Original Article

Proteome-wide identification and functional analysis of ubiquitinated proteins in Hepa1-6 cells by knockdown of E3 ubiquitin ligase SIAH1

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Background: Hepatocellular carcinoma (HCC) is an aggressive malignancy that poses a serious threat to human life. The conventional therapies for HCC cannot substantially improve overall survival (OS), disease duration, and prognosis. Therefore, it is important to study the underlying mechanism of HCC and seek better methods for HCC prevention and treatment. Ubiquitination is a post-translational modification that modulates great cellular function by cooperating with E1, E2, and E3 ligases. Yet, the ubiquitination and lysine residues in HCC are still elusive. Seven in absentia homolog 1 (*SIAH1*), as an important E3 ubiquitin ligase, regulates ubiquitin-mediated proteolysis to function as a tumor suppressor in HCC. In the present study, we downregulated *SIAH1* in the mouse HCC cell line Hepa1-6 and studied its function by using proteome-wide identification.

Methods: *SIAH1* was knocked down by *SIAH1* short hairpin RNA (shRNA) in mouse HCC cell line Hepa1-6 cells, and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was conducted to analyze the ubiquitinated proteins. Functional analysis was performed using Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) enrichment.

Results: The systematic profiling showed a total of 550 differently expressed proteins (DEPs), including 263 upregulated DEPs and 287 downregulated DEPs. Considering the amino acid sequences around the modified lysine residues, seven proteins were identified as conserved ubiquitination motifs in the peptides. The ubiquitinated proteins were mainly distributed in the cytoplasm, nucleus, and plasma membrane. Functional analysis suggested that the ubiquitinated proteins were mostly enriched in the nucleus, cytoplasm, and extracellular space; in addition, the ubiquitinated proteins were mostly attributed to the protein binding, and disease. The ubiquitinated proteins modulate HCC by mapping lysine modification sites.

Conclusions: The use of high-throughput characterization to identify novel and specific targets associated with *SIAH1* is of great significance in terms of functional weight. The results obtained in this paper from the

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analysis of proteomic data provided novel insights into ubiquitination regulation in HCC, which pave the way for further research and mechanism discovery of HCC.

Keywords: Hepatocellular carcinoma (HCC); *SIAH1* knockdown; ubiquitination

Submitted Jan 30, 2023. Accepted for publication Oct 20, 2023. Published online Jan 29, 2024.
doi: 10.21037/tcr-23-124
View this article at: https://dx.doi.org/10.21037/tcr-23-124

Introduction

Hepatocellular carcinoma (HCC) is an aggressive malignancy with an increasing mortality rate around the world; it represents a growing cause for concern. Infection of hepatitis B and C viruses, primary hemochromatosis, nonalcoholic fatty liver disease, and prolonged exposure to aflatoxin B1 have been reported as the risk factors for HCC (1). The conventional therapies for HCC include surgery, radiotherapy, and chemotherapy, but the overall survival (OS) rate and duration have not yet been improved. In recent years, even though the novel therapeutic modalities, such as immunotherapy, and targeted therapy, have been put forward, the prognosis for advanced HCC has remained poor. Therefore, it is important to study the pathogenesis of HCC and look for better methods for HCC prevention and treatment.

Ubiquitin is a group of small proteins consisting of 76 amino acids with a C-terminal diglycine tail (2). Ubiquitination is one kind of critical post-translational modifications, and plays a vital role in the cellular function by controlling the protein load in eukaryotic cells (3,4). Mechanically, the ubiquitination pathway exerts its biological function by cooperating with E1, E2, and E3 ligases in a 3-step cascade reaction (3,5). Explicitly, the C-terminal carboxyl is activated by ubiquitin-activation enzyme E1 and then binds a second ubiquitin under the help of ubiquitin-conjugating enzymes E2, and finally, the ubiquitin-protein ligases E3 mediates the transfer of ubiquitin from E2 to the ε-amino group of lysine residues (6-8). The E1-E2-E3 modification regulates a range of protein hydrolysis and signal transduction processes (9), among which E3 ubiquitin ligase has an important role as it can specifically recognize substrates among the ubiquitination modifications.

