Original Article

Frequent Intronic Insertion of ABL Gene Detected in Thai Chronic Myeloid Leukemia [CML] Patients Carrying Both e13a2 and e14a2 Variants

Kriangpol Wiriyaukaradecha MSc[1], Chakrit Bunyoo MSc[1], Wandee Udomchaiprasertkul MSc[1], Kamonwan Soonklang MSc[2], Narongrit Sritana MSc[1], Chirayu Auewarakul MD, PhD[3,4]

[1] Molecular and Genomic Research Laboratory, Division of Research and International Relations, HRH Princess Chulabhorn College of Medical Science, Bangkok, Thailand
[2] Data Management Unit, Division of Research and International Relations, HRH Princess Chulabhorn College of Medical Science, Bangkok, Thailand
[3] Faculty of Medicine and Public Health, HRH Princess Chulabhorn College of Medical Science, Bangkok, Thailand
[4] Division of Hematology, Department of Medicine, Faculty of Medicine Siriraj Hospital, Bangkok, Thailand

Background: Reverse-transcription polymerase chain reaction [RT-PCR] and quantitative RT-PCR [RQ-PCR] are valuable tools for investigating CML patients with BCR-ABL fusion gene. Several insertion and deletion mutations can also be identified.

Objective: To determine frequencies of e14a2 and e13a2 variants and explore existence of intronic ABL mutations in Thai CML patients.

Materials and Methods: The frequency of e14a2 and e13a2 variants was analyzed in 1,035 CML cases by RT-PCR and/or RQ-PCR. Cloning of pCRTM4-TOPO plasmid and sequencing were performed to confirm the insertion sequences of mutations.

Results: Collected samples of blood or bone marrow of CML patients were analyzed by RT-PCR (682 cases) and RQ-PCR (353 cases). The prevalence of e14a2, e13a2, and both variants in BCR-ABL positive cases were 61.9% (109/176), 32.4% (57/176), and 5.7% (10/176), respectively. Interestingly, an unexpected band larger than both e13a2 and e14a2 bands was detected in majority of the cases, which co-expressed both e13a2 and e14a2 sequences by RT-PCR. Sequencing analysis revealed that the 49-bp insertion was a part of ABL intron 1b. The insertion consisted of a stop codon, leading to only the BCR part of the fusion gene translated. This particular sequence of insertion had previously been reported only in one Japanese ALL patient.

Conclusion: The frequencies of e14a2 and e13a2 BCR-ABL variants were similar to those previously reported in Western and Asian studies. The intron 1b insertion was frequently observed in Thai CML patients carrying both variants in this study. However, its clinical importance should be further characterized.

Keywords: BCR/ABL, Chronic Myeloid Leukemia [CML], Intronic insertion

J Med Assoc Thai 2018; 101 [Suppl. 6]: S55-S61
Full text. e-Journal: http://www.jmatonline.com

The reciprocal translocation between the long arm of chromosome 9 and chromosome 22 t (9;22) (q34;q11) resulting in fusion BCR/ABL gene is a hallmark of chronic myeloid leukemia [CML][(1)]. The BCR breakpoint occurred between exon 13 and exon 14 or between exon 14 and exon 15 brings about e14a2 (b3a2) or e13a2 (b2a2) variants that can be translated into a 210 kDa (p210) fusion protein. These breakpoints were the major breakpoint cluster region (M-bcr); whereas, the minor break point region (m-bcr) of e1a2 variant is

Correspondence to:
Auewarakul C, HRH Princess Chulabhorn College of Medical Science, Chulabhorn Royal Academy, Bangkok 10210, Thailand.
Phone & Fax: +66-2-5766000
E-mail: chirayu.aue@pccms.ac.th

How to cite this article: Wiriyaukaradecha K, Bunyoo C, Udomchaiprasertkul W, Soonklang K, Sritana N, Auewarakul C. Frequent Intronic Insertion of ABL Gene Detected in Thai Chronic Myeloid Leukemia (CML) Patients Carrying Both e13a2 and e14a2 Variants. J Med Assoc Thai 2018;101;Suppl.6: S55-S61.

