

Persistent STAT5-mediated ROS production and involvement of aberrant p53 apoptotic signaling in the resistance of chronic myeloid leukemia to imatinib

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Abstract. The persistent activation of signal transducer and activator of transcription 5 (STAT5) may principally be attributed to breakpoint cluster region (BCR)-Abelson murine leukemia viral oncogene homolog 1 (ABL1), and have multi-faceted effects in the development of chronic myeloid leukemia (CML). The p53 protein network regulates important mechanisms in DNA damage repair, cell cycle regulation/checkpoints, and cell senescence and apoptosis, as demonstrated by its ability to positively regulate the expression of various pro-apoptotic genes, including B-cell lymphoma-2 (Bcl-2) and Bcl-2-associated X protein (Bax). In the present study, it was observed that the mRNA levels of STAT5A and STAT5B were upregulated in patients with imatinib-resistant CML and in the imatinib-resistant K562/G CML cell line. In addition, increased expression of STAT5 was observed in the BCR-ABL1 mutation group, compared with that in the non-BCR-ABL1 mutation group, regardless of patient imatinib resistance state. Elevated levels of reactive oxygen species (ROS) and DNA double-strand breaks were identified in K562/G cells using flow cytometric and phosphorylated H2AX (γ -H2AX) foci immunofluorescence assays, respectively, compared with the imatinib-sensitive K562 cells. The levels of intracellular ROS and γ -H2AX were decreased by the ROS scavenger (N-acetylcysteine), and ROS levels were also markedly reduced by STAT5 inhibitor (SH-4-54). In addition, imatinib significantly inhibited the proliferation of K562 and K562/G cells, with half maximal inhibitory concentration values of 0.17 ± 0.07 and $14.78 \pm 0.43 \mu\text{M}$, respectively, and the levels of apoptosis were significantly different between

K562 and K562/G cells following treatment with imatinib. The mRNA and protein levels of STAT5 and mouse double minute 2 homolog (MDM2) were upregulated, whereas those of Bax were downregulated in K562/G cells, as determined using western blot analysis. Additionally, although the two cell lines exhibited relatively low protein expression levels of p53, lower levels of p53 and TPp53BP1 transcripts were detected in the K562/G cells. Taken together, these findings suggest that the resistance of CML to the tyrosine kinase inhibitor, imatinib, may be associated with persistent STAT5-mediated ROS production, and the abnormality of the p53 pathway.

Introduction

Chronic myeloid leukemia (CML) is a malignant hematopoietic stem cell disorder characterized by the reciprocal translocation of chromosomes 9 and 22, resulting in the expression of a constitutively active breakpoint cluster region (BCR)-Abelson murine leukemia viral oncogene homolog 1 (ABL1) tyrosine kinase. The introduction of tyrosine kinase inhibitors (TKIs), including imatinib, nilotinib and dasatinib, has revolutionized the treatment of patients with CML and markedly improved their quality of life (1-3). A large proportion of patients with CML subjected to long-term TKI treatment exhibit high-level and stable molecular responses and, in certain cases, leads to undetectable minimal residual disease (4). However, up to 40% of patients show substantial resistance or intolerance to TKIs, typically during or following treatment, which seriously limits the attainment of treatment-free remission or a cure (5,6). The underlying mechanisms of drug resistance remain to be elucidated, although they are considered to be attributable mainly to mutations in the kinase domain (KD) of BCR-ABL1 (7) and to signaling pathways activated by BCR-ABL1, including RAS, phosphoinositide 3-kinase (PI3K), nuclear factor- κ B (NF- κ B) and signal transducer and activator of transcription 5 (STAT5) (8). In particular, STAT5 has at least four key effects in the initiation and progression of CML (9), and may be considered a crucial modulator of imatinib responsiveness (10).

Reactive oxygen species (ROS) are generated as a by-product of the normal oxidative metabolism in eukaryotic cells which, if generated to excess, can cause damage

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to cellular molecules, including DNA, RNA and proteins, resulting in oxidative stress. It is well established that ROS are important in a variety of pathophysiological processes, including cell differentiation, host defense, oxygen sensing, and cell proliferation and apoptosis. The generation of ROS is regulated by the STAT5 transcription factor, which is crucial in a number of hematological diseases and commonly functions downstream of certain kinases, including BCR-ABL1 in CML (11). It is known that ROS can trigger genomic stress, secondary genome instability and the potential accumulation of gene mutations within the basal p53-mediated DNA damage response (DDR) pathway. Therefore, there is scientific support for a link between STAT5, ROS production and BCR-ABL1 mutations.

p53 is considered to be a universal sensor of genotoxic stress, and is involved in different DNA repair mechanisms and in cell cycle checkpoint regulation through various signaling pathways. Generally, p53 and its downstream signaling pathways are involved in ROS-induced apoptosis. Numerous studies have reported crosstalk between p53 signaling and ROS metabolism during normal physiological conditions, based on the feedback loop between ROS and p53, whereby mitochondria-generated ROS promote p53 translocation and, in turn, trigger oxidative stress. A previous study also demonstrated that the progression of CML was frequently accompanied by increased inactivation of p53, and that p53 mutations were seldom observed in chronic phase CML, but were detected in ~30% of patients presenting with myeloid blast crisis (12). However, the mechanism of the interaction between p53 and STAT5-mediated ROS production in the resistance of CML to TKIs remains to be elucidated. Thus, in this study, we aimed to examine the role of STAT5-mediated ROS production and aberrant p53 apoptotic signaling in the resistance of CML to imatinib.

