

Insulin-like growth factor-binding proteins play a significant role in the molecular response to imatinib in chronic myeloid leukemia patients

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Abstract. Imatinib (IM) is successfully used in the majority of patients with chronic myeloid leukemia (CML), but some patients develop resistance to drug treatment. Insufficient apoptosis results in uncontrolled cell proliferation, which is closely associated with the occurrence of drug resistance. Therefore, it is crucial to identify new biomarkers related to drug resistance. This aim of the present study was to investigate the profile of apoptosis-related proteins in K562 and K562/G (IM-resistant K562 cells) cells, in order to identify new biomarkers. A human apoptosis antibody array was used to screen 46 proteins in the two cells lines, among which 20 proteins were found to be differentially expressed between K562 and K562/G cells. The major proteins included secreted caspase-8, insulin-like growth factor-binding protein (IGFBP)-1, IGFBP-2, IGFBP-3, caspase-3 and p27. IGFBP-1, IGFBP-2 and IGFBP-3 were selected for the follow-up study. Subsequently, reverse transcription-quantitative PCR analysis and western blotting were used to detect the expression levels of the IGFBPs. The results revealed that the expression levels of IGFBP-2 and IGFBP-3 in K562/G cells were significantly decreased compared with those in K562 cells, whereas the IGFBP-1 level was higher. Moreover, no significant correlation was observed between IGFBP-1 or IGFBP-2 and the level of the BCR-ABL fusion protein, whereas decreasing IGFBP-3 levels were associated with increasing BCR-ABL levels. These results suggested that IGFBP-1, IGFBP-2 and IGFBP-3 could be useful novel biomarkers for IM resistance in CML.

Introduction

Chronic myeloid leukemia (CML) is a clonal hematopoietic stem cell disease and its incidence among all adult leukemia

cases is 10-15% (1,2). CML is more common in middle-aged patients, and may be associated with malnutrition, night sweats, hematopenia and bleeding (3). CML may be divided into the chronic, accelerated and blast phases, and the majority of the patients are in the chronic phase at the time of diagnosis (4,5). Imatinib mesylate (IM) was the first tyrosine kinase inhibitor (TKI) to be used for the treatment of CML in clinical settings, and has provided a survival benefit by restoring normal hematopoiesis and achieving hematological, cytogenetic and molecular remission (6). However, despite the satisfactory efficacy of IM and second- and third-generation TKIs, a proportion of patients display varying degrees of resistance (7). Therefore, it is crucial to further investigate the molecular mechanism underlying the development of drug resistance and identify new targets to overcome this resistance.

The main components of the insulin-like growth factor (IGF) axis include the type 1 IGF receptor and insulin receptor, ligands (IGF-1 and IGF-2) and IGF binding proteins (IGFBPs) (8,9). IGF is a type of multifunctional cell proliferation regulator (10). IGFBPs play an essential role in the proliferation and differentiation of various cell types, and body development (11). It was previously demonstrated that the transmembrane tyrosine kinase receptor on the cell surface mainly mediates the biological functions of the IGF axis, and six IGDBPs mainly regulate its activity (12,13). Signal dysregulation has been associated with chemoresistance and radioresistance (14). The role of the IGF axis in tumors, such as malignant renal tumors, gastrointestinal cancer, breast cancer and hematological malignancies has been extensively investigated (15,16). However, it remains unclear whether the IGF axis plays a role in IM resistance of CML.

In the present study, protein microarray technology was used to assess differentially expressed proteins (DEPs) in K562 cells and K562/G (IM-resistant K562) cells. An apoptosis antibody array was used to screen 46 proteins in the cells, among which 20 proteins, differentially expressed between K562 and K562/G cells, were identified. Reverse transcription-quantitative (RT-q)PCR and western blot analyses were used to detect the levels of IGFBP-1, IGFBP-2 and IGFBP-3 in K562 and K562/G cells. In addition, the expression levels of IGFBP-1, IGFBP-2 and IGFBP-3 were detected in the peripheral blood (PB) of healthy individuals, patients with optimal response and patients with treatment failure. Furthermore,

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it was investigated whether there were correlations between IGFBP levels and BCR-ABL, in order to determine whether IGFBPs may be of value as a specific protein marker of imatinib resistance in CML. The findings of the present study may help identify novel targets for the treatment of CML.

