SIAH1-Mediated RPS3 Ubiquitination Contributes to Chemosensitivity in Epithelial Ovarian Cancer

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Research

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Abstract

Background: The E3 ligase SIAH1 is deregulated in human cancers and correlated with poor prognosis, but its contributions to chemoresistance in epithelial ovarian cancer (EOC) are not evident.

Methods: SIAH1, RPS3, NF- κ B (P65), FLAG and GFP protein levels were assessed by western blotting (WB) or immunohistochemistry (IHC). The mRNA levels were revealed by qRT-PCR. Colocalization of RPS3 and SIAH1 was detected by confocal microscopy and co-immunoprecipitation (CO-IP). RPS3 ubiquitination levels were detected by immunoprecipitation (IP). Cell functional experiments were performed and A2780 xenograft models were employed to expose the latent mechanisms of the SIAH1-RPS3-NF- κ B axis as well as the role of SIAH1 in inhibiting chemoresistance *in vitro* and *in vivo*.

Results: We show that SIAH1 is decreased in EOC tumour tissues and cell lines and negatively correlated with RPS3 level. SIAH1 overexpression suppresses tumour cell growth, colony formation, invasion, metastasis, and cisplatin resistance *in vivo* and *in vitro*. SIAH1 promoted RPS3 ubiquitination and degradation using the RING-finger domain, which was required for RPS3 localization to the cytoplasm for subsequent NF- κ B inactivation, thereby conferring chemosensitivity. Moreover, ectopic expression of RPS3 or depletion of RPS3 ubiquitination mediated by SIAH1 via the K214R mutant significantly impaired cisplatin-induced tumour suppression in cells stably expressing SIAH1.

Conclusions: Our findings reveal a tumour suppressor function of SIAH1 and provide evidence that the SIAH1-RPS3-NF- κ B axis may act as an appealing strategy to tackle treatment resistance in EOC.

Background

Largely due to the development of chemoresistance, most epithelial ovarian cancers (EOCs) are prone to treatment failure despite all current standard treatments (1), and these cancers are associated with the highest risk of death among gynaecological malignancies (2, 3). Hence, it is crucial to understand the regulatory mechanism of chemoresistance, which is the bottleneck of EOC treatment (4).

As a crucial player, the ubiquitin-proteasome pathway manages steady-state protein levels to supervise multifarious biological processes, including the cell cycle, cellular proliferation, apoptosis, and DNA damage response, which are linked with oncogenesis, cancer development, drug resistance and prognosis (5, 6). In the ubiquitination cascade, E3 ligases execute the terminal step and determine which proteins become ubiquitylated with the help of specifically binding the substrate protein. Give their capacity to regulate protein stability and functions, E3 ligases are considered to be promising drug targets (7). As a member of the E3 ubiquitin ligase family, seven in absentia homologue 1 (SIAH1) contains two zinc-finger domains, a RING-finger domain, and a substrate-binding domain. Of these domains, the RING-finger domain is critical to ubiquitin ligase activity (8). SIAH1 has been reported to behave as a tumour suppressor during tumorigenesis by binding to numerous proteins, thereby triggering their ubiquitylation and degradation via the ubiquitin–proteasome pathway, such as NcoR, TRAF, β -catenin, c-Myb, APC, and Kid (9–12). In addition, SIAH1 has been implicated in cancer-related

signalling pathways, including cell apoptosis (β -catenin, Sec6) (13, 14), DNA damage repair (c-Abl, HIPK2, and TRF2) (15–17) and the *in vivo* hypoxia response (PHD3, HIF-1 α , and IL-17) (13, 18, 19). Thus, SIAH1 has been implicated as a promising therapeutic target in cancer treatment (20).

As one of the eukaryotic ribosome 40S subunits, ribosomal protein S3 (RPS3) is critical for regulating the maturation of ribosomes and the initiation of translation with the eukaryotic initiation factors eIF2 and eIF3 (21, 22). Independent of the activities of ribosomes, RPS3 has a variety of extraribosomal functions, such as DNA repair (23–28), cell signalling (29–32), apoptosis/survival (33), host-pathogen interactions (34, 35) and transcriptional regulation (36–38). As reported, RPS3 interacts with the p65 subunit of nuclear factor kappa B (NF- κ B) through its K homology domain, which results in NF- κ B-induced transcriptional activation (38–40). NF- κ B adjusts the expression of genes (PTOV1, c-Myb, TRIM52) (41–43) related to numerous processes that play a pivotal role in the development and progression of cancer, such as proliferation, migration and apoptosis (44). Many findings have indicated that NF- κ B pathway activation promotes ovarian cancer chemotherapy resistance (41–43).

Above all, this project intends to explore the role of SIAH1 in EOC drug resistance, identify its interacting molecular chaperones, and regulate the NF- κ B pathway, aiming to identify new targets for EOC drug resistance reversal and prevention.

Methods

Cell culture and treatment

Human ovarian cancer cell lines (A2780, SKOV3) and human renal Epithelial Cell lines (HEK-293T) were obtained from Shanghai Institute of Cell Biology, China Academy of Sciences. Cells were cultured in RPMI medium modified supplemented with 10% fetal calf serum (Gibco BRL, Grand Island, NY), 100 U/mL penicillin and 100 μ g/mL streptomycin in a humidified atmosphere containing 5% CO₂ at 37°C.

Plasmids and lentivirus

The GFP-SIAH1, FLAG-SIAH1, SIAH1, GFP-SIAH1(Δ RING) (knock out the RING domain), sh-SIAH1, FLAG-RPS3, Cherry-RPS3, FLAG-RPS3(K214R) (point mutant), sh-RPS3 plasmids and their corresponding negative control plasmids were purchased from Genechem Co., Ltd (Shanghai, China). Lentivirus: SIAH1, sh-SIAH1, RPS3, RPS3(K214R) and their corresponding negative control lentivirus were purchased from Genechem Co. Ltd (Shanghai, China).

