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Research Article

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

Kazuma Ohyashiki[1,3]*, Kenichi Tadokoro[2], Toshikazu Yamaguchi[2], Seiichiro Katagiri[1], Tomohiro Umezu[3], Kazushige Ohtsuki[4], Tetsuzo Tauchi[1], Toshinori Yamamoto[2] and Junko H. Ohyashiki[4]

[1]*Department of Hematology, Tokyo Medical University, Japan*
[2]*Development of Clinical Genomics, BML. Inc., Japan*
[3]*Department of Molecular Science, Tokyo Medical University, Japan*
[4]*Division of Molecular Oncology, Institute of Medical Science, Tokyo Medical University, Japan*

*Corresponding author

Kazuma Ohyashiki, Department of Hematology, Tokyo Medical University, 6-7-1 Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan, Tel: 81-3-3342-1510; Fax: 81-5381-6651, E-mail: ohyashik@rr.iij4u.or.jp

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Keywords

- Chronic myeloid leukemia
- Imatinib
- *BIM* polymorphism

Abstract

Background: Accumulating evidence suggests that genetic variants, including deletion and single nucleotide polymorphism (SNP) have role in the genesis and progression of various cancers. *BIM* (also known as *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 *BIM* genetic variants in CML patients and clinical relevance.

Subjects and methods: We assessed known*BIM* polymorphic variants (*BIM* deletion polymorphism in intron 2 and silent SNP in *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 ($MR^{4.0}$) or more.

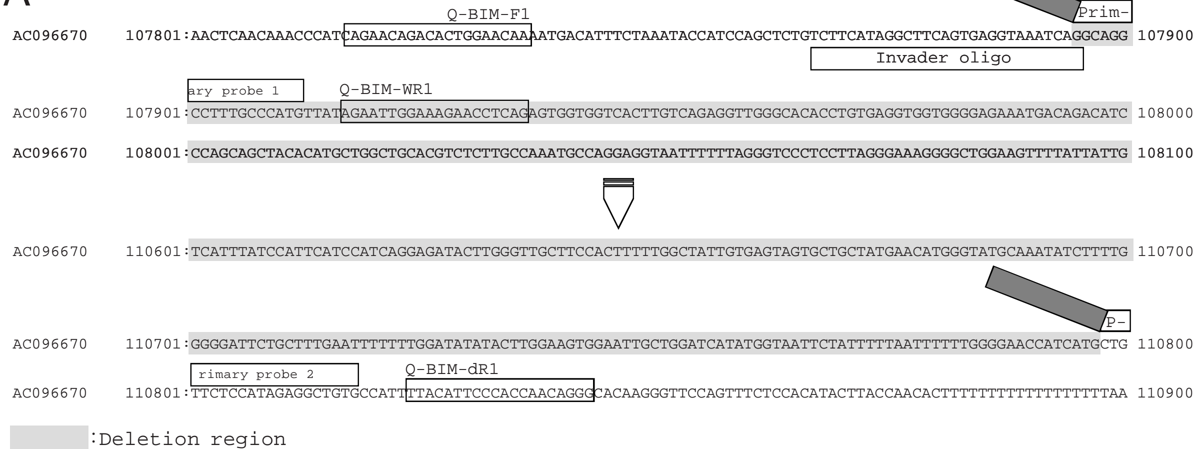
Results: The Q-Invader assay was able to detect *BIM* deletion polymorphism expanding approximately 2900 bp and *BIM*SNP at exon 5 (c465C>T). Six of 47 (12.8%) showed the BIM intron 2 deletion polymorphism and 11 of 47 (23.4%) CML patients did the SNP. In healthy volunteers, 4 of 20 (20%) had the BIM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

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.

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 *BCL-2* homology domain 3–only proteins, such as *BCL2-like 11* (*BCL2L11*, also known as *BIM*) [4]. Therefore, *BIM* plays a major role in TKI-induced apoptosis of *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 *BIM* (at intron 2) had inferior responses to TKIs

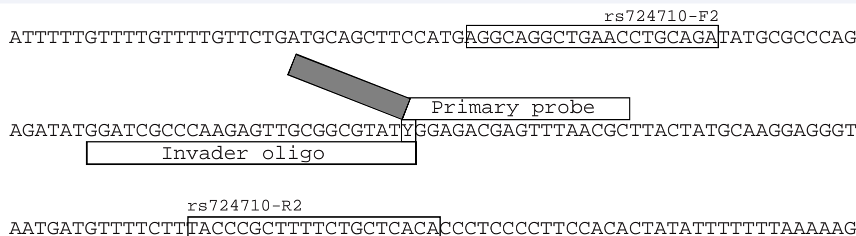
A



Invader probes
Primary probe 1: CGCGCCGAGGGGCAGCCTTTGCCCATG
Primary probe 2(for deletion): ACGGACGCGGAGCTGTTCTCCATAGAGGCTGTG
Invader oligo: TCTTCATAGGCTTCAGTGAGGTAAATCAA

