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Effects of PKM2 Knockdown on Proliferation and Apoptosis in Human Leukemia Cells and the Underlying Mechanisms: Postprint

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Date: 2018-11-25T00:00:00+00:00

Abstract

Objective: This study aimed to investigate the effects of the glycolytic enzyme pyruvate kinase M2 (PKM2) on the proliferation and apoptosis of human leukemia cells in vitro and the underlying mechanisms. Methods: Lentiviral vectors targeting PKM2 were transfected into human K562 cell lines (shPKM2 group), with an empty vector transfection group established as control (Vector group). qRT-PCR and Western blot were employed to detect PKM2 mRNA and protein expression and changes in autophagy markers in both groups; CCK-8 assay was used to assess in vitro cell proliferation capacity; flow cytometry was performed to analyze cell cycle and apoptosis; and Western blot was utilized to determine the expression levels of apoptosis-related proteins Bax and Bcl-2. Results: Following stable PKM2 interference, both PKM2 mRNA ($t=11.58$, $P=0.0003$) and protein levels ($t=11.88$, $P=0.0003$) were significantly reduced. Compared with the Vector group, the shPKM2 group exhibited significantly decreased in vitro proliferation capacity ($F=118.87$, $P<0.0001$). Additionally, PKM2 interference arrested the K562 cell cycle at the G1 phase and markedly increased the apoptosis rate ($t=37.23$, $P<0.0001$); it also increased pro-apoptotic protein Bax expression ($t=15.36$, $P=0.0001$) while decreasing anti-apoptotic protein Bcl-2 expression ($t=9.965$, $P=0.0006$). Moreover, PKM2 interference attenuated autophagy levels in K562 cells, as evidenced by reduced LC3II ($t_{LC3II}=10.32$, $P_{LC3II}=0.0005$) and elevated p62 levels ($t_{p62}=14.59$, $P_{p62}=0.0001$). Furthermore, autophagy inducers were found to reverse the shPKM2-induced reduction in leukemia cell proliferation capacity ($F=96.32$, $P<0.0001$). Conclusion: These findings demonstrate that PKM2 interference inhibits in vitro proliferation and promotes apoptosis of human leukemia K562 cells, potentially through a mechanism involving reduced PKM2-mediated autophagy activity, suggesting that PKM2 may represent a potential therapeutic target for leukemia diagnosis and treatment.

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Full Text

Effects of PKM2 Knockdown on Proliferation and Apoptosis of Human Leukemia Cells and Its Potential Mechanism

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Abstract

Objective: This study investigated the effects of the glycolytic enzyme pyruvate kinase M2 (PKM2) on the proliferation and apoptosis of human leukemia cells and explored the underlying mechanisms.

Methods: Lentiviral vectors targeting PKM2 were transfected into human K562 leukemia cells (shPKM2 group), with empty vector transfection serving as the control (Vector group). qRT-PCR and Western blot were used to detect PKM2 mRNA and protein expression levels in both groups. Cell proliferation was assessed using the CCK-8 assay, while cell cycle distribution and apoptosis rate were analyzed by flow cytometry. Apoptosis-related proteins Bax and Bcl-2 were measured by Western blot, and autophagy activity was evaluated by qRT-PCR and Western blot detection of autophagy markers.

Results: Stable knockdown of PKM2 significantly reduced both mRNA ($t=11.58$, $P=0.0003$) and protein ($t=11.88$, $P=0.0003$) expression levels in K562 cells. Compared with the Vector group, the shPKM2 group exhibited significantly inhibited cell proliferation ($F=118.87$, $P<0.0001$). Furthermore, PKM2 knockdown induced G1-phase cell cycle arrest and increased the apoptosis rate ($t=37.23$, $P<0.0001$). The shPKM2 group also showed upregulated pro-apoptotic Bax protein ($t=15.3$, $P=0.0001$) and downregulated anti-apoptotic Bcl-2 protein ($t=9.965$, $P=0.0006$) compared with the Vector group. Additionally, decreased PKM2 expression significantly downregulated LC3II levels (tLC3II=10.32, PLC3II=0.0005) and elevated p62 levels (tp62=14.59, Pp62=0.0001) in K562 cells. Finally, the autophagy activator rapamycin rescued the inhibitory effect on cell proliferation caused by PKM2 knockdown ($F=96.32$, $P<0.0001$).