translated into a 190 kDa (p190) in acute lymphoblastic leukemia [ALL][2]. The membrane tyrosine kinase domain of ABL can be controlled by tyrosine kinase inhibitor [TKI] such as imatinib[3,4]. In a previous study, approximately 20% of imatinib treatment patients did not respond to the drug[5]. The kinase domain mutations were found in 44% of imatinib resistant patients[6]. The second line kinase domain inhibitor dasatinib and nilotinib are provided for imatinib resistant patients[7]. This mutation could appear as a point mutation or an insertion or a deletion. Chromosome analysis, fluorescence in situ hybridization [FISH], and reverse-transcription polymerase chain reaction [RT-PCR] are used for the determination of BCR/ABL in CML patients[8]. Both Reverse-transcription polymerase chain reaction [RT-PCR][9] and quantitative RT-PCR [RQ-PCR][10] are sensitive tools for determining fusion gene or monitoring gene levels during TKI treatment.

In the present study, we determined the incidence of e14a2 and e13a2 frequencies and identified the intronic insertion in Thai CML patients.

Materials and Methods

Patient

Bone marrow or blood samples from 682 CML patients and 353 other patients were collected between 2009 and mid-year 2014 and were diagnosed by RT-PCR and RQ-PCR, respectively at Cancer Molecular Diagnostics Laboratory, Chulabhorn Hospital, Bangkok.

White blood cell collection

Red blood cell from bone marrow or blood samples were lysed using lysis buffer (NH_4Cl, $KHCO_3$ and EDTA) within 24 hours after collection. White blood cell was resuspended in 1 ml, TRIzol® REAGENT (Invitrogen, USA).

RNA isolation and cDNA synthesis

Total RNA was extracted from various dissected tissue using the TRIzol® REAGENT and then treated with RiboReseve™ RNA storage buffer (AMESCO, USA). The cDNA was synthesized from 1 µg of total RNA using by SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, USA).

RT-PCR for BCR-ABL

The p210 BCR-ABL fusion gene was detected using BART-F (5'CGGGAGCAGCAGAAGAAGTGT3') and BART-R (5'CGAAAAGGTTGGGGTCATTTTC3') primers. Beta-actin gene was amplified by using β-actin-F (5'GTGGGGCGCCCCAGGCACCA3') and β-actin-R (5'GTCCTTAATGTCACGCACGATTTC3') primers for internal control. The thermocycle condition were 95°C for five minutes followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for one minute. The final extension was carried out at 72°C for five minutes. The PCR product was detected by 2% TBE agarose gel electrophoresis.

RQ-PCR for BCR-ABL

Quantitative Real-time PCR for BCR-ABL gene were performed using condition of 95°C for 10 minutes followed by 60 cycles of 95°C for 10 seconds, 55°C for 20 seconds and 72°C for 20 seconds. Express qPCR SuperMixes® (invitrogen™, life technology, USA) was used together with primers and fluorescent probes for BCR/ABL and ABL gene amplification and detection. PCR was achieved using LightCycler® 480 Instrument (Roche™, USA).The percentage ratio of BCR-ABL gene per ABL gene was calculated (BCR-ABL/ABL x100).

Statistical analysis

Mann-Whitney U test was performed for analysis of the BCR/ABL RT-PCR results; while Kruskal Wallis Test was used for analyzing BCR/ABL variant results.

TA cloning and sequencing of RT-PCR product

The PCR products were removed from agarose gel and cloned into commercial pCR™ 4-TOPO® plasmid vector (Invitrogen®, USA). The recombinant plasmid was transformed into Mach1™-T1® *E. coli* stain (Invitrogen®, USA). After recombinant cell culture, the plasmid was extracted and commercially sequenced (First BASE Laboratories SdnBhd, Malaysia).

Sequence analysis

DNA sequences were analyzed using BLAST programs in the GenBank database. Multiple sequence aliments of nucleic acids were performed using. The deduced amino acids were archived using NCBI.

The protocol of this research was reviewed and approved by the Human research ethics committe Chulabhorn Research Institute No. 14/2554.

