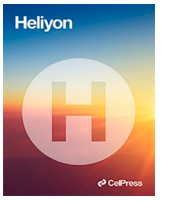
Heliyon 10 (2024) e33001

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Cytidine triphosphate synthase 1-mediated metabolic reprogramming promotes proliferation and drug resistance in multiple myeloma

Hanying Huang [a,b,1], Yanzhou Chen [a,1], Yang Li [a,b,1], Xinnan Zheng [a], Lingling Shu [a,b], Lin Tian [a], Huanxin Lin [a,c,**], Yang Liang [a,b,*,2]

[a] State Key Laboratory of Oncology in South China, Sun Yat-sen University Cancer Center, Guangzhou, 510060, PR China
[b] Department of Hematologic Oncology, Sun Yat-sen University Cancer Center, Guangzhou, 510060, PR China
[c] Department of Radiation Oncology, Sun Yat-sen University Cancer Center, Guangzhou, 510060, PR China

ARTICLE INFO

Keywords:
Bone marrow microenvironment
Bortezomib
CTPS1
Multiple myeloma
MYC

ABSTRACT

Upregulation of metabolism-related gene cytidine triphosphate synthase 1 (CTPS1) is associated with poor prognosis in multiple myeloma (MM). However, its role in MM remains unclear. In this study, bioinformatics analysis revealed significant differences in CTPS1 expression levels among various plasma cell malignancies. The patients with high CTPS1 expression had poor overall survival, progression-free survival, and event-free survival. CTPS1 was significantly correlated with sex, albumin, β2 microglobulin, lactate dehydrogenase, and advanced disease. *In vitro* experiments demonstrated that CTPS1-overexpressing (CTPS1-OE) cells proliferated faster than CTPS1-short hairpin RNA (CTPS1-sh) cells. NRG-SGM3 mice showed significantly accelerated tumor growth in the CTPS1-OE group. CTPS1-OE decreased sensitivity to bortezomib, whereas CTPS1-sh increased sensitivity to bortezomib in MM cell lines. Mechanistically, CTPS1 was primarily involved in metabolism processes. Additionally, CTPS1 was closely related to several co-expressed genes such as MYC and the bone marrow immune microenvironment. In conclusion, CTPS1 is a significant prognostic biomarker for patients with MM, suggesting a potential therapeutic target.

1. Introduction

Multiple myeloma (MM) originates from abnormal monoclonal plasma cells at the end of differentiation in the bone marrow. MM is the second most common malignant blood disease, accounting for approximately 10–15 % of all malignant blood diseases [1]. The global incidence of MM continued to increase from 1990 to 2016 [2]. Over the past two decades, the development of proteasome inhibitors (bortezomib, carfilzomib, ixazomib) and immunomodulators (thalidomide, lenalidomide, pomalidomide) has significantly improved the efficacy and survival of patients with MM [3–8]. Administration of bortezomib combined with lenalidomide and

* Corresponding author. State Key Laboratory of Oncology in South China, Sun Yat-sen University Cancer Center, Guangzhou, 510060, PR China.
** Corresponding author. State Key Laboratory of Oncology in South China, Sun Yat-sen University Cancer Center, Guangzhou, 510060, PR China.
E-mail addresses: linhx@sysucc.org.cn (H. Lin), liangyang@sysucc.org.cn (Y. Liang).
[1] These authors contributed equally.
[2] Lead contact.

https://doi.org/10.1016/j.heliyon.2024.e33001
Received 16 May 2024; Accepted 22 May 2024
Available online 20 June 2024

