



A novel human multiple myeloma cell line with a 1q21 gain genetic abnormality and CKS1B overexpression

Jie He^{1#}, Ke Yi^{1#}, Yajun Zhang¹, Hui Zhang², Jian Hou^{2,3}, Rong Li^{1,2}

¹Nuclear Radiation Injury Protection and Treatment Department, Navy Medical Center of PLA, Shanghai, China; ²Myeloma and Lymphoma Center, Department of Hematology, Changzheng Hospital, Navy Medical University, Shanghai, China; ³Department of Hematology, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

Contributions: (I) Conception and design: R Li, J Hou; (II) Administrative support: R Li, J Hou; (III) Provision of study materials or patients: J He, K Yi, H Zhang; (IV) Collection and assembly of data: J He, K Yi, Y Zhang; (V) Data analysis and interpretation: J He, K Yi, H Zhang, Y Zhang; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Rong Li, PhD. Nuclear Radiation Injury Protection and Treatment Department, Navy Medical Center of PLA, Huaihai West Road No. 338, Shanghai 200050, China. Email: lirong785@hotmail.com; Jian Hou, PhD. Department of Hematology, Renji Hospital, Shanghai Jiao Tong University School of Medicine, 145 Shandong Zhong Road, Shanghai 200001, China. Email: houjian@medmail.com.cn.

Background: Multiple myeloma (MM) is an incurable hematologic malignancy mainly due to its cytogenetic abnormalities. Therefore, it is important to establish permanent malignant MM cell lines as tools to develop more effective therapies.

Methods: Pleural effusion cells of a 70-year-old patient was collected to establish the CZ2 cell line. Characterization of CZ2 was determined with nephelometry, flow cytometry, fluorescence in situ hybridization (FISH), and chromosomal microarray analysis (CMA). Western blotting analysis was adopted to determine protein expression. Cell viability was measured by the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

Results: We established and characterized a new MM cell line, CZ2. Using nephelometry and flow cytometry, cells with typical plasma cell morphology but not classical plasma cell phenotype were found to be non-immunoglobulin-secreting cells. FISH analysis of cells revealed a unique characteristic, namely, that there was only gain of the 1q21 region (1q21+). No other common cytogenetic abnormalities in MM, such as deletion of 17p (17p-), deletion of 13q (13q-), or translocation of immunoglobulin heavy chain (IgH), were observed. In addition, the original cell line maintains its single cytogenetic abnormality. Meanwhile, we observed through western blotting that CDC28 protein kinase regulatory subunit 1B (CKS1B), an adverse prognostic gene located in the 1q21 region, was highly expressed in CZ2. Knockdown of CKS1B reduced cell viability and also increased the levels of cleaved-poly(ADP-ribose) polymerase (cleaved-PARP) and cleaved-caspase3.

Conclusions: CZ2 provides a suitable material for cellular and molecular studies of MM with only a 1q21 abnormality. This cell line is characterized by a gain of 1q21, and the high expression of CKS1B is an important model for studies of myeloma cell growth and drug resistance during therapy.

Keywords: Multiple myeloma (MM); cell line; 1q21 gain/amplification; CDC28 protein kinase regulatory subunit 1B (CKS1B)

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Introduction

Multiple myeloma (MM) is a hematopoietic disease that is characterized by the proliferation of plasma cells in the bone marrow (1). In recent decades, protease inhibitors, immunomodulators, autologous stem cell transplantation, and several elective treatment strategies have been developed to improve the prognosis of MM (2), and the median survival time has been extended to 5–7 years (3). However, MM is still considered an incurable disease, as patients inevitably relapse after a variable time of following therapy (4).

The incurability of MM is closely related to its biological characteristics. The International Myeloma Work Group (IMWG) suggested that molecular biological anomaly markers such as 1q21 (gain), translocation t(4;14), and del(17p) can be applied for the risk-stratification of MM patients (5–7). Among these cytogenetic abnormalities, 1q21 gain (≤ 3 copy numbers) or amplification (> 3 copy numbers), are relatively common types in MM patients (8). A 1q21 gain or amplification occurs in 30–40% of MM patients at the initial diagnosis and in 70% of patients at recurrence (9–11).