Conclusion: These results indicate that PKM2 knockdown inhibits cell proliferation and promotes apoptosis in leukemia cells, at least partially through modulation of autophagy activity, suggesting that PKM2 may represent a potential therapeutic target for leukemia treatment.

Key words: PKM2; leukemia; proliferation; apoptosis; autophagy

Introduction

Leukemia is a malignant proliferative disorder of hematopoietic stem cells that seriously threatens human health, with high morbidity and mortality rates and a five-year survival rate below 50% [1]. Current clinical treatments primarily involve chemotherapy and hematopoietic stem cell transplantation, which show good efficacy in some patients but are associated with high rates of adverse reactions and relapse. Therefore, identifying molecular targets for leukemia therapy represents an effective approach to improving treatment outcomes. Glucose metabolism is a crucial energy-supplying pathway in cellular metabolism. Pyruvate kinase (PK), a key rate-limiting enzyme in glycolysis, catalyzes the conversion of phosphoenolpyruvate to pyruvate [2]. Pyruvate kinase isoenzyme M2 (PKM2) is an M2 isoform of PK [3]. Beyond its classical role in glycolysis, PKM2 has been confirmed to function as a tumor-promoting factor that regulates tumorigenesis and progression [4]. While the non-metabolic oncogenic mechanisms of PKM2 in leukemia cells remain unclear, the role of autophagy in leukemia has attracted increasing attention in recent years [5]. To investigate whether autophagy is involved in PKM2-mediated leukemia promotion, this study examined the effects of PKM2 knockdown on the proliferation and apoptosis of K562 leukemia cells in vitro, providing experimental evidence for understanding the role of PKM2 in leukemia development and for targeted therapy research.

Materials and Methods

1.1.1 Cell Line The human leukemia cell line K562 was purchased from the Cell Bank of the Chinese Academy of Sciences Shanghai Life Sciences Institute and routinely maintained at the Key Laboratory of Laboratory Medical Diagnostics designated by the Ministry of Education, Chongqing Medical University.

1.1.2 Reagents RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco (USA). Lentiviral transfection reagents, shRNA lentiviral vectors targeting PKM2, and empty control lentiviral vectors targeting irrelevant sequences were constructed by Shanghai GeneChem Co., Ltd. Penicillin, streptomycin, isopropanol, chloroform, loading buffer, skim milk, DEPC water, phosphate-buffered saline (PBS), RIPA lysis buffer, and BCA protein quantification kits were obtained from Beyotime Biotechnology (Shanghai, China). TRIzol reagent for RNA extraction and RT-PCR kits were purchased from TaKaRa (Japan). Primers were synthesized by Shanghai Invitrogen. SYBR Fast qPCR kits were from KAPA Biosystems. Rabbit anti-human PKM2, LC3, and p62 monoclonal antibodies were from Cell Signaling Technology (USA). Rabbit anti-human β-actin polyclonal antibody was from Proteintech (USA). Rabbit anti-human Bax and Bcl-2 polyclonal antibodies were from Santa Cruz (USA). Cor-

responding goat anti-rabbit IgG secondary antibodies were from Proteintech (USA). ECL reagent and PVDF membranes were from Millipore (USA). The autophagy inducer rapamycin was from Selleck (USA). CCK-8 kits were from Dojindo (Japan). Cell cycle and apoptosis detection kits were from Thermo Fisher (USA).

1.2.1 Cell Culture Human leukemia K562 cells were cultured routinely in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in a 5% CO_2 incubator. Cells were collected for subsequent experiments when they reached logarithmic growth phase.

