

Expression of vaccinia-related kinase 1 (VRK1) accelerates cell proliferation but overcomes cell adhesion mediated drug resistance (CAM-DR) in multiple myeloma

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Objective: Vaccinia-related kinase 1 (VRK1) has been reported to participate in the development of a variety of tumors. However, the role of VRK1 in multiple myeloma (MM) has not been investigated. The present study was undertaken to determine the expression and biologic function of VRK1 in human MM.

Methods: First, we constructed a model of cell adhesion in MM, the mRNA and protein level of VRK1 in suspension and adhesion model was analyzed by RT-PCR and western blot. Then, flow cytometry assay and western blot were used to investigate the mechanism of VRK1 in the proliferation of MM cells. *In vitro*, following using shRNA interfering VRK1 expression, we performed adhesion assay and cell viability assay to determine the effect of VRK1 on adhesive rate and drug sensitivity.

Results: VRK1 was lowly expressed in adherent MM cells and highly expressed in suspended cells. In addition, VRK1 was positively correlated with the proliferation of MM cells by regulating the expression of cell cycle-related protein, such as cyclinD1, CDK2 and p27^{kip1}. Furthermore, VRK1 could reverse cell adhesion mediated drug resistance (CAM-DR) by down-regulating the ability of cell adhesion.

Conclusion and discussion: Our data supports a role for VRK1 in MM cell proliferation, adhesion, and drug resistance, and it may pave the way for a novel therapeutic approach for CAM-DR in MM.

Keywords: VRK1, Proliferation, Cell adhesion mediated drug resistance, Mitoxantrone, Multiple myeloma

Introduction

Multiple myeloma (MM) is one of the most common hematological malignancies, the majority of which remain incurable. Despite original responses to chemotherapy, MM patients finally develop drug resistance and become unresponsive to a wide spectrum of antineoplastic agents, a phenomenon called multidrug resistance (MDR).¹ Accumulating evidence reveals that the bone marrow (BM) microenvironment which consists of hematopoietic cells, stromal cells, and extracellular matrix (ECM) confers resistance to chemotherapy.²⁻⁴ Previous researches indicated that the direct adhesive interactions of myeloma cells with BM stromal cells and ECM components could transduce into the anti-apoptotic and cell cycle arrest

signals and ultimately lead to MDR.⁵⁻⁷ This protection phenomenon is termed as 'cell adhesion mediated drug resistance' (CAM-DR). It has been previously reported that adhesion of myeloma cells to fibronectin (FN) via beta1 integrin is associated with CAM-DR, which may induce perturbation in cell cycle progression and protect cells from apoptosis via increasing p27^{kip1} protein expression.⁸ Similar phenomenon also appears in lymphomas cells.⁹ In conclusion, these researches suggest that p27^{kip1} can induce a cell cycle arrest through BM ECM and plays an important role in cell adhesion mediated regulation of cell cycle progression. However, the underlying molecular mechanisms involved in regulating adhesion mediated MM cell cycle progression need to be further investigated.

Vaccinia-related kinase 1 (VRK1) is a mitotic kinase that exerts a vital role in cell cycle progression through participating in a variety of cell division process.^{10,11} It

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has been reported that VRK1, as a nucleosomal, or chromatin, kinase could directly and stably interact with a variety of chromatin proteins such as histone H3,¹² macroH2A1.2,¹³ and HP1.¹² Beyond that, VRK1 also interacts and phosphorylates some transcription factors such as c-Jun,¹⁴ ATF2,¹⁵ CREB,¹⁶ and p53,^{17–19} all of them participated in oncogenesis. VRK1 is required for exit G0 cell cycle phase and entry in G1,²⁰ acting as an early gene like MYC and FOS, but subsequently in mitosis is also required for chromatin condensation by phosphorylation of histone H3,^{12,21} nuclear envelope organization,²² and Golgi fragmentation.²³ Besides, VRK1 has been involved in responses to DNA damage induced by UV-light²⁴ and by ionizing radiation.²⁵ VRK1 shows abundant expression in proliferating tissues, for example fetal liver, testis, and thymus and in several cancer cell lines.^{11,26} However, no one has reported what the relationship is between VRK1 and cell cycle progression in MM.

In this report, we demonstrated for the first time the role of VRK1 in the proliferation and CAM-DR of MM cells. We found that adhesion of MM cells to ECM or HS-5 cells decreased VRK1 protein level. The decline of VRK1 was associated with significant arrest of cell growth through regulating the expression of cell cycle-related protein, which is indicative that VRK1 was responsible for promoting cell proliferation in MM. In addition, down-regulation of VRK1 expression resulted in induced CAM-DR phenotype, which was accomplished by enhancing the ability of cell adhesion. These data highlights the critical relationship between VRK1 and MM cells proliferation, adhesion, and drug resistance. These data also suggests that agents that promote the expression of VRK1 could be therapeutically useful to prevent MM progression.

Materials and methods

Cell cultures

The human MM cell lines RPMI 8226, U266, and BM stromal cell line HS-5 were gained from Cell Library, China Academy of Science. The cell lines RPMI8226, U266 were cultured in RPMI 1640 Medium (GibCo BRL, Grand Island, NY) while the HS-5 cells were maintained in F12 Medium (GibCo BRL, Grand Island, NY) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (GibCo BRL, Grand Island, NY) at 37°C and 5% CO₂.

