การตรวจวิเคราะห์สารพันธุกรรมในห้องปฏิบัติการสำหรับการรักษาผู้ป่วยโรคมะเร็งเม็ดเลือดขาวชนิดเรื้อรังแบบมัยอิลอยด์

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บทคัดย่อ

การตรวจวิเคราะห์ยีนลูกผสม *BCR*::*ABL1* มีบทบาทสำคัญสำหรับการดูแลรักษาผู้ป่วยโรคมะเร็งเม็ดเลือดขาวชนิดเรื้อรังแบบมัยอิลอยด์ (CML) ที่ได้รับการรักษาด้วยยายับยั้งไทโรซีนไคเนส (tyrosine kinase inhibitor) บทความนี้ มีวัตถุประสงค์ในการนำเสนอการตรวจวิเคราะห์ทางห้องปฏิบัติการในระดับเซลล์พันธุศาสตร์และอณูพันธุศาสตร์สำหรับวินิจฉัย และตรวจติดตามการรักษาโรค CML ได้แก่ (1) การตรวจโครโมโซมฟิลาเดลเฟีย ซึ่งเป็นวิธีมาตรฐานในการวินิจฉัยโรค (2) การตรวจยีนลูกผสม *BCR*::*ABL1* วิธี fluorescent *in situ* hybridization (FISH) ที่สามารถตรวจวิเคราะห์ความผิดปกติของยีนดังกล่าวที่มีตำแหน่ง breakpoint region ที่แตกต่างกันได้ (3) การตรวจปริมาณยีนลูกผสม *BCR*::*ABL1* ด้วยเทคนิค quantitative real-time PCR (qRT-PCR) มีบทบาทสำคัญสำหรับการตรวจติดตามโรค และประเมินการตอบสนองการรักษาต่อยา ซึ่งผู้ป่วยส่วนใหญ่ตรวจไม่พบยีนลูกผสม บ่งชี้ว่ามีการตอบสนองการรักษาได้ดี (deep molecular response) ดังนั้น การเลือกใช้เทคนิคที่มีความไวและจำเพาะสูงทำให้สามารถตรวจปริมาณยีนที่มีจำนวนน้อยได้ (4) เทคนิค droplet digital PCR สำหรับตรวจปริมาณยีนลูกผสมดังกล่าว ซึ่งช่วยลดข้อจำกัดของ qRT-PCR ได้ นอกจากนี้ บทความนี้นำเสนอ (5) เทคนิค direct sequencing สำหรับตรวจการกลายของยีนลูกผสม *BCR*::*ABL1* ในกรณีผู้ป่วยดื้อต่อยา รวมทั้ง (6) เทคนิค next-generation sequencing ที่ตรวจหาระดับการกลายที่มีปริมาณน้อย และยังสามารถตรวจหาการกลายของยีนชนิดอื่น ๆ ที่ทำให้ผู้ป่วยดื้อต่อยาได้ ดังนั้น การตรวจวิเคราะห์ทางห้องปฏิบัติการอย่างครอบคลุม และมีมาตรฐานจะส่งผลให้ผู้ป่วยได้รับการวินิจฉัยและการรักษาได้อย่างมีประสิทธิภาพสูงสุด

คำสำคัญ: มะเร็งเม็ดเลือดขาวชนิดเรื้อรังแบบมัยอิลอยด์ ยีนลูกผสม *BCR*::*ABL1* ยายับยั้งไทโรซีนไคเนส

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Genetic Testing for the Management of CML

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Abstract

Molecular analysis of the *BCR*::*ABL1* fusion transcripts is clinically important for the management of CML patients undergoing tyrosine kinase inhibitor (TKI) treatment. This review provides information about the fundamental and current molecular assays essential for managing CML. Chromosome analysis by standard karyotyping is recognized as a standard method for detecting Philadelphia chromosomes and identifying additional chromosomes to assess the patient's risk prior to therapy. Fluorescent *in situ* hybridization (FISH) has been used for the analysis of the transcriptional variants of *BCR*::*ABL1*. Since the level of *BCR*::*ABL1* transcript is reflected in the degree of response to TKIs, quantitative PCR, including traditional quantitative real-time PCR (qRT-PCR) and FDA-approved droplet digital PCR (ddPCR), have been extensively used for specific and sensitive detection of *BCR*::*ABL1* transcript during TKI treatment milestones. Sequencing technologies, including standard direct sequencing and recently high throughput next-generation sequencing (NGS), have been used to identify *BCR*::*ABL1* TKD mutations that contributed to establishing TKI resistance in patients. NGS has several benefits superior to direct sequencing, including detecting low levels of *BCR*::*ABL1* mutations, discrimination of compound/polyclonal mutations, and identifying more driving mutations besides *BCR*::*ABL1* TKD mutations associated with the establishment of TKI resistance.