1.2.2 Lentiviral Vector Construction and Transfection Cells in logarithmic growth phase were seeded in 6-well plates. When cell density reached 80%, lentiviral transfection was performed using polybrene according to the manufacturer' s instructions. After 48 hours of transfection, morphological changes were observed under an inverted optical microscope, and cells were collected for subsequent experiments. The lentiviral vector carrying PKM2-shRNA (PKM2-shRNA, 5' -CATCTACCACTTGCAATTA-3') and the empty control lentiviral vector targeting an irrelevant sequence were synthesized, packaged, and sequenced by Shanghai GeneChem Co., Ltd., with viral titers determined.

1.2.3 qRT-PCR Detection of mRNA Expression After 48 hours of culture, cells were transferred to pre-chilled 1.5 mL RNase-free EP tubes on ice. Cells were washed three times with PBS by centrifugation at 2000 r/min for 2 min each. Total RNA was extracted using TRIzol reagent. Chloroform (1/5 volume) was added, followed by centrifugation at 12,000 r/min for 15 min. The supernatant was collected, mixed with an equal volume of isopropanol, and centrifuged at 12,000 r/min for 10 min. After discarding the supernatant, the pellet was washed twice with 75% ethanol (12,000 r/min for 5 min each), air-dried at 37°C for 5 min, and dissolved in 10 L DEPC water. RNA concentration was measured and reverse-transcribed into cDNA using RT-PCR. β-actin served as the internal control for qRT-PCR amplification of target genes. Primer sequences are listed in .

Amplification conditions: 94°C for 5 min, followed by 39 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 50 s, with a final extension at 72°C for 10 min. Experiments were repeated independently three times. Relative quantification was calculated using the 2^(-ΔΔCt) method.

1.2.4 Western Blot Detection of Protein Expression Cells were collected and washed three times with ice-cold PBS (2000 r/min for 2 min each). Cell pellets were lysed in RIPA buffer on ice for 40 min, followed by centrifugation at 13,000 r/min for 40 min. Supernatants were collected, and protein concentrations were determined using the BCA assay. Protein samples (40 g) were separated on 12% SDS-PAGE gels (80 V for 30 min in stacking gel, 120 V

for 90 min in separating gel) and transferred to PVDF membranes using a wet transfer apparatus (210 mA for 1 h). Membranes were blocked with 5% skim milk at room temperature for 3 h, then incubated overnight at 4°C with primary antibodies against PKM2, Bax, Bcl-2, LC3, p62, and β-actin at appropriate dilutions. After washing twice with TBST and once with TBS (10 min each), membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Following the same washing procedure, target proteins were detected using ECL reagent and a Millipore chemiluminescence system. β-actin served as the loading control, and band intensities were quantified using Quantity One software.

1.2.5 CCK-8 Assay for Cell Proliferation Vector control, shPKM2, and autophagy inducer rapamycin (final concentration 5 M) treatment groups were seeded in 96-well plates at 1$×10^{3}$ cells per well (100 L cell suspension) with five replicate wells per group. At 0, 12, 24, 36, 48, and 60 h of culture, 10 L CCK-8 reagent was added to each well, followed by incubation at 37°C with 5% CO_2 for 3 h in the dark. Absorbance (OD) values at 450 nm were measured using a microplate reader to generate growth curves with culture time as the x-axis and OD450 values as the y-axis. Experiments were repeated independently three times.

1.2.6 Flow Cytometry Analysis of Cell Cycle Flow cytometry was used to detect cell cycle changes in K562 cells after PKM2 interference. shPKM2 and Vector group cells were collected, centrifuged at 1000 r/min for 5 min, washed twice with ice-cold PBS, and fixed overnight at -20°C in 75% ethanol while vortexing. After washing and centrifugation, cells were resuspended in ice-cold PBS. Each sample was treated with 190 L EDTA and 10 L RNase A (10 mg/mL) for 5 min at room temperature, followed by addition of PI staining solution and incubation at 4°C for 10 min in the dark before loading. Cell numbers in each cycle phase were detected, and results were analyzed using ModFit software to calculate phase distributions. Experiments were repeated independently three times.

