Detection of \textit{BIM (BCL2L11)} Polymorphic Variants in Chronic Myeloid Leukemia by Q-Invader Assay and Their Clinical Significance

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Abstract

\textbf{Background:} Accumulating evidence suggests that genetic variants, including deletion and single nucleotide polymorphism (SNP) have role in the genesis and progression of various cancers. \textit{BIM} (also known as \textit{BCL2L11}) is a proapoptotic protein that is essential in the tyrosine kinase inhibitor (TKI)-induced apoptosis in chronic myeloid leukemia (CML) cells. We therefore attempted to develop new detection assay system of the \textit{BIM} genetic variants in CML patients and clinical relevance.

\textbf{Subjects and methods:} We assessed known \textit{BIM} polymorphic variants (\textit{BIM} deletion polymorphism in intron 2 and silent SNP in \textit{BIM} exon 5) by using the Q-Invader method with molecular response by TKIs in 47 Japanese chronic myeloid leukemia (CML) patients who achieved 4-log reduction of molecular response (MR4.0) or more.

\textbf{Results:} The Q-Invader assay was able to detect \textit{BIM} deletion polymorphism expanding approximately 2900 bp and \textit{BIM} SNP at exon 5 (c465C>T). Six of 47 (12.8\%) showed the \textit{BIM} intron 2 deletion polymorphism and 11 of 47 (23.4\%) CML patients did the SNP. In healthy volunteers, 4 of 20 (20\%) had the \textit{BIM} deletion polymorphism and 4 of 20 (20\%) did the \textit{BIM} SNP: none of our subjects had the \textit{BIM} deletion polymorphism and SNP (c465C>T) concurrently. CML patients with \textit{BIM} polymorphic variants showed high frequencies of reduction of imatinib dose and switching to second-line TKIs.

\textbf{Conclusion:} TKIs. BIM is an essential protein for the apoptotic process in cancer cells with TKI therapy, the detection assay of polymorphic variants, such as Q-Invader assay, could be useful in clinical practice.

\section*{BACKGROUND}

Tyrosine kinase inhibitors (TKIs) are now used as the central therapeutic approach for treatment of several tumors, including chronic myeloid leukemia (CML), and the introduction of TKIs as a first-line treatment for CML has introduced new possibilities for curing CML [1,2], for example, the STOP imatinib (IM) study by Mahon et al. [3]. It is well-known that TKIs, including IM, activate proapoptotic \textit{BCL-2} homology domain 3–only proteins, such as \textit{BCL2-like 11} (\textit{BCL2L11}, also known as \textit{BIM}) [4]. Therefore, \textit{BIM} plays a major role in TKI-induced apoptosis of \textit{BCR-ABL1}–positive CML cells [5]. Recently, Ng et al. [6] demonstrated that East Asian CML patients with a common intronic deletion polymorphism in the gene coding \textit{BIM} (at intron 2) had inferior responses to TKIs...
compared with those without the genetic variation. Augis et al. [7] also found a single nucleotide polymorphism (SNP) c465C>T at BIM exon 5, without amino acid change, in French CML patients who did not respond to IM treatment. The detection of BIM genetic variants is critical for TKI therapy, not only for CML but also for other cancers [6].

Q-Invader assay is widely used to detect SNP in clinical practice [8,9]; however, this technique is not generally available to detect deletion polymorphism, such as BIM deletion polymorphism, because its deletion expands approximately 3 kb. In the current study, we ascertained that Q-Invader assay could detect BIM deletion polymorphism at intron 2 and SNP c465C>T at BIM exon 5. We used the Q-Invader assay and determined the clinical implications in CML patients with maintained major molecular response.

**PATIENTS AND METHODS**

**Patients**

To investigate genetic variations of BIM, we obtained DNA from 47 Japanese CML patients referred to the Tokyo Medical University Hospital who had achieved molecular remission (MR) of 4.0 or more [10]. Since all CML patients studied achieved MR4.0,
Figure 2 Schematic illustration of the Q-Invader assay in a duplex format for detecting BIM polymorphisms. Human DNA is amplified by two-step PCR. The denatured PCR products formed an invasive complex with the primary probe and Invader oligo. The released 5’-flap probe, a product of the first reaction, promotes cleavage of the fluorescence probe in the second Invader reaction, creating a detectable, amplified signal for detecting BIM polymorphisms. Finally, the PCR products were made into duplex DNA with DNA polymerase.

