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Research paper

Pak1 gene functioned differentially in different BCR-ABL subtypes in leukemiagenesis and treatment response through STAT5 pathway

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ABSTRACT

The BCR-ABL fusion gene (BCR-ABL) has different subtypes such as p210 and p190 with p190 appear to lead to a worse prognosis. To explore the mechanism of difference in pathogenesis and prognosis in different BCR-ABL subtype-related leukemia, expression profile microarray analysis was conducted between p190 and p210 patients and verified by RT-PCR. The p21-activated kinase (PAK1) gene was chosen and regulation of the PAK1-STAT5 biological axis and its influence on proliferation and apoptosis in leukemia cells were also analyzed. The results showed that PAK1 might be an important molecular mechanism of the pathogenic difference between different BCR-ABL subtypes. In P210 (+) chronic myelogenous leukemia (CML), down-regulated PAK1 gene expressions may lead to the suppression of cell proliferation and promotion of apoptosis through phosphorylation of STAT5, with a reverse effect in P190 (+) acute lymphoblastic leukemia(ALL), especially acute B lymphoblastic leukemia (B-ALL). Additionally, in P210 (+) CML, down-regulated PAK1 expression may enhance the effect of TKI, whereas the reverse is true in P190 (+) B-ALL, demonstrating that PAK1 might also be an important therapeutic target between different BCR-ABL subtypes.

1. Introduction

The Philadelphia chromosome (Ph), t (9;22)(q34;q11.2), transcribed as BCR-ABL, is one of the most common genetic abnormalities detected in leukemia, and it is clustered within three well-defined regions: the minor breakpoint cluster region (m-bcr), the major breakpoint cluster region (M-bcr), and μ-bcr, encode a 190 kDa chimeric protein (p190), a 210 kDa chimeric protein (p210) and a 230 kDa protein, respectively.

Many studies have indicated that the BCR-ABL fusion gene is a highly useful diagnostic tool for chronic myelogenous leukemia (CML), and recent research has shown that the BCR-ABL fusion gene can also be detected in 25–30% of adult and 2–5% of childhood cases of acute lymphoblastic leukemia (ALL), especially B-ALL [1,2]. Some reports [3–5] have shown that BCR-ABL in B-ALL indicated poor prognosis, and treatments such as allo-HSCT should be considered.

Our earlier study [6] further indicated that the M-bcr subtype and the m-bcr subtype occur differentially between CML and B-ALL patients: M-bcr occurs more frequently in classical CML patients and younger ALL patients, who show a good response to imatinib, and m-bcr occurs more frequently in unclassical CML patients and older B-ALL patients, who exhibit resistance to imatinib treatment with a poor prognosis. All of these studies showed that the BCR-ABL subtype may be significantly predictive for the long-term outcome of CML and B-ALL patients treated with first-line imatinib (IM).

The mechanism of BCR-ABL in inducing leukemia remains a hotspot in leukemia research, and the STAT5 pathway shows promise as a key point. Dalgic [7] have reported a different signature between CML with variant t (9;22) and classic translocation in the RAS-STAT5-MAPK pathway. Berger [8] described a critical role of STAT5a serine phosphorylation in STAT5a-driven leukemogenesis. Other reports [9–11] also indicated that STAT5 serine phosphorylation regulates the MAPK pathway and is required for BCR-ABL-induced leukemogenesis, suggesting that the phosphorylation of STAT5 is an important factor in tumor regulation, playing an important role in the development of leukemia. However, the mechanism and related genes between M-bcr

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CML and m-bcr B-ALL as well as their impact on prognosis remain unclear.

Hence, our study aims to find and verify different expressed genes and pathways between M-bcr CML and m-bcr B-ALL to provide some hints to studying the mechanism of leukemogenesis with BCR-ABL fusion genes of different subtypes. In this study, we performed gene expression profiling by microarrays to identify a differentially expressed genes between M-bcr CML and m-bcr B-ALL patients related to prognosis. The PAK1 gene was selected by microarrays and q-PCR verification for the study of gene function and regulation of STAT5 in different BCR-ABL subtypes. Our study confirmed that PAK1 expresses differentially between M-bcr CML and m-bcr B-ALL, and may plays a different role in the regulation of the Stat5 pathway, and is implicated in a wider range of cellular processes including cell proliferation and apoptosis in M-bcr CML and m-bcr B-ALL which may affect the therapeutic effect of TKI.

2. Material and methods

2.1. Patient and grouping

CML patients: A total of 190 chronic phase CML patients with M-BCR (p210) mRNA detected by RT-PCR in West China Hospital confirmed by clinical features, bone marrow cytology, molecular biology and cytogenetics according to 2008 WHO criteria were enrolled in this study.

B-ALL patients: A total of 129 B-ALL patients with m-BCR (p190) mRNA detected by RT-PCR in West China Hospital confirmed by clinical features, bone marrow cytology, molecular biology and cytogenetics according to 2008 WHO criteria were enrolled in this study.

Written informed consent was obtained from all included patients, and the study was approved by the Clinical Trials and Biomedical Ethics Committee of the West China Hospital, Sichuan University. The relevant Judgement's Reference Number is No. 199 (2014).

2.2. Gene expression profiling test and Validation

Of the M-BCR-positive CML and m-BCR-positive B-ALL patients enrolled, six cases of initial CML patients (group A) and 6 cases of initial B-ALL patients (group B) matched with age and sex were used for the gene expression profiling test (Supplementary TableS1).

QIAamp RNA Blood Mini-Kit (QIAGEN, Germany) was used for RNA extraction. Double-strand cDNA (ds-cDNA) was synthesized from total RNA using an Invitrogen SuperScript ds-cDNA synthesis kit. Human Gene Expression Array (Roche NimbleGen, Germany) was used for the gene expression profiling test in accordance with the NimbleGen Gene Expression Analysis protocol (NimbleGen Systems, Inc., Madison, WI, USA). Slides were scanned using an Axon GenePix 4000B scanner (Molecular Devices Corporation) piloted by Gene Pro 6.0 software (Axon). The scanned images were then imported into NimbleScan software (version 2.5) for grid alignment and expression data analysis. Expression data were normalized through quantile normalization and the Robust Multichip Average (RMA) algorithm included in the NimbleScan software. All gene level files were imported into Agilent GeneSpring GX software (version 11.5.1) for further analysis including hierarchical clustering, PATHWAY, Gene Ontology (GO) and protein-protein interaction (PPI) and protein-DNA interaction (PDI) networks.

The following conditions were set for screening differentially expressed genes for validation: a) the three genes with the highest increased or decreased differences; b) the three most differently expressed genes present in the PATHWAY and GO analyses; and c) all differentially expressed genes in the HUB analysis. Nine genes were chosen to be verified: Homo sapiens p21 protein (Cdc42/Rac)-activated kinase 1 (PAK1), Homo sapiens lymphocyte-specific protein tyrosine kinase (LCK), Homo sapiens CD79a molecule, immunoglobulin-associated alpha (CD79 A), Homo sapiens lymphoid enhancer-binding factor 1 (LEF1), Homo sapiens junction plakoglob in (JUP), Homo sapiens deoxynucleotidytransferase, terminal (DNTT), Homo sapiens integrin, alpha M (complement component 3 receptor 3 subunit) (ITGAM), Homo sapiens Mediterranean fever (MEFV), and Homo sapiens serpin peptidase inhibitor, clade B (ovalbumin), member 1 (SERPINB1).