1.2.7 Flow Cytometry Detection of Apoptosis Cells were collected, centrifuged at 1000 r/min for 5 min, washed three times with PBS, and resuspended in 500 L PBS. Annexin V-FITC/PI double staining was performed according to the apoptosis kit instructions. Annexin V-FITC(+)/PI(-) represents early apoptotic cells, Annexin V-FITC(+)/PI(+) represents late apoptotic cells, Annexin V-FITC(-)/PI(-) represents viable cells, and Annexin V-FITC(-)/PI(+) represents necrotic cells. The apoptosis rate was calculated as: (apoptotic cell number / total cell number) × 100%. Experiments were repeated independently three times.

1.2.8 Statistical Analysis SPSS 22.0 software was used for statistical analysis. All data represent three independent experiments and are expressed as

mean ± standard deviation ($\bar{x} \pm s$). Comparisons between two groups were performed using t-tests, while CCK-8 results were analyzed using two-way repeated measures ANOVA. The significance level was set at α=0.05, with P<0.05 considered statistically significant.

Results

2.1 PKM2 Expression in Leukemia K562 Cells After Interference qRT-PCR results showed that PKM2 mRNA levels were significantly reduced in the shPKM2 group compared with the Vector group (t=11.58, P=0.0003) [Figure 1a: see original paper]. Western blot results demonstrated that PKM2 protein band intensity was markedly decreased in the shPKM2 group [Figure 1b: see original paper], with significantly reduced relative protein expression (t=11.88, P=0.0003) [Figure 1c: see original paper]. These results confirm that lentiviral-mediated PKM2 interference successfully downregulated both mRNA and protein levels of PKM2 in K562 cells, establishing a stable cell line with PKM2 knockdown.

2.2 Effect of PKM2 Knockdown on K562 Cell Proliferation In Vitro CCK-8 assays revealed that OD450 values were significantly lower in the shPKM2 group compared with the Vector group at 12, 24, 36, 48, and 60 h of culture (F=118.87, P<0.0001) [Figure 2: see original paper], indicating that PKM2 knockdown inhibited the in vitro proliferation capacity of K562 cells.

2.3 Effect of PKM2 Knockdown on K562 Cell Cycle Flow cytometry analysis of cell cycle distribution after PKM2 interference showed that the shPKM2 group had a significantly higher percentage of cells in G1 phase (44.59$±0.15±1.65±0.76±0.30±0.62±$1.95%) (P<0.05) [FIGURE:3, TABLE:2]. These results demonstrate that PKM2 downregulation caused cell cycle arrest predominantly in the G1 phase.

2.4 Effect of PKM2 Knockdown on K562 Cell Apoptosis PKM2 interference significantly increased the apoptosis rate in K562 cells compared with the Vector group (t=37.23, P<0.0001) [FIGURE:4a, 4b]. Analysis of apoptosis-related proteins revealed increased Bax and decreased Bcl-2 expression in the shPKM2 group [Figure 4c: see original paper]. Quantification showed that Bax protein levels were significantly elevated (t=15.36, P=0.0001) while Bcl-2 levels were significantly reduced (t=9.965, P=0.0006) in the shPKM2 group [Figure 4d: see original paper]. These findings suggest that PKM2 knockdown promotes apoptosis in K562 cells by upregulating pro-apoptotic Bax and downregulating anti-apoptotic Bcl-2.

2.5 Effect of PKM2 Knockdown on Autophagy Activity in K562 Cells Given the regulatory role of autophagy in tumor cell growth [6], we examined

changes in autophagy markers (LC3II, p62) to assess the effect of PKM2 interference on autophagy activity. qRT-PCR showed that LC3II mRNA levels were significantly decreased ($t=4.219$, $P=0.0135$) while p62 levels were significantly increased ($t=6.301$, $P=0.0032$) in the shPKM2 group compared with the Vector group [Figure 5a: see original paper]. Western blot results confirmed reduced LC3II protein and increased p62 protein expression [Figure 5b: see original paper]. Quantitative analysis revealed significantly lower LC3II levels and higher p62 levels in the shPKM2 group (tLC3II=10.32, PLC3II=0.0005; tp62=14.59, Pp62=0.0001) [Figure 5c: see original paper]. These results indicate that PKM2 knockdown inhibits autophagy activity in leukemia cells.