Materials and methods

Cell culture and treatment. Human CML K562 cells were obtained from the Shanghai Institute of Life Sciences. The IM-resistant clone K562/G was obtained by constant exposure to increasing IM concentrations of up to 15 $\mu\text{mol/l}$. The K562 and K562/G cells were grown in Iscove's Modified Dulbecco's medium supplemented with 10% FBS (both purchased from Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin. In addition, the K562/G cells were cultured in the continuous presence of 1 $\mu\text{mol/l}$ imatinib. The methods for the detection of cell drug resistance, were as mentioned in an earlier study (17). The K562/G cells exhibited significantly higher resistance to IM, with >50-fold increase of the IC₅₀ value, compared with K562 cells.

Human subjects and blood samples. CML patients who were treated by chemotherapy at the Anhui Provincial Hospital between March 2018 and April 2019 were recruited in the present study. The study protocols were approved by the Institutional Review Board of Anhui Provincial Hospital and informed consent was obtained from all participants, according to the principles outlined in the Declaration of Helsinki. The patients were divided into different groups according to the therapeutic effect and time of treatment. A total of 19 healthy individuals served as control subjects. The patients were newly diagnosed and treated with IM 400 mg/day. All CML samples were subjected to cytogenetic analysis and RT-qPCR. The response to treatment was evaluated according to the relevant guidelines (18). Patients whose response lasted for at least 6 months, without discontinuing or changing drugs, were recruited into the corresponding group (optimal response or treatment failure). The remaining patients were divided into four groups according to the duration of treatment. Clinical data on all subjects are summarized in Tables I and II. PB samples were collected from the subjects and PB mononuclear cells were isolated using gradient centrifugation (400 x g for 20 min at room temperature) by layering on top of Ficoll (GE Healthcare). Total RNA was extracted and stored at -80°C until further use.

Apoptosis antibody array processing. An apoptosis antibody array (Ray Biotech, Inc.) was used to explore the differences in the apoptotic protein profile between K562 and K562/G cells. First, 100 μl of blocking solution was added to each well and incubated for 1 h at room temperature. Second, the blocking solution was removed, 100 μl sample was added to each well (500 $\mu\text{g/ml}$) and washed with Wellwash Versa chip (Thermo Fisher Scientific, Inc.). Third, 300 μl blocking solution and 70 μl biotin-labeled antibody were added to each well; after incubating for 2 h at room temperature, 70 μl of 1,500-fold diluted fluorescent dye-streptavidin conjugate was added to each well. Finally, the signal was scanned by an Axon MaPix laser scanner (Molecular Devices, LLC) and

Table I. Clinical characteristics of patients with optional responses and response failure.

Group	Optional responses (n=21)	Response failure (n=19)
Age	44.43±13.59	41.95±15.77
Sex		
Male	12	9
Female	9	10
White blood cells (10 ⁹ /ml)	9.06±4.36	63.55±65.13
BCR/ABL international scale (%)	0.03±0.08	19.17±17.61

data analysis was performed with software specific for Human Apoptosis Array G1 (AAH-APO-G1). DEPs were defined as those with fold-change >1.2 or <0.83, and a fluorescent value >150 according to the manufacturer's protocol to observe more differential proteins and further verify them in subsequent experiments. In order to analyze the related pathways that were enriched in the DEPs, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (<https://www.genome.jp/kegg/>) with the R package 'clusterProfiler' (v3.0.4) (19) was performed to link genomic information with higher-order functional information. Specifically, the method used was Fisher's precise inspection and the number was derived from R/Bioconductor according to the 'clusterProfiler' package. The selection criteria were the number of genes that fall on a certain term/pathway ≥ 5 and $P < 0.05$. The term/pathway obtained in the pathway is arranged in descending order according to the count value.

RNA extraction and RT-qPCR analysis. RNA was isolated and cDNA was prepared as described previously (20). The Power SYBR® Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for RT-qPCR and the PCR primers are shown in Table III. The mRNA levels were normalized to GAPDH. The changes in mRNA expression levels were calculated using the comparative C_q method, as follows: Fold-change = $2^{-\Delta\Delta C_q}$ (21). Healthy patients' data and untreated patients would be set to '1' and the other groups' expression levels shown 'relative' to that. The PCR conditions for all genes were as follows: Initial activation at 95°C for 30 sec, followed by 40 cycles at 95°C for 3 sec and at 60°C for 30 sec. Fluorescence determination at the melting temperature of the product for 20 sec was performed on a QuantStudio™ 5 Real-Time PCR instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.).

