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Comprehensive succinylome analyses reveal that hyperthermia upregulates lysine succinylation of annexin A2 by downregulating sirtuin7 in human keratinocytes

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ABSTRACT

Background and Objectives: Local hyperthermia at 44°C can clear multiple human papillomavirus (HPV)-infected skin lesions (warts) by targeting a single lesion, which is considered as a success of inducing antiviral immunity in the human body. However, approximately 30% of the patients had a lower response to this intervention. To identify novel molecular targets for anti-HPV immunity induction to improve local hyperthermia efficacy, we conducted a lysine succinylome assay in HaCaT cells (subjected to 44°C and 37°C water baths for 30 min). **Methods:** The succinylome analysis was conducted on HaCaT subjected to 44°C and 37°C water bath for 30 min using antibody affinity enrichment together with liquid chromatography-tandem mass spectrometry (LC-MS/MS). The results were validated by western blot (WB), immunoprecipitation (IP), and co-immunoprecipitation (Co-IP). Then, bioinformatic analysis including Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment, motif characterization, secondary structure, and protein-protein interaction (PPI) was performed. **Results:** A total of 119 proteins with 197 succinylated sites were upregulated in 44°C-treated HaCaT cells. GO annotation demonstrated that differential proteins were involved in the immune system process and viral transcription. Succinylation was significantly upregulated in annexin A2. We found that hyperthermia upregulated the succinylated level of global proteins in HaCaT cells by downregulating the desuccinylase sirtuin7 (SIRT7), which can interact with annexin A2. **Conclusions:** Taken together, these data indicated that succinylation of annexin A2 may serve as a new drug target, which could be intervened in combination with local hyperthermia for better treatment of cutaneous warts.

Key words: local hyperthermia, human papillomavirus, lysine succinylation, annexin A2, sirtuin7

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INTRODUCTION

Cutaneous wart, regarded as a benign

tumor, is a proliferative disease caused by human papillomavirus (HPV) infection.^[1]

The global prevalence of HPV infection

was 11.7%, while no completely effective treatment was determined.^[2,3] In the clinic, local hyperthermia at 44°C for 30 min could successfully clear cutaneous or anogenital warts as well as cervical HR-HPV infection. This treatment is characterized for the phenomenon that once the targeted lesion responded, other untargeted warts could fall off simultaneously.^[4-6] It can be predicted that hyperthermia plays a role in regulating antimicrobial immunity against HPV infection. However, approximately 30% of patients showed no response to this treatment. Potential mechanisms are required to be elucidated, which could provide a clue to find new drug targets and improve the treatment efficacy.

Lysine succinylation is a newly identified posttranslational modification (PTM) that describes the process of the transfer of succinyl group (-CO-CH₂-CH₂-CO₂H) from succinyl-CoA to the ε-amino group of lysine residues on the target protein in an enzymatic or nonenzymatic manner.^[7] Compared with several well-known PTMs, such as acetylation (42 Da) and methylation (14 Da), succinylation induces greater charge shifts (+1 to -1) and mass changes (100 Da) to the target proteins, indicating its greater potentials in cellular regulation.^[8,9] Recently, lysine succinylation has been reported in various biological processes, including reactive oxygen species (ROS) removal,^[10] inflammation,^[11] metabolism,^[11,12] autophagy,^[13] and immune response.^[14] A previous study showed that succinylation of mitochondrial antiviral signaling protein (MAVS) at lysine 7 could improve its activity in antiviral innate immunity by regulating retinoic acid-inducible (RIG-I)-like receptor (RLR)-mediated type I interferon (IFN) production.^[14] In addition, succinylation of pyruvate kinase isozyme type M2 (PKM2) at K311 could improve its transition from the cytoplasm to the nucleus, subsequently regulating the inflammatory response.^[11] However, whether lysine succinylation affects antimicrobial immunity to HPV infection is still unknown.

Succinylation, as a posttranslational modification, is typically regulated by catalyzing enzymes. Mammalian sirtuins (SIRT1 to SIRT7) are a family of seven proteins with a conserved nicotinamide adenine dinucleotide (NAD⁺)-dependent catalytic domain, which function as class III histone deacetylases (HDACs) in *Homo sapiens*.^[15] Sirtuins could remove the succinyl group from the lysine residue, which play roles as desuccinylases. Among these sirtuins, SIRT5 deficiency was confirmed to increase the level of succinylation on carbamoyl phosphate synthase 1 (CPS1) at K1291, suggesting that lysine succinylation (Ksucc) can be regulated by SIRT5 *in vivo*. *In vitro* studies have shown that SIRT5 desuccinylase activity is much higher than its deacetylase activity.^[16] SIRT7 is the only member that is primarily located in the nucleolus

with weak deacetylase activity.^[17] Recently, SIRT7 was found to act as a desuccinylase for H3K122 to regulate the chromatin remodeling process.^[18] As for SIRT3, the major mitochondrial protein deacetylase, recent evidence in cells has demonstrated that it may also be able to perform a desuccinylation reaction on histones.^[19] Carnitine palmitoyltransferase 1A (CPT1A) and lysine acetyltransferase 2A (KAT2A) possess succinyltransferase activities.^[20] CPT1A is a mitochondrial enzyme responsible for the formation of acyl carnitines.^[21] CPT1A has lysine succinyl-transferase (LSTase) activity both *in vivo* and *in vitro*.^[22] KAT2A, a member of the GCN5-related N-acetyltransferase (GNAT) superfamily, binds to succinyl-CoA and acts as a succinyltransferase for histone H3 on lysine 79.^[23]

In the present study, we carried out the first proteomic analysis of lysine succinylation in hyperthermia-induced HaCaT cells (subjected to 44°C and 37°C water baths for 30 min as control). A series of bioinformatic analyses were conducted to investigate the potential role of lysine succinylation in hyperthermia-mediated antimicrobial activities. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses demonstrated that 19 and 6 proteins related to immune processes and viral regulation were succinylated in response to heat treatment, respectively. Several lysine residues in annexin A2 were upregulated, while the protein level of desuccinylase SIRT7 was downregulated after hyperthermia. SIRT7 could directly interact with annexin A2, and the findings may provide novel insight into targeted drug design at the level of lysine succinylation that may potentially increase the cure rates or shorten the period of treatment of HPV infectious diseases. This study may improve our understandings of the mechanism underlying the breaking of microbial resistance as well as the successively elicited antimicrobial immunity induced by local hyperthermia in the skin.