Tissue samples

With the approval of the Jiangsu University ethics committee, serous ovarian cancer samples from patients with FIGO stage IIIC or IV were collected at Zhenjiang Maternal and Child Health Hospital (The Fourth Affiliated Hospital of Jiangsu University) and The Affiliated People's Hospital of Jiangsu University. All patients were treated with the standard care of platinum-based therapy after surgery, and informed consent was obtained from all patients. PFS was calculated from the time of surgery to the

time of progression or recurrence. Platinum resistance or platinum sensitivity was defined as a relapse or progression within 6 months or 6 months after the last platinum-based chemotherapy, respectively. Each group had more than 12 patient samples.

Transfection

The details are described in the Supplementary Methods.

Colony forming assay

The details are described in the Supplementary Methods.

Wound-healing assay

The details are described in the Supplementary Methods.

Assessing chemosensitivity to cDDP

The details are described in the Supplementary Methods.

Evaluation of apoptosis

The details are described in the Supplementary Methods.

Cycloheximide (CHX) chase assay

The details are described in the Supplementary Methods.

Immunohistochemistry

The details are described in the Supplementary Methods.

Western blotting analysis

The details are described in the Supplementary Methods.

Real-time Quantitative PCR

The details are described in the Supplementary Methods.

***in vitro* ubiquitination and co-immunoprecipitation**

in vitro ubiquitination, HEK293T cells were transfected plasmids as described previously for 36 h. Then, the cells were treated with 15 μ M MG132 (MedChemExpress, New Jersey, USA) for 6 h and lysed in buffer on ice for 30 minutes and 30 seconds oscillations at 5minutes intervals. Centrifuged for 10 min at 12,000g at 4 °C, followed by the collection of supernatants. A total of 0.5 mg of lysates was incubated overnight at 4 °C with PureProteome™ Protein A/G Magnetic Beads (Millipore, Massachusetts, USA) as

well as antibodies against SIAH1 (Abcam, Cambridge, UK), RPS3 (Abcam, Cambridge, UK), GFP (Proteintech, Rosemont, USA) or FLAG (Cell Signaling Technology, Massachusetts, USA). Using lysis buffer washed six times, the immune-precipitates were resuspended in 5× loading buffer, degenerated and further analyzed by western blotting as well as cell lysates. Immunoblot was performed using ubiquitin, SIAH1, RPS3, GFP, FLAG, GAPDH antibody.

Experimental animals

Female Specific pathogen Free BALB/C nude mice weighing 150 g were provided by the Shanghai SLAC experimental animal company of limited liability. The nude mice were housed at room temperature (26 ± 2 °C) with a humidity of 45–55% and a light duration of 12 h in the laboratory animal center of Jiangsu University. All experimental procedures involving nude mice were approved by the Institutional Animal Care and Use Committee of Jiangsu University.

Statistical analysis

All numeric data are presented as the means $\pm$ standard deviation (SD) of at least three independent experiments. The experimental results were analyzed by variance or a two-tailed Student's t-test at the significance level of $P < 0.05$ (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$). Prism 5 software (GraphPad Software, San Diego, CA, USA) was used to perform all statistical analyses. P value < 0.05 was considered statistically significant.

Results

SIAH1 deregulation was associated with drug resistance in EOC.

To determine the effect of SIAH1 on the development and progression of EOC, we explored the expression of SIAH1 in cDDP-sensitive EOC cells (A2780) and cDDP-resistant cells (SKOV3). The results showed that A2780 cells exhibited strong expression of SIAH1, whereas SKOV3 cells lacked SIAH1 expression (Figure 1A), demonstrating that SIAH1 may be associated with drug resistance in EOC. In addition, SIAH1 protein levels were obviously increased in tumours obtained from patients with progression-free survival (PFS) > 6 months (clinically described as cDDP sensitive), whereas its expression was reduced in tumours obtained from patients with PFS < 6 months (cDDP resistant; Figures 1B-C and S1B). This finding further suggested that SIAH1 may play a functional role in EOC.

To further clarify the role of SIAH1 in the progression of EOC, we transfected EOC cells with SIAH1, Vector, sh-SIAH1 and Ctl-shRNA respectively (Figure S1A), and discovered that SIAH1 overexpression decreased the quantity of cell colonies and cell viability, impaired cell migration and invasion, raised the apoptosis rate and declined the chemoresistance of EOC cells. In contrast, SIAH1 knockdown showed greater ability to restrain cell proliferation and cell viability, strengthen cell migration and invasion, inhibit cell apoptosis and enhance the drug resistance of EOC cells (Figures 1D-I and S1C-F). All these indicate that SIAH1 renders EOC cells sensitive to cDDP.

Identification of RPS3 as an interaction partner of SIAH1

To gain prime insight of SIAH1 expression patterns, we identified potential SIAH1-binding proteins by co-immunoprecipitation assays using the anti-SIAH1 antibody in HEK-293T cells transfected with SIAH1, Vector, sh-SIAH1 and Clt-shRNA plasmids respectively. Total immunoprecipitates were then disposed with ubiquitination mass spectrometry analysis (Ub-MS) (Figure 2A). The potential SIAH1-interacting proteins were selected on the basis of the following 2 criteria: (1) more than 2 unique protein peptides; (2) sequence coverage of proteins were greater than 70%. Consequently, 20 potential SIAH1-interacting proteins were distinguished (Supplementary Table 1). Interestingly, approximately one fourth (5 from 20) of them were from the ribosomal protein family. Additionally, it has been reported that RPS3 was essential to induce chemoresistance of cancer cells (45, 46). Therefore, we chose RPS3 as our research object. Using Co-IP assays, the interaction between SIAH1 and RPS3 was confirmed in HEK-293T cells transfected with SIAH1, and immunoprecipitates pulled down by anti-SIAH1 antibody were detected (Figure 2B). In the control, no RPS3 protein was detected in the immunoprecipitates that were pulled down using anti-IgG antibody.