Materials and methods

Cell culture. The human K562 CML cell line was purchased from the Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences (Shanghai, China) and imatinib-resistant K562 cells (K562/G) were obtained from the Department of Pharmacology at the Institute of Hematology, Chinese Academy of Medical Sciences (Tianjin, China). The cells were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1% penicillin and 1% streptomycin, in a 5% CO₂ incubator at 37°C. The cells were cultured in the presence or absence of 1 μ M imatinib. All cells were passaged every 2-3 days.

Clinical specimens. All patients were enrolled from Anhui Provincial Hospital of Anhui Medical University (Hefei, China) between July, 2015 and September, 2016. The present study was approved by the Ethics Committee of the Affiliated Provincial Hospital of Anhui Medical University. Peripheral blood mononuclear cells (PBMCs) were isolated from patients with CML during routine examinations following the provision of informed consent from all patients, in accordance with the Declaration of Helsinki. Aliquots of the PBMCs

were used for subsequent analysis. The molecular responses to imatinib were assessed according to the BCR-ABL/ABL ratio, standardized to the International Scale (13). According to the 2013 European Leukemia Net guidelines (<https://www.leukemia-net.org>), responses were assessed using standardized reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis at 3 and 6 months (14). BCR-ABL1 transcript levels $\leq 10\%$ at 3 months and $< 1\%$ at 6 months were defined as optimal responses, whereas levels $> 10\%$ at 6 months were defined as failed responses (14). BCR-ABL KD mutations and p53 mutations were assessed as described previously (15). The characteristics of the patients, and the results of the BCR-ABL KD mutations and p53 mutations are presented in Table I.

DNA damage signaling pathway PCR array. The K562 and K562/G cells lines ($\sim 1 \times 10^7$ cells) were collected, respectively. RNA extraction and first strand cDNA synthesis were performed via a routine protocol (16). A Human DNA Damage Signaling Pathway RT² Profiler™ PCR array was purchased from SABiosciences (Frederick, MD, USA). Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and RNA samples (1 μ g) were reverse transcribed into cDNA using the RT² PCR Array First Strand Synthesis kit (Qiagen). Subsequently, 91 μ l of ddH₂O was added to each 20 μ l cDNA synthesis reaction and mixed well. The following components were mixed in a 5-ml tube or a multi-channel reservoir: 1,050 μ l of 2X SuperArray PCR master mix, 105 μ l of the diluted first-strand cDNA synthesis reaction and 945 μ l of ddH₂O. The cocktails were then added to the PCR array. Real-time PCR detection was performed under the following thermocycling conditions: 95°C for 10 min; 40 cycles of 95°C for 15 sec and 60°C for 1 min. RT-qPCR and data analyses via the 2^{- $\Delta\Delta$ C_q} method were performed according to the manufacturer's protocol. The expression data were normalized to that of the housekeeping gene GAPDH. Differences in the levels of gene expression are presented as the fold increase/decrease, relative to the levels of the housekeeping gene (Table II).

Cell counting assay. A cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Shanghai, China) was used to determine the survival rate of the K562 and K562/G cells following incubation with imatinib. The cells were seeded in a 96-well plate at a density of 5×10^3 cells/well in IMDM containing 10% FBS. Subsequently, various concentrations of imatinib (0.02-4 μ M for K562 cells and 0.2-400 μ M for K562/G cells) were added. Following incubation of the cells at 37°C in 5% CO₂ for 24 h, 10 μ l of CCK-8 solution was added to each well and the cells were incubated for another 4 h. The absorbance was measured at 450 nm with a microplate reader. A well containing medium and CCK-8 solution only was used as a blank control. A control group of cells were incubated with cell culture medium and CCK-8 solution. The half maximal inhibitory concentration (IC₅₀) values of imatinib were determined as the mean of three independent experiments.

Cell apoptosis assessment using Annexin V-FITC and propidium iodide (PI) staining. Briefly, the cells were harvested and washed with cooled PBS at 4°C. Cell suspensions (5×10^4 cells each) were incubated with Annexin V-FITC (2 μ g/ml) and

Table I. Clinical characteristics of patients.

Characteristic	Chronic myeloid leukemia cases (n=63)	Non-IM-resistant (n=31)	IM-resistant (n=32)
Age at diagnosis (years)			
Median	37	40	36
Range	20.4-83.6	22.3-79.5	20.4-83.6
Sex			
Male	42	21	21
Female	21	10	11
White blood cells ($10^7/\text{ml}$)	49.16±69.68	12.17±6.55	84.99±83.52
International standard			
<1%	31	31	0
>1%	32	0	32
BCR/ABL mutation rate (%)	19.01	9.60	28.13
p53 mutation rate (%)	0.00	0.00	0.00

Non-IM-resistant patients had a BCR-ABL1/ABL1 ratio of 9.6%. BCR-ABL1 mutations, were D276G, F317L and F359I. IM-resistant patients had a BCR-ABL1/ABL1 ratio of 28.13%. BCR-ABL1 mutations were G250E, M244V, M387L, V299L, Y253H, E255K, E297K, F317L and G250E. IM, imatinib; BCR-ABL1, breakpoint cluster region-Abelson murine leukemia viral oncogene homolog 1.

PI (0.5 $\mu\text{g}/\text{ml}$) at room temperature for 15 min in the dark, and subsequently analyzed using flow cytometry (17). The cells were considered to be apoptotic if they exhibited Annexin V⁺/PI⁻ (early apoptotic) or Annexin V⁺/PI⁺ (late apoptotic) staining. The data are presented as the mean of three separate experiments, each performed in duplicate.