MATERIALS AND METHODS

Cell culture and hyperthermia treatment

HaCaT cells were purchased from American Type Culture Collection (Maryland, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Biological Industries, Beit-Haemek, Israel), penicillin (100 U/mL), and streptomycin (100 µg/mL) in a 37°C, 5% CO₂ constant temperature incubator.

For hyperthermia treatment, cell culture plates were sealed with parafilm and placed carefully in a circulating water bath for 30 min at 37°C for controls or 44°C for hyperthermia treatment. After these treatments, the cells were returned to a 37°C incubator and cultured for 0–24 h.

Sample preparation

Cells in the control group (37°C) and the heat-treated group (44°C) were harvested and washed with ice-cold PBS twice. Samples were sonicated on ice using a high-intensity ultrasonic processor (Scientz, Ningbo, China) in lysis buffer. The remaining debris was discarded by centrifugation at $12,000 \times g$ at 4°C for 10 min. Finally, the protein concentration was determined with a BCA kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. Finally, trypsin (Promega, York, USA) was added at a 1:50 trypsin-to-protein mass ratio for the first digestion overnight and a 1:100 trypsin-to-protein mass ratio for a second 4 h digestion.

High performance liquid chromatography fractionation and affinity enrichment

The peptides were fractionated into 60 fractions by high pH reverse-phase high performance liquid chromatography (HPLC) using a Thermo Betasil C18 column (5 μ m particles, 10 mm ID, 250 mm length). Then, the peptides were combined into 6 fractions and dried by vacuum centrifugation.

The peptides dissolved in NETN buffer (100 mmol/L NaCl, 1 mmol/L ethylene diamine tetraacetic acid (EDTA), 50 mmol/L Tris-HCl, and 0.5% NP-40, pH 8.0) were incubated with prewashed pan-antisuccinyl antibody beads (PTM Biolabs, Hangzhou, China) at 4°C overnight. Then, the beads were washed with NETN buffer and H₂O. The bound peptides were eluted from the beads with 0.1% trifluoroacetic acid (TFA) and vacuum-dried.

Liquid chromatography-tandem mass spectrometry analysis

For liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, the resulting peptides were desalted with C18 ZipTips (Millipore, Mass, USA). The peptides were subjected to NSI source followed by tandem mass spectrometry (MS/MS) using Q Exactive™ Plus (Thermo Fisher Scientific, Mass, USA) coupled online to the UPLC.

Database search

The resulting mass spectrometry data were processed using the MaxQuant search engine v.1.5.2.8 (Max Planck Institute of Biochemistry, Munich, Germany). Tandem mass spectra were searched against the SwissProt Human database concatenated with the reverse decoy database. Carbamidomethyl on Cys was specified as a fixed modification, and succinylation on Lys was specified as a variable modification.

Bioinformatics analysis

GO annotation was obtained from the UniProt-GOA database ([www. http://www.ebi.ac.uk/GOA/](http://www.ebi.ac.uk/GOA/)),

InterProScan database (<http://www.ebi.ac.uk/interpro/>), and DAVID database (<https://david.ncifcrf.gov/>). The subcellular localization was analyzed by WoLF PSORT software. For the functional descriptions of the protein domain, the InterProScan database was used. Protein pathways were annotated by the KEGG database and KEGG online service tools KEGG mapper. Motif-x was used to predict specific Ksucc motifs. Analysis of secondary structure was conducted by NetSurfP v1.0 (DTU Health Tech, Lyngby, Denmark).

Isolated tissue and hyperthermia treatment

Fresh condyloma acuminata (CA) tissues were obtained from patients undergoing surgical treatment at the First Hospital of China Medical University. The lesion was equally divided into two parts and separately placed in cell culture dishes containing DMEM plus 10% fetal bovine serum. For hyperthermia management, the tissue was treated in the same way as HaCaT cells described above. Subsequently, tissue samples were fixed with paraformaldehyde 4 h after hyperthermia treatment and then used for immunohistochemical assays. This part of the study was approved by the Ethics Committee of the First Hospital of China Medical University (Approval No. 2018-135-2), and informed consent was obtained from the patients.

Western blotting

Cultured cell samples were treated with RIPA lysis buffer (Beyotime, Shanghai, China) containing protease inhibitors and phosphatase inhibitors (Beyotime, Shanghai, China) and centrifuged at $15,000 \times g$ at 4°C for 10 min. The supernatant was carefully aspirated, mixed with SDS-PAGE loading buffer after protein quantification by BCA assay, boiled at 100°C for 5 min, and then subjected to SDS-PAGE detection. After being transferred to a polyvinylidene difluoride membrane (PVDF), nonspecific binding was blocked with QuickBlock blocking solution (Beyotime, Shanghai, China) for 15 min. The blots were probed with the following primary antibodies: fatty acid synthase (FASN) antibody (Cell Signaling Technology, Mass, USA), KATA2 antibody (Cell Signaling Technology, Mass, USA), succinyl lysine antibody (PTM Biolabs, Hangzhou, China), SIRT5 antibody (Cell Signaling Technology, Mass, USA), SIRT3 antibody (Cell Signaling Technology, Mass, USA), SIRT7 antibody (Cell Signaling Technology, Mass, USA) and annexin A2 antibody (Proteintech, Wuhan, China).

Plasmid construction and transfection

SIRT7 overexpression plasmids were constructed by Shanghai Heyuan Biotechnology Co., Ltd. According to the protocols provided by the manufacturer, plasmid DNA was transfected with Lipofectamine 3000 Transfection Reagent (Thermo Fisher Scientific, Mass, USA), and cells

were harvested 48 h after transfection.

Co-immunoprecipitation

Cultured cells were lysed by immunoprecipitation (IP) buffer (0.25% sodium deoxycholate, 50 mmol/L Tris, 1 mmol/L EDTA, 1% Triton X-100, 1% NP-40, and 150 mmol/L NaCl) after washing twice with ice-cold PBS. The supernatants of digested cells were transferred into new EP tubes, mixed with primary antibody and magnetic beads (Bimake, Houston, USA), and incubated overnight on a rotator at 4°C. The next day, the magnetic bead particles were washed 5 times with cold IP buffer, and then protein loading buffer was added and boiled at 100°C for 5 min.