Western blot analysis and antibodies

Western blot was performed according to methods described previously.^{27,28} The antibodies used in this research included: anti-VRK1 (anti-mouse, monoclonal, sc-271061, 1:500, Santa Cruz Biotechnology),

anti-p27^{kip1} (anti-mouse, monoclonal, sc-1641, 1:500, Santa Cruz Biotechnology), anti-CDK2 (anti-rabbit, polyclonal, sc-163, 1:500, Santa Cruz Biotechnology), anti-cyclinD1 (anti-rabbit, polyclonal, sc-753, 1:500, Santa Cruz Biotechnology), and anti-GAPDH (anti-rabbit, polyclonal, G9545, 1:1000, Sigma).

Transient transfection

The full-length VRK1 was constructed using the human cDNA library and cloned into cDNAp3.1-HA (Invitrogen, Carlsbad, CA), generating the HA-VRK1 vector. The VRK1 shRNA and control shRNA were designed and synthesized by GenechemCo (Genechem Co. Ltd, Shanghai, China). The shRNA targeting VRK1 sequence was 5'-ATAATAACTGACATGGCAA-3'. Transfection experiments were performed using lipofectamine 2000 according to the manufacturer's instructions. Six hours following transfection, cells were subsequently resuspended in normal medium culture at a density of 10⁶/ml and prepared for the next experiments.

RT-PCR

Total RNA was isolated from the indicated MM cells using TRIzol (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany). RT-PCR was performed using a PCR Master Mix (Promega) and an appropriate pair of primers. The RT-PCR sequences of the specific primers pairs for VRK1: sense, 5'-GAGGCCATACAGACCCGTTCC-3'; antisense, 5'-TCCACCTCGCAAGACTCACA-3'. GAPDH was used as an internal control and was detected using the following primers: sense, 5'-TGATGACATCAAGAAGGTGGTGAAG-3'; antisense, 5'-TCCTTGGAGGCCATGTGGGCCAT-3'.

Cell cycle analysis

To analyze cell cycle, the MM cells were fixed in 70% ethanol for at least 24 hours at 4°C after harvested and then incubated with 1 mg/ml RNase A for 30 minutes at 37°C. Subsequently, cells were stained with propidium iodide (50 mg/ml PI) (Becton–Dickinson, SanJose, CA, USA) in phosphate-buffered saline (PBS), 0.5% Tween-20, and analyzed using a Becton–Dickinson flow cytometer BD FACScan (Becton–Dickinson).

Cell viability assay

To evaluate the effect of transfection of VRK1-shRNA, the MM cells were seeded on a 96-well cell culture cluster at a density of 5 × 10⁴/well in a volume of 100 µl and grew overnight. Cell Counting Kit (CCK)-8 reagents (Dojindo, Kumamoto, Japan)

were added to the different subset wells at due time and then incubated at 37°C and 5% CO₂ for an additional 1 hour. In the end, the absorbance was quantified at a test wavelength of 490 nm using an automated plate reader.

Adhesion assays and detection of adhesion rate

Adhesion of MM cell lines to soluble FN was achieved as previously described.¹ In co-culture experiments, HS-5 stromal cells were seeded first of all to different well of a 96-well plate and incubated overnight at 37°C and 5% CO₂. The following day, stromal cells were washed once with serum-free medium, and MM cell lines were added to HS-5 cells to adhere for 2 hours in serum-free RPMI1640. Then non-adhered cells were removed and RPMI 1640 supplemented with 10% fetal bovine serum was added for an additional 24 hours. In order to detect the adhesion rate, MM cells were incubated with 5µM of Calcein-blueAM (Santa Cruz Biotechnology) for 30 minutes, washed and incubated for 45 minutes to allow unbound dye to diffuse out of the cells. Labeled cells were allowed to adhere for 2 hours and non-adherent cells were removed with three washes in PBS. The absorbance was quantified by a 96-well plate reader at 490 nm wave length.

Drug cytotoxicity assay

For drug cytotoxicity assays, MM cells were washed once after transfection and adhered to FN or stromal cells as previously described. After 24 hours, control-supplemented media or chemotherapy agents were added and incubated for an additional 48 hours, then MM cells were resuspended and seeded in new 96-well plates. The survival cells were assessed by CCK-8 assay.

Cell apoptosis assay

Flow cytometry assay was performed to measure the degree of apoptosis using an ApoScreen AnnexinV kits (Southern Biotechnology, Birmingham, AL). After washing with cold binding buffer, MM cells were stained with Annexin V and propidium iodide. Then, the number of stained cells was analyzed though FCM (BD FACS AriaII).

Statistical analysis

Each experiment consisted of at least three replicates per condition. All values were expressed as mean ± SEM. One-way ANOVA followed by Tukey *post hoc* multiple-comparisons tests was used for statistical analysis. $P < 0.05$ was considered statistically significant.

Results

The expression of VRK1 is decreased in adherent MM cell lines

A hypothesis was proposed that VRK1 might be essential during the interaction of MM cells with ECM and therefore could have an influence on tumor progression and survival. In order to verify the role of VRK1, we first built a model of cell adhesion using stromal cell HS-5 or FN, then RT-PCR (Fig. 1A and B) and western blot analysis (Fig. 1C and D) was performed to investigate whether FN or HS-5 mediated adhesion had an effect on VRK1 expression in MM cells, including RPMI 8226 and U266 cells. We found that VRK1 protein and mRNA level were both obviously diminished in adherent cells compared with cells grown in suspension, which suggested for the first time that the existence of an adhesion dependent regulation of VRK1 expression.