Keywords: Chronic myeloid leukemia, *BCR*::*ABL1* fusion transcripts, Tyrosine kinase inhibitor

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Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm, and the global incidence data is ranged from 0.7 to 1.5 per 100,000 persons by the American Institute for Cancer Research.[1] CML is defined by the presence of balanced chromosomal translocation, t(9;22)(q34;q11.2), the so-called Philadelphia (Ph) chromosome. The disease frequently has three phases of progression, including the initial chronic phase, the transitional accelerated phase, and the progressive blast crisis phase (Table 1).[2] The central pathogenesis of CML is that Ph chromosome produces *BCR*::*ABL1* oncoprotein with high constitutive tyrosine kinase activity that alters several biological pathways such as STAT, RAS, RAF, MYC, and JUN, leading to the formation of CML leukemic cells.[3] Since the discovery of imatinib, the first tyrosine kinase inhibitor (TKI) approved for targeting *BCR*::*ABL1* oncoprotein, treatment outcomes of patients with CML have been dramatically changed by improving overall survival and quality of life of the patients.[4] Although it has an unclear picture illustrating the national trend of CML in Thailand, the number of accumulated CML cases will reach 180,000 cases in the United States by 2030-2040.[5] Hence, alongside the improvement in CML therapy, laboratories investigation of CML, monitoring assays of the patient during treatment, and the detection of measurable residual disease (MRD) during the remission stage are crucial for the effective management of patients with CML.

Imatinib has been recognized as the first TKI approved by the US Food and Drug Administration (FDA) that has been introduced to treat CML. The drug specifically blocks the interaction between *BCR*::*ABL1* fusion proteins and adenosine triphosphate (ATP). This interaction blocks the transformation efficacies and induces apoptosis of CML clones without harming the normal counterpart of blood cells and hematopoietic stem cells (HSCs).[4, 6] Subsequently, second-generation TKIs (2G-TKIs), including nilotinib, dasatinib, and bosutinib have been approved for treating CML with different indicators and reported outcomes.[2] Recently, the generic imatinib has been widely used with significant benefits for cost savings for both patients and healthcare systems. However, generics are not inferior to the original imatinib in terms of efficacy, with an acceptable toxicity profile that needs further investigation.[7]

The high efficacy of currently available TKIs to target *BCR*::*ABL1* makes newly diagnosed CML patients respond very well and results in life expectancy compared with age-matched normal individuals.[8] Practical laboratory tools for diagnosis, prognostic scoring, and monitoring of patients' response to TKIs are crucial components of the effective management of CML. The recent opinions from CML experts suggested that prognostic factors at baseline in CML patients have to be

considered to assess the long-term probability of disease-related death, the possible toxicity, and the projected long-term overall survival.[9] Those include the patient's age at diagnosis and additional cytogenetic abnormalities.[9] Thus, besides the beneficial use of chromosome analysis (karyotyping) for CML diagnosis by identifying Ph chromosome, karyotyping could provide additional information for the CML prognosis.

At present, it still has controversy in data regarding the prognostic significance of CML patients and the transcriptional variants of major *BCR*::*ABL1* (p210). From the perspective of the laboratory for the management of CML, it is essential to assess the *BCR*::*ABL1* transcriptional subtypes to prevent the chance of false negative results conducted by quantitative real-time polymerase chain reaction (qRT-PCR), which is specifically amplified only typical p210 *BCR*::*ABL1*. Thus, in our setting, we perform reverse transcription polymerase chain reaction (RT-PCR) assay to assess the transcription variants of *BCR*::*ABL1* in all samples derived from patients with CML and to monitor atypical *BCR*::*ABL1* transcripts in CML and other leukemia. Similarly, fluorescent *in situ* hybridization (FISH) has been introduced for the identification of *BCR*::*ABL1* fusion, especially in the laboratory that could not perform standard karyotyping. While its applications could not suitable for CML monitoring compared with qRT-PCR, the assay is meaningful for the tracking of patients positive for atypical *BCR*::*ABL1* transcripts.

