Original Article

# Endoplasmic reticulum stress-MMRN1 positive feedback contributes to cisplatin resistance in small cell lung cancer

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**Background:** Small cell lung cancer (SCLC) accounts for 15% of all lung cancers and presents early metastasis and poor prognosis. Chemotherapy with cisplatin (CDDP) remains one of the standards of care in first-line treatment. However, the emergence of acquired resistance to CDDP causes disease progression and cancer recurrence. A comprehensive understanding of the CDDP-resistant mechanisms aids in defining accurate biomarkers and developing potential strategies.

**Methods:** The liquid chromatograph mass spectrometer (LC-MS/MS) was conducted to analyze the differential exosomal proteins from plasma samples of SCLC patients with non-treatment or resistance to CDDP. The online RNA-seq data with clinicopathological information on SCLC patients were downloaded from the Gene Expression Omnibus (GEO) database for further prognostic analysis. The SCLC cell line model of acquired CDDP resistance was established to investigate the role of platelet protein multimerin-1 (MMRN1) in CDDP resistance.

**Results:** MMRN1 was increased in CDDP-resistant SCLC patients and cell line models. Reduction of MMRN1 recovered the sensitivity to CDDP while overexpression of MMRN1 conferred CDDP resistance. The CDDP-resistant SCLC cells disseminated resistant to the CDDP-sensitive SCLC cells via the exosomal MMRN1. Additionally, CDDP treatment induces endoplasmic reticulum (ER) stress and subsequent upregulation of MMRN1. Increasing MMRN1 interacted with binding immunoglobulin protein (BiP) in the ER, maintaining the ER stress in SCLC cells.

**Conclusions:** The present study identified exosomal MMRN1 as a potential biomarker for CDDP resistance in SCLC. MMRN1 sustains ER stress via interaction with BiP and subsequently facilitates CDDP resistance, which might be a promising therapeutic target to overcome CDDP resistance.

**Keywords:** Small cell lung cancer (SCLC); cisplatin resistance; endoplasmic reticulum stress (ER stress); multimerin-1 (MMRN1); binding immunoglobulin protein (BiP)

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## Introduction

Lung cancer is a leading cause of mortality worldwide, with 2,480,301 new cases diagnosed in 2022 (1). According to the cell of origin, lung cancer can be categorized into small cell lung cancer (SCLC) and non small cell lung cancer (NSCLC), representing approximately 15% and 85%, respectively (2). SCLC arises from poorly differentiated neuroendocrine cells and is characterized by rapid proliferation, aggressive metastasis, high recurrence risk, and poor prognosis (3). Standard of care treatment for SCLC includes surgery, radiotherapy, chemotherapy, and immunotherapy, often involving a combination of these treatments (4). In the past decades, the standard frontline therapy for newly diagnosed patients with SCLC is etoposide with either cisplatin (CDDP) or carboplatin (4). Although the initial objective response rate (ORR) for platinum drugs is high, the emergence of acquired resistance causes a five-year survival rate of less than 10% (5). Therefore, it remains crucial to understand the molecular mechanisms of CDDP resistance in SCLC and identify accurate biomarkers to predict resistance.

The underlying mechanisms of CDDP resistance have been enormously investigated in past decades and include reduced drug uptake, increased drug efflux, cellular detoxification, DNA damage repair, autophagy, apoptosis evasion, ferroptosis, and many others (6). Endoplasmic reticulum (ER) stress is induced by unbalanced proteostasis with the accumulation of misfolded/unfolded proteins in ER (7). ER stress activates unfolded protein response (UPR) to restore proteostasis by switching on the ER chaperone immunoglobulin heavy chain binding protein (BiP)-mediated downstream signaling pathways, including inositol-requiring enzyme 1 (IRE1), double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6) (8). Emerging evidence suggests that ER stress contributes to cancer initiation, progression, and therapeutic resistance. CDDP induces ER stress and ER stress enhances CDDP resistance (9,10). Specifically, the CDDP resistance associated with ER stress correlates with the downstream pathways of the UPR or the altered biological functions of BiP (11). However, the interplay between CDDP resistance and ER stress in SCLC is sophisticated and remains unclear.

The exosome is a subtype of extracellular vehicles (EVs) with an average diameter of 100 nm (12). Exosomes transmit numerous components, which including nucleic acids, proteins, metabolites, and others (12). Different tumor-related exosomes and its exosomal contents are secreted by different tumor cells under multi-conditions, which can be transferred from the parent cells to recipient cells and be one of the key determinants of cellular communication. It has been proved that exosome inhibition could improve responses to the first-line therapy in SCLC, which hints the vital functions of exosomes in SCLC (13).

### Highlight box

**Key findings**

- Higher levels of exosomal multimerin-1 (MMRN1) in the plasma of cisplatin (CDDP)-resistant small cell lung cancer (SCLC).
- Intercellular transmission of MMRN1 via exosomes spread CDDP resistance.
- Endoplasmic reticulum (ER) stress elevates intracellular and exosomal MMRN1.
- MMRN1 binds to BiP and maintains constant ER stress.

**What is known and what is new?**

- CDDP induces ER stress and ER stress enhances CDDP resistance.
- Exosomal MMRN1 transmits CDDP resistance via maintaining constant ER stress.

**What is the implication, and what should change now?**

- Since SCLC is more aggressive but uncommon, only six patients were enrolled in this study which also lacked the self-comparison before/after CDDP resistance form. More clinical samples are needed to verify the predictive value of exosomal MMRN1 in CDDP-resistant SCLC.
- Further studies employing in vitro protein-protein interaction experiments and relevant cell line/patient-derived SCLC mouse models are required to strengthen the rationale and understanding of mechanisms and potential for targeting MMRN1/BiP/ER stress axis in SCLC.

Multimerin-1 (MMRN1) is a glycoprotein belonging to the EMILIN/multimerin family and is mainly expressed in platelets, megakaryocytes, endothelial cells, and the extracellular matrix but undetectable in normal plasma (14,15). In recent years, MMRN1 in cancer has come to notice. High levels of MMRN1 are observed in various types of cancer, including acute myeloid leukemia, epithelial ovarian cancer, and head and neck squamous cell carcinoma (HNSCC), and are associated with poor prognosis (16-18). High level of MMRN1 is also detected in liquid biopsies, such as saliva samples of epithelial ovarian cancer, serum samples of prostate cancer, urine samples of cervical cancer, and exosomal samples of urothelial bladder cancer (19). Additionally, low levels of MMRN1 are also reported in breast cancer, hepatocellular carcinoma, NSCLC, and plasma of cervical cancer (19-21). The differential expression of MMRN1 in distinct types of cancer indicates that the role of MMRN1 in cancer initiation and progression is context-dependent. However, little is known about the detailed mechanisms underlying the pro- or anti-tumoral function of MMRN1.

In the present study, we conducted a proteomic analysis of exosomal proteins in plasma collected from SCLC patients with non-treatment and CDDP-resistance and identified MMRN1 as a potential biomarker for CDDP resistance. We hypothesized that cancer cell-derived MMRN1 might contribute to CDDP resistance in SCLC patients. In support of this hypothesis, we discovered higher intracellular and exosomal MMRN1 levels in acquired CDDP-resistant SCLC cells established in our lab. Genetic knockdown of MMRN1 recovered sensitivity to CDDP *in vivo* and *in vitro*. We found that intercellular transfer of MMRN1 by exosomes transmitted CDDP resistance. Moreover, MMRN1 induced by ER stress bound to BiP and facilitated adaption to ER stress, which eventually led to CDDP resistance. Our collective findings could provide a potential biomarker for monitoring CDDP resistance and a promising therapeutic target for recovering CDDP sensitivity in SCLC patients. We present this article in accordance with the ARRIVE and MDAR reporting checklists (available at https://jtd.amegroups.com/article/view/10.21037/jtd-24-1477/rc).