DNAs obtained from peripheral blood from CML patients are of representative germ-line change rather than somatic CML cells. There were 10 patients who maintained MR for more than 12 months after discontinuation of IM, 3 patients who experienced relapse within 4 months after cessation of IM, 16 patients who maintained MR for 24 consecutive months while using TKIs, and 18 patients treated with TKIs who showed fluctuating MR (but maintained major molecular response) over the course of 24 months. We also studied DNA obtained from 20 healthy volunteers used as the Japanese control group. This study was approved by the Institutional Review Board of Tokyo Medical University (no. 1655; approved on January 28, 2011). KCL22 was used as a positive control and K562 was used as a negative control for the BIM deletion polymorphism of intron 2 [6,10].

Detection of BIM deletion polymorphism and SNP by Q-Invader technique

Preparation of genomic DNA was performed with a robotic workstation (Magtration System, 6GC; Precision System Science, Chiba, Japan) with the EZ1 DNA Blood 350 μl kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. The genomic DNA was subjected to polymerase chain reaction amplification using primer designed to detect a deletion site (2903 bp) in intron 2 (deletion polymorphism) (Figure 1-A) and a silent SNP in exon5 (c465C>T) (Figure 1-B), separately. Thus, Two fluorescence signals (carboxyfluorescein or FAM for non-deletion polymorphism in intron 2 and C allele in exon 5; RED and RED or RED for deletion polymorphism in intron 2 and T allele in exon5) could be detected in each single reaction with a Universal General Purpose Reagent (Hologic, Bedford, MA, USA), including Cleavase and FRET mix with two common fluorescence probes (Figure 2). Template DNA was added to a 15-μl reaction mixture containing 500 nM primers (Sigma Aldrich Japan, Tokyo, Japan) for amplification BIM gene, 600 nM of each primary probe (Sigma Aldrich Japan), 70 nM Invader oligo (Sigma Aldrich Japan), Universal General Purpose Reagent (Hologic) and FRET mix (Table 1). The reaction mixture was preheated in a 384-PCR plate (Roche, Basel, Switzerland) at 95°C for 2 min, and a two-step PCR reaction was carried out for 40 cycles (95°C for 15 sec, 65°C for 60 sec) in a LightCycler 480 (Roche) [8,9]. Fluorescence values of FAM (wavelength/bandwidth: excitation, 465 nm; emission, 510 nm) and RED (excitation, 533 nm; emission, 610 nm) were measured at end of the incubation/extension step at 65°C for each cycle and by standard real-time PCR. By analyzing the results, a crossing point (Cp) can be obtained by an endpoint genotyping method in the Light Cycler 480 software.