One hundred and twenty CML patients and eighty B-ALL patients treated with imatinib and achieved MMR(BCR-ABL < 0.1%)were enrolled in the validation (Supplementary Table S2). Reverse transcription (RT) and Q-PCR were used for the gene expression verification. Reverse transcription was performed with a cDNA Synthesis Kit (Takara) according to the manufacturer's instructions. The resulting cDNA was used as the template of real-time PCR reaction, which was performed in a LightCycler 480 real-time fluorescence PCR detection system, and the concentration of mRNA was quantified by normalizing their amplification to the house-keeping gene (ABL) by relative concentration.

2.3. BCR-ABL detection and subtyping

BCR-ABL p210 mRNA and BCR-ABL p190 mRNA form both CML and ALL patient were detected by one-step RT-PCR kits(Yuanqi, Shanghai)using ligthcycler 480 (Roche, Switzerland).

2.4. Construction and verification of the Lentivirus for PAK1 interference and PAK1 over-expression

The si-PAK1 fragments were obtained by annealing and connected with pSIH1-H1-copGFP vector (NTCC, China) to construct PAK1 interfering plasmid. Lipofectamine 2000 (Invitrogen, USA) was used to transfect PAK1 interfering plasmids into 293 T cells with three pSRL-PACK plasmids (Tianjin Saier Biotech, China). Lentiviruses were collected and infected with K562 cells.

The target fragment of PAK1 overexpression was obtained by enzymatic digestion and connected with the pCDH1-MCS1-EF1-copGFP (NTCC, China) vector to construct PAK1 overexpressed plasmid. Lipofectamine 2000 (Invitrogen, USA) was used to transfect PAK1 overexpressed plasmids into 293 T cells with three pSRL-PACK plasmids (Tianjin Saier Biotech, China). Lentiviruses were collected and infected with sup-B15 cells.

The mRNA detection results for K562 cells transfected with PAK1 interference lentivirus and negative lentivirus, sup-B15 cells transfected with PAK1 overexpression lentivirus and negative lentivirus were shown in Supplementary Figure S5 and the WB detection results were shown in Supplementary Figure S6. Both results showed that K562 cells transfected with PAK1 interference lentivirus have PAK1 expression decreased, while sup-b15 cells transfected with PAK1 overexpression lentivirus have PAK1 expression increased, which was in line with the expectation,

2.5. Cell line and treatment

The M-BCR-positive human CML cell line K562 and the m-BCR-positive human ALL cell line Sup-b15 were maintained in RPMI 1640 and treated as follows according to the gene expression file and validation result:

K562:

A PAK1 interference negative: treated with PAK1 interference negative lentivirus;
B PAK1 interference: treated with PAK1 interference lentivirus;
C TKI treatment: treated with 1 μM/L Imatinib;
D PAK1 interference negative with TKI: treated with PAK1 interference negative lentivirus and 1 μM/L Imatinib
E PAK1 interference with TKI: treated with PAK1 interference lentivirus and 1 μM/L Imatinib;

SUP-B15:

A PAK1 over-expression negative: treated with PAK1 over-expression negative lentivirus;
B PAK1 over-expression: treated with PAK1 over-expression lentivirus;
C TKI treatment: treated with 5 μM/L Imatinib;
D PAK1 over-expression negative with TKI: treated with PAK1over-expression negative lentivirus and 5 μM/L Imatinib;
E E: PAK1 over-expression with TKI: treated with PAK1 over-expression lentivirus and 5 μM/L Imatinib.

2.6. Stat5 and MAPK pathway phosphorylation detection

The K562 cell line and the Sup-b15 cell line treated as before were used for phosphorylation detection. The STAT5 and MAPK pathway-related protein phosphorylation was analyzed by Western blot. Protein extracts were prepared by solubilizing the cells. The proteins were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes which were then blocked with 3–5% BSA and probed with primary Abs against STAT-1, phosphotyrosine-STAT-1 (Ser727; Upstate Biotechnology), ERK, phosphotyrosine-ERK, JNK, phosphotyrosine-JNK, p38, phosphotyrosine-p38, CDK1, phosphotyrosine-CDK1, CDK2, and phosphotyrosine-CDK2 (Abcam, UK). Filters were properly developed with secondary Ig Abs conjugated to HRP and detected by the ECL-Plus Detection System.

2.7. PAK1 function analysis

K562 cell and Sup-b15 cell treated as before were used for phosphorylation detection. BrdU was used to detect cell proliferation, and MTT was used to detect cell apoptosis. Flow cytometry was used to detect cell apoptosis and the proliferation index.

2.8. Statistical analysis

Differentially expressed genes with statistical significance by T-test were identified through Volcano Plot filtering. GO analysis and Pathway analysis were performed using the standard enrichment computation method by the Fisher exact test.

Validation measurements were performed in triplicate at a minimum, and the results were expressed as the range with median or the means ± SD. One-way ANOVA in SPSS software (version 17.0, SPSS Inc., USA) was used for the statistical analysis. The differences were considered significant if $p < 0.05$.

3. Results

3.1. Gene expression profiling test and validation

Using the NimbleGen microarray platform, the transcription profiles of M-BCR-positive CML (group A) patients and m-BCR-positive B-ALL (group B) patients were analyzed.

3.1.1. Differently expressed genes screening

A total of 4218 transcripts were identified as being significantly differentially expressed (group B vs. group A with $p < 0.05$), and 3234 of them had $P < 0.01$. The number of genes demonstrating decreased expression levels was greater than the number of genes demonstrating increased expression levels (2361 vs. 1857 genes, respectively, Table1, Supplementary Figure S1).

3.1.2. Pathway analysis

A total of 84 pathways showed significantly differential expression (group B vs. group A with $p < 0.05$), with 48 up-regulated and 36 down-regulated pathways, with the highest 10 differentially expressed pathways with their Enrichment Score showed (Supplementary TableS3, Supplementary Figs. 2).

3.1.3. GO analysis

The significant enrichment analysis of the GO terms for the differently expressed genes using the R language package software revealed that these genes are involved in many important biological processes, cellular components and molecular functions (Supplementary TableS4) including apoptosis and the regulation of B cell differentiation.

3.1.4. HUB analysis

The PPI and PDI networks were inferred from the differentially expressed gene list, and the top12 hub genes ranked by the MCC method are as follows: PAK1, LCK, ZAP70, MAPK1, PTPN6, POT1, HSPA4, LCP2, LAT, VHL, CDKN1 A and ATN1 (Supplementary Figs. 3).

