1 **Nrf-2/Gst- α Mediated Imatinib Resistance through**
2 **Rapid 4-HNE Clearance**

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Abstract.

The advent of imatinib mesylate (IM) has dramatically improved the outcome of patients with chronic myeloid leukemia, but drug resistance, particularly in advanced stage of disease, portends eventual relapse and progression. To identify the candidate molecule responsible for resistance during IM treatment, an IM-resistant K562 cell line was generated by culturing in gradually increasing dose of IM. The expression of Nrf-2 and its downstream target, Gst- α , were significantly induced in these cells. GST- α , in turn, mediated cell survival by maintaining intracellular low level of 4-HNE. Inhibition of Nrf-2 effectively reduced the expression of Gst- α , resulting in accumulation of 4-HNE and elevated sensitiveness to IM. Moreover, in IM-sensitive K562 cells enforced Gst- α expression strikingly protected cells from the insult of IM. Finally, we also examined the levels of Nrf-2 in clinical bone marrow samples. Nrf-2 and Gst- α were more abundant in bone marrow of CML patients compared with that of healthy donors. In addition, Nrf-2 and Gst- α were further up-regulated in samples of patients with weak response to IM. In conclusion, our study shows that rapid clearance of 4-HNE by Nrf-2/GST may represents a novel molecular basis of IM resistance in CML.

Key words. Chronic myeloid leukemia (CML), TKI resistance, nuclear factor erythroid-2 related factor 2 (Nrf-2), glutathione S-transferase (GST), 4-Hydroxynonenal (4-HNE)

1. Introduction

Chronic myeloid leukemia (CML) is a hematopoietic stem cell disorder associated with an acquired chromosomal translocation, t (9,22) which leads to the formation of *bcr/abl* fusion gene, coding for Bcr/Abl fusion protein[1]. This tyrosine kinase with constitutive activity is capable of activating multiple downstream signaling pathways, resulting in deregulation of cell cycle control and resistance to apoptosis. The advent of imatinib mesylate (IM), a specific tyrosine kinase inhibitor (TKI) that targets Bcr/Abl fusion protein, has revolutionized the treatment of CML[2]. Despite the great achievement, CML is currently considered incurable in most patients largely due to the development of drug resistance[3]. IM resistance resulted from mutations in the *bcr/abl* gene could be overcome by application of the more potent second-generation TKIs, such as nilotinib [4] and dasatinib[5]. The outcome of TKIs treatment is limited in patients with blast crisis CML (BC-CML) in which the leukemic cells may not depend solely on Bcr/Abl tyrosine kinase for survival, as a result of additional molecular alternations[6]. For instance, Hsp-70 over-expression, which is independent of Bcr/Abl in some patients without mutations in *bcr/abl* gene, contributes to cell survival of the TKIs treatment, even the tyrosine kinase was fully blocked[7]. Therefore, TKIs alone may not be sufficient to eliminate cancer cells since Bcr/Abl is not necessary for their survival. Targeting multiple pathways simultaneously by partnering existing TKIs with novel agents will bring new hope for overcoming drug resistance and finally eradicate the entire leukemic cell population.