RPS3 confers chemoresistance in EOC

To clarify the role of RPS3 in EOC drug resistance, RPS3 expression was assessed in primary EOC specimens originated from 24 EOC patients. cDDP-resistant patients (PFS<6) showed higher RPS3 expression, and cDDP-sensitive patients (PFS>6) exhibited lower RPS3 expression (Figures 3A-B and S2B). Analogous to high SIAH1 expression, low RPS3 expression was associated with longer PFS. Additionally, we observed high RPS3 expression in cDDP-resistant EOC cells (Figure 3C). To further confirm the clinical relevance of SIAH1 and RPS3, we assessed SIAH1 and RPS3 protein expression in serum obtained from 12 subjects, including 6 idiopathic cases of EOC patients (EOCs) and 6 age-matched healthy female controls (HCs). The results showed higher SIAH1 expression in HCs and higher RPS3 expression in EOCs (Figure 3D). After the promotion or inhibition of RPS3 expression by transfection with RPS3-specific plasmids (Figure S2A), the short-term impact of RPS3 on cell survival was evaluated using a cell viability assay (CCK-8 assay). RPS3 overexpression significantly enhanced cDDP resistance in EOC cells, suggesting that RPS3 confers chemoresistance in EOC cells (Figure 3I-J). To evaluate the long-term impact of RPS3 on cell proliferation after treatment with cDDP, a colony formation assay was performed. The number of colonies in the presence of cDDP was significantly increased by RPS3 overexpression and was greatly decreased by RPS3 knockdown (Figures 3E and S2C). Furthermore, overexpression of RPS3 inhibited cell apoptosis, strengthened cell migration and invasion, and enhanced cDDP resistance (Figures 3F-H and S2D-F).

SIAH1 sensitizes EOC cells to cDDP through regulation of RPS3

Next, we sought to further confirm that SIAH1-mediated EOC chemotherapy sensitization is related to its partner, RPS3. EOC cells were transfected with SIAH1, SIAH1+RPS3, sh-SIAH1 and sh-SIAH1+sh-RPS3 plasmids respectively. The reduction of cell viability, impairment of cell migration and invasion, increase of apoptosis rate and decline of chemoresistance were detected in SIAH1-expressing EOC cells, and these

effects were mitigated upon RPS3 transfection. In contrast, increase of cell proliferation and cell viability, reinforcement of cell migration and invasion, inhibition of cell apoptosis and enhancement of chemoresistance in EOC cells transfected sh-SIAH1 were repaired by sh-RPS3 (Figure 2C-H). All these indicate that the SIAH1-induced sensitivity of EOC cells to cDDP was attenuated by RPS3.

SIAH1 facilitates degradation of RPS3 by ubiquitin-proteasome pathway

To detect whether RPS3, which was reduced by SIAH1, was involved in proteasome-dependent degradation, A2780 cells transfected with SIAH1 plasmid were handled with MG132 (proteasome inhibitor), and the expression levels of RPS3 were detected in SIAH1-expressing cells. The results showed that SIAH1-mediated down-regulation of RPS3 protein expression was attenuated by MG132 treatment (Figure 4A), and SIAH1 had no effect on RPS3 gene expression (Figure 4B). Furthermore, the half-life of RPS3 was decreased by SIAH1 in EOC cells treated with CHX (Figure 4C-D). These above results indicated that SIAH1 destabilizes RPS3 proteins through the ubiquitin-proteasome pathway in EOC cells. Consistently, the co-localization of SIAH1 and RPS3 was discovered by confocal microscopy in EOC cells (Figure 4E). Moreover, the results in Figure 4F indicated that SIAH1 interacted with both endogenous and exogenous RPS3, further supporting the above conclusion.

To confirm whether SIAH1 can induce RPS3 ubiquitination, we conducted immunoprecipitation and western blotting experiments. The outcomes showed that SIAH1 markedly induced endogenous and exogenous RPS3 ubiquitination, which was revealed by immunoprecipitation analysis (Figure 5A-B). On the contrary, knockdown of SIAH1 resulted in inhibition of RPS3 ubiquitination (Figure 5C).

SIAH1 ubiquitinates RPS3 at its K214

To identify the potential chief ubiquitin site of RPS3, we analysed the results of Ub-MS again, and 11 potential ubiquitination sites were found (Supplementary Table 2). Based on their scores, P values and ubiquitination levels in the context of SIAH1 overexpression or knockdown, K214 was selected as the most outstanding site. Then, we mutated the lysine residues (K214) to arginine and measured ubiquitination, which verified the ubiquitin modification of RPS3 at K214 (Figure 5D). Additionally, ubiquitin did not increase with SIAH1 knockdown (Figure 5E). The above data suggest that SIAH1 ubiquitinates RPS3 at K214 in a proteasome-dependent manner.

RING finger domain of SIAH1 is required for RPS3 ubiquitination

To determine the key domain of SIAH1 mediating RPS3 ubiquitination, we co-transfected RPS3 with SIAH1 lacking the RING domain (GFP-SIAH1 Δ RING) and therefore could not realize E3-mediated ubiquitin transfer on its targets for demonstrating the direct impact of SIAH1 E3 ligase activity. We discovered that the deletion of RING domain abolished the ability of SIAH1 to ubiquitinate RPS3 (Figure 5F). These consequences implied that the RING finger domain of SIAH1 is required for RPS3 ubiquitination and are consistent with the notion that SIAH1 acts as a RING finger E3 ubiquitin ligase.