The seven in absentia homolog (*SIAH*) family belongs to the RING finger ubiquitin ligases of E3 ubiquitin ligases. *SIAH1*, a mammalian homolog of Drosophila *Siah1*, is a p53 target gene, and regulates various physiological reactions, such as hypoxia, apoptosis, DNA damage response, tumorigenesis, and neural functions, by binding proteins (10,11). *SIAH1* regulates ubiquitin-mediated proteolysis by using its RING finger domain (12-14). RING is located on chromosome 16q12-q13, a region that has a high frequency of the loss of heterozygosity (LOH) in tumors arising in various tissues such HCC (15), suggesting that *SIAH1* might function as a tumor suppressor. Furthermore, *SIAH1* can interact with adenomatous polyposis coli, a tumor suppressor gene, and accelerate *β-catenin* degradation (16,17). In HCC, *SIAH1* interacts with paternally expressed gene 10 (*PEG10*) to mediate cell death (18). E3 ligases can regulate the degradation of tumor promoters or repressors and are involved in regulating some common signaling pathways in HCC (19,20). Among them, the important E3 ligase *SIAH1* has a complex function in HCC and can be involved in the development and progression of

Highlight box

Key findings
- The detailed differently expressed proteins (DEPs) regulated by seven in absentia homolog 1 (*SIAH1*) in hepatocellular carcinoma (HCC) are provided.
- Ubiquitination sites and motifs of *SIAH1* in the Hepa1-6 cells are identified.
- Proteomic/ubiquitylomic analyses and functional analysis of ubiquitinated proteins regulated *SIAH1* are conducted.

What is known and what is new?
- *SIAH1* might function as a tumor suppressor in HCC.
- We studied the 550 DEPs related to *SIAH1* and identified the ubiquitination sites and motifs. Furthermore, we conducted the proteomic/ubiquitylomic analyses and functional analysis of DEPs.

What is the implication, and what should change now?
- The study provided novel insights into ubiquitination regulated by *SIAH1* in HCC.
- Further study should be conducted to explore the detailed function and molecular mechanism of *SIAH1* on HCC development.

HCC by targeting the *Wnt/β-catenin* pathway (20). In the present study, to further identify the role of *SIAH1* on HCC, we downregulated *SIAH1* with short hairpin RNA (shRNA) in the mouse HCC cell line Hepa1-6, studied its cell function by using proteome-wide identification, and detected alterations in the expression levels of hypoxia-, proliferation-, apoptosis-, and autophagy-related proteins. Our data raise novel insights for hepatocarcinogenesis and treatment of HCCs. We present this article in accordance with the MDAR reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-124/rc).

Methods

Cell culture

Mouse HCC cell line Hepa1-6 cells (#SCSP-512) were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China), and cultured in Dulbecco's modified Eagle medium (DMEM; Keygen Biotech, Nanjing, China) supplemented with 10% fetal bovine serum (FBS; Beyotime, Shanghai, China) at 37 ℃ under 5% CO_2. The cell line used for the experiment was the second passage cell.

For *SIAH1* knockdown, cells were planted on a 6-well plate with the density of 3×10^5 cells per well. After being cultured at 37 ℃ under 5% CO_2 for 24 hours, cells were treated with shRNA targeting cellular *SIAH1* using Lipofectamine® 2000 transfection reagent (Thermo Fisher, Waltham, MA, USA) according to the manufacturer's instructions. The shRNA sequence against *SIAH1* was (5' to 3') CCTTGTAAATATGCCTCTTCT.

The cells transfected with green fluorescent protein (*GFP*) served as a negative control. After 24 hours, cells were collected for the subsequent experiment.

Protein extraction

Frozen samples were lysed with 300 μL lysis buffer supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), followed by centrifugation at 12,000 g for 15 minutes at 4 ℃. After removing the insoluble particles and other precipitation, the proteins were collected from the supernatant, and proteins of each sample were acquired and separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and equal amount of protein was collected for the following experiment. Before storage at −80 ℃, the protein concentration was detected by using the bicinchoninic acid (BCA; Thermo Fisher) method.

Western blotting

Proteins in cell and liver samples were extracted with radioimmunoprecipitation assay (RIPA) buffer. Proteins were separated by SDS-PAGE according to standard procedures. Polyvinylidene fluoride (PVDF) membranes were closed with 5% skimmed milk powder for 2 hours at room temperature and then incubated with death-associated protein 1 [*DAP1*; 2282S; Cell Signaling Technology (CST), Danvers, MA, USA], *RGS3* (66790-1-Ig; Proteintech, Rosemont, IL, USA), *UBE2Q2* (12581-1-AP, Proteintech), *H1-2* (19649-1-AP, Proteintech), and *β-actin* (81115-1-RR, Proteintech) antibodies overnight at 4 ℃. The next day, the membranes were washed with membrane wash and incubated with secondary antibodies at room temperature for 2 hours. The target bands were visualized by enhanced chemiluminescence (ECL) solution after membrane wash and the relative expression of the target bands was quantified by Image J (National Institutes of Health, Bethesda, MD, USA) using β-actin as the internal reference.