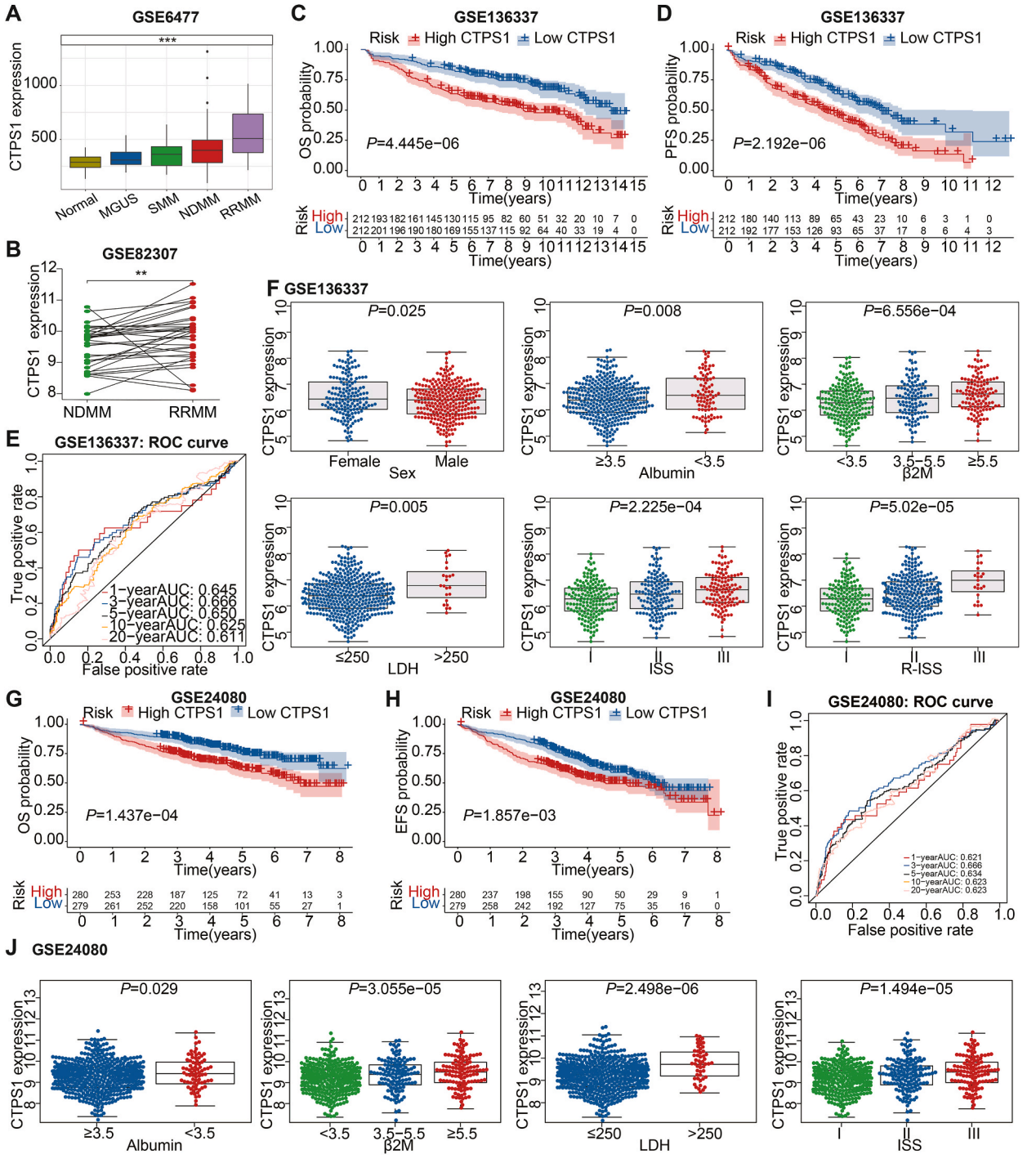


Fig. 1. CTPS1 expression in plasma cell malignancy. (A–B) Expression levels of CTPS1 at different stages of disease progression in plasma cell malignancy. (C–D) The effect of CTPS1 on OS and PFS in the GSE136337 dataset. (E) CTPS1 predicted 1-, 3-, 5-, 10-, and 20-year survival rates in the GSE136337 dataset. (F) Correlation between CTPS1 and clinical indicators in the GSE136337 dataset. (G–H) The effect of CTPS1 on OS and EFS in the GSE24080 dataset. (I) CTPS1 predicted 1-, 3-,5-, 10-, and 20-year survival rates in the GSE24080 dataset. (J) Correlation between CTPS1 and clinical indicators in the GSE24080 dataset. Abbreviations: Cytidine triphosphate synthase 1, CTPS1; MM, multiple myeloma; NDMM, newly diagnosed multiple myeloma; MGUS, monoclonal gammopathy of undetermined significance; SMM, smoldering myeloma; RRMM, relapsed/refractory multiple myeloma; PCL, plasma cell leukemia; BTZ, bortezomib; OS, Overall survival; PFS, Progression-free survival; EFS, event-free survival; β2M, β2 microglobulin; LDH, lactate dehydrogenase; ISS, International Staging System; R–ISS, Revised International Staging System.

dexamethasone followed by hematopoietic stem cell transplantation has better remission and survival rate [9]. With the advent of new drugs, such as anti-cluster of differentiation (CD) 38 monoclonal antibody, exportin 1 inhibitor, chimeric antigen receptor T-cell immunotherapy, have also shown promising efficacy in the treatment of relapsed/refractory MM [10–12]. Although the overall treatment of MM has significantly improved, it remains incurable, and relapse of drug resistance is inevitable in almost all patients with MM [13,14].

Gene mutations are key features of malignant tumors that can generate activated oncoproteins or inactivated tumor suppressor proteins, leading to cancer progression or drug resistance. In our previous study, we screened the key genes of MM using the Gene Expression Omnibus (GEO) database and found that cytidine triphosphate synthase 1 (CTPS1) was associated with poor prognosis in MM [15]. In addition, previous studies have reported that CTPS1 is a more significant enzyme than CTPS2 [16–18]. CTPS1, located on chromosome 1p34.2, is a cytosol-associated CTP synthetase involved in the de novo synthesis of CTP. It uses L-glutamine to catalyze the adenosine triphosphate-dependent transfer of amide nitrogen from glutamine to the C-4 position of uridine triphosphate to produce CTP molecules, which is a precursor required for DNA, RNA, and protein [19].

This study aimed to conduct an in-depth investigation on the role of CTPS1 in MM, reveal the effects of CTPS1 on MM drug resistance and its potential molecular mechanisms, and provide an important reference for tumor-specific targeted therapy of MM.

2. Results

2.1. CTPS1, not CTPS2, was a poor prognostic gene in MM

According to the Gene Expression Profiling Interactive Analysis (GEPIA) and Oncomine databases, CTPS is upregulated in various cancers, including leukemia and diffuse large B-cell lymphoma (as reported by online sources). However, CTPS expression levels in plasma cell malignancies remain unknown. CTPS includes CTPS1 and CTPS2. In plasma cell malignancy samples from the GEO datasets (Table S1), the expression levels of CTPS1 showed significant clinical variability (Fig. 1 A, B). In newly diagnosed MM (NDMM), the group with high CTPS1 expression (grouped by median value-) had a lower overall survival (OS) and progression-free survival (PFS) rates than that in the group with low CTPS1 expression (Fig. 1 C, D). The single gene CTPS1 predicted the 1-, 3-, 5-, 10-, and 20-year survival rates in MM, with an accuracy and specificity of >61 % (Fig. 1 E). Furthermore, CTPS1 was positively correlated with female sex, low albumin levels, high β2 microglobulin levels, high lactate dehydrogenase levels, and advanced disease in the GSE136337 dataset (Fig. 1F–Table S1). Independent validation was conducted using the GSE24080 dataset. The group with high CTPS1 expression had lower OS and event-free survival (EFS) rates than those of the group with low CTPS1 expression (Fig. 1 G, H). Additionally, CTPS1 alone accurately predicted 1-, 3-, 5-, 10-, and 20-year survival rates in MM, with >62 % accuracy and specificity (Fig. 1 I). Furthermore, CTPS1 was positively correlated with low albumin levels, high β2 microglobulin levels, high lactate dehydrogenase levels, and advanced disease in the GSE24080 dataset (Fig. 1J–Table S2).