3.1.5. Validation of differently expressed genes

The following nine genes were chosen for validation: PAK1, LCK, CD79 A, LEF1, JUP, DNTT, ITGAM, MEFV and SERPINB1. The result (Table 2) showed that in P190 (+) B-ALL patients, PAK1 gene expression decreased significantly compared with P210 (+) CML patients. Patients after treatment till MMR have PAK1 expression increased in P190 (+) B-ALL patients, whereas Patients after treatment till MMR have PAK1 expression decreased in P210 (+) CML patients. The Pak1 protein in the CML, B-ALL and healthy control groups were also tested (Supplementary Figure S4) and had the same tendency as RNA expression.

3.2. PAK1 affects Stat5 and MAPK pathway phosphorylation differently in p210+ CML cells and p190+ ALL cells

Among the five groups of K562 cells (Fig. 1A), the STAT5 phosphorylation level was highest in the negative control group and lowest in the Imatinib + PAK1 interference group. PAK1 expression decreased after transfection with PAK1 interfering lentivirus, followed by a phosphorylation reduction in STAT5 and its downstream pathway including ERK, JNK and CDK. After the Imatinib treatment, the phosphorylation level of STAT5 decreased significantly, and the phosphorylation levels of ERK, JNK and CDK were also significantly reduced while phosphorylation of P38 showed no significant change. These results indicated that in the P210-positive CML cell line interference with PAK1 expression may lead to a decrease in the phosphorylation level of STAT5 and downstream pathways and may have a synergistic effect with TKI in phosphorylation reduction.

Among the five groups of SUP-B15 cells (Fig. 1B), the STAT5 phosphorylation level was highest in the negative control group and lowest in the Imatinib + PAK1 over-expression group. PAK1 expression increased after transfection with PAK1 over-expression lentivirus, followed by a phosphorylation reduction in STAT5 and its downstream pathway including ERK, JNK and CDK. After the Imatinib treatment, the phosphorylation level of STAT5 decreased significantly, and the phosphorylation levels of ERK, JNK and CDK were also significantly reduced while phosphorylation of P38 showed no significant change. These results indicated that in the P190-positive B-ALL cell line PAK1 over-expression may lead to a decrease in the phosphorylation level of STAT5 and downstream pathways and may have a synergistic effect with TKI in phosphorylation reduction.

3.3. PAK1 gene differentially affects cell proliferation and apoptosis in p210+ CML cells and p190+ ALL cells

3.3.1. BrdU test

In the K562 cell line (P210 + CML, Fig. 2A), after culturing for 24, 48 and 72 h, the proliferation rate was highest in the PAK1 interference negative group and lowest in the Imatinib + PAK1 interference group. The proliferation rate decreased after PAK1 interference and was most

Table 1
The number of genes differentially expressed and the 10 genes with the highest differences in gene expression by microarray analyses.

	Number of differentially expressed genes	The 10 genes with the highest differences in expression (values in fold change)
Induced	1857	MDK(5.42); HBA1(2.81); ZCCHC7(4.54); IGHM(5.30); DNTT(53.98); HBA2(3.01); GPR110(11.30); CD99(4.37); JUP(6.65); SCARB1(4.12)
repressed	2361	CEACAM1(14.97); CTN1(26.85); SPI1(4..06); LYZ(12.03); ITGAM(14.36); MEFV(9.45); IFI44L(17.56); ARG1(14.28); UNQ3033(24.09); CLEC12A(23.44)

Table 2
The verification result of nine genes between CML and ALL patient.

Gene	CML-Before treatment X ± SD	CML-After treatment X ± SD	ALL-Before treatment X ± SD	ALL-After treatment X ± SD	*P* value CML vs ALL	*P* value CML before and after treatment	*P* value ALL before and after treatment
PAK1	**8.17 ± 2.82**	**3.69 ± 1.24**	**0.30 ± 0.11**	**1.63 ± 0.52**	**0.007**	**0.03**	**0.021**
LCK	4.01 ± 3.87	24.75 ± 14.45	3.17 ± 2.23	9.45 ± 5.67	0.048	0.00	0.008
CD79A	3.79 ± 1.92	12.98 ± 7.67	17.84 ± 9.32	6.68 ± 4.17	0.008	0.00	0.007
LEF	14.38 ± 9.13	36.05 ± 14.39	23.13 ± 13.43	6.89 ± 4.12	0.023	0.00	0.001
JUP	2.01 ± 1.02	3.65 ± 2.02	3.67 ± 1.93	0.49 ± 0.27	0.046	0.14	0.021
DNTT	0.14 ± 0.09	0.46 ± 0.31	0.01 ± 0.01	0.30 ± 0.19	0.013	0.01	0.046
ITGAM	41.55 ± 26.33	98.86 ± 52.43	5.69 ± 3.12	31.66 ± 21.03	0.004	0.00	0.001
MEFV	9.40 ± 6.14	83.79 ± 48.31	2.15 ± 1.43	12.18 ± 7.33	0.011	0.00	0.008
SERPINB1	151.72 ± 80.43	248.50 ± 139.48	17.43 ± 9.34	92.47 ± 51.43	0.009	0.02	0.001

obvious after the 72 h culture, from 100% to 77.89%. In Imatinib-treated K562 cells the proliferation rate of PAK1 interference was also decreased compared with that of Imatinib alone, and the decline was most obvious after 72 h, decreasing from 48.12% to 23.81%. The results indicate that in P210 (+) CML cells, interference with PAK1 expression may inhibit cell proliferation and have synergistic effects with TKI. Meanwhile, in the SUP-B15 cell line (Fig. 2B), the proliferation rate was the highest in the PAK1 over-expression negative group and the lowest in the Imatinib + PAK1 over-expression group. The proliferation rate decreased after PAK1 over-expression and was most obvious after the 72-h culture, from 100% to 76.61%. In the Imatinib-treated SUP-B15 cells, the proliferation rate after PAK1 over-expression was also decreased compared with that of Imatinib alone, and the decline was most obvious after 72 h, decreasing from 59.39% to 30.45%. The results indicate that in P190 (+) B-ALL cells, over-expression of PAK1 may inhibit cell proliferation and have synergistic effects with TKI.

3.3.2. MTT test

In the K562 cell line (Fig. 3A), after culturing for 24, 48 and 72 h, the cell-inhibiting rate was lowest in the PAK1 interference negative group and highest in Imatinib + PAK1 interference group. The cell-inhibiting rate increased after PAK1 interference and was most obvious after the 72-h culture, from 0% to 23.20%. In Imatinib-treated K562 cells, the cell-inhibiting rate of PAK1 interference was also increased compared with that of Imatinib treated alone, and the increase was most obvious after 72 h, increasing from 39.16% to 52.12%. The results indicate that in P210 (+) CML cells, interference with PAK1 expression may increase cell inhibition and have synergistic effects with TKI. Meanwhile, in the SUP-B15 cell line (Fig. 3B), the cell-inhibiting rate was lowest in the PAK1 over-expression negative group and highest in the TKI + PAK1 over-expression group. The cell-inhibiting rate increased after PAK1over-expression and was most obvious after the 72-h culture, from 0% to 22.56%. In the TKI-treated SUP-B15 cells, the cell-inhibiting rate after PAK1 over-expression was also increased compared with that of TKI alone, and the increase was most obvious after 72 h, increasing from 36.76% to72.46%. The results indicate that in P190 (+) B-ALL cells over-expression of PAK1 may increase cell inhibition and have synergistic effects with TKI.