Numerous clinical studies have demonstrated that patients with 1q21 gain or amplification exhibit poor prognosis (12). Moreover, various genes located in 1q21 are involved in MM, especially CDC28 protein kinase regulatory subunit 1B (CKS1B), which is overexpressed in malignant tumors including MM, and is thought to be a factor of poor prognosis in refractory/recurrent MM patients (13,14). Collecting a bulk of neoplastic cells from MM patients for research is an inconvenient and time-consuming process. Therefore, determining the underlying mechanism of MMs with 1q21 gain or amplification is critical for the development of precise and efficient treatments to cure MM patients. For this, permanent malignant hematopoietic cell lines, which act as effective

tools, are needed. In general, immortalized cell lines are capable of renewing themselves in artificial culture indefinitely. In the present study, we established a novel MM cell line, CZ2, which is characterized by only 1q21 gain, in hope that it can be utilized in MM research and the development of more effective therapies. We present the following article in accordance with the MDAR reporting checklist (available at <https://atm.amegroups.com/article/view/10.21037/atm-22-5741/rc>).

Methods

Patient

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of Changzheng Hospital (No. AF-HEC-055) and informed consent was obtained from the patient. Our patient was a 70-year-old male who was diagnosed with stage III MM according to the International Staging System (ISS) and stage IIIA MM based on the Durie-Salmon (DS) staging system. Bone marrow aspiration showed that plasma cells accounted for 97.5% of the bone marrow cells. The patient's myeloma cells secreted a monoclonal protein of immunoglobulin D (IgD), the kappa chain. Also, his serum immunoglobulin G (IgG) was 3.39 g/L, immunoglobulin A (IgA) was 0.26 g/L, and immunoglobulin M (IgM) was 0.18 g/L. For this experiment, we used pleural effusion cells at the cell collection stage.

Cell culture and establishment of cell line

Pleural effusion specimens were obtained from the patient and the mononuclear cells were separated by Ficoll density sedimentation. CD138⁺ cells were isolated using CD138 magnetic beads and cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), penicillin (100 U/mL), and streptomycin (100 µg/mL). The cells were cultured in an incubator under an atmosphere with 5% carbon dioxide (CO₂) at 37 °C, and the culture medium was exchanged every 2–3 days to maintain optimal cell growth.

Morphology and cytogenetic analysis

Cytospin preparations were routinely prepared and harshly treated (87 °C for 10 minutes, then cooled to 70 °C), and

Highlight box

Key findings

- Novel cell line with a 1q21 gain only was established.

What is known and what is new?

- Permanent MM cell lines act as effective tools.
- A novel MM cell line, CZ2, which is characterized by only 1q21 gain and CKS1B overexpression was established.

What is the implication, and what should change now?

- CZ2 is an important model for studies of myeloma cell growth.

then stained using Giemsa to regularly assess the cell type morphology and the presence of mitoses. Cell morphology was examined using light microscopy.

Fluorescence in situ hybridization (FISH) analysis

FISH was performed on interphase CZ2 using a Fluorescent *in situ* Hybridization Kit (CytoCell, UK), according to the manufacturer's instructions. The commercial FISH probes targeting 1q21, 13q14 (*RB1* gene), 17p13 (*TP53* gene), and translocation t(4;14) were used for this assay. The probe used in the FISH test was obtained from the p53/D13S319/RB1/1q21/IgH/(IgH/CCND1)/(IgH/MAF)/(IgH/MAFB)/(IgH/FGFR3) DNA Probe kit (F.01367-01, AnBiPing, China). At least 200 interphase cells with intact nuclei were evaluated for each probe. Positive nuclear staining in more than 20% of the cells was considered positive.

Chromosomal microarray analysis (CMA)

The experiment was performed as previously reported (15). Hybridization-based genomic profiling arrays were blindly performed on the CytoSan HD array platform, which contains 2.6 million and 1.8 million combined copy number variation (CNV) and single nucleotide polymorphism (SNP) markers, respectively, with a median inter-marker distance of 500–600 bases (Affymetrix, Inc., Santa Clara, CA, USA). Hybridizations were performed according to the manufacturer's protocols. CEL files obtained from the CytoScan HD array platform were analyzed using the Chromosome Analysis Suite software package (Affymetrix) and the Nexus copy number software (Biodiscovery Inc., Hawthorne, CA, USA). The GRCh37 (hg19) genome version was applied for annotations. Measures achieving the manufacturer's quality cut-off -were included in the analysis.