Detection of intracellular ROS. The accumulation of intracellular ROS was detected using 2',7'-dichlorofluorescein diacetate (DCFH-DA) probes (Molecular Probes[®]; Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. In brief, 2×10^5 cells/ml of K562 and K562/G cells were stained with 2.5 μM DCFH-DA at 37°C for 15 min. The samples were then washed and resuspended in phosphate-buffered saline (PBS), and the fluorescence intensity was analyzed using flow cytometry (FACSCanto II) using BD FACSDiva software v6.1.3 (both from BD Biosciences, San Jose, CA, USA). Additionally, K562/G cells were pretreated with 5 mM N-acetylcysteine (NAC; Meilunbio, Dalian, China) or 20 μM SH-4-54 (Selleck Chemicals, Houston, TX, USA) for 24 h in a humidified atmosphere at 37°C and 5% CO₂, and then subjected to the same analysis. For each sample, $\sim 10^4$ cells contained in the gated regions were counted. ROS-positive cells were stained with DCFH-DA. The experiments were repeated

three times, and all data were analyzed with BD FACSDiva software.

RT-qPCR analysis. Total RNA was extracted from the cultured cell lines and PBMCs of the patients with CML using TRIzol reagent (Thermo Fisher Scientific, Inc.) and quantified with a NanoDrop spectrophotometer (Amoy Diagnostics, Xiamen, China). A total of 0.5 μg of the isolated RNA was used for cDNA synthesis and qPCR analysis was subsequently performed on a 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with a SYBR-Green reaction kit (Invitrogen; Thermo Fisher Scientific, Inc.). GAPDH was used as a housekeeping gene to normalize the levels of target gene expression. The changes in mRNA expression levels were calculated using the comparative Cq method, as follows: Fold change = $2^{-\Delta\Delta Cq} = [(Cq \text{ gene of interest} - Cq \text{ internal control}) \text{ sample A} - (Cq \text{ gene of interest} - Cq \text{ internal control}) \text{ sample B}]$ (18). The primer sequences used are listed in Table III.

Immunofluorescence analysis of the expression of phosphorylated H2AX (γ -H2AX). γ -H2AX forms microscopically visible foci, and the number of γ -H2AX foci has been found to correlate well with the number of DNA double-strand breaks (DSBs) (19). The K562 and K562/G cells (5×10^4) were plated on glass slides via a cell concentrator and fixed with 4% paraformaldehyde for 30 min at room temperature. Following three washes with PBS containing 0.2% Tween-20, the cells were permeabilized with PBS containing 0.3% Triton X-100 at room temperature for 30 min, and then blocked with blocking buffer containing 5% goat serum (SL038; Solarbio, Beijing, China) and 0.3% Triton X-100 in PBS for 1 h at room temperature. Incubation with primary γ -H2AX antibody (1:200; monoclonal rabbit anti-H2AX; cat. no. 9718; Cell Signaling Technology, Inc., Danvers, MA, USA) was performed in blocking solution overnight at 4°C. The cells were then washed three times and incubated with anti-rabbit IgG antibodies (1:600; cat. no. A-1101; AlexaFluor V[®] 488 goat anti-mouse; Molecular Probes[®]) at room temperature for 1 h. The nuclei were stained with 4',6-diamidino-2-phenylindole and images were captured with a laser scanning confocal microscope (DMI6000B TCS SP5; Leica Microsystems GmbH, Mannheim, Germany), using microscope imaging software (LAS AF6500; Leica Microsystems GmbH, Wetzlar, Germany).

Western blot analysis. The K562 and K562/G cells were collected and lysed with RIPA buffer containing a protease inhibitor cocktail. The lysates were centrifuged at 12,000 $\times g$ for 10 min, 4°C and the supernatant was collected. The total protein concentration in the supernatant was determined using a BCA protein assay. Equal quantities of protein (30 μg) were subjected to 10-12% SDS-polyacrylamide gel electrophoresis at a constant voltage of 80 V for 30 min and 120 V for another 1.5 h. The resolved proteins were electrophoretically transferred onto PVDF membranes (EMD Millipore, Billerica, MA, USA), and the membranes were blocked in 5% skimmed milk for 1 h. Subsequently, the membranes were incubated overnight at 4°C with primary monoclonal antibodies at 1:1,000 dilutions. The following day, the membranes were exposed to

Table II. mRNA fold-differences in DNA damage response genes between K562 and K562G cells.

Gene	Abbreviation	K562G/K562 (fold-difference)
Bloom syndrome, RecQ helicase-like	BLM	-1.53
Calcium and integrin binding 1 (calmyrin)	CIB1	-2.02
Growth arrest and DNA-damage-inducible, α	GADD45A	-1.51
Nth endonuclease III-like 1	NTHL1	-1.89
Cell cycle checkpoint protein RAD17	RAD17	-1.55
Tumor protein p53	TP53	-2.56
Tumor protein p53 binding protein 1	TP53BP1	-1.93
Hypoxanthine phosphoribosyltransferase 1	HPRT1	-1.73

horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (1:1,000 dilution; A21010, A21020-1; Abbkine, Wuhan, China) for 2 h at room temperature. To visualize the protein bands, the membranes were treated with an enhanced chemiluminescence kit solution (GE Healthcare Life Sciences; Chalfont, UK) and digitalized by scanning (Fusion Solo3 v 16.12; Fusion FX Vilber Lourmat, France). The primary antibodies against STAT5 (94205S), phosphorylated (p-)STAT5 (9359S), p53 (2524S), B-cell lymphoma-2 (Bcl-2) (2870S), mouse double minute 2 homolog (MDM2; 86934) and Bcl-2-associated X protein (Bax) (5023T) were obtained from Cell Signaling Technology, Inc., and anti- β -actin was purchased from Santa Cruz Biotechnology, Inc. (sc-47778; Dallas, TX, USA).

