USP25 Regulated Multiple Myeloma Cell Cycle Through STAT3 Ubiquitination

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Research Article

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Abstract

Background

Constitutively activated STAT3 (Signal transducer and activator of transcription 3) has been seen in Multiple Myeloma (MM). However, STAT3 regulator in MM remains enigmatic.

Methods

Herein, we applied public dataset analysis and identified USP25 (Ubiquitin carboxyl-terminal hydrolase 25) was a potential regulator of STAT3. We further applied western blot and IP to confirm the relation between USP25 and STAT3. Furthermore, we used cell cycle assay to assess the effect USP25 on MM cell cycle.

Results

USP25 highly expressed in MM CD138+ cells, and support MM cell proliferation. In protein level, USP25 take part in IL-6/USP25/STAT3 axis and could directly down-regulated STAT3 ubiquitination. Using truncated form of USP25, we also proved UCH (Ubiquitin carboxyl-terminal hydrolase) domain of USP25 is critical for USP25-STAT3 binding, UIM (Ubiquitin interacting motif) domain is required for STAT3 ubiquitination, we further proved cell cycle changed by USP25 required STAT3 and cyclinD1, suggesting USP25 inhibition is promising in STAT3, cyclinD1 abnormal MM patients.

Introduction

Multiple myeloma (MM) is a malignant disease caused by the accumulation of clonal plasma cells in the bone marrow, resulting in symptoms such as bone pain, nausea, and constipation[1]. Currently, MM remains incurable, and although various therapies, such as target therapy and immunomodulation, can alleviate patients' symptoms, most patients experience recurrence and eventually become resistant to treatment[2]. Knowledge of the critical pathway in MM would help in understanding the mechanism of MM tumorigenesis and developing new therapeutic targets.

Signal transducer and activator of transcription 3 (STAT3) and the ubiquitination pathway are important in MM[3]. The STAT3 protein belongs to the STAT family and contains six domains, including the Src homology 2 (SH2) domain, DNA-binding domain, and amino-terminal domain (NTD)[4]. Among all STAT proteins, STAT3 is the most studied, and many factors, including interleukin (IL)-6, can activate kinases such as JAK; then, STAT3 would form a dimer, move into the nucleus, and induce transcription of downstream genes[5, 6]. Unlike its controlled status in normal cells, STAT3 is constitutively activated in tumor cells and specifically participates in tumor cell growth and invasion[7].

Another important pathway is the ubiquitin modification pathway. Ubiquitin is a highly conserved polypeptide composed of 76 amino acid residues, and ubiquitin modification involves E1-E2-E3 cascade reaction-mediated ubiquitin binding and the ubiquitin-specific protease (USP)-mediated deubiquitination

process[8]. Several ubiquitination enzymes have been demonstrated to be closely related to tumors; for example, the ubiquitin ligase SKP2 could identify tumor suppressors p21, p27, and FOXO1, and induce ubiquitin degradation[4]. USP9X suppresses the K48 ubiquitination process of MCL-2, which leads to imatinib resistance in chronic myeloid leukemia patients [9]. USP25 belongs to the USP family, but its relationship with MM has not been studied.

In this study, we first confirmed the relationship between USP25 and STAT3 in MM, in which USP25 could function as an oncogene; demonstrated that USP25 could regulate STAT3 ubiquitination and its downstream gene cyclin D1 expression; and revealed that the effect of USP25 on the MM cell cycle requires STAT3 and cyclin D1.

Material And Methods

Patients samples

The bone marrow of MM patients was obtained from Ningbo Beilun People's Hospital, and the sample extraction protocol followed the Declaration of Helsinki and was approved by the Institutional Ethics Board of Ningbo Beilun People's Hospital, all patients were informed and consented to the use of their tumor tissue. After extraction of MM bone marrow, CD138 + cells were isolated using the EasySep Human CD138 Positive Selection Kit II (STEM CELL Technologies, Cambridge, MA, USA).