During the treatment, complete blood count (CBC) including differential cell counts are recommended every two weeks (or more frequently in a case with hematologic toxicity) until a complete hematological response.[8] It has been clear that quantitative analysis of *BCR*::*ABL1* mRNA level (qRT-PCR), which is reported to be the percent international scale (%IS) by multiplying with a specific conversion factor (CF), is dynamically reflected in the molecular response (MR) of patients. Remarkably, qRT-PCR is currently used for defining the degree of MR during TKI treatment. This is very helpful for managing patients, including providing decision-making by the clinician for the appropriate selection of TKIs, optimal drug dosage adjustment, and defining the treatment-free remission.

Although the treatment outcomes of CML have dramatically improved over the development of TKIs, approximately 20-30% of patients become resistant to TKIs, leading to treatment failure and disease relapse.[10] At present, acquired mutations of the tyrosine kinase domain (TKD) in the *BCR*::*ABL1* coding sequence have been known to be a major cause of TKI resistance in CML.[10]

Direct sequencing has been broadly used as a standard method for the analysis of *BCR*::*ABL1* TKD mutations during TKI treatment. Direct sequencing could detect all mutations and novel mutations spanning

BCR::*ABL1* TKD. However, the assay has low sensitivity, and is laborious compared to current high throughput methods such as mass spectrometry and next-generation sequencing (NGS). Additionally, evidence indicates that driving mutations in genes associated with the tumorigenesis of myeloid leukemia could result in the establishment of CML with TKI-resistant phenotypes. Those genetic aberrations include mutations of *AXSL1, IDH1,* and *SETBP1.*[11-12] Therefore, the comprehensive use of currently available molecular genetic assays for analyzing genetic alterations in CML is essential for effectively managing the disease.

Here, we reviewed the current genetic laboratory tests available for the effective management of CML. Furthermore, we provide information on the future direction of using frontier molecular genetic tools such as NGS to explore the biology of CML and the potential use of those methods for clinical practices.

Current Terms and Definitions Used in Molecular Genetic Tests for the Management of CML

In the US, there is approximately 50% of a patient diagnosed with CML without signs of the disease (asymptomatic).[5] The definition of disease phases, including chronic, accelerated, and blastic phases, was unchanged following the previous publication by the European LeukemiaNet (ELN).[2] The details of the criteria for disease phase definitions are indicated in Table 1. Similarly, the baseline prognostic risk assessment of the patient prior to therapy also followed the guideline from previous reports. There were three prognostic systems currently used to predict the outcomes of the treatment of CML, including Sokal, Euro, and EUTOS (Table 2).[13-15] To assess the complete cytogenetic response (CCyR) in CML patients, as the sensitivity of cytogenetic analysis conferred by the number of analyzed metaphase cells, the recommendation for cytogenetic analysis by G-banding method should perform the analysis on a minimum of 20 metaphase cells. FISH was recommended to be used only to define the achievement of CCyR (≤ 1% *BCR*::*ABL1*-positive nuclei in a total of at least 200 interphase cell nuclei).[2] To assess the major molecular response (MMR) and deep molecular response (DMR), qRT-PCR has been recommended to assess the level of *BCR*::*ABL1* transcript, which reports into the %IS by multiplying with the laboratory-specific (in-house assay) or batch-specific (commercial kit) CFs. The recommended control genes (housekeeping genes) used in qRT-PCR for the calculation of the *BCR*::*ABL1* ratio included *ABL1, GAPDH,* and *GUSB.* The qRT-PCR report must be represented as %IS on the log reduction scale, where 1%, 0.1%, 0.01%, 0.0032%, and 0.001% IS conferred to a reduction of 2, 3, 4, and 5 log reduction, respectively (Table 3).[8] In brief, *BCR*::*ABL1* ≤ 1% is equivalent to CCyR.[16] *BCR*::*ABL1* transcript level ≤0.1% was defined