Recently, contribution of cellular anti-oxidation in chemo-resistance has been intensively studied. Generation of reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide are considered inevitable in aerobic life. Overproduction of ROS promotes peroxidation of lipid in cellular membranes. 4-HNE (4-Hydroxynonenal) is one of most bioactive products and has been widely accepted as a cytotoxic molecule by forming covalent adducts with cellular macromolecules, such as proteins, nucleic acids to affect their functions[8]. Several signaling pathways have been shown to be modulated by 4-HNE, for example, 4-HNE induces caspase-3 and -9 activation and of release of cytochrome c from mitochondria [9]. Moreover, 4-HNE suppresses AKT activity and induces P53 expression [10,11]. In order to counteract the insults of ROS, mammals have evolved a battery of defensive enzymes, GST (glutathione S-transferase), one of the best documented enzymes, exerts detoxifying role by catalyzing the formation of 4-HNE-GSH conjugate, promoting its extracellular export [12]. The CNC (cap' n' collar) family member Nrf-2 (nuclear factor erythroid-2 related factor 2) transcription factor is the master regulator of GST genes. Under basal circumstance, Nrf-2 maintains inactive and is interacts with Keap-1 (Kelch-like ECH-associated protein 1) in cytoplasm [13]. In response to oxidative stress, the interaction equilibrium is disrupted and Nrf-2 trans-locates into nucleus to trigger targeted genes transcription by binding to ARE regulatory DNA sequence [14]. Various studies have demonstrated that constitutively activated Nrf-2 confers resistance to chemotherapies [15-18]. Therefore, the role of Nrf-2 in IM resistant CML cells is of great interest.

In the present study, we focused on the abundance of Nrf-2 in IM resistant K562 cells and clinical bone marrow samples from patients with CML at different stages, our data suggested that Nrf-2 may serve as a novel target for treatment of CML with drug resistance.

2. Materials and methods

2.1. Main reagents

Imatinib mesylate was purchased from Selleck, antibodies to 4-HNE was purchased from Abcam (Cambridge, MA, USA), Keap-1, Nrf-2, c-Abl, H2AX, γ -H2AX were purchased from cell signaling technology (Danvers, MA,

USA). Gst- α , Gst- μ , Gst- π and HPR-conjugated secondary antibodies were products of Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cell counting kit CCK-8 was purchased from Selleck Chemicals (Houston, TX, USA). Co-Immunoprecipitation Kit was purchased from Pierce™ (Rockford, IL, USA).

2.2. Cell culture

K562 were purchased from BeNa Culture Collection (BNCC, Beijing, China), cells were grown in RPMI-1640 medium (Gibco, Life technologies, NY) supplemented with 10% FBS (Gibco, Life technologies, NY) in 37 °C, 5% CO₂. For K562-R cells, 1 μ M imatinib was added to maintain the resistance.

2.3. Generation of IM resistant K562 cells

Parental K562 were gradually exposed to increasing concentrations of IMATINIB at a rate of 0.1 μ M increment every week. After approximately 10 weeks, sublines of cells growing in 1 μ mol/L imatinib were maintained continuously in culture in this dose of the inhibitor. Sensitive K562 cells were grown in parallel medium without STI571.

2.4. Clinical samples

Bone marrow mononuclear cells (BM-MNCs) were isolated using human bone marrow mononuclear cell isolation kit (Tbd science, Tianjin, China), sample information was listed in supplementary table 1. Informed consent forms were signed by all patients and healthy donors to allow the use of their cells for experiments and the experiments were performed in accordance with the regulation of institutional ethics committee of the First affiliated hospital of Xi'an Jiaotong university. Lineage negative cells were separated using human lineage cell depletion set BD Pharmingen (San Diego, CA, USA) according to users' manual.

2.5. Keap-1 and Gst- α overexpression

Targeted genes (Keap-1, Gst- α) were cloned and inserted into pFlag-CMV3 vector and transformed into DH5 α cells, the plasmids were next analyzed by PCR and double enzyme digestion, and the positive candidates were further validated by sequencing. For transfection of 1×10^6 cells, 2 μ g plasmids were mixed with 6 μ L FuGENE 6 (Roche, Basel, Switzerland) according to the user's manual. Cells were collected at indicated times for viability, protein experssion analysis.

2.6. Nrf-2 knockdown

Three siRNAs against Nrf-2 and the negative control were designed and synthesized by RiboBio (Guangzhou, China), transfected into K562 cells with Lipofectamine 2000 (Invitrogen, NY, USA) according to the user's manual. Cells were collected at indicated times for viability, protein expression analysis.