SIAH1 inhibits NF- κ B pathway via RPS3 downregulation

Considering that RPS3 can increase the transcriptional activity of NF- κ B by binding its p65 subunit, we investigated whether SIAH1-mediated RPS3 degradation has an effect on the activity of NF- κ B. Consistently, with RPS3 overexpression or knockdown, the level of NF- κ B protein had an analogous trend with RPS3 in EOC cells (Figure 6A), whereas SIAH1 expression led to the downregulation of NF- κ B, which was rescued by RPS3 overexpression (Figure 6B). Moreover, an inhibitor of NF- κ B (PDTC) had no effect on SIAH1 and RPS3 expression (Figure 6C). All of these indicate that SIAH1 inhibits NF- κ B pathway via RPS3 downregulation. Not surprisingly, PDTC enhanced the sensitization effect of SIAH1 and weakened the tumour-promoting effect of sh-SIAH1 (Figure 6D-I). These findings suggest that SIAH1-mediated RPS3 degradation sensitizes EOC cells to cDDP partially by inhibiting the NF- κ B pathway.

SIAH1 enhances the sensitivity of EOC cells to cDDP by down-regulating RPS3 *in vivo*.

The above data jointly indicated that SIAH1 targeting represents an efficient anti-EOC therapy *in vitro*. To verify this hypothesis in a preclinical animal model, we developed an *in vivo* tumour model (Figure 7A). We subcutaneously and intraperitoneally injected A2780 cells transfected respectively with lentivirus of Vector, SIAH1, CIt-shRNA, sh-SIAH1 and RPS3 stable knockdown A2780 cells transfected respectively with lentivirus of SIAH1+FLAG-RPS3, SIAH1+FLAG-RPS3 (K214R) into female BALB/c nude mice, aiming to produce appropriately sized tumours (n=6). Once tumours were formed (approximately a week), cDDP treatment was applied to these mice 3 times a week 8 times. Then, these mice were sacrificed, and the tumours were collected for analysis. Subsequently, we evaluated animal survival, tumour histology and immunostaining analyses.

SIAH1 overexpression resulted in smaller tumors volume, fewer tumors, less RPS3 protein expression, higher apoptosis rates and lower proliferation rates. Knockdown of SIAH1 had the opposite biological effect (Figure 7B-G left panel). Compared with the SIAH1+FLAG-RPS3 group, transfection with SIAH1+FLAG-RPS3(K214R) resulted in larger tumour volume, more tumours, more RPS3 protein expression, lower apoptosis rates and higher proliferation rates (Figure 7B-G right panel).

These results further support the hypothesis that SIAH1 enhances the sensitivity of EOC cells to cDDP by down-regulating RPS3 *in vivo*. Together, these *in vivo* results strongly favour the efficacy of SIAH1 overexpression combined with cDDP in anti-EOC therapy.

Discussion

The development of acquired chemoresistance and enhanced metastasis is considered to be the major contributor to clinical mortality in EOC (2, 47). As reported, cisplatin resistance in tumours is associated with increased drug efflux, alteration of intercellular signalling, tubulin mutation, and overexpression of β -tubulin isotype composition (48, 49). It has been reported that the ubiquitin-proteasome system (UPS) may be involved in the regulation of tumour occurrence and metastasis (50–52). However, whether UPS increases the occurrence of EOC and its cisplatin resistance is unclear. Here, we investigated the contributions and the clinical relevance of SIAH1, which is an E3 ubiquitin ligase, in the context of chemoresistance. Here, we provide further information on the functional role of SIAH1, a significant

conditioner of the UPS, in EOC resistance and provided conclusive evidence that SIAH1 was down-regulated in drug-resistant EOC cells, patient serum and cancer tissues. Low SIAH1 protein expression was significantly related to the proliferation ability, invasiveness, migration and drug resistance of EOC cells. With regard to the mechanisms, RPS3 was identified as a new substrate of SIAH1 and was targeted by SIAH1 for ubiquitin proteasome-dependent degradation, which further inhibited the NF- κ B pathway and led to the chemosensitization of EOC (Fig. 8).

As a member of the highly conserved family of E3 ubiquitin ligases, increasing research has revealed the key role of SIAH1 in cancer development and tumour resistance. As documented, SIAH1 overexpression induced the apoptosis of cancer cells and inhibited the progression of cancer in many cancers, such as breast cancer and hepatocellular carcinoma (53, 54). Our research confirmed that exogenous overexpression of SIAH1 suppressed EOC cell proliferation, invasion, migration, drug resistance and tumour growth both *in vitro* and *in vivo*. In addition, compared with sensitive EOC patients, SIAH1 was significantly reduced in the serum and cancer tissues of drug-resistant EOC patients. Furthermore, SIAH1 overexpression obviously enhanced the effect of cDDP on apoptosis and tumour growth, whereas SIAH1 knockdown had the opposite effect.

RPS3 was identified as a potential SIAH1 binding protein by Ub-MS. Furthermore, our results also showed that SIAH1 played an important biological role in the human body by inducing RPS3 ubiquitination and degradation. Here, the ribosomal protein RPS3 was described for the first time as an important substrate of SIAH1 and an important medium for SIAH1 to regulate EOC cell proliferation, migration, invasion and drug resistance. As a ribosomal component, RPS3 plays a pivotal role in certain human tumours. YongJoong Kim et al. discovered that the level of secreted RPS3 was increased in more malignant cells and suggested that the secreted RPS3 protein was a poor prognostic indicator of malignant tumours (55). Our results indicated that RPS3 may act as a tumour resistance promoter downstream of SIAH1 by promoting NF- κ B-induced transcriptional activation which has been considered to contribute to EOC chemotherapy resistance. In addition, RPS3 overexpression could compensate for the reduction of EOC cell proliferation, migration, invasion and drug resistance caused by overexpression of SIAH1, indicating that SIAH1 and RPS3 jointly regulate the development and resistance of EOC. Further exploring its mechanism, we found that SIAH1, serving as a proverbial E3 ubiquitin ligase, induced RPS3 ubiquitination and degradation based on its RING domain. Consistent with this notion, the canonical E3 ubiquitin ligases, which contain the RING finger domain, induces the correlative substrate for ubiquitination and degradation via its conserved Cys and His residues (56).