Immunohistochemical analysis

Immunohistochemical (IHC) staining was performed to detect the expression of SIRT7 in CA tissues after *in vitro* hyperthermia. Briefly, CA tissues (hyperthermia-treated group or controls) were fixed with 4% paraformaldehyde and then embedded in paraffin. Tissue sections were prepared for antigen retrieval with 0.01 mol/L sodium citrate antigen retrieval solution in a pressure cooker for 2 min. Endogenous peroxidase activity was inactivated using 3% H₂O₂. Then, nonspecific interactions were blocked with 10% goat serum. SIRT7 antibody at a dilution of 1:200 was incubated with the slides overnight at 4°C. The following steps were performed according to the protocols of the EliVision kit (Maxim, Fuzhou, China). The slides were examined using light microscopy (Olympus IX51, Tokyo, Japan).

Statistical analysis

Two-tailed Fisher's exact tests were applied to GO, KEGG, and protein domain enrichment analyses. Categories with a corrected *P* value < 0.05 were considered significant.

RESULTS

Proteome-wide analysis of succinylation proteins and sites identified in HaCaT cells.

Hyperthermia clears multiple remote cutaneous warts by targeting a single lesion through eliciting anti-HPV immune responses.^[24] The clinical treatment of children's perianal warts showed that local hyperthermia removed multiple lesions by treating one place of lesions (Figure 1A). Lysine succinylation is a novel PTM and has already been confirmed to be involved in multiple cellular processes. Here, we conducted a comprehensive succinylome in HaCaT cells (both the control and hyperthermia-treated groups) and identified 387 proteins with 969 succinylated lysine sites altogether (Figure 1B), of which 119 proteins with 197 lysine sites were upregulated in response to hyperthermia treatment (44°C) (Figure 1C).

The peptides contained different numbers of succinylated sites varying from 1 to 21. Of all succinylation proteins identified in our data, 51.9% (201/387) had a single site, 18.6% (72/387) contained two succinylated sites, 10.6% (41/387) possessed three modified sites, and 3.6% (14/387) had 10 or more succinylation peptide sites (Figure 1D).

Among the 387 identified proteins, the most extensively succinylated was HSPD1, with 21 modified lysine sites. In addition, endoplasmic (HSP90B1), alpha-enolase (ENO-1), annexin A2, and serine beta-lactamase-like protein (LACTB) contained 14, 12, 10, and 7 succinylated sites, respectively.

Functional annotation and enrichment analysis of identified succinylated proteins

To investigate the potential roles of succinylated proteins in HaCaT cells, we annotated all the identified proteins (387) using gene ontology (GO) functional characterization based on biological process (BP), cellular component (CC), and molecular function (MF) (Figure 2A). In terms of the biological process analysis, the two largest categories were related to cellular process (345 proteins) and metabolic process (284 proteins). Notably, 155 and 46 succinylated proteins were involved in response to stimuli and immune system processes, indicating that succinylated proteins may play a role in the immune response to exogenous stimuli. Within the molecular function category, the two largest groups belonged to binding (230 proteins) and catalytic activity (207 proteins), suggesting that lysine succinylation may alter the structure and functional activity of modified proteins.

The subcellular localization may provide a clue for predicting protein function.^[25] We found that most succinylated proteins were in the mitochondria (47.29%), followed by the cytoplasm (29.46%) and nucleus (8.01%) (Figure 2B). All the above results indicated that succinylated proteins were distributed extensively and affected various cellular processes in HaCaT cells.

The KEGG enrichment analysis showed that succinylated proteins were more likely to be enriched in the thermogenesis, carbon metabolism, oxidative phosphorylation, glycolysis/gluconeogenesis, and fatty acid metabolism pathways (Figure 2C). It can be predicted that lysine succinylation of proteins may influence the thermal process and energy metabolism in HaCaT cells.

Motif analysis and structural properties of identified succinyl proteins

To identify the specific succinyl-peptide patterns, we characterized the frequencies of position-specific amino acids flanking succinyllysine (10 amino acids upstream