Trypsin digestion

For trypsin digestion, a protein sample was reduced via reducing buffer [10 mM dithiothreitol (DTT), 8 M urea, 100 mM triethylammonium bicarbonate (TEAB), pH 8.0] and incubated at 60 ℃ for 1 hour. After cooling at room temperature, the protein sample was treated by indole-3-acetic acid (IAA) with the final concentration of 50 mM in darkness at room temperature for 40 minutes. The protein sample was then diluted by 100 μL 300 mM TEAB and centrifuged at 12,000 rpm for 20 minutes and this step was repeated twice. After washing, the samples were incubated with 100 μL 300 mM TEAB and followed with 3 μL sequencing-grade trypsin (1 μg/μL). After digestion at 37 ℃ for 12 hours, samples were centrifuged at 12,000 rpm for 20 minutes, and the solutions were collected and lyophilized.

Nano liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

The lyophilized peptides were re-suspended in 2% acetonitrile containing 0.1% formic acid, and 4 μL aliquots and then loaded into a ChromXP C18 (3 μm, 150 Å) trap column. The chromatography was conducted using

an Ekspert nanoLC 415 system (SCIEX, Concord, ON, Canada). Briefly, the peptides were carried out with 100% solvent A for 5 minutes and then eluted with the gradient of 5–85% solvent B over 67 minutes. Mass spectrum (MS) techniques were conducted on a Triple TOF 6600 tandem mass spectrometer (SCIEX) fitted with a Nanospray III ion source to acquire tandem data. An Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher) coupled to EASY-nLC 1000 was conducted to performLC-MS/MS analysis. A total of three individual biological replicates were performed on each sample.

Enrichment of phosphopeptides

To enriched phosphopeptides, titanium dioxide beads (TiO_2) was used. Briefly, samples were centrifuged at 12,000 rpm for 3 minutes, the precipitate peptides were collected and resolved using enrichment kit loading buffer. After vortex for 15 minutes, the fluid on the wall was collected by centrifuge, and then the TiO_2 beads were added (protein:TiO_2 =1:4, m/m) and vortex for 15 minutes. The sediment was collected after centrifugation, and 400 µL wash buffer 1 [0.5% trifluoroacetic acid (TFA)/50% acetonitrile (can)] was added. After centrifugation, the sediment was washed by 10% $NH_3 \cdot H_2O$, and the phosphor-peptides were washed off in the solution. The final enriched phosphor-peptides solution, which could be vacuum concentrated for further use, was collected followed by centrifugation for 5 minutes at 12,000 rpm.

Enrichment of ubiquitin-modified peptides

Ubiquitin-modified peptides were dissolved in immunoaffinity purification (IAP) buffer at pH =8.0, consisting of 50 mM NaCl, 50 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.5% NP-40. Afterward, the solution was incubated by anti-K-ε-GG antibodies crosslinked on agarose beads from PTMScan Ubiquitin Remnant Motif Kit (Cell Signal Technology, USA). After centrifugation, the beads were washed with intragenic antimicrobial peptide (IAP) and double-distilled H_2O (ddH_2O) twice times, individually. After elution with 0.1% trifluoroacetic acid, peptides were vacuum-dried and stored at –80 ℃ for LC-MS/MS analysis.

Bioinformatic analysis

Differently expressed proteins (DEPs) were identified with a reference threshold: fold change =1.2 and P value <0.05. Gene Ontology (GO) analysis was performed via the Gene Ontology Database (http://www.geneontology.org) using Blast2GO (version 3.3.5). GO functions of DEPs including biological process (BP), molecular function (MF), and cellular component (CC) were analyzed. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment of DEPs was conducted to analyze the main functional pathways. KAAS (https://www.genome.jp/tools/kaas/) was used to annotate the KEGG database, and KEGG Mapper was used to map the annotation result on the KEGG pathway database (https://www.genome.jp/kegg/). The Motif-X software (http://meme-suite.org/tools/momo) was used to find conserved Lys ubiquitination motifs.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM) and analyzed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). The *in vitro* experiments were repeated at least 3 times. Comparisons between two groups were performed with unpaired 2-tailed Student's *t*-tests. A P value <0.05 was considered statistically different.

Results

DEPs in the Hepa1-6 cells in response to sh-SIAH1

In this study, a total of 550 DEPs were identified in Hepa1-6 cells between *SIAH1* knockdown and GFP groups by proteome analysis, including 263 upregulated and 287 downregulated DEPs (*Figure 1A*). The detailed DEPs are provided in the Tables S1,S2. Furthermore, we validated the expressions of representative DEPs by western blotting. Congruent with the expectations, *SIAH1* knockdown inhibited the protein level of *DAP1*, *RGS3* (regulator of G-protein signaling 3), and *UBE2Q2* (ubiquitin conjugating enzyme E2 Q2), and promoted *H1-2* (histone H1.2) protein level (*Figure 1B*). For functional analysis, the KEGG enrichment mostly attributed DEPs candidates to 40 pathways, including the top 20 upregulated DEPs, and the top 20 downregulated DEPs. As illustrated in *Figure 1C*, KEGG enrichment of upregulated DEPs mainly annotated in 'alcoholism, autophagy-animal, coronavirus disease-COVID-19, oocyte meiosis, progesterone-mediated oocyte maturation, ribosome'; whereas downregulated DEPs were significantly enriched in 'estrogen signaling pathway, focal adhesion, human cytomegalovirus infection, pathways