Western blot analysis. Cells were prepared for protein extraction using RIPA buffer containing a protease inhibitor cocktail (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. The protein concentration was estimated by the Enhanced BCA Protein Assay kit (Beyotime Institute of Biotechnology). Protein (40 μg) samples were separated on 12% SDS-PAGE, followed by transfer to a PVDF membrane; the membranes were then blocked in 5% skimmed milk for

Table II. Clinical characteristics of patients taking medicine for different durations.

Group	Untreated	<6 months	6-12 months	>12 months
Age	47.15±18.74	43.20±16.56	44.14±17.27	39.74±11.74
Sex				
Male	11	9	8	15
Female	9	6	5	12
White blood cells (10 ⁹ /ml)	75.97±80.21	34.37±48.45	24.88±54.14	7.48±7.57
BCR/ABL international scale (%)	66.46±65.34	10.08±15.36	3.12±5.65	3.12±10.51

Table III. Primers for reverse transcription-quantitative PCR.

Gene	Primer sequence (5'-3')
IGFBP-1	F: CACAGGGTATGGCTC R: CTTCTGGGTCTTGGG
IGFBP-2	F: CGATGCTGGTGCTTCTCA R: GGGGTCTTGGGTGGG
IGFBP-3	F: CTCTCCCAGGCTACACCA R: GAAGTCTGGGTGCTGTGC
GAPDH	F: GAGCGAGATCCCTCCAAAT R: GGCTGTTGTCATACTTCTCATGG

IGFBP, insulin-like growth factor-binding protein.

1.5 h at 37°C. The membrane was subsequently incubated with specific antibodies: IGFBP-1 (1:1,000; cat. no. 31025T; Cell Signaling Technology, Inc.), IGFBP-2 (1:1,000; cat. no. 3922S; Cell Signaling Technology, Inc.), IGFBP-3 (1:1,000; cat. no. 13216S; Cell Signaling Technology, Inc.) and β -actin (1:100,000; cat. no. AC026; Abclonal). On the following day, TBS containing 0.1% Tween 20 was used to wash the membrane again for 15 min. Then, the membranes were incubated with horseradish peroxidase conjugated-Goat Anti-Rabbit IgG (H+L) (1:50,000; cat. no. A21020; Abbkine Scientific Co., Ltd.) for 1 h at 37°C before washing the membrane for 15 min once again. Finally, the membranes were treated with BeyoECL Moon (P0018FS; Beyotime Institute of Biotechnology) and digitalized by scanning (Fusion Solo3 v 16.12; Fusion FX; VilberLourmat).

Statistical analysis. Protein microarray data were statistically analyzed with the 'R' programming language (R version 3.6.2) (22). After raw data were normalized by the software, DEPs were screened by fold-change and P-value. Data are expressed as the mean \pm SD of three independent experiments. All statistical analyses were performed with the SPSS software, version 17.0 (SPSS, Inc.). Differences between two cells lines were determined by Student's t-test. One-way ANOVA and Bonferroni's post hoc test were used in the analysis of the differential expression of IGFBPs in human subjects. Non-parametric Spearman's correlation was used to analyze the association between two indicators. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Analysis of apoptosis antibody array data. In the present study, a total of 46 proteins associated with apoptosis were detected, 20 of which were found to be differentially expressed between K562 and K562/G cells. To identify DEPs, the fold-change of each protein was analyzed individually between two groups. It was observed that the levels of BCL-w, TRAILER-1, HTRA, caspase-8, SMAC, HSP27, BID, Stnf-R1, IGFBP-1 and IGF-II were increased in K562/G cells, while those of IGFBP-3, Stnf-R2, P27, Survivin, TRAILER-4, caspase-3, IGFBP-2, FAS, cytoC and CD40L were decreased. Among these DEPs, IGFBP-1, IGFBP-2 and IGFBP-3 were significantly different between K562 and K562/G cells (Fig. 1A). Furthermore, enrichment of the KEGG pathway with the R package 'clusterProfiler' was performed to analysis these pathways mainly enriched in the DEPs. A total of 12 pathways were mainly enriched in the DEPs, including apoptosis, apoptosis-multiple species and the p53 signaling pathway (Fig. 1B).