Statistical analysis. All data in the present study are presented as the mean \pm standard deviation of three independent experiments unless stated otherwise. The results were analyzed using one-way analysis of variance and unpaired Student's t-tests to evaluate the significance of differences between groups. $P < 0.05$ was considered to indicate a statistically significant difference. Data analyses were performed using Prism software version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Elevated levels of STAT5 correlate with BCR-ABL1 mutation and imatinib sensitivity in vivo and in vitro. Several previous studies have reported that the activation of STAT5 contributes to imatinib resistance in BCR/ABL1 CML. In a previous study, Warsch *et al* revealed that imatinib-resistant patients had upregulated levels of STAT5 in leukemic cells (10). In the present study, it was found that patients with CML and imatinib resistance exhibited higher mRNA expression levels of STAT5A and STAT5B ($P = 0.0093$ and $P = 0.0091$) (Fig. 1A). Of note, the expression levels of STAT5 were significantly

Table III. Primer sequences used in gene expression analysis.

Gene	Primer sequence (5'-3')	Product length (bp)
GAPDH	F: GGAGCGAGATCCCTCCAAAAT R: GGCTGTTGTCATACTTCTCATGG	197
STAT5A	F: GCAGAGTCCGTGACAGAGG R: CCACAGGTAGGGACAGAGTCT	106
STAT5B	F: CAGAACACGTATGACCGCTG R: CTGGAGAGCTACCAATTGTTGG	106
p53	F: CCAGCAGCTCCTACACCGGC R: GAAACCGTAGCTGCCCTG	99
Bcl-2	F: CATGTGTGTGGAGAGCGTCAA R: GCCGGTTCAGGTACTCAGTCA	147
Bax	F: GCGTCCACCAAGAAGCTGAG R: ACCACCCTGGTCTTGATCC	113
MDM2	F: GAATCATCGGACTCAGGTACATC R: TCTGTCTCACTAATTGCTCTCCT	167

STAT5, signal transducer and activator of transcription 5; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein; MDM2, mouse double minute 2 homolog; F, forward; R, reverse.

higher in patients with BCR-ABL1 mutations, compared with those without BCR-ABL1 mutations (sensitive, $P = 0.02$ and $P = 0.009$; resistant $P = 0.01$ and $P = 0.009$), and this effect was independent of the state of imatinib resistance (Fig. 1B). Additionally, the K562/G cells exhibited elevated mRNA levels of STAT5A and STAT5B, compared with the imatinib-sensitive K562 cells ($P < 0.001$ and $P < 0.001$) (Fig. 1C). Consistently, the results of the western blot analysis demonstrated that the protein expression levels of STAT5 and p-STAT5 were markedly increased in the K562/G cells, compared with those in K562 cells (Fig. 1D).

ROS accumulation contributes to enhanced levels of DNA DSBs in K562/G cells. Significantly higher levels of ROS were detected in the imatinib-resistant K562/G cells, compared with those in the imatinib-sensitive K562 cells ($P < 0.001$). Following treatment with 5 mM NAC, a scavenger of ROS, the levels of ROS in the K562/G cells were significantly decreased ($P = 0.323$) (Fig. 2A). Additionally, the present study investigated whether STAT5 contributed to the accumulation of ROS by using the STAT5 inhibitor, SH-4-54. As shown in Fig. 2B, exposure to 20 μ M SH-4-54 markedly reduced the levels of ROS in the K562/G cells ($P = 0.872$ vs. K562; $P = 0.01$ vs. K562/G). It is well established that ROS can induce DNA damage, including DNA DSBs, and is generally recognized as an inducer of resistance mutation; γ -H2AX is frequently observed in regions of histone lesions resulting from ROS damage and may be used to quantify DSBs (20). The present study showed that higher levels of γ -H2AX were detected in the K562/G cells than in the K562 cells, which suggested that higher levels of ROS and secondary DNA