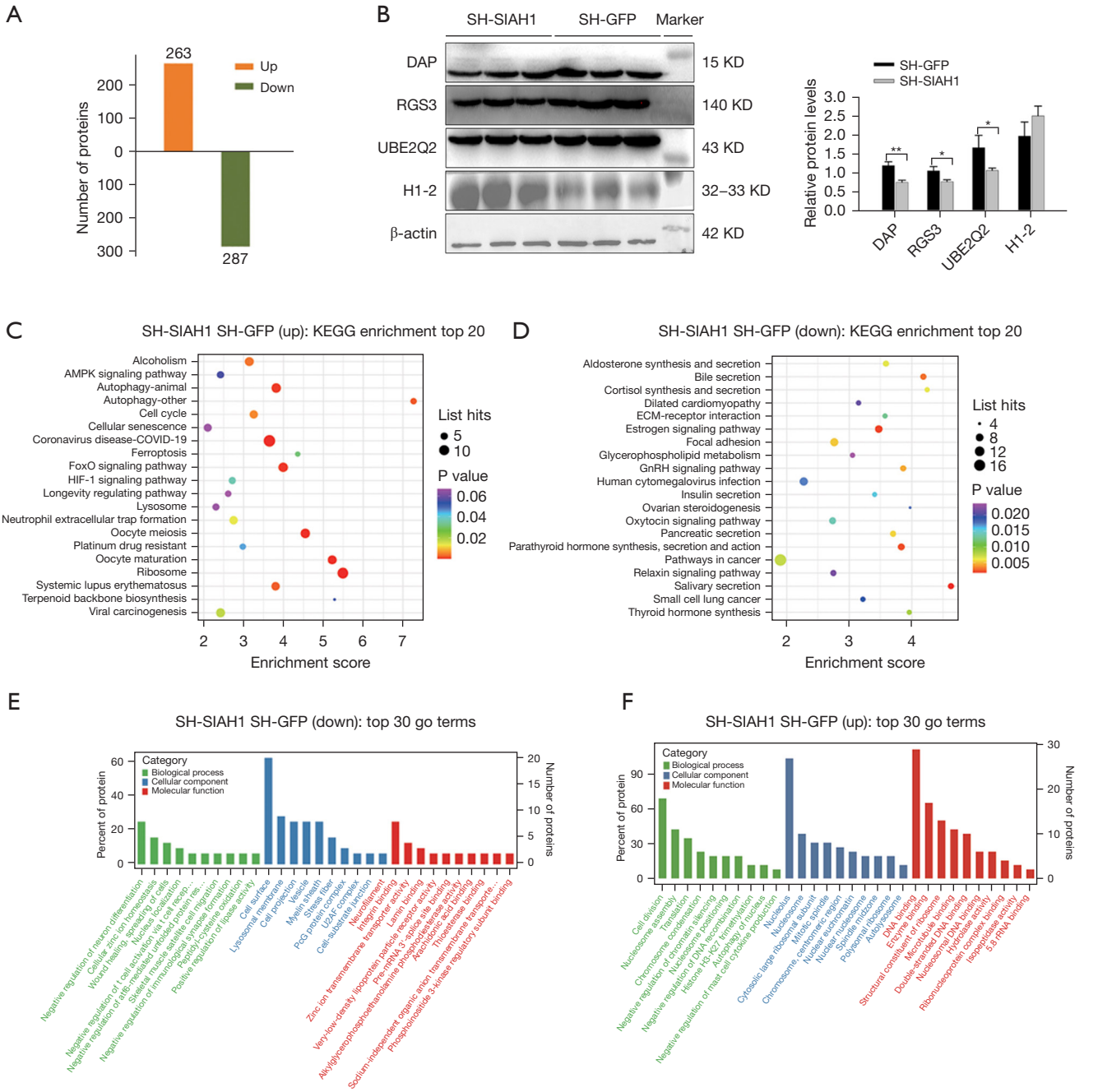

Figure 1 Comparative analyses on differentially expressed genes in Hepa1-6 cells in response to *SIAH1* knockdown. (A) Number of DEPs in response to *SIAH1* knockdown. (B) Protein levels of *DAP1*, *RGS3*, *UBE2Q2*, and *H1-2*. (C) KEGG enrichment for upregulated DEPs. (D) KEGG enrichment for downregulated DEPs. (E) GO enrichment analysis for downregulated DEPs. (F) GO enrichment analysis for upregulated DEPs. *, P<0.05; **, P<0.01 *vs.* SH-GFP. DEPs, differently expressed proteins; KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; COVID-19, coronavirus disease 2019; ECM, extracellular matrix.