Results

Clinical data of patients with and without BCR/ABL transcript

Six hundred eight-two CML patients and 353

Table 1. Clinical parameters of patients with positive and negative BCR/ABL fusion gene

Variables	BCR/ABL		*p*-value
	Positive	Negative	
No. of patients	176	177	
WBC (x10^9/ L)	138.2±170.7*	81.2±540.7*	<0.001
	69.5 (1.9 to 997.0)$^+$	21.5 (0.6 to 7,130)$^+$	
Hb (g/dl)	10.2±6.4*	11.0±5.0*	0.043
	9.8 (1.8 to 3,233)$^+$	10.1 (4.8 to 60.4)$^+$	
Platelet (x10^9/ L)	564.6±492.7*	509.6±493.3*	0.038
	451.0 (1.8 to 3,233)$^+$	311 (1.7 to 2,364)$^+$	
Hct (%)	29.6±7.5*	33.2±10.1*	0.005
	30 (9.1 to 56)$^+$	31.8 (14.9 to 65)$^+$	

Mann-Whitney U test
* Mean ± SD; $^+$Median ± SD

Table 2. Clinical parameters of patients carrying each variant

Variables	BCR/ABL (positive)			*p*-value
	e13a2	e14a2	e13a2 and e14a2	
No. of patients	57	109	10	
WBC (x10^9/ L)	146.3±205.9*	137.5±155.8*	99.8±92.7*	0.913
	38.2 (3.1 to 997)$^+$	73.3 (2.1 to 808)$^+$	62.6 (1.9 to 227)$^+$	
Hb (g/dl)	11.7±10.8*	9.5±2.4*	9.6±2.1*	0.182
	10.2 (4.5 to 86)$^+$	9.6 (2.8 to 16.0)$^+$	10.2 (6.4 to 13.3)$^+$	
Platelet (x10^9/ L)	458.2±465.8*	633.6±513*	417.1±208.6*	0.150
	303 (1.8 to 2,342)$^+$	572 (2 to 3,233)$^+$	451 (141 to 744)$^+$	
Hct (%)	30.5±7.8*	29.1±7.4*	30.7±8.2*	0.538
	30.5 (13.9 to 46.5)$^+$	29.5 (9.1 to 56)$^+$	30.6 (18 to 45.9)$^+$	

Kruskal Wallis test
* Mean ± SD; $^+$Median ± SD

unclassified patients were analyzed by RT-PCR and RQ-PCR, respectively, between 2009 and mid-year 2014. The data from RT-PCR showed 176 patients (49.9%) were positive for BCR/ABL fusion gene (Table 1). Significant difference of white blood cell count (WBC) was found between positive and negative BCR/ABL transcripts (138.2 vs. 81.2 x10^9/L, p<0.001), but Hemoglobin [Hb], Hematocrit [Hct], and Platelet Count were similar. In addition, e14a2, e13a2 and both variants were detected in 109 (61.9%), 57 (32.4%), and 10 (5.7%) samples, respectively (Table 2). There was no difference of WBC, Hb, Hct, and platelet counts between the transcript variants. Meanwhile, RQ-PCR data presented the percentage ratio between <0.01 to 103.23, while the reducing trend of percentage ratio in TKI treatment patients was not different from e14a2 and e13a2 patients. Mutation of ABL kinase domain with co-expressed e14a2 and e13a2 variants.

We found nine patients with both e13a2 and e14a2 variants. e14a2 and e13a2 was separately found in one patient and two patients, respectively, at first diagnosis. Interestingly, for RT-PCR, all samples with co-expression of both e13a2 and e14a2 appeared to have an unexpected band larger than both variants (Figure 1). After sequencing this PCR product, the insertion was found between BCR exon 14 (e14) and ABL exon 2 (a2) in BCR/ABL fusion transcript (Figure 2). This 49-bp insertion was a part of ABL intron 1b. The insertion consisted of stop codon; therefore, it could be translated only the BCR part of the fusion

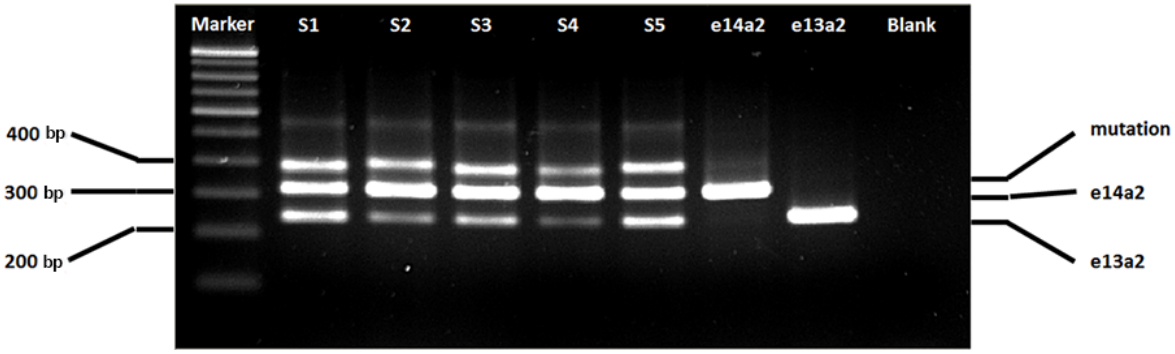

Figure 1 Ethidium-bromide-strained 2% agarose gel electrophoresis presenting the band of BCR-ABL fusion gene by RT-PCR; lane S1 to S5, co-expression of e13a2 and e14a2 variant with intronic insertion BCR/ABL band.

