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An Uncommon E450G Mutation within the BCR/ABL Kinase Domain in a Chronic Myeloid Leukemia Patient Presenting with Resistance to Imatinib and Nilotinib [Version 1, Awaiting Peer Review]

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Abstract

Backgroud: Chronic myelogenous leukemia (CML) is a myeloproliferative neoplasm that is characterized by the constitutive expression of the oncogenic protein BCR-ABL tyrosine kinase. ABL1 kinase domain mutation is a common mechanism of TKI resistance. Ninety different amino acid mutations at more than 60 positions have been identified in the ABL kinase domain.

Case Presentation: Here, we report an uncommon mutation (E450G) identified in a tyrosine kinase inhibitor (TKI)-resistant CML patient. Most features of our patient were consistent with those previous reported for CML cases, which include fatigue, splenomegaly, leukocyte abnormalities. BCR/ABL fusion gene sequencing revealed the rarely reported E450G mutation in the ABL kinase in this patient. The patient was treated with hydroxyurea and interferon at first and complete hematological remission (CHR) was achieved. Two years later, clinical characteristic and bone marrow examination showed accelerated phase CML. The BCR-ABL transcript was 28.9% by qPCR. He started with imatinib and maintained CHR. However, the BCR/ABL transcript was present as 21%. The treatment strategy was changed to nilotinib and the BCR/ABL transcript level remained 22%.

Conclusion: To the best of our knowledge, mechanism by which E450G mutation affects the interaction with TKIs and causes nilotinib-resistance has not yet been reported. In order to address this unsolved issue, circular dichroism(CD) was applied to determine the dynamic structure of protein, while intrinsic fluorescence, 1-Naphthalenesulfonicacid and 8-phenylamino (ANS) fluorescence spectra analyses were used to investigate the mechanism of resistance.

Keywords

Chronic Myelogenous Leukemia; Mutation; E450G; Tyrosine Kinase Inhibitor; Dynamic Structure; Mechanism

Abbreviations

CML-Chronic Myelogenous Leukemia; TKI-Tyrosine Kinase Inhibitor; CHR-Complete Hematological Remisiion; CD-Circular Dichroism; ANS-1-Naphthalenesulfonicacid and 8-phenylamino; ALL-Acute Lymphoblastic Leukemia; PCR-Real-Time Quantitative Polymerase Chain Reaction; WT-Wild-Type; NAMD-Nanoscale Molecular Dynamics; (Glu)-Glutamate; (Gly)-Glycine; (Trp)-Tryptophan

Competing Interests

The authors declare that they have no competing interests.

Background

CML is a hematological malignancy caused by spontaneous fusion of the BCR gene with the ABL1 gene resulting in a constitutively active tyrosine kinase (BCR-ABL) and cell transformation [1]. Specific tyrosine kinase inhibitors (TKIs) of BCR-ABL are the first line therapy for CML and are highly effective in reducing disease burden, delaying disease progression, and prolonging overall patient survival, however, TKI-resistance has become an important cause of treatment failure [2-3].

ABL1 kinase domain mutation is a common mechanism of TKI resistance, and methods for detection of these mutations are available for clinical use. Ninety different amino acid mutations at more than 60 positions have been identified in the kinase domain [4]. Here, we report a new mutation (E450G) identified in a TKI-resistant CML patient who responded poorly to imatinib and nilotinib. E450G is an uncommon mutation that has been reported in combination with T315I or V299L [4-5]. An independent mutation at this position (E450K) has been reported to be associated with resistance to imatinib in transformed Baf3 cells, with a half maximal inhibitory concentration (IC_{50}) of 2.3 μM [6]. A Philadelphia (Ph)-positive acute lymphoblastic leukemia (ALL) with the rare E450G mutation showed high rates of regression and weak selective effects [7]. However, the mechanism by which the E450G mutation affects the interaction with TKIs and causes nilotinib-resistance has not yet been reported. To explore the mechanism of the E450G mutation caused TKI-resistance, the dynamic structure of protein was determined using a combination of CD, intrinsic fluorescence and ANS fluorescence spectra analyses. The results indicated that the E450G mutation affects both the secondary and tertiary structure of ABL.