3.3.3. Cell apoptosis

Cell apoptosis was detected through flow cytometry. The results (Fig. 4A) indicated that in the K562 cell line the proportion of apoptotic cells (LR) increased from 10.2% (negative control) to 17.3% after PAK1 interference while the proportion of normal cells (LL) decreased from 85.1% to 78.5%.The proportion of apoptotic cells in the K562 cells treated with TKI was further increased to 29.5%. The proportion of apoptotic cells in the TKI + PAK1 interference group was the highest among the five groups, reaching 36.5% while the proportion of normal cells decreased to 59%. The result indicated that PAK1 inference may increase the apoptosis of P210 (+) CML cells, and the effect of increasing cell apoptosis may have a synergistic effect with TKI drugs. In the SUP-B15 cell line (Fig. 4B), the proportion of apoptotic cells (LR) increased from 3.93% (negative control) to 12.9% after PAK1 over-expression while the proportion of normal cells (LL) decreased from 91.9% to 85.2%. The proportion of apoptotic cells in the SUP-B15 cells treated with TKI was further increased to 15.5%, and the proportion of apoptotic cells in the TKI + PAK1 over-expression group was the highest among the five groups, reaching 30.2%; meanwhile, the proportion of normal cells decreased to 56.9%. The result indicated that PAK1 over-expression may increase the apoptosis of P190 (+) B-ALL cells, and the effect of increasing cell apoptosis may have a synergistic effect with TKI drugs.

3.3.4. Proliferation index

The proliferation index (PI) was calculated as the following formula: $PI = (S + G2/M)/(G0/G1 + S + G2/M) \times 100\%$. In K562 cells, the cell

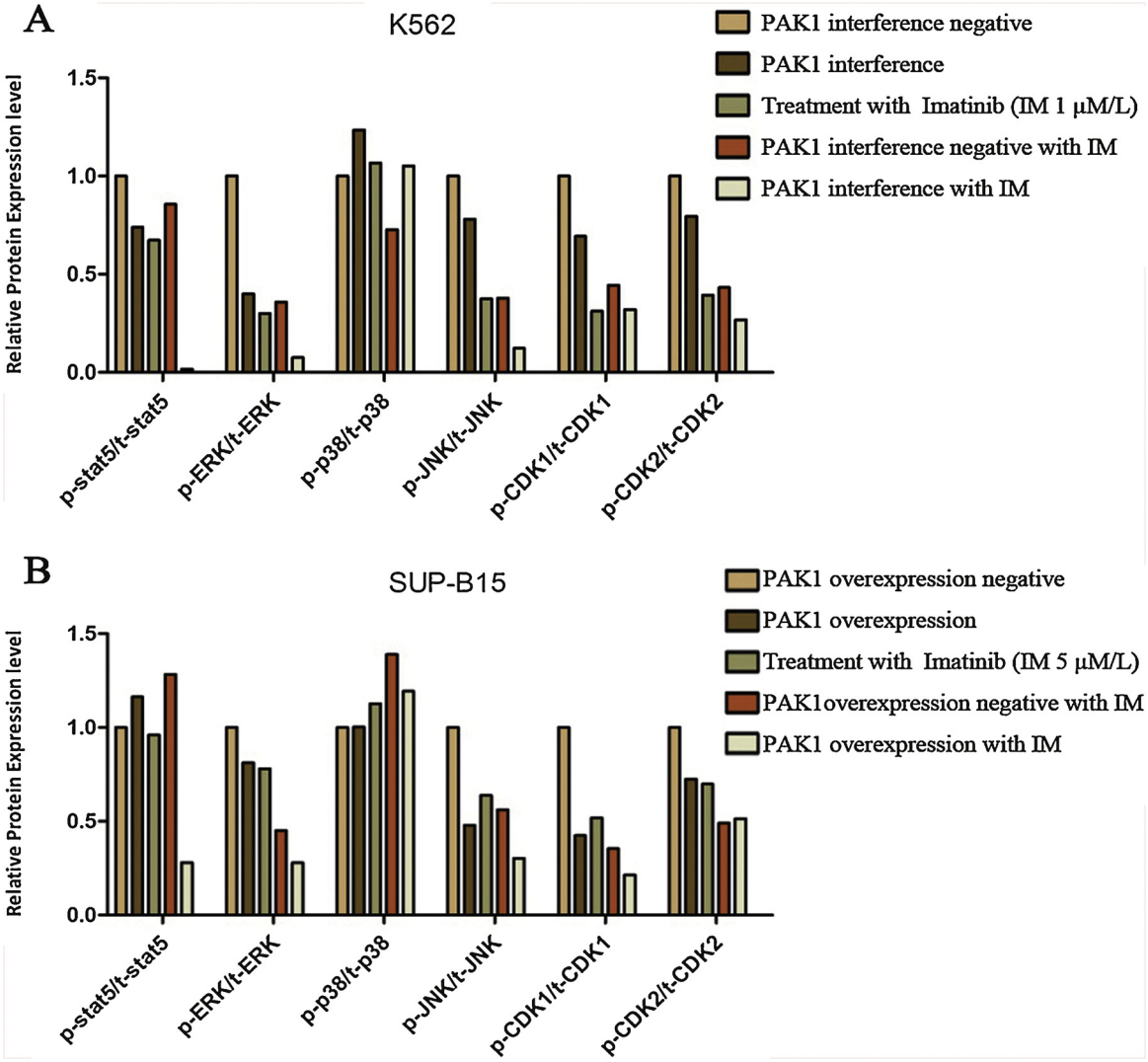

Fig. 1. Histogram of phosphorylation for STAT5 Pathway A: K562 cell line B: SUP-B15 cell line.

cycle distributions (Fig. 5A). The PI showed that in the K562 cell line -the cell proliferation index decreased from 56.86% to 52.98% after PAK1 interference and decreased to 38.05% after TKI treatment. In all 5 groups of cells, the TKI treatment + PAK1 interference group had the lowest PI with 30.05%. The results above suggest that in P210-positive CML cells, interfering with PAK1 expression may inhibit cell proliferation and have synergistic effects with TKI. In SUP-B15 cells, the cell cycle distributions in different groups are shown in (Fig. 5B) and the PI showed that in the SUP-B15 cell line, the cell proliferation index decreased from 57.79% to 45.17% after PAK1 over-expression and decreased to 30.20% after TKI treatment. In all 5 groups of cells, the TKI treatment + PAK1 over-expression group had the lowest PI of 24.00%.The results above suggest that in P190-positive B-ALL cells, PAK1 overexpression may inhibit cell proliferation and have synergistic effects with TKI.