The expression levels of CTPS2 in plasma cell malignancies at various stages of disease progression were not significantly different

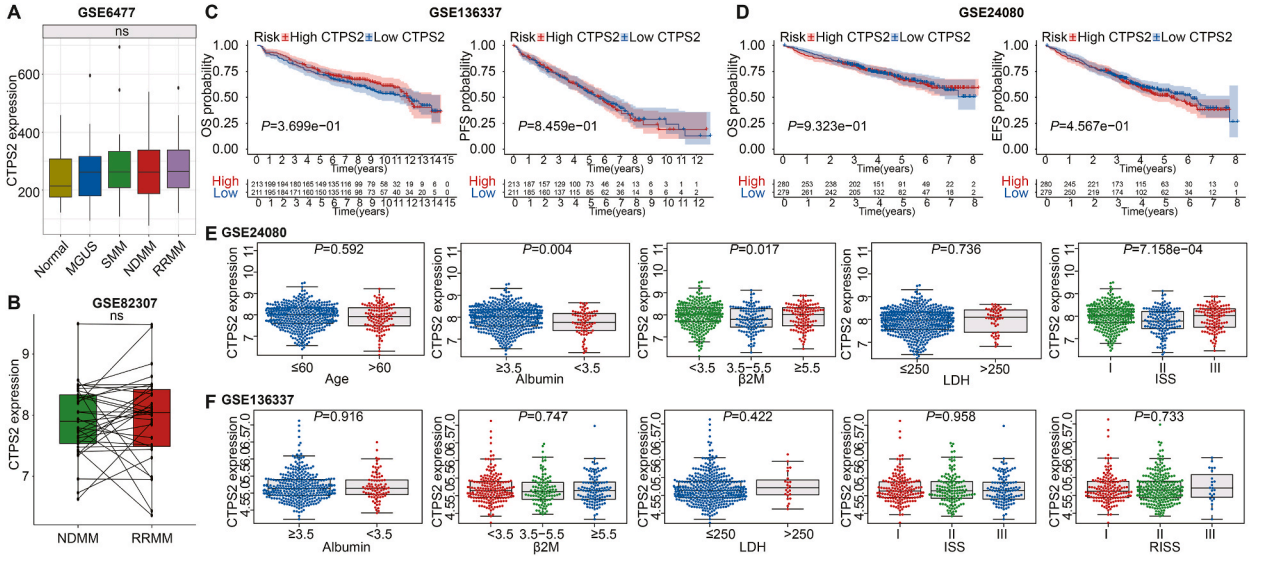


Fig. 2. Cytidine triphosphate synthase 2 (CTPS2) expression in plasma cell malignancy. (A–B) Expression levels of CTPS2 at different stages of disease progression in plasma cell malignancy. (C–D) The effects of CTPS2 on overall survival (OS), progression-free survival (PFS), or event-free survival (EFS). (E–F) Correlation between CTPS2 and clinical indicators. Abbreviations: MM, multiple myeloma; NDMM, newly diagnosed multiple myeloma; MGUS, monoclonal gammopathy of undetermined significance; SMM, smoldering myeloma; RRMM, relapsed/refractory multiple myeloma; PCL, plasma cell leukemia; BTZ, bortezomib; OS, Overall survival; PFS, Progression-free survival; EFS, event-free survival; β2M, β2 microglobulin; LDH, lactate dehydrogenase; ISS, International Staging System; R–ISS, Revised International Staging System.