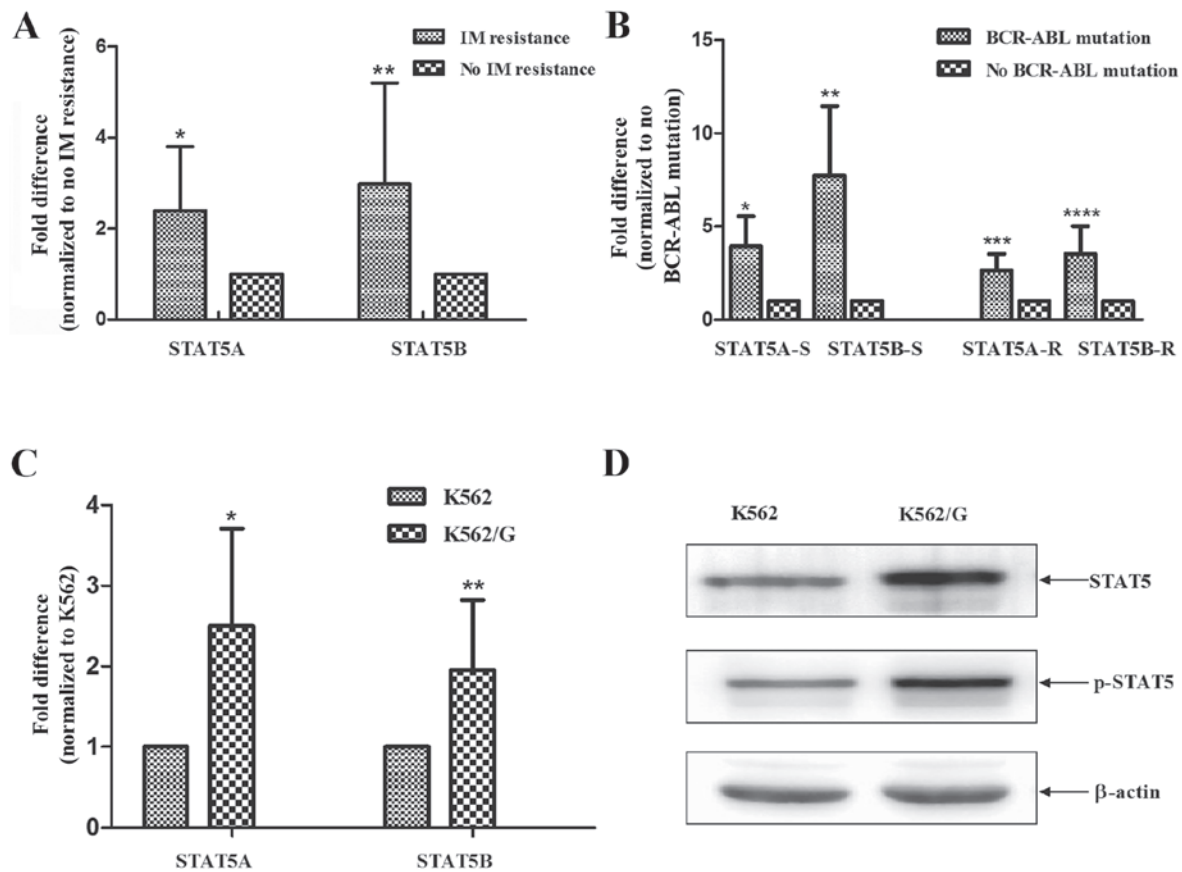


Figure 1. Expression levels of STAT5 in patients with CML and CML cell lines. (A) mRNA levels of STAT5A and STAT5B in patients with CML with IM resistance (n=32) and in patients without IM resistance (n=31; *P=0.0093 and **P=0.0091 vs. no IM resistance). (B) Expression levels of STAT5 in IM-sensitive patients (*P=0.02 and **P=0.009 vs. no mutation) and IM-resistant patients (***P=0.01 and ****P=0.009 vs. no mutation). (C) mRNA expression levels of STAT5A and STAT5B in the K562 and K562/G cell lines (*P<0.001 and **P<0.001 vs. K562). (D) Immunoblot analysis of protein expression levels of STAT5 and p-STAT5 in K562 and K562/G cells. Similar results were obtained from six replicates (three independent experiments performed in duplicate). S, sensitive to IM; R, resistant to IM. STAT5, signal transducer and activator of transcription 5; CML, chronic myeloid leukemia; IM, imatinib; p-, phosphorylated; BCR-ABL, breakpoint cluster region-Abelson murine leukemia viral oncogene homolog 1.

damage were present in the K562/G cells. To confirm the link between STAT5-mediated ROS production and DSB generation, the K562/G cells were pretreated with NAC. As expected, NAC pretreatment decreased the level of γ -H2AX staining (Fig. 2C).

Involvement of the p53 apoptotic pathway in the reduced apoptotic rate and resistance of K562/G cells to imatinib. Imatinib, as a first generation TKI, is used as a principle treatment for patients with CML (4). A significant difference in the rate of ABL mutation was observed between the imatinib-resistant group and the non-resistant group (28.13 vs. 9.60%) (Table I). The imatinib-resistant K562/G CML cell line was used in the present study, and the resistance of the K562/G cells was first characterized and compared with that of normal K562 cells. The K562 and K562/G cells were treated with imatinib, and a CCK-8 assay was used to assess the cytotoxic effects of various concentrations of imatinib on the cell lines, for which the IC_{50} values were determined. The results showed that the ratio of IC_{50} values between the K562/G and K562 cells was 87:1 (14.78 ± 0.43 vs. 0.17 ± 0.07) (Fig. 3). Therefore, the K562/G cell line was resistant to imatinib and maintained a high level of drug resistance, which confirmed the efficacy of the imatinib-resistant cell model. The apoptotic rates of

the K562 and K562/G cells in response to different concentrations of imatinib (0.1, 1, 5, 10 and 15 μ M) were measured using flow cytometric analysis. The results showed that the rate of apoptosis in the K562/G cells was significantly lower, compared with that in the K562 cells at all concentrations of imatinib (P<0.05). This effect was evident in early and late apoptosis (Fig. 4A-C). Previous evidence indicates that ROS-induced apoptosis is characterized by the upregulation of tumor suppressor protein p53 (21), delocalization of cytochrome c, caspase activation, DNA fragmentation, and suppressed expression of histone deacetylase in CML cells (22). In the present study, using a human DNA damage signaling pathway array, the downregulated transcription levels of DDR genes were found in K562/G cells. Among these downregulated genes, tumor protein p53 and tumor protein p53-binding protein 1 were involved in the apoptotic pathway (Table II). Although the K562 and K562/G cells exhibited low protein expression levels of p53, the mRNA level of p53 was significantly lower in the K562/G cells, compared with that in the K562 cells (P<0.01) (Fig. 4D and E). Similarly, the expression levels of Bax were significantly decreased in the K562/G cells (P<0.01), whereas those of Bcl-2 and MDM2 were significantly increased (P<0.01) (Fig. 4D). The p53 mutation was not found in the CML patients (Table I).

