CRISPR/Cas9-mediated microRNA-21 Cleavage Sensitizes the Treatment Response to Dasatinib in Chronic Myeloid Leukemia Cell Line K562

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Abstract

Background: Tyrosine kinase inhibitors (TKIs) targeting BCR-ABL1 kinase are effective in treating chronic myeloid leukemia (CML), but TKI resistance occurs in a significant number of patients, and the underlying molecular mechanisms of this resistance remain largely unknown. As an oncomiR, microRNA-21(miR-21) functions directly in drug resistance, but its relationship with TKI resistance in CML is rarely reported. As a novel and effective gene editing tool, clustered regularly interspaced short palindromic repeats–CRISPR-associated protein 9(CRISPR/Cas9) has certain advantages in completely knocking out target genes at the gene level.

Methods: We successfully constructed lentiviruses LV-VMP1-sgRNA (045001) and human chronic myeloid leukemia K562 cells were transducted with them. Single-cell-derived clones were screened for miR-21 deletion by genomic DNA PCR and Sanger sequence. RQ-PCR assays was used to confirm the knockout of miR-21. 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide(MTT) and colony formation assays were applied to assess the cell growth inhibition. Imatinib and dasatinib sensitivity was determined by MTT assay and Annexin-V APC/7-AAD double staining flow cytometry. Western blot assay was performed to measure the

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levels of Phosphatase and tensin homolog(PTEN), Phosphatidylinositol 3-kinase(PI3K), Serine/threonine Kinase(AKT), p-AKT, BCR-ABL(P210), p-BCR-ABL (p-P210) .

Result: miR-21 knockout inhibited proliferation of K562 cells, promoted their apoptosis and increased their sensitivity to dasatinib. Further mechanism studies suggest that this is achieved by inhibiting the PI3K/AKT signaling pathway and destroying BCR-ABL.

Conclusions: Our study reveals the efficacy of CRISPR/Cas9 gene editing to miR-21 in K562 and indicates miR-21 as a potential target in sensitizing dasatinib treatment for CML patients with pool response to the TKI targeting therapy.

Key words

hsa-mir-21 microRNA, Myeloid Leukemia, Chronic, tyrosine kinase inhibitor, CRISPR-Cas Systems, dasatinib

Background

Chronic myeloid leukemia (CML) is a chronic myeloproliferative disorder of the hematologic system characterized by the Philadelphia (Ph) chromosome[1] giving rise to the BCR-ABL fusion oncogene[2], which generates a protein with elevated tyrosine kinase activity[3-5]. The emergence of BCR-ABL targeted tyrosine kinase inhibitors (TKIs) has significantly improved 10-year overall survival (OS) of CML from 20% to 80-90%[5, 6], turning it from a malignant fatal disease into a chronic and manageable one. However, only about 10% of patients in chronic phase (CP) CML can stop treatment with TKIs and remain in therapy-free remission without relapse[7]. The remaining CP patients who need prolonged treatment with TKIs may face serious adverse events, lack of treatment compliance and high costs, among which approximately 20-30% may develop primary or secondary resistance to TKIs[8]. The remaining patients who fail to find a stem-cell donor will likely die of leukemia.

Multiple mechanisms are involved in TKI resistance in CML, which can be divided into two categories. One category is a BCR-ABL–dependent mechanism, including mutations of/outside the BCR-ABL kinase domains, compound mutations, defective DNA repair mechanisms, and amplification and/or overexpression of

BCR-ABL. The other category is a BCR-ABL–independent mechanism resulting from activation of alternate prosurvival signaling pathways, drug influx–efflux activity, epigenetic modifications, inherently resistant stem cells, the bone marrow stromal microenvironment, and elevated levels of inhibitors of apoptosis proteins[9]. BCR-ABL–dependent mechanisms have been extensively investigated, but BCR-ABL–independent factors are also essential, and their molecular foundations are poorly understood.

Numerous studies have shown that miR-21 whose gene is located in chromosome 17q23.2c[10], as an oncomiR, is essential in the proliferation and apoptosis of various tumors[11-17] and participates in the regulation of various downstream effectors related to tumors[18]. Some studies have found that miR-21 is relevant to the sensitivity of CML cells to chemotherapy drugs, such as triptolide[19], daunorubicin[20], arsenic trioxide[21] and arabinosylcytosine[22]. However, the relevance between miR-21 and TKI resistance in CML has rarely been reported. In a study that predicted which patients would achieve the optimal response with TKIs, miR-21 expression levels were found to be the highest in patients who did not respond optimaliy after 6 and 12 months of treatment[23]. In this case, miR-21 may be a novel therapeutic strategy to reverse TKIs resistance in CML.

In recent decades, precise and effective genome editing technology has developed rapidly and has brought new opportunities to life science. Compared with other genomic modification technologies such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), CRISPR-Cas (clustered regularly interspaced short palindromic repeats–CRISPR-associated) has become the first choice of gene editing technology due to its advantages of simple design, quick implementation, and low cost as well as high efficiency and specificity[24-27]. In this study, the targeted knockout of miR-21 in CML cell line K562 was performed using CRISPR/Cas9 technology, laying a foundation for subsequent studies on the relevance between miR-21 and TKI resistance in CML.

Mmethods

Construction of the lentiviral vectors

The CRISPR/Cas9-mediated miR-21 gene knockouting vector LV-VMP1-sgRNA (045001) and the

non-targeting control sgRNA vector sgRNA-CON251 were obtained from the company (Shanghai Genechem Co., LTD). The sgRNA sequence targeting miR-21 is ATGTTGACTGTTGAATCTCA; the non-targeting sgRNA sequence is CGCTTCCGCGGCCCGTTCAA. The sgRNA were subcloned into the Lenti-CAS9-sgRNA tag vectors. The linked products were transformed into the TOP10 competent cells, and the positive clones were obtained by colony PCR and sequenced, to obtain the lentivirus plasmids with correct expression of sgRNA. Lentiviral particles carrying the miR-21 targeting sgRNA or the non-targeting control were packaged in 293T cells by Genechem.