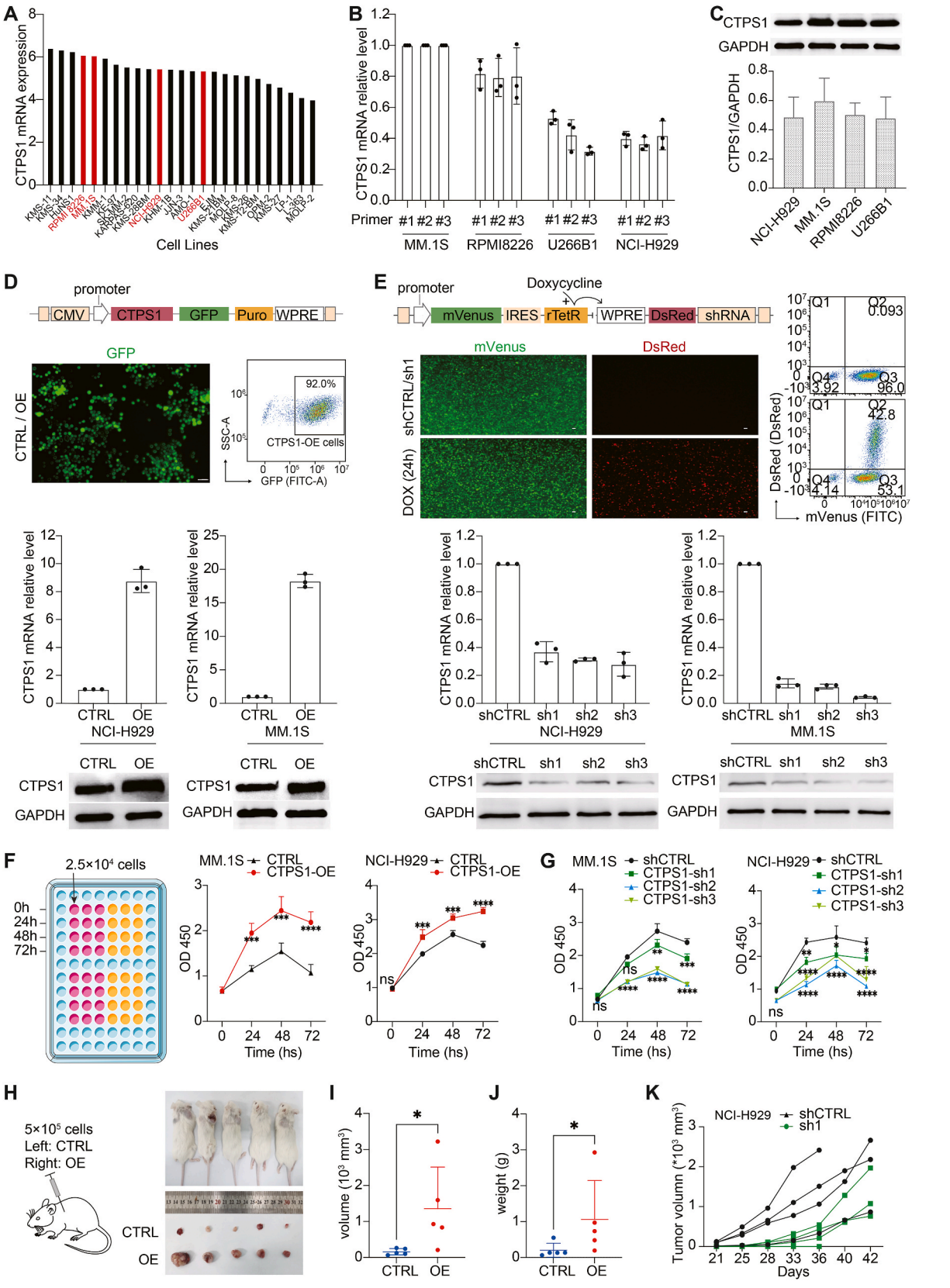


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Fig. 3. CTPS1 promoted multiple myeloma (MM) proliferation. (A) CTPS1 transcript levels in 25 MM cell lines. (B–C) CTPS1 expression levels in four MM cell lines. (D) Validation of CTPS1-OE cells (scale bar = 50 μm). (E) Validation of CTPS1-sh cells (scale bar = 50 μm). (F–G) *In vitro* cell proliferation of the CTPS1-OE/sh group. (H) Tumor growth in mice of the CTPS1-OE group. (I–J) Tumor volume and weight of CTPS1-OE mice. (K) Tumor growth in mice of the CTPS1-sh group. Abbreviations: Cytidine triphosphate synthase 1, CTPS1; CTPS1-OE, CTPS1-overexpressing; CTPS1-sh, CTPS1-short hairpin RNA.

between the two datasets (Fig. 2 A, B). In the NDMM group, CTPS2 expression did not exhibit statistical significance for OS, PFS, or EFS (Fig. 2 C, D). Additionally, no significant correlation was observed between CTPS2 and clinical variables (Fig. 2 E, F, Table S3).

2.2. *CTPS1 promoted MM proliferation and affected drug sensibility*

The expression levels of CTPS1 were analyzed in 25 myeloma cell lines using the Cancer Cell Line Encyclopedia (CCLE) data (Fig. 3 A). In four MM cell lines (NCI–H929, MM.1 S, U266B1, and RPMI8226), both CTPS1 mRNA and protein expression levels were examined (Fig. 3 B, C, Table S4). To further investigate the role of CTPS1, CTPS1-overexpressing (CTPS1-OE) and CTPS1-short hairpin RNA (CTPS1-sh) cells were cultured (Fig. 3 D, E, Tables S5 and S6). *In vitro* cell proliferation assays showed a higher proliferation rate in the CTPS1-OE group and a lower proliferation rate in the CTPS1-sh group (Fig. 3 F, G). *In vivo* experiments demonstrated that the CTPS1-OE group exhibited faster tumor growth, larger tumor volume, and heavier tumors compared with those in the control group on

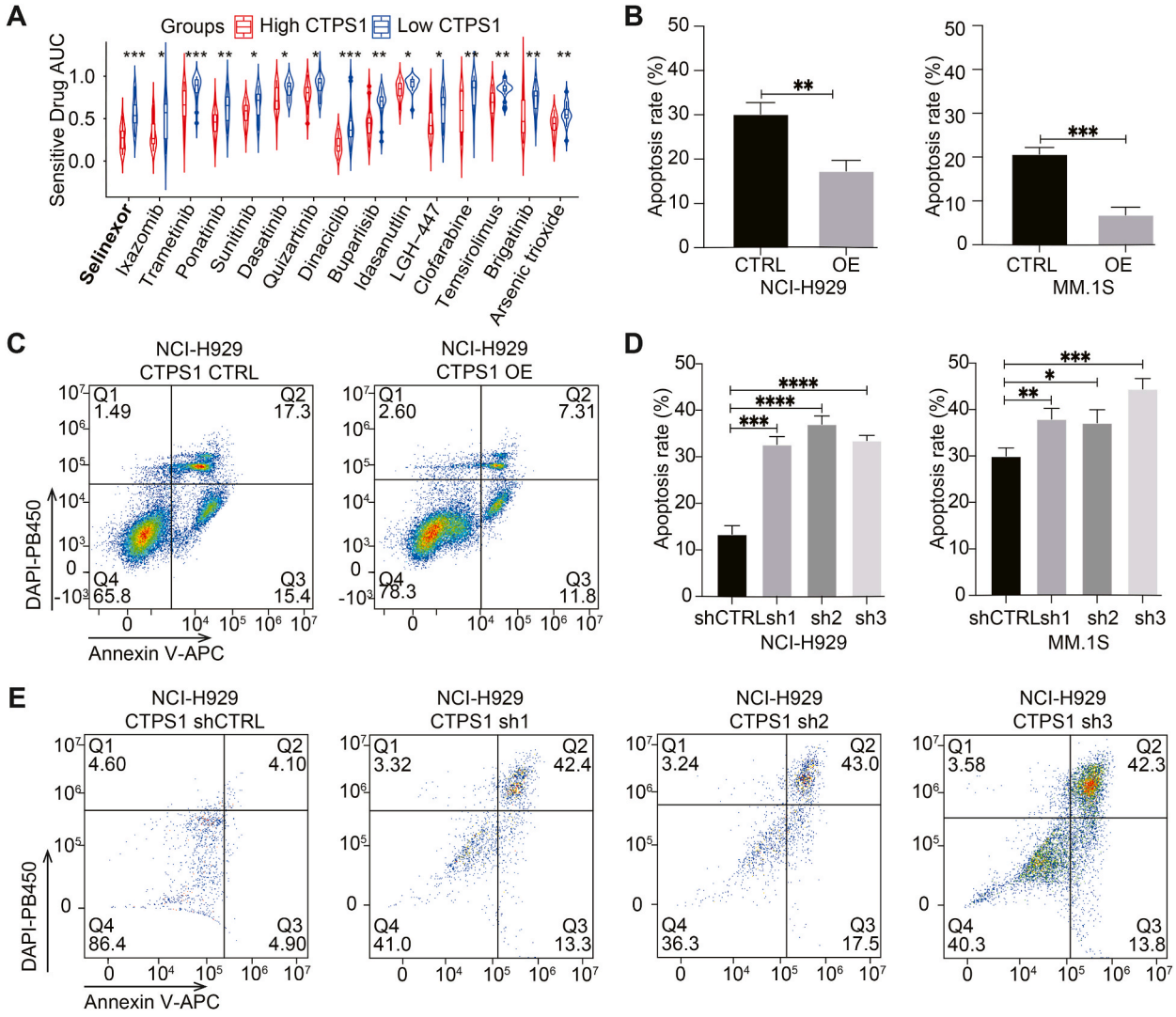

Fig. 4. CTPS1 promoted multiple myeloma (MM) drug resistance. (A) Effects of CTPS1 on drug sensitivity. (B) Apoptosis rate of the CTPS1-OE group 24 h after 20 nM bortezomib treatment. (C) Apoptosis rate of the CTPS1-sh group 24 h after 20 nM bortezomib treatment. Abbreviations: Cytidine triphosphate synthase 1, CTPS1; CTPS1-OE, CTPS1-overexpressing; CTPS1-sh, CTPS1-short hairpin RNA.

day 33 (Fig. 3 H, I, J). Moreover, the tumor growth was slower in the CTPS1-sh group than in the CTPS1-shCTRL group (Fig. 3 K).