2.7. CCK-8 assay

CCK-8 assay was applied to calculate the IC₅₀ of cells with indicated treatment described above. Cells were transfected with siRNA or plasmids for 6-8h, plated in 96-well plate (5×10^4 cells per well), imatinib was added at

indicated concentrations (total volume, 200μL). After 24h culturing, 20μL CCK-8 was added. The absorbance of 450 nm was measured by BioTek ELx800 micro-plate reader (Biotek Winooski, Vermont, USA).

2.8. Extracellular 4-HNE quantification

Extracellular 4-HNE was quantified using 4-HNE ELIAS kit (R&D Systems, Minneapolis, MN, USA) according to users' manual. Briefly, mediums were centrifuged and diluted, incubated with antibody, enzyme labeled, developed, A450 was measured by micro-plate reader.

2.9. Western blotting

Proteins were separated by SDS-PAGE, and transferred to PVDF membrane, blocked in 5% fat-free milk. Indicated proteins were incubated with corresponding primary antibodies overnight at 4 °C, followed by incubation with HRP-conjugated secondary antibodies and detected by ECL reagent (Merck Millipore, Billerica, MA, USA). The images were captured with GelDoc XR system (Bio-Rad, Hercules, CA, USA).

2.10. Co-Immunoprecipitation

Co-IP was described previously[19]. Briefly, according to the manufacturer's guide, cells were lysed, and the lysate was pre-cleaned using control agarose resin followed by incubation with HA or Keap-1 antibodies to form immune complex which was then captured by protein A/G plus agarose, and centrifuged to separate the uncombined Nrf-2. The pellets were washed and re-suspended with non-reducing lane marker sample buffer, boiled and centrifuged to collect elution for western blotting analysis.

2.11. Statistical analysis

The data were expressed as $\bar{X} \pm SD$ (n=3). The difference between each group was evaluated using Student's t-test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Generation and characterization of K562R cells

To mimic the development of resistance to TKIs during the process of treatment, we constructed an IM-resistant K562 (K562R) cell line by culturing in gradually increasing dose of IM. The initiating concentration of IM was 0.1 μM. To calculate to resistance index (RI), CCK-8 assay was then performed. IC₅₀ of K562 and K562R cells were 0.93 and 6.14, respectively (Fig.1a); the RI of K562R was 6.6. FCM results showed significant reduction of apoptotic rate in K562R cells treated with 1μM IM for 24h in comparison with K562 cells (K562S) (Fig. 1b). Next, we analyzed the expression of Bcr/Abl fusion protein which was elevated in K562R cells, and the level decreased when IM was added (Fig. 1c), however, we found a remarkable increase of Nrf-2 in K562R cells compared with K562S cells (Fig. 1c). We further investigated the status of potential targets of Nrf-2, the RT-PCR results showed an enforced Gst-α transcription along with Nrf-2 up-regulation (Fig.1d). This result was then validated at protein level (Fig. 1e).

3.2. Nrf-2 knockdown inhibits 4-HNE scavenging

We first analyzed the amount of 4-HNE in cells exposed to IM (1 μ M, 12 h). Western blotting results showed that three major molecules were adducted with 4-HNE, the MW were 130, 55 and 25 *Kd*, respectively. IM markedly increased the production of 4-HNE in K562S cells, but not in its resistant counterpart. In addition, 4-HNE levels were lower in K562R cells without IM treatment than that in K562S cells (Fig. 2a). To confirm that the reduced 4-HNE levels was a consequence of enhanced ability to export in K562R cells, extracellular 4-HNE in medium was next investigated, higher 4-HNE levels was detected in medium of K562R cells at all indicated time points under IM circumstance (Fig. 2b), which was coincided with less intracellular 4-HNE. To explore the contribution of Nrf-2 in IM resistance, siRNAs was employed to inhibit its expression. Our results indicated that siRNA 1 among 3 candidates effectively reduced Nrf-2 (Fig. 2c), in turn, expression Gst- α was declined as well, moreover, the inhibition of expression was not affected by IM addition (Fig. 2d). Consequently, intracellular 4-HNE accumulation was detected (Fig. 2e) and the capacity to clean 4-HNE was strikingly impaired (Fig. 2f).