3.4. PAK1 expression and its relationship with the clinical features in different BCR-ABL subtypes

3.4.1. P210 + CML group

One hundred ninety p210+ CML patients were enrolled and divided into three groups according to the expression of PAK1s: low value group [0.25–5.57], median group [5.59–13.16], high-value group [13.20–32.80]. The results (Table3) indicate no difference between the ages and the sexes among different groups. The relative concentrations of BCR-ABL in the three groups were 59.27%, 77.18% and 94.85%, respectively, with statistical significance, indicating a positive correlation between PAK1 expression and BCR-ABL expression. The white blood cell count (WBC) was significantly different between the three groups, 129.56 * 10 ^ 9 / L, 150.86 * 10 ^ 9 / L, and 185.04 * 10 ^ 9 / L, respectively, indicating a positive correlation with PAK1 expression; meanwhile, the other indicators showed no association with PAK1 expression. In clinical symptoms, fever and splenomegaly may be correlated with PAK1 expression, but other symptoms had no correlation with PA1 expression.

3.4.2. P190 + ALL group

One hundred twenty-nine B-ALL patients were enrolled and divided into three groups according to the expression of PAK1s: low-value group [0.0029–0.0935], median group [0.0943–0.237], and high-value group [0.245–0.94]. The results (Table3) indicated no difference between the ages and the sexes among the different groups. The relative concentrations of BCR-ABL in the three groups were 87.14%, 72.56% and 65.44%, respectively, with statistical significance, indicating a negative correlation between PAK1 expression and BCR-ABL expression. The WBC count (WBC) was also significantly different among the three groups, 36.36 * 10 ^ 9 / L, 42.48 * 10 ^ 9 / L, and 56.74 * 10 ^ 9 / L, respectively, indicating a positive correlation with PAK1 expression;

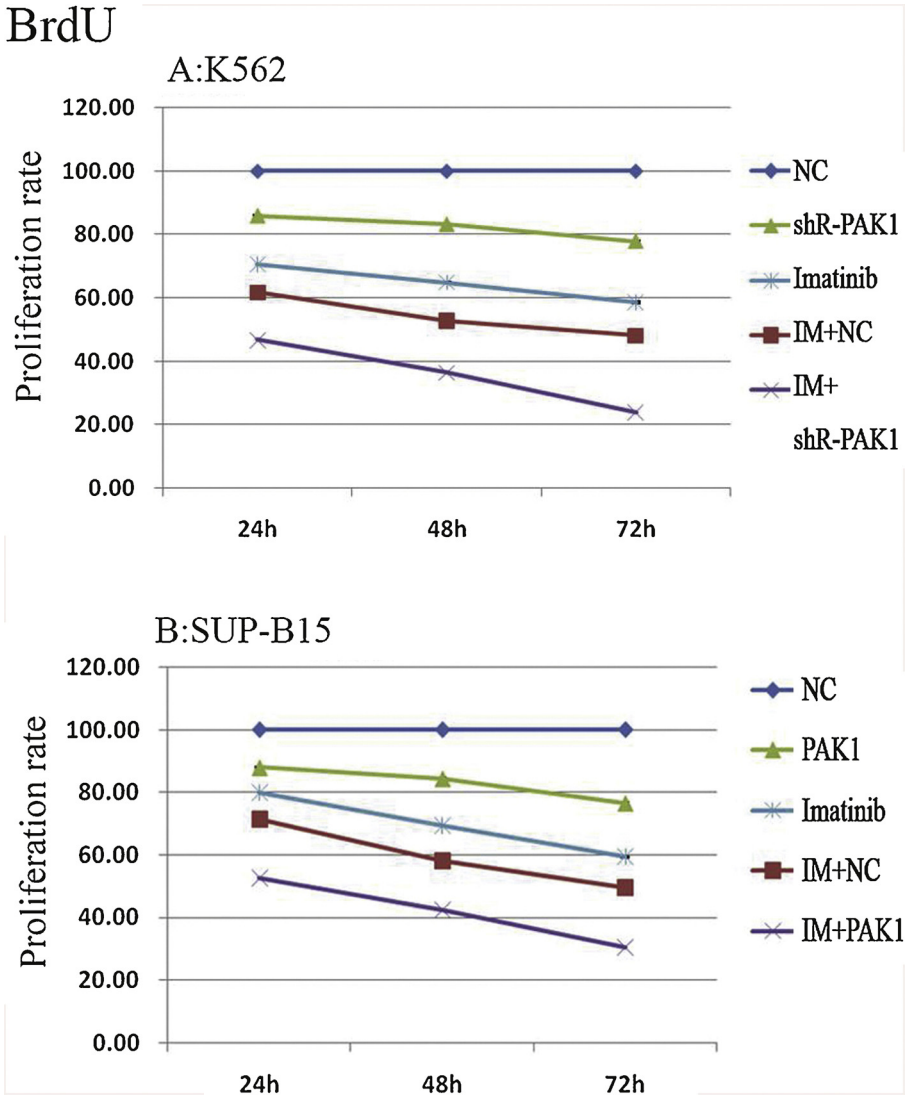

Fig. 2. line chart of BrdU test A: K562 cell line B: SUP-B15 cell line.

meanwhile, the other indicators showed no association with PAK1 expression. Additionally, no symptoms appear to be correlated with PAK1 expression.

4. Discussion

The BCR-ABL fusion gene associated with the Ph chromosome is one of the most frequently detected abnormal genes in leukemia. 90–95% of CML patients, 25%–30% of adult ALL patients and 2–5% of child ALL patients started with the characteristic cytogenetic t (9;22) (q34;q11) abnormality. The BCR-ABL fusion gene encoding of different fracture sites produces abnormal fusion proteins of different sizes, which may affect the phenotype of the disease. Furthermore, a difference in the prognosis between P190 and P210 subtype may exist, and the prognosis of the P190 subtype was worse than that of the P210 subtype. When treated with TKI drugs, the P190 patients reaching main molecular biologic remission (MMR) were more likely to relapse [12–14]. Some reports have suggested P190 to be the deletion mutant of P210, the lack of which has the function of "escalator" DH - pH domain structure, and it is often accompanied by other mutations, which may lead to the characteristics of fast onset and worse prognosis [15]. However, few studies have discussed the different genes and regulatory mechanism between the P190 and P210 subtypes directly. Exploring the regulatory mechanism involved in the leukemia development process in different BCR-ABL subtypes may have important significance in further understanding BCR–ABL-related pathogenesis, searching for new markers, and improving the level of diagnosis and treatment of leukemia related to the BCR-ABL fusion gene.

In this research, gene expression profiling chips were used for screening differentially expressed genes between the BCR - ABL subtypes. Age and gender were matched between groups to minimize interference factors that may influence the results. By chip screening, thousands of differentially expressed genes were discovered between different BCR-ABL subtypes. Our results coincide with the study by Reckel [16], which discussed the difference signal network between P210 and P190 by proteomics, hinting at the existence of multiple network differences among different BCR-ABL subtypes associated with many genes and signaling pathways which may produce different effects in different types of cell functions. According to the RT-PCR validation, the average relative concentration of the PAK1 gene in P210 + CML was 27.23 times that in P190+ B-ALL before treatment, and it decreased to 2.26-fold after treatment with significant statistics. The result not only confirmed that PAK1 is differentially expressed between different BCR-ABL subtypes, but it also indicated that PAK1 has different responses to the treatment and is one of the important differentially expressed genes between different BCR-ABL subtypes.