IGFBP-1, IGFBP-2 and IGFBP-3 are differentially expressed in cells and CML patients. After apoptosis antibody array analysis, RT-qPCR and western blot analyses were conducted to validate microarray data. The results of the RT-qPCR analysis revealed that the expression levels of the IGFBP-1 were significantly increased in K562/G cells compared with K562 cells ($P < 0.05$; Fig. 2A); conversely, the levels of IGFBP-2 and IGFBP-3 were lower. The results of western blotting were consistent with those of RT-qPCR analysis (Fig. 2B). Furthermore, significantly increased IGFBP-1 expression was observed in patients with treatment failure, compared with that in patients with optimal responses and healthy individuals ($P < 0.05$). In addition, the mRNA expression of IGFBP-2 and IGFBP-3 in the patients with optimal response was significantly increased compared with that in healthy individuals and patients with treatment failure (Fig. 2C).

Expression of IGFBP-1, IGFBP-2 and IGFBP-3 by medication time and association with BCR-ABL. The patients were divided into four groups to further investigate the changes of IGFBPs in PB of CML patients with prolonged treatment time. RT-qPCR was used to detect the expression of IGFBP-1, IGFBP-2 and IGFBP-3 in the PB of patients in each group. The present study compared each treatment group with the untreated group. The results revealed that IGFBP-1 was significantly increased in untreated patients compared with patients who had been receiving IM for >12 months ($P < 0.05$).