Cell culture

The human MM cell line XG 7, RPMI8226 was cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) at 37°C and 5% CO₂.

Public dataset analysis

In this study, GSE19784 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE19784>), GSE9782 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE9782>), and GSE5900 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE5900>) were retrieved from the Gene Expression Omnibus (GEO) database using the R 4.3.1. GEOquery was used for normalization, survival analysis was conducted using the survival package, and correlations between STAT3 and other genes were determined by the `cor.test()` function in R. High correlation of a gene to STAT3 was defined as $r^2 > 0.4$, $p < 0.05$.

qRT-PCR

The mRNA expression level was detected by qRT-PCR. First, mRNA was extracted by Trizol buffer (Invitrogen, Carlsbad, CA, USA), and then mRNA was reverse transcribed into cDNA using PrimeScript RT Reagent Kit with gDNA Eraser (Clontech, Dalian, China). Finally, mRNA gene expression was detected by SYBR® Premix Ex Taq II (Clontech, Dalian, China). The following primers were used: control gene GAPDH, sense 5'-TATGCTCTCCTCATGCATTG - 3', antisense 5'-GGGACGACCTTCGATCTACC - 3'; and USP25,

sense 5'- GTTCTATGGCAGATTCCTGGCTG – 3', antisense 5'-GCAGCTTCTAGGCACTCATGCA-3'. The relative expression of USP25 was determined by the $2^{-\Delta\Delta Ct}$ method.

Cell apoptosis, proliferation, and cell cycle assays

RPMI8226 cells were first transfected with scramble and USP25-shRNA for 48 h. (1) Apoptosis was analyzed using the Annexin V APC/PI kit. Cells were first washed three times with phosphate-buffered saline, suspended in Annexin V binding buffer, and labeled with Annexin V APC/propidium iodide (PI; BD Biosciences, San Jose, CA, USA) for 10 min using a BD Calibur machine to detect changes in cell apoptosis. (2) Cell proliferation was determined using a CCK8 kit (Dojindo Molecular Technologies, Rockville, MD, USA). Cells were seeded in a 96-well plate; after attachment, CCK8 reagent was added, and cells were cultured for 3 h. Then, cell proliferation was determined by a microplate reader as the optical density (OD) at 450 nm, and the proliferation index was calculated as the ratio of OD 450 nm at a specific time point to that at 0 h. (3) For cell cycle analysis, cells were stained with a high concentration of PI (50 $\mu\text{g/mL}$) for 30 min and then detected using a BD Calibur machine.

Western blot

For western blotting, cells were lysed in RIPA buffer for 30 min on ice, and then 2× sample buffer was added. After 15 min of boiling in the water bath, constant-voltage electrophoresis (50 V) was performed. After the bromophenol blue reached the demarcation line, constant electrophoresis at 140 V was applied until the bromophenol blue reached the bottom. The protein was then transferred to a polyvinylidene fluoride membrane and blocked with 5% bovine serum albumin labeled with the corresponding primary antibodies, GAPDH, USP25, STAT3, p-STAT3 (phosphorylated STAT3), Flag, Myc, CDK2, CDK4, cyclin E, cyclin A, cyclin D1 (Abcam, Cambridge, UK), and secondary antibody (ZhongShan Golden Bridge Biotechnology, Beijing, China). Finally, enhanced chemiluminescence solution was used for exposure, and protein expression was quantified using Image J software (Pierce Biotechnology, Chicago, IL, USA).