VRK1, associated with the proliferation of MM cells

Previous studies have demonstrated that cell adhesion could induce a cell growth arrest.^{9,29} Here, to investigate whether the expression of VRK1 was associated with cell cycle in MM cells, RPMI 8226 cells were cultured in serum-free conditioned medium for 72 hours and then recovered serum refeeding. We observed that cells were arrested in the G1 phase by serum deprivation. After serum re-addition, cells were released from the G1 phase and re-entered the S phase (Fig. 2A). Upon serum addition and releasing from G1, Western blot assay was performed to analyze the expression of VRK1 and p27^{kip1} (Fig. 2B and C). As expected, VRK1 content was gradually increased with an accordant down-regulation of cyclin-dependent kinases (CDK) inhibitor p27^{kip1}, a major regulator of G1-S transition in the cell cycle progression after serum stimulation. Therefore, VRK1 might be involved in the proliferation of MM cells.

Effect of meddling in expression of VRK1 on the cell cycle progression in MM

To booster the claim above, we firstly meddled with VRK1 in the MM cell lines and tested the expression of VRK1 after intervention by Western blot analysis and RT-PCR (Fig. 3A and B). Then, HA-VRK1 was transfected into MM cells, which significantly up-regulated the expression of VRK1 in cells, confirmed by Western blot analysis (Fig. 3C). After that, FACS assay was performed to investigate the percentage of cells with different expression level of VRK1 in the G1 phase when RPMI 8226 cells were subjected to serum starvation and serum refeeding. We observed that the percentage of cells with

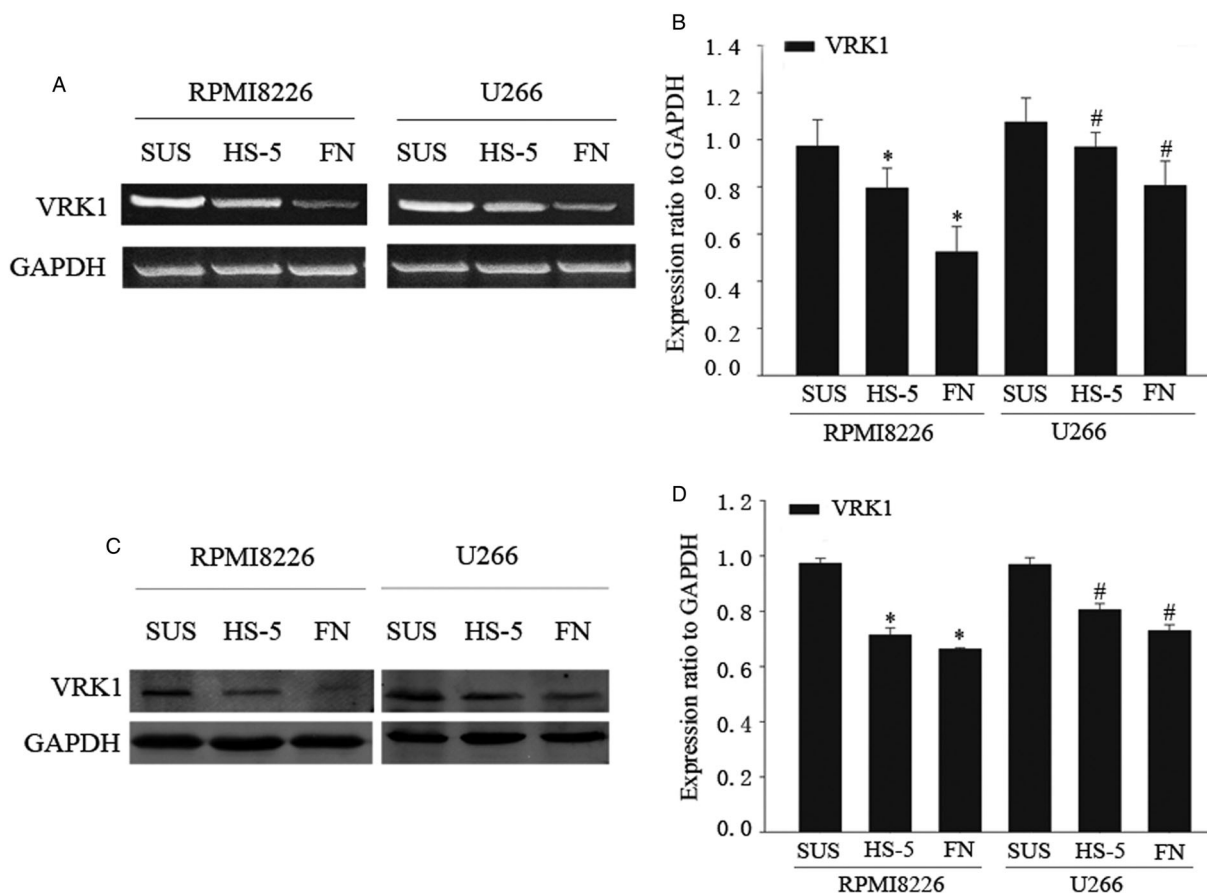


Figure 1 The expression of VRK1 in cell adhesion model. (A, B) The mRNA levels of VRK1 in RPMI 8226 and U266 cells when adhere to HS-5 cells and FN for 48 hours or cultured in suspension were measured by real-time PCR as described in 'Materials and methods'. GAPDH was measured as control for sample integrity and equal loading. Integrated band densities were obtained by densitometric scanning. The data are mean \pm SEM, (*, # $P < 0.05$ compared with suspension). (C, D) Western blot analysis of expression of VRK1 in a cell adhesion model of RPMI 8226 and U266 cells adhered to HS-5 cells or FN. Cells in suspension were used as negative control. The bar chart demonstrated the ratio of VRK1 to GAPDH for the above by densitometry. Data are mean \pm SEM (*, # $P < 0.05$ compared with suspension).