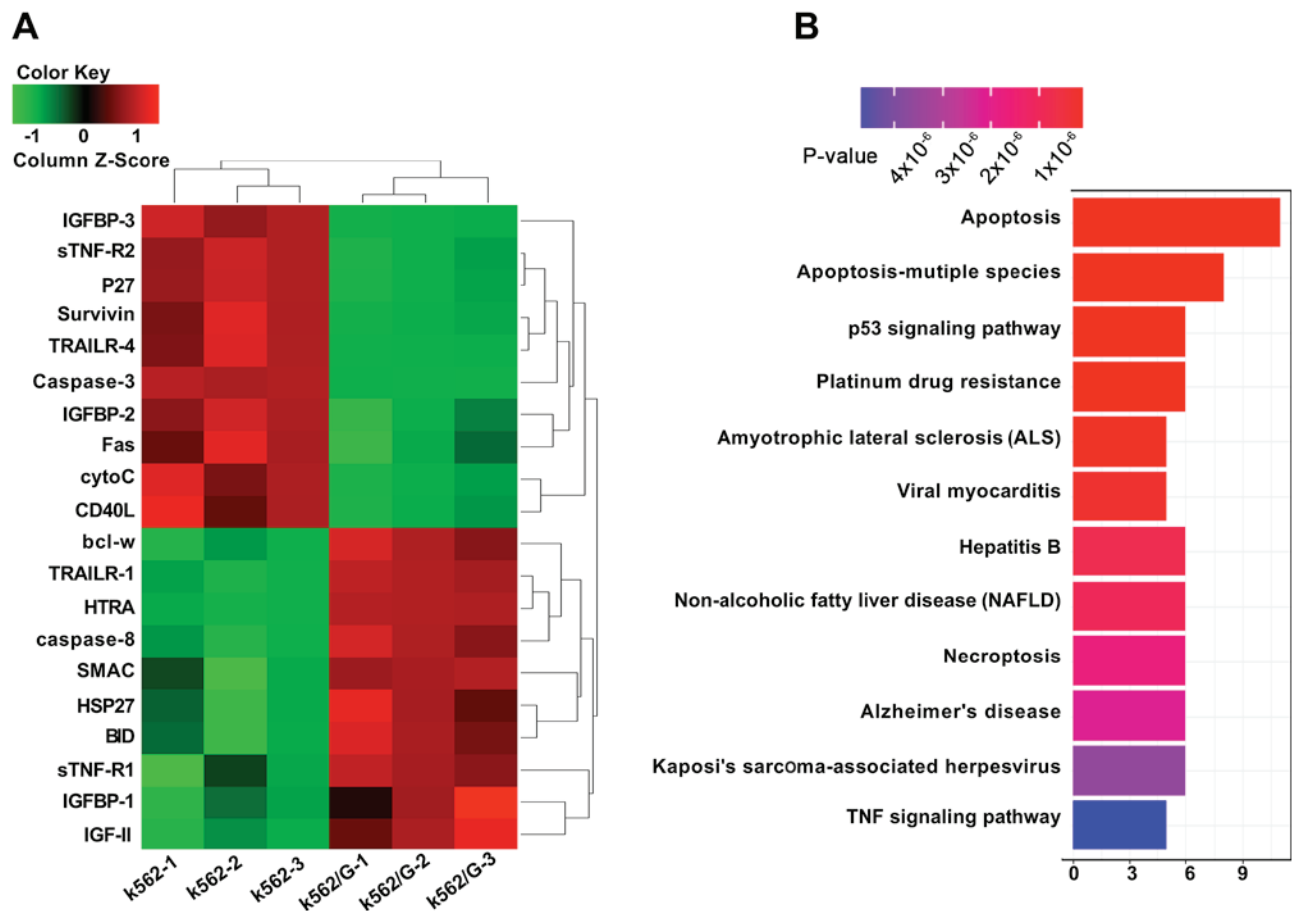


Figure 1. Results of apoptosis antibody array data. (A) Heat maps of differentially expressed proteins between K562 and K562/G cells, the cut-off value was set as follows: Fold-change <1.2 or <0.83, fluorescent value >150 and P<0.05 (K562/G VS K562). (B) Kyoto Encyclopedia of Genes and Genomes pathway analysis of differentially expressed proteins.

Conversely, IGFBP-2 and IGFBP-3 were decreased in the PB of untreated patients compared with those treated (Fig. 3A). There was no significant difference among the groups whose medication time was <12 months. No significant correlation was found between IGFBP-1 or IGFBP-2 and the level of BCR-ABL ($r=0.144$, $P>0.05$ and $r=-0.112$, $P>0.05$, respectively). However, a decrease in IGFBP-3 levels was observed with increasing BCR-ABL levels ($r=-0.602$, $P<0.0001$; Fig. 3B).

Discussion

IM is currently the main agent used for the treatment of CML. IM can inhibit the proliferation of leukemic cells and promote their apoptosis by targeting the BCR-ABL fusion gene and inhibiting the activity of the BCR-ABL fusion protein (23,24). The advent of imatinib has markedly improved the survival rate of CML patients and certain shortcomings were overcome by the second- and third-generation TKIs (25,26). Unfortunately, IM resistance in CML is a frequent occurrence and poses a major clinical challenge in the successful treatment of CML. The most common cause of TKI resistance is gene mutations of BCR-ABL1, which usually occur at a frequency of 40-90% (27). Other drug resistance mechanisms include abnormal expression of Src family kinase, an epigenetic mechanism, abnormal expression of tumor drug

resistance-associated proteins, increased telomerase activity and the presence of leukemic stem cells (28-32). The research for targets apart from BCR-ABL1 is crucial for the treatment of CML.

To the best of our knowledge, the present study is the first to propose a high-throughput robust protein array method, which has enough clinical specificity and sensitivity, for identifying new protein targets. As an advanced new tool, protein chip technology has developed rapidly in recent years (33,34). Its basic principle is that all types of proteins are fixed on various carriers in an orderly manner. The designated, labeled antibodies can be matched by chromatin immunoprecipitation (ChIP). The fluorescent antibody is matched to the corresponding protein and the corresponding signal indicates the expression level of the protein. All other antimicrobial agents that are not complementary are washed away and the samples are evaluated by fluorescence scanners or laser scanning techniques. By analyzing the fluorescence intensity of each point on the chip and the interaction between proteins, the expression level of the related proteins can be evaluated. A total of 46 apoptosis-related proteins were detected and 20 DEPs were screened out and enriched with KEGG, and these proteins were found to play a role in 12 regulatory pathways, including the apoptosis signaling pathway, the p53 signaling pathway and the tumor necrosis factor signaling pathway.