Immunophenotypic analysis by flow cytometry

The following antibodies were used for immunophenotypic characterization of the CZ2 cells: CD1a, CD19, CD20, CD21, CD25, CD3, CD28, CD33, CD38, CD45, CD56, CD117, CD138, CD4, CD13, CD27, CD5, CD8, CD9, CD10, CD24, CD15, CD34, CD44, CD89, CD95, CD98, CD96, CD105, CD184, HLA-DR; and cytoplasmic κ and γ light immunoglobulin chains (Invitrogen, USA).

Immunoglobulin secretion

To measure the secretion of free immunoglobulin light chains in the serum and culture medium of CZ2, the nephelometric technique was applied and performed on a Siemens BNII Nephelometer (Siemens Healthcare Ltd., Newark, DE, USA).

Virus detection

The presence of Epstein-Barr virus (EBV) was examined using an EBV polymerase chain reaction (PCR) Fluorescence Detection Kit (Huirui Biotechnology, Shanghai, China). PCR was carried out using a LightCycler480 II (Roche, USA) for 40 cycles.

Western blotting analysis

The whole cell protein from CZ2 and other MM cells was collected and resolved in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and then electrotransferred to polyvinylidene difluoride membranes (Millipore, USA). Rabbit polyclonal anti-CKS1B antibody (1:500, Invitrogen, CA, USA), rabbit polyclonal anti-poly(ADP-ribose) polymerase (anti-PARP) antibody (1:1,000, Beyotime Biotech. Inc., Shanghai, China), and rabbit polyclonal anti-caspase3 antibody (1:1,000, Beyotime Biotech. Inc., Shanghai, China) were used as primary antibodies, while horseradish peroxidase-conjugated goat anti-rabbit IgGs (1:3,000, Santa Cruz, CA, USA) were used as secondary antibodies. After treatment with the secondary antibodies, the protein bands were detected by enhanced chemiluminescence.

Cell viability analysis

The 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to measure the cell viability of CZ2 and other MM cells. These cells were seeded in 96-well plates and transfected with CKS1B small interfering RNA (siRNA) or control siRNA (Shanghai GenePharma Co., Ltd., Shanghai, China). The target sequences of siRNAs are listed in Supplemental *Table 1*. After 48 or 72 h of treatment, 10 μ L of the MTT viability reagent was added to each well. The plates were incubated in an incubator at

Table 1 CMA analysis of the CZ2 cell line

Location	Cytogenetic abnormality	Copy number
Chromosome 1	dup(1)(p36.33p34.3)	3
	dup(1)(p34.3p32.3)	4
	dup(1)(p32.3p13.3)	3
	dup(1)(p13.3p12)	4
	dup(1)(p11.2q23.3)	3
	dup(1)(q23.3q25.3)	4
	dup(1)(q25.3q44)	3
Chromosome 2	dup(2)(p25.3q23.2)	3
	dup(2)(q23.2q32.1)	4
	hmz(2)(q32.1q37.3)	–
Chromosome 3	dup(3)(p26.3p26.1)	3
	dup(3)(p26.1p25.1)	4
	dup(3)(p25.1p21.31)	3
	hmz(3)(p26.3q29)	–
Chromosome 4	dup(4)(p16.3q32.1)	3
	del(4)(q32.1q32.2)	1
	dup(4)(q32.2q35.2)	3
Chromosome 5	dup(5)(p15.33q11.2)	4
	dup(5)(q11.2q31.3)	3
	dup(5)(q32q34)	3
	dup(5)(q34q35.3)	3
Chromosome 6	dup(6)(p25.3p12.3)	4
	dup(6)(p12.3q13)	3
	dup(6)(q13q15)	4
	dup(6)(q23.3q27)	3
	hmz(6)(6q15q23.3)	–
Chromosome 7	dup(7)(p22.3p11.2)	3
	dup(7)(q11.21q36.3)	4
Chromosome 8	dup(8)(p23.3p22)	3
	dup(8)(p11.23q24.3)	3
	hmz(8)(p23.3p11.23)	–

Table 1 (continued)**Table 1** (continued)