in cancer' (*Figure 1D*). GO enrichment analysis showed that the downregulated DEPs were mostly enriched in cell division under the subcategory of BP. As for CC, DEPs showed enrichment in nucleolus. MF showed that those genes predominantly enriched at DNA binding (*Figure 1E*). Meanwhile, the upregulated DEPs were predominantly involved in negative regulation of neuron differentiation under the subcategory of BP, cell surface under the subcategory of CC, and integrin binding under the subcategory of MF (*Figure 1F*).

Ubiquitination sites and motifs in the Hepa1-6 cells

To further understand the structural context of Hepa1-6 cells in response to sh-*SIAH1*, we first examined the amino acid sequences around the modified lysine residues. Herein, we found that among 696 peptides, 7 were conserved motifs, which were designated as ……T…K……, …… L..K……, ……AK……, ……LK……, ……SK……, …… TK……, ……K.L……..(*Figure 2A*). Those ubiquitination motifs showed different abundance, and ……K.L…… was widely distributed, followed by ……L..K…… and …… LK……. (*Figure 2B*).

Next, ubiquitylomic analysis was performed. Functional analysis showed that GO identification enriched those ubiquitinated proteins in ubiquitin-dependent protein catabolic process under the subcategory of BP, cytosol under the subcategory of CC, and identical protein binding under the subcategory of MF. Among which, the proteins targeted for ubiquitination were predominantly involved in the subcategory of CC (*Figure 2C*). To obtain insights into KEGG enrichment, those ubiquitinated proteins were mainly enriched in amyotrophic lateral sclerosis, coronavirus disease, endocytosis, pathways of neurodegeneration, ribosome, and other processes (*Figure 2D*). Subcellular localization prediction presented that those ubiquitinated proteins were mainly distributed in the cytoplasm, nucleus, and plasma membrane (*Figure 2E*).

Proteomic and ubiquitylomic analyses on proteins of different patterns

To further elucidate the regulation of ubiquitinated proteins, we performed integrative analyses on the proteome and ubiquitylome data. As shown in *Figure 3A*, within the 1,287 ubiquitinated proteins, 1,210 proteins overlapped with the proteins identified from the proteome. Furthermore, the 1,287 ubiquitinated-proteins and 263 up-regulated DEPs were identified, 38 of which were overlapped (*Figure 3B*). In addition, among 1,287 ubiquitinated proteins and 287 down-regulated DEPs, 27 were down-regulated and also ubiquitinated (*Figure 3C*).

Functional analysis of ubiquitinated proteins

To further conduct the functional analysis of ubiquitinated proteins, GO enrichment analysis on the proteome and ubiquitylome data were performed. As shown in *Figure 4A*, the main GO functions associated with proteome and ubiquitylome under the subcategory of BP were enriched in negative/positive regulation of transcription by RNA polymerase II, and apoptotic process (*Figure 4A*). Under the subcategory of MF, the ubiquitinated and proteomic proteins were mostly enriched in the identical protein binding, ATP binding, and metal ion binding (*Figure 4B*). Moreover, the ubiquitinated and proteomic proteins were mostly enriched in the cytoplasm, cytosol, and nucleus under the subcategory of CC (*Figure 4C*). The KEGG analysis indicated that the ubiquitinated and proteomic proteins were mostly enriched in the pathways of neurodegeneration-multiple diseases, and amyotrophic lateral sclerosis (*Figure 4D*).

Discussion

Treatment for HCC at the initial stage includes surgery, radiotherapy, and chemotherapy; there are limited treatments for patients at advanced stage. However, most HCC patients are diagnosed at advanced stage, leading to the high mortality rate. In addition, the high rate of recurrence and metastasis also contribute to the poor prognosis. Therefore, there is an urgent need to explore novel prognostic molecular markers and search for potential effective therapeutic insights for HCC to improve patients' OS rate. Recently, the development of high-throughput identification followed by high-resolution mass spectrometry analysis has made it possible to enrich the novel proteins in various diseases. Therefore, it is interesting to identify the functional importance of HCC-related novel and specific protein by using high-throughput identification.