as a major molecular response (MMR) or MR.[3] A *BCR*::*ABL1* transcript level ≤ 0.01% or undetectable disease in cDNA with more than 10,000 *ABL1* (control gene) transcripts was defined as MR.[4] A *BCR*::*ABL1* transcript level ≤ 0.0032% or by undetectable disease in cDNA with > 32,000 *ABL1* transcripts in the same volume of cDNA used to test for *BCR*::*ABL1* was defined as MR.[4,5] Finally, the recent ELN guideline defined the consensus terms recommended for the monitoring of treatment milestones of CML during TKI treatment, including optimal, warning, and failure responses which are described in more detail in Table 4.[8]

1. Cytogenetic Analysis

The diagnosis of CML is confirmed by cytogenetic analysis (karyotyping) for the identification of t(9;22)(q34;q11.2) or Philadelphia (Ph) chromosome in bone marrow cells.[8] To perform karyotyping, heparinized bone marrow specimens derived from CML patient is subjected to short-term culture. The cultured metaphase cells were then harvested and spread on a glass slide. G-banding was performed to stain the metaphase chromosomes. The guideline for analyzing chromosomes is based on the International System for Human Cytogenetic or Cytogenomic Nomenclature (ISCN) (the current ISCN guideline is now in version 2020).[17] For monitoring TKI therapy, a reduction in the number of Ph-positive cells is reflected in response to treatment. The ELN 2013 guideline recommended that cytogenetic analysis should be carried out at the 3, 6, 12, and every 12 months until achieving CCyR. The definition of each CyR level is shown in Table 3. Importantly, chromosome analysis has been used for the identification of additional chromosome aberrations (ACA), which have been reported to be associated with prognostic significance in CML. Currently, high-risk ACA includes +8, a second Ph-chromosome (+Ph), i(17q), +19, -7/7q-, 11q23, or 3q26.2 aberrations, and complex karyotype abnormalities.[5] Furthermore, the cytogenetic analysis could be used to monitor CML patients during treatment in cases with rare or atypical *BCR*::*ABL1* transcripts that qRT-PCR cannot detect.

2. Fluorescent *in situ* hybridization (FISH)

FISH has been used to compensate for the limitation of standard karyotyping to detect *BCR*::*ABL1* rearrangement, especially in a case with insufficient metaphase cells. To determine the *BCR*::*ABL1* rearrangement by FISH analysis, a bone marrow or peripheral blood sample is collected and prepared for the interphase cells on the glass slide. The dual fluorescent probes are then hybridized to the locus-specific region on *BCR* and *ABL1* genes. *BCR*::*ABL1*-positive nuclei (of at least 200 nuclei) are counted under the fluorescent microscope and reported as % *BCR*::*ABL1*-positive cells.

Table 1 Definitions of Criteria for Accelerated Phase and Blastic Phase Recommended by WHO and ELN Criteria.[2, 30]

Phase	Definition	
	WHO	**ELN**
AP	- Blast cells in blood or bone marrow 10-19%	- Blasts in blood or marrow 15-29%; blasts plus promyelocytes in blood or marrow > 30%, with blasts < 30%
	- Basophils in blood ≥ 20%	- Basophils in blood ≥ 20%
	- Persistent thrombocytopenia (platelet count < 100 x 10^9/L) unrelated to therapy	- Persistent thrombocytopenia (platelet count < 100 x 10^9/L) unrelated to therapy
	- Increasing spleen size and increasing WBC count unresponsive to therapy	- Not included
	- Cytogenetic evidence of clonal evolution (the appearance of additional genetic abnormalities that were not present at the time of diagnosis)	- Clonal chromosome abnormalities in Ph+ cells (CCA/Ph+), major route, on treatment
BP	- Blasts in blood or marrow ≥ 20%	- Blasts in blood or marrow ≥ 30%
	- Extramedullary blast proliferation, apart from spleen	- Extramedullary blast proliferation, apart from spleen
	- Large foci or clusters of blasts in the bone marrow biopsy	- Not included