Location	Cytogenetic abnormality	Copy number
Chromosome 9	del(9)(p21.3p21.2)	0
	del(9)(p21.1)	0
	dup(9)(p21.1p13.2)	3
	dup(9)(p13.2p13.1)	5
	dup(9)(q22.31q34.3)	3
	hmz(9)(p24.3p13.1)	–
	hmz(9)(q21.11q34.3)	–
Chromosome 10	dup(10)(p15.3p12.1)	3
	dup(10)(p12.1p11.23)	4
	dup(10)(p11.23q21.1)	3
	dup(10)(q21.1)	4
	dup(10)(q21.1q23.1)	3
Chromosome 11	hmz(10)(q23.1q26.3)	–
	dup(11)(p15.5p14.2)	3
	dup(11)(p14.2p14.1)	4
	dup(11)(p14.1q14.1)	3
Chromosome 12	dup(11)(q14.1q25)	4
	dup(12)(p12.1q24.33)	3
	hmz(12)(p13.33p12.1)	–
Chromosome 13	dup(13)(q31.3)	13
	hmz(13)(q11q34)	–
Chromosome 14	dup(14)(q24.2q32.33)	3
	hmz(14)(q11.2q32.33)	–
Chromosome 15	dup(15)(q25.1q26.3)	3
Chromosome 16	dup(16)(p13.3q23.1)	3
	dup(16)(q23.1)	4
	dup(16)(q23.1q24.1)	5
Chromosome 17	dup(16)(q24.1q24.3)	4
	dup(17)(q11.1q25.3)	3
	hmz(17)(p13.3p11.2)	–

Table 1 (continued)

Table 1 (continued)

Location	Cytogenetic abnormality	Copy number
Chromosome 18	dup(18)(p11.32p11.22)	4
	dup(18)(p11.22q11.2)	3
	dup(18)(q11.2q12.1)	5
	dup(18)(q12.1q12.3)	4
Chromosome 19	dup(19)(p13.3q13.43)	3
Chromosome 20	dup(20)(q11.1q13.33)	3
	hmz(20)(p13p11.1)	–
Chromosome 21	dup(21)(q11.2q21.2)	3
	dup(21)(q21.2q22.3)	4
Chromosome 22	dup(22)(q11.1q11.21)	4
	dup(22)(q11.21)	11
	dup(22)(q11.21q11.23)	13
	hmz(22)(q11.23q13.33)	–
Chromosome X	dup(X)(p22.33)	3
	hmz(X)(p22.33q28)	–

GMA, chromosomal microarray analysis.

37 °C for 2 h. The medium was then discarded and 200 μ L dimethyl sulfoxide (DMSO) was added to each well. The absorbance at 570 nm was then determined with a microplate reader (Tecan, Spark, Switzerland).

Statistical analysis

All experiments were independently replicated at least three times with replicate samples. Statistical analysis was performed using the Student's *t*-test or paired *t*-test.

Results

Establishment and biological characterization of the CZ2 MM cell line

The separated cells from the patient's pleural effusion were cultured in RPMI-1640 supplemented with 10% FBS and grown as single cells or in small clusters in a suspension. The cell doubling time was about 48 h; hence, they could be passaged every 2 or 3 days. Following 20 months (68 passages), the CZ2 cell line displayed stable proliferation. The cells were then frozen in liquid nitrogen and proliferated after revival. Using FACScan (Beckman

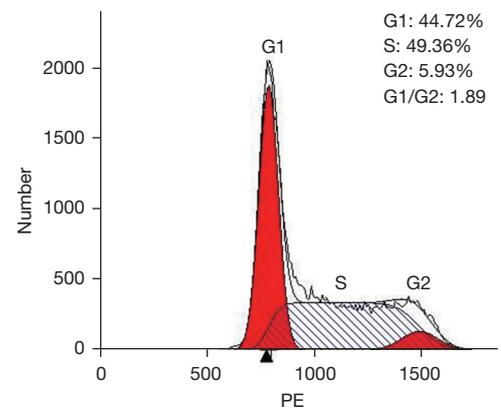


Figure 1 The cell cycle of the CZ2 cell line. The CZ2 cell cycle was analyzed using flow cytometry. PE, phycoerythrin.