Cell culture and lentiviral transduction

Human chronic myeloid leukemia (CML) K562 cells were preserved by Fujian Institute of Hematology and authenticated. K562 cells were cultured in RPMI 1640 medium (Hyclone, UK) containing 10% fetal bovine serum (GEMINI, CA, USA) in a humidified incubator with 5% CO_2 at 37℃. For transduction, K562 cells were incubated into a 96-well plate with the density of 5,000 cells/well in 100 μL of culture medium and cultured to a confluency of 70%-80%. The corresponding volumes of viral suspension LV-VMP1-sgRNA when MOI=100, MOI=200, and MOI=400 were added to the cell suspension in each well and thoroughly mixed, followed by a 72 hr incubation.

Surveyor nuclease assay

We use the surveyor nuclease assay to determine the mutagenesis ability of sgRNA. Genomic DNA was extracted from K562 cells after three days of transduction using QuickExtract™ DNA Extraction Solution (Lucigen, US). Thirty-five cycles of PCR reaction was used to amplify the targeted locus by a pair of primers, miR-21-F (TGGGGTTCGATCTTAACAGG) and miR-21-R (TTTCAAAACCCACAATGCAG). The PCR products were re-hybridized by denaturing and annealing to generate the mismatched pair of DNA double strain. Then the mismatched site was recognized digested by surveyor nuclease (Integrated DNA Technologies). The digested products including the uncleaved DNA and cleaved DNA were separated by electrophoresis with 2%

agarose gel. Image J software was used to quantify the DNA bands and calculate the mutation rate of the cell population.

Identification of mutant single clones

K562 cells transduced with miR-21 targeting CRISPR/Cas9 lentivirus were incubated into 96-well plates with a concentration of 1 cell/well. We have picked up part of cells from each colony for DNA extraction and the remaining cells were retained for further culture. PCR reaction was performed as mentioned above, followed by electrophoresis on a 2% agarose gel to detect mutations. We also sent the PCR product of the picked clones for Sanger sequencing to further verify the genome of clones..

qRT-PCR

To evaluate the knockout cells, we conducted quantitative real-time PCR to quantify the miR-21 level. We use the miRcute miRNA isolation kit (TIANGEN, CN) to extract RNA and the miScript RT Kit II (Qiagen) for reverse transcription. The quantified PCR was performed with 1ul cDNA using the miScript SYBR Green PCR Kit (Qiagen) and appropriate primers (Qiagen). The RNU6B was used as an internal control. The relative expression level was calculated by $2^{-\Delta\Delta Ct}$ and all the reactions were done in parallel triplicate.

MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

K562 cells were seeded with a concentration of 3000 cells/100μl/well in triplicate in 96-well plates.. The plates were then incubated for 24, 48, 72 and 96 hours respectively at 37℃ in a 5% CO_2 incubator. Before detection, 20 μl 5 mg/ml MTT was added to each well, after further incubation for 4 hours, the supernatant was removed and replaced with 100 μl DMSO to each well. The plates were then shaken on an orbital shaker for 10 min until formazan crystals were solubilized. The absorbance value (OD value) was then measured with dual wavelengths of 480 nm and 630 nm on the Synergy HTX microplate reader (Bio-Tek, US). The assays were repeated three times.

Colony formation assay

We used methylcellulose for the colony formation assay. First 200 cells were suspend in RPMI-1640 media containing 10% fetal bovine serum by volume and 0.8 g/L methyl cellulose each well on 24-well plates. After 7 - 14 days of incubation, number of colonies were counted under a 400× optical microscope. The cloning formation rate were calculated using the following formula and the experiment was repeated three times. cloning formation rate= (clone number/200) ×100%.

Imatinib/dasatinib treatment and apoptosis assay

Various concentrations of TKI were added to the cell culture medium respectively in 96-well plates, including imatinib (0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 μM) and dasatinib (0, 8, 16, 32, 64, 100, 128,150, 256 and 512 nM). MTT assay was used to evaluate cell viability at 48 hours after TKI treatment.

To analysis apoptosis level of induced by TKI treatment, K562 cells ($5x10^5$ cells/well) were incubated into 6-well plates and treated with imatinib (0, 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 μM) or dasatinib (0, 32, 64, 128 and 256 nM) for 48 hours. Then we washed cells twice with cold PBS and stained with the Annexin V-APC/7-AAD Apoptosis Detection Kit (BD Pharmingen™, US). The stained cells were incubate away form light for 15 min at 37℃. The we evaluate the cell apoptosis level on a FACS Calibur Flow Cytometer (BD Biosciences, US) . The results were analyzed using FlowJo V10 software (BD).

RNA-Seq analysis

RNA-seq analysis was performed by the company (Beijing BerryGenomics Co., LTD). Sequence libraries were generated and the index codes were added following manufactory's instruction to attribute sequences to each sample. After clustering, the library preparations were sequenced on an Illumina Hiseq 2500/2000 platform and 50 bp single-end reads were generated. DESeq2 methods were used to analyze the differential expression of

the two conditions/groups. The P-values were adjusted using the Benjamini and Hochberg method. KEGG enrichment analysis were used to analyze target gene candidates of differentially expressed mRNA.