Ubiquitination reaction is involved in various human physiological processes, such as embryogenesis, cell growth, and oxidative stress. The abnormal response of ubiquitination often leads to important events such as tumorigenesis (21,22). The mass spectrometry (MS)-

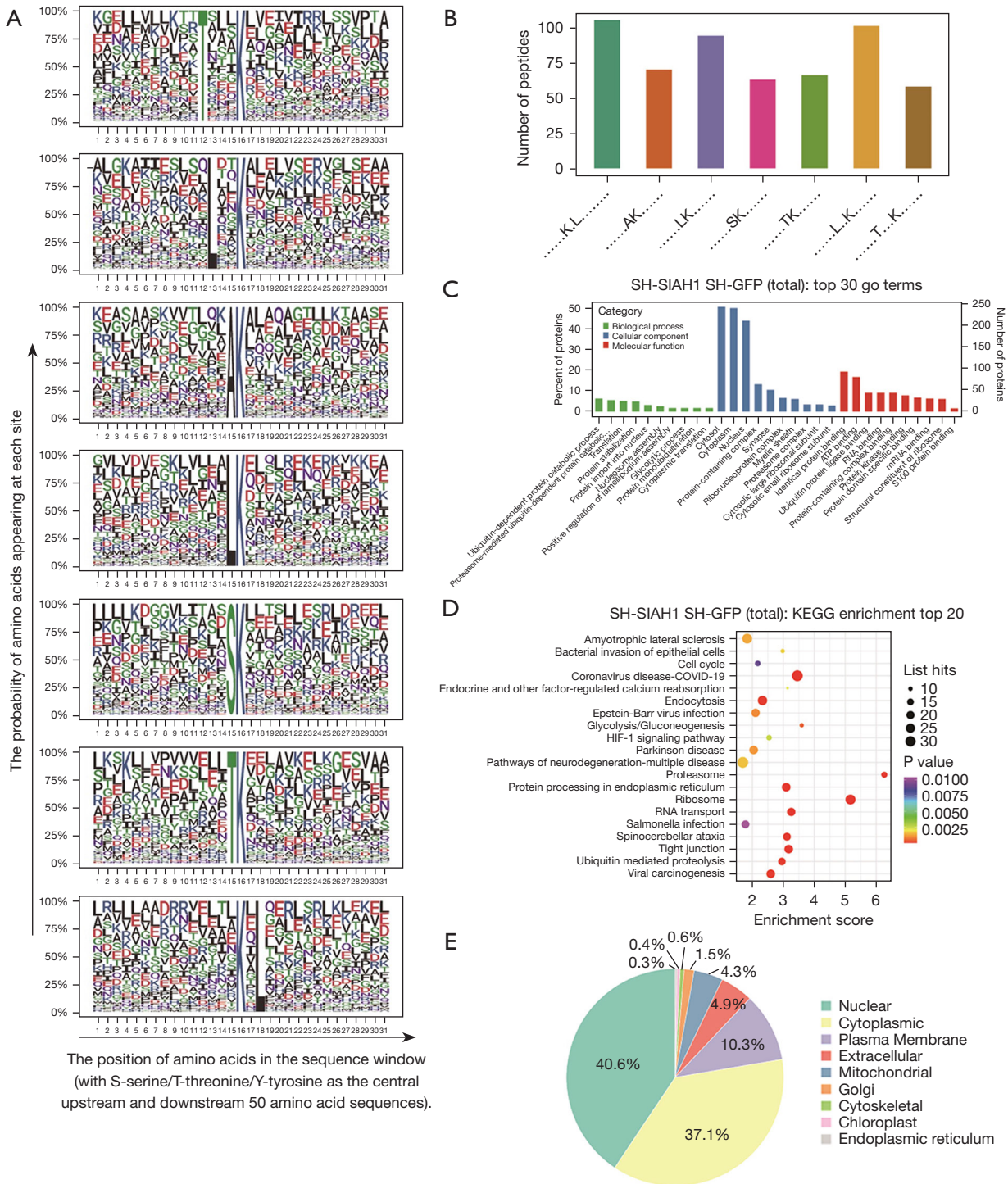

Figure 2 Sequence characteristics of ubiquitinated proteins in Hepa1-6 cells in response to *SIAH1* knockdown. (A) Conserved motifs of ubiquitinated proteins. The middle K represents ubiquitinated Lys, and the height of each letter indicates the frequency of amino acid residues at this position. The capital letter E represents glutamate (Glu); the capital letter D represented aspartic acid (Asp); and the capital letter G represents glycine (Gly). (B) Number of identified peptides containing the indicated ubiquitinization motifs. (C) GO enrichment analysis of the ubiquitinated protein substrates. (D) KEGG enrichment of the ubiquitinated proteins. (E) Predicted subcellular localization of the ubiquitinated proteins. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; COVID-19, coronavirus disease 2019.