## Methods

### *Patients and clinical samples*

A total of six SCLC patients were enrolled in this study at Sichuan Cancer Hospital (Chengdu, China) from September 2020 to September 2021. All patients were pathologically confirmed as SCLC and detailed clinicopathologic information was included in Table S1. Three SCLC patients did not receive any antitumor treatment and were assigned as the non-treatment cohort. Three SCLC patients were diagnosed with disease progression after receiving CDDP treatment for four cycles. The blood was centrifuged for 20 min at 1,500 g at 4 ℃ and 3,000 g at 4 ℃ for 20 minutes to separate the plasma. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Ethics Committee of Sichuan Cancer Hospital (SCCHEC-02-2022-047). All patients signed a written consent for using blood and cancer tissue for scientific research.

### *Animals experiments*

Experiments were performed under a project license (No. SCCHEC-04-2022-002) granted by the Ethics Committee of Sichuan Cancer Hospital, in compliance with Laboratory Animal Guideline for ethical review of animal welfare (No. GB/T 35892-2018) for the care and use of animals. The four-week-old male nude mice were purchased from Huafukang Animal Centre (Beijing, China) and allowed free access to food and water under specific pathogen-free (SPF) feeding conditions. A total of 100 µL serum-free medium containing H446-R-shNC/H446-R-sh2 cells ($1\times10^7$) and 100 µL High Concentration Matrigel (Cat.354248; Corning, NY, USA) were inoculated subcutaneously into the right dorsal flanks of the mice (N=6/group) (22). When the tumor volume reached 280–300 $mm^3$ (day 0), the mice were treated as previously described (23). The mice were treated with CDDP every other week (5 mg/kg in saline, intraperitoneal) for two weeks and the tumor volume was monitored every four days. The mice were sacrificed, and tumors were excised immediately. The tumors were washed in saline, measured, and fixed with 4% paraformaldehyde for further analysis.

### *Exosome isolation*

Exosomes were isolated by Exosome Precipitation Solution (Urine) (Cat.114909; MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) according to the manufacturer's instruction. Briefly, the conditional medium from the cell culture was centrifuged at 4,000 ×g for 15 min to remove cell debris. The supernatant was transferred to a new tube and supplemented with 0.4 volumes of

Exosome Precipitation Solution (Urine). The samples were then mixed thoroughly via vortex and incubated at 4 ℃ for 30 min. Subsequently, centrifuge the samples at 4,000 ×g for 15 min to precipitate the exosomes. The exosome was resuspended in either RNase-free $H_2O$ or Radioimmunoprecipitation Assay (RIPA) buffer (Cat. P0013B; Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitor cocktail (Cat.B14001; Selleck Chemicals, TX, USA) and phosphatase inhibitor (Cat.B15001; Selleck Chemicals) for further experiments.

### *Transmission electron microscopy (TEM)*

The sample was absorbed by 10 μL and deposited on the copper mesh for 1 min. The float was absorbed by filter paper. 10 μL of the uranyl acetate was added to the copper net to precipitate for 1 min, and the filter paper absorbed the floating liquid. Dry at room temperature for a few minutes. Electron microscopy imaging was performed at 100 kv.2.4 liquid chromatograph mass spectrometer (LC-MS/MS) analysis.

### *Plasma exosomes isolation (ultra-centrifugation)*

The plasma sample was centrifuged at 2,000 g, 4 ℃ for 30 min. The supernatant was carefully moved to a new centrifuge tube for 10,000 g, 4 ℃ for 45 min to remove the larger vesicles. The supernatant was obtained and filtered by 0.45 μm filter membrane and collected. The filtrate was centrifuged at 4,100,000 g, 4 ℃ for 70 min to remove the supernatant. After being re-suspended with 10 mL of pre-cooled 1× PBS, it would be centrifuged at 4,100,000 g, 4 ℃ for 70 min again. The supernatant was removed and re-suspended with 150 μL pre-cooled 1× PBS. The exosomes were frozen at –80 ℃.

### *Differential proteins expressions analysis*

Plasma exosomal proteomic data of patients in this study were analyzed by using the DESeq2 R package, and proteins were selected as differential expression proteins with a satisfactory of $|\log_2(FC)| > 1$ and $P<0.05$.

### *Bioinformatics analysis of public datasets*

The SCLC RNA-seq data was obtained from the GEO database (GSE60052), comprising a total of 83 SCLC samples. Survival curve analysis is analyzed through the R package survival.

### *Immunohistochemical (IHC) analysis*

IHC was performed on SCLC tumor tissue slides as described previously (24). The slides were incubated with Anti-MMRN1 rabbit pAb (1:50, Cat.ab244451, Abcam, Cambridge, UK) at 4 ℃ overnight.

### *Cell culture and establishment of CDDP-resistant cells*

HEK293T cells and human SCLC cells (NCI-H446 and NCI-H1688) were purchased from Guangzhou Cellcook Biotech Co., Ltd. (Guangzhou, China). SCLC cells were cultured in RPMI 1640 medium (Cat.C11875500BT; Gibco, MA, USA) supplemented with 10% fetal bovine serum (FBS; Cat.F101-01; Vazyme Biotech, Jiangsu, China), 100 U/mL penicillin, and 100 μg/mL streptomycin (Cat. CB010; Yamei Bio, Shanghai, China). HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Cat.C11995500BT; Gibco) supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were cultured at 37 ℃ in a humidified atmosphere containing 5% $CO_2$. All cell lines were authenticated every 6 months via STR profiling (Beijing Tsingke Biotech Co., Ltd., Beijing, China).

H446 cells were treated with increasing doses of CDDP (Cat.S1166; Selleck Chemicals) for three days and recovered in a medium without CDDP for 14 days. The survival clones were continually cultured in 1 μM CDDP for more than 90 days. The half-maximal inhibitory concentration ($IC_{50}$) of CDDP was determined by CCK-8 assay.

### *Generation of MMRN1 overexpression cells and knockdown cells*

HEK293T cells were transfected with envelop vector (pMD2G), packaging vector (psPAX2), and transfer vector (GV492) with or without full-length human MMRN1 cDNA (Gene ID: 22915) to generate lentiviral particles. H446 and H1688 cells were transfected with lentiviral particles and selected by puromycin.

One lentiviral plasmid containing non-target control sequence (shNC) and two lentiviral plasmids containing short hairpin RNAs (shRNA) specifically targeting different sites of MMRN1 were purchased from Shanghai Genechem Co., Ltd. The shRNA lentiviral particles were generated as above. The CDDP-resistant H446-R cells were transfected

with lentiviral particles and selected by puromycin.

### *Cell counting kit 8 (CCK-8) assay*

CCK-8 assay was performed to determine the $IC_{50}$ of CDDP according to the manufacturer's instruction (Cat.B34302; Selleck Chemicals). Briefly, 5,000 cells/well of indicated cells were seeded in 96-well plates and incubated at the 37 ℃ incubator overnight. The cells were treated with CDDP at different concentrations for 72 h. The CCK-8 reagents were prepared in fresh medium and added to each well. After 2 h incubation, the optical density at a wavelength of 450 nm was detected by using the spectrophotometer (Molecular Device, CA, USA). The $IC_{50}$ value was calculated and graphed via GraphPad software (MA, USA).

### *Western Blot (WB)*

Intracellular and exosomal proteins were lysed by RIPA buffer with protease and phosphatase inhibitors. Protein was quantified using the BCA Protein Quantification kit (Cat.E112-02; Vazyme) and resolved by SDS-PAGE gels. The protein was transferred to the polyvinylidene fluoride (PVDF) membrane (Cat.IPVH00010; EMD Millipore, MA, USA). The membranes were incubated with the indicated primary antibody at 4 ℃ overnight, followed by 1 h incubation with the corresponding HRP-linked secondary antibody at room temperature. A chemiluminescence substrate (Cat. E423-01; Vazyme) was used to visualize the protein. Antibodies used for WB: MMRN1 (Cat.YN4274; 1:1,000; Immunoway, CA, USA), HRP goat anti-rabbit IgG (Cat.AS014; 1:5,000; ABclonal, Hubei, China).