Case Presentation

Patient

A 23-year-old man presented to our hospital in May, 2010 with 6-month history of fatigue. On physical examination, his spleen was 5 cm below the costal margins. His leukocyte count was 1.90×10^6/ml with 1.70×10^6/ml neutrophilic granulocytes, and elevated eosinophilic and basophilic granulocytes. Bone marrow examination supported a diagnosis of CML, and was confirmed by Ph chromosome and BCR/ABL fusion gene examination positivity.

The BCR-ABL gene transcript was identified as the p210 variant. By BCR/ABL fusion gene sequencing, we found that the A nucleotide at position 1349 is replaced with an G nucleotide, leading to the Glu 450 to Gly (E450G) variation. The patient was treated with hydroxyurea and interferon (300 million U, every two days) and complete hematological remission was achieved. Two years later, enlargement of his spleen recurred and his leukocyte count was elevated to 333.2 $\times10^6$/ml. Bone marrow examination showed active hyperplasia, blasts accounting for 12%, and accelerated phase CML. The BCR-ABL transcript was

28.9% by real-time quantitative Polymerase chain reaction (PCR). The patient received one cycle of chemotherapy (Homoharringtonine 4 mg QD for 5 days, cytarabine 150 mg QD for 7 days). He suffered severe pulmonary infection during chemotherapy but achieved complete hematological remission. He was started with imatinib 400 mg/d in March 2013. His clinical manifestations of CML disappeared and CHR was maintained after imatinib therapy; however, the BCR/ABL fusion gene transcript was still present at high levels (21%), indicating that the patient presented as imatinib-resistant. Although the treatment strategy was changed to nilotinib (600 mg per day), the BCR/ABL fusion gene transcript level remained high (22%) after 3 and 6 months of therapy, indicating a poor response to nilotinib. DNA extracted from the patient's peripheral blood cells was used to analyze the ABL kinase sequence when he presented as imatinib-resistant.

This study was carried out in strict accordance with recommendations of the Regulations on human and animal experimentation of Central South University (China). All experimental protocols were approved by the ethics committee of the Second Xiang-ya Hospital of Central South University (China). Written informed consent was obtained from the patient for publication and any accompanying images. A copy of the written consent is available for review by the Editor-in-Chief.

Crystal Structure Analysis

The location of the amino acid residue E450G in the ABL Src homology 2 (SH2) domain was determined by comparison with the crystal structure of the wild-type (WT) ABL SH2 domain (PDB: 2HYY and 3CS9) [8- 9]. The 3-dimensional structure of the ABL E450G mutant was obtained using the software Modeller 9.11 [10] and AutoDock 4.2 [11]. Simulations were then performed using the Nanoscale Molecular Dynamics (NAMD) analysis package developed by the Theoretical and Computational Biophysics Group at the University of Illinois at Urbana-Champaign (USA) [12]. Highly hydrophilic cavities inside the protein were filled with water molecules using the program DOWSER [12], and the protein was enclosed in a water box with 10 Å padding. Sodium and chloride ions were added to neutralize the system. All simulations were performed using the CHARMM force field [13] with particle mesh Ewald electrostatics and periodic boundary conditions. The structures of all mutant ABLs were created as described previously [8, 9].