Furthermore, MM subjects with high CTPS1 expression exhibited increased drug sensitivity, such as selinexor, as indicated by lower area under the curve (AUC) values in the dataset GSE148715 (Fig. 4 A). In addition, our previous findings observed that MM with high expression of CTPS1 was resistant to bortezomib [15]. *In vitro* testing of the apoptosis rate at 20 nM bortezomib revealed that the CTPS1-OE group exhibited a lower apoptosis rate (Fig. 4 B, C), whereas the CTPS1-sh group showed an increased apoptosis rate (Fig. 4 D, E).

2.3. CTPS1 was associated with cell metabolism and senescence

Using NDMM transcription sequencing, a clear distinction was identified in the gene sets between high and low expressions of CTPS1 (Fig. 5 A, B). In the low-expression group, this distinction encompassed genes such as PPBP and CD79A, whereas in the high-expression group, it included genes such as CTPS1, MYC, and FABP5 (Fig. 5 C, D). The Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed that CTPS1 was associated with various mechanisms, including cell cycle, DNA replication, purine

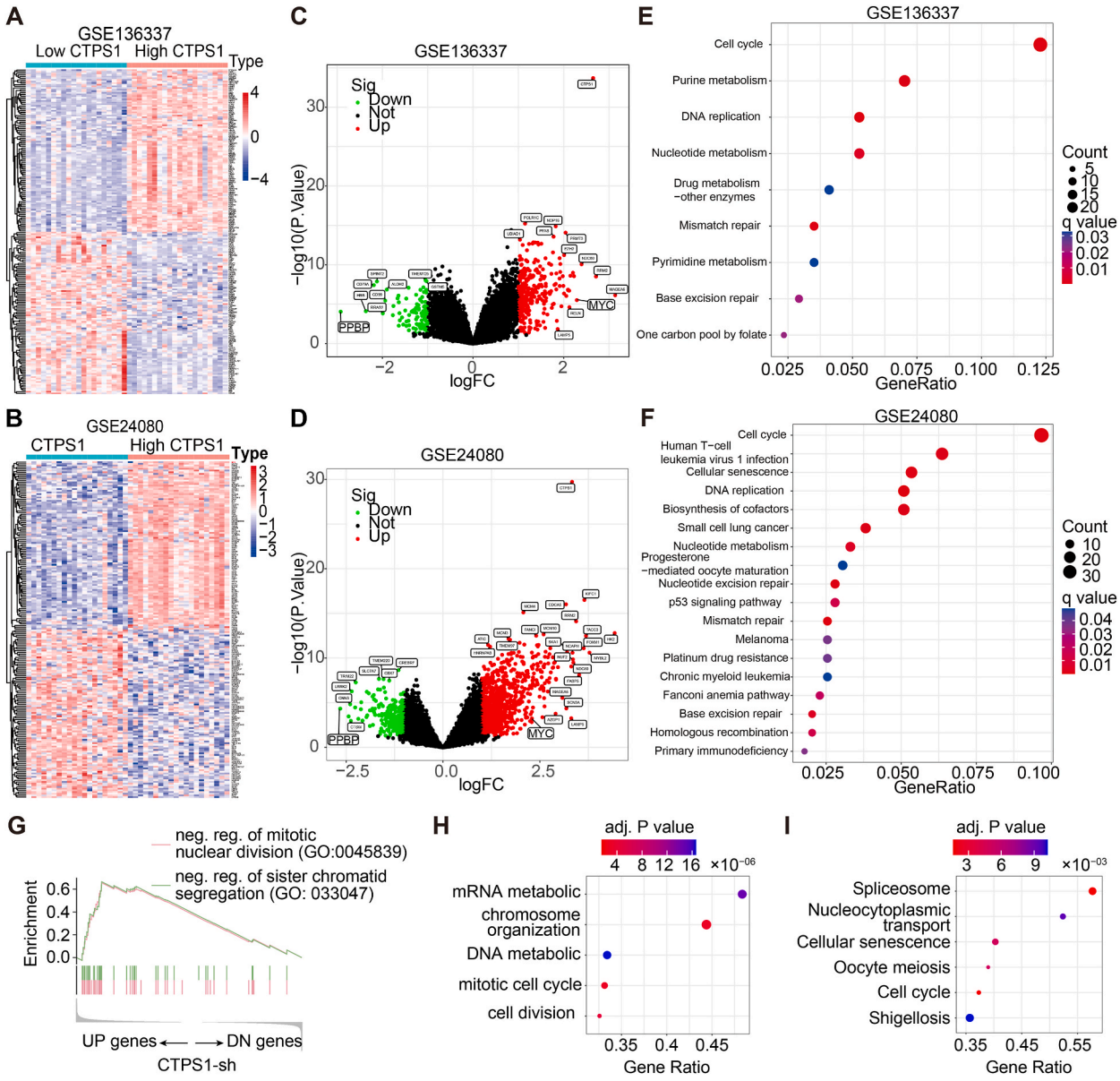

Fig. 5. CTPS1 was associated with cell metabolism and senescence. (A–B) Heat map of differentially expressed genes. (C–D) Volcano map of differentially expressed genes. (E–F) KEGG enrichment analysis. (G–I) GO and KEGG enrichment analyses in cell transcriptome sequencing. Abbreviations: Cytidine triphosphate synthase 1, CTPS1; Kyoto Encyclopedia of Genes and Genomes, KEGG; Gene Ontology, GO.

metabolism, pyrimidine metabolism processes, nucleotide metabolism, enzymatic drug metabolism, and p53 signaling pathway (Fig. 5 E, F). Transcript sequencing of CTPS1-sh cell lines revealed a close association between CTPS1 and various aspects of the cell cycle, including mRNA metabolism, DNA metabolism, chromosome assembly, and cell division cycle (Fig. 5 G, H). Additionally, KEGG enrichment analysis of CTPS1-sh cell lines indicated that CTPS1 was associated with cell senescence and cell cycle (Fig. 5 I).