Immunoprecipitation (IP)

In the IP experiment, truncated forms of USP25, including the UIM1 + 2 domain, UCH domain, USP25 with UCH deletion, USP25 with UIM1 deletion, and USP25 with UIM2 deletion were designed by Jinsirui Company (Shanghai, China). (1) The harvested cells were decomposed on ice, and (2) a small amount of lysate was used for western blot analysis. Then, 1 μg of corresponding antibody and 10–50 μg protein A/G-beads (Sigma Aldrich, St. Louis, MO, USA) were added to the remaining lysate. (3) After IP, protein A/G-beads were centrifuged at 3,000 $\times g$ at 4 °C for 5 min, the supernatant was carefully absorbed, protein A/G-beads were washed 3–4 times with 1 mL cracking buffer, and then 15 mL of 2× sodium dodecyl sulfate buffer was added and boiled for 10 min. (4) Western blotting analysis was conducted.

Statistics

Significance levels in this study were determined by Student's t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Results

USP25 was positively correlated with STAT3 in the public dataset

The IL-6-STAT3 pathway is a well-known pathway in MM. To determine which gene regulates STAT3, we first employed the Pearson correlation coefficient algorithm in the GSE19784, GSE9782, and GSE5900 datasets. After combining genes with high correlations to STAT3 in the three datasets, USP25 was confirmed as one of the common genes in these datasets (Fig. 1A). In GSE19784, we divided MM patients into USP25 high- and low-expression groups. Using GSEA, we found that the GO: 0042509 pathway, which was defined as the regulation of tyrosine phosphorylation of STAT protein, was highly enriched in patients with high USP25 expression (Fig. 1B).

USP25 interacted with STAT3 and was highly expressed in MM patients

To further demonstrate the interaction between USP25 and STAT3, we transfected myc-USP25 and flag-STAT3 vectors into 293 cells. After 48 h, using IP, we successfully detected USP25 binding to STAT3 protein (Fig. 1C) and found that USP25 and STAT3 were also colocalized in 293 cells (Fig. 1D). As STAT3 is an oncogenic factor in MM, genes that are highly correlated with STAT3 might have similar functions; therefore, we next detected the role of USP25 in the survival of MM patients and found that high USP25 expression indicated poor prognosis in patients with MM (Fig. 1E). In-house data also showed that USP25 was highly expressed in the CD138 + cells of 11 MM patients (Fig. 1F), which further confirmed that USP25 might act as an oncogene.

USP25 inhibition induced apoptosis and G1 cell cycle arrest and suppressed proliferation in MM cells

Because of the abnormal expression of USP25 in MM and its relationship with STAT3, we examined the role of USP25 in the MM cell line RPMI8226. USP25-shRNA transfection efficiently inhibited USP25 expression at the protein level (Fig. 2A). Consistent with USP25 inhibition, apoptosis of XG 7 cells (Fig. 2B) and RPMI8226 cells (Fig. 2C). G1 arrest in cell cycle were induced in RPMI8226 cells (Fig. 2D). According to G1 arrest, USP25 inhibition also inhibited cell proliferation (Fig. 2E). Using a transwell assay, we demonstrated that inhibition of USP25 reduced the migration ability of RPMI8226 cells (Fig. 2F), furthermore, by using in vivo experiment, we confirmed that down regulation of USP25 could suppress tumor growth and immune suppression signal in vivo (Fig. 3A, C and E), and overexpression of USP25 increased tumor growth and immune suppression signal in vivo (Fig. 3B, D and F). Based on these results, USP25 widely participates in MM cell function.

IL-6-induced USP25 could activate STAT3

Given the strong correlation between USP25 and STAT3, we attempted to determine the role of USP25 in the IL-6–STAT3 pathway. In RPMI8226 cells, IL-6 induced USP25 expression and STAT3 phosphorylation

