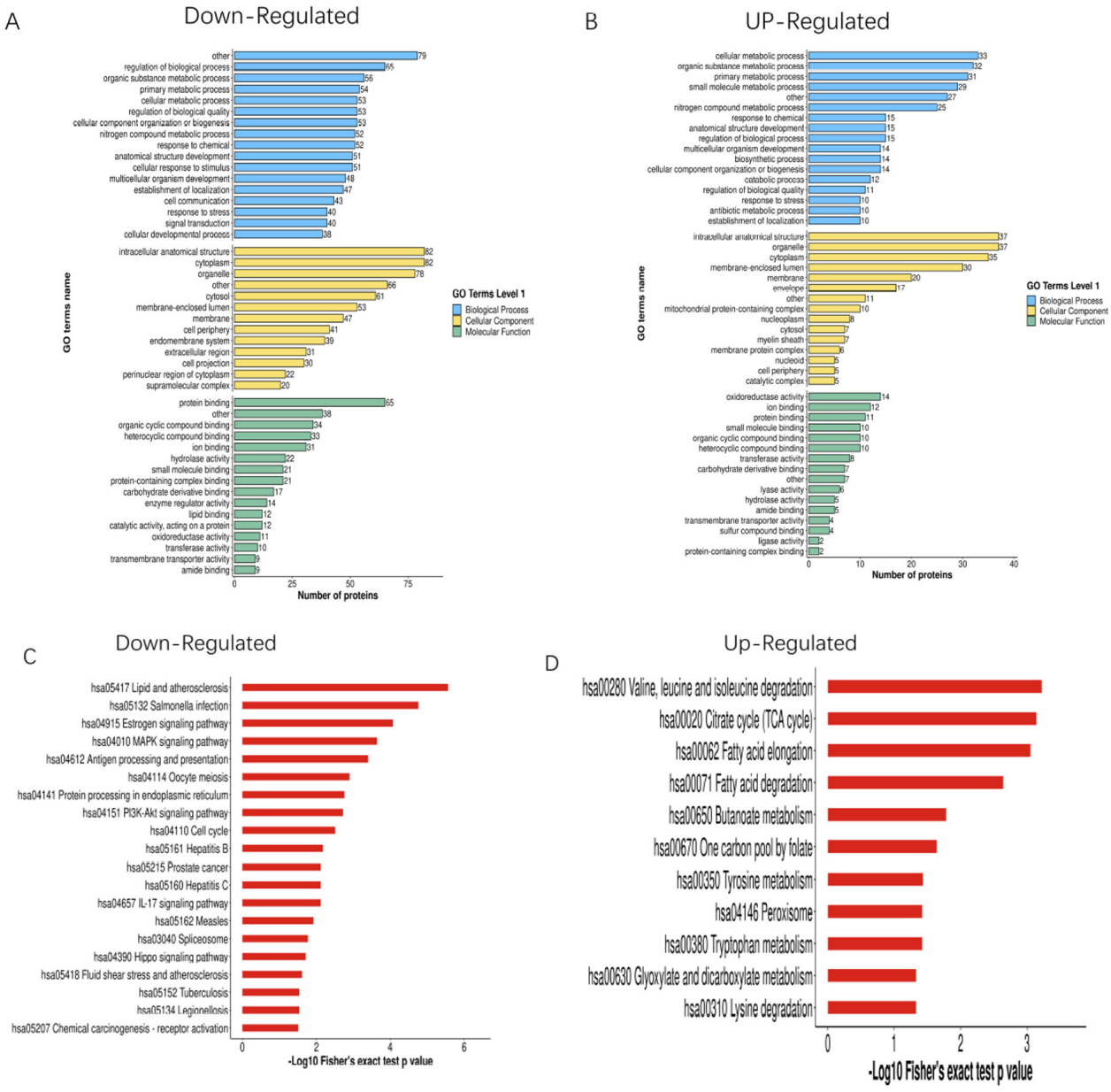


Figure 3. Functional analysis of lysine succinylation group in synovial fibroblasts of rheumatoid arthritis. (A) up- and (B) downregulated succinylated proteins were examined by GO functional enrichment; (C) up- and (D) downregulated succinylated proteins were examined by KEGG pathway analysis.