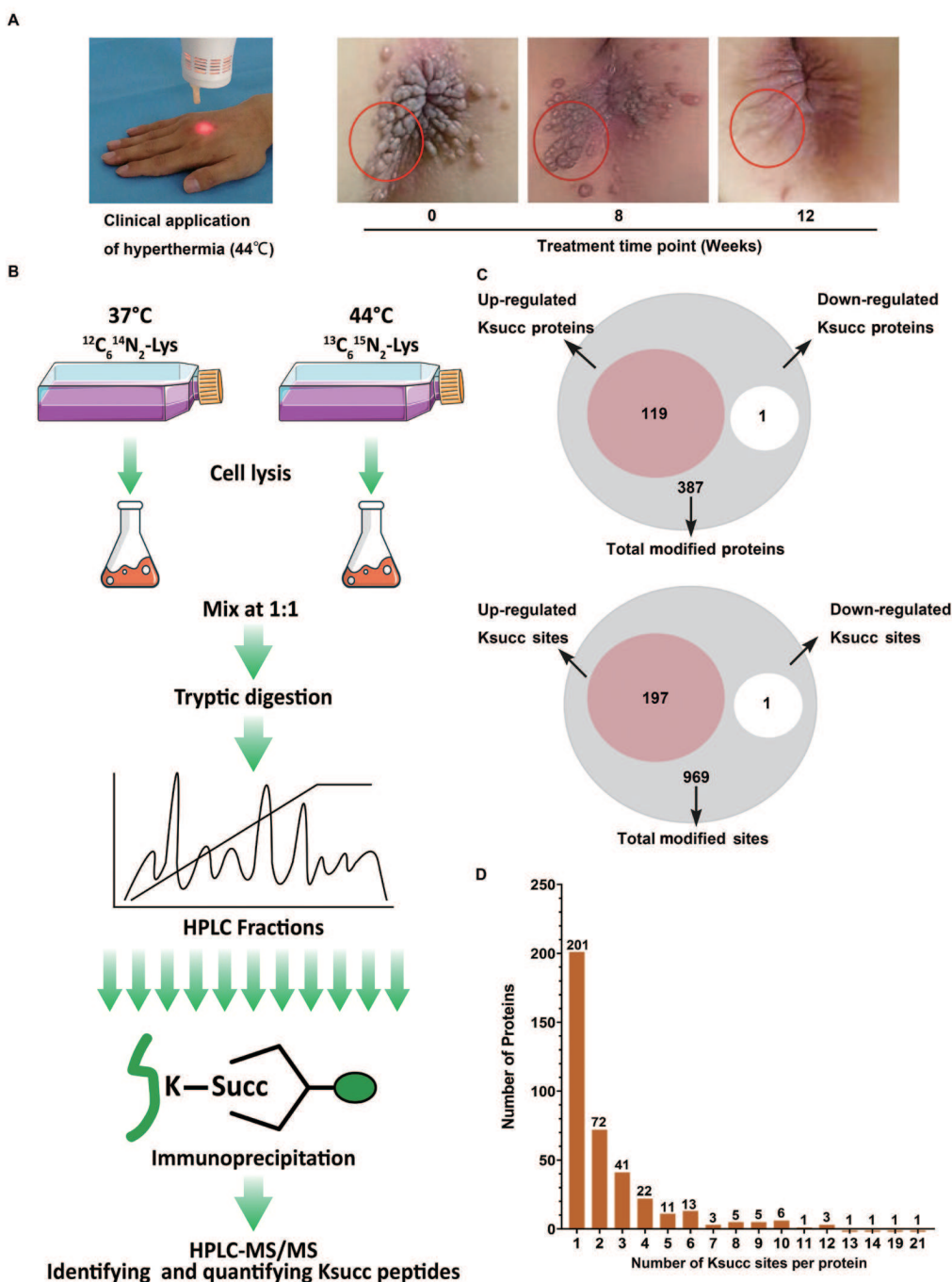


Figure 1: The clinic treatment of cutaneous warts and total profiling of lysine succinylome in HaCaT cells after response to hyperthermia. (A) Local hyperthermia treatment in children’s perianal warts. The red circle is therapeutic target for multiple verrucous lesions. (B) Experimental workflow for the identification of succinylated proteins and sites in HaCaT cells (with or without 44°C treatment). (C) Quantification of succinylated sites of HaCaT cells in response to hyperthermia. (D) Distribution of succinylation proteins based on their number of succinylated sites. Ksucc: lysine succinylation; MS/MS: mass spectrometry.

and downstream of the centered Ksu site) by the Motif-X program. Two preferred motifs, I* Ksu and V* Ksu, were identified (* indicates a random amino acid residue and Ksu indicates a succinylated lysine site). Nearly 84 and 94 succinylated peptides possessed these two motifs, respectively (Figure 2D). Surrounding succinylated lysine

(K) at sites –1 and –2, the basic amino acids arginine (Arg) and another K were less frequent. Four residues, alanine (Ala), aspartic acid (Asp), isoleucine (Ile), and valine (Val), were commonly preferred around the modified lysine (Figure 2E).

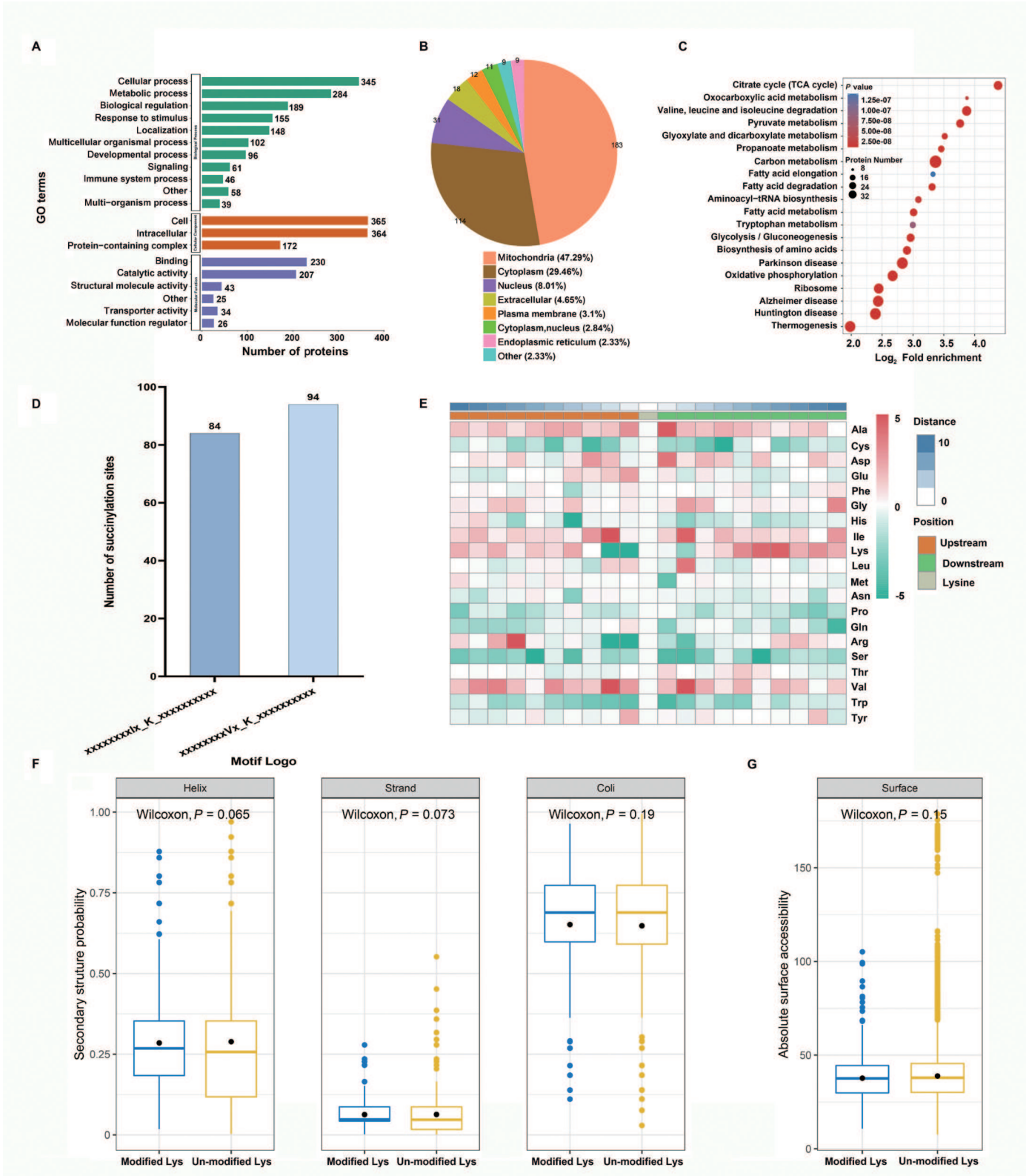


Figure 2: Functional enrichment analysis of upregulated Ksucc proteins and structural analysis of identified succinyl proteins in HaCaT cells in response to hyperthermia. (A) GO characterization of succinylated proteins based on BP, CC, and MF. (B) The subcellular location of succinylated proteins. The enrichment analysis of succinylproteins in terms of (C) KEGG pathway. (D) Number of succinylation peptides in each motif. (E) Heat map of the amino acid surrounding succinyl-lysine. Properties of modified lysine based on (F) secondary structure probability and (G) absolute surface accessibility. Helix: alpha helix; Strand: beta-strand; Coil: coiled coil; BP: biological process; CC: cellular component; MF: molecular function; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