In addition, some reports suggest that PAK1 plays an important regulatory role in the occurrence and development of tumors with the

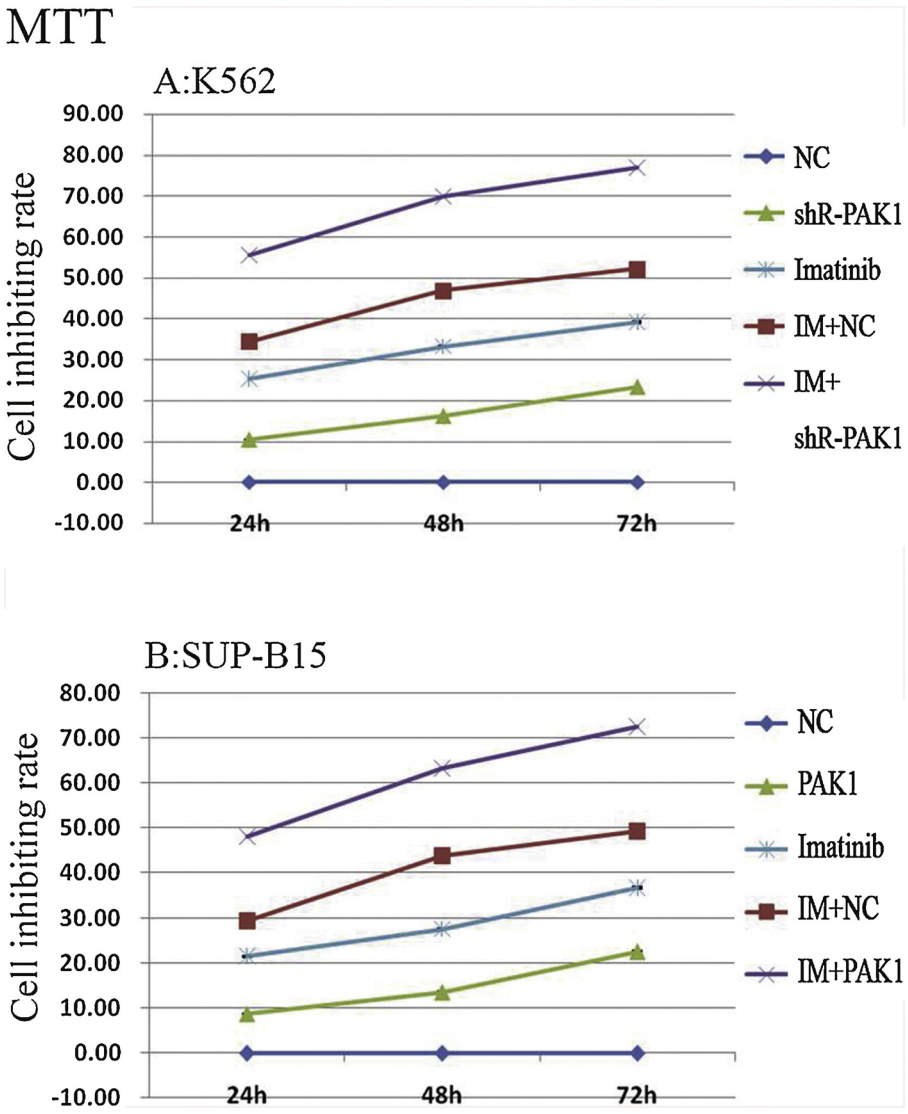

Fig. 3. line chart of MTT test A: K562 cell line B: SUP-B15 cell line.

function of the central node of the complex network of signal transformation, and excessive expression of PAK1 may affect cell tumor and survival mode conversion [17], whereas PAK1 phosphorylation helps to protect cells from apoptosis. Wang showed that the PAK1 gene, a group I p21-activated kinase confirmed to be a breast cancer oncogene [18,19], co-regulated multiple signaling pathways leading to malignant transformation and is the regulatory factor of STAT5. The PAK1 gene connects the Rac and STAT5a signal transduction pathways, and it is implicated in a wider range of cellular processes including cell proliferation, apoptosis, migration and adhesion to the extracellular matrix [20,21]; it may play a role in regulating the BCR-ABL fusion gene in CML. Therefore, PAK1 is selected for further study.

To further explore the effect of PAK1 on the STAT5 pathway in different BCR-ABL subtypes, the regulation of the phosphorylation of the STAT5-MAPK biological axis was analyzed by a cytological experiment. The results indicated that PAK1 could positively regulate the phosphorylation of STAT5, ERK and JNK in the P210 (+) CML cell line, and it also had positive regulatory effects on CDK. In addition, after TKI treatment, the phosphorylation level of STAT5, MAPK and CDK all decreased, consistent with earlier reports [22–24]that the expression of STAT5 decreased significantly after dasatinib treatment in K562 cells, indicating that STAT5 is an important treatment target. When the PAK1 interference lentivirus was transfected after TKI treatment, the phosphorylation level of STAT5 and the downstream MAPK and CDK decreased further, indicating that in the K562 cell line the inhibition of PAK1 may enhance the effect of TKI in inhibiting phosphorylation in STAT5-MAPK pathways. This is consistent with the reports of Hazlehurst [25] and others research results, and it demonstrated that the PAK1-STAT-5-MAPK biological axis plays an important role in BCR-ABL P210 subtype-mediated CML and is necessary for P210 + CML. However, in the sup-b15 cell line the results suggest that PAK1 has an opposite regulation on STAT5 to that in K562, which is contrary to other reports on CML [20]. Although Dalgic suggested that the P210 and P190 subtypes play a similar role in the early precursor cells of the red line and that STAT5 is an important regulator in both subtypes, a functional proteomics analysis [16] indicated that P190 had a network different from the P210 subtypes caused by the positioning differences. The P190 subtype showed stronger phosphorylation activity, which may lead to "wiring" in the network system of cellular pathways and finally lead to the difference in STAT5 phosphorylation. The difference in the intrinsic structure in different subtypes of BCR-ABL has been speculated to lead to differences in locating in cells, resulting in "wiring" to the signal network system; this may be the main reason for the difference in PAK1 in the BCR-ABL subtypes. However, there's no significant difference in p38. This may be related to the different function of P38, ERK, and JNP. P38, for example, is present in most cells and is

