

Downregulation of Chemokine CCL20 Involved in Myeloma Cells Resistant to Elotuzumab and Lenalidomide

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Abstract

Background: Immunotherapy has received an increasing amount of attention in the field of multiple myeloma treatment because it has low-level toxicity however, there are very few studies on immunotherapy drug resistance. This study aimed to initially explore the relevant factors and possible mechanisms of immunotherapy drugs Elotuzumab (Elo) and Lenalidomides' resistance both in vivo and in vitro.

Methods: Cell models which are resistant to Elotuzumab and Lenalidomide were constructed; Different expression genes in UW, UR, UE, and URE cell lines were detected by using gene expression microarray; RT-qPCR validated CCL20 mRNA expression of four cell lines and patient samples; Bioinformatics Analysis of CCL20 expressions in NDMM and RRMM; ELISA detected the presence of CCL20 in the plasma of MM patients; Recovered CCL20 levels could increase the sensitivity of drug-resistant cell lines to immunomodulatory drugs; Constructed lenalidomide-resistant (UR) mouse subcutaneous xenograft model to explore whether or not CCL20 and lenalidomide treatment influenced the tumor volume's growth.

Results: Cell models of drug resistance were successfully constructed and we found that the mRNA expression of CCL20 was down-regulated in resistant UR, UE, and URE cell lines; RT-qPCR confirmed these, and also identified the downregulation of CCL20 in RRMM patients compared with NDMM; bioinformatics analysis found that the mRNA expression of CCL20 was also down-regulated in RRMM patients; Furthermore, RRMM patients were found to have lower levels of CCL20 protein in their plasma when compared to NDMM. CCL20 could increase the sensitivity of UR,UE, and URE cell lines to immunomodulatory drugs in vitro and in vivo.

Conclusions: The expression of CCL20 was decreased in lenalidomide and elotuzumab resistant U266 cells and RRMM patients.CCL20 could possibly increase the sensitivity of lenalidomide in vitro and in vivo.

Background

Multiple myeloma (MM) is the second common hematologic malignancy and is commonly seen in the elderly. In recent years, new drugs have greatly improved the response rates and overall survival rate of MM patients, however, the disease is still incurable [1]. Many patients eventually progress to relapse/refractory MM (RRMM). Immunotherapy has attracted increasing attention in the treatment of multiple myeloma due to its advantages of targeting and low toxicity [2]. While there are few studies on the mechanism of immunotherapy resistance. Because of the ability to regulate the immune function, lenalidomide, as the second generation of immunomodulators, has been widely used in the treatment of multiple myeloma. Elotuzumab (ELO) is the second monoclonal antibody drug approved for the treatment of RRMM. In 2015, the FDA approved ELO for use in combination with lenalidomide and dexamethasone (E-RD regimen) in the treatment of relapsed/refractory multiple myeloma patients [3, 4]. The therapeutic target of Elotuzumab is a member of the signal lymphocyte activating molecule family 7 (slamf7, also

known as cd319, CS1, etc.). CS1 is a cell surface glycoprotein molecule, which has shown to be highly expressed on the surface of myeloma cells and NK cells, but not on the surface of normal tissues and hematopoietic stem cells, and is involved in the regulation of bone marrow microenvironment [5]. Elotuzumab can directly activate NK cells and induce CD16 mediated cytotoxicity to kill myeloma cells with high expressions of CS1, but has little effect on normal tissues. Although the E-RD regimen achieved satisfactory results, some patients still developed drug resistance. In this present study, myeloma cell lines resistant to erlotuzumab, and lenalidomide were constructed in order to explore the mechanism of monoclonal antibody and immunotherapy resistance. We aim to provide new targets for immunotherapy and provide new ideas for reversing drug resistance.

Methods

Myeloma cells culture

Bone marrow samples of MM patients were sorted by CD138 and magnetic beads were collected from Shengjing Hospital of China Medical University and approved by the ethics committee of Shengjing Hospital (approval No.: ps270k).

