Case report

Clinical application of molecular testing for the diagnosis of primary myelofibrosis clinicopathologically mimicking chronic myelogenous leukemia: a case report

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Background Chronic myelogenous leukemia (CML) and primary myelofibrosis (PMF) are both myeloproliferative neoplasms that have overlapped clinical manifestations, but their prognosis and treatment are different. Besides conventional chromosome analysis, molecular testing is required for the correct diagnosis in some patients.

Objective To present a case report that applied molecular techniques to diagnose PMF in a patient with CML-like presentation.

Results The patient was a 66 year-old male who presented with splenomegaly and marked leukocytosis with left shift. CML was suspected, but chromosome analysis did not demonstrate a Philadelphia chromosome. Reverse transcription- polymerase chain reaction (RT-PCR) and real-time quantitative PCR (RQ-PCR) also were negative for the identification of BCR-ABL1. Analysis of positive JAK2 V617F mutations as well as increased reticulin fibers in a bone marrow biopsy supported the diagnosis of PMF.

Conclusion Molecular diagnostic techniques are required for the diagnosis of MPNs, especially in patients with overlapping clinical manifestations of CML and PMF. **Chiang Mai Medical Journal 2015;54(1):39-45.**

Keywords: primary myelofibrosis, chronic myelogenous leukemia, RT-PCR for BCR-ABL1, RQ-PCR for BCR-ABL1, JAK2 V617F mutation analysis

Introduction

Chronic myelogenous leukemia (CML) and primary myelofibrosis (PMF) are both myeloproliferative neoplasms (MPNs) that commonly present with splenomegaly[1,2]. However, the prognosis, and treatment are different. CML derives from an abnormal pluripotent stem cell that is associated with the BCR-ABL1 fusion gene. Most patients with CML have translo-

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cation of chromosome 9 and 22, which results in the Philadelphia (Ph) chromosome[3]. Treatment with tyrosine kinase inhibitors (TKIs), for example imatinib, is very effective and leads to improved survival[4,5].

PMF is a clonal MPN that is associated with reactive bone marrow fibrosis and extramedullary hematopoiesis[6]. JAK2 V617F mutation is found in approximately half of PMF patients. The prognosis is poor with median survival of 3-7 years[6]. The treatment is mainly symptomatic such as hydroxyurea for symptomatic splenomegaly[7].

Since CML and PMF have overlapped clinical manifestations, complete blood count (CBC) and peripheral blood smear that show leukocytosis with multi-stage differentiation of myeloid series can be found in both disorders[3,6]. As a result, further laboratory investigations are required in order to make a definite diagnosis.

Herewith, the authors report the application of molecular tests including the amplification-refractory mutation system (ARMS-PCR) for identifying JAK2 V617F mutation, and reverse transcription- polymerase chain reaction (RT-PCR) and real-time quantitative PCR (RQ-PCR) for expressing BCR-ABL1 and diagnosing PMF in a patient that had CML-like presentation.

Methods

BCR-ABL1 (p210) gene expression

Total RNA was extracted from 10 mL of EDTA-blood using the trizol method (Invitrogen, CA, USA). Two µg of total RNA was reverse-transcribed to cDNA in the SuperScript® III First-Strand Synthesis System (Invitrogen, CA, USA). Two µL of cDNA was amplified for RT-PCR using sequence specific primers (Invitrogen, CA, USA) for p210 BCR-ABL transcripts. Glyceraldehyde-3-phosphate dehy-drogenase (GAPDH) was used as an endogenous control gene and amplified in a separate reaction. The primers used for RT-PCR were as follows: Bcr-Abl (p210)-forward (F), 5'-GAT GCT GAC CAA CTC GTG TG-3'; Bcr-Abl (p210)-reverse (R), 5'-AAC GAA AAG GTT GGG GTC AT-3'; GAPDH-F, 5'-ACC ACA GTC CAT GCC ATC AC-3' and GAPDH-R; 5'-TCC ACC ACC CTG TTG CTG TA-3'. PCR reactions were performed in 25 µL of total volume with 5 mM of $MgCl_2$, 0.2 mM of dNTP mixture, 0.32 µM of each primer and 1 unit of Taq DNA Polymerase (Invitrogen, CA, USA). The cycle conditions were as follows: initial denaturation at 95°C for 5 minutes, followed by 40 cycles at 95°C for 30 sec, 58°C for 30 sec and 72°C for 45 sec. PCR products were loaded onto 2% agarose gel for gel electrophoresis before staining with ethidium bromide. The 194-bp, 265-bp and 400-bp PCR products represented b2a2, b3a2 and GAPDH, respectively.