(Figure 3A), fatty acid acyl-CoA and fatty acid derivatives were bound, and acyl-CoA dehydrogenase activity was significantly upregulated (Figure 3B). For biological processes, metabolic processes, such as citrate metabolism, tricarboxylic acid metabolism, aerobic respiration, and fatty acid oxidation, were significantly upregulated (Figure 3B), while biological processes, such as protein localization in the nucleus, positive regulation of organelles, and regulation of cell cycle phase transition, were significantly downregulated (Figure 3A).

KEGG signaling pathway enrichment analysis showed that related metabolic processes, such as branched chain amino acids, the tricarboxylic acid (TCA) cycle, fatty acid extension and degradation, and one-carbon metabolism, were significantly enriched (Figure 3D), and the estrogen signaling pathway, the Mitogen-Activated Protein Kinase (MAPK) signaling pathway, endoplasmic reticulum protein processing, and cell cycle signaling pathway were significantly downregulated (Figure 3C).

3.4. Differentially expressed protein network interaction analysis

Protein–protein interaction (PPI) network analysis of succinylated groups was conducted. Upregulated genes in the PPI network were mainly mitochondrion-related metabolic enzymes, while the downregulated proteins were closely related to cytoskeletal assembly, protein synthesis, endoplasmic reticulum stress, and other processes. Expression of DUT, SHMT2 (nucleotide synthesis related), NNT, SDHA (mitochondrial respiratory chain related), HMGCL, IVD (leucine catabolic metabolism related), ACAD9, UQCRB (oxidative phosphorylation), IDH2, and CS (tricarboxylic acid cycle) were obvious among upregulated proteins (Figure 4). Thus, it is highly likely that *Sirt5* affects RA progression by affecting succinylation of these molecules.

4. Discussion

In this study, novel label-free proteomic quantitative technology was used to quantitatively analyze RA FLSs, and the potential mechanism of succinylation in the occurrence and development of rheumatoid arthritis was investigated by quantitative succinylation analysis after *Sirt5* silencing. Compared with the treatment group, 436 differentially expressed proteins with 1,548 succinylation sites were found. By GO and KEGG analysis of different succinylated proteins, it was found that high expression of succinylated proteins was mainly associated with mitochondria, suggesting that succinylation affects mitochondrial function. In addition, KEGG pathway enrichment analysis showed that the up-regulated pathway was mainly concentrated in mitochondria-related metabolism, such as branched-chain amino acid degradation, TCA cycle, fatty acid metabolism, *etc.* According to previous studies (*9*), high succinylation in brain injury after cerebral hemorrhage mainly affects

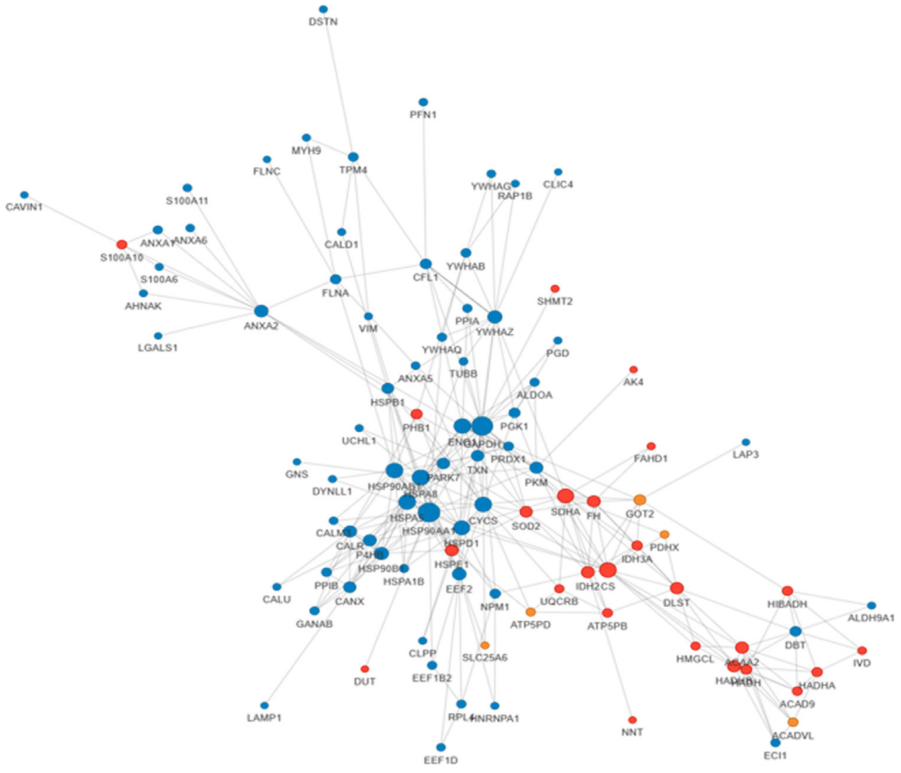


Figure 4. Protein–protein interaction network of succinylated proteins in synovial fibroblasts of rheumatoid arthritis.