Myeloma U266 cell line and Lenalidomide resistant cell line (U266 / R10R, UR) were presented by Professor Orlowski Robert in the MD Anderson Cancer Center. Lenalidomide sensitivity (U266 / WT, UW) and single resistance UR were co-cultured with human peripheral blood mononuclear cells (PBMC) respectively. They were then added with a great amount of concentration of elotuzumab and cultured for 48 hours. Then, the surviving cells were collected and sorted with CD138 antibody while the myeloma cells were collected by flow cytometry. The drug stimulation was continued and the above process was repeated until the cell lines were stable with Elo single resistance (U266 / elor, UE) and double resistance (U266 / r10r / elor, URE).

Cell viability assay

Four kinds of cells (UW, UR, UE, URE) were detected. Different concentrations of erlotuzumab (0,1,10,50,100,500,1000ug / ml) with PBMC were treated. According to the manufacturer's instruction, cells were processed with CCK-8 kits to detect cell proliferation.

Gene expression profiling

Illumina gene expression microarray data were obtained as described previously.

Real-time RT-PCR to detect the CCL20 gene expression

To detect CCL20 mRNA expressions in cell lines and MM patient samples, qPCR was performed on an Applied Biosystems Step One Plus Real Time PCR system. CCL20
Forward:ATGTGCTGTACCAAGAGTTGC;

CCL20 Reverse:CCAATTCCATTCCAGAAAAGCC.

Enzyme-linked immunosorbent assay to detect CCL20 protein expression

To detect CCL20 protein expression in the plasma of MM patients, we used CCL20 (MIP 3alpha) (peprotech, USA) KIT following the instruction.

Bioinformatics analysis

R language was used to download the chip expression spectrum data of GSE16791, GSE31504, and GSE51317 for probe conversion, data standardization, and batch correction. To analyze the expression of CCL20, the Ggpurb package was used.

Xenograft model

Female NOD-SCID mice aged 4–5 weeks were purchased from Beijing Hua Fukang Bioscience Company (Beijing, China) and were housed and monitored in a pathogen-free environment. All animal studies were approved by the Research Ethics Committee of China Medial University. 1×10^7 U266/R10R cells were injected subcutaneously into NOD-SCID mice. The animal treatment was initiated after the detection of palpable tumors, approximately 12 days following injection. Mice were randomized in two groups using a computer based random order generator, three mice per group, control group (lenalidomide + NS) and experimental group (lenalidomide + CCL20) to observe the effect of CCL-20 reverse the lenalidomide resistance. Investigators could not be blinded to the mouse. Tumor growth was monitored every 3 days for about 3 weeks. Serial measurements of xenograft growth were performed, and tumor volume (V) was calculated using the formula: $V=0.5\times a\times b^2$, where a and b represent the longest and shortest tumor diameters, respectively. At the end of study, animals were killed and tumors were collected.

Statistical analysis

To analyse the data, the Spss23.0 software was used to analyse the difference between the two samples, and an independent sample t-test was used. To analyze the difference between multiple groups, the one way ANOVA was used $P < 0.05$ was regarded as statistically significant.

Results

Generation of Elotuzumab and lenalidomide resistant myeloma cells

The single drug resistance (U266 / elor, UE), and dual drug resistance (U266/ r101r/ elor, URE) were constructed by using the continuous stimulation culture of elotuzumab. With the increasing concentration of elotuzumab, the cell viability of sensitive cell lines (UW) decreased gradually, while the cell viability of erlotuzumab resistant cell line (UE) did not change significantly (Figure 1A); The cell viability of the dual drug-resistant cell line (URE) has lower cell viability compared with lenalidomide resistant but ELO sensitive cell line (UR) treated with different concentration of elotuzumab by adding

PBMC (Figure 1B). The results showed that the single ELO resistant cell line UE and the dual drug-resistant cell line URE had stable resistance to Erlotuzumab.