Use of the Applied Biosystems 7,500 Fast Real Time PCR System and 2 µL of cDNA were amplified for RQ-PCR using the same sequence-specific primers (Invitrogen, CA, USA) as those for RT-PCR and TagManfluorogenic probe FAM-AGA CCC TGA GGC TCA AAG TCA GAT GCT ACT–TAMRA (AITbiotech, Singapore) for p210 BCR-ABL transcripts. ABL was used as an endogenous control to correct the expression of p210 BCR-ABL genes. ABL primers (Invitrogen, CA, USA) and probes (AITbiotech, Singapore) were as follows: ABL-F, 5'-GCC TCA GGG TCT GAG TGA AG-3'; ABL-R, 5'-ACA CCA TTC CCC ATT GTG AT-3'; and ABL-probe, FAM-AGA GTG TTA TCT CCA CTG GCC ACA AAA TCA–TAMRA. Plasmid cDNAs of p210 BCR-ABL and ABL were employed as standard at concentrations of 10^2, 10^3, 10^4, 10^5 and 10^6 copies per µL. PCR reactions were performed in 20 µL of total volume with 0.32 µM of each primer, 0.1 µM of probe and 1xPCR TaqMan Gene Expression Master Mix (Life Technologies, CA, USA). The cycle conditions were as follows: initial denaturing at 95°C for 10 minutes, followed by 50 cycles at 95°C for 15 sec, 58°C for 15 sec and 72°C for 30 sec.

JAK2 V617F mutation analysis

Genomic DNA from 2-mL of EDTA-Blood was extracted using the Genomic DNA Mini Kit (Geneaid Biotech Ltd, Sijhih City, Taiwan) by following the manufacturer's instructions. The purified DNA was subjected to JAK2-V617F mutation analysis by using amplification-refractory mutation system-polymerase chain reaction (ARMS-PCR)[8]. This technique permits the normal and mutant allele, plus a DNA quality control sequence, to be detected in a single PCR reaction by using tetra-primers as follows; forward outer (FO), 5'-TCC TCA GAA CGT TGA TGG CAG-3'; reverse outer (RO), 5'-ATT GCT TTC CTT TTT CAC AAG AT-3'; forward inner-wild-type-specific (Fwt), 5'-GCA TTT GGT TTT AAA TTA TGG AGT ATa TG-3'; and reverse inner-mutant-specific (Rmt), 5'-GTT TTA CTT ACT CTC GTC TCC ACA aAA-3'. The intended mismatches were introduced at the 3' end of wild-type/mutant-specific primers (indicated in lowercase) to maximize discrimination of the 2 alleles. Two outer primers flanking exon 14 of the JAK2 gene enabled generation of a 463-bp band that controlled DNA quality in all cases. The forward outer primer and reverse inner-mutant -specific

primer amplified a 279-bp band from the JAK2 V617F allele. The forward inner-wild-type-specific primer and reverse outer primer amplified a wild-type allele resulting in a normal band of 229-bp.

PCR reactions were performed in 25 μL of total volume with 100 ng of DNA, 1.5 mM of $MgCl_2$, 0.12 mM of each dNTP, 0.4 μM of FO, 0.3 μM of RO, 0.5 μM of Fwt, 1.0 μM of Rmt, and 1.0 U of GoTaq® DNA Polymerase (Prome-ga, Madison, WI). Amplifications were conducted using 2-min initial denaturation at 95°C, followed by 40 cycles of 30 sec at 94°C, 45 sec at 58°C, 45 sec at 72°C and a 10-min final step at 72°C. The 5-μL of PCR products were analyzed on 2% agarose gel, with ethidium bromide staining.