in a dose-dependent manner (Fig. 4A), indicating that USP25 is involved in the IL-6 pathway. And transfection of USP25-shRNA down regulated the expression of P-STAT3 and total STAT3, treatment of IL-6 could restore the expression of these 3 proteins (Fig. 4B). After pretreatment with 20 μ M of the STAT3-specific inhibitor SH-4-54, IL-6 failed to increase the phosphorylation and total expression of STAT3, but it could still induce USP25 expression (Fig. 4C). Using another STAT3 inhibitor WP1066 we get the similar results (Fig. 4D). Furthermore, USP25 inhibition decreased the phosphorylation and total expression of STAT3 in XG 7 cells (Fig. 4E), and overexpression of USP25 increased the phosphorylation and total expression of STAT3 in XG 7 cells (Fig. 4F). In combination with USP25 belonging to deubiquitinating enzymes (DUBs), which can cleave ubiquitin from the target protein, we then used western blotting to detect the total amount of protein bound to ubiquitin, and found that the amount of ubiquitin-bound protein increased after USP25 inhibition (Fig. 4G). IP also indicated that ubiquitin binding to STAT3 increased after USP25 inhibition (Fig. 4H). These results demonstrated the IL-6/USP25/STAT3 axis in MM cell lines.

USP25 regulated the ubiquitination of STAT3

Based on previous results, we preliminarily revealed that USP25 could act as an upstream regulator of STAT3. USP25 contains two ubiquitin-interacting motif (UIM) domains and one ubiquitin carboxyl-terminal hydrolase (UCH)[10]. We then introduced a truncated mutation type of USP25, as shown in Fig. 4A. The UIM1 + 2 domain of USP25 failed to pull down STAT3, but the UCH domain of USP25 did achieve pull down, indicating that UCH of USP25 is critical for binding with STAT3 (Fig. 5A). In addition, using the deletion form of USP25, we demonstrated that deletion of the UCH domain only slightly decreased the level of ubiquitin-binding STAT3; however, deletion of UIM1 significantly decreased the amount of ubiquitin-binding STAT3, and UIM2 was more important for this process (Fig. 5B). USP25 could induce K63- and K48-linked deubiquitination. To determine which type of deubiquitination occurs in the USP25 and STAT3 interaction, we transfected K63R (Fig. 5C) and K48R (Fig. 5D) mutants of ubiquitin in RPMI8226 cell lines. K63R-transfected MM cells failed to induce STAT3 ubiquitination after USP25 knockdown, which revealed that USP25 regulated STAT3 ubiquitination through K63 polyubiquitination.

USP25 could regulate the cell cycle through STAT3 and cyclin D1

STAT3, related to the cell cycle marker cyclin D1, has already been studied in published data. In our study, we also demonstrated that among multiple cell cycle markers, cyclin D1 decreased most after USP25 inhibition (Fig. 5E), and other cell cycle markers also been modified such as Cyclin E, Cyclin A, CDK1, Cyclin B in XG 7 and RPMI8226 cell lines, indicating that USP25 widely involved in the cell cycle change in MM cells(Fig. 5E, F, G, H).

Discussion

In this study, using a combination of public dataset analyses and in-house experiments, we demonstrated that USP25 participates in the IL-6/STAT3 pathway and further regulates the MM cell cycle. This conclusion is supported by the following observations. (1) In the GSE19784, GSE9782, and GSE5900 datasets, the mRNA levels of USP25 and STAT3 were significantly correlated, and they bound to each other in protein form. (2) USP25 is highly expressed in MM patients, and high expression of USP25 is important for MM cell line proliferation and cell cycle progression. (3) The IL-6/USP25/STAT3 axis was confirmed. (4) The UCH domain of USP25 is critical for USP25 binding to STAT3, and the UIM domain is responsible for STAT3 ubiquitination. (5) STAT3 and cyclin D1 are important for USP25-mediated changes in the cell cycle.