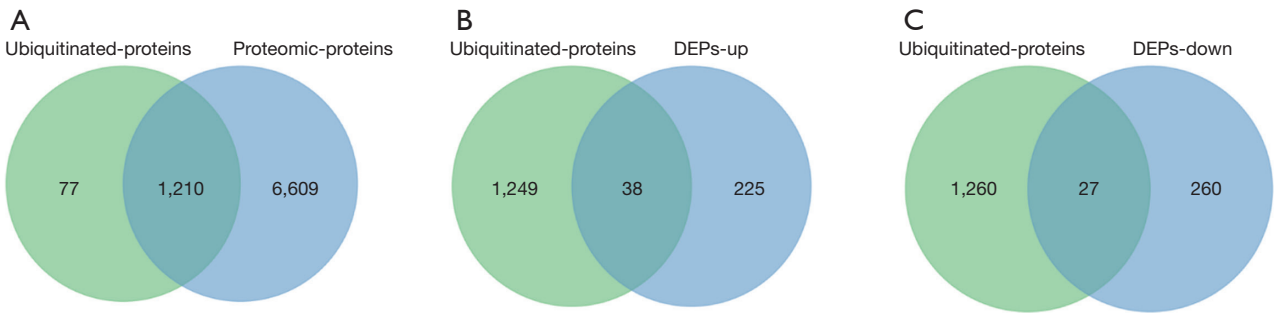

Figure 3 Comparative analysis on the differentially expressed genes and ubiquitinated proteins. (A) Venn diagrams between total identified proteins and ubiquitinated sites. (B) Venn diagrams of the connection between upregulated DEPs and ubiquitinated proteins. (C) Venn diagrams of the connection of between downregulated DEPs and ubiquitinated proteins. DEPs, differently expressed proteins.