CCL20 expression decreased in resistant myeloma cell

We used the gene expression microarray to detect the gene expression differences of four myeloma cells: UW, UR, UE, URE in order to further explore the possible mechanism of resistance. The results showed that the expression of CCL20 was significantly down-regulated in drug-resistant cell lines (Figure 2A). RT-qPCR data further confirmed this result (Figure 2B). We then detected CCL20 expressions in MM patients. We collected 27 samples of bone marrow fluid from patients with newly diagnosed mm (NDMM), and 5 samples of bone marrow fluid from patients with relapsed refractory MM (RRMM) exposed to lenalidomide. The results showed that compared with NDMM, the expression level of CCL20 mRNA in RRMM was lower ($p < 0.01$) (Figure 2C). Furthermore, the data from the GEO database showed that the expression of CCL20 in bone marrow plasma cells of RRMM patients was lower than that of NDMM patients, especially the MM exposed to lenalidomide, which was consistent with PCR results (Figure 2D). Finally, fresh plasma was collected from 5 NDMM patients and 5 RRMM exposed to lenalidomide. The average content of CCL20 was 24.44 ± 0.88 pg/ml in the plasma of patients with NDMM and 8.97 ± 1.88 pg/ml in RRMM ($p < 0.01$) (Figure 2E). These data supported the CCL20 expression decreased in resistant myeloma cells

CCL20 increased the sensitivity of drug-resistant myeloma cells to immunomodulatory drugs

The experimental units were divided into three groups, namely, lenalidomide, elotuzumab, and lenalidomide + erlotuzumab. The results showed that in each group, the cell viability with CCL20 adding were significantly lower than that without CCL20 (Figure 3A), which indicated that CCL20 could partially restore the drug sensitivity of drug-resistant myeloma cells. All the group are added PBMC.

CCL20 increases the sensitivity of resistant myeloma mice to lenalidomide in vivo.

We constructed UR resistant NOD-SCID mice in order to verify the effect of CCL20 on the myeloma resistance in vivo. With the prolongation of the lenalidomide treatment, the tumor volume growth rate of mice in the experimental group (lenalidomide + CCL20) was significantly slower than that in the control group (lenalidomide + NS) (Figure 3B). The tumor volume of mice in the experimental group was significantly smaller than that in the control group (Figure 3C).

Discussion

In recent years, the emergence of new drugs and new therapies have brought hope for myeloma patients. However, most patients eventually progress to relapse and refractory states due to the occurrence of drug resistance. Compared to traditional chemotherapy, the effect of immunotherapy is improved while the side effects are decreased. However, there are still patients who are resistant to immunotherapy. It is therefore paramount to explore the mechanism of immunotherapy resistance in order to improve the

response to immunotherapy. Two vital immunotherapeutic drugs are immunomodulators and monoclonal antibodies. Lenalidomide is a widely used immunomodulator, which can inhibit the secretion of inflammatory factors, directly inducing the phosphorylation of CD28 on T cells, leading to the activation of downstream target factors, and then activating T cells and enhancing various mechanisms mediated by NK cells to achieve the anti-tumor effect. Elotuzumab can target and label CS1 protein on the surface of multiple myeloma cells, inhibit the adhesion between myeloma cells and bone marrow stromal cells, regulate the MM bone marrow microenvironment and reduce the growth-stimulating factors on myeloma cells[6-9]. A phase III clinical trial included 646 patients with multiple myeloma (RRMM) [10], the E-Rd (Elotuzumab, lenalidomide plus low-dose dexamethasone) has obvious advantages compared with Rd: the median progression-free survival (PFS) is 14.9 (Rd) months vs.19.4 months (E-Rd) ($p < 0.001$). However, the E-Rd regimen is still not sensitive to some patients. The mechanism of resistance to Elotuzumab and lenalidomide is not clear yet.