Results

Clinical case

A 66-year-old man presented with 2 weeks of progressive fatigue and weight loss of 8 kilograms over the past 4 months. Physical examination revealed moderately pale conjunctivae, no jaundice, 12 cm liver span, and splenomegaly 15 cm below the left costal margin. CBC showed anemia and leukocytosis as shown in Table 1. His blood smear revealed nucleated red cells (NRCs) and marked leukocytosis with left shift, slightly eosinophilia and basophilia (Figure 1). Other laboratory tests are shown in Table 2.

At first, accelerated phase of CML on top of underlying thalassemia trait or intermedia was suspected. Hb analysis confirmed the diagnosis of beta-thalassemia trait. Bone marrow aspiration revealed only bone marrow fluid. A bone marrow biopsy showed hypercellularity (90%) that was composed mainly of granulocytic component with preserved maturation (Figure 2A). The myeloid to erythroid (M:E) ratio was more than 10:1. Megakaryocytes were increased slightly without striking pleomorphism. Rare dwarf megakaryocytes were seen. Reticulin stain showed dense reticulin with extensive intersections compatible with grade MF-3 bone marrow fibrosis accompanied by minimal osteosclerosis (Figure 2B). These findings suggested MPN; differential diagnoses being PMF vs CML with marrow fibrosis.

To differentiate between CML and PMF, chromosome analysis from the bone marrow was performed, showing 46, XY and no Ph chromosome detection. The RT-PCR of peripheral blood for b2a2 and b3a2 expression was negative (Figure 3), with RQ-PCR for BCR-ABL1 transcript being undetectable. The RT-PCR and RQ-PCR assays used in this study could detect at least 100 (Figure 3, lane 8) and 20 copies (data not shown), respectively. JAK2

Table 1. Serial complete blood count of the patient

Date	1/10/2013 First presentation	1/1/2014 Admitted due to septicemia (6 weeks after starting hydroxyurea treatment)	1/7/2014 Last follow-up (no hydroxyurea treatment)
Hemoglobin (g/dL)	7.56	6.1	8
Hematocrit (%)	22.8	19.9	25.2
White blood count (/μL)	104,000	93,600	134,000
Myeloblast (%)	12	2	11
Promyelocytes (%)	6	1	6
Myelocytes (%)	5	1	12
Metamyelocytes (%)	5	0	5
Band (%)	13	8	9
PMN (%)	41	69	35
Lymphocytes (%)	8	4	7
Monocytes (%)	10	11	5
Eosinophils (%)	6	3	4
Basophils (%)	4	1	6
NRC (per 100 WBC)	11	6	22
Platelets (/μL)	104,600	34,000	61,300

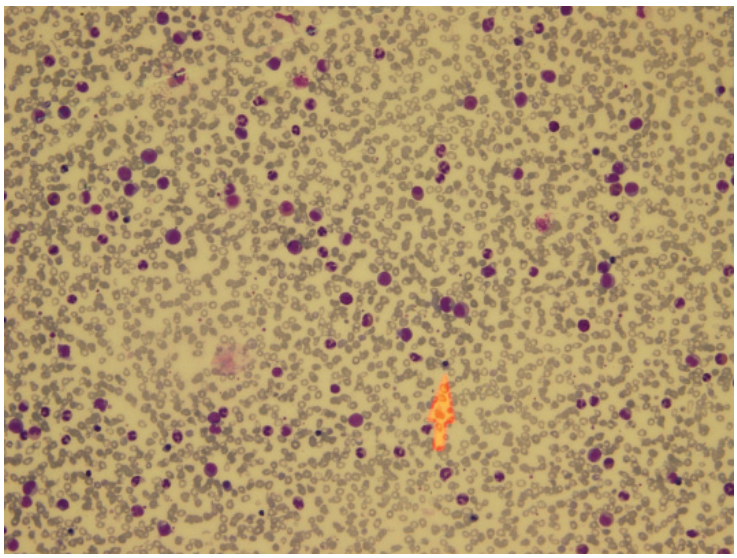

Figure 1. Blood smear of the patient. Peripheral blood smear showing hypochromic microcytic red cells with anisocytosis and poikilocytosis that were compatible with thalassemia trait. Some tear drop cells and nucleated red cells (NRCs; arrow) were found as well. The major findings were marked leukocytosis with left shift and circulating myeloblasts that could be found in both chronic myelogenous leukemia (CML) and primary myelofibrosis (PMF)