fatty acid metabolism, and most of the subcellular localization of succinylation occurs in mitochondria. This suggests that succinylation affects disease development primarily by affecting mitochondria-related metabolism. Metabolic disorders involve almost all the pathogenesis stages of RA, and the humoral metabolome related to RA indicates that the disorder of RA metabolism may be related to maintenance of the inflammatory environment, which leads to an increased demand for biological energy and biosynthesis (*10*). This may require changes in mitochondrial metabolic pathways to meet the chronic inflammation of RA. The TCA cycle is the main productive pathway in mitochondria, and the raw materials required for the tricarboxylic acid cycle can be obtained through the degradation of branched-chain amino acids, which may be the reason for elevation of the two metabolic pathways after the succinylation of RA. Mitochondria play an important role in maintaining the normal life activities of cells. Dysfunction will lead to the release of inflammatory mediators and the aggregation of inflammatory cells, aggravate the inflammation of RA, inhibit the apoptosis of synovial cells, and promote the invasion of synovial cells (*11*).

Sirt5 is a desuccinylase. According to relevant literature reports, the expression of sirt5 is down-regulated in activated macrophages, which indicates that the decreased expression of *sirt5* may be related to the increase of RA and play a protective role in RA (*12*). Therefore, the molecules that are up-regulated in the succinylation data after the knockdown of sirt5 may play a role in RA. We will continue to study these molecules. Through PPI analysis, it was shown that upregulated proteins such as HSPE1, DUT, NNT, HMGCL, IDH2, IVD, ACAD9, UQCRB, SHMT2, SDHA, HADHA, HADHB and CS were all metabolic enzymes related to mitochondria. SHMT2, a binding protein of pyridoxal phosphate, plays an important role in catalytic catabolism of serine and promotes cancer cell proliferation (*13*). Succinylation activity of SHMT2 is regulated by *Sirt5* desuccinylase, and an increase in *Sirt5* expression downregulates SHMT2 enzymatic activity, thereby inhibiting cancer cell proliferation (*14*). IDH2 is a reduced nicotinamide adenine nucleotide phosphate (NADPH)-dependent enzyme, which catalyzes the conversion of isocitric acid into α-ketoglutaric acid through oxidative decarboxylation. NAPDH produced in this process is a cofactor, which maintains cell oxidation homeostasis, plays a role in maintaining cell REDOX function in cancer cell metabolism, and promotes tumor cell invasion by affecting mitochondrial dynamics (*15*). According to previous studies, sirt5 can promote IDH2 desuccinylation, reduce oxidative stress of cardiomyocytes, and maintain cell REDOX homeostasis (*16*). CS is a rate-limiting enzyme in the tricarboxylic acid cycle, and the enzyme activity of CS is regulated by succinylation. Previous studies have confirmed that *Sirt5* can desuccinylate CS at K393 and K395, thus affecting the proliferation and migration of colon cancer cells (*17*). SDHA is mainly involved in the TCA cycle and oxidative phosphorylation. Studies have shown that SDHA interacts with sirt5 to desuccinylate SDHA, and the desuccinylation of SDHA will weaken the interaction with SDH5 and promote the proliferation of renal cancer cells (*18*). Therefore, targeting these metabolism-related molecules has potential for research in rheumatoid arthritis.

The pathogenesis of rheumatoid arthritis is extremely complex. However, abnormal energy metabolism plays a major role in the process of rheumatoid arthritis (*19*). Mitochondria are the main sites of energy supply and participate in various metabolic pathways. They also play an important role in maintaining the internal environment of synovial cells (*20*). Studies of molecular interactions and functions in mitochondria contribute to understanding the pathogenesis of rheumatoid arthritis, and proteins with differential succinylation are likely to be potential biomarkers of rheumatoid arthritis.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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