### *Quantitative real-time polymerase chain reaction (qRT-PCR)*

Messenger RNAs were extracted using the RNA isolation kit V2 (Cat.RC112-01; Vazyme), followed by the synthesis of cDNA using the HiScript III All-in-one RT SuperMix Perfect for qPCR kit (Cat. R333-01; Vazyme). The ChamQ SYBR qPCR Master Mix kit (Cat. Q311-02; Vazyme) and the Bio-Rad CFX96 Real-Time system (CA, USA) were used for qRT-PCR reactions. Relative mRNA expression was quantified by using the $-\Delta\Delta Ct$ method. Primers used for qRT-PCR:

MMRN1: forward: 5'-CAGAAGGAGTGGTCAAGTTACAG-3'; reverse: 5'-GCTGAGGTATGTTTCCCGTGG-3'.

Actin: forward: 5'-GGCATTCACGAGACCACCTAC-3'; reverse: 5'-CGACATGACGTTGTTGGCATAC-3'.

### *Molecular docking analysis*

Rigid protein-protein docking was performed via the GRAMM web server (https://gramm.compbio.ku.edu/gramm) as previously described (25,26). The protein sequences and structures of MMRN1 and BiP were obtained from the Uniprot database (www.uniprot.org) and Alphafold database (https://alphafold.ebi.ac.uk/), respectively. The protein-protein interaction was analyzed and visualized using Pymol (version 2.4) and PDBePISA (https://www.ebi.ac.uk/pdbe/pisa/).

### *Coimmunoprecipitation (Co-IP)*

Protein was lysed by IP lysis buffer (Cat.87787; Thermo Fisher, MA, USA) supplemented with protease and phosphatase inhibitors. BiP was immunoprecipitated using the anti-BiP antibody (Cat.11587-1-AP; Proteintech, IL, USA) and Protein A/G Magnetic Beads for IP (Cat.B23201; Selleck Chemicals). MMRN1-Flag was immunoprecipitated with anti-Flag antibody (Cat.AE005; ABclonal) and Protein A/G Magnetic Beads. The anti-IgG (Cat.sc-2025; Santa Cruz, CA, USA) was used as the corresponding isotype control. The interacting proteins were detected using WB.

### *Statistical analysis*

In this study, GraphPad Prism 9.0 and Microsoft Excel 2023 software were used for statistical analysis. Student's *t*-test was used to analyze the difference between the two groups of data. Two-way ANOVA (or mixed model) test was used to analyze the differences between multiple sets of data between the two groups. $P<0.05$, $P<0.01$ and $P<0.001$ are represented as *, **, and ***, respectively, which are statistically significant differences in different confidence intervals. Figures are representative of three independent experiments at least.

## Results

### *Elevated MMRN1 is detected in CDDP-resistant SCLC patients and correlated with poor prognosis*

To investigate mechanisms for CDDP resistance, we first

isolated exosomes in the plasma of three SCLC patients before CDDP treatment (non-treatment cohort) and three SCLC patients with disease progression after CDDP treatment (resistant cohort) in our hospital (*Figure 1A*). The exosomes were identified using TEM (*Figure 1B*). The exosomal proteins were processed for proteomic analysis. As shown in *Figure 1C*, the proteomic analysis detected 16 increased proteins and 8 decreased proteins in the plasma from the resistant cohort compared to the non-treatment cohort. The expression pattern of all altered proteins in the non-treatment and resistant cohorts was shown in the heat map (*Figure 1D*). Among all the differentially expressed proteins, exosomal MMRN1 was significantly upregulated in the plasma from CDDP-resistant SCLC patients. The level of MMRN1 in the tumor tissues from the non-treatment and resistant cohorts was also evaluated using IHC. Compared to the non-treatment SCLC patient (#1), MMRN1 was higher in the tumor tissue from the CDDP-resistant SCLC patient (#4) (*Figure 1E*). These findings imply that MMRN1 is elevated in CDDP-resistant tissues and released in exosomes, which might contribute to CDDP-resistance development in SCLC. Additionally, compared to those with a low level of MMRN1, those with a high level of MMRN1 had a greater hazard of death in SCLC patients (P<0.0001) (*Figure 1F*). These results suggest that MMRN1 could be both CDDP resistant biomarker and a prognostic biomarker for SCLC patients, which needs further identification.

### *MMRN1 contributed to CDDP resistance in SCLC cells*

To investigate the role of MMRN1 in CDDP resistance, we established acquired CDDP-resistant H446 cells, namely H446-R (*Figure 2A*). H446-R cells responded with morphological change as compared with their parental H446 cells (*Figure 2B*). The $IC_{50}$ against CDDP in H446 cells was 2.390 μM, while the $IC_{50}$ increased to 12.880 μM in H446-R cells (*Figure 2C*). Then we examined the expression level of MMRN1 in both H446 and H446-R cells. As shown in *Figure 2D,2E*, MMRN1 mRNA and protein expression were significantly upregulated in H446-R cells compared to its parental counterpart. Immunofluorescent staining showed that MMRN1 was mainly localized in the ER (*Figure 2F*).

To address the contribution of MMRN1 on CDDP resistance in SCLC cells, we established SCLC cells with stable overexpression of MMRN1 (*Figure 2G,2H*). The CCK-8 assay showed that MMRN1 led to an increase of $IC_{50}$ against CDDP in both H446 and H1688 cells (*Figure 2I,2J*). We also silenced MMRN1 in H446-R cells via two shRNAs specifically targeting different regions of MMRN1. The knockdown efficiency was confirmed by WB and qRT-PCR (*Figure 3A*). The $IC_{50}$ against CDDP dropped from 11.950 μM to 5.916 μM and 4.382 μM in two H446-R with stable knockdown of MMRN1 (*Figure 3B*). We further established the xenograft tumor models by subcutaneously injecting H446-R-shNC and H446-R-sh2 cells into the dorsal surfaces of BALB/c-nu mice, who were treated with CDDP to investigate whether targeting MMRN1 could alter CDDP sensitivities of SCLC *in vivo*. Following the treatment of CDDP to xenograft tumor models, it was shown that the tumor weight and volume of H446-R-sh2 cell-derived tumors were much smaller than those of H446-R-shNC tumors (*Figure 3C-3E*). The H&E staining was also conducted and H446-R-sh2 cell-derived tumor tissue was shown with more necrotic lesions (*Figure 3F*). Collectively, these findings illustrate that MMRN1 induces CDDP resistance in SCLC cells and inhibition of MMRN1 might be a promising strategy for SCLC treatment.

### *Exosomal MMRN1 contributed to CDDP resistance in SCLC cells*

Next, we studied whether MMRN1 was specifically packed into exosomes. Exosomes were extracted from the medium of parental H446 and CDDP-resistant H446-R cells and processed for WB analysis. The MMRN1 level was markedly increased in exosomes secreted by H446-R cells (*Figure 4A*). The purified exosomes were marked by PKH67 and supplemented into H446 cells (*Figure 4B*). CCK-8 assay showed that H446 cells became insensitive to CDDP after the incubation with the exosomes derived from H446-R cells (*Figure 4C*). The MMRN1 level in exosomes derived from H446 and H1688 cells with exogenous MMRN1 was also upregulated compared to control cells (*Figure 4D,4E*). Additionally, the exosomes secreted by H446 and H1688 cells with exogenous MMRN1 decreased sensitivity to CDDP in H446 and H1688 cells, respectively (*Figure 4F,4G*).