Table 1: Differentially expressed Ksucc proteins related to both antiviral and immune process

Protein accession	Protein description	Gene name	Position	heat/control ratio	heat/control P value	Regulation
P07355	Annexin A2	<i>ANXA2</i>	K47	1.516	0.000415	Up
			K152	1.533	0.000537	Up
			K227	1.545	0.00537	Up
			K81	1.521	0.0113	Up
P11142	Heat shock cognate 71 kDa protein	<i>HSPA8</i>	K257	1.596	0.00327	Up
			K108	1.633	0.00462	Up
			K246	1.580	0.0107	Up
			K597	1.540	0.0198	Up
			K319	2.005	0.0383	Up
Q03135	Caveolin-1	<i>CAV1</i>	K26	1.698	0.0136	Up

Table 2: Top 30 upregulated succinylated sites

Protein accession	Protein description	Gene name	Position	heat/control ratio	heat/control P value	Regulation
P83111	Serine beta-lactamase-like protein, mitochondrial	<i>LACTB</i>	K266	7.357	0.000347	Up
			K243	4.750	0.0003	Up
			K252	4.626	0.00783	Up
			K259	4.384	0.0236	Up
			K283	3.267	0.0291	Up
P41250	Glycine-tRNA ligase	<i>GARS</i>	K284	3.204	0.000126	Up
			K219	2.414	0.000158	Up
P07954	Fumarate hydratase, mitochondrial	<i>FH</i>	K204	2.404	0.0403	Up
			K66	2.247	0.000822	Up
P34897	Serine hydroxymethyltransferase, mitochondrial	<i>SHMT2</i>	K61	2.204	0.0000278	Up
			K302	2.101	0.00186	Up
P60709	Actin, cytoplasmic 1	<i>ACTB</i>	K326	2.020	0.00147	Up
P11142	Heat shock cognate 71 kDa protein	<i>HSPA8</i>	K319	2.005	0.0383	Up
P23284	Peptidyl-prolyl cis-trans isomerase B	<i>PPIB</i>	K67	1.968	0.0218	Up
P41250	Glycine-tRNA ligase	<i>GARS</i>	K224	1.956	0.00424	Up
			K646	1.911	0.00557	Up
P34897	Serine hydroxymethyltransferase, mitochondrial	<i>SHMT2</i>	K181	1.856	0.00617	Up
P23284	Peptidyl-prolyl cis-trans isomerase B	<i>PPIB</i>	K41	1.843	0.00162	Up
P60709	Actin, cytoplasmic 1	<i>ACTB</i>	K50	1.816	0.0373	Up
Q9Y6N5	Sulfide:quinone oxidoreductase, mitochondrial	<i>SQRDL</i>	K365	1.796	0.000387	Up
P23284	Peptidyl-prolyl cis-trans isomerase B	<i>PPIB</i>	K209	1.729	0.000408	Up
P55084	Trifunctional enzyme subunit beta, mitochondrial	<i>HADHB</i>	K254	1.728	0.00428	Up
P23284	Peptidyl-prolyl cis-trans isomerase B	<i>PPIB</i>	K129	1.727	0.00197	Up
P07954	Fumarate hydratase, mitochondrial	<i>FH</i>	K94	1.717	0.00153	Up
Q9Y6N5	Sulfide:quinone oxidoreductase, mitochondrial	<i>SQRDL</i>	K125	1.696	0.0022	Up
P07954	Fumarate hydratase, mitochondrial	<i>FH</i>	K122	1.685	0.0034	Up
P60709	Actin, cytoplasmic 1	<i>ACTB</i>	K113	1.685	0.00944	Up
P34897	Serine hydroxymethyltransferase, mitochondrial	<i>SHMT2</i>	K469	1.674	0.00315	Up
P30101	Protein disulfide-isomerase A3	<i>PDIA3</i>	K494	1.655	0.0044	Up
P55084	Trifunctional enzyme subunit beta, mitochondrial	<i>HADHB</i>	K272	1.643	0.0499	Up