To further improve the efficacy of immunotherapy and in order to clarify the mechanism of immunotherapy resistance, the myeloma cell model of resistance to Elotuzumab, and dual resistance to both Elotuzumab and lenalidomide were firstly constructed. We then found that the CCL20 decreased expression in drug-resistant cells. We also verified CCL20 down-regulation involved in the development of drug resistance in immunotherapy of myeloma cells both in vivo and in vitro. CCL20 (chemokine (C-C motif) ligand 20; macrophage infectious protein-3 α, mip-3 α) is one of the important members of chemokine family. The gene is located on chromosome 2. CCL20 mRNA encodes and synthesizes 96 amino acid precursor proteins, which are finally cut into 70 amino acid mature proteins [11]. CCL20 is mainly expressed in immune cells, activated T cells, dendritic cells and monocytes. Additionally, it can also be expressed in endothelial cells. Its receptor is CCR6, which can be expressed in lymphoid tissue, liver, and lung [12]. The expression of CCL20 is regulated by a variety of cytokines. Research shows that TNF-α, IL-17, and IFN-γ can induce the expression of CCL20, causing the chemotaxis of inflammatory cells, and affecting the proliferation and metastasis of tumor cells [13, 14]. An increasing number of studies have shown that CCL20 has abnormal expression in a variety of cancers such as; esophageal cancer, lung cancer, colorectal cancer, etc. Studies have also shown that CCL20 has increased expression in paclitaxel chemotherapy in triple-negative breast cancer, thus activating NF-κB, promoting the self-renewal of breast cancer stem cells/stem like cells, and participating in the occurrence of drug resistance [15-17]. Recently, it has been shown that CCL20 is related to the regulation of bone marrow microenvironment of myeloma cells and participates in the occurrence of myeloma osteopathy [18, 19]. Besides, it has been discovered that the increase of CCL20 can promote T-cell-mediated antitumor immunity through CCL20-CCR6-dependent dendritic cell regulation, thus providing a new target for immunotherapy [20]. We discovered that increasing CCL20 can enhance the anti-myeloma effect of lenalidomide. We speculated that the mechanism may be through increasing lymphocyte chemotaxis to tumor area and assisting in cell-mediated immunity. In RRMM, the down-regulation of CCL20 results in the decrease of the immunotherapeutic effect.

Progressive attention has been paid to the role of CCL20-CCR6 axis in immune regulation and mechanism in autoimmune diseases. Our study preliminarily found the role of CCL20-CCR6 axis in the

resistance of immunotherapy of myeloma. In order to provide a new target for immunotherapy, reverse the resistance of myeloma cells to immunotherapy, and improve the efficacy and safety of RRMM treatments, further studies on its downstream effector cells and mechanism will be expected.

Conclusions

We found that the expression of CCL20 was decreased in lenalidomide and elotuzumab resistant U266 cells and RRMM patients. CCL20 could possibly increase the sensitivity of lenalidomide in vitro and in vivo. Our study preliminarily found the role of CCL20-CCR6 axis in the resistance of immunotherapy of myeloma and may provide a new target for immunotherapy, reverse the resistance of myeloma cells .

Abbreviations

Multiple myeloma (MM)

relapse/ refractory MM (RRMM)

newly diagnosed mm (NDMM)

Elotuzumab (ELO)

peripheral blood mononuclear cells (PBMC)

Declarations

Ethics approval and consent to participate

The studies involving client-owned animals were approved by The Ethic Committee of Shengjing Hospital of China Medical University (approval No.: ps270k). The client or owner provided their written informed consent to participate in this study.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Study conception and design: HW and AL. Quality control: AL. Statistical analysis: HS and XH. Manuscript preparation: WY and HW. Manuscript review: WY, AL, and HW. All authors contributed to the article and approved the submitted version.

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Figures

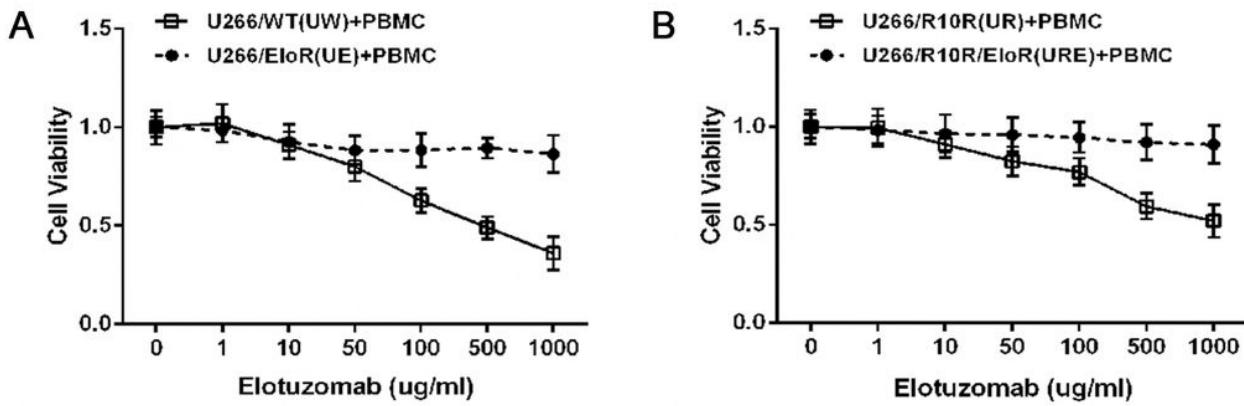


Figure 1

Cell viability of myeloma cells to Elotuzumab A. The cell viability of sensitive (U266 /WT, UW) and elotuzumab resistant (U266 / elor, UE) cell lines to elotuzumab (ELO) coculture with peripheral blood mononuclear cells (PBMC). B. The cell viability of lenalidomide resistance (U266 / r10r, UR) and lenalidomide plus elotuzumab double resistance (U266 / r10r / elor, URE).