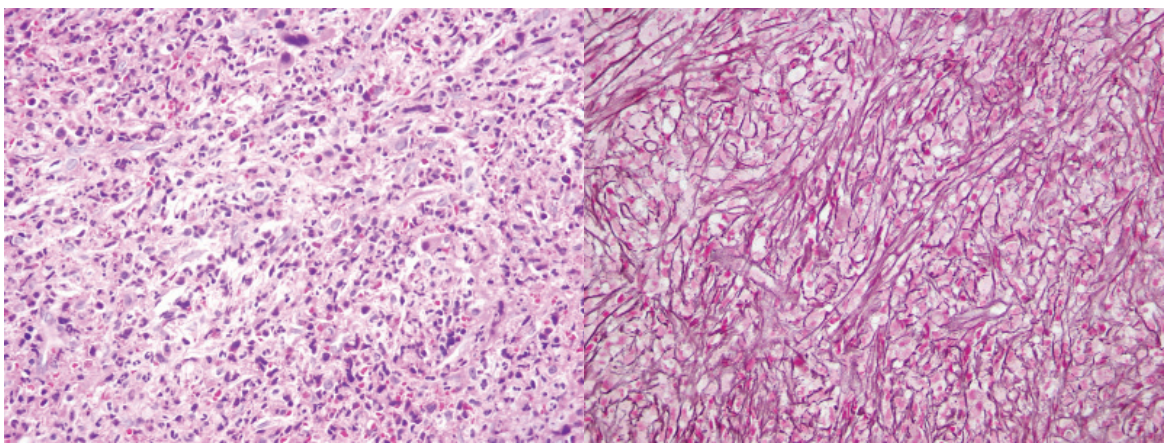

Figure 2. A (left) and 2B (right) Bone marrow biopsy. Cellularity is increased due mainly to granulocytic proliferation with preserved maturation. Small megakaryocytes are observed without marked pleomorphism (A; Hematoxylin and Eosin stain, 400x magnification). Reticulin stain shows a diffuse and dense increase in reticulin fibers with extensive intersections (B; Reticulin stain, 400x magnification).

V617F mutation analysis was positive (Figure 4). These results supported the diagnosis of PMF.

The patient in this study received hydroxyurea at 500 mg/day for treating symptomatic splenomegaly. However, 6 weeks after commencement of treatment, he was admitted to hospital with septicemia. Antibiotic therapy was given and hydroxyurea discontinued, due to severe infection. It was planned to restart hydroxyurea if the patient had significant symptoms of splenomegaly. His serial CBC is shown in Table 1.

Table 2. Other laboratory results from the patient

Laboratory	Results	Normal range
Blood urea nitrogen	26 mg/dL	6-20
Serum creatinine	1.7 mg/dL	0.7-1.2
Liver function test:		
Albumin	3.8 g/dL	3.5-5.2
Globulin	3.6 g/dL	3.1-3.5
Alkaline phosphatase	230 U/L	40-129
Aspartate aminotransferase	31 U/L	0-40
Alanine aminotransferase	13 U/L	0-40
Total bilirubin	2.07 mg/dL	0.0-1.2
Direct bilirubin	0.81 mg/dL	<0.3
Serum uric acid	4.5 mg/dL	3.4-7.0
Serum lactate dehydrogenase	1,230 U/L	150-249