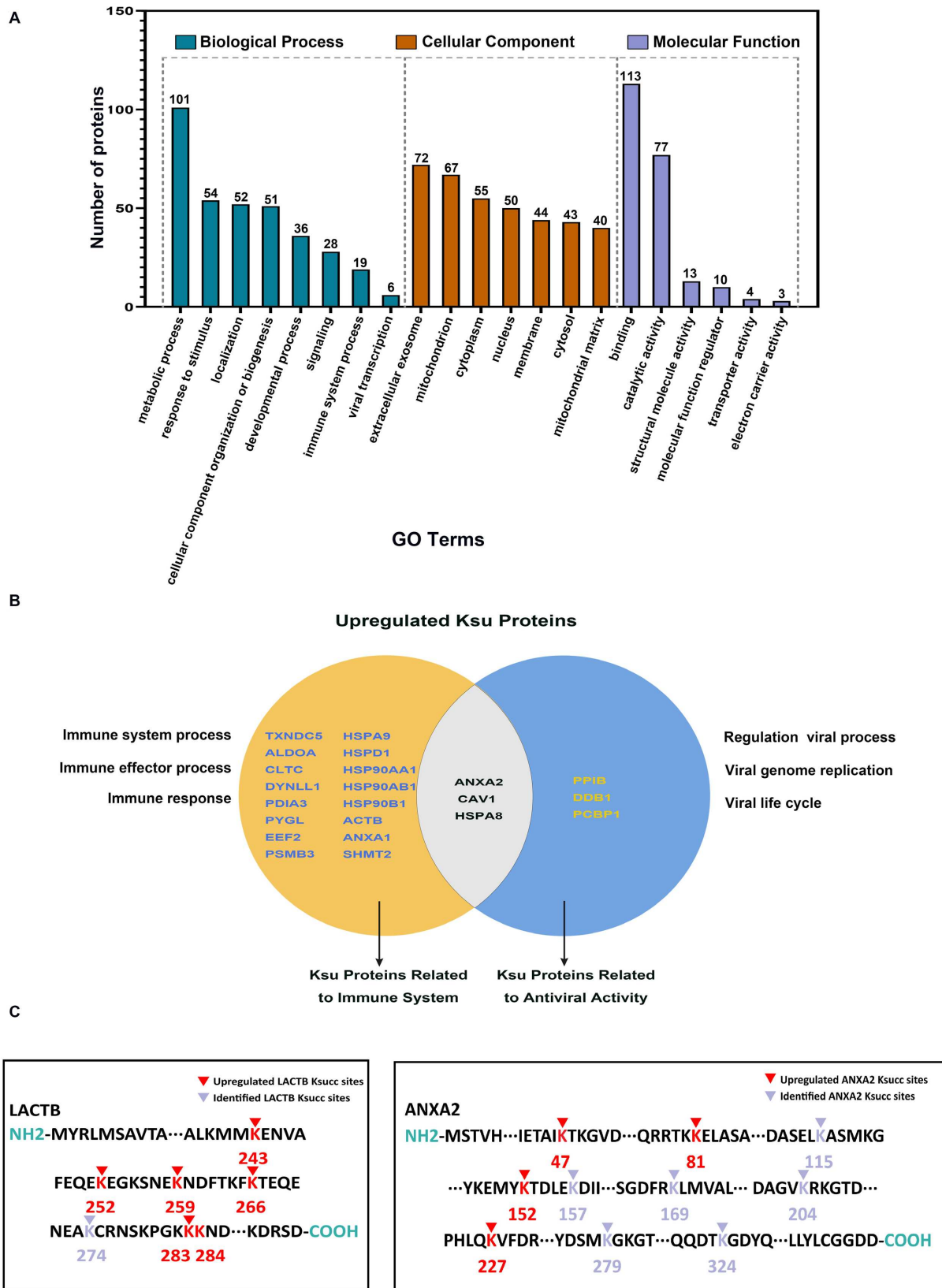


Figure 3: Characterization description of differentially succinylated proteins in response to hyperthermia in HaCaT cells. (A) Functional annotation of differentially succinylated proteins in response to hyperthermia treatment. (B) The integration of upregulated succinylated proteins in immune system process and viral transcription. (C) The Ksucc sites analysis of annexin A2 and LACTB. GO: Gene Ontology; LACTB: serine beta-lactamase-like protein.

Furthermore, we analyzed the secondary structure of the identified succinylation proteins to investigate whether lysine succinylation had a tendency for specific structural regions. Approximately 34.8% succinylated sites were detected in the ordered secondary structure. Among them, 28.5% were found in the α -helix, and 6.3% were in the β -sheet. The remaining sites (65.2%) were located at the regions of random coils, indicating that succinylated peptides are more frequently occurred in the unstructured regions of the protein (Figure 2F). In addition, compared with all lysine residues, succinylated sites did not show moderate bias for the protein surface (Figure 2G).

Functional annotation of differentially succinylated proteins in response to hyperthermia treatment

To determine the potential role of lysine succinylation in HaCaT cells in response to hyperthermia treatment, all upregulated succinylated proteins (119 proteins with 197 sites) were submitted to GO analysis (Figure 3A). Based on the classification of MF, the most of succinylation proteins belonged to binding and catalytic activity. Within the CC category, most proteins were related to extracellular exosomes, mitochondria, and cytoplasm, showing that succinylated proteins existed broadly in HaCaT cells. In terms of BP, proteins connected with metabolic process (101 proteins) and response to stimulus (54 proteins) were more likely to be succinylated, which was consistent with the CC analysis that most proteins were in metabolism-related regions. In addition, 19 and 6 proteins were involved in the immune system process and viral transcription, respectively, indicating that the treatment effect of hyperthermia for HPV-infected skin warts could be achieved by regulating lysine succinylation on these proteins.

Moreover, three succinylated proteins, annexin A2, caveolin

1 (CAV1), and HSPA8, were involved in both the immune system process and viral transcription (Figure 3B). Among them, annexin A2 possessed four upregulated succinylated lysine sites (K47, 81, 152, 227) (Figure 3C, Table 1).

We sorted the proteins according to the upregulation ratio of succinylation sites in response to hyperthermia treatment. Among them, LACTB, mitochondria ranked first, and multiple sites were upregulated (K243, 259, 266, 283, 284) by more than 3 times (Figure 3C, Table 2).

Succinylation of global proteins was affected by hyperthermia and showed an obvious increase in annexin A2 and LACTB proteins after heat treatment

To verify the results of proteome analysis, we examined the succinylation of global proteins at different time points in HaCaT cells after hyperthermia treatment. The western blot results showed that the succinylation level of global proteins increased at 30 min after hyperthermia and gradually recovered to untreated levels at 6 h (Figure 4A).

According to proteome analysis, LACTB, annexin A2, and FASN were the most evident molecules in the upregulation ratio of succinylation. Therefore, their expression was also verified by WB. The detection of protein levels showed that LACTB and FASN were not significantly changed in HaCaT cells after hyperthermia, but annexin A2 was decreased (Figure 4B). The succinylation of LACTB, FASN, and annexin A2 was examined by co-immunoprecipitation prior and post to hyperthermia (after 4 h). The data showed that the succinylation of LACTB and annexin A2 was significantly increased after hyperthermia compared to that of the untreated group, which was consistent with the results of our succinylome analysis (Figure 4C). However, the succinylation of FASN

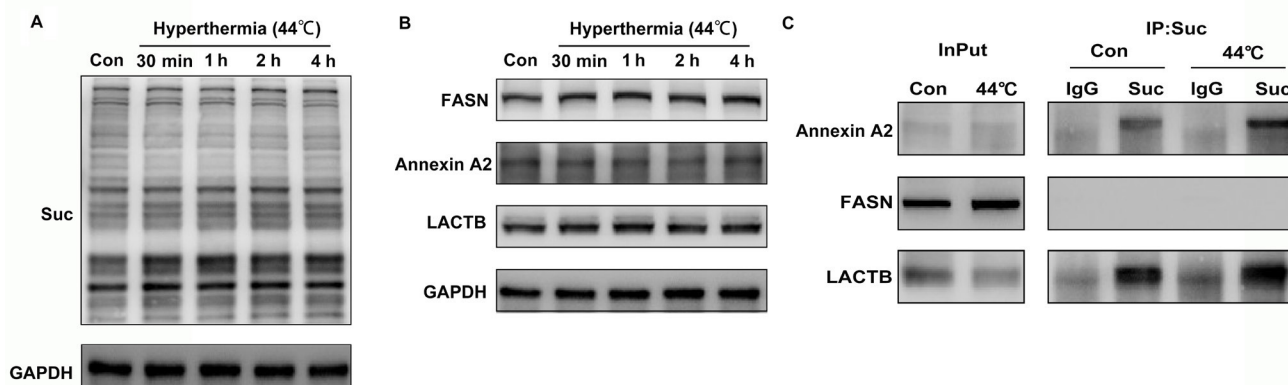


Figure 4: Hyperthermia increase the succinylation levels of total proteins in HaCaT cells. (A) Western blot analysis of the succinylation of total proteins in HaCaT cells in different time points after hyperthermia. (B) Western blot analysis of the expression of LACTB, FASN, and annexin A2 in HaCaT cells in response hyperthermia. (C) Co-immunoprecipitation analysis of the succinylation of LACTB, FASN, and annexin A2 before and after hyperthermia. Suc indicates the lysine succinylation. FASN: fatty acid synthase; LACTB: serine beta-lactamase-like protein; IgG: immunoglobulin G; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