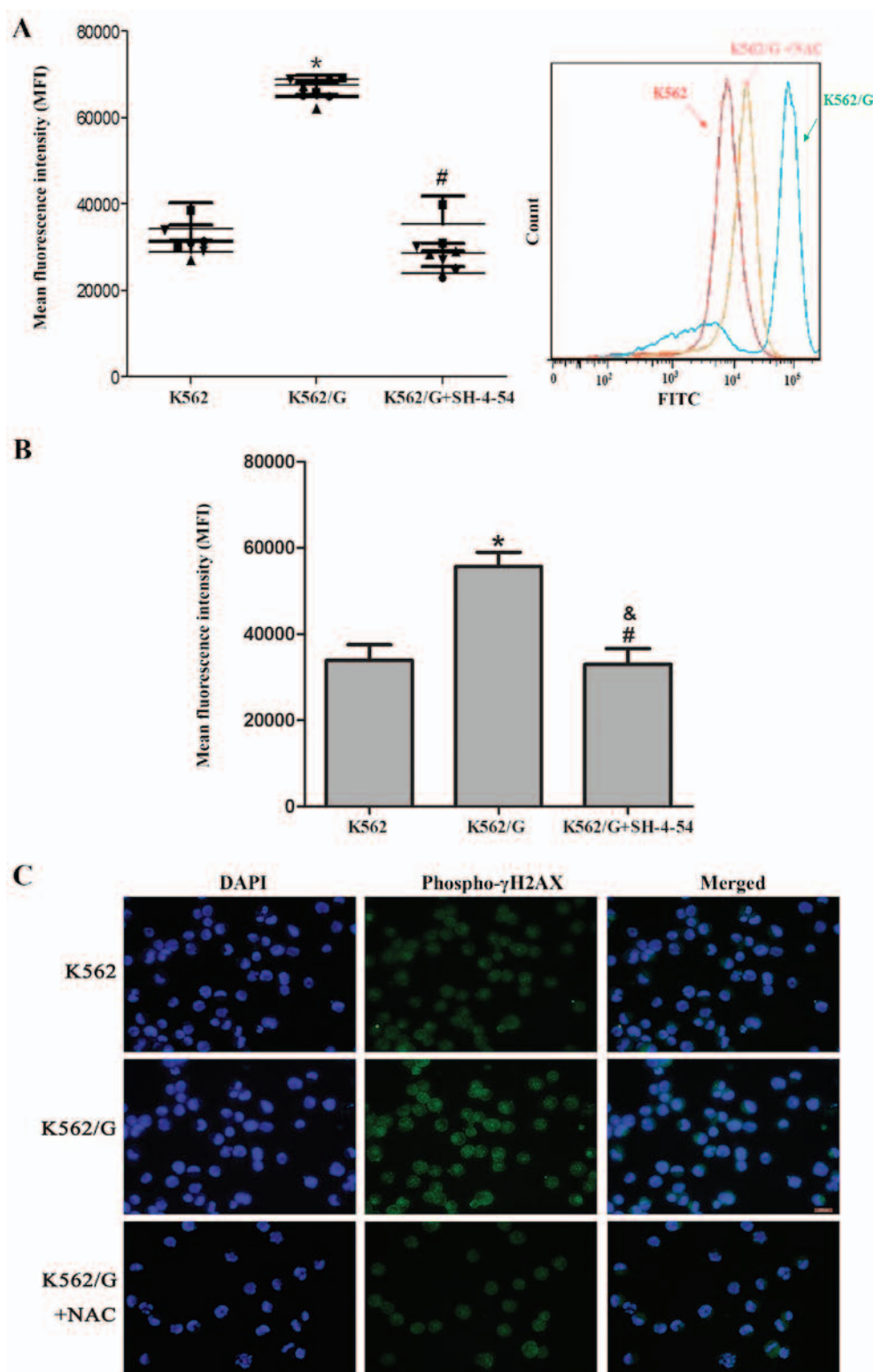


Figure 2. Generation of DNA double-strand breaks mediated by STAT5-mediated ROS production. Cells were incubated in the presence or absence of (A) NAC (* $P < 0.001$ and $\#P = 0.323$ vs. K562) or (B) SH-4-54 (* $P = 0.012$ and $\#P = 0.872$ vs. K562; * $P = 0.01$ vs. K562/G) for 24 h, the levels of ROS were measured in the cell groups using FACS analysis. (C) Immunofluorescence staining. K562/G cells were left untreated or were treated with NAC (5 mM) for 24 h, and then stained for phosphorylated H2AX (γ -H2AX). The cells were subsequently stained with FITC-conjugated secondary antibody (green), and counterstained with DAPI (blue). Original magnification, x400. STAT5, signal transducer and activator of transcription 5; ROS, reactive oxygen species; NAC, N-acetylcysteine; DAPI, 4',6-diamidino-2-phenylindole.

Discussion

CML is commonly considered to be a typical model for understanding the molecular pathogenesis of malignancy.

Although the treatment and prognosis of CML have markedly improved since the development of TKIs, including imatinib, which is now used as a standard first-line therapeutic agent, not all patients with CML respond well. Even following

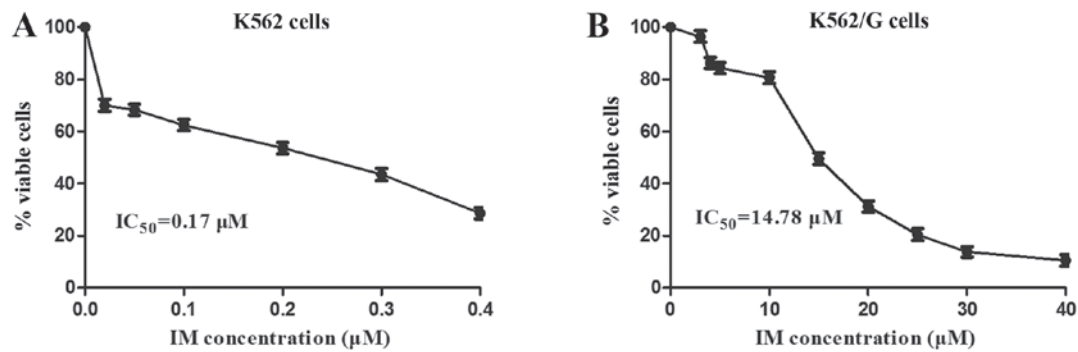


Figure 3. IM decreases the viability of (A) K562 and (B) K562/G cells. K562 and K562/G cells were treated with the indicated concentrations of IM for 24 h. Cell viability was evaluated using a cell counting kit-8 assay. Data are presented as the mean \pm standard deviation of three independent experiments. IM, imatinib; NAC, N-acetylcysteine; IC_{50} , half maximal inhibitory concentration.