3.3. Nrf-2 knockdown reduces IM resistance

The effects of Nrf-2 knockdown on survival of K562R cells were tested next. CCK-8 assay showed the IC₅₀ of IM was dropped to 1.36 μ M (Fig. 3a), and the apoptosis rates at 12 h and 24 h were reached to 21% and 38%, respectively (Fig. 3b). The apoptotic cells were also detected morphologically by wrights' staining (Fig. 3c). As a product of lipid peroxidation, 4-HNE triggers DNA damage that was characterized by elevated γ -H2AX level, the histone H2A variant, which undergoes rapid phosphorylation in response to DNA damage [20]. We therefore measured γ -H2AX by immunofluorescence, the result showed notable increase of γ -H2AX amount in K562R cells treated with combination of siRNA and IM (Fig. 3d), this result was further confirmed by western blotting (Fig. 3e). Finally, we determined the levels of pro-apoptotic members of Bcl-2 family and the status of caspase which may be responsible for apoptosis, 3 of 4 tested proteins were clearly up-regulated, and the cleaved caspase-3 and its substrate PARP were also detected in siRNA plus IM group (Fig. 3f).

3.4. Keap-1 up-regulation reduces nuclear portion of Nrf-2

Keap-1 is a natural inhibitor of Nrf-2. Engagement with Keap-1 assists degradation of Nrf-2 through ubiquitination. Using pFlag-CMV3-HA-Keap-1 vector (HA tagged Keap-1), the amount of Keap-1 was raised markedly (Fig. 4a), and corresponding fall in Nrf-2/Gst- α was detected (Fig. 4b). Further, we found decrease of Nrf-2 amount was mainly in nucleus (Fig. 4c), which was then validated morphologically by immunofluorescence (Fig. 4e). Additionally, enforced Keap-1 expression was accompanied with sharply increase in intracellular 4-HNE levels (Fig. 4d). Co-IP assay was conducted to analyze the association between Keap-1 and Nrf-2, as the data indicated, precipitated Nrf-2 in K562R/Keap-1 cells was significantly lower than control cells (Fig. 4f). And lastly, we determined the influences of Keap-1 over-expression on IM resistance, Keap-1 over-expression alone caused about 20% apoptosis, which was doubled with IM administration (Fig. 4g).

3.5. Gst- α over-expression facilities K562S cells resistance to IM

As a targeted gene downstream of Nrf-2, Gst- α might play a key role in 4-HNE rapid scavenging. We therefore exogenously expressed Gst- α in K562S by transfection with pFlag-CMV3-Gsta vector. Gst- α levels was tripled (Fig. 5a), and the IC₅₀ of K562S/ Gst- α cells was 3.55 μ M (Fig. 5b). Meanwhile, a significant decline in apoptosis

rate was found in K562S/ Gst- α cells treated with IM at 1 μ M for 24 h (Fig. 5c). These results indicated that GSt- α participated at least, partly, in IM resistance. In order to further identify the connection between GSt- α mediated resistance and 4-HNE export, intra/extracellular 4-HNE were next measured, GSt- α promoted 4-HNE export under no drug condition, and this action was further enhanced with addition of IM(Fig. 5d). Accordingly, total intracellular 4-HNE levels were decreased in K562S/Gst- α cells (Fig. 5e).