Primers
Q-BIM-F1: CAGAACAGACACTGGAACAA
Q-BIM-WR1: CTGAGGTTCTTTCCAATTCT
Q-BIM-dR1: CCCTGTTGGTGGGAATGTAA

B



Invader probes
Primary probe 1: CGCGCCGAGGCGGAGACGAGTTTAACGC
Primary probe 2: ACGGACGCGGAGTGGAGACGAGTTTAACGC
Invader oligo: GGATCGCCCAAGAGTTGCGGCGTATC

Primers
rs724710-F2 : AGGCAGGCTGAACCTGCAGA
rs724710-R2 : TGTGAGCAGAAAAGCGGGTA

Figure 1 Design used to detect *BIM* genetic variants. A: Cleavage point for the Q-Invader assay at BIM deletion polymorphism. Cleavage points in polymerase chain reaction amplicon (white boxes). Deletion area at intron 2 (shaded area). B: Cleavage point for the Q-Invader assay at rs724710. Cleavage point in polymerase chain reaction amplicon (white box).

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 $MR^{4.0}$,

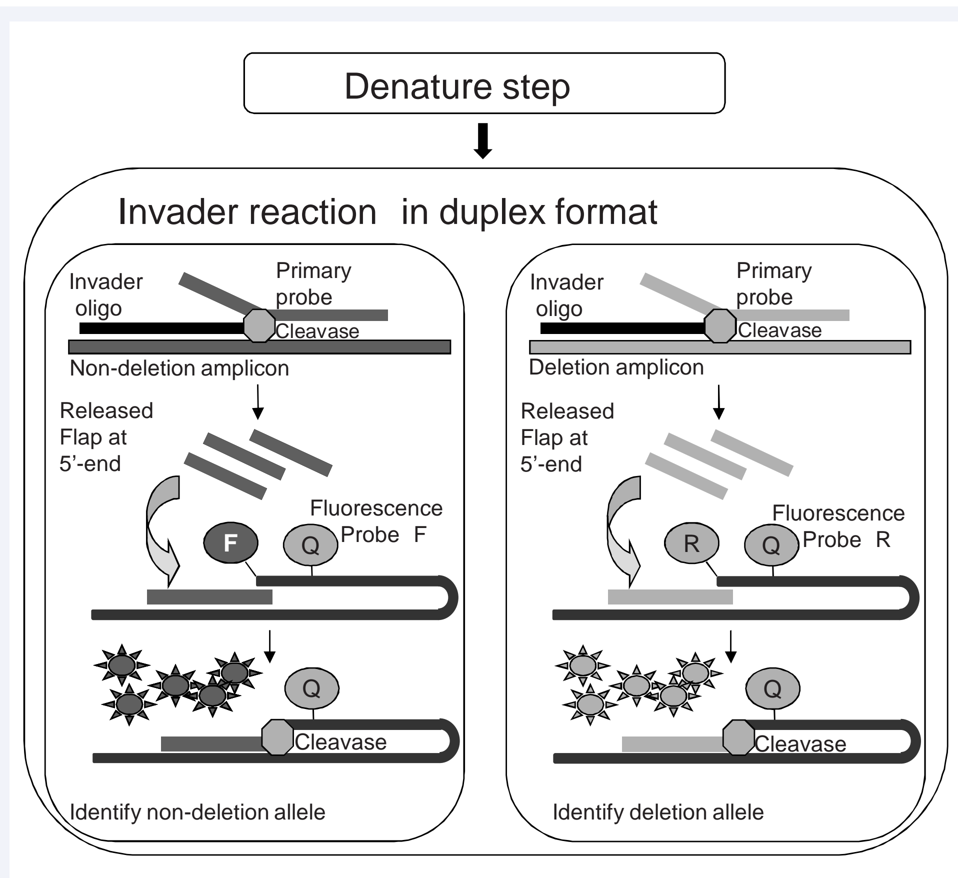


Figure 2 Schematic illustration of the Q-Invader assay in a duplex format for detecting BIM polymorphisms. Human DNA is amplified by two-step PCR. The denatured PCR products formed an invasive complex with the primary probe and Invader oligo. The released 5'-flap probe, a product of the first reaction, 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 (Magtraction 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; REDmond 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.