Statistical analysis

The results were statistically analyzed with GraphPad Prism.
Table 1: Polymerase−chain−reaction primer for detection of BIM deletion polymorphism and BIM single−nucleotide−polymorphism.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence 1</th>
<th>Sequence 2</th>
<th>Sequence 3</th>
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<tbody>
<tr>
<td>BIM-F1 (polymorphism)</td>
<td>5'-CCTGGTGAAAGCCGACAAAACTAT-3'</td>
<td>5'-CGCGCCGAGGCGGAGACGAGTTTAACGC-3'</td>
<td>5'-CGCGCCGAGGCGGAGACGAGTTTAACGC-3'</td>
</tr>
<tr>
<td>BIM-W1 (polymorphism)</td>
<td>5'-GGCCADGRTAACCRCGGGGTC-3'</td>
<td>5'-ACGGACGCGGAGCTGTTCTCCATAGGTAATC-3'</td>
<td>5'-ACGGACGCGGAGCTGTTCTCCATAGGTAATC-3'</td>
</tr>
<tr>
<td>BIM-dR1 (polymorphism)</td>
<td>5'-CCTGGGCTCAGCCTGACGATGGA-3'</td>
<td>5'-CCTGGGCTCAGCCTGACGATGGA-3'</td>
<td>5'-CCTGGGCTCAGCCTGACGATGGA-3'</td>
</tr>
<tr>
<td>asBIMdel-p1</td>
<td>5'-CGGCGGACGGGGAGCGCTTTCGCTG-3'</td>
<td>5'-CGGCGGACGGGGAGCGCTTTCGCTG-3'</td>
<td>5'-CGGCGGACGGGGAGCGCTTTCGCTG-3'</td>
</tr>
<tr>
<td>asBIMdel-p2</td>
<td>5'-ACGGACGCGGAGCTGTTCTCCATAGGTAATC-3'</td>
<td>5'-ACGGACGCGGAGCTGTTCTCCATAGGTAATC-3'</td>
<td>5'-ACGGACGCGGAGCTGTTCTCCATAGGTAATC-3'</td>
</tr>
<tr>
<td>asBIMdel-i0</td>
<td>5'-CCTGGGCTCAGCCTGACGATGGA-3'</td>
<td>5'-CCTGGGCTCAGCCTGACGATGGA-3'</td>
<td>5'-CCTGGGCTCAGCCTGACGATGGA-3'</td>
</tr>
<tr>
<td>as-rs724710-p1</td>
<td>5'-CGGCGGACGGGGAGCGCTTTCGCTG-3'</td>
<td>5'-CGGCGGACGGGGAGCGCTTTCGCTG-3'</td>
<td>5'-CGGCGGACGGGGAGCGCTTTCGCTG-3'</td>
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<tr>
<td>as-rs724710-p2</td>
<td>5'-ACGGACGCGGAGCTGTTCTCCATAGGTAATC-3'</td>
<td>5'-ACGGACGCGGAGCTGTTCTCCATAGGTAATC-3'</td>
<td>5'-ACGGACGCGGAGCTGTTCTCCATAGGTAATC-3'</td>
</tr>
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</table>

RESULTS

Using the Q-Invader assay, the fluorescent intensity of FAM increases when no deletion polymorphism or no SNP (c465), whereas the intensity of Red increases when the sample has the intron 2 BIM deletion polymorphism or SNP (c465>T). These two fluorescent intensities plot the x-axis and the y-axis, respectively, and the polymorphism is identified by the scatter plot (Figure 3). Data for the BIM deletion polymorphism were confirmed by standard polymerase chain reaction assay and gel electrophoresis, as described previously [10]. Q-Invader assay was used for assessment of BIM polymorphic variants in the Japanese population.

None of the CML patients or healthy subjects had the BIM deletion polymorphism and SNP (c465>c>T) concurrently; thus, we analyzed BIM deletion polymorphism variants separately and then combined as a group of BIM genetic variants. None of our subjects showed T/T genotype of the c465c>T SNP. Six of 47 (12.8%) Japanese CML patients and 4 of 20 (20%) Japanese volunteers showed the BIM intron 2 deletion polymorphism. Regarding the SNP (c465c>T), using the Q-Invader technique, 11 of 47 (23.4%) CML patients and 4 of 20 (20%) volunteers showed the SNP. As reported previously, 3 of 13 CML patients who stopped IM had the BIM deletion polymorphism, and all 3 patients with BIM deletion had molecular relapse within 4 months, whereas none of the remaining 10 patients without the BIM deletion maintained molecular response after stopping IM [10]. Although we found three CML patients with BIM SNP at c465c>T in the group that stopped using IM, none of them had molecular relapse. Of the 34 CML patients who experienced MR of 4.0 while still taking TKIs, 3 of 34 (8.8%) had BIM deletion polymorphism and 8 of 34 (23.5%) had BIM SNP (c465c>T).

We next assessed clinical features of 44 CML patients showing the BIM deletion polymorphism or BIM SNP (c465c>T; BIM polymorphism variants). Of the 47 CML patients with BIM status, three were excluded from the clinical analysis because of inappropriate IM intake (one with previous chemotherapy and two with low adherence). Thirty patients were treated with first-line IM without previous alpha-interferon therapy. None of the 28 CML patients without BIM genetic variants switched to second-line TKIs, whereas CML patients with either BIM SNP (2/10 versus 0/34; P = 0.1722) or BIM deletion polymorphism (2/6 versus 0/38; P = 0.0262) had a history of switching to second-line TKIs. When we combined genetic variants of the BIM gene (intron 2 deletion polymorphism and SNP [c465c>T]), a significantly high frequency of switching to second-line TKIs in CML patients with BIM genetic variants was again noted (4/16 versus 0/28; P = 0.0055) (Table 2). Switching to second-line TKIs in our CML patients was attributable to hematologic or non-hematologic adverse events, but not to IM resistance. We next assessed IM dose for these CML patients. The frequency of continuing the IM dose of 400 mg was significantly higher in CML patients without BIM genetic variants than in those with genetic variants (25/28 versus 6/16; P = 0.0003). These observations indicate that Japanese CML patients without BIM genetic variants (deletion polymorphism at intron 2 and SNP [c465c>T]) could benefit from the standard IM dose without switching to second-line TKIs. This tendency was also evident in 30 CML patients administered first-line IM therapy (data not shown).