3.6. Nrf-2 expression in CML patients' bone morrows

Finally, we wondered if the Nrf-2/Gst- α scenario reflects the natural development of drug resistance. Bone marrow mononuclear cells from 10 patients with CML at different stage and 5 healthy donors (HD) were evaluated for Nrf-2 abundance. As expected, both Nrf-2 and GSt- α were up-regulated in CML group (Fig. 6a). We further selected two samples from patents in advanced stage with a weak response to IM (No.4,5) and a good responder in chronic phase (No.2) to examine Nrf-2/Gst- α levels after IM treatment (1 μ M, 24h). Nrf-2/Gst- α levels increased notably in samples No.4 and No.5, but changed slightly in sample No.2 in which total 4-HNE levels increased (Fig. 6b). These results are in line with what we found in K562R cells. Considering the high heterogeneity of the BM cells, we speculated that Nrf-2/Gst- α levels may vary in different population, even in chronic samples. Thus, we compared Nrf-2/Gst- α levels in lineage negative cells with more mature cells from 4 chronic patients (No. 6, 8, 9, 10), and found Nrf-2/Gst- α were significantly higher in Lin⁻ cells (Fig. 6c), indicating that Nrf-2/Gst- α levels diminished as cell differentiation.

4. Discussion

In this study, we have identified the abnormal activity of Nrf-2 and its implication in IM resistant cell line and bone marrow of CML patients. As a pivotal sensor of chemical-induced oxidative stress, Nrf2 functions to restore homeostasis by up-regulating antioxidant and other cytoprotective enzymes. Dysfunction of Nrf-2 and its implication in chemoresistance have been intensively studied during the past decade. Indeed, several lines of investigation suggest that Nrf-2 pathway activation is closely related to chemotherapy resistance, particularly in solid tumors [15, 16, 21, 22]. A recent study demonstrated that genetic alternation occurs frequently in Keap-1 gene in non-small-cell lung cancer, leading to loss of Keap-1 function and constitutive activation of Nrf-2([23]). Besides, hypermethylation of promoter region that resulting in silence of the Keap-1 gene is an alternative mechanism of increased Nrf-2 activity[24]. Our data revealed the involvement of Nrf-2 in IM resistance, which is accordance with the former studies, and the molecular mechanism of Nrf-2 will be our next concern.

A cluster of genes is induced by Nrf-2. Hereby, we confirmed that a target GSt- α was responsible for reduction of the toxicity of oxidative stress by rapid clearance of 4-HNE, although our study did not exclude a role for other Nrf-2 targets, such as HO-1[25], ALDH[25]. Future studies will be necessary to determine the capacity of these molecules to convert 4-HNE to less reactive chemical agent.

Robust 4-HNE generation can provoke cell death through varies signaling. For example, 4-HNE is proposed to form adduct with Fas[26], which subsequently activates related downstream signaling, including ASK, JNK and caspase-3. In addition, 4-HNE modification of mitochondrial proteins impairs mitochondrial metabolism. Approximately, 30% 4-HNE target proteins locates in mitochondria, some enzymes are responsible for energy metabolism, such as ATP5B[27]. 4-HNE overproduction significantly reduces the enzymatic activities. In our western blotting analysis, three bands were found to form adducts with 4-HNE. Although we could not identify the related pathway in which these proteins were involved due to the limitation of application of 4-HNE antibody, significant increase was measured after exposure to IM in K562S but not in K562R cells, we suppose that IM

mediated generation of 4-HNE may serve as an intermediate to give rise to the activation of downstream pro-apoptotic proteins. As for K562R cells, rapid removing of 4-HNE minimizes the toxic effects of IM. Thereby, repression of 4-HNE clearance may represent a therapeutic opportunity to reverse drug resistance.

CML is a stem cell originated disorder, and there is an increasing interest in the characters of CML stem cells, which, attributed to the independence of Bcr/Abl tyrosine kinase activity, is generally accepted as source of disease recurrence[28, 29]. Our data also suggested that, compared with lineage committed cells, Nrf-2 in CML progenitors was more abundant. Unfortunately, as limited by scarceness of isolated lin⁻ cells, we could not further separate the CD34⁺/CD38⁻ population, the most primary cells to investigate the contribution of Nrf-2 in IM resistance. Based on its differentiated expression, dysfunction of Nrf-2 is nevertheless a potential molecular basis for TKI resistance in CML stem cells, and is worth further study using more clinical samples and an *in vivo* study using mouse CML-like disease model.