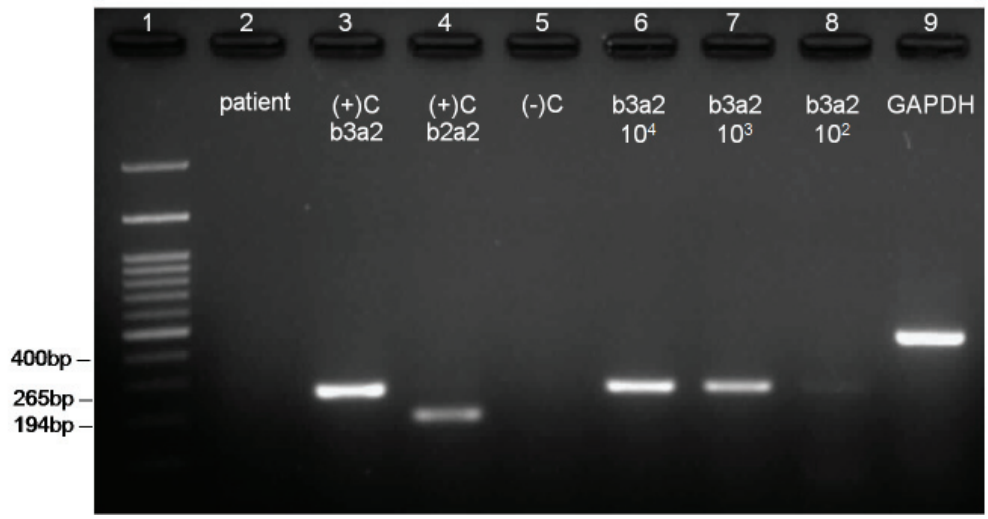

Figure 3. Negative RT-PCR for p210 BCR-ABL1 (Lane 2). Lane1, ladder; Lane2, patient; Lanes 3-4, positive control for b3a2 (265 bp) and b2a2 (194 bp), respectively; Lane 5, negative control; Lanes 6-8, plasmid cDNA (b3a2) 10^4, 10^3 and 10^2 copies, respectively; and Lane 9, GAPDH house-keeping gene (400 bp).

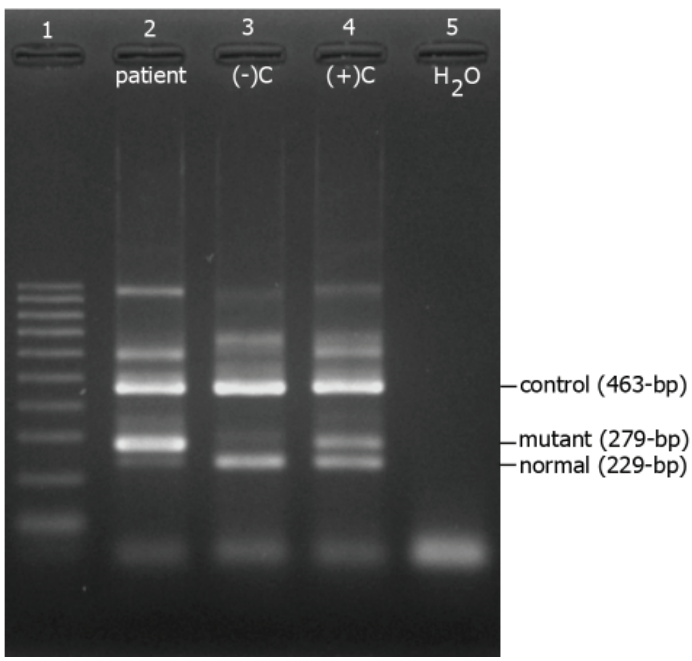

Figure 4. Positive ARMS-PCR for JAK2 V617F mutation (Lane 2). Lane1, ladder; Lane2, patient; Lane 3 negative control with control band (463 bp) and normal band (229 bp); Lane 4, positive control with control band (463 bp), normal band (229 bp) and JAK2-V617F mutant band (279 bp).

Discussion

This patient demonstrated the usefulness of molecular techniques to distinguish between PMF and CML. Clinical manifestations in this patient, including huge splenomegaly, constitutional symptoms, leukocytosis with left shift and anemia can be found in both disorders[3,6]. Minimal to advanced bone marrow fibrosis also has been detected in up to 30 percent of CML patients[9]. Although the morphology of megakaryocytes is helpful in differentiating between CML and PMF, typical findings in PMF such as bulbous, "cloud-like" or "balloon-shaped" nuclei[6] were not found in the bone marrow of this patient. Megakaryocytes with small hypolobated nuclei, or "dwarf" megakaryocytes, typically seen in CML[3] were not found with any significance. The correct diagnosis of CML is important due to the good

outcome from TKI treatment. In this case, absence of the Ph chromosome as well as BCR-ABL1 expression from both RT- and RQ-PCR results made CML less likely.