Gene Cloning, Expression and Purification

The fragment of the WT and mutant ABL kinase domain was obtained by the RT-PCR and site directed mutagenesis. The ABL kinase domain consists of residues 218–500 of the 1130 amino acids of full-length human c-Abl, with an N-terminal His tag and a factor Xa cleavage site. Proteolytic cleavage with factor Xa leaves four extraneous amino acids at the restriction sites BamHI and HindIII. Automated sequencing of plasmid DNA confirmed the expected sequence of the plasmid pFB-HTA-Abl500. Recombinant baculovirus FB-HTA-Abl500 was generated using the conventional Bacto- Bac method from Invitrogen. Recombinant protein was expressed in Sf9 cells cultured in Excell 400 medium and 5% FCS in an 8 l total volume bioreactor (FairMenTec GmbH, Germany). The O2 level was 36% saturation and the culture temperature was maintained at 301 K. At 72 h post-infection, cells were pelleted and stored at 203 K. The WT and the mutant AKs was expressed and purified as described previously [Structural biology contributions to the discovery of drugs to treat chronic myelogenous leukaemia]. The Bradford's method was used to determine the protein concentration.

Fluorescence Analysis

The intrinsic fluorescence of WT and mutant ABLs was determined with an F-4500 spectrofluorometer using a 1-cm path-length cuvette. For the ANS fluorescence measurements, a 50-fold molar excess of ANS was added to WT and mutant ABLs samples. The samples were then equilibrated for 30 min in the dark, and extrinsic fluorescence was measured on an F-4500 spectrofluorometer. Far-UV CD spectra were recorded on a Jasco 715 spectrophotometer with a 1-mm path-length cell. All the experiments were carried out at 25°C.

Results

E450G Mutant ABL Kinase in the Patient

DNA sequencing revealed the rarely reported E450G mutation in the ABL kinase in this patient (Figure 1).

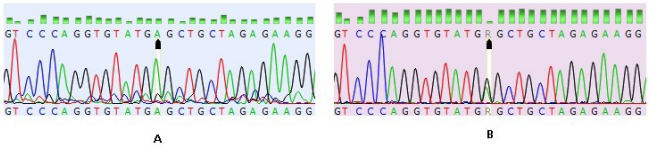

Figure 1: The left panel shows the amino acid sequence at E450 within the ABL kinase domain of the control (left panel). The right panel shows sequencing of the mutation at time of the patient relapse. The A nucleotide at position 1349 is replaced with an G nucleotide, leading to the Glu 450 to Gly (E450G) variation. The arrow shows the point mutation.

Crystal Structure Analysis of the E450G Mutant ABL Kinase

The crystal structure of BCR/ABL showed that E450 is located in the middle of the fifth α-helix of the ABL kinase domain. Glutamate (Glu) is a large acidic amino acid residue that interacts with K454 by hydrogen bonding, which is important to sustain the stability of alpha (α)-helices and the kinase domain. When it was substituted by glycine (Gly), which is a much simpler amino acid residue that cannot form hydrogen bonds, the disruption of the stability of the α-helices and kinase domain may affect kinase activity through changes in the domain spatial structure (Figure 2).

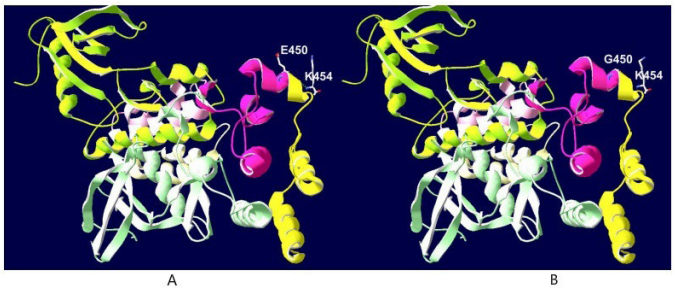

Figure 2: Homology modeling of mutated the ABL kinase domain indicating that E450 interacts with K454 through hydrogen bond formation, which is important to the stability of alpha helices and kinase domains. Hydrogen bond formation is prevented by the substitution of Glu for Gly, causing disruption of the stability of alpha helices and changes in the structure of the kinase domain that affect kinase activity.