Western blot

K562 cells were lysed on ice in RIPA buffer (ThermoFisher Scientific) for 30 min after being washed twice in cold PBS. Following centrifugation at 13,000 g for 15 min at 4ºC, the cell lysate supernatants were extracted as total protein and quantified on an ultraviolet-visible spectrophotometer (ThermoFisher Scientific). Subsequently, the protein (80 μg) from each sample was separated by SDS-PAGE and then transferred onto a nitrocellulose membrane (Pall Life Sciences, US). After blocking with 5% non-fat milk in TBST for 1 h, the membranes were washed three times with TBST (7 min/wash). The membranes were then incubated with primary antibodies overnight at 4ºC. After washing with TBST three times, the membranes were incubated with horseradish peroxidase labeled secondary antibodies at room temperature for 1 h. The primary antibody GAPDH were obtained from Santa Cruz (sc-32233, US) and the PTEN (#9188), PI3K (#4257), p-AKT (#4060), AKT (#2920), p-BCR-ABL (p-P210, #2864), BCR-ABL (P210, #2862) antibodies were from cell signaling technology (CST, US). For secondary antibodies either anti-rabbit (#14708) or anti-mouse (#14709) IgG conjugated with horseradish peroxidase (CST) were used. Western Lightning ECL (Bio-Eed) reagents were used for fluorescence production and a ChemiDoc™ Imaging System (Bio-Rad Laboratories, US) was used to visualize the protein detected.

Statistical analysis

Data are expressed as the mean ± standard error of the mean. Comparisons between the K562 miR-21 KO clone and the K562-WT were performed using unpaired t test. Differences were considered to be significant at $P<0.05$. Data was analyzed using GraphPad Prism program version 7.0.

Results

CRISPR/Cas9-mediated disruption of the microRNA-21 gene in K562 cells

To test whether CRISPR/Cas9 lentivirus is effective in editing miRNA, we used CRISPR/Cas9 lentivirus to transduce chronic myeloid leukemia (CML) K562 cells. We used K562 cells transduced with non-targeting control as the negtive control (labeled as K562-WT) and 293T cells transduced with miR-21 targeting sgRNA as positive control (labeled as 293T miR-21-KO) . After three days of transduction of K562 cells with miR-21 CRISPR/Cas9 lentivirus at different MOI values (100, 200, 400), genomic DNA of the transduced cells was extracted, and mutation rates of genomic DNA of the cells were detected with a surveyor nuclease assay. The results of agarose gel electrophoresis showed that CRISPR/Cas9 could specifically shear genomic DNA, and create cleavage bands. We used indel ratiao (indel%) to evaluate the mutation efficiency, which is the ratio of gray value of the cleaved bands versus the total gray value (cleaved + uncleaved bands). The induced mutation efficiency was the highest when MOI is 200, about 21.88 (Fig. 1A).

We then isolated single cell clones. The single-cell cloning formation rate of K562 cells after CRISPR/Cas9 transfection was 3% (18/600). Genomic DNA was isolated from each single cell clones (numbers on top) and PCR screening was performed on the obtained single-cell clones using the same primers for surveyor assay. The PCR results of three clones (#1, #2, #5) showed only one amplification fragment shorter than wild type, indicating that these three clones might be homozygous knockout clones (Fig. 1B).

Subsequently, mature miR-21 was determined by qRT-PCR for further verification. qRT-PCR results showed that in the clones containing short fragment PCR products, the expression level of mature miR-21 in #5 was as low as 0.003% of wild type, and the expression level of the other clones (#1, #2) was also significantly reduced (Fig. 1C).

We further did sanger sequencing to confirm the isolated single clones, the sequences were shown in Fig.1D. Compared to WT (430bp), the length of three clones were 393bp (#1), 412bp (#2), 411bp (#5) respectively. The deletion of the sequences in each clone was consistant with the PCR result.

miR-21 knockout affected cell growth, apoptosis and the drug susceptibility

First we analyzed the cell viability of K562 cells after miR-21 knockout. The MTT assay were used to measure the cell growth rates and all the three clones with the loss of miR-21 grew much slower compared to parental K562 cells (Fig. 2A). The clonogenic rates of methyl cellulose colony formation assay of each group were 73.94%±3.15% (WT), 32.39%±3.69% (#1), 23.52±1.72% (#2) and 41.11±2.05% (#5) ($P<0.05$), indicating that miR-21 knockout cells generated many fewer colonies (Fig. 2B and Fig. 2C). Thus, knockout of miR-21 impaired the proliferation ability in K562 cells.

Next we investigated whether knockout of miR-21 affect the chemotherapeutic sensitivity to TKI therapy in K562 cells. The miR-21 knockout and the wild-type cells were treated with dasatinib at various concentrations ranging from 8 to 512 nM, and their viability was measured by an MTT assay to calculate the IC50 of dasatinib. The IC50 of WT and clones #1, #2, #5 were 206.273±6.945 nm/ml, 99.362±1.631 nm/ml, 84.693±3.462 nm/ml and 101.492±2.469 nm/ml respectively, indicating that miR-21 knockout significantly increased drug sensitivity of dasatinib ($P<0.05$). (Fig. 3A). However, we did not observe that miR-21 knockout sensitized the treatment response to imatinib (Fig. 3B).

We also analyzed the apoptosis level induced by dasatinib in miR-21 knockout and wild-type cells treated or not treated with dasatinib; the apoptotic rate was calculated by the percentage of AnnexinV+ population. We compared the apoptotic rate of dasatinib and imatinib between miR-21 KO and wild-type cells. The apoptotic rate of dasatinib and imatinib are shown in Fig. 3 C&D. For both dasatinib and imatinib, the apoptotic rate increased as the treatment dose increased, which revealed a dose-related response pattern. For dasatinib, at the same dose, the apoptotic rates were higher in the three miR-21 knockout clones. However, for imatinib, there were two clones (#1 and #2) with more apoptotic cells upon TKI treatment. Therefore, miR-21 knockout increased the early dasatinib-induced apoptosis in K562. Combined with the MTT results, loss of miR-21 increased the apoptotic rate and the sensitivity to dasatinib in K562 cells. But for imatinib, we do not have enough evidence to show that miR-21 knockout increases sensitivity.