Coulter, CA, USA), we found that in the exponential growth phase, 44.72% of the cells were in the G1 phase, 5.93% were in the G2 phase, and 49.36% were in the S phase (Figure 1). Wright-Giemsa staining showed that the cells exhibited a typical plasma cell morphology, which had large nuclei, 2–3 nuclei/cell, and a basophilic cytoplasm (Figure 2).

Immunophenotypic and immunoglobulin analysis

The CZ2 cells did not express CD38, which is a typical plasma cell phenotype. In addition, the expressions of CD19, CD45, and CD117 were negative, whereas those of CD43, CD28, CD40, CD58, and β 2 were positive. Cells were partly positive for CD10 and c-MYC and marginally positive for CD7, CD15, and CyclinD1. Moreover, the CZ2 cells were also found to be non-secretory cells after several generations. Furthermore, the PCR results of the cells showed them to be EBV-negative.

Cytogenetic characterization

Classic cytogenetic analysis revealed that 65–67 chromosomes were found with the following aberrations in the 41st generation of the cells: 65–67<3n>,XX,+1,+del(1)(q?),+del(1)(p?),+2,+5,+5,+6,+7,+7,+del(7)(p?),+del(8)(p?),9p+,+del(9)(q?),+10,+10,+del(10)(q?),+11,+11,+12,+12,-13,-14,+15,+16,+16,+del(17)(p?),+del(17)(p?),del(18)(q?),+19,+21(cp10) (Figure 3).

Given that FISH results have been included in the Revised International Staging System (RISS), FISH analysis

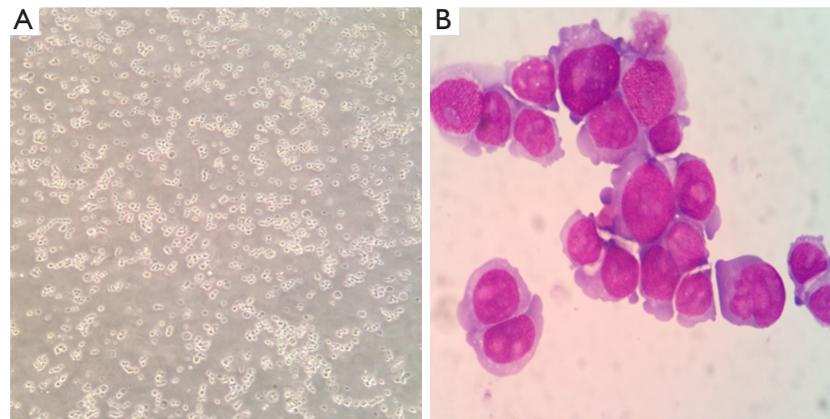


Figure 2 Cell morphology of the CZ2 cell line. (A) Cultures of the CZ2 cell line under the inverted microscope (100× magnification). (B) Giemsa-stained cytospin slide preparations in 30th generation culture of the CZ2 cell line (1,000× magnification).

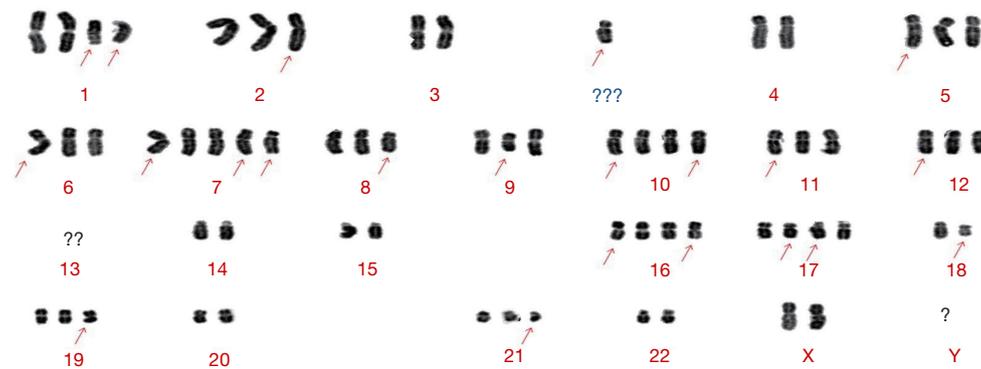


Figure 3 The karyotype of the CZ2 cell line. An example of classical cytogenetic analysis. (Chromosomal structural and numerical changes are indicated by arrows). The question mark means that the chromosome can not be fully identified.

is necessary during the diagnosis and treatment of MM. Using the common MM probes [i.e., the 1q21 probe, the TP53/CEP 17 FISH probe, the D13S319 (13q14.3) probe, and the immunoglobulin heavy chain (IgH) dual color probe], we found that the only cytogenetic abnormality was a 1q21 region (1q21+) gain; no other common cytogenetic abnormalities in MM, such as deletion of 17p (17p-), deletion of 13q (13q-), and translocation of IgH (Figure 4), were observed.