Polymerase–chain–reaction primer
Q-BIM-F1 (polymorphism): 5'-CCTCGTGGAAGGCGACAACCTAT-3'
Q-BIM-WR1 (polymorphism): 5'-GGCCADGGRTACCCRGGCTG-3'
Q-BIM-dR1 (polymorphism): 5'-CCTGGGCTCAGCCYGGGTA-3'
rs724710-F2 (SNP): 5'-AGGCAGGCTGAACCTGCAGA-3'
rs724710-R2 (SNP): 5'-TGTGAGCAGAAAAGCGGGTA-3'
Invader probes
asBIMdel-p1: 5'-CGCGCCGAGGGGCAGCCTTTGCCCATG-3'
asBIMdel-p2: 5'-ACGGACGCGGAGCTGTTCTCCATAGAGGCTGTG-3'
asBIMdel-io: 5'-TCTTCATAGGCTTCAGTGAGGTAAATCAA-3'
as_rs724710-p1: 5'-CGCGCCGAGGCGGAGACGAGTTTAACGC-3'
as_rs724710-p2: 5'-ACGGACGCGGAGTGGAGACGAGTTTAACGC-3'
as_rs724710-io: 5'-GGATCGCCCAAGAGTTGCGGCGTATC-3'

p1: primary probe (FAM)
p2: primary probe (RED)
io: Inavaderoligo
5'–prime of the p1 probe (CGGCCGAGG) and 5'–prime of the p2 probe (ACGGACGCGGAG) are FLAP sequence.
3'-prime of P1 and P2 probes are amino modification
io: Invader oligo, p1: primary probe (FAM), p2: primary probe (Red), SNP: single nucleotide polymorphism.

5 (GraphPad Software, La Jolla, CA, USA). The profiles of two groups were analyzed by chi-square test. $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Using the Q-Invader assay, the fluorescent intensity of FAM increases when the sample has 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 (c465C>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 (c465C>T) concurrently; thus, we analyzed *BIM* 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

Table 2: Correlation between *BIM* genetic variants and response to tyrosine kinase inhibitors.

	CML patients	Maintain imatinib	Imatinib dose	Switch to second
		400 mg	reduction	TKIs
BIM SNP (c465c>T)	10/44	4/10	4/10	2/10
P value				
BIM deletion polymorphism	2/44	2/6	2/6	2/6
P value		0.032	0.4	0.0262
BIM SNP or deletion	16/44			
Polymorphism				
P value		0.0003	0.0341	0.0055
No genetic variants	28/44	25/28	3/28	0/28

P values were calculated by chi-square test.
CML: chronic myeloid leukemia, SNP: single nucleotide polymorphism, TKI: tyrosine kinase inhibitor.