Alteration of gene expression level in miR-21 deleted K562 cells

Given the fact of K562 cells lacking miR-21 expression resulted in poorer cell viability as well as higher sensitivity to dasatinib treatment, we conducted RNA-seq to look for what downstring genes or pathways might be altered in miR-21 knockout K562 cells. To quantify gene expression and analyze differential genes, we used an unsupervised clustering heat map and volcano plots to evaluate the gene expression differentiation between the wild type K562 cells and the miR-21-KO cells (Fig. 4A and 4B). We identified a total of 347 genes with significant differential changes, of which 302 were up-regulated and 45 down-regulated. The KEGG pathway analyses of differentially expressed mRNAs can provide hints about the process of resistance to TKIs in CML. The first 20 pathways with the the lowest q value, which indicated the most significant change in the KEGG pathway enrichment were shown in Fig. 4C. The KEGG pathway enrichment results revealed changes in systemic lupus erythematosus, alcoholism, PI3K-AKT signaling pathway, Ras signaling pathway, pathways in cancer, etc.

The PTEN/PI3K/AKT pathway was participated in miR-21 regulated signaling

Among the activated or inhibited pathways, we found that PTEN/PI3K/AKT signaling were down regulated after miR-21 knockout. We thus conducted a western blot assay to validate the RNA sequencing results (Fig. 5). We found a robust decrease of total PI3K level. Meanwhile, the phosphate AKT was significantly inhibited in miR-21 knockout cells without any alteration of the total AKT protein level. In addition, there was no difference in PTEN level between miR-21 wild-type and miR-21 knockout K562 cells. Moreover, when we looked at the level of BCR-ABL fusion protein, we found that both P-210 and p-P210 levels were significantly down regulated, indicating that the BCR-ABL fusion proteins were disrupted by miR-21 knockout.

Discussion

Overexpression of miR-21 functions directly in various biological processes in human diseases[28], including drug resistance[29]. However, the mechanism of miR-21 in CML resistance to TKI has not been identified. In this study, we successfully obtained complete miR-21 knockout K562 cell clones using the

CRISPR/Cas9 lentivirus, which demonstrated that the knockout is effective in disrupting miR-21 in K562. Screening and isolation of individual clones do not require drug selection, which not only reduced the toxicity of transfection on cells, but also avoided other factors that might affect changes in the gene expression profile. Further functional studies showed that the miR-21 gene knockout mediated by CRISPR/Cas9 led to decreased proliferation and increased apoptosis of K562 cells.

CRISPR/Cas9 technology has become a powerful gene-editing tool for gene knockout research and functional genomic screening since 2013 when researchers first used it to achieve efficient fixed-point editing of genomes in mouse and human cells[30]. However, editing efficiency and off-target effects are still critical problems in the process of CRISPR/Cas9. There are significant differences in cutting efficiency between different sgRNAs[31], and both sgRNA and Cas9 factors cause off-target effects. Off-target effects caused by CRISPR-Cas9 editing in vivo will lead to many unpredictable gene mutations[32]. Researchers have identified many ways to improve the editing efficiency of CRISPR/Cas9[33] and reduce the off-target effects[34].

Previous drug resistance mechanisms have involved redistribution of drug accumulation in cells, modification of drug target molecules, increased DNA damage repair as well as ,inhibition of drug-induced apoptosis and so on[35]. More and more studies have shown that miRNA-mediated drug resistance is another mechanism of drug resistance, and may be essential in drug resistance by targeting multiple signaling pathways. The results of Darido et al.[36], Zheng et al.[37], and Chai et al.[38] all showed that miR-21 promoted cell proliferation by activating PTEN-dependent PI3K/Akt in cancer cells. The PI3K/AKT/mTOR pathway has been validated that having a role in imatinib related resistance[39]. PTEN is also found having a function as a tumor suppressor protein[40] and the downregulation of PTEN is considered to be the most important role of miR-21 in CML cells[41].

In this study, we confirmed that knockout of miR-21 inhibited the PI3K/Akt signaling pathway. Compared with wild-type, p-AKT and PI3K were significantly down-regulated in miR-21 knockout K562 cell clones, which was consistent with previous reports[20, 42]. However, there was no significant change in PTEN, which may be related to the involvement of other small molecular RNAs targeted by PTEN in TKI resistance of

leukemia cell lines, or that the crosstalk between the PI3K signaling pathway and other tumor-causing signaling pathways, such as those involving Ras, p53 or mTOR, led to such change.

Espadinha et al. used microarray-based miRNA expression profiling in CML model K562 cells and found that oncoprotein BCR-ABL1 is dependent on the presence of two STAT binding sites on the miR-21 promoter and the phosphorylation of STAT5 to regulate the expression of miR-21[43]. Wang et al. found that emodin showed anti-tumor activity in vivo and in vitro by inactivating PTEN/PI3KAKT and deleting BCR-ABL to induce apoptosis[44]. In our experiment, the expression of BCR-ABL and p-BCR-ABL in miR-21 knockout cell clones was also significantly down-regulated. These results indicate a regulatory relationship between miR-21 and BCR-ABL, but the specific regulatory mechanism needs to be further explored.