To further interrogate the contribution of exosomal MMRN1 on CDDP resistance, GW4869 was adopted to suppress exosome secretion. GW4869 treatment caused accumulation of MMRN1 in cells (*Figure 4H*). The conditional medium from H446-R treated with GW4869 could not increase $IC_{50}$ against CDDP in H446 (*Figure 4I*). Similarly, the conditional medium from MMRN1

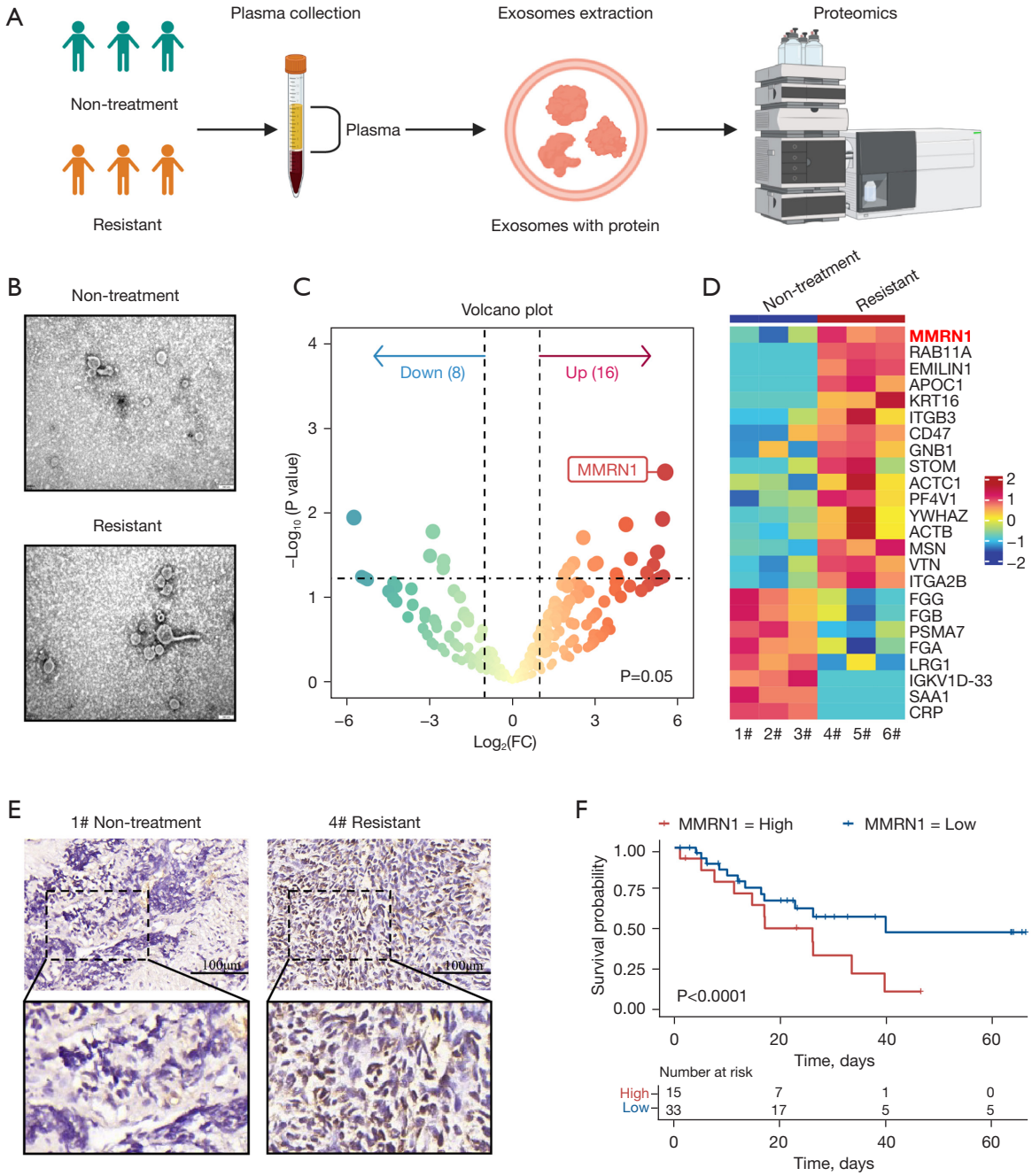



**Figure 1** MMRN1 is elevated in plasma exosomes and tumor tissues from CDDP-resistant SCLC patients. (A) Overview of plasma collection from SCLC patients before treatment (non-treatment cohort, N=3) and with disease progression after CDDP treatment (resistant cohort, N=3). The workflow for processing the proteomic data was shown, including plasma separation, exosome isolation, and the LC-MS/MS analysis. (B) The TEM images of the plasma exosomes. Scale bar =200 nm. (C) Volcano plot of differentially expressed exosomal proteins generated from plasma from non-treatment and CDDP resistant SCLC patients. $|Log_2 (FC)=1|$, P=0.05. (D) Heatmap of differentially expressed exosomal proteins generated from plasma from non-treatment and CDDP resistant SCLC patients.(E) Representative image of IHC analysis of MMRN1 from non-treatment (1#) and CDDP resistant (4#) SCLC patients. Scale bar =100 μm. 4.9× magnifications are shown. The zoom image shows the selected region from the core. (F) Kaplan-Meier plots of the survival analysis of SCLC patient data from the GSE60052 dataset (N=83, P<0.0001). SCLC, small cell lung cancer; CDDP, cisplatin; LC-MS, liquid chromatograph mass spectrometer; TEM, transmission electron microscopy; FC, fold change; IHC, immunohistochemistry.