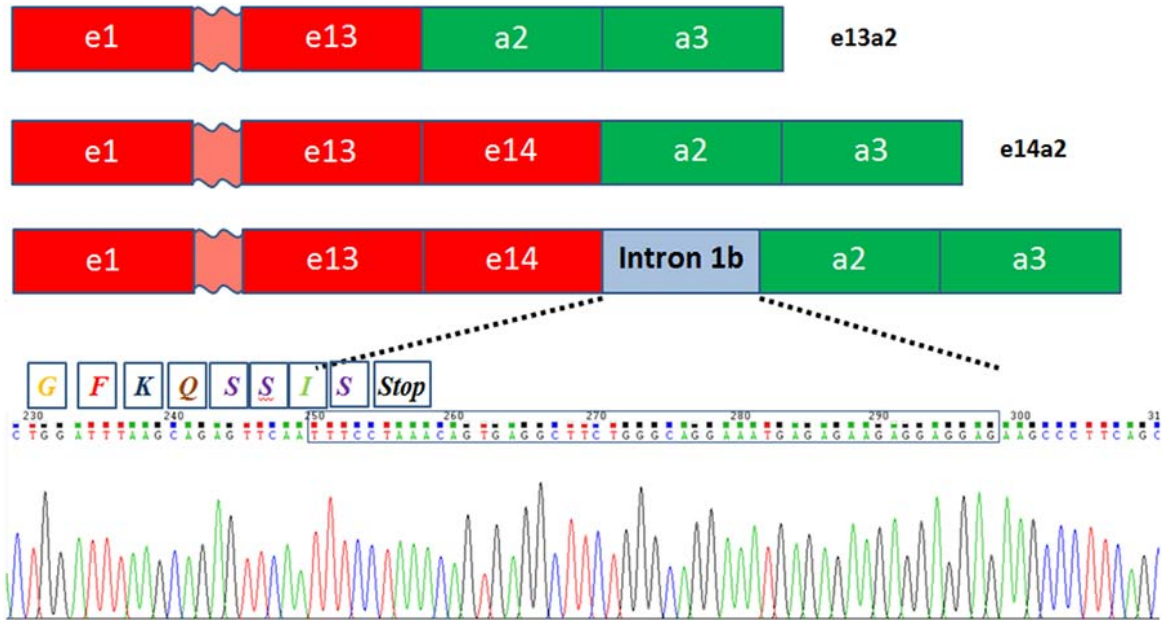

Figure 2 Diagram show BCR-ABL variant, insertion sequence and deduced amino acid; Blue box, intronic 1b insertion between exon14 of BCR and exon2 of ABL fusion gene; italic in box, the amino acid sequence encoded; underneath box show intronic 1b nucleotide sequence.

protein. Since this translated protein showed no tyrosine kinase activity, it may not be considered as an oncoprotein.

Discussion

The qualitative RT-PCR is the technique for determining BCR/ABL gene that has been used together with chromosome analysis or FISH in CML patient[11,12]. This report showed that 49.9% of BCR/ABL fusion gene was detected in CML patient at this laboratory. The frequency of e14a2 and e13a2 were found to be 61.9% and 32.4%, respectively. This result is also similar to the Asian and Thai CML patient's data[13-15]. Moreover, we found 5.7% of co-expression e13a2 and e14a2 variant. In the case of RT-PCR with co-expression, we identified a 49-bp insertion from ABL intron b1 in e14a2 BCR/ABL fusion gene. Despite not being the first report due to a previous report on this sequence of insertion in ALL patients[16]. The BCR/ABL with insertion in this patient did not appeared on