It was also interesting to find that miR-21 knockout clones enhanced the sensitivity to dasatinib, but the sensitivity to imatinib did not change significantly. Ferreira and colleagues also suggested that the TKI inhibitor dasatinib affects the expression of miR-let-7d, miR-let-7e, miR-15a, miR-16, miR-21, miR-130a and miR-142-3p, while imatinib affects the levels of miR-15a and miR-130a[45]. It is possible that miR-21 is a dasatinib-specific sensitive factor that didn't affect imatinib. Further studies are needed to resolve such differences and the specific mechanisms involved.

Conclusions

Our study reveals the efficacy of CRISPR/Cas9 gene editing to miR-21 in K562 and indicates miR-21 as a potential target in sensitizing dasatinib treatment for CML patients with pool response to the TKI targeting therapy.

List of abbreviations

TKIs: Tyrosine kinase inhibitors; CML: chronic myeloid leukemia; CRISPR/Cas9: clustered regularly interspaced short palindromic repeats–CRISPR-associated protein 9; sgRNA: single guide RNA; MTT: 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide; PTEN: Phosphatase and tensin homolog;

PI3K: Phosphatidylinositol 3-kinase; AKT: Serine/threonine Kinase; Ph: Philadelphia; OS: overall survival; CP: chronic phase; ZFNs: zinc-finger nucleases; TALENs: transcription activator-like effector nucleases; miR-21: microRNA-21; KO: knockout; cDNA: complement deoxyribonucleic acid; qRT-PCR: quantitative real-time polymerase chain reaction; MOI : multiplicity of infection; WT: Wild type; OD: Optical density.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The data that support the findings of this study are available from the corresponding author, B. C., upon reasonable request.

Competing interest

The authors declare no confict of interest.

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Author Contributions

B. C. and J. H. conceived and designed the experiment; Z. Y., L. W. and J. L. operated the experiment and organized the data; P. J. and M. L.analyzed the data; Z. Y., L. W. and J. L.wrote the paper; B. C. and J. H. reviewed the paper. Manuscript is approved by all authors for publication.

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Figures

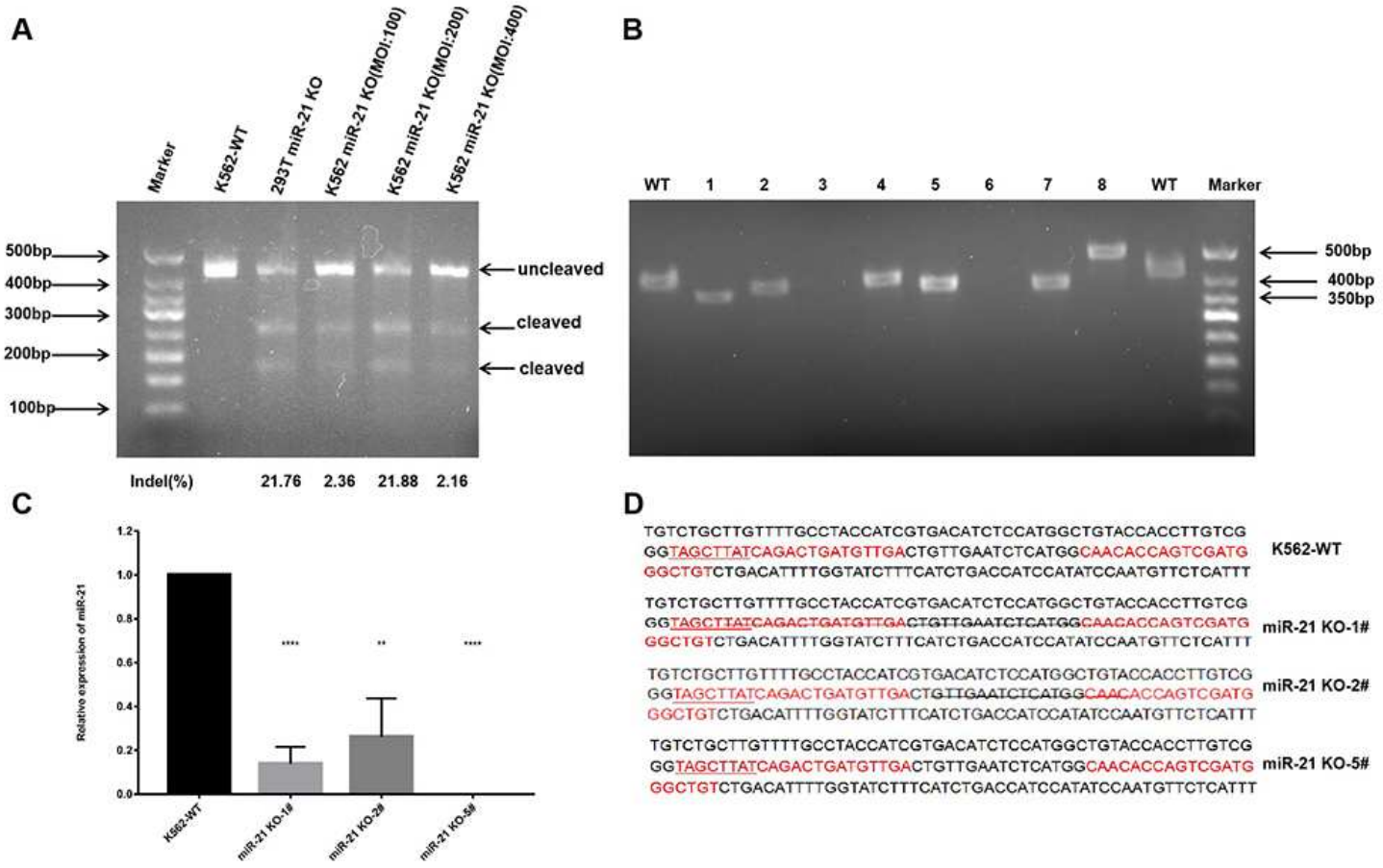

Figure 1

A. T he induced mutation efficiency was the highest when MOI is 200, about 21.88. B. The PCR results of three clones (#1, #2, #5) showed only one amplification fragment shorter than wild type, indicating that these three clones might be homozygous knockout clones. C. Subsequently, mature miR-21 was determined by qRT-PCR for further verification. qRT-PCR results showed that in the clones containing short fragment PCR products, the expression level of mature miR-21 in #5 was as low as 0.003% of wild type, and the expression level of the other clones (#1, #2) was also significantly reduced. D. We further did sanger sequencing to confirm the isolated single clones