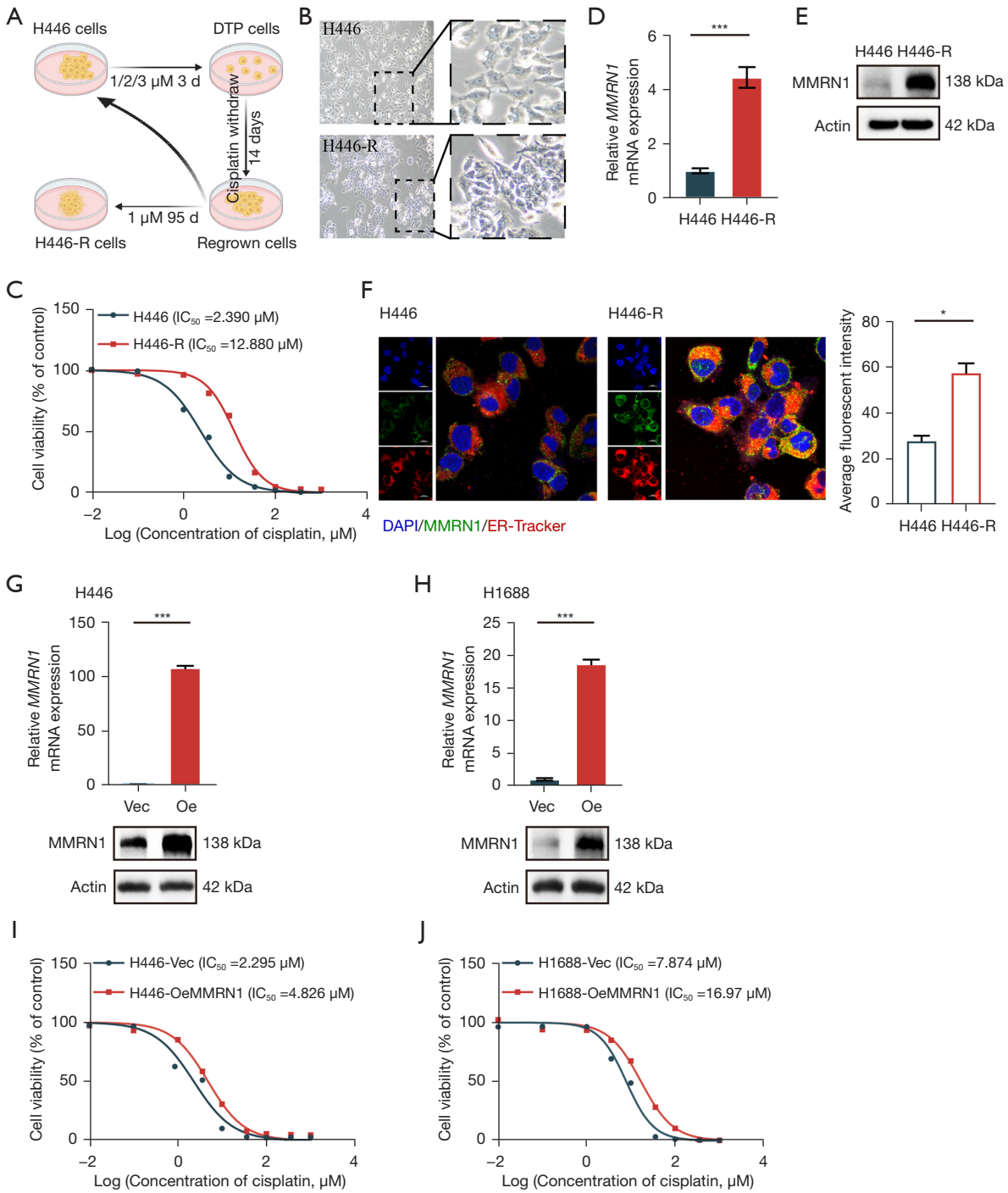



**Figure 2** MMRN1 reduced the sensitivity of SCLC cells to CDDP. (A) The schematic diagram of establishing CDDP-resistant H446 cells (H446-R) (created with Biorender.com). (B) Morphological changes of CDDP-resistant cancer sub-lines (H446-R) represent elongated and irregular morphology. ×6. (C) CCK-8 assay was conducted to determine the $IC_{50}$ of CDDP in parental H446 and CDDP-resistant H446-R cells. (D) qRT-PCR was performed to measure MMRN1 mRNA expression in parental H446 and CDDP-resistant H446-R cells. (E) WB was performed to measure MMRN1 protein expression in parental H446 and CDDP-resistant H446-R cells. (F) Immunofluorescent staining of MMRN1 (green), ER-tracker (red), and nuclear (DAPI, blue) was performed to analyze the expression and localization of MMRN1. (Left panel) Representative image; (right panel) semi-quantitative measurement of mean fluorescence intensity of MMRN1. ×9.

(G,H) qRT-PCR and WB were performed to measure MMRN1 expression in H446 and H1688 stably transfected with control plasmid (Vec) or MMRN1 (Oe). mRNA expression was normalized to Vec. (I,J) CCK-8 assay was conducted to determine the $IC_{50}$ of CDDP in H446 and H1688 stably transfected with Vec or Oe. *, P<0.05; ***, P<0.001. SCLC, small cell lung cancer; CDDP, cisplatin; CCK-8, cell counting kit 8; $IC_{50}$, half-maximal inhibitory concentration; qRT-PCR, quantitative real-time polymerase chain reaction; WB, Western Blot; ER, endoplasmic reticulum.

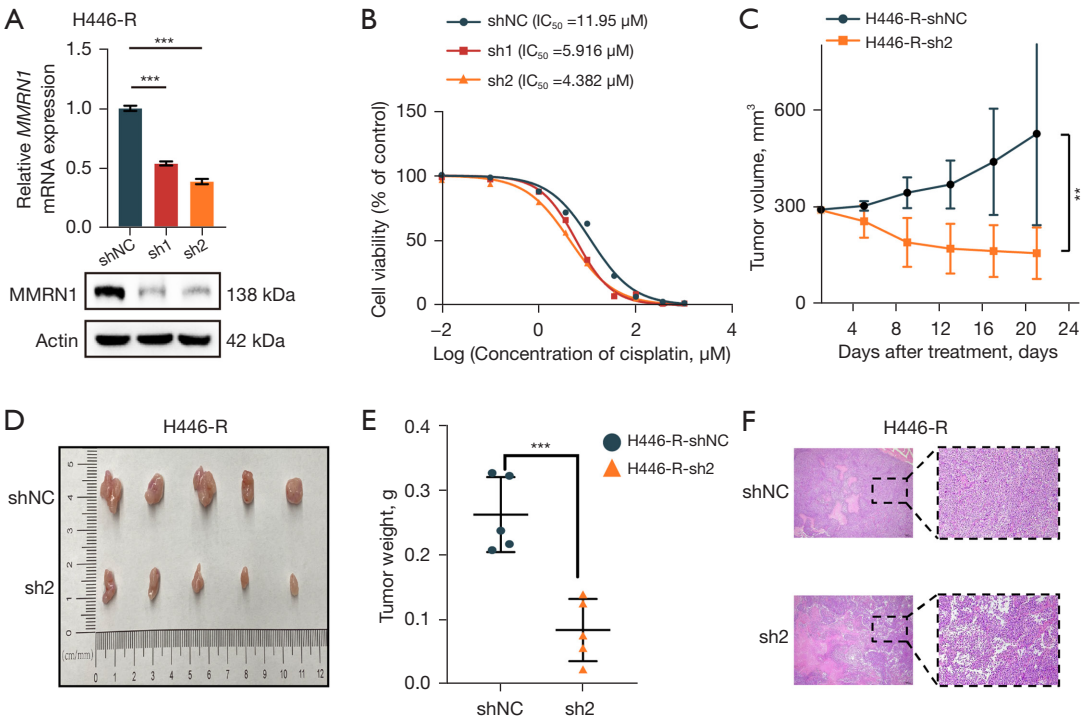



**Figure 3** Downregulation of MMRN1 recovers the CDDP sensitivity in SCLC. (A) qRT-PCR and WB were performed to measure MMRN1 expression in H446-R stably transfected with non-target control (shNC) or sh1/sh2 specifically targeting MMRN1. mRNA expression was normalized to shNC. (B) CCK-8 assay was conducted to determine the $IC_{50}$ of CDDP in H446-R stably transfected with shNC or sh1/sh2 specifically targeting MMRN1. (C) The volume of subcutaneous tumors after treatment. (D) Images of subcutaneous tumors at day 22 and (E) tumor weight. (F) H&E staining of tumors from different groups. Scale bar =100 μm. 16× magnifications are shown. **, P<0.01; ***, P<0.001. Data represent the mean ± SD in (E). P values were determined using Student's *t*-test in (C,E). CDDP, cisplatin; SCLC, small cell lung cancer; qRT-PCR, quantitative real-time polymerase chain reaction; WB, Western Blot; CCK-8, cell counting kit 8; $IC_{50}$, half-maximal inhibitory concentration; SD, standard deviation.

overexpressing SCLC cells treated with GW4869 could not alter the sensitivity to CDDP (*Figure 4J,4K*). These observations indicate that the exosomal MMRN1 might transmit CDDP resistance to sensitive SCLC recipient cells.

### *ER stress-MMRN1 feedback induced CDDP resistance*

Since MMRN1 is a secreted protein and localized in the ER, we speculated whether MMRN1 induces CDDP resistance through ER stress. We analyzed ER stress-related proteins in parental H446 and CDDP-resistant H446-R cells, as well as MMRN1 overexpressing H446 and H1688 cells. As shown in *Figure 5A*, the BiP expression, phosphorylated PERK, and phosphorylated ERK were elevated in H446-R cells. Compared to their control cells, higher BiP expression and higher PERK and ERK phosphorylation were observed in MMRN1 overexpressing H446 and H1688 cells (*Figure 5B,5C*). Additionally, exosomes secreted by H446-R and H446 with exogenous MMRN1 gave rise to BiP and phosphorylation of PERK and ERK in H446 cells