Effects of E450G Mutations on BCR/ABL

To further confirm the effects of mutations on BCR/ABL, we generated four single point-mutants (E450D, E450G, E450V, and E450A) and one double mutant (E450D/K454R) by site-specific mutagenesis. Spectroscopic analyses were conducted to determine the effects of mutations on the secondary and tertiary structures of ABL. The mean residue ellipticity values at 222 nm ([θ222] MRW) of the E450G, E450V and E450A mutant ABLs were smaller than that of the WT ABL (Figure 3A). The E450G mutation showed the smallest ellipticity at 222 nm. This result suggests that E450G decreases the content of the regular secondary structure in the WT ABL. It can be speculated that this change disrupts the regular secondary structure of the α-helices and kinase domain.

Concerning the tertiary structure, the emission maximum of the intrinsic fluorescence spectra (Emax) displayed red shift to 331 nm for E450D, 334 nm for E450D/K454R, 336 nm for E450V, 339 nm for E450A and 340nm for E450G, when compared to that of WT ABL (330nm). This result indicated that tryptophan (Trp) residues in E450G E450V and E450A mutant ABLs are more exposed to water than that of the WT ABL (Figure 3B). Since nine of four Trp residues are located in the ABL domains, these changes might be caused by disruption of the compact structure of the α-helices and kinase domain by the mutations.

Furthermore, the ANS fluorescence intensity changes of the mutant ABLs compared with the WT (Figure 3C) showed similar trend to that of the Emax. This result implies that the E450G ABL mutant has more solvent-exposure of hydrophobic regions than the WT ABL and allows more binding of ANS molecules (Figure 3C). Combined with the results of previous studies, we deduced that the E450G mutation might affect the secondary and tertiary structure of ABL. Furthermore, the free energy minimization of the WT BCR/ABL was 31748.56 KJ, while that of the E450G mutant was -897.25 KJ.

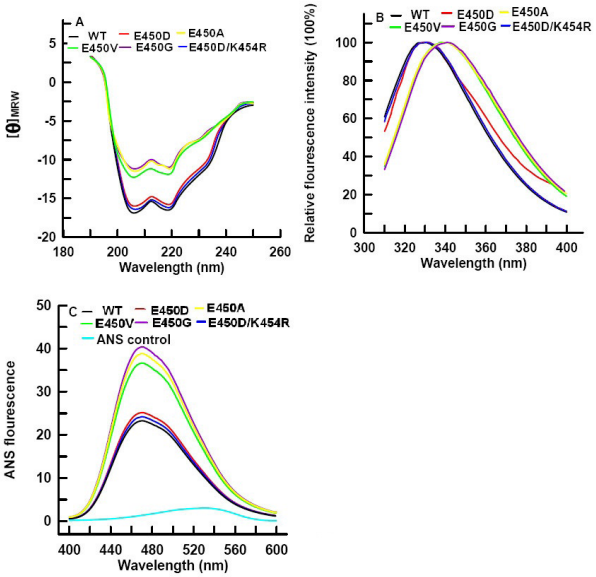

Figure 3: Effects of mutations on the structure of BCR-ABL detected by circular dichroism (CD) (left panel), intrinsic fluorescence spectra (middle panel) and ANS fluorescence spectra (right panel). (A) CD analysis results indicate that E450G mutation decreases the secondary structure content through disruption of the E450/K454 interaction. (B) Intrinsic fluorescence spectra show that the mutation affects the special structure directly for loosen it. (3) CANS fluorescence spectra further suggest that the mutation affect the tertiary structure resulting in internal hydrophobic amino acid exposure.

Discussion

The E450G mutation of the ABL kinase has been reported previously [5, 7]. In a study on outcomes of CML patients treated with TKIs reported by Russo Rossi, the E359V-E450G double mutant was detected in 2 of 82 patients at the time of imatinib failure; however, more details of the patients and investigation of the effects of the E450G mutation in TKI-resistance were not reported [5]. Jones performed KD mutational analysis of the ABL kinase in patients with Ph-positive ALL (n = 113) or TKI-resistant CML (n = 870) [7]. The study showed that the median time to development of uncommon KD mutations was after 41 months of TKI treatment, and the E450G mutation was reported in a Ph positive ALL patients at relapse along with the T315I mutation. In current case, the E450G mutation was detected as an isolated mutation at the time imatinib failure, thus implicating this mutation in the mechanism underlying TKI-resistance. The effects of the E450G mutation on the ABL kinase domain have not been analyzed in previous studies. Therefore, we aimed to elucidate the mechanism of this mutation caused TKI-resistance resistance by means of crystal structure analysis.