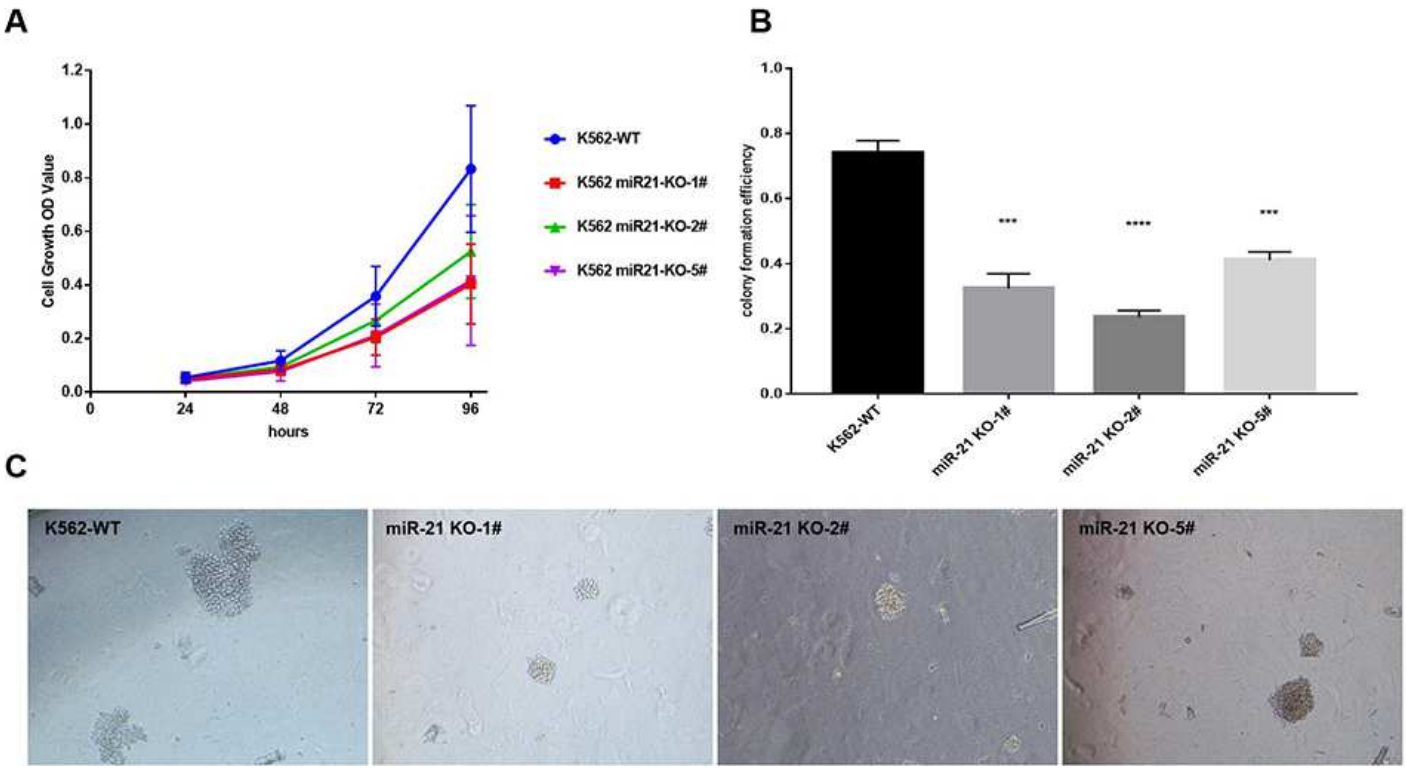

Figure 2

A. First we analyzed the cell viability of K562 cells after miR-21 knockout. The MTT assay were used to measure the cell growth rates and all the three clones with the loss of miR-21 grew much slower compared to parental K562 cells. B-C. The clonogenic rates of methyl cellulose colony formation assay of each group were 73.94%±3.15% (WT), 32.39%±3.69% (#1), 23.52±1.72% (#2) and 41.11±2.05% (#5) ($P<0.05$), indicating that miR-21 knockout cells generated many fewer colonies