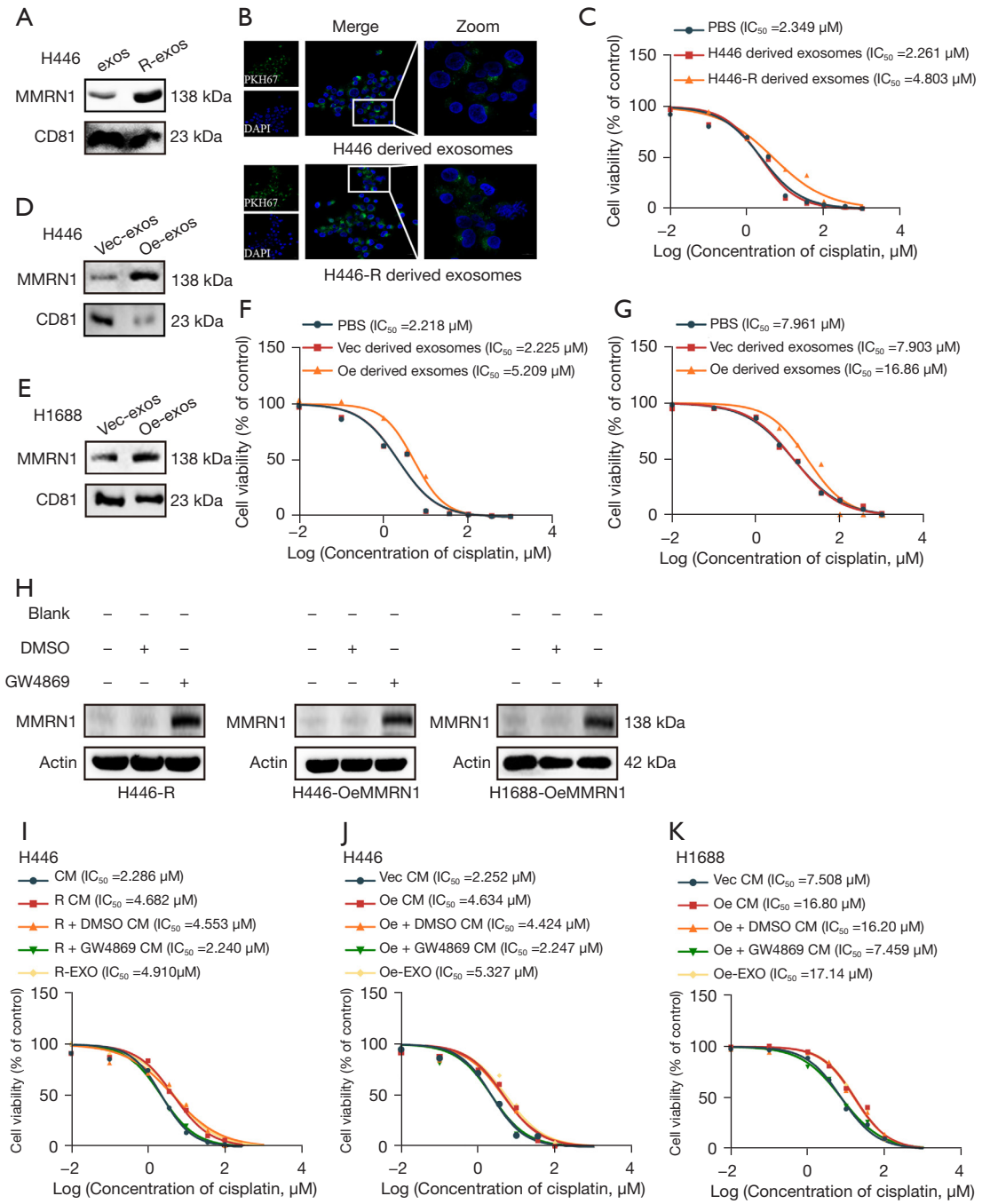
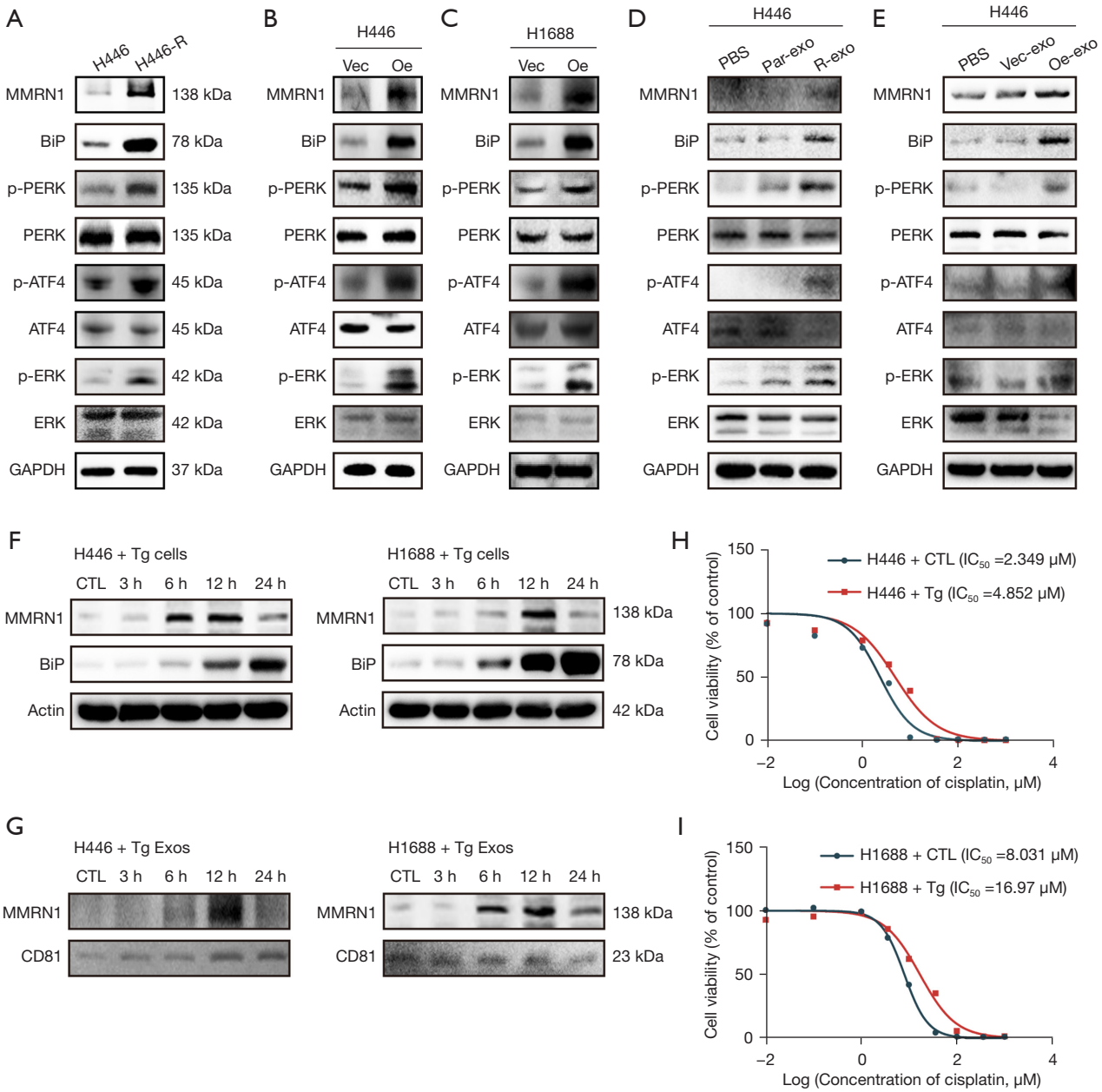



**Figure 4** Exosomal MMRN1 promoted CDDP resistance. (A) WB was performed to measure MMRN1 protein expression in exosomes extracted from parental H446 and CDDP-resistant H446-R cells. (B) Exosomes were marked with PKH67 (green) and added to the medium of H446 cells. Immunofluorescent staining of exosomes (green) and nuclear (DAPI, blue) was performed to visualize the uptake of exosomes. ×10. (C) CCK-8 assay was conducted to determine the $IC_{50}$ of CDDP in H446 incubated with PBS, or exosomes purified from parental H446 or CDDP-resistant H446-R cells for 48 h before CDDP treatment. (D) WB was performed to measure MMRN1 protein expression in exosomes extracted from H446 stably transfected with control plasmid (Vec) or MMRN1 (Oe). (E) WB was performed to measure MMRN1 protein expression in exosomes extracted from H1688 stably transfected with control plasmid (Vec) or MMRN1 (Oe). (F) CCK-8 assay was conducted to determine the $IC_{50}$ of CDDP

in H446 incubated with PBS, or exosomes purified from H446 control (Vec) or MMRN1 overexpressing (Oe) cells for 48 h before CDDP treatment. (G) CCK-8 assay was conducted to determine the $IC_{50}$ of CDDP in H1688 incubated with PBS, or exosomes purified from H1688 control (Vec) or MMRN1 overexpressing (Oe) cells for 48 h before CDDP treatment. (H) WB was performed to measure intracellular MMRN1 protein expression in CDDP-resistant H446-R and MMRN1 overexpressing cells treated with or without 10 μM GW4869 for 24 h. (I-K) CCK-8 assay was conducted to determine the $IC_{50}$ of CDDP in H446 or H1688 incubated with CM or exosomes derived from cells treated with or without 10 μM GW4869 for 24 h. CDDP, cisplatin; WB, Western Blot; CCK-8, cell counting kit 8; $IC_{50}$, half-maximal inhibitory concentration; CM, conditional medium.