Abbr.: WHO, World Health Organization; ELN, European LeukemiaNet; AP, accelerated phase; BP; blastic phase

Table 2 Calculation of Prognostic Score and Risk Definition.[8, 13-15]

Prognostic system	Calculation Formula	Risk definition
Sokal	Exp 0.0116 × (age in years-43.4) + 0.0345 × (spleen-7.51) + 0.188 × [(platelet count/700)^2 -0.563] + 0.0887 × (blast cells-2.10)	- Low risk < 0.8 - Intermediate risk 0.8-1.2 - High risk > 1.2
Euro/Hasford	0.666 when age > 50 years + (0.042 × spleen) + 1.0956 when platelet count > 1500 × 10^9/L + (0.0584 × blast cells) + 0.20399 when basophils > 3% + (0.0413 × eosinophils) × 100	- Low risk < 780 - Intermediate risk 781-1480 - High risk > 1480
EUTOS	(7 × basophils) + (4 × spleen size), where the spleen was measured in centimeters below the costal margin and basophils as percentage ratio	- Low risk < 87 - High risk > 87

Table 3 Definitions of Hematologic Response, Cytogenetic Response, and Molecular Response According to ELN Recommendation.[2, 30]

Response	Definition
Hematologic response (HR)	
- Complete (CHR)	- White blood cell < 10 x 10^9/L - Basophil < 5% - No myelocytes, promyelocytes, myeloblasts in the differential - Platelet count 450 x 10^9/L - Spleen nonpalpable
Cytogenetic response (CyR)	
- Complete (CCyR)	- No Ph+ metaphases by CBA or < 1% *BCR*::*ABL1*+ nuclei by iFISH out of ≥ 200 cellls
- Partial (PCyR)	- 1% to 35% Ph+ metaphases by CBA
- Minor (mCyR)	- 36% to 65% Ph+ metaphases by CBA
- Minimal (minCyR)	- 66% to 95% Ph+ metaphases by CBA
- None (noCyR)	- > 95% Ph+ metaphases by CBA
Molecular response (MR)	
- Major (MMR)	- *BCR*::*ABL1* transcript level ≤ 0.1% IS
- Deep MR:	
- MR^4	- *BCR*::*ABL1* transcript level ≤ 0.01% IS or *BCR*::*ABL1* not detectable with at least 10,000 *ABL1* or 24,000 *GUSB* transcripts
- $MR^{4.5}$	- *BCR*::*ABL1* transcript level ≤ 0.0032% IS or *BCR*::*ABL1* not detectable with at least 32,000 *ABL1* or 77,000 *GUSB* transcripts

Abbr. :Ph+, Philadelphia positive; *BCR*::*ABL1*+, *-BCR*::*ABL1* positive; CBA, chromosome banding analysi; iFISH, interphase fluorescent *in situ* hybridization; IS, international scale, *GUSB*, beta-glucuronidase

Table 4 Treatment Milestones of CML Undergoing TKIs Expressed as *BCR*::*ABL1* on the International Scale (IS)[8]

	Optimal	Warning	Failure
Baseline	NA	High-risk ACA, high-risk ELTS score	NA
3 months	≤ 10%	> 10%	> 10% if confirmed within 1-3 months
6 months	≤ 1%	> 1-10%	> 10%
12 months	≤ 0.1%	> 0.1-1%	> 1%
Any time	≤ 0.1%	> 0.1-1%, loss of ≤ 0.1% (MMR)[a]	> 1%, resistance mutations, high-risk ACA

For patients aiming at TFR, the optimal response (at any time) is *BCR*::*ABL1* ≤ 0.01% (MR4)

A change of treatment may be considered if MMR is not reached by 36-48 months.

NA; not applicable, ACA; additional chromosome abnormalities in Ph+ cells, ELTS; EUTOS long term survival score.