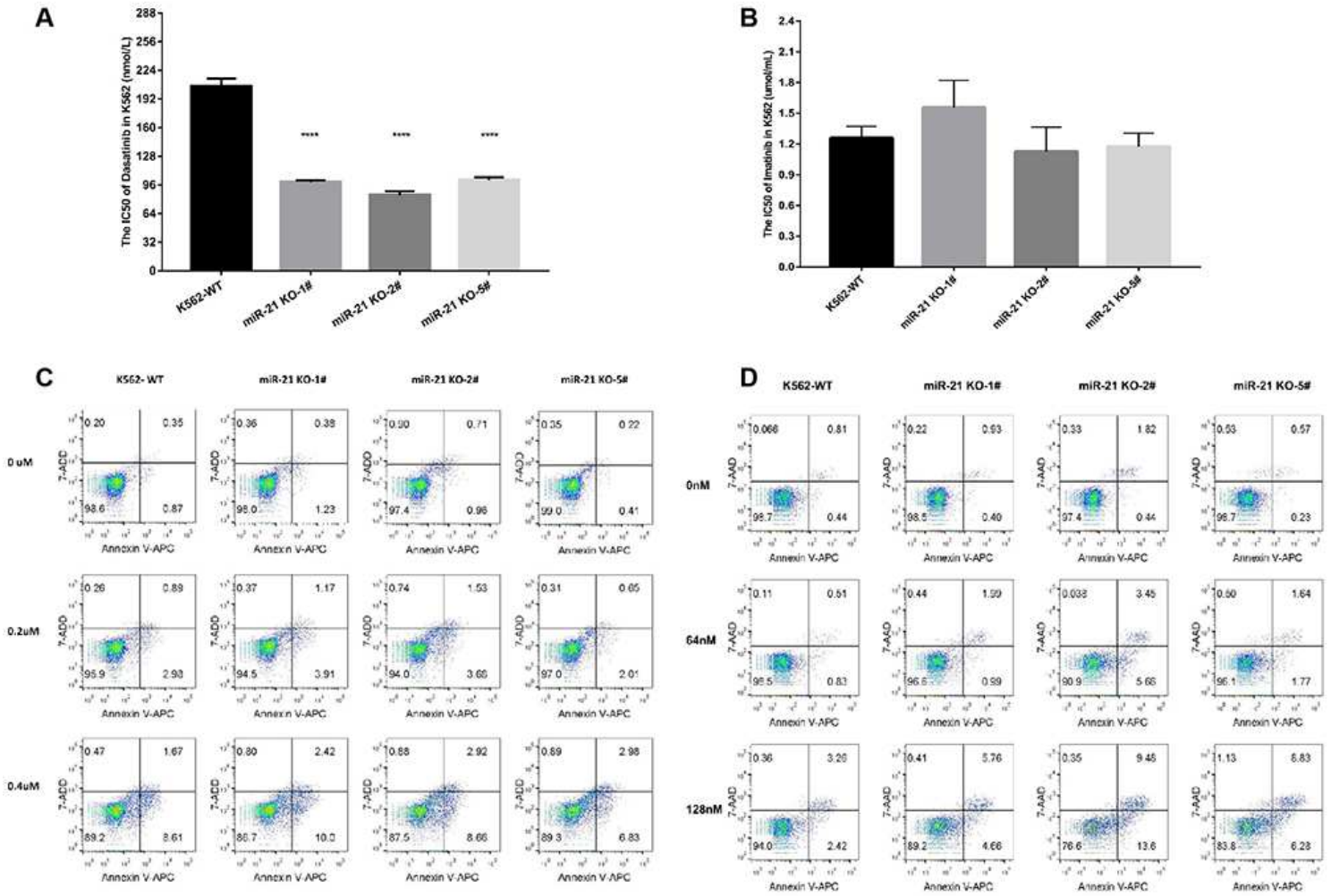

Figure 3

A. The IC50 of WT and clones #1, #2, #5 were 206.273±6.945 nm/ml, 99.362±1.631 nm/ml, 84.693±3.462 nm/ml and 101.492±2.469 nm/ml respectively, indicating that miR-21 knockout significantly increased drug sensitivity of dasatinib ($P<0.05$). B. We did not observe that miR-21 knockout sensitized the treatment response to imatinib. C&D. The apoptotic rate of dasatinib and imatinib