HA-VRK1 in the G1 phase was decreased obviously. Meanwhile depleted VRK1 increased the percentage (Fig. 3D). All these results indicated that VRK1 is an important factor in proliferation of MM cells.

Inhibition of VRK1 silencing on proliferation in MM cells by up-regulating p27^{kip1} expression

To further investigate the potential effect of VRK1 on the proliferation of MM cells, CCK-8 assay was used to measure cell viability of VRK1 knocked down cells (Fig. 4A and B). It showed that knockdown of VRK1 resulted in an obvious inhibition of cell growth rate in MM cells RPMI 8226 and U266. To explore the mechanism of the decreased cell growth affected by VRK1 shRNA, cell cycle distributions of cells transfected with VRK1 shRNA or control shRNA were determined by FACS. The percentage of cells in the S phase of VRK1 knocked down cells was obviously decreased as compared with that of control shRNA cells while G1 phase was just the opposite (Fig. 4C and D), suggesting that VRK1 might be able to promote the G0/G1-S transition and thus the cell growth. After that, we examined

the levels of expression of several cell cycle-related molecular. In consistent with the previous serum starvation and release experiments, knockdown of VRK1 resulted in inversely increase of p27^{kip1}, but was correlated with decreased expression of CDK2 and CyclinD1 (Fig. 4E and F). In a word, these data suggested that the expression of VRK1 might associate with the expression of cell cycle regulator and promote the G1/S transition, which might be responsible for the MM cells proliferation.

VRK1 affect the adhesive capacity of MM cell to ECM

Previous studies indicated that adhesion of hematologic malignancies cells to FN leads to a reversible cell cycle arrest, which in turn promotes CAM-DR.^{30,31} Hence, it is necessary for us to elucidate the correlation between cell adhesion and the expression of VRK1. We discovered that the cell adhesion rate significantly increased when FN or HS-5 cells existed (Fig. 5A), and overexpression of VRK1 causes the decline of the cell adhesion rate in MM cells RPMI 8226 and U266 (Fig. 5B and C). Inversely,

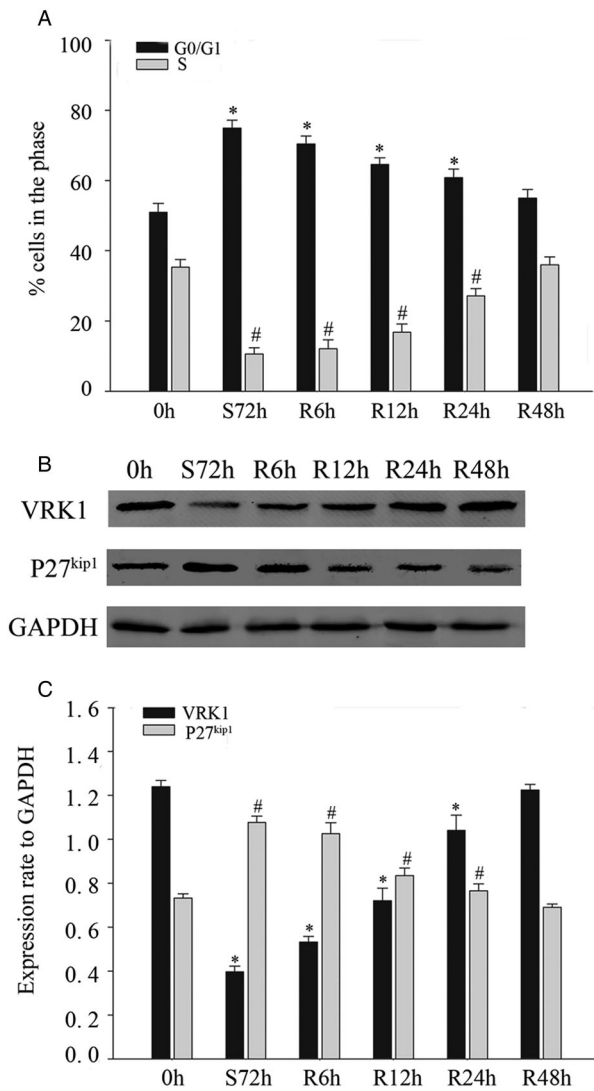


Figure 2 The expression of VRK1 and p27^{kip1} in proliferating RPMI 8226 cells. (A) Flow cytometry was used to analyze the cell cycle progression of RPMI 8226 cells that were subjected to serum starvation (S) for 72 hours and refeeding (R) for 0, 6, 12, 24, and 48 hours. (B) Western blot images showed that the expression of VRK1 and p27^{kip1} in RPMI 8226 cells that were subjected to serum starvation (S) for 72 hours and refeeding (R) for 0, 6, 12, 24, and 48 hours. GAPDH was used as a control for protein load and integrity. (C) A bar chart demonstrated the relative protein expression of VRK1 and p27^{kip1} in RPMI 8226 cells at different time points, as measured by Western blot analysis. Data are presented as mean \pm SEM of three independent measurements. (*, # $P < 0.05$ versus control).

knockdown of VRK1 further promoted the increased cell adhesion in MM cells RPMI 8226 and U266 (Fig. 5D and E). Taken together these data suggested that VRK1 expression might negatively correlated with the cell adhesion. It was an important reason for VRK1 to affect the CAM-DR in MM.