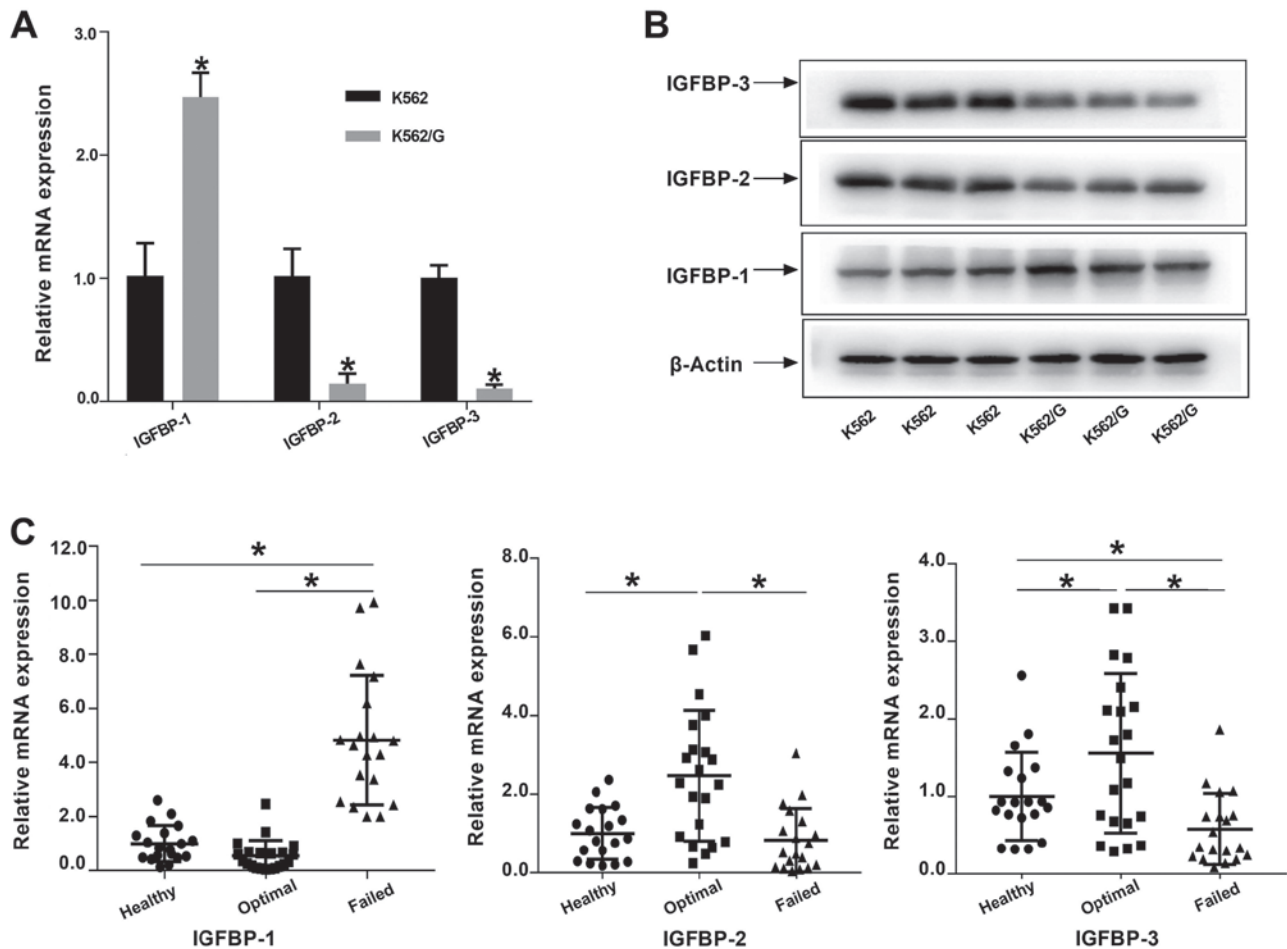


Figure 2. Results of RT-qPCR and western blot analyses. (A) RT-qPCR analysis for the mRNA levels of IGFBP-1, IGFBP-2 and IGFBP-3 in K562 and K562/G cells. The mRNA levels were normalized to GAPDH. * $P < 0.05$ vs. K562 group. (B) Western blotting was performed to analyze the protein expression levels of IGFBP-1, IGFBP-2 and IGFBP-3 in K562 and K562/G cells. (C) RT-qPCR was performed to detect the IGFBP-1 (left), IGFBP-2 (middle) and IGFBP-3 (right) levels in patients with chronic myeloid leukemia with optimal response to imatinib mesylate ($n=21$) or treatment failure ($n=19$), and healthy subjects ($n=19$). GAPDH mRNA expression was used as an internal control. * $P < 0.05$. IGFBP, insulin-like growth factor-binding protein; RT-qPCR, reverse transcription-quantitative PCR.

The authors previously demonstrated that the p53 signaling pathway that regulates redox status plays an essential role in IM resistance (17). The study had revealed that IGFBP-1 acts as a negative regulator of BAK-dependent apoptosis and its expression is involved in the transcriptional and mitochondrial functions of the p53 tumor suppressor protein (35). However, the role of IGFBP in CML resistance is unclear. IGFBP-1, IGFBP-2 and IGFBP-3 proteins, which were significantly different in the results of a protein chip, were selected and a follow-up study was performed.

The expression of IGFBP-1, IGFBP-2 and IGFBP-3 in the K562 and K562/G cell lines was detected by RT-qPCR and western blot analyses to confirm the results of the protein ChIP analysis. The results demonstrated that the level of IGFBP-1 in the drug-resistant cell line K562/G was increased compared with that in K562, while the levels of IGFBP-2 and IGFBP-3 were lower, consistent with the results of the antibody array.