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Fig. 1. Generation and characterization of K562R cells. Cells were treated with IM at 1 μ M for 24h. (a) CCK-8 assay was applied to calculate IC₅₀ to IM of K562S and K562R cells. (b) Apoptosis was measured by FCM in K562S and K562R cells under exposure to IM at 1 μ M for 24h. (c) levels of Nrf-2 were measured by western blotting. (d) upregulation of Gsta were measured by RT-PCR, and (e) validated at protein level by western blotting. *p<0.05, **p<0.01.

Fig.2. Nrf-2 knockdown inhibits 4-HNE scavenging. (a) 4-HNE protein adducts were significantly increased in K562S but slightly decreased in K562R cells after IM treatment (1 μ M, 12h). (b) Extracellular 4-HNE in medium was measured by ELISA. (c) siRNA mediated Nrf-2 silencing in K562R cells was confirmed by western blotting. (d) Down-regulation of Nrf-2 was not restored by IM stimulation in K562R cells (1 μ M, 24h). (e) IM combined with siRNA increased formation 4-HNE protein adducts, (1 μ M, 12h). (f) IM combined with siRNA reduced extracellular 4-HNE (1 μ M, 12h). *p<0.05, **p<0.01.

Fig. 3. Nrf-2 knockdown reduces IM resistance. (a) siRNA 1 reduced IC₅₀ of K562R to 1.36 μ M (24h). (b) siRNA1 promoted IM (1 μ M, 24h) stimulated apoptosis in K562R cells. (c) Apoptotic cells were detected by wrights' staining (bar: 10 μ M). (d) γ -H2AX was detected by immunofluorescence (bar: 10 μ M) and (e) western blotting. (f) IM (1 μ M, 24h) plus siRNA1 induction of pro-apoptotic members of Bcl-2 family and activation of caspase-3, PRRP. *p<0.05, **p<0.01.

Fig.4. Keap-1 up-regulation reduces nuclear portion of Nrf-2. (a) pFlag-CMV-HA-Keap-1 mediated Keap-1 overexpression in K562R cells. (b) Levels of Nrf-2 and Gsta were decreased in Keap-1 overexpressed K562R cells. (c) Keap-1 overexpression reduced nuclear portion of Nrf-2 and confirmed by (d) immunofluorescence (bar: 10 μ M). (e) Keap-1 increased 4-HNE protein adducts in K562R cells. (f) Keap-1 interacted with Nrf-2. (g) Keap-1 overexpression promoted K562R apoptosis provoked by IM (1 μ M, 24h). *p<0.05, **p<0.01.

Fig.5. Gsta over-expression facilitates K562S cells resistance to IM. (a) pFlag-CMV-Gsta mediated Gsta overexpression in K562S cells. (b) Gsta overexpression increased IC₅₀ of K562S cells to IM (24h). (c) Gsta overexpression inhibited apoptosis in K562S provoked by IM (1 μ M, 24h). (d) Gsta overexpression facilitated 4-HNE export, with or without IM (1 μ M, 12h). (e) Gsta overexpression decreased 4-HNE protein adducts in K562S cells, with or without IM (1 μ M, 12h). *p<0.05.

Fig.6. Nrf-2 expression in CML patients' bone marrow. (a) Differential expression of Nrf-2/Gsta in bone marrow from healthy donors and CML patients. (b) Changes of Nrf-2/Gsta levels and 4-HNE protein adducts in BM-MNCs exposed to IM (1 μ M, 12h) in patients with weak response to imatinib mesylate (No. 4, 5) and with well response (No. 2). (c) Nrf-2/Gsta levels in lin⁻ cells were higher than that in lin⁺ cells in 4 patients that clinically responded well to imatinib mesylate. *p<0.05.

a **b** **c** **d** **e** **f**

Resistant

Sensitive