IL-6/STAT3 is a well-known abnormal pathway in MM. Through the hexamer formed by two IL-6, two IL-6Ra, and two gp130 subunits, IL-6 could efficiently activate JAK family proteins such as JAK1, JAK2, and TYK2, leading to activation of STAT3 [11]. Activated STAT3 in MM could induce BCL-2-mediated suppression of apoptosis[3] and cyclin D1-mediated increase in proliferation[12], and it could even increase levels of miRNAs, such as miR-21, and thus inhibit MM cell apoptosis[13]. Based on the important role of STAT3 in MM, STAT3 inhibitors have already been developed. These inhibitors can be divided according to their targets: the SH2 domain, DNA-binding domain, and N-terminal domain[14]. Among them, SH2 inhibitors have been widely used; they can inhibit STAT3 Y705 phosphorylation, dimer formation, and subsequent transcription[15]. However, they remain in the pre-clinical research stage for two reasons. (1) The pharmacology of SH2 inhibitors involves the inhibition of dimer formation; however, it is difficult for small molecules to inhibit this process due to the large area of protein-protein interactions in dimer formation[16, 17]. (2) The SH2 domain widely exists in tyrosine proteins, and direct inhibition of this domain can cause serious side effects[18].

To address this dilemma, identifying the key regulator in the STAT3 pathway is urgent. In this study, we first confirmed that USP25 could function as a regulator of STAT3 in MM. USP25, which belongs to the DUB family, mediates target protein deubiquitination. In USP25^{-/-} cells, the activation of transcription factors IRF3 and NF-κB was inhibited, and USP25^{-/-} mice were more sensitive to H5N1 and HSV-1 infection[19]. In non-small-cell lung cancer, USP25 is a direct target of miRNA-200c, which regulates cell invasion and metastasis[20]. In addition, in our study, we showed that USP25 was highly expressed in MM and could support MM cell proliferation.

Although relationships between USP molecules and STAT3 have already been examined, most previous studies treated USP as a downstream gene of STAT3; for example, STAT3 could increase colon cancer cell proliferation through USP7 expression and further downregulate p53 expression[21]. In our study, inhibition of STAT3 did not change USP25 expression, and USP25-shRNA transfection downregulated STAT3 expression and phosphorylation. Moreover, similar to USP28 and USP37, USP25 contains the UCH domain and UIM domain[10]. We confirmed that STAT3 ubiquitination requires the UIM domain and not the UCH domain.

After we demonstrated the IL-6/USP25/STAT3 axis, we determined whether USP25 could also regulate STAT3 downstream genes. STAT3 can directly transcribe the cell cycle marker cyclin D1, and constitutively activated STAT3 is required for cyclin D1 overexpression and tumor cell growth[12]. We screened multiple cell cycle markers and confirmed that USP25 influenced cyclin D1 most. Thus, by inhibiting cyclin D1 and STAT3, the effect of USP25 on the MM cell cycle is offset. However, since cyclin D1 is also deubiquitinated by USP2[22], whether USP25 could directly affect cyclin D1 ubiquitination still needs to be studied.

Conclusion

In summary, suppression of USP25 could significantly affect STAT3 and the MM cell cycle. Therefore, the application of a USP25 inhibitor in patients with abnormal expression of STAT3 and cyclin D1 is promising.

Declarations

Ethics approval and informed consent

The protocol followed the Declaration of Helsinki and was approved by the Institutional Ethics Board of Ningbo Beilun People's Hospital.

Conflict of interest

None

Acknowledgments

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Figures

Figure 1

USP25 positively correlated with STAT3, and highly expressed in multiple myeloma patients. (A) Though combination of genes which highly correlated with STAT3 in GSE19784, GSE9782, GSE5900 datasets, USP25 was confirmed as common gene correlated with STAT3. (B) In GSE19784 dataset, GO: 0042509 pathway was highly enriched in USP25 high expression patients by using GSEA analysis. (C) 293 cells were transfected with myc-USP25 or myc-USP25+Flag-STAT3, then using IP to detect the interaction

between USP25 and STAT3. (D) Colocalization of USP25 and STAT3 was confirmed by IF assay. (E) In GSE9782 and GSE19784 dataset, USP25 highly expression related with MM patients' poor prognosis. (F) In 3 cases of healthy donors' bone marrow mononuclear cells and 11 cases of MM patients' CD138+ cells, USP25 expression level was determined by QPCR.