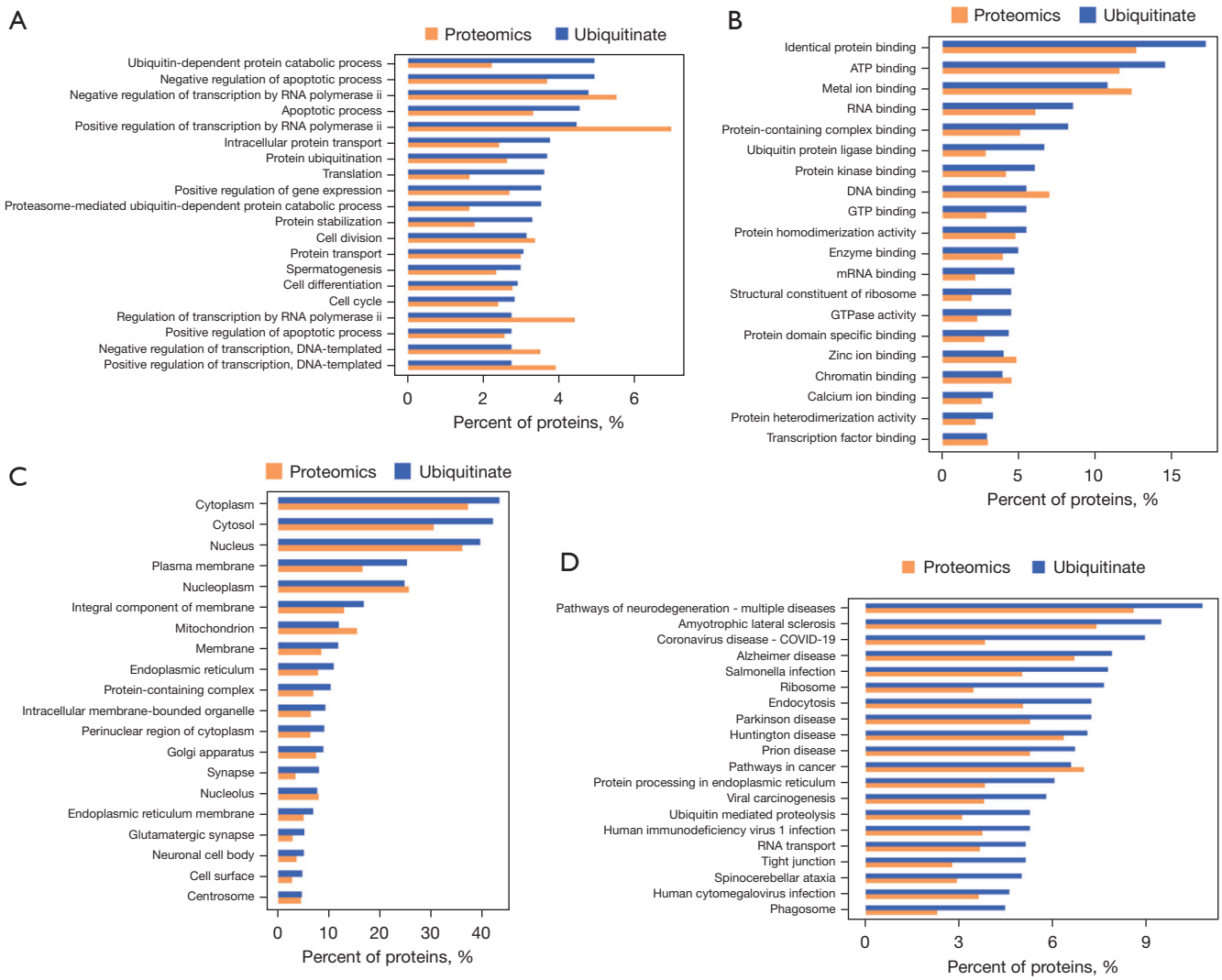

Figure 4 Analysis of ubiquitinated proteins compared to global proteome. Top 20 GO enrichment between ubiquitinated proteins and global proteome in (A) biological process, (B) molecular function, and (C) cellular component. (D) Top 20 KEGG pathways between ubiquitinated proteins and global proteome. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; COVID-19, coronavirus disease 2019.

based high-throughput proteomic approach is a crucial technique for large-scale protein characterization, and could quantify peptides or intact proteins, which make it possible for the research into basic biology on human disease. Ubiquitination analysis based on the high-resolution LC-MS/MS has allowed researchers to systematically comprehend the potential biological mechanism. Ubiquitination is a critical post-translational protein modification on regulating the lysine modification sites. Further, ubiquitination sites and the E3 ligases might act as the attractive therapeutic targets (23,24). In addition, hundreds of E3 ligases mediate the function and structure of its target proteins, and E3-substrate interactions often induce target degradation. In recent years, a number of different degradation signals have been identified to analyze ubiquitinated protein targets (25). In the present study, to identify the role of E3 ligases on HCC, we selected *SIAH1* as the target E3 ligase, and found that *SIAH1* knockdown in Hepa1-6 cells altered 550 proteins. Functional analysis illustrated that those proteins were mainly enriched in the diseases, and located at the CC. These data indicated that these processes are regulated by *SIAH1*. Additionally, to further identify the ubiquitin modification that was regulated by *SIAH1*, the KEGG and GO analysis were performed to analyze their function. The results showed that ubiquitinated proteins were mostly regulated diseases, and manifested cytosol, cytoplasm, and nucleus. The results further confirmed that E3 ligases mediated various human diseases, which could be considered as a therapeutic target for further clinic treatment (26). The functions of ubiquitinated proteins were reported to relate to their lysine modification sites. Herein, *SIAH1* knockdown altered great ubiquitination motifs with different abundance, and motif analyses of peptides showed that ……KL…… was widely distributed, followed by ……L..K…… and ……LK……., indicating that *SIAH1* modulates HCC by mapping the lysine modification sites.

Taken together, this study revealed that *SIAH1* mediates HCC by regulating great protein expression, and those ubiquitinated proteins are mainly located at the nucleus, cytoplasm, and extracellular space. Those ubiquitinated proteins are mostly enriched in diseases, protein binding, and virus. In addition, the ubiquitinated proteins modulate HCC by mapping lysine modification sites. Based on this, further study should be conducted to explore the role of *SIAH1* on HCC in the clinical setting and to search for possible mechanisms and potential targets for *SIAH1* to affect HCC, providing great basis for HCC therapy.

Conclusions

The use of high-throughput identification followed by high-resolution mass spectrometry analysis to identify novel and specific targets associated with *SIAH1* is of great significance for HCC treatments. The results obtained in this paper provide novel insights into ubiquitination regulation by *SIAH1* in HCC, which pave the way for further research and mechanism disclosure of HCC.

Acknowledgments

Funding: This work was supported by grants from National Natural Science Foundation of China [Nos. 81900791 (to Z.W.) and 82100929 (to Z.Z.)]; The Scientific and Technological Projects of Henan Province of China [Nos. 192102310322 (to Z.W.) and 202102310449 (to Z.Z.)]; Henan Medical Science and Technology Research Plan Joint Co-construction Project [No. 2018020080 (to Z.Z.)]; and The Guangzhou Municipal Science and Technology Project [No. 202102010286 (to J.L.)].

Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-124/rc

Data Sharing Statement: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-124/dss

Peer Review File: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-124/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-124/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Cite this article as: Hu X, Zhu Z, Liu K, Liu J, Li J, Wang Z. Proteome-wide identification and functional analysis of ubiquitinated proteins in Hepa1-6 cells by knockdown of E3 ubiquitin ligase SIAH1. Transl Cancer Res 2024;13(1):102-111. doi: 10.21037/tcr-23-124