Ubiquitin, which is attached to the lysine residue on a substrate, will be recognized and degraded by proteasome pathway (57, 58). The specific lysine site of RPS3 for binding to ubiquitin was identified by Ub-MS and was replaced with arginine. We concluded that lysine-214 of RPS3 is required for SIAH1-induced ubiquitination and degradation. Considering animal tumour formation experiments, we verified the effects of SIAH1 and RPS3 on tumour growth and development *in vivo*. RPS3 compensated for the inhibitory effect induced by SIAH1, whereas RPS3 (K214R) had a more obvious compensation effect than the former because it was not degraded by SIAH1. All of these further taken our focus from RPS3 to its

lysine site K214. In the foreseeable future, lysine-214 of RPS3 may be a key site to predict chemoresistance and prognosis of EOC on the basis of SIAH1 deregulation.

Conclusions

Collectively, these results revealed SIAH1 as an extraordinary and pivotal regulator of chemoresistance in EOC and stressed the potential of the SIAH1-RPS3-NF- κ B axis, which acts as a promising therapeutic target in response to chemotherapy in EOC patients, especially in those with decreased SIAH1 expression.

Abbreviations

EOC: Epithelial ovarian cancer; SIAH1: seven in absentia homolog 1; RPS3: Ribosomal protein S3; NF- κ B: nuclear factor kappa B; cDDP: cisplatin; NcoR: nuclear receptor co-repressor; TRAF: TNF receptor associated factor; c-Myb: c-MYB proto-oncogene; MDR: Multidrug resistance; IC50: Half maximal inhibitory concentration; Co-IP: Co-immunoprecipitation; IP: Co-immunoprecipitation; WB: western blotting; Ub-MS: Ubiquitination Mass Spectrometry analysis.

Declarations

Ethics approval and consent to participate

All Clinical research were performed with the approval of the Jiangsu University Ethics Committee, and informed consent was obtained from all patients. All the animal experiments were manipulated according to guidelines approved by the Jiangsu University Medical Experimental Animal Care Commission.

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Lu Chen and Xiaolan Zhu accomplished the study concept and design, drafted the manuscript, obtained funding and performed experiments. Lu Chen, Wujiang Gao and Li Lin performed acquisition of data, analysis and interpretation of data. Yuefeng Li and Wenlin Xu got the clinical data. Qi Chen, Hong Wei, Taoqiong Li and Chunli Sha gave the administrative and technical support. Jie Xing, Mengxue Zhang and Shijie Zhao gave the material support. All authors discussed the results and commented on the manuscript. All authors read and approved the final manuscript.

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References

1. Liu D, Zhang XX, Li MC, Cao CH, Wan DY, Xi BX, et al. C/EBPbeta enhances platinum resistance of ovarian cancer cells by reprogramming H3K79 methylation. *Nat Commun*. 2018;9(1):1739.
2. Konecny GE, Wang C, Hamidi H, Winterhoff B, Kalli KR, Dering J, et al. Prognostic and therapeutic relevance of molecular subtypes in high-grade serous ovarian cancer. *J Natl Cancer Inst*. 2014;106(10).
3. Agarwal R, Kaye SB. Ovarian cancer: strategies for overcoming resistance to chemotherapy. *Nat Rev Cancer*. 2003;3(7):502-16.
4. Wang A, Li J, Zhou T, Li T, Cai H, Shi H, et al. CUEDC2 Contributes to Cisplatin-Based Chemotherapy Resistance in Ovarian Serious Carcinoma by Regulating p38 MAPK Signaling. *J Cancer*. 2019;10(8):1800-7.
5. Du L, Li CR, He QF, Li XH, Yang LF, Zou Y, et al. Downregulation of the ubiquitin ligase KBTBD8 prevented epithelial ovarian cancer progression. *Mol Med*. 2020;26(1):96.
6. Angers S, Thorpe CJ, Biechele TL, Goldenberg SJ, Zheng N, MacCoss MJ, et al. The KLHL12-Cullin-3 ubiquitin ligase negatively regulates the Wnt-beta-catenin pathway by targeting Dishevelled for degradation. *Nat Cell Biol*. 2006;8(4):348-57.
7. Humphreys LM, Smith P, Chen Z, Fouad S, D'Angiolella V. The role of E3 ubiquitin ligases in the development and progression of glioblastoma. *Cell Death Differ*. 2021;28(2):522-37.
8. Tang AH, Neufeld TP, Kwan E, Rubin GM. PHYL acts to down-regulate TTK88, a transcriptional repressor of neuronal cell fates, by a SINA-dependent mechanism. *Cell*. 1997;90(3):459-67.
9. Xiao JH, Ghosn C, Hinchman C, Forbes C, Wang J, Snider N, et al. Adenomatous polyposis coli (APC)-independent regulation of beta-catenin degradation via a retinoid X receptor-mediated pathway. *J*

Biol Chem. 2003;278(32):29954-62.