[a]Loss of MMR (*BCR*::*ABL1* > 0.1%) indicates failure after TFR

Approximately 5% of CML patients exhibited culture failure (no metaphase cell) during the karyotyping process, yielding misdiagnoses in those patients or false negative results. Moreover, the classification of the disease phases and the determination of CyR could not be performed without cytogenetic data. Thus, by using specific probes to detect *BCR*::*ABL1* fusion sequences, FISH was introduced to detect the *BCR*::*ABL1* fusion gene in the interphase cells derived from bone marrow or peripheral blood of CML patients. Notably, FISH has been recommended to be used for the detection of *BCR*::*ABL1* rearrangement in atypical CML with Ph negative/*BCR*::*ABL1*- positive.[18]

3. Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR has been widely used for qualitative analysis of *BCR*::*ABL1* transcript in CML. The assay has higher sensitivity than karyotyping and FISH assays, and could determine subtypes of *BCR*::*ABL1* transcripts. The majority of CML patients have either typical e13a2 or e14a2 (also called b2a2 and b3a2) transcript types which generate p210 *BCR*::*ABL1* fusion protein. However, approximately 2-4% of these patients have atypical *BCR*::*ABL1* transcription variants, which result from the other breakpoints on *BCR* or *ABL1* sequences such as the e1a2 (p190, m-BCR), e19a2 (p230, μ-BCR).[8] The different subtypes of *BCR*::*ABL1* are associated with the diversity in clinical features and the response to TKI in CML. While a lower observed frequency of the p190 transcript is reported in CML (about 2-3%), it associates with monocytosis and poorer response to TKIs.[19] Thus, the assessment of *BCR*::*ABL1* transcription isoforms is very important and necessary for all CML patients prior to TKI administration.

For almost the past three decades, standard RT-PCR has been used for the monitoring of minimal residual disease (MRD) in CML patients. Alongside the discovery of effective TKI, imatinib, the CML experts group had published the BIOMED-1 protocol with highly specific and sensitive to monitoring *BCR*::*ABL1* transcripts.[20] Currently, this protocol is still widely used as a method of choice for monitoring *BCR*::*ABL1* transcript, especially in a laboratory setting where qRT-PCR is not available. Furthermore, multiplex RT-PCR has recently been developed by combining different primers specific to frequent *BCR*::*ABL1* isoforms. Similar to other settings, we have developed the multiplex RT-PCR for assessing *BCR*::*ABL1* p210, p190, and p230 transcripts. Nevertheless, we could not establish multiplex RT-PCR for the analysis of other rare *BCR*::*ABL1* isoforms. That was due to their naturally proceeding long fusion sequence, which cannot succeed in amplifying by our PCR system. Therefore we prefer to use FISH in particular cases that might harbor atypical *BCR*::*ABL1* fusions.

4. Quantitative real-time polymerase chain reaction (qRT-PCR)

Molecular monitoring allows the significantly important results providing the determined MR for managing CML patients undergoing TKI therapy. Standard qRT-PCR had been developed, consensus by a panel of CML experts, Europe against Cancer Network, and widely used to monitor the level of *BCR*::*ABL1* fusion transcripts during TKI treatment.[21] This method is designed to specifically amplify major *BCR*::*ABL1* isoform (p210) and co-amplification of control housekeeping genes (*ABL1*, *GAPDH*, and *GUSB*) in cDNA samples generated from RNA derived from blood or bone marrow samples of CML patients during TKI therapy. The details of the assay protocol included the validated primer sets used in the real-time PCR reaction, sample operation, RNA extraction method, cDNA synthesis, PCR cycle condition, and the calculation of *BCR*::*ABL1* copy ratio.[22] In line with the improvement in treatment outcomes in patients with CML, qRT-PCR has been familiar and widely used in laboratories worldwide as the assay shows the ability to detect low levels of *BCR*::*ABL1* copy number, which is now known as the deep molecular response (DMR). Additionally, the measurement of dynamic change in copy number of *BCR*::*ABL1* in patients during therapy (treatment milestone) is correlated with the degree of response in individual patients, especially in TKI failure that needs dose adjustment or TKI switch. Therefore, laboratory standardization and quality control systems regarding the interpretation and assay performances of qRT-PCR for *BCR*::*ABL1* are crucial for the effective management of CML in the TKIs era.