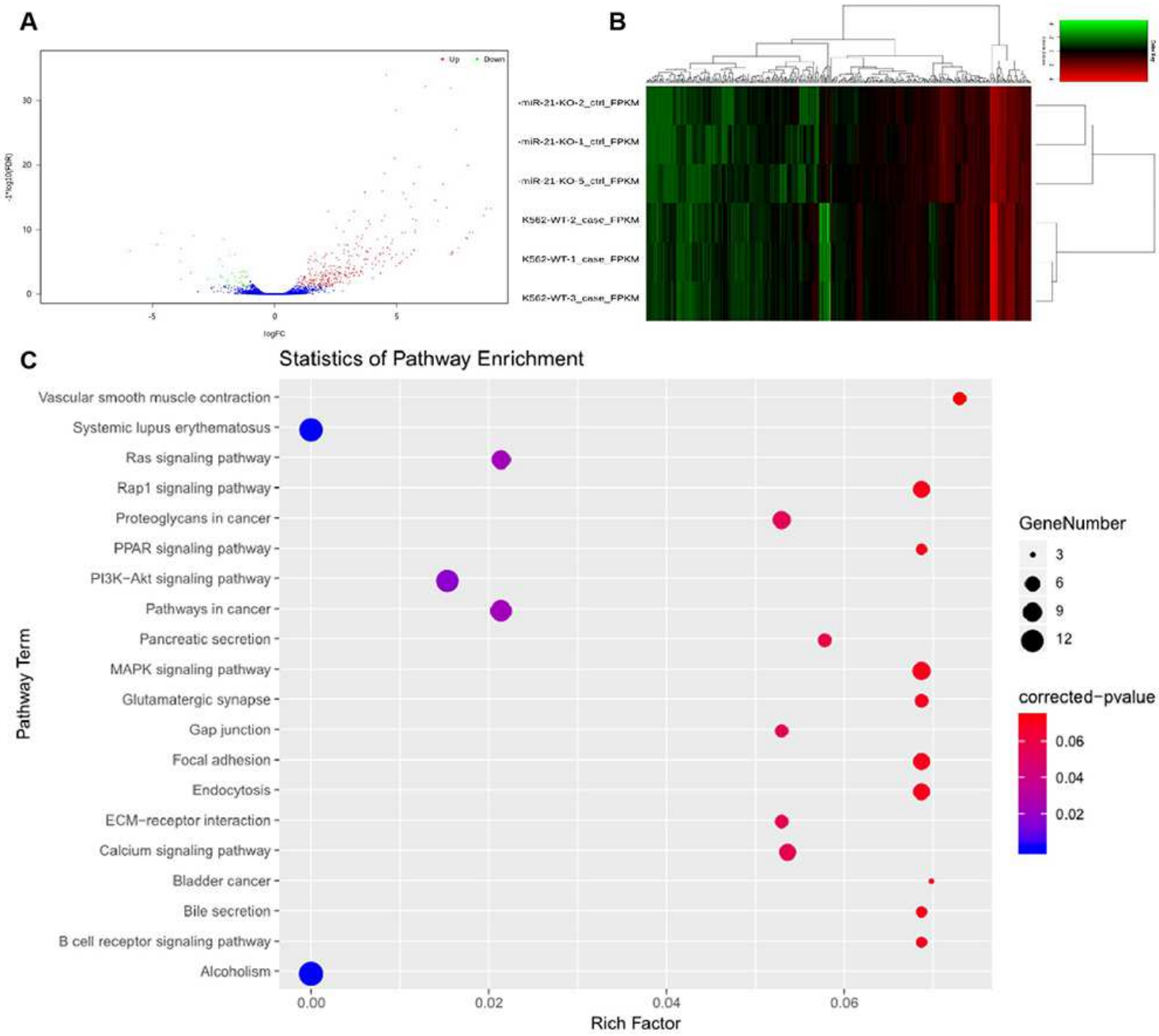

Figure 4

A & B. To quantify gene expression and analyze differential genes, we used an unsupervised clustering heat map and volcano plots to evaluate the gene expression differentiation between the wild type K562 cells and the miR-21-KO cells. C. The first 20 pathways with the the lowest q value, which indicated the most significant change in the KEGG pathway enrichment

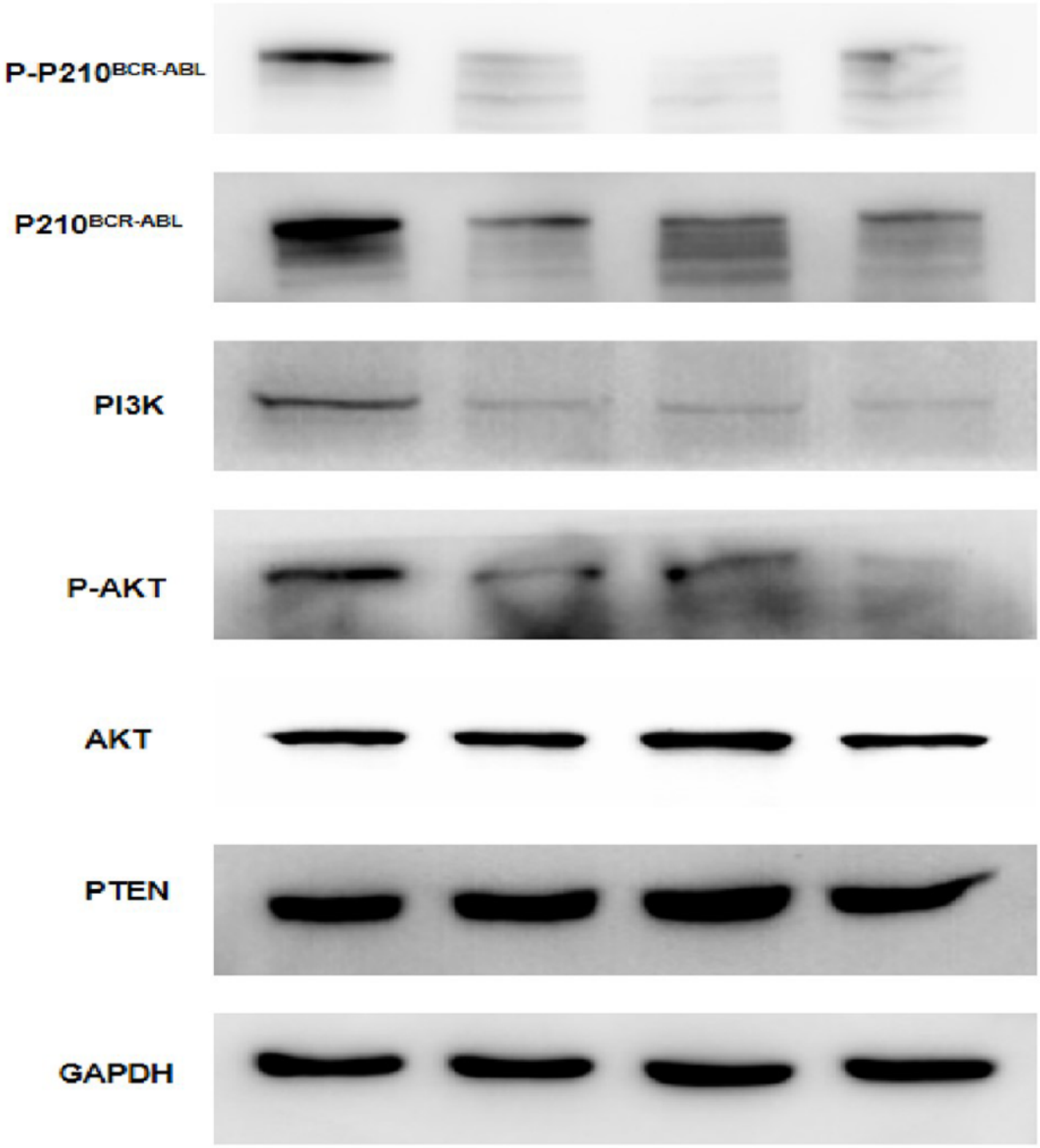

Figure 5

A western blot assay to validate the RNA sequencing results