10. Hu G, Fearon ER. Siah-1 N-terminal RING domain is required for proteolysis function, and C-terminal sequences regulate oligomerization and binding to target proteins. *Mol Cell Biol.* 1999;19(1):724-32.
11. Gwak J, Song T, Song JY, Yun YS, Choi IW, Jeong Y, et al. Isoreserpine promotes beta-catenin degradation via Siah-1 up-regulation in HCT116 colon cancer cells. *Biochem Biophys Res Commun.* 2009;387(3):444-9.
12. Bruzzoni-Giovanelli H, Fernandez P, Veiga L, Podgorniak MP, Powell DJ, Candeias MM, et al. Distinct expression patterns of the E3 ligase SIAH-1 and its partner Kid/KIF22 in normal tissues and in the breast tumoral processes. *J Exp Clin Cancer Res.* 2010;29:10.
13. Xiao Z, Wei Z, Deng D, Zheng Z, Zhao Y, Jiang S, et al. Downregulation of Siah1 promotes colorectal cancer cell proliferation and migration by regulating AKT and YAP ubiquitylation and proteasome degradation. *Cancer Cell Int.* 2020;20:50.
14. Tanaka T, Iino M. Sec6 regulated cytoplasmic translocation and degradation of p27 via interactions with Jab1 and Siah1. *Cell Signal.* 2014;26(10):2071-85.
15. Reuven N, Adler J, Porat Z, Polonio-Vallon T, Hofmann TG, Shaul Y. The Tyrosine Kinase c-Abl Promotes Homeodomain-interacting Protein Kinase 2 (HIPK2) Accumulation and Activation in Response to DNA Damage. *J Biol Chem.* 2015;290(27):16478-88.
16. House CM, Moller A, Bowtell DD. Siah proteins: novel drug targets in the Ras and hypoxia pathways. *Cancer Res.* 2009;69(23):8835-8.
17. Fujita K, Horikawa I, Mondal AM, Jenkins LM, Appella E, Vojtesek B, et al. Positive feedback between p53 and TRF2 during telomere-damage signalling and cellular senescence. *Nat Cell Biol.* 2010;12(12):1205-12.
18. Shi H, Zheng B, Wu Y, Tang Y, Wang L, Gao Y, et al. Ubiquitin ligase Siah1 promotes the migration and invasion of human glioma cells by regulating HIF-1alpha signaling under hypoxia. *Oncol Rep.* 2015;33(3):1185-90.
19. Matsui-Hasumi A, Sato Y, Uto-Konomi A, Yamashita S, Uehori J, Yoshimura A, et al. E3 ubiquitin ligases SIAH1/2 regulate hypoxia-inducible factor-1 (HIF-1)-mediated Th17 cell differentiation. *Int Immunol.* 2017;29(3):133-43.
20. Wong CS, Moller A. Siah: a promising anticancer target. *Cancer Res.* 2013;73(8):2400-6.
21. Westermann P, Heumann W, Bommer UA, Bielka H, Nygard O, Hultin T. Crosslinking of initiation factor eIF-2 to proteins of the small subunit of rat liver ribosomes. *FEBS Lett.* 1979;97(1):101-4.
22. Tolan DR, Hershey JW, Traut RT. Crosslinking of eukaryotic initiation factor eIF3 to the 40S ribosomal subunit from rabbit reticulocytes. *Biochimie.* 1983;65(7):427-36.
23. Seong KM, Jung SO, Kim HD, Kim HJ, Jung YJ, Choi SY, et al. Yeast ribosomal protein S3 possesses a beta-lyase activity on damaged DNA. *FEBS Lett.* 2012;586(4):356-61.
24. Kim Y, Kim HD, Kim J. Cytoplasmic ribosomal protein S3 (rpS3) plays a pivotal role in mitochondrial DNA damage surveillance. *Biochim Biophys Acta.* 2013;1833(12):2943-52.

25. Kim TS, Kim HD, Kim J. PKCdelta-dependent functional switch of rpS3 between translation and DNA repair. *Biochim Biophys Acta*. 2009;1793(2):395-405.
26. Kim SH, Lee JY, Kim J. Characterization of a wide range base-damage-endonuclease activity of mammalian rpS3. *Biochem Biophys Res Commun*. 2005;328(4):962-7.
27. Kim J, Chubatsu LS, Admon A, Stahl J, Fellous R, Linn S. Implication of mammalian ribosomal protein S3 in the processing of DNA damage. *J Biol Chem*. 1995;270(23):13620-9.
28. Hegde V, Wang M, Deutsch WA. Human ribosomal protein S3 interacts with DNA base excision repair proteins hAPE/Ref-1 and hOGG1. *Biochemistry*. 2004;43(44):14211-7.
29. Kim SH, Kim J. Reduction of invasion in human fibrosarcoma cells by ribosomal protein S3 in conjunction with Nm23-H1 and ERK. *Biochim Biophys Acta*. 2006;1763(8):823-32.
30. Kim HD, Kim TS, Kim J. Aberrant ribosome biogenesis activates c-Myc and ASK1 pathways resulting in p53-dependent G1 arrest. *Oncogene*. 2011;30(30):3317-27.
31. Joo YJ, Kim JH, Kang UB, Yu MH, Kim J. Gcn4p-mediated transcriptional repression of ribosomal protein genes under amino-acid starvation. *EMBO J*. 2011;30(5):859-72.
32. Jang CY, Lee JY, Kim J. RpS3, a DNA repair endonuclease and ribosomal protein, is involved in apoptosis. *FEBS Lett*. 2004;560(1-3):81-5.
33. Sen N, Paul BD, Gadalla MM, Mustafa AK, Sen T, Xu R, et al. Hydrogen sulfide-linked sulfhydration of NF-kappaB mediates its antiapoptotic actions. *Mol Cell*. 2012;45(1):13-24.
34. Wan F, Weaver A, Gao X, Bern M, Hardwidge PR, Lenardo MJ. IKKbeta phosphorylation regulates RPS3 nuclear translocation and NF-kappaB function during infection with Escherichia coli strain O157:H7. *Nat Immunol*. 2011;12(4):335-43.
35. Gao X, Wan F, Mateo K, Callegari E, Wang D, Deng W, et al. Bacterial effector binding to ribosomal protein s3 subverts NF-kappaB function. *PLoS Pathog*. 2009;5(12):e1000708.
36. Yadavilli S, Mayo LD, Higgins M, Lain S, Hegde V, Deutsch WA. Ribosomal protein S3: A multi-functional protein that interacts with both p53 and MDM2 through its KH domain. *DNA Repair (Amst)*. 2009;8(10):1215-24.
37. Wier EM, Neighoff J, Sun X, Fu K, Wan F. Identification of an N-terminal truncation of the NF-kappaB p65 subunit that specifically modulates ribosomal protein S3-dependent NF-kappaB gene expression. *J Biol Chem*. 2012;287(51):43019-29.
38. Wan F, Anderson DE, Barnitz RA, Snow A, Bidere N, Zheng L, et al. Ribosomal protein S3: a KH domain subunit in NF-kappaB complexes that mediates selective gene regulation. *Cell*. 2007;131(5):927-39.
39. Youn H, Son B, Kim W, Jun SY, Lee JS, Lee JM, et al. Dissociation of MIF-rpS3 complex and sequential NF-kappaB activation is involved in IR-induced metastatic conversion of NSCLC. *J Cell Biochem*. 2015;116(11):2504-16.
40. Yang HJ, Youn H, Seong KM, Jin YW, Kim J, Youn B. Phosphorylation of ribosomal protein S3 and antiapoptotic TRAF2 protein mediates radioresistance in non-small cell lung cancer cells. *J Biol*

Chem. 2013;288(5):2965-75.