2.4. Co-expressed gene set of CTPS1

MYC expression significantly correlated with CTPS1 expression (Fig. 5). In plasma cell malignancy, higher levels of MYC expression were associated with more severe disease (Fig. S1 A, B). Moreover, MYC and CTPS1 expression levels were positively correlated (Fig. S1 C, D).

To identify key co-expressed genes of CTPS1, a scale-free network of Weighted Gene Co-expression Network Analysis (WGCNA) was performed on the CTPS1 high- and low-expression groups of the GSE136337 dataset (Fig. S2 A). The scale-free topological scale was verified, and k was negatively correlated with p(k) ($R^2 = 0.9$), indicating that the selected soft threshold was able to establish a gene scale-free topological network (Fig. S2 B). A co-expression network was constructed based on the optimal soft threshold to partition the genes into different modules (Fig. S2 C). Among these, the MEblue and MEturquois modules were strongly correlated with CTPS1 (Fig. S2 D). A positive correlation between the MEblue and MEturquois modules and CTPS1 was found in scatter plots, and the potential co-expressed genes of CTPS1 were listed (Fig. S2 E, F). These gene modules suggested that the role of CTPS1 may involve multiple mechanisms in MM.

2.5. Correlation between CTPS1 and bone marrow immune microenvironment

The infiltration of bone marrow immune cells was analyzed using the GSE136324 dataset (Fig. S3 A). The immune cells closely related to plasma cells were monocytes, resting mast cells, neutrophils, M0 macrophages, $CD8^+$ T cells, and memory B cells (Fig. S3 B). In addition, CTPS1 expression is statistically positive for monocytes, naive B cells, activated natural killer cells, resting mast cells, eosinophils; negative for memory B cell, activated mast cells, resting natural killer cells, activated dendritic cells, resting dendritic cells; but no connection with others (Fig. S3 C).

3. Discussion

Although significant improvements have been made in the treatment of MM, recurrence remains inevitable in almost all patients, making it an incurable problem. The pathogenesis of MM is highly complex and involves various driving factors, such as hyperdiploidy, translocation of immunoglobulin heavy chain gene loci, copy number abnormalities of key genes, somatic mutations, alterations in signaling pathways, epigenetic abnormalities, and changes in the bone marrow microenvironment [20]. These genetic events interact with changes in the bone marrow microenvironment, leading to regulation of the cell cycle, inhibition of apoptosis, and promotion of tumor cell proliferation. However, drug resistance is a major cause of recurrent and incurable MM. Drug resistance can occur through multiple mechanisms, including intraclonal heterogeneity, drug efflux pumps, changes in drug targets, inhibition of apoptosis, increased DNA repair, release of cell adhesion molecules and soluble factors, increased complement proteins, and effects and changes in the bone marrow microenvironment [21,22]. In addition, the identification of cancer stem cells is crucial for treating MM. Therefore, to develop new diagnostic and treatment and specific targeted drugs for MM, it is of great clinical value to conduct in-depth studies on the molecular mechanisms of MM pathogenesis and drug resistance and to identify tumor-specific targets.

Our previous study showed that metabolism-related genes (CTPS1, FABP5, NSDHL, SLC25A5, FLNA, UBE2C, and CISH) in the bone marrow microenvironment were predictive of MM [15]. Among these, upregulation of CTPS1 was associated with poor prognosis in MM and drug resistance recurrence. CTPS1 is mainly involved in cytidine metabolism and nucleic acids metabolism. CTPS1 uses glutamine to catalyze the transfer of adenosine-dependent amide nitrogen from glutamine to uridine triphosphate to produce glutamate and CTP, thereby promoting nucleic acid and protein synthesis [19].

Other studies published in Nature have revealed that CTPS1 plays a crucial and specific role in the immune system [23]. It is responsible for maintaining lymphocyte proliferation during the activation of the immune response. This finding suggests that CTPS1 can be targeted by immunosuppressive drugs to specifically inhibit lymphocyte activation, making it a potential therapeutic target.

One study found that the expression level of CTPS1 in epithelial ovarian cancer tissues was significantly higher than that in normal ovarian tissues and that high expression of CTPS1 was closely related to poor tumor prognosis [24]. Inhibition of CTPS1 activity inhibits the proliferation and invasion of epithelial ovarian cancer cells and increases apoptosis [24]. CTPS1 has a potential role in non-small cell carcinoma; however, its specific role is unclear [25]. High CTPS1 expression is associated with poor disease-free survival and OS in patients with triple-negative breast cancer [26]. *In vitro* and *in vivo* experiments have shown that silencing CTPS1 significantly inhibits the proliferation, migration, and invasion of breast cancer cells and induces apoptosis [26]. In addition, the transcription factors MYC and YBX1 were found to bind to the CTPS1 promoter and promote its transcription [26,27].

Another study showed that MYC positively regulated CTPS1 expression and that elevated CTPS1 expression levels predicted poor OS and PFS in mantle cell lymphoma and had independent prognostic significance [28]. The inhibition of CTPS1 may induce immune-related responses and prevent mantle cell lymphoma tumor growth by activating the dsDNA-cGAS-STING pathway [28]. Notably, Step Pharma has developed an inhibitor called STP938, which targets CTPS1, and is currently undergoing phase I/II clinical trials for relapsed/refractory B-cell and T-cell lymphomas (NCT05463263). Preclinical data indicated that CTPS1 was a novel therapeutic target in lymphoid malignancy [29].

Although CTPS1 has been studied in several cancers, its role and significance in MM have not yet been clear. Recently, CTPS1 has been reported as an important prognostic gene for MM and a potential novel therapeutic target for MM [18], which is consistent with our findings. In addition, the project found that CTPS1 inhibitors synergizes with inhibition of CHEK1, ATR or WEE1 [18]. Therefore, our study focused on the prognostic value and potential role of CTPS1 in patients with MM. Through bioinformatics analysis and experiments, it was found that CTPS1 was highly expressed in MM and closely related to poor biochemical indicators, stage, PFS, EFS and OS. These findings suggest that CTPS1 may serve as a biomarker for MM. Overexpression of CTPS1 promoted tumor proliferation, and knockdown of CTPS1 cooperated with bortezomib to induce cell apoptosis. This suggests that CTPS1 can be used as a potential biomarker and therapeutic target in MM. Mechanistically, CTPS1 is involved in biosynthesis and metabolism. RNA-sequencing (seq) analysis showed that CTPS1 was associated with cell cycle, anabolism, and multiple genes (such as PPBP, CD79A, MYC, FABP5).