Figure 2

USP25 inhibition induced apoptosis, G1 cell cycle arrest, and inhibited cell growth in MM cell lines. (A) XG7 and RPMI8226 were transfected with Scramble and USP25-shRNA vector, after 48h, USP25 expression was detected by western blot. Using Annexin V APC/PI double staining to detect cell apoptosis

change in XG 7(B) and RPMI8226 (C). (D) Using high concentration PI staining to detect cell cycle in RPMI8226 cell lines. (E) CCK8 assay to determine cell proliferation in RPMI8226 cells. (F) Transwell assay to determine cell migration in RPMI8226 cells.

Figure 3

Regulation of tumor growth in vivo by USP25. (A-B) Applying in vivo image system to detect the change of tumor proliferation in RPMI8226 CON and USP25 shRNA (A) and XG 7 CON, USP25 (B) cells. (C-D) Summary of tumor volume in RPMI8226 CON and USP25 shRNA (C) and XG 7 CON, USP25 (D) group. (E-F) Using IF to detect the change of immune suppression signal(CD206 and CD163) in the primary tumor of RPMI8226 CON and USP25 shRNA (E) and XG 7 CON, USP25 (F).

Figure 4

IL-6 induced USP25 could regulate STAT3 degradation through STAT3 ubiquitination. (A) RPMI8226 was treated by 50 ng/ml IL-6 as indicated time, then USP25, p-STAT3, total STAT3 expression were detected by western blot. (B) RPMI8226 cells was transfected with CON or USP25-shRNA, then RPMI8226 USP25-shRNA were treated with 50 ng/ml IL-6 for 12h, and using western blot to detect the change of p-STAT3, total STAT3 and USP25. (C) RPMI8226 was pretreated with 20 μ M SH-4-54, then RPMI8226 was treated

with 50 ng/ml IL-6, then USP25, p-STAT3, total STAT3 expression were detected by western blot. (D) RPMI8226 was pretreated with 3 uM WP1066, then RPMI8226 was treated with 50 ng/ml IL-6, then USP25, p-STAT3, total STAT3 expression were detected by western blot. (E) RPMI8226 was transfected with USP25-shRNA, then USP25, STAT3 and p-STAT3 were detected by western blot. (F) RPMI8226 was transfected with full length of USP25, then Cyclin D1, USP25, STAT3 and p-STAT3 were detected by western blot. (G) Using western blot to detect ubiquitin binding protein in Scramble and USP25-shRNA RPMI8226 cells. (H) Using IP to detect ubiquitin binding STAT3 in Scramble and USP25-shRNA RPMI8226 cells.

Figure 5

UCH domain of USP25 bind with STAT3 and induced K63 ubiquitination of STAT3 (A) USP25 UIM1+2, UCH domain and full length were transfected in 293 cells and using IP to detect their interaction with STAT3. (B) Truncated form of USP25, which contain USP25 UCH deletion form, UIM1 deletion form, UIM2 deletion form, were transfected in 293 cells, then using IP to assess ubiquitin binding STAT4. (C) HA-Ubiquitin(K63R), Flag-STAT3, USP25-shRNA were transfected in RPMI8226 cells, then using IP to detect

ubiquitin binding STAT3 change. (D) HA-Ubiquitin(K48R), Flag-STAT3, USP25-shRNA were transfected in 293 cells, then using IP to detect ubiquitin binding STAT3 change. XG 7 cells were transfected with (E) USP25-shRNA (G) USP25 for 48h, then Cyclin E, Cyclin A, Cyclin D1, CDK1, Cyclin B change in protein level were detected by western blot. RPMI8226 cells were transfected with (F) USP25-shRNA and (H) USP25 for 48h, then Cyclin E, Cyclin A, Cyclin D1, CDK1, Cyclin B change in protein level were detected by western blot.

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