DISCUSSION

In the current study, we determined BIM genetic variants in Japanese CML patients using the Q-Invader assay. The current Q-Invader assay could clarify which subjects have BIM SNP as well as the long-distance deletion, such as BIM deletion polymorphism at intron 2, separately. Therefore in clinical setting, the Q-Invader assay should be performed twice on the same patient sample, since none of our sample showed...
concomitant positivity for these two genetic variants. The BIM genetic variants were detected in 40% of the Japanese control population; 20% had intron 2 deletion polymorphism and 20% had exon 5 SNP of the BIM gene. Ng et al. [6] reported that 12.3% of East Asian healthy individuals had BIM intron 2 deletion polymorphism. They reported that CML patients without the BIM deletion polymorphism had a significantly high optimal response compared with those with the BIM genetic variant because of switched BIM splicing from exon 4 to exon 3, resulting in lack of the proapoptotic BCL-2 homology domain 3 [6]. Augis et al. [7] also reported another BIM genetic variant at exon 5 in French CML patients. This genetic variant was significantly associated with a late achievement of major molecular response thereby leading frequent BCR-ABL1 tyrosine kinase domain mutations [7]. Our previous study also demonstrated that CML patients with BIM deletion polymorphism are IM-dependent [10], suggesting that BIM dysfunction attributable to polymorphism variants is essential in TKI treatment for CML patients. Since approximately 45% of CML patients who showed resistant to IM treatment had no BCR-ABL1 domain mutation, BIM genetic variants, including BIM-SNP, may have some role in such patients who resist to TKIs.

It has also been demonstrated that intron 2 BIM deletion polymorphism in epidermal growth factor receptor (EGFR) mutation-positive nonsmall cell lung cancer showed intrinsic resistance to EGFR TKIs [6,11], and the resistance was overcome by the combination of TKIs and histone deacetylase inhibitor because of restoration of the aberrant splicing of BIM and upregulation of BCL-2 homology domain 3-containing BIM isoforms to resestitize to EGFR TKIs [11]. However, the correlation between BIM SNP at exon 5 (c465C>T) and the inferior response to TKIs in non-small cell lung cancer has not been reported. Furthermore, biological implication of BIM-SNP at exon 5 (c465C>T) should be clarified in various neoplasias. The BIM deletion polymorphism is uncommon in Caucasians and African Americans, whereas BIM SNP at exon 5 (c465C>T) has been found in the Caucasian cohort [7]. Moreover, BIM haploinsufficiency may affect the immune system [12-14]; for example, BIM dysfunction affects interleukin-15–mediated survival of natural killer cells [13]. Immune surveillance is now considered to be a key element for prevention of cancer proliferation, including CML. Since Augis et al. demonstrated a reduction of Bim mRNA levels in the circulating mononuclear cells of healthy controls with c465C>T genotype [7], further information regarding BIM genetic variants and immune function should be accumulated.

CONCLUSIONS

Our results show that a high-throughput assay like the current Q-Invader assay could be a powerful tool to delineate the clinical relevance of BIM dysfunction in various cancers. Finally, the Q-Invader assay has clearly identified individuals with either BIM deletion polymorphism or BIM SNP.

ACKNOWLEDGEMENTS

Kazuma Ohyashiki received research support and honoraria for lecture fees from Bristol-Meyers Squibb KK and Novartis KK.

Author’s contributions

K.O.: designed and wrote the manuscript; K.T. and T.Y.: developed and performed Q-Invader assay; S.K. and T.T.: collected patient samples; T.U. and K.O.: performed the polymerase chain reaction technique; Y.Y.: supported this study; and J.H.O.: reviewed the manuscript.

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