VRK1 acts as a negative factor of CAM-DR in MM Accumulating evidence has proved that the cell adhesion to ECM or stromal cells is a main reason which leads to drug resistance in MM probably

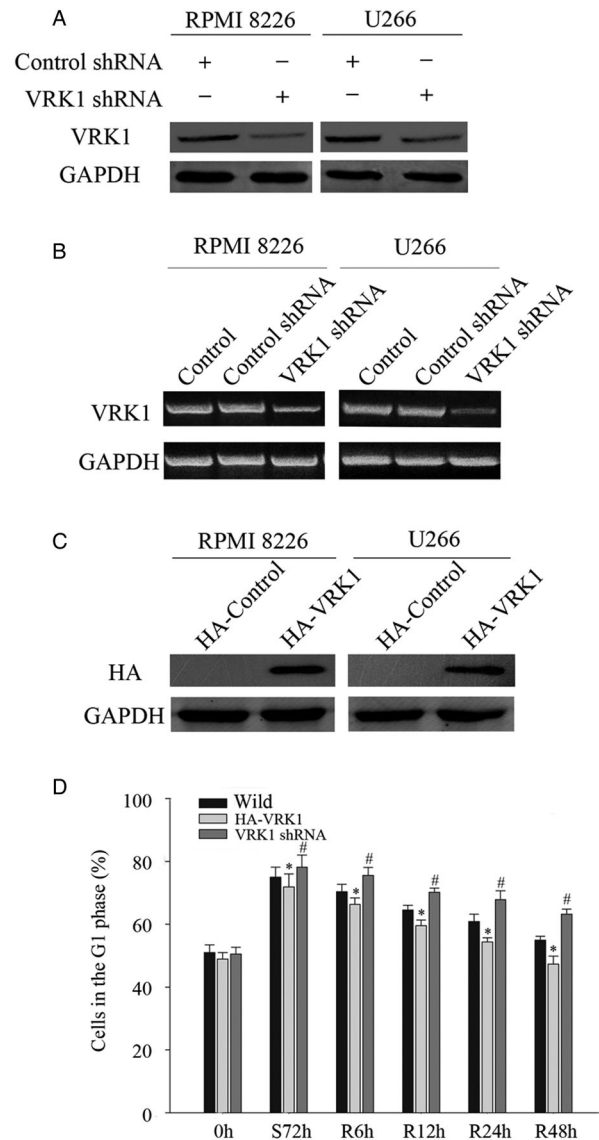


Figure 3 Meddling in expression of VRK1 has an effect on the cell cycle progression of MM cells. (A) MM cells were transfected with either VRK1-shRNA or a scrambled sequence (control shRNA) as mentioned in 'Materials and Methods'. (B) Representative PCR images showed that the mRNA expression of VRK1 in MM cells was decreased by treatment of VRK1-shRNA. (C) A representative Western blot image showed that the VRK1 expression in MM cells was increased by treatment of HA-VRK1. (D) A bar chart demonstrated the percentage of cells with different expression level of VRK1 in the G1 phase when RPMI 8226 cells at different time points, which measured by Flow cytometry (*, # $P < 0.05$ versus control).

through inducing the cell cycle arrest.^{6,32,33} Consistent with existing studies, the sensitivity of adherent RPMI 8226 and U266 cells to mitoxantrone in different concentrations is lower than that of cells in suspension (Fig. 6A and B). Meanwhile, we discovered that the cytotoxic effect was the largest with 2 μ M mitoxantrone, so the concentration of 2 μ M would be applied to the following experiments. In this study, we have demonstrated that adhesion of MM cells to FN or HS-5 cells attenuated VRK1