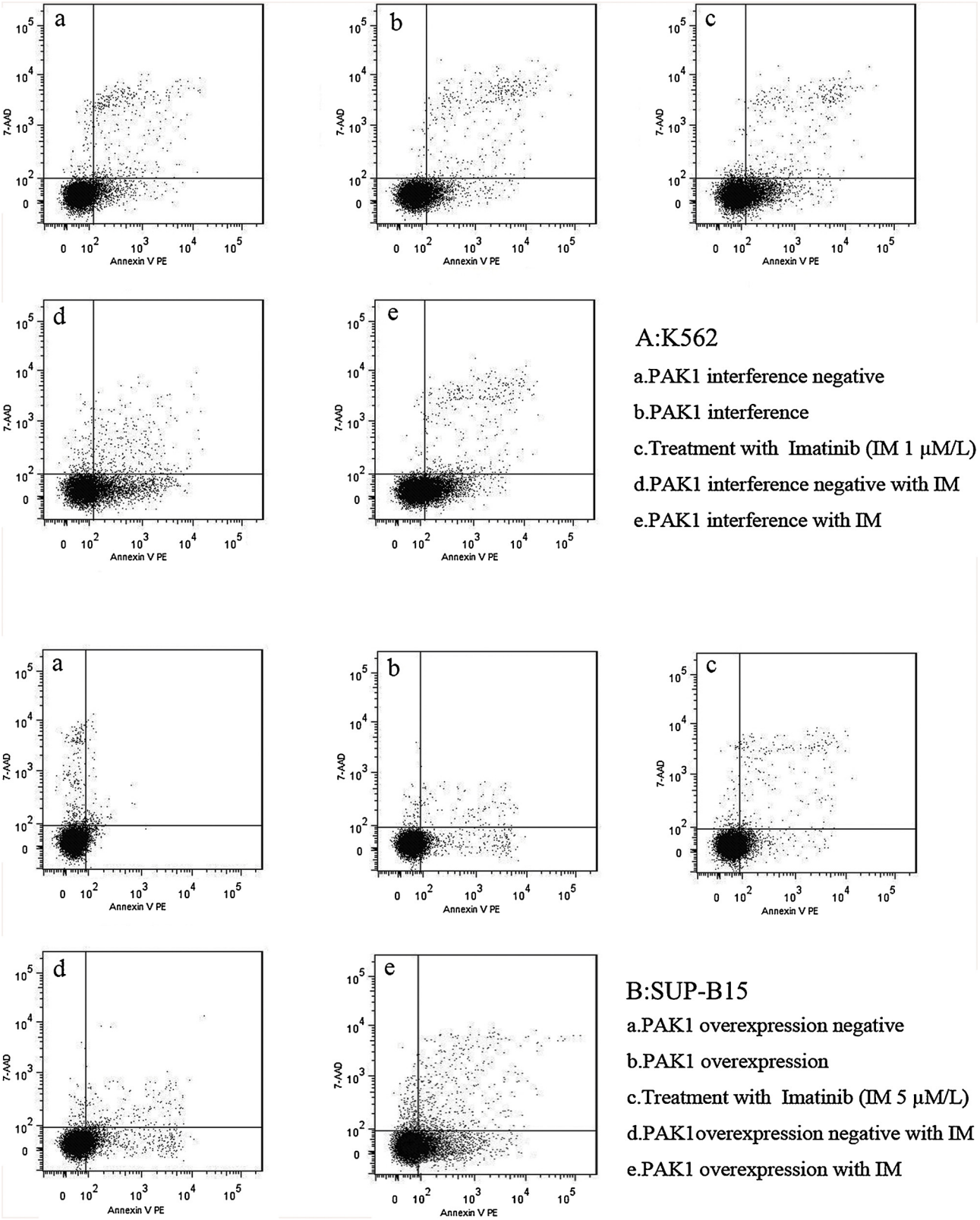

Fig. 4. Flow Cytometry Scatter Diagram for Cell Apoptosis A: K562 cell line B: SUP-B15 cell line.

an important signal system that transfer the extracellular signal to the intracellular response in eukaryotic cells, which may further affect the inflammatory response and cell metabolism. Second, the effects of PAK1 on the proliferation and apoptosis of different BCR-ABL subtypes of leukemia cells were studied by cytological experiments. The results suggest that in the K562 cell line interference of PAK1 expression can inhibit cell proliferation and promote cell apoptosis, which is advantageous to the inhibition of leukemia cells; this is in accordance with the report of Deacon [26] that the activation of PAK1 can promote cell proliferation through mitosis. Otherwise, some reports [18,27] have

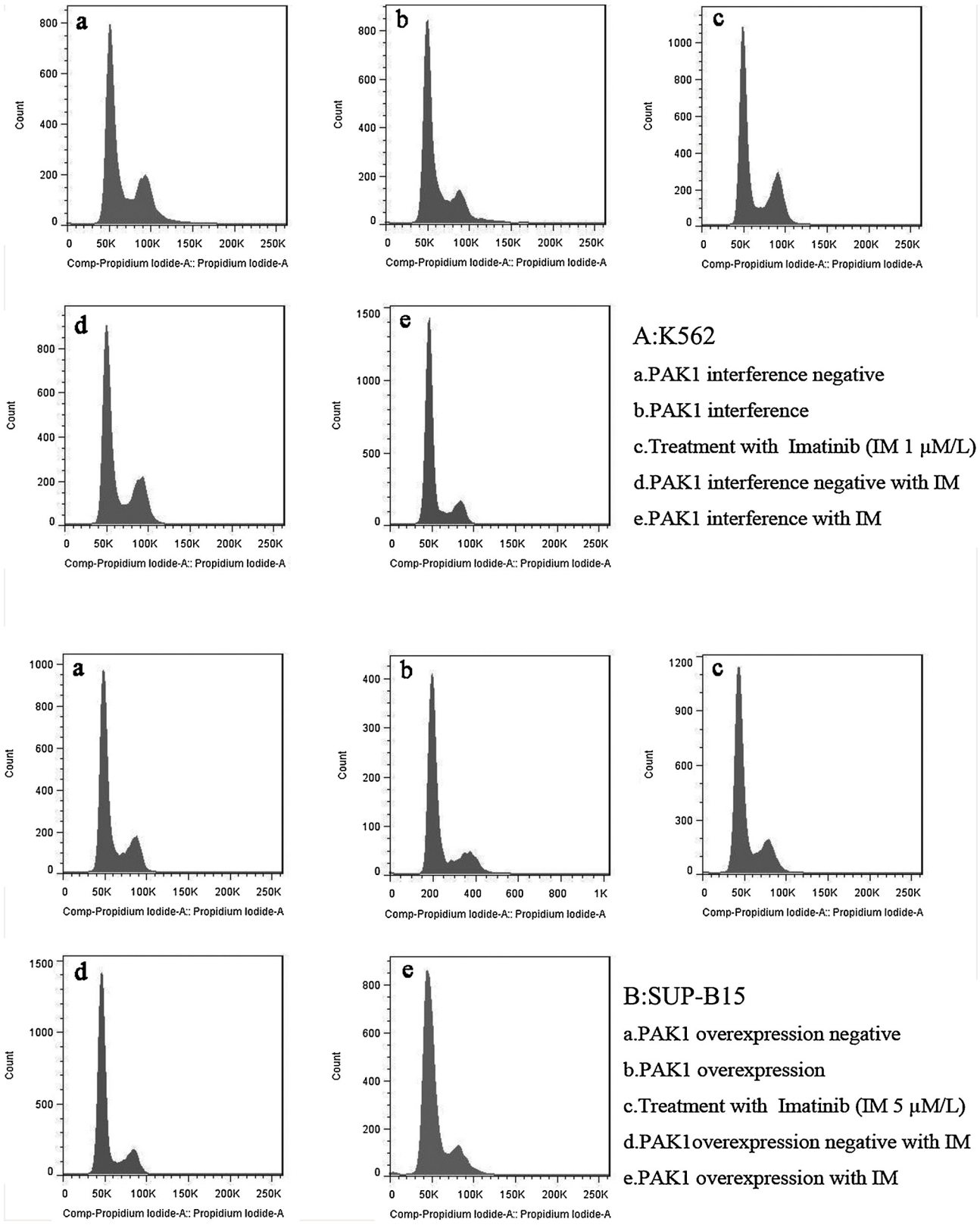

Fig. 5. Cycle Distributions for Proliferation Index(PI) calculation A: K562 cell line B: SUP-B15 cell line.