41. Yang W, Liu L, Li C, Luo N, Chen R, Li L, et al. TRIM52 plays an oncogenic role in ovarian cancer associated with NF-kB pathway. *Cell Death Dis.* 2018;9(9):908.
42. Tian M, Tian D, Qiao X, Li J, Zhang L. Modulation of Myb-induced NF-kB -STAT3 signaling and resulting cisplatin resistance in ovarian cancer by dietary factors. *J Cell Physiol.* 2019;234(11):21126-34.
43. Shen H, Liao B, Wan Z, Zhao Y, You Z, Liu J, et al. PTOV1 promotes cisplatin-induced chemotherapy resistance by activating the nuclear factor kappa B pathway in ovarian cancer. *Mol Ther Oncolytics.* 2021;20:499-507.
44. Dolcet X, Llobet D, Pallares J, Matias-Guiu X. NF-kB in development and progression of human cancer. *Virchows Arch.* 2005;446(5):475-82.
45. Sun MY, Xu B, Wu QX, Chen WL, Cai S, Zhang H, et al. Cisplatin-Resistant Gastric Cancer Cells Promote the Chemoresistance of Cisplatin-Sensitive Cells via the Exosomal RPS3-Mediated PI3K-Akt-Cofilin-1 Signaling Axis. *Front Cell Dev Biol.* 2021;9:618899.
46. Huang Z, Zhang Y, Li H, Zhou Y, Zhang Q, Chen R, et al. Vitamin D promotes the cisplatin sensitivity of oral squamous cell carcinoma by inhibiting LCN2-modulated NF-kappaB pathway activation through RPS3. *Cell Death Dis.* 2019;10(12):936.
47. Sun C, Li N, Yang Z, Zhou B, He Y, Weng D, et al. miR-9 regulation of BRCA1 and ovarian cancer sensitivity to cisplatin and PARP inhibition. *J Natl Cancer Inst.* 2013;105(22):1750-8.
48. Vergara D, Tinelli A, Iannone A, Maffia M. The impact of proteomics in the understanding of the molecular basis of Paclitaxel-resistance in ovarian tumors. *Curr Cancer Drug Targets.* 2012;12(8):987-97.
49. Kavallaris M. Microtubules and resistance to tubulin-binding agents. *Nat Rev Cancer.* 2010;10(3):194-204.
50. Li J, Zhang W, Gao J, Du M, Li H, Li M, et al. E3 Ubiquitin Ligase UBR5 Promotes the Metastasis of Pancreatic Cancer via Destabilizing F-Actin Capping Protein CAPZA1. *Front Oncol.* 2021;11:634167.
51. Herskho A, Ciechanover A. The ubiquitin system. *Annu Rev Biochem.* 1998;67:425-79.
52. Chen D, Li Y, Zhang X, Wu H, Wang Q, Cai J, et al. Ubiquitin ligase TRIM65 promotes colorectal cancer metastasis by targeting ARHGAP35 for protein degradation. *Oncogene.* 2019;38(37):6429-44.
53. Zhao J, Wang C, Wang J, Yang X, Diao N, Li Q, et al. E3 ubiquitin ligase Siah-1 facilitates poly-ubiquitylation and proteasomal degradation of the hepatitis B viral X protein. *FEBS Lett.* 2011;585(19):2943-50.
54. Wen YY, Yang ZQ, Song M, Li BL, Yao XH, Chen XL, et al. The expression of SIAH1 is downregulated and associated with Bim and apoptosis in human breast cancer tissues and cells. *Mol Carcinog.* 2010;49(5):440-9.
55. Kim Y, Kim HD, Youn B, Park YG, Kim J. Ribosomal protein S3 is secreted as a homodimer in cancer cells. *Biochem Biophys Res Commun.* 2013;441(4):805-8.

56. Deshaies RJ, Joazeiro CA. RING domain E3 ubiquitin ligases. *Annu Rev Biochem.* 2009;78:399-434.
57. Hou Y, Zhang Z, Xu Q, Wang H, Xu Y, Chen K. Inhibitor of growth 4 induces NFkappaB/p65 ubiquitin-dependent degradation. *Oncogene.* 2014;33(15):1997-2003.
58. Hou Y, Moreau F, Chadee K. PPARgamma is an E3 ligase that induces the degradation of NFkappaB/p65. *Nat Commun.* 2012;3:1300.

Figures

Figure 1

Figure 1

SIAH1 sensitizes ovarian cancer cells to cDDP. (A) Western blotting for SIAH1 in SKOV3 and A2780 cells. (B) Representative images of immunohistochemistry staining for SIAH1 in tumor specimens from ovarian cancer patients with PFS $\geq$ 6 months vs PFS $<$ 6 months (x 200 and x 400 magnification). (C) Staining was assessed and scored on a scale of 0 ($<$ 5% staining) to 4 ($>$ 75% staining). Quantification of IHC staining (n = 24; PFS $\geq$ 6, n = 12; PFS $<$ 6, n = 12) was shown. Cell colonies assay (D) and Transwell assay (x 100

magnification) (E) of A2780 cells transfected with Vector, SIAH1, Clt-shRNA and sh-SIAH1 were shown. The wound-healing assay was used to assess the effects of SIAH1 on cellular motility over time as shown (0, 24, 48, 72 and 96 h; original magnification, x100) (F), Cell Edu assay (x 200 magnification) (G), cell viability (H, I left panels), IC50 for cDDP (H, I right panels) were analyzed in A2780 cells respectively.*p < 0.05, **p < 0.01, ***p < 0.001.