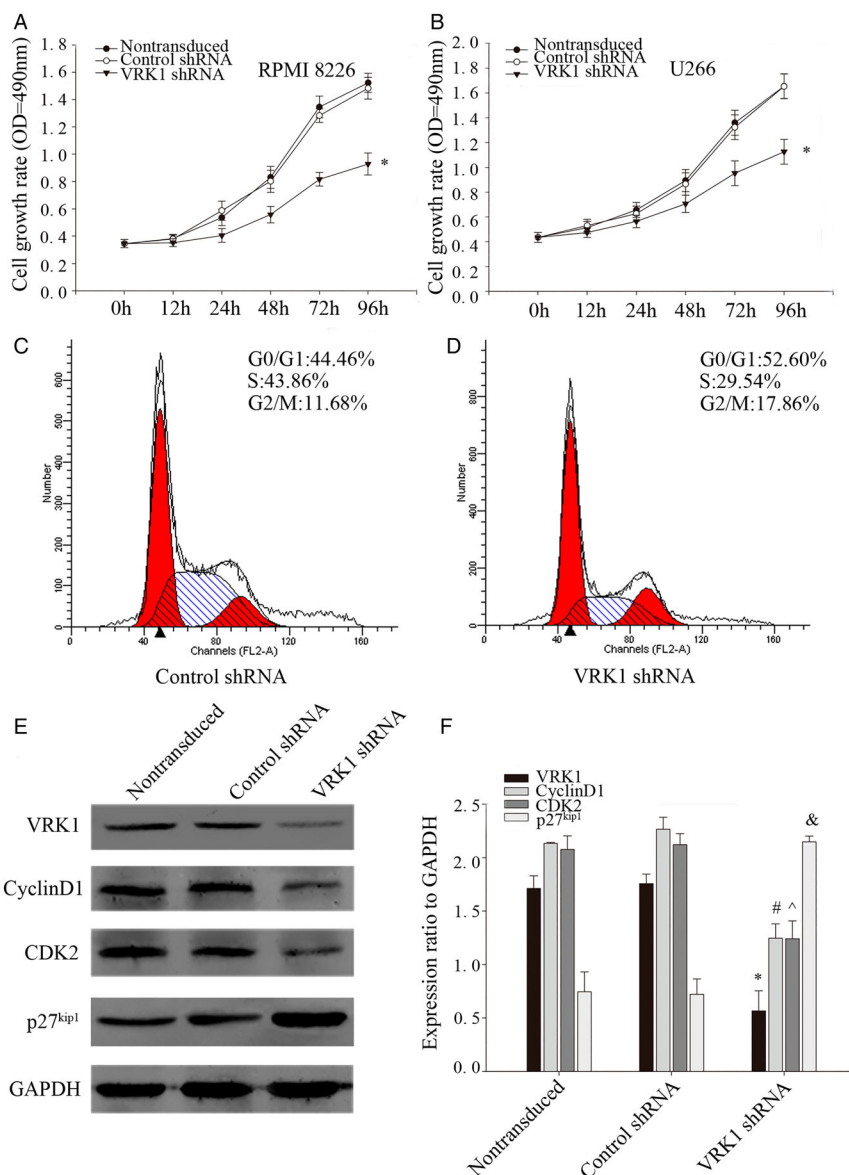


Figure 4 VRK1 knockdown inhibited the proliferation of MM cells. (A, B) Cell growth rate was examined by CCK-8 assay at the indicated time. Data show mean \pm SEM of triplicates from one experiment representative of three experiments performed (* $P < 0.05$ compared with control or cells transfected with control shRNA). (C, D) RPMI 8226 cells transfected with VRK1-shRNA or control shRNA were stained with PI for DNA content analysis by FACS. (E) A representative Western blot image showed the expression of VRK1, cyclinD1, CDK2, and p27^{kip1} in Control shRNA and VRK1-shRNA treated cells, respectively. GAPDH was used as internal control. (F) The bar chart demonstrates the ratio of VRK1, cyclinD1, CDK2, and p27^{kip1} protein to GAPDH for the above by densitometry. The data are mean \pm SEM of three independent experiments (*, #, & $P < 0.05$ compared with the control group).

expression and the decline of VRK1 level exhibited perturbations in cell cycle progression and enhanced the capacity of adhesion. Therefore, the exact role that VRK1 plays in regard to drug resistance requires further investigation. As expected, the cell viability was inhibited after transfecting with HA-VRK1 and promoted after interfering with shRNA VRK1 in adherent MM cells (Fig. 6C and D). Besides, flow cytometry also showed that overexpression of VRK1 yielded a drastic increase of cell death, while suppression of VRK1 resulted in significant decrease of cell death following an addition of 2 μ M mitoxantrone in adherent MM cells (Fig. 6E). In a word,

these data supported a role of down-regulation of VRK1 in conferring drug resistant through cell-adhesion mechanisms.

Discussion

MM is a potentially lethal form of plasma cell malignancy owing to MDR. Recent studies have verified that the BM microenvironment which provides a niche that promotes MM cells survival must be considered when evaluating drug response.³⁴ Specially, MM cells adhered to FN or stromal cells conferred a multidrug resistant phenotype, known as CAM-DR, owing to the cell cycle arrest.⁸ Therefore, a hypothesis was