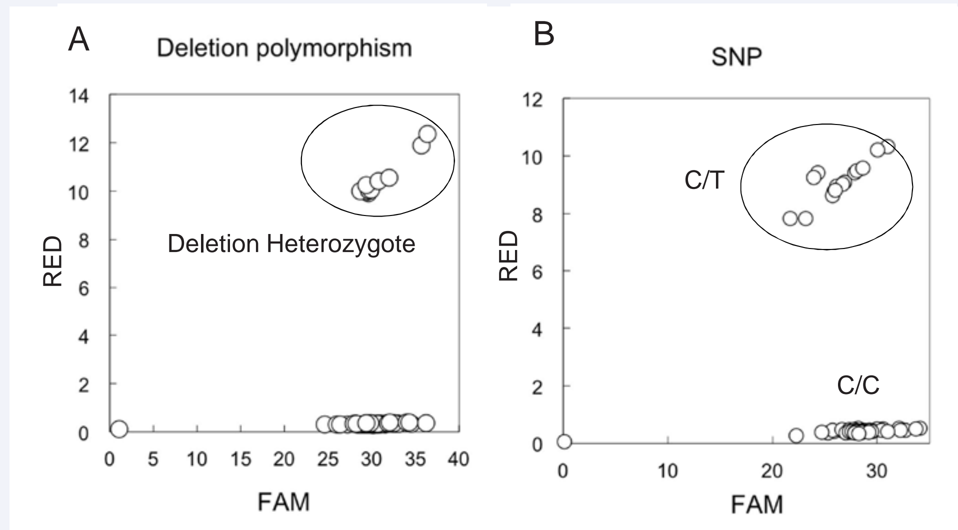


Figure 3 Scatter plot of the fluorescent intensity of FAM and Red. The fluorescent intensity of FAM increases when the sample has no deletion polymorphism or no SNP (c465), whereas the intensity of Red increases when the sample has *BIM* deletion polymorphism (A) or *BIM* SNP (c465C>T) (B). These two fluorescent intensities plot the *x*-axis and *y*-axis, respectively, and the polymorphism is identified by the scatter plot. **A**: *BIM* deletion heterozygote (circle) and non-*BIM* deletion homozygote. **B**: Scatter plot of rs724710 polymorphism for the patients. C/T heterozygote (circle) and C/C homozygote.

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 resensitize 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 haplo* insufficiency 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.

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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.

REFERENCES

1. Baccarani M, Deininger MW, Rosti G, Hochhaus A, Soverini S, Apperley JF, et al. European LeukemiaNet recommendations for the management of chronic myeloid leukemia. Blood. 2013; 122: 872-884.

2. Mauro M. Striving to achieve safe, permanent treatment

discontinuation in chronic myeloid leukemia. Leuk Res. 2013; 37: 1395–1403.

3. Mahon FX, Réa D, Guilhot J,Guilhot F, Huguet F, Nicolini F, et al. Discontinuation of imatinib in patients with chronic myeloid leukaemia who have maintained complete molecular remission for at least 2 years: the prospective. Multicenter Stop Imatinib (STIM) trial. Lancet Oncol. 2010; 11: 1029-1035.

4. Kuroda J, Puthalakah H, Cragg MS,Kelly PN, Bouillet P, Huang DC, et al. Bim and Bad mediate imatinib-induced killing of Bcr/Abl+ leukemic cells and resistance due to their loss is overcome by a BH3 mimetic. ProcNatlAcadSci USA. 2006; 103:14907-14912.

5. Kuribara R, Honda H, Matsui H, Shinjyo T, Inukai T, Sugita K, et al. Roles of Bim in apoptosis of normal and Bcr-Abl-expressing hematopoietic progenitors. Mol Cell Biol. 2004; 24: 61724-61783.

6. Ng KP, Hillmer AM, Chuah CT, Juan WC, Ko TK,Teo AS, et al. A common BIM deletion polymorphism mediates intrinsic resistance and inferior responses to tyrosine kinase inhibitors in cancer. Nat Med. 2012; 18: 521-528.

7. Augis V, Airiau K,Josselin M, Turcq,Mahon FX, Belloc F. A single nucleotide polymorphism in the cBIM is associated with a slow achievement of major molecular response in chronic myeloid leukaemia treated with Imatinib. Plos ONE. 2013; 8: e78582.

8. Tadokoro K, Kobayashi M, Suzuki F,Tanaka C, Yamaguchi T, Nagano M,et al. Comparative quantitative analysis of hepatitis C mutations at amino acids 70 and 91 in the core region by the Q-Invader assay. J Virol Meth. 2013; 189: 221-227.

9. Tadokoro K, Yamaguchi T, Egashira T, and Hara T. Quantitation of viral load by real-time PCR-monitoring Invader reaction. J Virol Meth. 2009; 155: 182-186.

10. Katagiri S, Umezu T, Ohyashiki JH, Ohyashiki K. The BCL2L11 (BIM) deletion polymorphism is a possible criterion for discontinuation of imatinib in chronic myeloid leukaemia patients. Br J Haematol. 2013; 160: 269-271.

11. Nakagawa T, Takeuchi S, Yamada T, Ebi H, Sano T, Nanjo S, et al. EGFR-TKI resistance due to BIM polymorphism can be circumvented in communication with HDAC inhibition. Cancer Res. 2013; 73: 2428-2434.

12. Bouillet P, Purton JF, Godfrey DI,Zhang LC, Coultas L, Puthalakath H, et al. BH3-only family member Bim is required for apoptosis of autoreactive thymocytes. Nature. 2002; 415: 922-926.

13. Huntington ND, Puthalakath H, Gunn P, Naik E, Michalak EM, Smyth MJ, et al. Interleukin-15 mediated survival of natural killer cells is determined by interacting among Bim, Noxa, and Ncl-1. Nat Immunol. 2007; 8: 856-863.

14. Sandalova E, Wei C-H, Masucci MG, Levitsky V. Regulation of expression of Bcl-2 protein family Bim by T cell receptor triggering. Proc Natl Acad Sci USA. 2004; 101: 3011-3016.

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