In addition, CMA pinpointed various pathogenic CNVs in the CZ2 cell line (Table 1), including the CNVs in 1q21. Furthermore, the CMA also confirmed that there were no other common cytogenetic abnormalities of MM, such as 17p-, 13q-, and translocation of IgH was present in the CZ2 cell line.

Molecular characterization

Considering that the MM cell line CZ2 exhibited 1q21+ as the only cytogenetic abnormality, we focused on the 1q21 region of the cells. There are numerous MM-associated genes located in the 1q21 chromosomal region, among which CKS1B is the most widely studied, and is a promising immunotherapeutic target of MM. Through western blotting analysis, we found that the CKS1B protein expression in CZ2 cells was significantly higher than that in peripheral blood mononuclear cells (PBMCs) of normal people (Figure 5). Meanwhile, compared with the other MM cell line RPMI 8226, CZ2 cells expressed higher levels of the CKS1B protein (Figure 5).

To explore the role of CKS1B in MM cells, we knocked

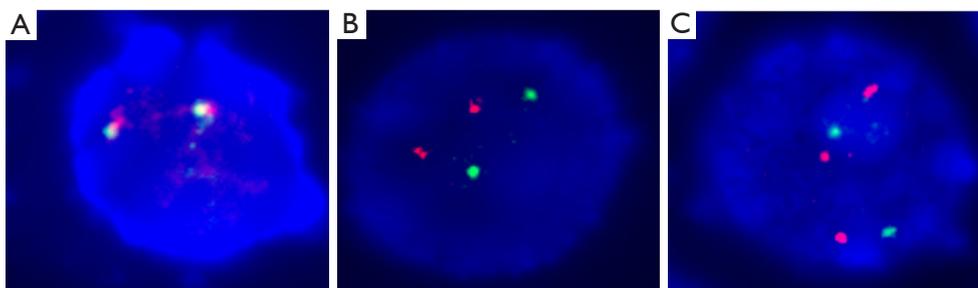


Figure 4 Results of the CZ2 cells detected by FISH using the IgH, P53, D13S319, RB1, and 1q21 probes. (A) Cells in this image showed that IgH was normal (two yellow signals). (B) This image shows the normal P53 and D13S319 (two green and two red signals). (C) This image shows the normal RB1 and abnormal 1q21 (two green and three red signals) (original magnification: 10×100). FISH, fluorescence in situ hybridization; IgH, immunoglobulin heavy chain.

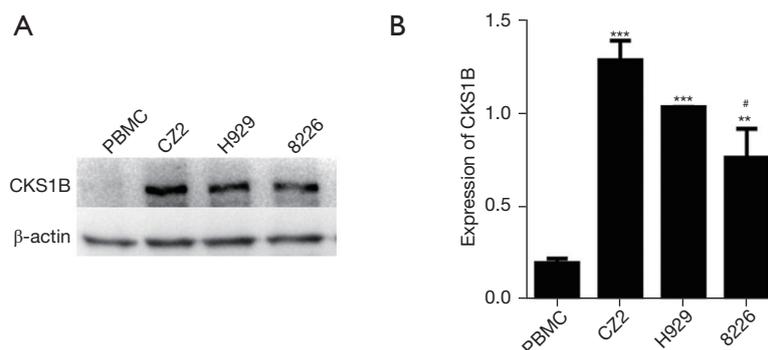


Figure 5 CKS1B is highly expressed in MM. (A) Representative immunoblot analysis of CKS1B. β -actin was used as the internal standard and control for protein loading. (B) Summarized data of the respective protein expressions (n=3 per group). Data are shown as the mean \pm SEM. ***, $P < 0.001$, **, $P < 0.01$, vs. PBMC; #, $P < 0.05$ vs. CZ2. CKS1B, CDC28 protein kinase regulatory subunit 1B; PBMC, peripheral blood mononuclear cell; MM, multiple myeloma; SEM, standard error of the mean.

down the expression of CKS1B by siRNA (Table S1) technology in the CZ2, H929, and RPMI 8226 cell lines (Figure 6A). The MTT assay results revealed that cell viability was significantly impaired in RPMI 8226, H929, and CZ2 after 48 h of CKS1B siRNA interference, and in H929 and CZ2 after 72 h of CKS1B siRNA interference, indicating that CKS1B is vital for MM cell survival (Figure 6B).