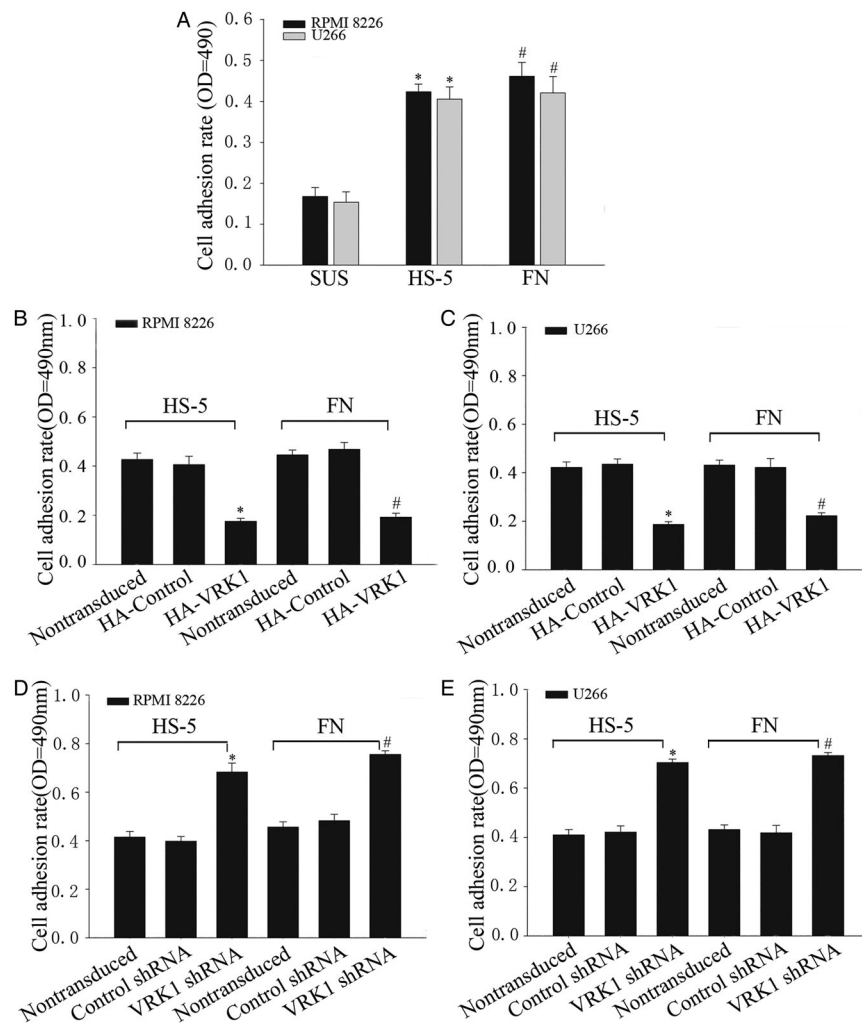


Figure 5 The expression of VRK1 was associated with the adhesion ability. (A) RPMI 8226 and U266 cells were adhered to HS-5 cells or FN for 4 hours and the cell adhesion rate was measured. (B, C) The cell adhesion rate of RPMI 8226 and U266 cells transfected with HA-control or HA-VRK1 was measured as described above. (D, E) RPMI 8226 and U266 cells transfected with shRNA targeting either VRK1 or scrambled sequence (control shRNA) as described above and the cell adhesion rate was measured. A representative of three independent experiments was shown (*, # $P < 0.05$ compared with the control group).

proposed that abnormal expression of cell cycle regulators might be conducted to CAM-DR, then we focused on identifying them as targets to overcome CAM-DR.

In the current study, we initially built a model of cell adhesion consisting of MM cell lines and stromal cell HS-5 or FN. Then, we started to investigate the molecular mechanisms and biological consequences of adherent MM cells. We determined for the first time that the level of VRK1 protein declined in MM cell lines RPMI 8226 and U266 when they were adhered to HS-5 cells or FN. It has been reported that VRK1 contributes to a poorer tumor prognosis which can be partly due to its role in proliferation. VRK1 is regulated in cell cycle and its impact is mediated by the contribution to the coordination of several processes required for cell division.¹⁰ However, the role of VRK1 in MM, especially in CAM-DR, has never been reported. Consequently, these findings motivated us to examine the function of VRK1 protein in CAM-DR of MM cells.

Our study has firstly demonstrated that VRK1 was low-expressed in adhered cells. The low-expression of VRK1 suggested a potential role of this protein in the MM pathophysiology. In this study we aimed to elucidate the role of VRK1 in CAM-DR by meddling in its expression using VRK1 shRNA and HA-VRK1 in RPMI 8226 and U266 cell lines. Amounting evidence showed that cell adhesion could induce cells growth arrest, therefore, VRK1 may be involved in cell cycle progression of MM. In order to verify the hypothesis, RPMI 8226 cells were subjected to serum starvation and serum refeeding. We discovered that the protein level of VRK1 increased along with the proliferation of RPMI 8226 cells. At the same time, CCK8 assay was used to evaluate cell proliferation and revealed a dramatic decrease in proliferation of cells transduced with VRK1 shRNA compared to control shRNA. Changes in cell proliferation are always associated with modulation in the cell cycle, so cell cycle was characterized by flow cytometry analysis to explore the mechanism underlying the action of VRK1