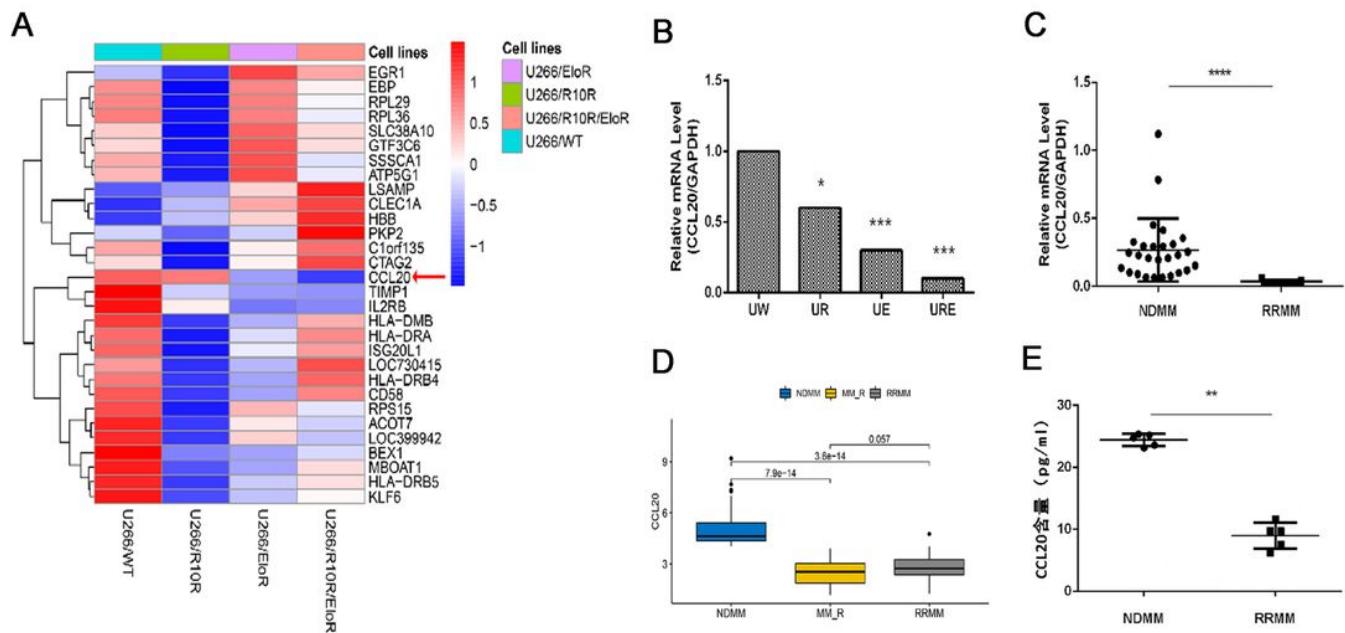


Figure 2

CCL20 expression decreased in resistant myeloma cell A. Heatmap of gene expression differences of four myeloma cells: UW, UR, UE, URE. B. RT-qPCR to verify the relative expression of CCL20 gene in myeloma cell lines. C. CCL20 gene expression in MM patients samples, 27 samples of bone marrow fluid from patients with newly diagnosed mm (NDMM) and 5 samples from relapsed refractory MM (RRMM)

exposed to lenalidomide. D. Bioinformatics analysis CCL20 relative gene expression in NDMM and RRMM patients samples. MM_R is the patients who resistant to lenalidomide.E. CCL20 protein expression in plasma of NDMM and RRMM to lenalidomide patients by ELISA.