Table 3. The intronic insertion was found in CML and ALL patient

Gender	Age (year)	Disease	Variant	Size of ins. (bp)	Source of insert	Mutation	Country	Reference
Female	69	ALL	e14a2	49	Intron 1b	Premature Stop codon	Japan	16
Male	31	CML	e3a2	44	Intron 1b	Inflame insertion	Spain	17
Female	14	CML	e18a2	10	Intron 1b	Translate to 130 aa (>p120)	Netherlands	18
Male	44	CML	e14a2	42	Intron 1b	In-frame insertion of 15 new amino acids	France	19
Male	55	CML	e8a2	55	Intron 1b	Frameshift mutation result in premature termination seven codons	Australia	20
Male	15, 60, 50	CML		35	Intron 8 insert between ABL exon 8	Premature translational stop codon after 10 intron-encodedamino acids	USA	21

the first diagnosis, it only appeared as e1a2 variant. This fusion gene with intronic insertion occurred after patient treatment for 8 months. Our report is; nonetheless, the first time in CML patients with co-expression of e14a2 and e13a2 BCR/ABL transcripts. After analyzing the deduced amino acid from this insert variant, the protein expression may not affect TKI treatment in patients since this protein does not present any tyrosine kinase domain. In case of quantitative RT-PCR detected by Real-time PCR, it may show higher BCR/ABL value than actual because BCR/ABL with insertion was also amplified. Moreover, there have been several reports shown the intronic insertion in CML patients. Some insertions are between exon18 in BCR and exon2 in ABL, exon3 in BCR and exon2 in ABL, exon14 in BCR and exon 2 in ABL exon8 in BCR and exon 2 in ABL (Table 3). Some insertions cause in-frame insertion or frame shift insertion. All these BCR/ABL with insertion were not apparently co-expressed and these can cause hematologic malignancies[16-20].

This study showed that the three CML TKI resistant patients have the intronic insertion different from other reports. These patients were found to have insertion of intron 8 with in exon 8 of the ABL of fusion gene. This report presents that some intronic insertion may lead to TKI resistance if the insertion is in the tyrosine kinase domain[21]. Although the intronic insertion from this report do not represent any tyrosine kinase domains, clinical results in this group of patients should be further studied.

Conclusion

In conclusion, the frequency of e14a2 and e13a2 BCR-ABL variants was similar to that of the previously reported Western and Asian studies. The intron 1b insertion was frequently observed in Thai CML patients carrying both variants in this study. Its clinical importance needs to be further characterized.

What is already known on this topic?

RT-PCR and RQ-PCR are used for BCR/ABL gene detection. BCR/ABL was found approximately 50% in CML patient. The e14a2 variant had a higher frequency than the e13a2 variant.

What this study adds?

The intron 1b insertion was found frequently with e14a2 and e13a2 variant in Thai CML patient. This BCR/ABL with insertion may not express tyrosine kinase activity due to a stop codon in the insert sequence.

Acknowledgements

The authors would like to extend our special thanks to Chulabhorn Royal Academy for the kind assistance and support towards the success and accomplishment of this research study. The authors thank Dr. Danupon Nantajit for his assistance in reviewing the manuscript.

Potential conflicts of interest

None.