In addition, CTPS1 is closely associated with the bone marrow immune microenvironment, including monocytes, resting mast cells, neutrophils, M0 macrophages, $CD8^+$ T cells, memory B cells, and naive B cells. One study reported a homozygous loss-of-function mutation in CTPS1 (rs145092287) that resulted in life-threatening immunodeficiency that impaired the ability of activated T and B cells to proliferate in response to antigen receptor-mediated activation [23].

In conclusion, CTPS1 is a significant poor prognostic factor for MM. In this study, we elucidated the biological function of CTPS1 in MM and provided new insights into potential biomarkers and targeted therapies.

4. Limitations of the study

Although this study provides valuable insights into the role of CTPS1 in MM prognosis and drug resistance, it has some limitations that should be addressed. First, it is important to validate our findings using primary MM samples to further understand the role of CTPS1. Second, additional experiments using various approaches should be conducted to verify the functional role of CTPS1. Furthermore, to gain a better understanding of the effects of CTPS1 on drug resistance, it would be beneficial to use humanized mouse models to construct more MM models.

5. Inclusion and diversity

We support inclusive, diverse, and equitable conduct of research.

6. Star methods

The detailed methods are provided in the online version of this paper and include the following:
KEY RESOURCES TABLE.

7. Resource availability

7.1. Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yang Liang (liangyang@sysucc.org.cn).

7.2. Materials availability

This study did not generate new unique reagents.
Data and code availability.

- This study analyzed existing publicly available data. Accession numbers of the datasets are listed in the **key resources table.**
- All codes used for bioinformatics analysis in this study were derived from existing software and algorithms, as listed in the **key resources table**. This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

8. Experimental model and study participant details

8.1. Human samples

All RNA-seq data and clinical characteristics of the human samples in this study were obtained from the GEO (http://www.ncbi.nlm.nih.gov/geo/) public databases. A summary of the information for all GEO datasets is presented in Table S1.

8.2. Cell lines

Human MM cell lines (including NCI–H929, MM.1 S, U266B1, RPMI 8226, and 293 T) were purchased from American Type Culture Collection (Manassas, VA, USA). Details of the cell lines are listed in Table S4. NCI–H929, MM.1 S, U266B1, and RPMI 8226 cell lines

were cultured in the Roswell Park Memorial Institute medium (Gibco, Cat#C11875500BT). The 293 T cell line was cultured in Dulbecco's Modified Eagle Medium (Gibco, Cat#C11995500BT) and passaged using 0.25 % trypsin-ethylenediaminetetraacetic acid (Gibco, Cat#25200072). All cells were cultured in a humidified incubator containing 5 % CO_2 at 37 °C. All cell lines were cultured under controlled conditions at 37 °C in 5 % CO_2 and supplemented with 10 % fetal bovine serum (WISENT, Cat#086–150), 1 % penicillin (with a final concentration of 100 U/mL), and 1 % streptomycin (with a final concentration of 100 mg/mL) (Gibco, Cat#15140122). All cell lines were identified using short tandem repeat analysis. All the cell lines were tested for mycoplasma contamination. All the cell lines used in the experiments were thawed for 15 generations.

8.3. Mouse models

The CTPS1-control (CTRL) or CTPS1-OE or CTPS1-sh of NCI–H929 cells were mixed with an equal volume of Matrigel (CELLada, Cat#CELLada-OM-2) and implanted by subcutaneous injection into 3–4-week-old humanized NRG-SGM3 mice (NOD.Cg-Rag1$^{tm1\text{-}Mom}$Il2rg^{tm1Wjl}Tg(CMV-IL3,CSF2,KITLG)1Eav/J, Jackson Lab, Cat#024099) (5×10^5 cells/50μL/mouse). The CTPS1-CTRL cells were injected on one side of the back, and CTPS1-OE or CTPS1-sh cells were injected on the other side. The volume of the subcutaneous tumor was measured every 3 days, and the tumor sizes were calculated using the following formula: $4/3 \times \pi \times (\text{width}/2) \times (\text{width}/2) \times (\text{length}/2)$. The animals were maintained and handled according to the principles of laboratory animal care under protocols approved by the Institutional Animal Care and Use Committee. This study was reviewed and approved by the Animal Experiment Ethics Committee of our center (approval number: L025501202206012). There was no blinding, and randomization was not applied because the mice were homologous in sex and age.

9. Method detailsdetails

9.1. Data source and differential expression analysis

The GEO is an international public repository for data sharing. Relevant datasets are listed in Table S1. The gene expression matrix and clinical data were organized according to different microarray annotation platforms, while ensuring no sample duplication between datasets. The GEPIA and Oncomine databases were used to reveal the expression of genes in different cancers. Gene expression in all myeloma cell lines was mined from the CCLE database.

Packages of RStudio software version 3.6.3 (Posit Software Inc., USA) were used for the analysis. The "ggplot2" and "reshape2" R packages were used for comparison between groups. The "ggpubr" R package was used for paired comparisons. The "survival" and "survminer" R packages were used to analyze OS, EFS, or PFS rates. The accuracy and specificity in predicting prognosis were quantified by calculating time-dependent receiver operating characteristic (ROC) curves and AUC values using the "survivalROC" R package. The "beeswarm" R package was used for clinical correlation analysis. The *t*-test was used to analyze the relationship between CTPS1 and clinical covariates and the differences between samples.

9.2. GO and KEGG enrichment analyses

The 20 samples with the highest CTPS1 expression level and the 20 samples with the lowest CTPS1 expression level in the GSE136337 dataset were used for the enrichment analysis. Differential genes were analyzed using the "pheatmap" R package. The volcanoes were plotted using the "ggplot2" R package. GO and KEGG enrichment analyses of the differentially expressed genes were performed to analyze the potential mechanisms mediated by CTPS1 in MM.