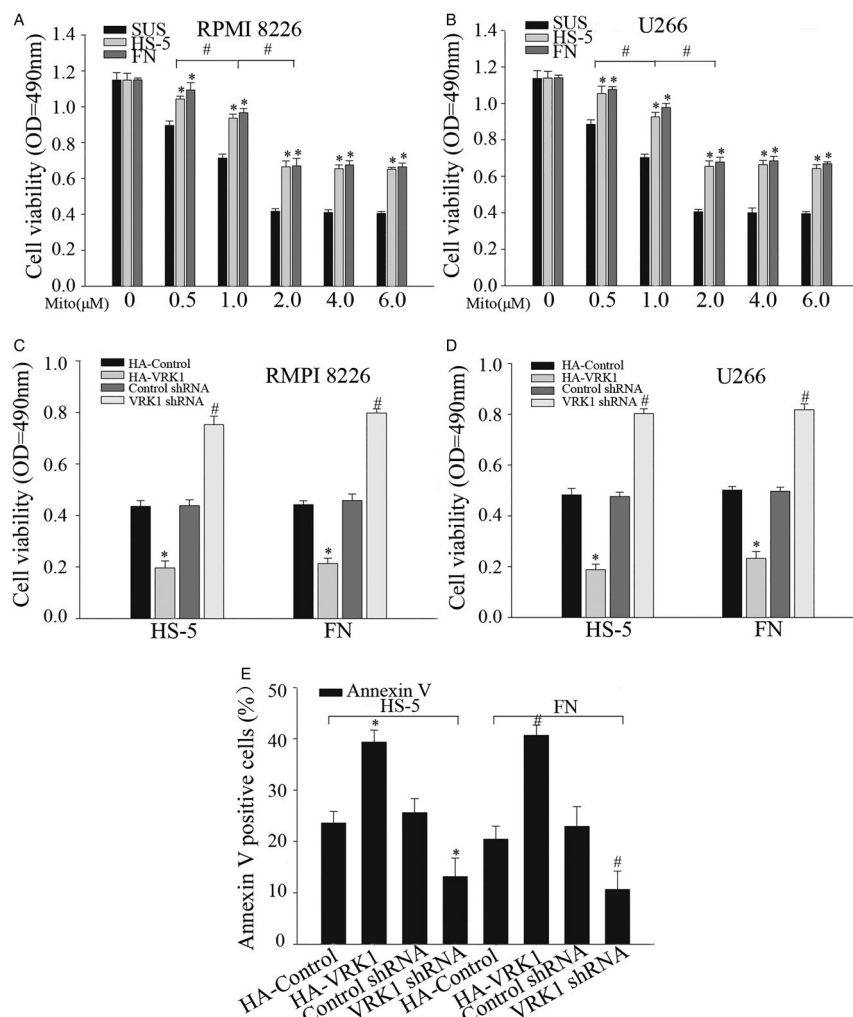


Figure 6 The role of VRK1 in CAM-DR of MM. (A, B) Cell viability of RPMI 8226 and U266 after incubated for 48 hours with mitoxantrone (* $P < 0.05$ compared with the group of 0 μM mitoxantrone, # $P < 0.05$ compared with the former group). (C, D) RPMI 8226 and U266 cells with VRK1 over-expressed or low-expressed were adhered to HS-5 cells or FN for 24 hours. Cell viability was analyzed after being treated with mitoxantrone for 48 hours as described above (*, # $P < 0.05$ compared with the control group). (E) Quantification of the results from the flow cytometry-based study showed that VRK1 positively regulates the number of Annexin V + cells after the drug stimulation (*, # $P < 0.05$ compared with the control group). Data were collected from three independent experiments.

expression in regulating cell proliferation, We observed significant decrease in S phase cells after transduced with VRK1 shRNA as compared to cells with control shRNA. There was a concomitant increase in G1 phase cells. These findings suggested that VRK1 played a role in cell cycle progression and probably the G1-S transition arrest was the reason for the anti-proliferative effect of VRK1 suppression in myeloma cells. Having observed the accumulation of cells in G1 phase in VRK1 silenced cells, we examined the levels of expression of several cell cycle-related molecules. We observed that expression of p27^{kip1} was up-regulated in VRK1 silenced cells and inversely correlated with CDK2 and cyclinD1 that play major roles in S phase. Nevertheless it should be noted that overexpression of p27^{kip1} has been reported to protect cells from apoptosis induced by chemotherapeutic drugs. Cell adhesion can mediate p27^{kip1} elevation and it is associated with cell cycle arrest.^{9,35,36} Our study, for the first time, demonstrated that the negative correlation

between VRK1 and p27^{kip1} expression might be the reason for cell cycle arrest and CAM-DR in MM cells.

Previously, our laboratory has discovered that adhesion of MM cells to FN can confer a form of drug resistance, and the adhesion status of MM cells may be of outstanding significance for their response to therapeutic drugs.^{30,31,37} In this research the relationship between VRK1 expression and adhesive ability of MM cells was investigated. We found that down-regulation of VRK1 induced cell adhesion, thus protected MM cells from mitoxantrone-induced apoptosis. Up-regulation of VRK1 was just the opposite. Hence, VRK1 appeared to have specific effects on CAM-DR which was similar to apoptotic proteins.

In conclusion, this study revealed that VRK1 exhibited decreased expression in the MM cell adhesion model and increased expression among proliferating cells. Down-regulation of VRK1 might decrease MM cell proliferation, but lead to induction of adhesion,

which in turn results in drug resistance. The relationship of these two characteristics of VRK1 should be taken into account in terms of MM treatment.

Acknowledgement

This work was supported by the National Natural Science Foundation of China Grants (grant number 81373223, 81172879, 81201858, 81372537) and the Natural Science Foundation of Jiangsu province Grant (grant number BK20131206, BK2012231); a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD)

Disclaimer statements

Contributors Jing Liu and Yuchan Wang are co-first authors of this article who were responsible for study design, data analysis, manuscript writing and revision. Song He, Xiaohong Xu, Yuejiao Huang, Jie Tang reviewed and revised the manuscript. Yaxun Wu, Xiaobing Miao, Yunhua He, Qiru Wang, Li Liang assisted in completing the statistical analysis. Chun Cheng is the guarantor.

Funding None.

Conflict of interest All the authors declare no conflict of interest.

Ethics approval Ethical approval was not required for this original research.

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