References

1. Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ, Porwit A, et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood 2009; 114: 937-51.
2. Hermans A, Heisterkamp N, von Linden M, van Baal S, Meijer D, van der Plas D, et al. Unique fusion of bcr and c-abl genes in Philadelphia chromosome positive acute lymphoblastic leukemia. Cell 1987; 51: 33-40.
3. Druker BJ. Translation of the Philadelphia chromosome into therapy for CML. Blood 2008; 112: 4808-17.
4. Roy L, Guilhot J, Krahnke T, Guerci-Bresler A, Druker BJ, Larson RA, et al. Survival advantage from imatinib compared with the combination interferon-alpha plus cytarabine in chronic-phase chronic myelogenous leukemia: historical comparison between two phase 3 trials. Blood 2006; 108: 1478-84.
5. Sherbenou DW, Hantschel O, Kaupe I, Willis S, Bumm T, Turaga LP, et al. BCR-ABL SH3-SH2 domain mutations in chronic myeloid leukemia patients on imatinib. Blood 2010; 116: 3278-85.
6. Vaidya S, Vundinti BR, Shanmukhaiah C, Chakrabarti P, Ghosh K. Evolution of BCR/ABL gene mutation in CML is time dependent and dependent on the pressure exerted by tyrosine kinase inhibitor. PLoS One 2015; 10: e0114828.
7. O'Hare T, Eide CA, Deininger MW. Bcr-Abl kinase domain mutations, drug resistance, and the road to a cure for chronic myeloid leukemia. Blood 2007; 110: 2242-9.
8. Hughes T, Deininger M, Hochhaus A, Branford S, Radich J, Kaeda J, et al. Monitoring CML patients responding to treatment with tyrosine kinase inhibitors: review and recommendations for harmonizing current methodology for detecting BCR-ABL transcripts and kinase domain mutations and for expressing results. Blood 2006; 108: 28-37.
9. Dobrovic A, Trainor KJ, Morley AA. Detection of the molecular abnormality in chronic myeloid leukemia by use of the polymerase chain reaction. Blood 1988; 72: 2063-5.
10. Bolufer P, Sanz GF, Barragan E, Sanz MA, Cervera J, Lerma E, et al. Rapid quantitative detection of BCR-ABL transcripts in chronic myeloid leukemia patients by real-time reverse transcriptase polymerase-chain reaction using fluorescently labeled probes. Haematologica 2000; 85: 1248-54.
11. Cox MC, Maffei L, Buffolino S, Del Poeta G, Venditti A, Cantonetti M, et al. A comparative analysis of FISH, RT-PCR, and cytogenetics for the diagnosis of bcr-abl-positive leukemias. Am J Clin Pathol 1998; 109: 24-31.
12. Schoch C, Schnittger S, Bursch S, Gerstner D, Hochhaus A, Berger U, et al. Comparison of chromosome banding analysis, interphase- and hypermetaphase-FISH, qualitative and quantitative PCR for diagnosis and for follow-up in chronic myeloid leukemia: a study on 350 cases. Leukemia 2002; 16: 53-9.
13. Udomsakdi-Auewarakul C, Pratya Y, Boonmoh S, Vatanavicharn S. Detection of molecular variants of BCR-ABL gene in bone marrow and blood of patients with chronic myeloid leukemia by reverse-transcriptase polymerase chain reaction (RT-PCR). J Med Assoc Thai 2000; 83: 928-35.
14. Inokuchi K, Inoue T, Tojo A, Futaki M, Miyake K, Yamada T, et al. A possible correlation between the type of bcr-abl hybrid messenger RNA and platelet count in Philadelphia-positive chronic myelogenous leukemia. Blood 1991; 78: 3125-7.
15. Deb P, Chakrabarti P, Chakrabarty S, Aich R, Nath U, Ray SS, et al. Incidence of BCR-ABL transcript variants in patients with chronic myeloid leukemia: Their correlation with presenting features, risk scores and response to treatment with imatinib mesylate. Indian J Med Paediatr Oncol 2014; 35: 26-30.
16. Hirota M, Hidaka E, Ueno I, Ishikawa M, Asano N, Yamauchi K, et al. Novel BCR-ABL transcript containing an intronic sequence insert in a patient with Philadelphia-positive acute lymphoblastic leukaemia. Br J Haematol 2000; 110: 867-70.
17. Roman J, Parziale A, Gottardi E, De Micheli D, Cilloni D, Tiribelli M, et al. Novel type of BCR-ABL transcript in a chronic myelogenous

leukaemia patient relapsed after bone marrow transplantation. Br J Haematol 2000; 111: 644-6.
18. van der Velden VH, Beverloo HB, Hoogeveen PG, Zwaan ChM. A novel BCR-ABL fusion transcript (e18a2) in a child with chronic myeloid leukemia. Leukemia 2007; 21: 833-5.
19. Sorel N, Mayeur-Rousse C, Deverriere S, Roy L, Brottier-Mancini E, Guilhot F, et al. Comprehensive characterization of a novel intronic pseudo-exon inserted within an e14/a2 BCR-ABL rearrangement in a patient with chronic myeloid leukemia. J Mol Diagn 2010; 12: 520-4.
20. Branford S, Rudzki Z, Hughes TP. A novel BCR-ABL transcript (e8a2) with the insertion of an inverted sequence of ABL intron 1b in a patient with Philadelphia-positive chronic myeloid leukaemia. Br J Haematol 2000; 109: 635-7.
21. Laudadio J, Deininger MW, Mauro MJ, Druker BJ, Press RD. An intron-derived insertion/truncation mutation in the BCR-ABL kinase domain in chronic myeloid leukemia patients undergoing kinase inhibitor therapy. J Mol Diagn 2008; 10: 177-80.