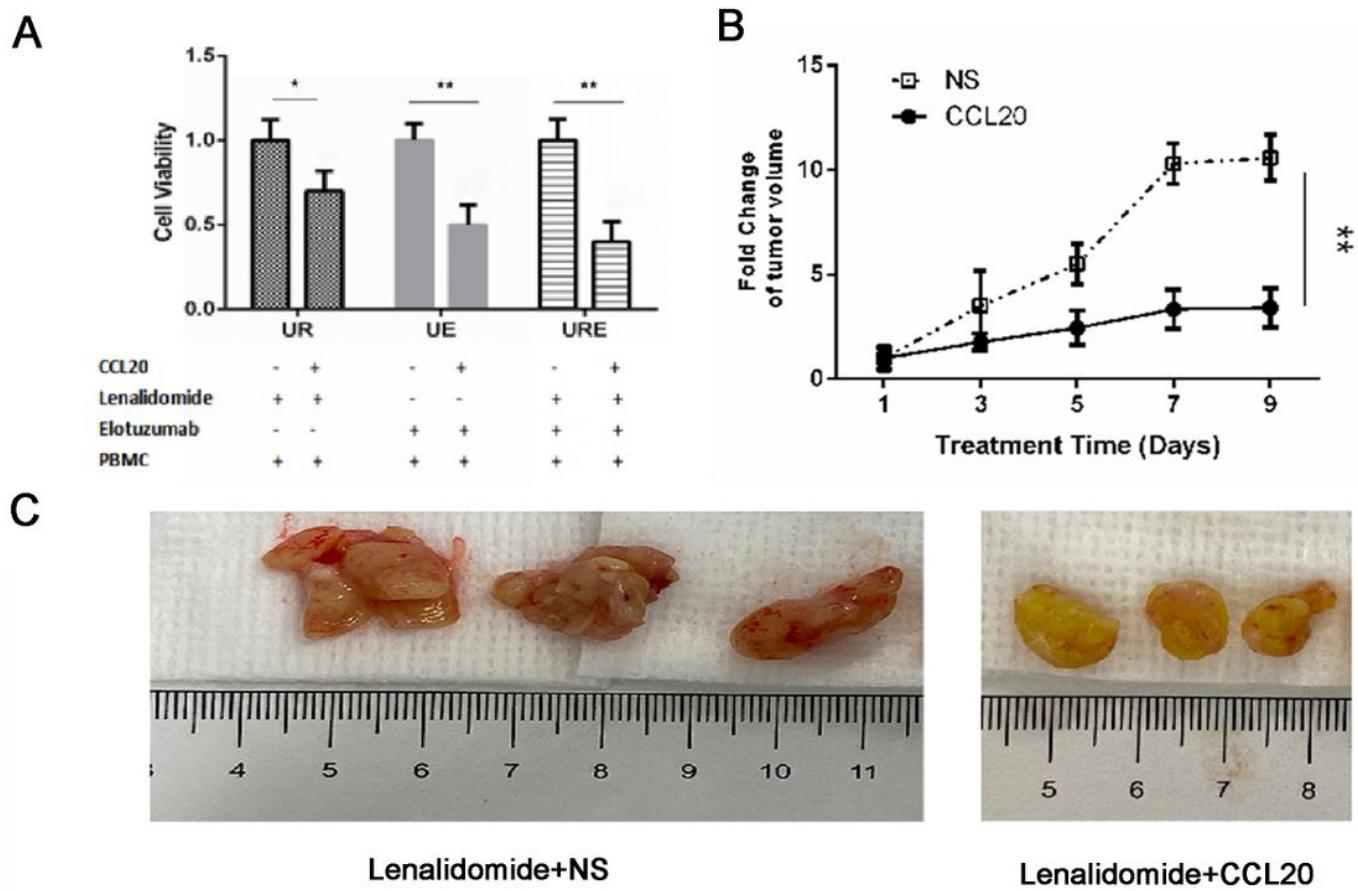


Figure 3

CCL20 increases drug sensitivity in vitro and in vivo. A. Cell viability of UR,UE,URE cells treated with lenalidomide or elotuzumab with or without CCL20. B.Tumor volume growth rate of UR xenograft mice in the experimental group (lenalidomide + CCL20) and control group (lenalidomide + NS) C. Tumor volume (blank control group on the left, experimental group on the right).