To investigate the role of CKS1B in cell apoptosis, we examined the expressions of cleaved-PARP and cleaved-caspase3. Our results showed that the expression of cleaved-caspase3 and cleaved-PARP significantly increased in the CKS1B-knockdown CZ2, H929, and RPMI 8226 cell lines (n=3) ($P < 0.05$), suggesting that the overexpression of CKS1B reduced cell apoptosis (Figure 6C).

Discussion

In this study, we established a unique myeloma cell line, CZ2, which possesses the genetic abnormality of 1q21 gains only, from the pleural effusion of a patient with MM. The CZ2 cells were found to be non-immunoglobulin-secreting cells. Meanwhile, through western blotting, we also observed that CKS1B, an adverse prognostic gene that is located in the 1q21 region, was highly expressed in CZ2 (Figure 5). Knockdown of CKS1B reduced cell viability and increased the levels of cleaved-PARP and cleaved-caspase3 (Figure 6). It has been reported that several MM cell lines are eventually transformed into original lymphoid cell lines due to EBV infection (16,17). Using PCR, we confirmed

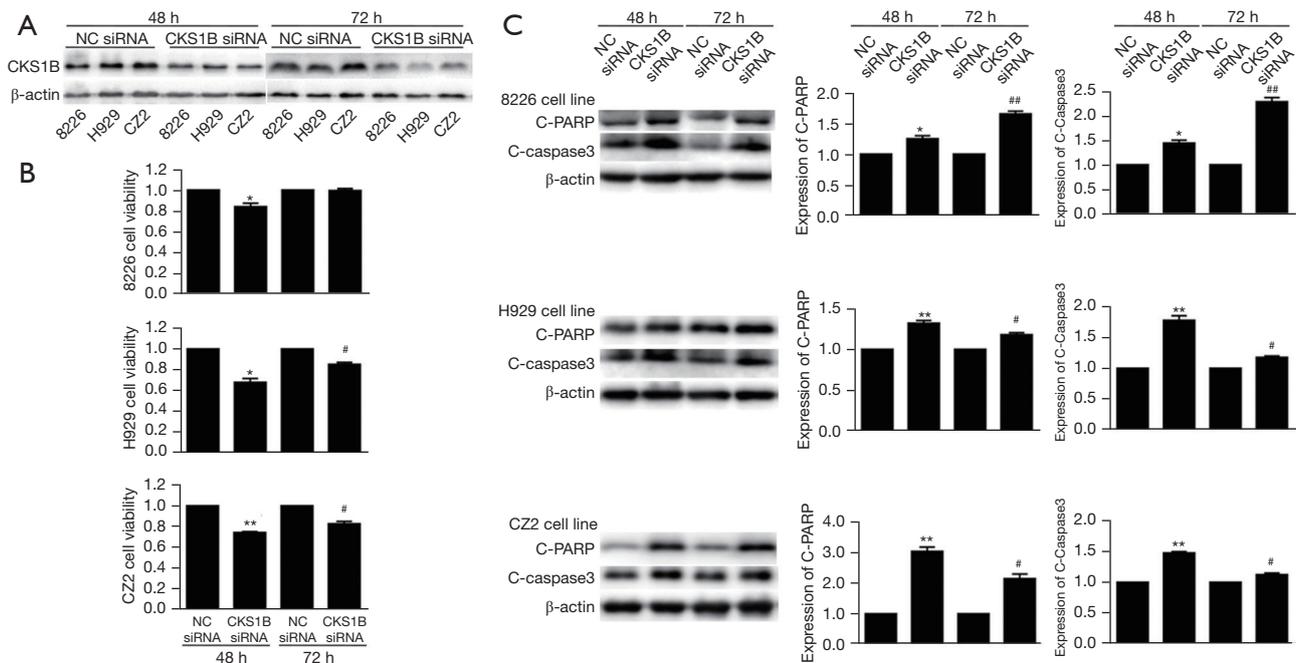


Figure 6 MM cell viability and apoptosis after CKS1B-knockdown. (A) The CKS1B protein expression in 8226, H929, and CZ2 cells decreased after 48 and 72 h of CKS1B siRNA transfection compared to the NC siRNA. (B) The cell viability of the 8226, H929, and CZ2 cell lines was impaired after CKS1B-knockdown, as measured by MTT (n=3 per group). (C) The expression of C-PARP and C-Caspase3 in 8226, H929, and CZ2 cells were increased after 48 and 72 h of CKS1B siRNA transfection compared to the NC siRNA (n=3 per group). Data are shown as the mean \pm SEM. **, P<0.01, *, P<0.05 vs. the 48 h-transfected NC siRNA; ##, #, P<0.01, #, P<0.05 vs. the 72 h-transfected NC siRNA. CKS1B, CDC28 protein kinase regulatory subunit 1B; NC, negative control; siRNA, small interfering RNA; C-PARP, cleaved-PARP; C-caspase3, cleaved-caspase3; MM, multiple myeloma; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; SEM, standard error of the mean.

that CZ2 are EBV-negative cells. Drexler *et al.* proved that the typical MM cells highly express CD138, CD38, CD28, and CD58, and negatively express CD10 and CD19 (16). However, we observed that CZ2 did not express CD38.