Figure 2

Identification of RPS3 as an interaction partner of SIAH1 The heatmap showed that protein level of RPS3 was down-regulated with SIAH1 overexpression in EOC cells. (B) Pull down the RPS3 protein with SIAH1 antibody and IgG antibody in A2780 cells transfected with SIAH1. Cells were treated with 10 μ M MG132 for 6 h before cell lysis. A2780 cells were separately transfected with SIAH1, RPS3, sh-SIAH1 or sh-RPS3 for 48 h, the number of cell colonies (C), the cell number of transwell (D), the calculation of wound closure percentage (0, 24, 48, 72 and 96 h) (E), Edu positive rates (F), cell viability (G, H left panels), IC50 for cDDP (G, H right panels) were measured in A2780 cells respectively. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 3

SIAH1 down-regulates the protein level of RPS3. (A) Representative images of immunohistochemistry staining for RPS3 in tumor specimens from ovarian cancer patients with PFS ≤ 6 months vs PFS > 6 months (x 200 and x 400 magnification). (B) Staining was assessed and scored on a scale of 0 (<5% staining) to 4 (>75% staining). Quantification of IHC staining (n = 24; PFS > 6, n = 12; PFS < 6, n = 12) was shown. (C) Protein level of RPS3 in SKOV3 and A2780 cells. (D) Protein level of SIAH1 and RPS3 levels in serum samples from healthy controls (HC) and EOC patients (EOC). A2780 cells were separately transfected with Vector, RPS3, Ctl-shRNA and sh-RPS3 for 48 h, the number of cell colonies (E), the cell number of transwell (F), the calculation of wound closure percentage (0, 24, 48, 72 and 96 h) (G), Edu positive rates (H), cell viability (I, J left panels), IC50 for cDDP (I, J right panels) were measured in A2780 cells respectively. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 4

SIAH1 induces degradation of RPS3 protein. (A) A2780 cells were transfected with Vector, SIAH1. Cells were treated with or without 10 μ M MG132 for 6 h before cell lysis. Cell lysates were subjected to Western blotting. (B) RPS3 mRNAs level was detected in A2780 cells with SIAH1 overexpression. (C, D) RPS3 protein half-life was assayed by using CHX (30 μ g/ml) in HEK293T cells transfected with GFP or GFP-SIAH1 plasmid. The relative remaining RPS3 protein levels following CHX treatment at each time point

was calculated accordingly. (E) Colocalization of RPS3 and SIAH1. The GFP-SIAH1 or Cherry-RPS3 plasmids were transfected into A2780 cells. Cells were treated with or without 10 μ M MG132 for 6 h before cell lysis. GFP-SIAH1 was detected using a fluorescence microscope with an excitation wavelength of 488 nm. Cherry-RPS3 was detected with an excitation wavelength of 556 nm. The cell nuclei were stained with DAPI. Scale bar, 50 μ m. (F) A2780 cells were transfected with plasmids as indicated, pull down the RPS3 protein with GFP antibody. Cell lysates were subjected to Western blotting. *p < 0.05, ***p < 0.001.

Figure5

Figure 5

SIAH1 is an E3 ligase to induce RPS3 ubiquitination. (A, B, C, D, E, F) HEK293T cells were transfected plasmids as indicated for 36 h. Cell lysates were subjected to denatured immunoprecipitation and Western blotting. Cells were treated with MG132 (10 μ M) for 6 h before cell lysis. (A) SIAH1 over expression induced ubiquitination and degradation of endogenous RPS3. (B) SIAH1 over expression induced ubiquitination and degradation of exogenous RPS3. (C) Knock down SIAH1, the ubiquitination level of RPS3 was significantly reduced. (D) Upon SIAH1 expression, FLAG-RPS3 (K214R) had a significantly lower ubiquitination level compared with wild type (FLAG-RPS3). (E) With SIAH1 KD, FLAG-RPS3 (K214R) had a significantly lower ubiquitination level compared with wild type (FLAG-RPS3). (F) In SIAH1 inhibited A2780 cells, mutation of the RING finger domain of SIAH1 significantly reduced the ubiquitination level of RPS3.

Figure6
A

and sh-SIAH1 for 48 h, and treated with PDTTC for 24 h. The number of cell colons (D), the cell number of transwell (E), the calculation of wound closure percentage (0, 24, 48, 72 and 96 h) (F), Edu positive rates (G), cell viability (H, I left panels), IC50 for cDDP (H, I right panels) was measured in A2780 cells respectively. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure7

Figure 7

SIAH1 enhances the sensitivity of ovarian cancer cells to cDDP by down-regulating RPS3 in vivo. A2780 cells were transfected respectively with lentivirus of Vector, SIAH1, CIt-shRNA, sh-SIAH1, SIAH1+FLAG-RPS3 and SIAH1+FLAG-RPS3 (K214R) subcutaneously and intraperitoneally injected into female BALB/c nude mice, and potential tumors were allowed to grow for a week(n=6). All the groups were administered DDP (5 mg/kg) by intraperitoneal injection three times a week for 8 times before sacrificed. (A) Flow chart of the animal experiment. (B) Representative images of the excised tumors on day 28 after tumor cell injection. (C) Tumor volumes of the excised tumors on day 35 after tumor cell injection. (D) Typical pictures of tumors in the abdominal cavity after intraperitoneal injection in each group of mice. (E) The level of SIAH1 and RPS3 proteins were assessed by Western blotting in mouse xenograft tumor tissues. (F) The apoptosis level of tumor tissue in each group detected by TUNEL. (G) Immunohistochemistry analyses for KI-67, SIAH1, RPS3 and FLAG staining were carried out on A2780 xenograft tumor sections. Representative staining is shown (x 400 magnification). **p < 0.01, ***p < 0.001.

Figure 8

Schematic diagram of this study.

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