The results of RT-PCR and RQ-PCR for BCR-ABL1 and the bone marrow biopsy showed hypercellularity with fibrosis, and raised the probable diagnosis of PMF. The firm diagnosis of PMF requires all 3 major criteria and 2 minor ones[6,10]. The major criteria includes, (1) presence of megakaryocyte proliferation and atypia accompanied by collagen fibrosis, (2) inability to meet WHO criteria for other myeloid neoplasms, and (3) demonstration of JAK2 V617F mutation or no evidence of secondary bone marrow fibrosis. Minor criteria comprise leukoerythroblastosis, increase in serum LDH, anemia, and splenomegaly. This patient had all of 4 minor criteria and at least the first two major criteria. Therefore, positive JAK2 V617F mutation in this case fulfilled the diagnosis of PMF.

This patient had many high risk features of PMF, including age of more than 65 years, constitutional symptoms, leukocytosis greater than 25,000/μL, anemia with Hb less than 10 g/dL, circulating blasts greater than 1%, and necessary transfusion, which brought him into the high risk category according to IPSS[11], DIPSS[12], and DIPSS-plus[13]. Therefore, the prognosis of this patient was poor with estimated survival of around 2 years.

In conclusion, molecular diagnostic techniques are important for diagnosing MPNs, especially in patients with overlapping clinical manifestations of CML and PMF. Accurate diagnosis provides appropriate management for the patients.

Ethics

This case report was approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Chiang Mai University, Thailand.

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Conflict of interest statement

There is no conflict of interest

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การนำการตรวจในระดับโมเลกุลมาใช้ในการวินิจฉัยโรคพังผืดในไขกระดูกซึ่งมีลักษณะทางคลินิกคล้ายกับมะเร็งเม็ดเลือดขาวเรื้อรังชนิดมัยอีลอยด์: รายงานผู้ป่วย

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ความเป็นมา โรคมะเร็งเม็ดเลือดขาวเรื้อรังชนิดมัยอีลอยด์และโรคพังผืดในไขกระดูกจัดเป็น myeloproliferative neoplasms (MPNs) ซึ่งมีลักษณะทางคลินิกที่คล้ายกันแต่มีการพยากรณ์โรคและการรักษาแตกต่างกัน นอกเหนือจากการตรวจโครโมโซมแบบทั่วไปแล้ว การตรวจในระดับโมเลกุลมีความจำเป็นในผู้ป่วยบางราย

วัตถุประสงค์ เพื่อรายงานผู้ป่วยซึ่งนำการตรวจในระดับโมเลกุลมาใช้ในการวินิจฉัยโรคพังผืดในไขกระดูกซึ่งมีลักษณะทางคลินิกคล้ายกับมะเร็งเม็ดเลือดขาวเรื้อรังชนิดมัยอีลอยด์

ผลการศึกษา ผู้ป่วยชายอายุ 66 ปี มาด้วยม้ามโตและพบว่ามีเม็ดเลือดขาวสูงมากและมี left shift การวินิจฉัยเบื้องต้นเป็นมะเร็งเม็ดเลือดขาวเรื้อรังชนิดมัยอีลอยด์แต่การตรวจโครโมโซมแบบทั่วไปไม่พบ Philadelphia chromosome การตรวจ reverse transcription- polymerase chain reaction (RT-PCR) and real-time quantitative PCR (RQ-PCR) สำหรับยีน BCR-ABL1 ให้ผลลบ การตรวจการกลายพันธุ์ของยีน JAK2 V617F ให้ผลบวกร่วมกับการตรวจทางพยาธิวิทยาของไขกระดูกซึ่งพบว่ามี reticulin fibers เพิ่มขึ้น สนับสนุนการวินิจฉัยโรคพังผืดในไขกระดูก

สรุปผลการศึกษา การตรวจในระดับโมเลกุลมีความจำเป็นต่อการวินิจฉัยโรคในกลุ่ม MPNs โดยเฉพาะผู้ป่วยซึ่งมีอาการทางคลินิกคล้ายกันระหว่างโรคมะเร็งเม็ดเลือดขาวเรื้อรังชนิดมัยอีลอยด์และโรคพังผืดในไขกระดูก
เชียงใหม่เวชสาร 2558;54(1):39-45.

คำสำคัญ: primary myelofibrosis, chronic myelogenous leukemia, RT-PCR for BCR-ABL1, RQ-PCR for BCR-ABL1, JAK2 V617F mutation analysis