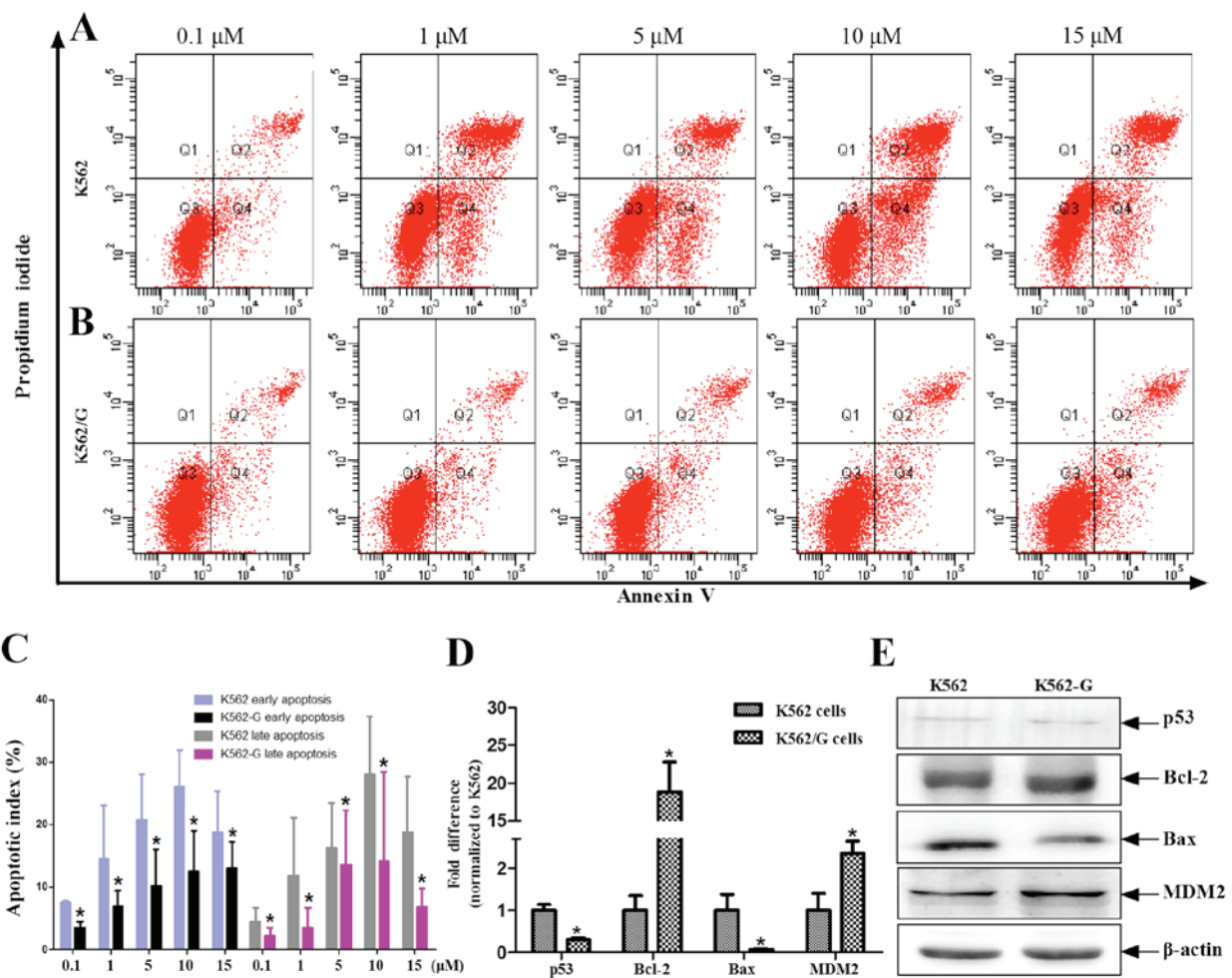


Figure 4. IM induces different rates of apoptosis in K562 and K562/G cells. (A) K562 and (B) K562/G cells were treated with different doses of IM for 24 h, following which PI and Annexin V-FITC were added to the culture medium. Apoptotic cells were detected by Annexin V-FITC/PI staining followed by flow cytometric analysis. (C) Data are expressed as the mean \pm standard deviation of four independent experiments and were analyzed using Student's t-test. * $P < 0.05$ K562/G vs. K562. Analysis of p53 and other apoptosis-related genes. (D) Reverse transcription-quantitative polymerase chain reaction analysis was performed to detect the mRNA expression levels of p53, Bcl-2, Bax and MDM2. (E) Changes in protein expression levels of p53, Bax, Bcl-2 and MDM2 in whole cell lysates (K562 and K562-G cells) were detected using western blot analysis. Similar results were obtained from six replicates (three independent experiments performed in duplicate). * $P < 0.01$ K562/G vs. K562. IM, imatinib; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; MDM2, mouse double minute 2 homolog; PI, propidium iodide.