Recently, batch-specific CF have been developed and used as a calibrator for calculating *BCR*::*ABL1* copy numbers into the % international standard (%IS).[23] Then the %IS is translated to the log reduction score and plotted into the graph directly to the time of monitoring recommended by the standard guideline. This improvement has a significant change in the management of CML to become more international and resolve the problem of using different assay platforms in various laboratories. In brief, the calculation formula of %IS is shown below.

$$\%\textit{BCR}::\textit{ABL1}\ \text{p210}/\textit{ABL1}\ \text{ratio} = (\textit{BCR}::\textit{ABL1}\ \text{p210 copy number}/\textit{ABL1}\ \text{copy number}) \times 100$$

$$\%\text{IS} = \%\textit{BCR}::\textit{ABL1}\ \text{p210}/\textit{ABL1}\ \text{ratio} \times \text{conversion factor}$$

qRT-PCR analysis is recommended to perform every three months until the patient achieves MMR (≤ 0.1% IS) and then every 3 to 6 months (ELN2013).[2] Although qRT-PCR is a very highly sensitive method used to assess MR, the assay has several

drawbacks, such as the inconsistency in the limit of detection (LOD) from different analysis settings/platforms, the required batch-specific CFs, and the difficulty in the preparation of standard calibrators. Presently, many qRT-PCR commercial kits are available and designed to solve several limitations of qRT-PCR, as mentioned above. These commercial kits provide all reagents needed in the qRT-PCR assay, including the batch-specific CF and instant-friendly used analysis software.

5. Droplet digital PCR (ddPCR)

Recently, absolute quantitative ddPCR has been developed to measure the level of *BCR*::*ABL1* transcript in CML patients during TKI therapy. ddPCR is known as the third generation of PCR technology, which is principally based on reaction partitioning (mimicking limiting dilution) into approximately a thousand droplets (each droplet is equivalent to an independent PCR reaction). The assay does not require the standard curve plot for copy number calculation and has now become widely used to assess TKI response.[24, 25] The ddPCR is similar to qRT-PCR with monitoring target genes by specific primer-Taqman probe technology and quantifying by the fluorescent intensity in the reactions. After PCR amplification, copy numbers of the target gene are calculated by Poisson distribution and the ratio of positive droplets to total droplets. Therefore, ddPCR assay allows direct absolute quantitation of nucleic acid targets without needing calibration curves.[24] For the ddPCR process, one microgram of RNA extracted from the patient sample is used to perform cDNA in conjunction with the %IS calibrators, positive, negative RT, and no template control. ddPCR master mix is then prepared for all sample types and aliquot to each reaction well. Droplets from each reaction well are generated for approximately 20,000 droplets by a droplet generator machine and then put into a thermal cycler for target amplification. PCR product is finally counted for total droplets, including identifying the droplet type (positive droplet with the fluorescent signal, negative droplet with no fluorescent signal) by the droplet reader instrument. Copy numbers of *BCR*::*ABL1* and *ABL1* are calculated by Poisson distribution, and the result is reported as %IS value.[25]

Currently, there was evidence in the study of the comparison in assay performance of using the FDA-approved commercially available ddPCR assay and the standard qRT-PCR for the measurement of *BCR*::*ABL1* p210 transcript.[26] ddPCR had comparable results in assay performances when compared with qRT-PCR and could be used in the clinical laboratory for the management of CML.[26] ddPCR trend to provide a shift to a more profound molecular response class than qRT-PCR. Additionally, ddPCR showed an advantage in detecting deep molecular response and reduced interlaboratory variations. This suggests the potential usefulness of ddPCR for

clinical trials regarding the assessment of achieving and maintaining treatment-free remission.[27] More recently, the assay has been introduced to measure the level of *BCR*::*ABL1* in a case undetected by qRT-PCR in the clinical trial of discontinued TKIs.[28]

Besides using ddPCR for monitoring treatment response in CML patients undergoing TKI treatments, the assay has been extended and used for the analysis of hotspot clinically significant *BCR*::*ABL1* tyrosine kinase domain (TKD) mutations. Remarkably, T315I mutation is the gatekeeper associated with several available TKI resistant. ddPCR showed several advantages in detecting a low level of T315I mutations. Additionally, the assay could be used to identify the mutations in second-generation TKIs resistant. However, ddPCR cannot detect compound/polyclonal mutations of *BCR*::*ABL1* TKD.[29]

6. Direct sequencing analysis

Although TKIs have shaped the world in the improvement of CML therapy, making the quality of life of patients similar to age-match normal, approximately 20-30% of CML patients become TKIs resistant.[5] TKI resistance is categorized into primary (patient lack of response to TKI therapy) and secondary resistance (patient has disease progression after undergoing the initial response to treatment), which has two major mechanisms, including *BCR*::*ABL1*-independent or *BCR*::*ABL1*-dependent. The *BCR*::*ABL1*-independent mechanisms include aberrant drug influx/efflux expression, the dysregulation of alternative signaling pathways, and epigenetic dysregulations.