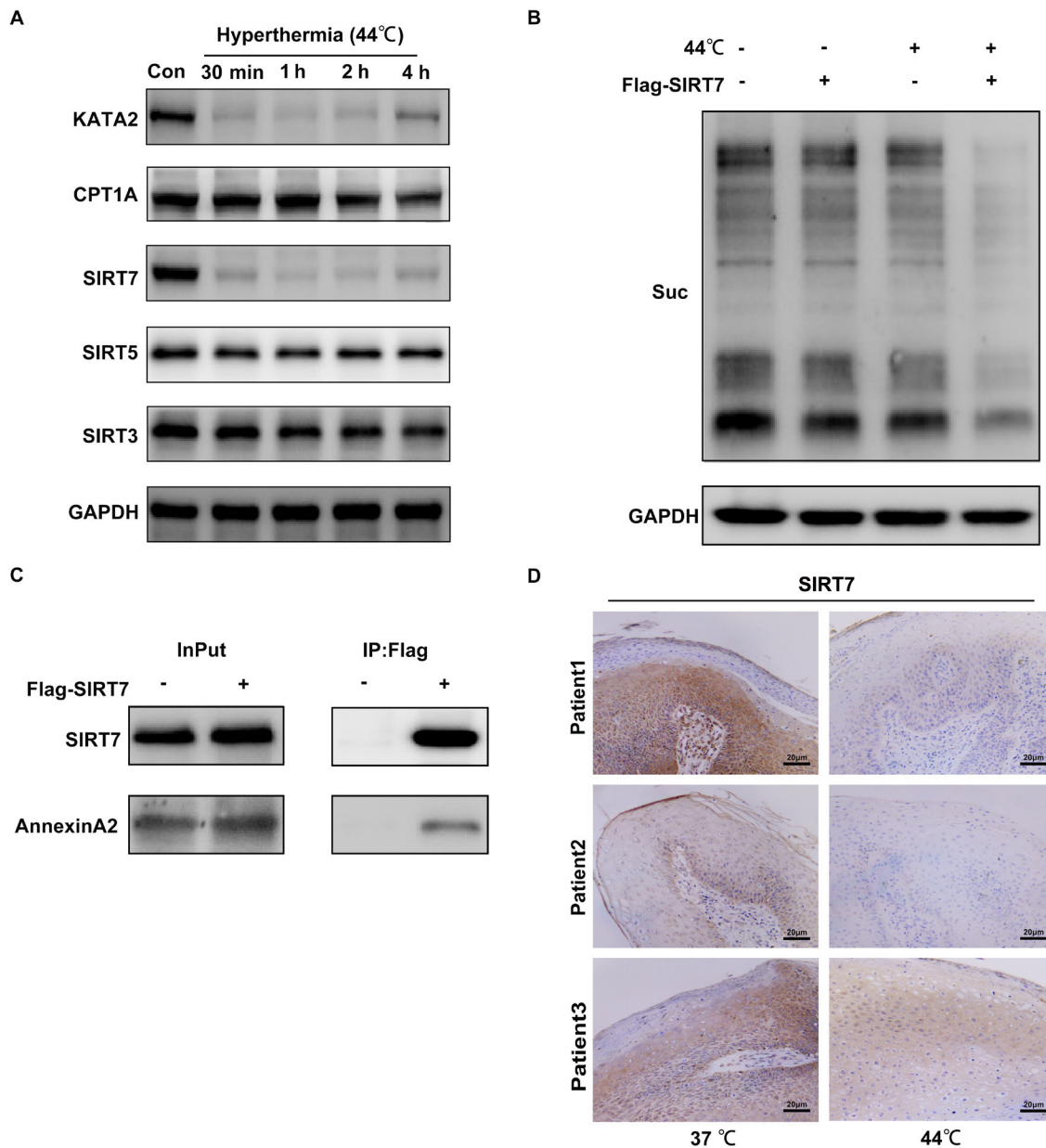


Figure 5: Hyperthermia influencing the succinylation of global proteins by downregulating SIRT7 in HaCat cells. (A) Western blot analysis of the expression of succinylated enzymes after hyperthermia. (B) Western blot analysis of the succinylation of global proteins in overexpression SIRT7. (C) Co-immunoprecipitation analysis of the interaction of SIRT7 with LACTB, FASN, and annexin A2. (D) The immunohistochemical staining of SIRT7 in condyloma acuminatum tissues. Suc indicates the lysine succinylation. - means empty vector plasmid. + means SIRT7 plasmid. Original magnification: 200×. FASN: fatty acid synthase; SIRT7: sirtuin7; LACTB: serine beta-lactamase-like protein; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

was not detected, and it is possible that FASN did not undergo succinylation in HaCaT cells.

Hyperthermia affects succinylation by influencing the expression of SIRT7

First, we examined the expression levels of succinylated enzymes after 44°C hyperthermia treatment. The results indicated that KATA2 and SIRT7 were apparently decreased in expression compared with nonheated cells (Figure 5A). Specifically, KATA2 and SIRT7 were highly

decreased at 1 h after heat treatment, gradually began to recover at 4 h, and finally returned to the base level at 12 h (Supplementary Figure 1).

To explore the possible reasons for hyperthermia influences on the succinylation of HaCaT cells, SIRT7 overexpression plasmids were constructed. We observed that SIRT7 affected the succinylation of global proteins in HaCaT cells, and succinylation was lowered in the SIRT7 overexpression group than that in the control group (transfected vector

plasmid), and this difference was more significant in cells treated with hyperthermia (Figure 5B).

HPV was shown to interact with annexin A2 at both the extracellular plasma membrane and endocytic vesicles, indicating the role of annexin A2 as a receptor for HPV infection, especially in virus attachment, internalization, and trafficking.^[26–28] To investigate whether SIRT7 might be the regulatory enzyme of annexin A2, co-immunoprecipitation of SIRT7 with annexin A2 was performed, and the results showed that SIRT7 interacted with annexin A2 (Figure 5C).