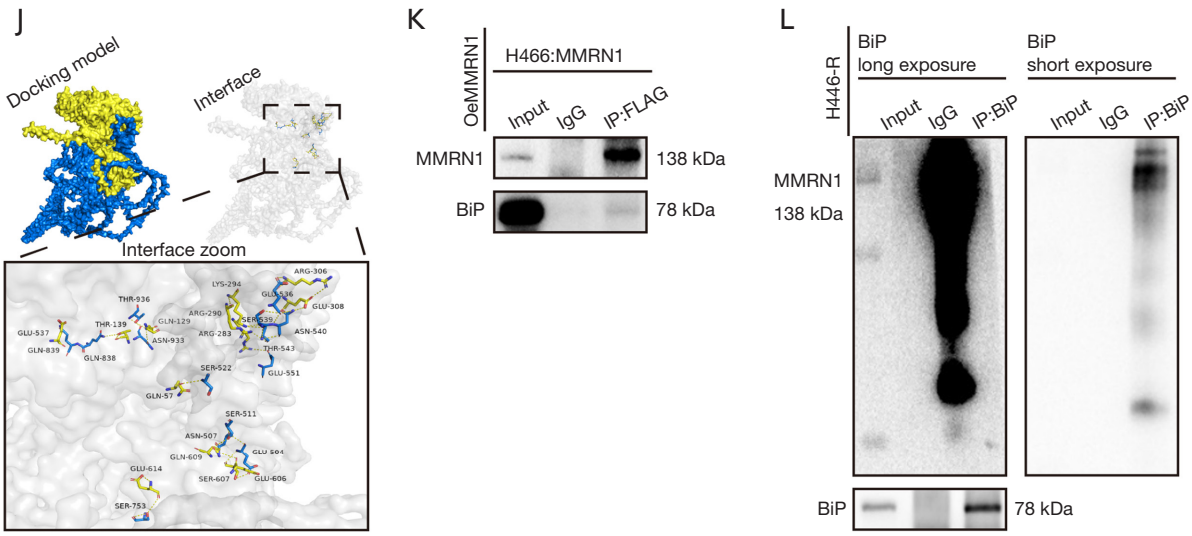



**Figure 5** Endoplasmic reticulum stress-MMRN1 mediates CDDP resistance through direct interaction with BiP. (A) WB was performed to measure ER stress-associated proteins in parental H446 and CDDP-resistant H446-R cells. (B) WB was performed to measure ER stress-associated proteins in H446 stably transfected with control plasmid (Vec) or MMRN1 (Oe). (C) WB was performed to measure ER stress-associated proteins in H1688 stably transfected with Vec or MMRN1. (D) WB was performed to measure H446 incubated with PBS or exosomes derived from parental H446 or H446-R for 48 h. (E) WB was performed to measure H446 incubated with PBS or exosomes derived from H446 or MMRN1 overexpressing H446 for 48 h. (F) H446 and H1688 cells were treated with 1 μM Tg for 0, 3, 6, 12, and 24 h. WB was performed to measure MMRN1 and BiP expression. (G) Exosomes were extracted from H446 and H1688 cells treated with 1 μM Tg for 0, 3, 6, 12, and 24 h. WB was performed to measure exosomal MMRN1 expression. (H) CCK-8 assay was conducted to determine the $IC_{50}$ of CDDP in H446 pre-treated with 1 μM for 12 h. (I) CCK-8 assay was conducted to determine the $IC_{50}$ of CDDP in H1688 pre-treated with 1 μM for 12 h. (J) Surface diagram of the docking model. The interface zoom diagram shows the interfacing residue between MMRN1 (blue) and BiP (yellow). The dotted line represents the hydrogen bond. (K) Cell lysates of MMRN1 overexpressing H446 were immunoprecipitated with anti-Flag antibody or IgG. IP products were probed with anti-MMRN1 and anti-BiP antibodies. (L) Cell lysates of H446-R were immunoprecipitated with anti-BiP antibody or IgG. IP products were probed with anti-MMRN1 and anti-BiP antibodies. Blots of short and longer exposures for MMRN1 are illustrated. CDDP, cisplatin; BiP, binding protein; WB, Western Blot; ER, endoplasmic reticulum; CTL, control; Tg, thapsigargin; CCK-8, cell counting kit 8; $IC_{50}$, half-maximal inhibitory concentration; IP, immunoprecipitation.

(*Figure 5D,5E*). These data indicated that MMRN1 maintained ER stress in CDDP-resistant SCLC cells.

To further investigate whether CDDP resistance has resulted from ER stress in SCLC cells, H446 and H1688 were treated with ER stress inducer thapsigargin (Tg). WB was conducted to analyze MMRN1 and BiP expression at different time points. As shown in *Figure 5F*, BiP showed a time-dependent increase at 6, 12, and 24 h for Tg treatment. The peak Tg-induced intracellular and exosomal MMRN1 expression occurred at 12 h, with decreased expression by 24 h (*Figure 5F,5G*). Additionally, the CCK-8 assay revealed that the $IC_{50}$ value of CDDP for ER-stressed SCLC cells was about two times higher than it for unstressed cells (*Figure 5H,5I*). These data implied that ER stress upregulated MMRN1 expression and reduced CDDP sensitivity in SCLC cells.

The ER chaperone BiP is a key regulator of ER function (27). To investigate whether MMRN1 maintains ER stress via direct interaction with BiP, we conducted rigid protein-protein docking using the GRAMM web server (26). As shown in *Figure 5J*, hydrogen bonds through amino acid residue sites, such as Arg306-Glu536, Lys294-Ser539, Gln57-Ser522, etc., were detected between BiP and MMRN1, which indicated that BiP and MMRN1 formed a stable protein docking model. Co-IP results also showed that MMRN1 could interact with BiP (*Figure 5K,5L*). Overall, these findings suggested that ER stress-induced MMRN1 could directly interact with BiP, which maintained the ER stress and ultimately promoted CDDP resistance in SCLC cells (*Figure 6*).