shown that in breast cancer cells the inhibition of PAK1 can inhibit the PAK1-RAC biological axis through mediated apoptosis, which significantly improved the efficacy of cancer chemotherapy. These reports are consistent with our study showing that interference with PAK1 expression can strengthen TKI function in the K562 cell line. However, in the SUP-B15 cell line, increased PAK1 expression is advantageous to the inhibition of leukemia cells, and over-expression of PAK1 may enhance TKI pharmacological function in inhibiting proliferation and promoting apoptosis, which is contrary to the effect on the K562 cell line. This may also be caused by "wiring" in the network system of

Table 3
expression and clinical feature in BCR-ABL-p210 CML and BCR-ABL-p190 ALL.

subtype	items	PAK-low	PAK-mid	PAK-high	*P*
BCR-ABL p210 + CML	age/median	41.2	42.5	42.8	0.984
	gender	male : 39 female : 24	male:40 Female : 23	male : 36 female : 28	0.683
	BCR-ABL/average	**59.27%**	**77.18%**	**94.85%**	**< 0.001**
	WBC/average	**129.56 × 10^9/L**	**150.86 × 10^9/L**	**185.04 × 10^9/L**	**0.012**
	RBC/ average	3.68 × 10^12/L	3.48 × 10^12/L	3.72 × 10^12/L	0.112
	PLT/ average	422.93 × 10^9/L	362.02 × 10^9/L	519.27 × 10^9/L	0.343
	fever/number	**3**	**1**	**9**	**0.014**
	splenomegaly / number	**47**	**46**	**54**	**0.018**
	dizzy / number	24	27	36	0.051
	anemia/ number	0	0	1	0.369
	Pain/ number	0	0	1	0.369
	Weakness/ number	40	39	44	0.538
BCR-ABL p190 + ALL	age/median	40.56	37.07	37.74	0.886
	gender	male : 21 female : 22	male:23 Female : 20	male : 21 female : 22	0.816
	BCR-ABL/ average	**87.14%**	**72.56%**	**65.44%**	**< 0.001**
	PLT/ average	**36.36 × 10^9/L**	**42.48 × 10^9/L**	**56.74 × 10^9/L**	**0.024**
	RBC/ average	2.95 × 10^12/L	3.13 × 10^12/L	2.86 × 10^12/L	0.126
	WBC/ average	82.20 × 10^9/L	48.87 × 10^9/L	57.79 × 10^9/L	0.132
	fever/number	20	24	18	0.430
	dizzy / number	11	10	9	0.777
	bleeding / number	26	16	16	0.063
	Blast cell percentage/median	83.97%	80.35%	76.43%	0.726
	anemia/ number	26	26	32	0.376
	Pain/ number	22	27	22	0.542
	Weakness/ number	19	20	17	0.724

cellular pathways and the difference in STAT5 phosphorylation caused by cellular pathway "wiring."

Finally, the study group discussed the relationship between the expression of PAK1 mRNA and the clinical manifestations of different leukemia caused by different BCR-ABL subtypes. The result suggests that in the P210 (+) CML group, PAK1 expression has a positive correlation with poor prognosis factors (BCR-ABL relative quantitative, the WBC) which indicated that high expression of PAK1 is likely to be a poor prognosis factor in CML. This is in accordance with the reports that PAK1 expression led to a number of centrosome and spindle structure changes, causing aneuploidy and resulting in the loss of tumor suppressor genes and the accumulation of oncogenes, which eventually led to the occurrence of a tumor [22,23]. In the P190 (+) B-ALL group, PAK1 expression has a negative correlation with poor prognosis factors (BCR-ABL relative quantitative) and a positive correlation with good prognosis factors (PLT), demonstrating that low expression of PAK1 is likely to be a poor prognosis factor in Ph + ALL patients. These results confirmed our hypothesis that the BCR-ABL subtype is an important factor for PAK1 expression, and understanding the PAK1 mechanism in different BCR-ABL subtypes is helpful to further understand the pathogenic mechanism of different BCR-ABL subtypes.

This study suggests that the distinct differences in the structure the BCR - ABL subtype can lead to regulatory differences in PAK1-STAT5–MAPK, which may further imply the regulation of the proliferation and apoptosis of leukemia cells, leading to the differences in the generation and prognosis of leukemia. The result indicated that PAK1 may be not only an important molecular mechanism in the process of the development of leukemia but also the important molecular mechanism of the differences in the pathogenesis and drug response between the P210 and P190 subtypes. It can be speculate that different regulation of pak1 according to different subtypes of BCR-ABL may help improve the effect of TKI on leukemia with different BCR-ABL subtypes, which give a hint to the development of new targeted drugs. Therefore, the research on PAK1 regulation is also of great importance. Current research [28] has shown that the expression of PAK1 can also be regulated by miRNA through bioinformatic analysis, finding that microRNA7 (miR-7), which has a complementary sequence with PAK1 in the 3′- UTR region, may be a potential regulator of PAK1, providing a further direction for our research. Also, there are some limitations in our study. First, p190 CML and p210 ALL were not involved because the limitation of patients and cell line. Second, other different expressed genes, such as CD79a and LEF1, were not further studied, which will be the direction for our further study.

5. Conclusions

Our findings delineate that PAK1 plays an importance role in regulation of STAT5 pathway between different BCR-ABL subtypes, which may influence Cell proliferation and apoptosis and even take effect in therapeutic effect of TKI and thus influence patient prognosis.

Contributions

Yuanxin Ye managed the research and wrote the paper. Yanhong Zhou designed the detailed experiment and guide the experiment. Qin Zheng provided the data of complete blood count and bone marrow smear. Sishi Tang analysed the data. Yang Dai provided the data of clinical feature. Yi Zhou performed the experiment of flow cytometry. Minjin Wang performed the experiment of Cytology experiment. Juan Zhou performed the experiment of molecular biology. Xiaojun Lu guide the experiments and helped designing the research study. Lanlan Wang provided the foundation. Binwu Ying designed the research study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.leukres.2019.01.012.

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