2.6 Effect of PKM2 Knockdown Combined with Autophagy Inducer on K562 Cell Proliferation To further validate the role of autophagy in PKM2-mediated cell proliferation, we treated PKM2-knockdown K562 cells with the autophagy inducer rapamycin and observed changes in proliferation capacity. CCK-8 results showed that while PKM2 knockdown significantly reduced cell proliferation compared with the Vector group, treatment with rapamycin markedly restored proliferation capacity compared with the shPKM2 group ($F=96.32$, $P<0.0001$) [Figure 6: see original paper]. These findings demonstrate that autophagy induction can reverse the proliferation inhibition caused by PKM2 downregulation.

Discussion

PKM2 is a key enzyme in the glycolytic pathway, and most research on PKM2 in cancer has focused on solid tumors [7]. Recent studies have reported PKM2 involvement in hematological malignancies. Wang et al. [8] found that PKM2 is highly expressed in leukemia cell lines and normal hematopoietic stem cells, while Panchabhai et al. [9] demonstrated that high PKM2 expression predicts poor clinical prognosis in multiple myeloma patients. This study investigated the effects of PKM2 on proliferation and apoptosis in the human leukemia cell line K562 and explored the underlying mechanisms. We first established a stable K562 cell line with PKM2 knockdown using RNA interference technology, similar to Lin et al. [10] who successfully constructed cervical cancer cell lines (HeLa, SiHa) with stable PKM2 downregulation using lentiviral shRNA vectors. Subsequent proliferation assays showed that PKM2 knockdown significantly reduced the in vitro proliferation capacity of K562 leukemia cells, consistent with Goldberg et al. [11] who reported that PKM2 downregulation decreased survival of hepatocellular carcinoma and ovarian cancer cells. Since cell proliferation is regulated through cell cycle progression, we examined the effect of PKM2 knockdown on the K562 cell cycle. The results showed that PKM2 interference caused cell cycle arrest predominantly in the G1 phase, thereby inhibiting K562 cell proliferation. Zheng et al. [12] found that high PKM2 expression upregulated cyclin CCND1 and downregulated CDKN1A, promoting cell cycle

progression and enhancing ovarian cancer cell growth and survival. We also observed that PKM2 knockdown significantly increased the apoptosis rate of K562 cells, accompanied by increased pro-apoptotic Bax and decreased anti-apoptotic Bcl-2 expression, suggesting that PKM2 may promote leukemia progression by enhancing proliferation and inhibiting apoptosis.

Autophagy, also known as cellular "self-digestion" [13], has gained increasing attention in hematological malignancies as its molecular regulatory mechanisms have been elucidated. Therefore, we examined the effects of PKM2 knockdown on LC3II and p62 expression in K562 cells. We found that PKM2 interference decreased LC3II levels while increasing p62 levels, indicating reduced autophagy activity in leukemia cells. To further verify the importance of autophagy in mediating leukemia cell proliferation, we performed a rescue experiment. Although PKM2 knockdown inhibited leukemia cell growth, treatment with the autophagy inducer rapamycin reversed this proliferation inhibition. Polak et al. [14] reported that treatment of acute lymphoblastic leukemia cells with the autophagy inhibitor hydroxychloroquine suppressed autophagy activity and reduced leukemia cell proliferation. Recently, Piya et al. [15] found that bone marrow mesenchymal cells could induce autophagy activity in leukemia cells, leading to chemoresistance against cytarabine and idarubicin, while silencing the autophagy-related gene ATG7 increased leukemia cell sensitivity to chemotherapy. Our results, together with previous studies, suggest that targeting PKM2 combined with autophagy inhibition may represent a potential therapeutic strategy for leukemia. However, the specific mechanisms by which PKM2 regulates autophagy will be the focus of our future research.

In summary, PKM2 knockdown inhibits the in vitro proliferation of human leukemia K562 cells, induces G1-phase cell cycle arrest, and promotes apoptosis. These effects may be mediated through reduced autophagy activity regulated by PKM2. Future studies will investigate the role of PKM2 in leukemia development using animal models and clinical samples. In-depth investigation of PKM2 mechanisms may provide a novel therapeutic target for leukemia diagnosis and treatment.

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