9.3. Cloning and production of CTPS1-OE MM cells

Lentiviral vectors, CMV-MCS-CTPS1-EF1-copGFP-T2A-Puro were purchased from RuiBioTech Co., Ltd. (Guangzhou, China). The lentiviral vectors were co-transfected with packaging vectors psPAX2 and VSVG (Addgene) into 293 T cells for lentiviral production. To establish stable cell lines, the cells were transduced using the above lentiviruses with polybrene (8 mg/mL, Sigma). Moreover, 72 h after transduction, copGFP-positive cells were selected by flow sorting (Beckman, USA).

9.4. Cloning and production of CTPS1-sh MM cell

The desalted oligonucleotide was cloned into the Tet-on-DsRed-PGK-mVenus (Addgene Cat#111176) plasmid vector containing the XhoI/EcoRI restriction site purchased from the public plasmid library. The shRNA was packaged in a lentiviral vector by co-transfection with the packaging vectors psPAX2 and VSVG (Addgene) into 293 T cells for lentiviral production. To establish stable cell lines, the cells were transduced using the above lentiviruses with polybrene (8 mg/mL, Sigma). After 72 h of transfection, doxycycline was added to induce CTPS1 knockdown, and DsRed-positive cells were selected by flow sorting (Beckman, USA). The shRNA sequences of the CTPS1 gene are listed in Table S5.

9.5. Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction was performed, as previously described [30]. Gene expression was evaluated by

the $2^{-\Delta\Delta Ct}$ analysis. All primer sequences designed and synthesized by RuiBioTech Co., Ltd. (Guangzhou, China) are listed in Table S6.

9.6. Western blotting

Western blotting was performed, as previously described [30,31]. Relative protein grayscale values were analyzed using the ImageJ software version 1.8.0 (National Institutes of Health, USA). Detailed information on all the antibodies is listed in Table S7.

9.7. Cell viability assay

Cell Counting Kit-8 (K1018, APExBIO) was used, according to the manufacturer's instructions. Cells were seeded in 96-well plates at 2.5×10^4 cells/well. Data were analyzed using Prism 9.5 (GraphPad Software Inc., San Diego, CA, USA).

9.8. Cell apoptosis assay

Annexin V-APC (MultiSciences, Hangzhou, China) and DAPI-Pacific Blue (Beyotime, Shanghai, China) staining were performed, according to the manufacturer's instructions. Cells were seeded at 2.5×10^6 cells/well in 6-well plates with phosphate-buffered saline or bortezomib (20 nM, Qilu, China), and the cells were incubated for 24 h at 37 °C. Fluorescence was measured using a CytoFLEX LX flow cytometer (Beckman Coulter), and the data were analyzed using the FlowJo software version 10.8.1 (BD Biosciences).

9.9. RNA-sequencing

CTPS1-sh cell lines were collected, and total cellular RNA was extracted using an RNA extraction kit (No. RN001, ESscience), according to the manufacturer's instructions. cDNA synthesis was performed using the RevertAid First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Sequencing data were used to analyze genes co-expressed with CTPS1 and their potential mechanisms or pathways.

9.10. WGCNA

Original microarray data from the GSE136337 dataset were used to construct a co-expression network and screen for key genes. The high CTPS1 group and low CTPS1 GSE136337 dataset of differentially expressed genes (false discovery rate <0.025, $|\log2FC| > 0.1$) were screened. WGCNA was performed for differentially expressed genes. Scale-free co-expression networks were developed using the WGCNA algorithm [32]. In brief, gene expression data from the GSE136337 dataset (20 subjects with highest or lowest CTPS1 expression, respectively) were used to construct a "signed network adjacency" matrix using a soft threshold of 4 to ensure scale-free topology and provide sufficient node connections. The adjacency matrix was transformed into a "topological overlap matrix (TOM)" to minimize the effects of noise and spurious associations, and the corresponding dissimilarity was calculated as "1-TOM." Differences were hierarchically clustered using an "average" linkage approach to produce gene cluster trees in which the branches of gene cluster groups are highly interconnected. Gene network modules were identified by cutting branches from the cluster tree using the "DynamicTreeCut" R package, with the cut height value set to 0.90, the depth split to 2, and the minimum module size to 60. The key genes associated with CTPS1 were identified based on the Spearman correlation coefficient between the modules and the gene expression profiles in each module.

9.11. Bone marrow immune microenvironment

Infiltration of bone marrow immune cells was analyzed in the GSE136324 dataset. The normalized Gene expression profiles of each sample were converted to the proportions of 22 immune cells using the "CIBERSORT" R package [33]. The following 22 types of immune cells were obtained: naive B cells, memory B cells, plasma cells, $CD8^+$ T cells, naive $CD4^+$ T cells, $CD4^+$ resting memory T cells, $CD4^+$ memory-activated T cell, follicular helper T cells, Tregs, gamma delta T cells, resting natural killer cells, activated natural killer cells, monocytes, M0 macrophages, M1 macrophages, M2 macrophages, resting dendritic cells, activated dendritic cells, resting mast cells, activated mast cells, eosinophils, and neutrophils.

9.12. Quantification and statistical analysis

Prism 9.5 (GraphPad Software Inc., San Diego, CA, USA) and RStudio software version 3.6.3 (Posit Software Inc., USA) were used for statistical analysis. A two-sided P value of <0.05 was considered statistically significant. Each result is presented as the mean and standard deviation of more than three independent experiments, with **** representing $P < 0.0001$, *** representing $P < 0.001$, ** representing $P < 0.01$, and * representing $P < 0.05$.

CRediT authorship contribution statement

Hanying Huang: Writing – original draft. **Yanzhou Chen:** Writing – original draft. **Yang Li:** Writing – original draft. **Xinnan Zheng:** Methodology. **Lingling Shu:** Conceptualization. **Lin Tian:** Conceptualization. **Huanxin Lin:** Supervision. **Yang Liang:**

Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Y. Liang was supported in part by Sun Yat-sen University Start-up Funding (Grant No. 201603). H-X. Lin was supported by the National Natural Science Foundation of China (Grant No. 81773103) and Natural Science Foundation of Guangdong Province (Grant No. 2017A030313617). T. Lian was supported by the National Natural Science Foundation of China (Grant No. 82173278). L-L. Shu was supported by Natural Science Foundation of Guangdong Province (Grant No. 2022A1515010290). H–Y. Huang was supported by Fostering Program for NSFC Young Applicants (Tulip Talent Training Program) of SYSUCC (Grant No. TTP - SYSUCC -202406).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33001.

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