The ABL kinase has a highly plastic structure, which exists in equilibrium between conformations that favor catalytic activity or quiescence [14]. TKIs bond to the unique conformations of this kinase domain. The co-crystal structure of TKIs and the

ABL kinase domain revealed that the drug achieves specificity by trapping the kinase in an ABL-specific inactive conformation [15]. Point mutation can favor kinase constitutive activation, cell transformation, and affect either intramolecular regulatory interactions or binding of cellular inhibitors that might confer drug resistance through novel mechanisms.

Crystal structure analysis is a very valuable method used to elucidate the molecular basis of TKI-resistance. Kuang. et al. used crystal structure analysis to demonstrate a novel I293MP mutation within the BCR-ABL kinase domain in a Ph-positive acute lymphoblastic leukemia patient presenting with resistant to imatinib. [16]. This group analyzed the major structural differences between the WT and mutant ABL kinase domains and showed that obvious shifts in the α-helix and A- and C-loops influence the binding of imatinib with the α-helix (E286) and C-loop (I360, H361). Analysis of the E450G crystal structure showed that the mutation does not affect the structure of the imatinib and nilotinib binding site directly, however, changes in the secondary and tertiary structures of the ABL kinase lead to TKI-resistance. As we seen from the mimic structure of the WT and the E450G mutant ABL kinase domain, the E450 could interaction with the K454 which might play important roles in keeping the open conformation for the TKI interaction with the WT Bcr-Abl. When the E450 was replaced by the G450 which destructed the interaction between E450, the K454 and finally may lead the conformation of the ABL kinase domain into the closed state, thus caused the TKI resistance. Consistenting to our hypothesis, the regular secondary structure of the α-helices and kinase domain was decreased. Meanwhile the tertiary structure of the kinase domain in much looser conformations could also indicate E450G mutation caused the TKI drug resistance. Comparing with previous studies based on direct structural analysis, in this study, we conducted a structure simulation analysis combined with endogenous and exogenous fluorescence analysis. This is the first time that structural details, energy landscape, dynamic behaviors, and other properties of E450G mutation have been determined by using molecular dynamic simulation. Our findings provide a “first conjecture” approach to clarification of the role of the E450G ABL kinase mutation in the mechanism of TKI-resistance.

Conclusion

We report a new mutation (E450G) identified in a TKI-resistant CML patient who responded poorly to imatinib and nilotinib. By using a combination of circular dichroism(CD), intrinsic fluorescence , 1-Naphthalenesulfonicacid and 8-phenylamino (ANS) fluorescence spectra, we deduced that the E450G mutation might affect the secondary and tertiary structure of ABL, and the free energy minimization of the WT BCR/ABL was 31748.56 KJ, while that of the E450G mutant was -897.25 KJ. Our clinical observations and molecular simulation represent the basis of a useful and convenient approach to select the most effective treatments for CML patients and for further investigation of the mechanisms of TKI-resistance associated with ABL kinase mutations as well as development of more *efficient* therapies.

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Availability of Data and Materials

The datasets supporting the conclusions of this article are included within the article and its additional file. More details are available on request.

Authors’ Contributions

Si-Si Xie: Acquisition of data and writing the paper

Zhao Cheng: Analysis of data and writing the paper

Yi-Fang Yi: Acquisition of data

Ya-Fei Yin: Analysis and interpretation of data

Hong Sun: Analysis and interpretation of data

Hong-ling Peng: Design study and modification of the paper

Si-Si Xie and Zhao Cheng contributed equally to this study and share the first authorship

Consent for Publication

Written informed consent for publication was obtained from the patient of this Case report and any accompanying images.

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