Similarly, the level of IGFBP-1 in the PB of patients with drug resistance was increased compared with that in IM-sensitive patients and healthy subjects, while the levels of IGFBP-2 and IGFBP-3 were lower. Previous mechanistic investigations revealed that IGFBP-1 plays a role via

a mechanism involving the extracellular signal regulated kinase (ERK)/c-Jun pathway (36). IGFBP2 induced the apoptosis of tumor cells through the PI3K/protein kinase B/inhibitor of NF- κ B kinase subunit β pathway (37). However, IGFBP3 gene silencing mediated inhibition of ERK/mitogen associated protein kinase signaling pathway on proliferation, apoptosis, autophagy and cell senescence (38). In addition, the content and ratio of IGFBP-1 and IGFBP-3 play an important role in the occurrence, development and prognosis of the tumor (39). In the present study, the levels of IGFBP-1 in patients who have been receiving IM for >12 months were lower compared with those in the untreated group, whereas IGFBP-2 and -3 exhibited the opposite results. Such differences may arise from the change of cellular components in CML patients receiving long-term IM treatment, which should be further confirmed in future studies. No significant correlation between IGFBP-1 or IGFBP-2 and the level of BCR-ABL was observed. A decrease in the levels IGFBP-3 was observed with increasing BCR-ABL levels. These results indicate that the role of IGFBP-3 in IM resistance may depend on the level of BCR-ABL, which is not the case for IGFBP-1 and IGFBP-2. This suggests that IGFBPs may