long-term imatinib treatment, stem cells in the majority of patients with CML continue to express BCR-ABL mRNA. Additionally, following long-term therapy, it has been reported that 60% of patients experience relapse at the molecular level,

whereas 15% of patients developed drug resistance and subsequently discontinued imatinib following a sustained complete molecular response (23). The most frequently reported cause for TKI resistance is a gene mutation in the KD of BCR-ABL1,

which typically occurs at a frequency of 40-90% (24). Other mechanisms may include the upregulation of BCR-ABL1, increased expression of drug transporter ABCB1, elevated levels of granulocyte-macrophage colony-stimulating factor, and inactivation of TP53 (25,26). In the present study, 63 blood samples from imatinib-resistant (n=32) and non-resistant (n=31) patients were analyzed by DNA sequencing, and 28% of the imatinib-resistant cases were found to harbor mutations within the KD of BCR-ABL1 (Table I).

It is generally accepted that STAT5 exerts key effects in various hematological malignancies, including anti-apoptotic effects and growth-stimulatory functions (27). To date, STAT5 has been confirmed to support BCR-ABL1-triggered CML via four key mechanisms: Cell cycle control, cell viability maintenance, ROS generation and TKI resistance (28). The importance of STAT5 in the pathogenesis of CML is further highlighted by findings that STAT5 is upregulated during disease progression and that high STAT5 levels can significantly decrease imatinib sensitivity. In the present study, significantly increased expression levels of STAT5 were consistently found in the blood samples of imatinib-resistant patients and in imatinib-resistant K562/G cells. This was in agreement with a previous study that identified significantly increased mRNA levels of STAT5A in patients presenting with secondary imatinib resistance without BCR-ABL1 mutations, compared with newly diagnosed imatinib responders (29). The present study investigated the potential connection between the transcription factor STAT5 and the occurrence of BCR-ABL1 mutations. The data showed that significantly higher levels of STAT5 were expressed in the BCR-ABL1 mutation group, compared with those in the non-BCR-ABL1 mutation group, regardless of TKI resistance state.

ROS are considered to be potent signaling mediators, and may interfere with gene regulatory pathways, including the mitogen-activated protein kinase and hypoxia-responsive element/hypoxia-inducible factor pathways, which are also known to be regulated by STAT5 transcription factors. In the present study, as expected, the STAT5 inhibitor SH-4-54 caused a marked reduction in the levels of ROS in K562/G cells. However, under environmental stress, ROS levels increase markedly, which can cause significant damage to cell structures, and ultimately mutation. In BCR-ABL1⁺ cells, high levels of STAT5 have been associated with elevated levels of endogenous ROS (30). In the present study, the levels of ROS in K562/G cells were elevated, compared with those in K562 cells. Previous studies have shown that certain chemotherapeutic drugs can induce cell death by increasing the generation of intracellular ROS (31,32). The production of ROS can lead to DNA damage (33). DNA DSBs are considered to be the most destructive form of DNA damage, and have a high probability of resulting in cumulative mutations. In the present study, it was observed that ROS increased the levels of γ -H2AX, which was alleviated by NAC.

The function of p53 as a tumor suppressor has been attributed to its ability to promote cell death or permanently inhibit cell proliferation. p53 is subject to a wide range of post-translational modifications, including phosphorylation, acetylation, methylation and ubiquitination. Regulation of the gene expression of p53 usually occurs mainly at the protein level (34). Somatic TP53 mutations have been identified in several types

of cancer, at various frequencies depending on the cancer type. Overall, TP53 mutations are found in 5-10% of *de novo* cases of myelodysplastic syndrome MDS and acute myeloid leukemia cases, and have been associated with complex karyotypes and reduced survival rates (35,36). p53 mutations are considered to be high-risk factors for the development of leukemia, and indicators of a poor response to chemotherapy and poor prognosis (37). In the patients with CML enrolled in the present study, no p53 mutations were detected. However, whether the contributions of p53-associated TKI resistance occur mainly via mutations or epigenetic modifications remains to be elucidated. In a previous study, the deletion of p53 was associated with the progression of CML, and the wild-type p53 protein was present in the KBM5 chronic phase cell line, whereas the low expression or absence of p53 was observed in K562 cells (an advanced stage CML cell line) (38). In the present study, the results of the Human DNA Damage Signaling Pathway Array revealed downregulated transcription levels of DDR genes, including p53 and p53-binding protein 1, in the K562/G cells. The expression levels of p53, Bcl-2 and Bax, proteins of the p53 apoptotic pathway, were assessed using western blot analysis, and the downregulation of Bax was observed in K562/G cells. These experimental results are consistent with the findings of a previous study involving the deletion of p53 in K562 and K562/G cells (38).

In conclusion, the present study identified elevated levels of STAT5 in patients with imatinib-resistant CML and K562/G cells. In addition, the high levels of STAT5 and subsequent accumulation of ROS were associated with increased chronic oxidative damage to DNA, as evidenced by increased DSBs within the DNA of K562/G cells, which may be a cause of gene mutations associated with resistance. It was also found that aberrant regulation and expression of the p53 pathway was involved in imatinib resistance. The expression of p53 can be modulated by changes in transcriptional and translational events, and the activity and protein levels of p53 are negatively regulated by the E3 ubiquitin ligase MDM2 (39). The present study detected lower mRNA and protein expression levels of MDM2 in K562 cells, compared with those in the K562/G cells. Therefore, a time profile of the induced expression of p53 and its epigenetic modification (methylation status, post transcriptional regulation by microRNAs) during the establishment of resistance to imatinib require further examination in future investigations.

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