Hyperthermia downregulates the expression of SIRT7 in CA tissues

Immunohistochemical staining of CA tissue samples showed that SIRT7 was reduced in heat-treated tissue samples as compared with untreated samples (Figure 5D). This result was consistent with the *ex vivo* experiments in HaCaT cells. In summary, our results implied that hyperthermia reduces the expression of SIRT7 in CA tissues, which in turn affects the succinylation levels of HPV infection-related molecules.

DISCUSSIONS

In this work, we explored the impact of hyperthermia therapy on the succinylation level and its possible role on antiviral immunity. The succinylome assay identified 119 proteins with 197 lysine sites that were upregulated in response to hyperthermia treatment. Proteomics analysis showed that differential succinylation proteins were related to antimicrobial resistance and changes in bioresponsiveness, indicating that lysine succinylation could participate in antiviral defense activities.

PTMs are important components regulating microbial resistance and immunity.^[29,30] New emerging evidences support that PTMs serve as interfaces between viruses, restriction factors, and cellular defense mechanisms, providing potential drug targets for future immunoregulatory therapy.^[31] Many restriction factors of viruses, such as IFITM3/2, APOBEC, and SAMHD1, can be subjected to phosphorylation.^[32–34] Moreover, ubiquitination constitutes a main regulatory mechanism allowing viruses to evade the action of restriction factors.^[35–37] Although it is well documented that ubiquitination and phosphorylation are increasingly associated with antiviral responses, the role of succinylation and other modifications in antiviral activity are still poorly understood.^[38]

SIRT7 exhibits desuccinylase activity in histones and maintains chromatin compaction and genome stability.^[18] The expression of SIRT7 was significantly decreased after hyperthermia. SIRT7 reduced the succinylation of

global proteins. It was implied that hyperthermia regulated the succinylation of overall proteins through SIRT7 in HaCaT cells. Meanwhile, decreased expression of SIRT7 was observed in condyloma acuminata tissues after hyperthermia. The pathological characteristics of warts were hyperplasia of the dermis and epidermis,^[39] which could be regarded as benign tumors. A study showed that SIRT7 was significantly increased in highly proliferating tissues and a variety of human cancer cells.^[40] Hyperthermia was likely to inhibit HPV-induced hyperplasia of epidermal cells by decreasing SIRT7.

Although no relationship has been discovered between HPV and SIRT7 according to current research, the latest studies have shown that SIRT1, the same family protein of SIRT7, is closely related to the life cycle and replication of HPV. TopBP1 is the pivotal binding partner of E2, and it is essential for E1-E2 replication and the viral life cycle. SIRT1 deacetylates the TopBP1 protein, which can lead to the functional switch from DNA replication to protein repair and cell cycle arrest.^[41] In addition, SIRT1 directly regulates E1-E2-mediated DNA replication of HPV16 through PTMs. SIRT1, as a component of the E1-E2 DNA replication complex, is carried into the viral genome through the E1-E2 protein during replication and plays a stabilizing role in the E1-E2 complex.^[42] Like SIRT1, SIRT7 could also have an impact on antimicrobial immunity and other bioresponsive factors by influencing succinylation, possibly. Hence, hyperthermia might improve antimicrobial immunity by regulating SIRT7.

Annexin A2 is a multicompartamental protein whose functions are generally influenced by subcellular localization. It often exists as a monomer and participates in various cellular processes, including endocytosis,^[43] exocytosis,^[26] cytoskeletal rearrangement,^[44] and membrane trafficking through lipid microdomain formation.^[45] On the plasma membrane, it can form a heterotetrametric complex with S100A10.^[46] Several studies have reported that heat stress could stimulate cell surface translocation by phosphorylating annexin A2 at the tyrosine 23 site through Src-like tyrosine kinases.^[47,48] Moreover, HPV has been reported to result in Tyr23 phosphorylation and extracellular translocation of annexin A2 to the outer plasma membrane by inducing EGFR-dependent Src kinase activation.^[26] We found that the succinylation of annexin A2 was significantly increased after hyperthermia. It was suggested that succinylation could influence the interaction of annexin A2 with HPV as phosphorylation. Phosphorylation and extracellular translocation of annexin A2 allow HPV16 to interact with annexin A2 and S100A10 as heterotetramers, causing HPV16 to enter host cells.^[26] Moreover, annexin A2, A2t (composed of two annexin A2 monomers), and S100A10 control the progression of

HPV infection from early endosomes to late multivesicular endosomes, inhibits capsid decomposition, and formed complexes with CD63.^[27] Hence, succinylation may play regulatory roles in annexin A2-assisted virus entry into host cells. Therefore, the Ksucc sites of annexin A2 may be a potential target for drug design against HPV infection.

However, there are currently no suitable in-vivo models with intact immune functions for HPV infection; moreover, cervical cancer cell lines that contain HPV genome copies are also inadequate for their different epidermal origins, so we found it difficult to tell a appropriate story of succinylation regarding hyperthermia therapy with the use of current available models. In this study, alterations in succinylation were detected by co-immunoprecipitation in LACTB, annexin A2, and FASN due to the lack of site-specific succinylated antibodies for these molecules. In the next step, we will design the site-specific antibodies of succinylation for more comprehensive and in-depth research.

CONCLUSIONS

In this study, we observed the effect of hyperthermia on succinylation through succinylation proteomics and preliminarily explored the possible mechanisms of succinylation in virus defense and antimicrobial immunity. Pan-proteome-wide succinylation was upregulated in HaCaT cells in response to hyperthermia. After hyperthermia, the desuccinylase SIRT7 was decreased in HaCaT cells and condyloma acuminatum tissues, which implied that hyperthermia was likely to affect succinylation by downregulating SIRT7. Subsequent functional analysis indicated that differential proteins were involved in viral replication, life cycle regulation, and the immune system, especially annexin A2, a key molecule for HPV endocytosis. In summary, this study was the first to explore the relevance of succinylation with hyperthermia in an antimicrobial immunity context, and the promising results suggested that lysine succinylation sites of annexin A2 possess huge potential available to drug targets for eliminating HPV in hyperthermia treatment of cutaneous warts.

Availability of Data and Materials

The datasets generated and analyzed during this study are available from the corresponding author on reasonable request.

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Ethics Approval and Consent to Participate

The study was approved by the Ethics Committee of the First Hospital of China Medical University (Approval No. 2018-135-2), and informed consent was obtained from the patients.

Conflict of Interest

None declared.

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