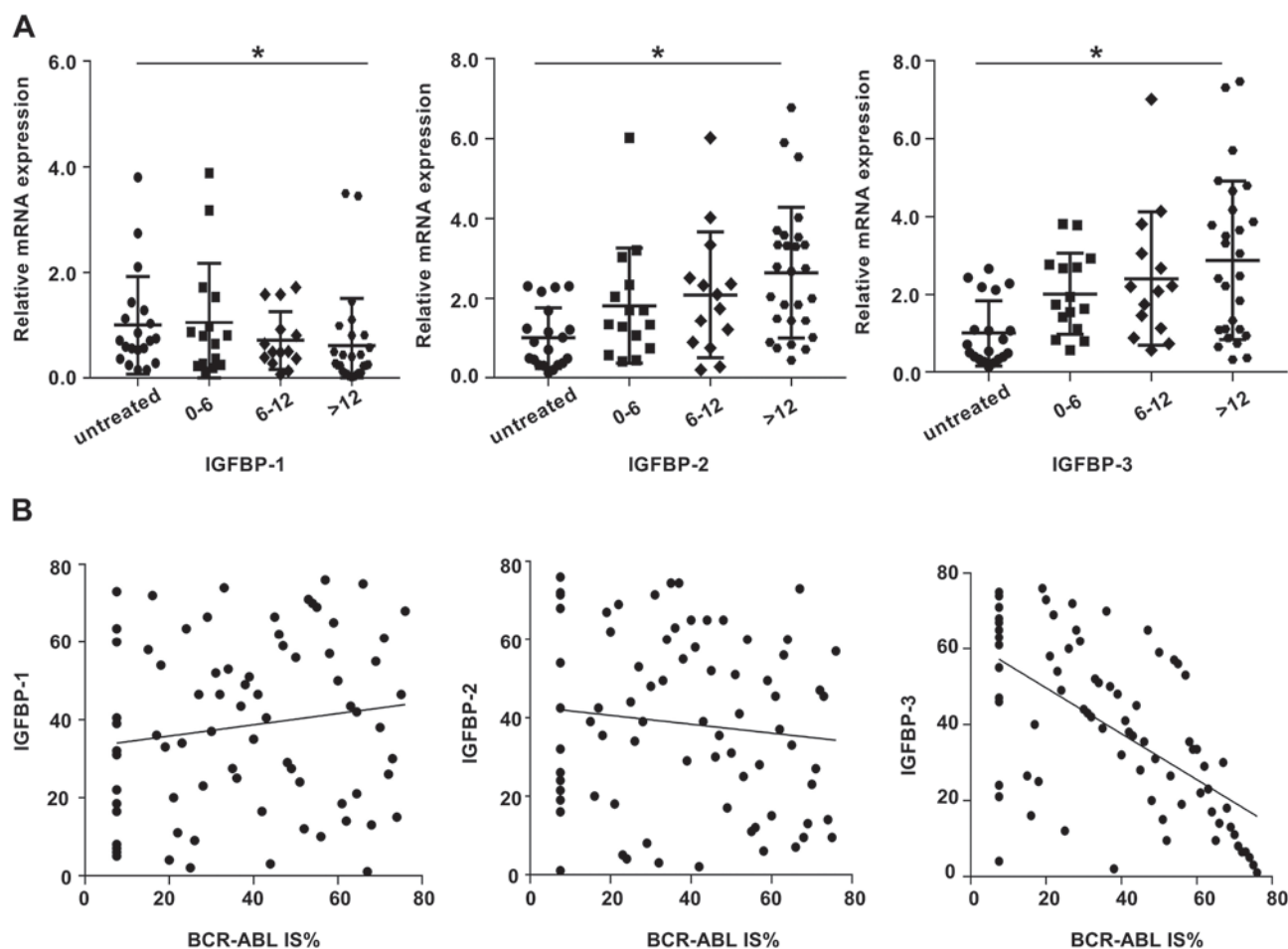


Figure 3. Change of levels of IGFBPs with prolonged treatment time and results of correlation analysis. (A) Reverse transcription-quantitative PCR analysis for mRNA levels of IGFBP-1 (left), IGFBP-2 (middle) and IGFBP-3 (right) in the four groups (untreated, 0-6 months, 6-12 months and >12 months). The mRNA levels were normalized to GAPDH. * $P < 0.05$. (B) Correlation analysis of IGFBP-1 (left), IGFBP-2 (middle) and IGFBP-3 (right) with BCR-ABL levels. IGFBP, insulin-like growth factor-binding protein.

play a role in IM resistance, but it is not clear which IGFBP plays the dominant role. However, these results indicate that the protein array is a powerful tool for biomedical discovery. IGFBPs may play a key role in the development of drug resistance in CML and IGFBP-1, IGFBP-2 and IGFBP-3 may represent potential therapeutic targets. However, the specific underlying mechanism requires further verification.

IGFBPs are a family of proteins binding to IGFs, including 6 high-affinity IGFBPs, namely IGFBP-1 through IGFBP-6 (40). IGFBP family members may be useful prognostic biomarkers in various malignancies and have been indicated to be involved in the development and progression of tumors (41). Previous studies validated the role of IGFBPs in the diagnosis and prognosis prediction of certain solid tumors, including rectal, ovarian and pancreatic (42-44). IGFBP-1 is abundantly expressed in the liver and decidualized endometrium, and mainly functions in the intracellular and pericellular compartments to regulate cell growth and survival (45,46). In addition, IGFBP-1 can perform IGF-independent functions, such as transcriptional regulation, nuclear localization, modulation of other growth factors and binding to non-IGF molecules involved in tumorigenesis, and tumor growth, metastasis and progression (47,48). In humans, IGFBP-2 is the second most abundant IGFBP in the blood (49). High levels of IGFBP-2

have been detected in the serum of cancer patients with a poor prognostic outcome (50,51). In addition to its role as a secretory protein, the intracellular oncogenic functions of IGFBP-2 promote cancer cell proliferation, invasion, metastasis and drug resistance (52,53). IGFBP-3 is the most abundant IGFBP and accounts for 80% of all IGF binding (54). Moreover, low serum IGFBP-3 levels or free IGFBP-3 levels, which are assessed by the molar difference of IGFBP-3 and IGF-1, increase the risk of tumors (55,56). The present study demonstrated that the IGFBP-1 level was higher in the PB of patients with drug resistance compared with IM-sensitive patients and healthy subjects, whereas the levels of IGFBP-2 and IGFBP-3 were lower, suggesting that IGFBP-1, IGFBP-2 and IGFBP-3 may be novel biomarkers of IM resistance.

In summary, the present study assessed K562 and K562/G cells by protein arrays. RT-qPCR and western blot analyses were used to confirm the results. Of note, the results were obtained from clinical samples and they suggest that IGFBPs may represent novel targets for the treatment of IM resistance in CML.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YR and XX contributed to study design and preparation for the manuscript. YR, SY and YL contributed to the acquisition, the analysis and interpretation of data. All authors read and approved the manuscript.

Ethics approval and consent to participate

The study protocols were approved by the Institutional Review Board of Anhui Provincial Hospital and informed consent was obtained from all participants, according to the principles outlined in the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

All the authors declare that they have no competing interests.

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