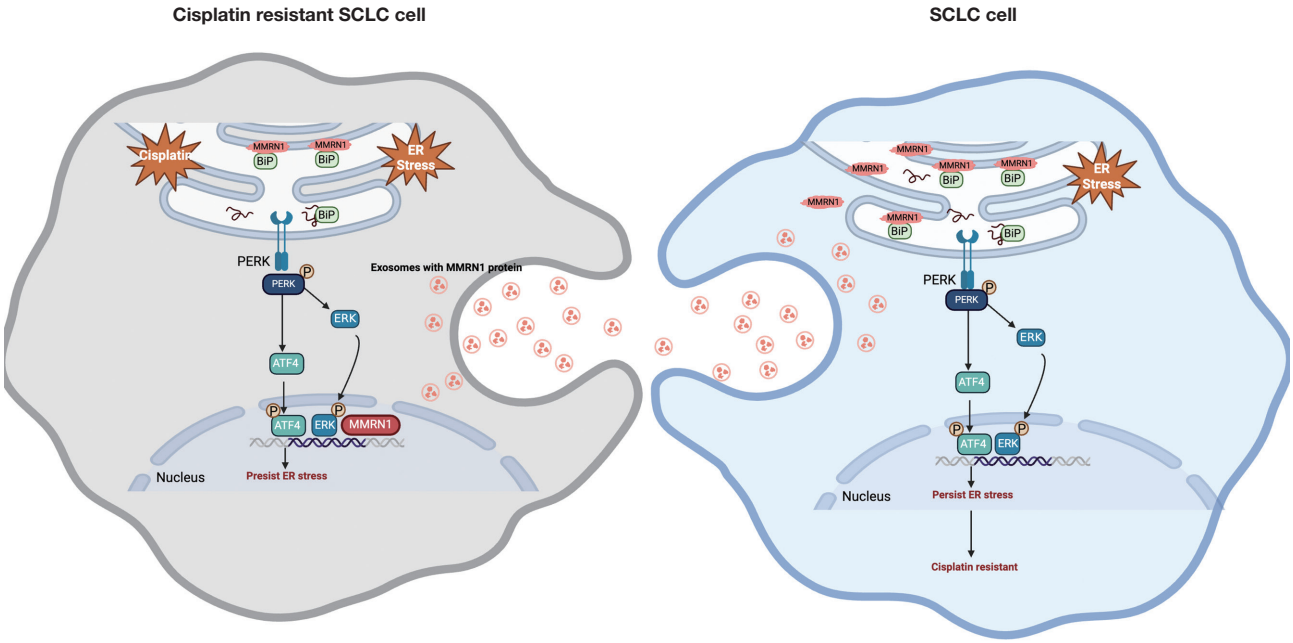



**Figure 6** A schematic diagram of MMRN1/BiP/ER stress axis-based CDDP resistance in SCLC. MMRN1 promotes CDDP resistance in ER stress SCLC cells by binding to BiP, resulting in an increase of phosphorylated PERK and phosphorylated ERK. Moreover, exosomal MMRN1 secreted by CDDP resistance SCLC cells could transmit CDDP resistance to recipient cells. Created with Biorender.com. CDDP, cisplatin; SCLC, small cell lung cancer; ER, endoplasmic reticulum.

## Discussion

CDDP resistance is a challenge for effective therapy for extensive-stage SCLC patients. Although the mechanisms of CDDP resistance have been studied over decades in various types of cancer, accumulating studies have confirmed the multifaceted characteristics of CDDP resistance (6). A comprehensive understanding of the underlying mechanism might provide insights into potential biomarkers and promising therapeutic strategies to obstruct compensatory pathways. Accumulating studies suggest that enriched exosomes in liquid biopsy play vital roles in physiological and pathological processes and are novel biomarkers with great potential (28). With the emerging literature highlighting the impact of exosomes in liquid biopsy on tumorigenesis, metastasis, angiogenesis, immune evasion, and therapeutic resistance, we sought to investigate the mechanisms of CDDP resistance in SCLC by analyzing the circulating exosomes in plasma and their derived component. By proteomic analysis of exosomal proteins from plasma samples, we identified MMRN1 as a biomarker of CDDP resistance in SCLC patients, which is confirmed in cancer biopsies and the SCLC cell models of acquired CDDP resistance generated in our lab. Of note, we observed a robust increase in both intracellular and secreted MMRN1, and SCLC patients with high levels of MMRN1 show poor prognosis, suggesting its use as a biomarker of CDDP resistance in future clinical practice in SCLC. Since SCLC is more aggressive but uncommon, only six patients were enrolled in this study which also lacked the self-comparison before/after CDDP resistance form. More clinical samples are needed to verify the predictive value of exosomal MMRN1 in CDDP-resistant SCLC.

As a secreted protein, MMRN1 with distinct cytoplasmic expression in megakaryocytes, thrombocytes and endothelial cells prefers to deposit to the extracellular matrix. Thus, we supposed that CDDP related ER stress might negatively affect the normal secretory pathways of MMRN1 protein, induced the upregulation of MMRN1 and promoted MMRN1 secreted with exosomes. Increasing studies have identified high levels of MMRN1 in the liquid biopsies of various types of cancer. A recent study reported that MMRN1 might promote ovarian cancer progression via DNA damage response and repair pathways via proteomic analysis of ovarian cancer cells with MMRN1 silence (29). In this study, we observed a two to three-fold

decrease of $IC_{50}$ against CDDP after silencing MMRN1 in CDDP-resistant SCLCs. Similarly, about two-fold increase of $IC_{50}$ was seen in two SCLC cell lines with MMRN1 overexpression. These findings indicate the inducing role of MMRN1 in CDDP resistance in SCLC.

ER is a key organelle that strictly regulates protein synthesis, folding, and post-translational modification. The accumulation of misfolded or unfolded proteins caused by diverse stimuli leads to ER stress (7). Genetic/epigenetic alteration and metabolic aberration collaborate to induce ER stress and trigger a state of constant ER stress, which thereby promotes cancer progression, including tumor growth, metastasis, and therapeutic resistance (30). Multiple studies have indicated that CDDP generates ER stress in different cancer cells, including lung cancer cells and colorectal cancer cells (9,31,32). In oral squamous cell carcinoma cells, CDDP directly binds to the ER membrane protein p22phox, which blocks the entry of CDDP to the nucleus and induces CDDP resistance (33). Concurrently, constant ER stress confers to CDDP resistance (32,34-36). In nasopharyngeal carcinoma, CDDP treatment induces the secretion of ER-resident protein 44 (Erp44) via exosomes that strengthen and transmit CDDP resistance to adjacent cells (33). Despite Erp44, other ER-related proteins, such as BiP, have been reported to advance CDDP resistance (36-38). Preclinical studies in two/three dimensions cell models have demonstrated that the inhibition of BiP by a small molecule reverses CDDP resistance (36). In this study, we detected persistent ER stress in both CDDP-resistant and MMRN1 overexpressing SCLC cells. Consistent with previous studies, the ER stress inducer upregulated intracellular and exosomal MMRN1 and reduced sensitivity to CDDP in SCLC cells. Moreover, we observed the interaction between MMRN1 and BiP and indicated that MMRN1-BiP might be a competitive binding mechanism maintaining the adaptive ER stress, which also need further investigation to understand the binding region and detailed mechanisms in the future.

## Conclusions

Our studies identified exosomal MMRN1 in plasma as a promising non-invasive biomarker of CDDP resistance in SCLC patients, which requires further clinical evaluation of its accuracy by expanding the samples' quantity. Moreover, our study revealed that ER stress increased MMRN1, which in turn bound to BiP to maintain the persistent ER stress and ultimately enhanced CDDP resistance in SCLC. The CDDP resistance could be disseminated through the intercellular transfer of exosomal MMRN1. Further studies employing *in vitro* protein-protein interaction experiments and relevant cell line/patient-derived SCLC mouse models are required to strengthen the rationale and understanding of mechanisms and potential for targeting MMRN1/BiP/ER stress axis in SCLC.

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## Footnote

*Reporting Checklist:* The authors have completed the ARRIVE and MDAR reporting checklists. Available at https://jtd.amegroups.com/article/view/10.21037/jtd-24-1477/rc

*Data Sharing Statement:* Available at https://jtd.amegroups.com/article/view/10.21037/jtd-24-1477/dss

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*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at https://jtd.amegroups.com/article/view/10.21037/jtd-24-1477/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. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Ethics Committee of Sichuan Cancer Hospital (SCCHEC-02-2022-047). All patients signed a written consent for using blood and cancer tissue for scientific research. Experiments were performed under a project license (No. SCCHEC-04-2022-002) granted by the Ethics Committee of Sichuan Cancer Hospital, in compliance with Laboratory Animal- Guideline for ethical review of animal

welfare (No. GB/T 35892-2018) for the care and use of animals.



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