Cell lines represent a convenient and readily available model to explore disease mechanisms, especially tumors. Many advanced understandings of MM and other malignant hematopathies have been achieved using immortal hematopathic cell lines. At present, more than 100 MM cell lines have been established (18). Nonetheless, various characteristics of the cell lines have not been probed clearly due to technological limitations at the time, especially cytogenetic and molecular characteristics. Moreover, cytogenetic analysis results have shown that numerous MM cell lines exhibit complicated cytogenetic abnormalities. In contrast, our CZ2 cell line not only has detailed cytogenetic results detected by karyotyping and FISH, but also only exhibits 1q21 gains, without other common MM genetic

abnormalities. Therefore, CZ2 is an ideal model to study the underlying mechanism of 1q21 gains in MM.

Immunotherapy is thought to be a promising treatment for tumors including MM (19,20). Exploring a suitable tumor-specific antigen, which is expressed in tumors but not in other normal tissues, or a tumor-associated antigen, which is overexpressed in tumors but poorly expressed in normal cells, is crucial in active immunotherapy. Notably, *CKS1B*, *MCL1*, *IL6R*, *BCL9*, *ILF2*, as well as several MM-associated antigens are located in the 1q21 chromosomal region. In this study, we found that CKS1B was overexpressed in the CZ2 and other MM cell lines (Figure 5); in addition, CKS1B-knockdown decreased cell viability (Figure 6).

The role of CKS1B in MM has been explored for a long time. Bock *et al.* reported that the median progression-free survival (PFS) of the CKS1B and the control groups were 15.0 and 33.0 months (P=0.002), respectively (21).

Moreover, CKS1B was thought to be an adverse prognostic factor in refractory or recurrent MM (13). Zhan *et al.* and Huang *et al.* found that the overexpression of CKS1B in MM could increase the degradation of cyclin-dependent kinase inhibitors, such as P27 and P21, concluding that CKS1B induced the growth and drug resistance of MM (22,23).

Conclusions

In summary, we established CZ2, which is a novel human MM cell line with only 1q21 gains, providing a characteristic model for pathological and therapeutic research of MM. The unique chromosomal abnormalities of CZ2 allow for this cell line to be utilized to better understand the genetic and molecular mechanisms of 1q21 gains in MM.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at <https://atm.amegroups.com/article/view/10.21037/atm-22-5741/rc>

Data Sharing Statement: Available at <https://atm.amegroups.com/article/view/10.21037/atm-22-5741/dss>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://atm.amegroups.com/article/view/10.21037/atm-22-5741/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). The study was approved by the Ethics Committee of Changzheng Hospital (No. AF-HEC-055) and informed consent was taken from the patient.

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