For the *BCR*::*ABL1*-dependent mechanism, the *BCR*::*ABL1* TKD mutation is the most common abnormality involving TKI resistance. More than a hundred different mutation types have been reported, leading to a structural conformation change of protein and inhibiting the TKI binding. Those include mutation spanning in; 1) activation loop (A-loop) mutations, 2) catalytic domain mutations, 3) ATP-binding loop (P-loop) mutations, and 4) ATP-binding site mutations.[12] In addition, *BCR*::*ABL1* amplification and clonal evolution of CML leukemic cells are also included in the *BCR*::*ABL1*-dependent mechanism.[30] Recently, the ELN guideline has recommended the clinical relevance of *BCR*::*ABL1* TKD mutations regarding the establishment of specific TKI resistance and optimal management of the patient after mutations occurred.[8] Thus, laboratory methods for identifying *BCR*::*ABL1* TKD mutations are necessary for managing CML to achieve better therapeutic outcomes in patients undergoing TKI therapy.

To date, the Sanger sequencing or direct sequencing method is recognized as a standard method and widely used for identifying *BCR*::*ABL1* TKD mutation in samples derived from patients with CML who exhibit signs of resistance. Direct sequencing could screen all

mutations spanning in the *BCR*::*ABL1* TKD with a limit of detection of about 20% mutational burden.[31] Moreover, the assay could detect multiple mutation patterns that were reported to be associated with several TKI resistant in CML. Additionally, the assay has been used to confirm mutation identified by other methods such as ddPCR, allele specific-PCR (AS-PCR), pyrosequencing, denaturing high-performance liquid chromatography (dHPLC), and NGS.

RNA is isolated from blood/bone marrow samples derived from CML patients and reverse transcribed to cDNA. RT-PCR is then performed using a specific primer set to amplify the *BCR*::*ABL1* fusion transcripts in the first round of PCR. The *ABL1* kinase domain region is amplified in the second round of PCR using the first PCR product as a template. After checking for the expression of the *ABL1* kinase domain, purification of the second-PCR product, cycle sequencing reaction, and sequencing product purification are performed and subsequently sequenced with the genetic analyzer. The sequencing result from the patient is analyzed compared to the wild-type *ABL1* sequence.[32]

Although standard direct sequencing is frequently used and could detect all mutation types in the *BCR*::*ABL1* kinase domain, the assay has relatively low sensitivity (about 20%) to detect *BCR*::*ABL1* TKD mutation, especially in a case with a low level of mutation.[5, 8, 10] Besides the limitation of direct sequencing to detect *BCR*::*ABL1* mutations drawn to the low level of mutational burden, the assay has several cons, including being laborious, time-consuming, expensive, and cannot distinguish polyclonal mutation patterns. Hence, to overcome several disadvantages of direct sequencing, high throughput NGS technology, with higher precision, higher sensitivity, able to discriminate polyclonal mutations, has been introduced for routine screening and monitoring of *BCR*::*ABL1* TKD mutations.

7. Next-generation sequencing (NGS)

Recently, NGS has been introduced for both baseline characterization and monitoring of *BCR*::*ABL1* TKD mutations. NGS showed several advantages over traditional direct sequencing and other molecular methods, such as sensitivity, the length of mutations detected, cost, automation, and assay throughput. Importantly, NGS could discriminate between polyclonal (mutations by the different Ph+ clones) and compound mutations (different mutation patterns in the same Ph+ clones) of *BCR*::*ABL1* TKD in an individual patient.[33] These could explain the clonal diversity of CML leukemic cells in the patient during TKI therapy.[34] Furthermore, NGS could detect the CML clones with TKI resistant harboring low-level mutations experience selective expansion if the TKI is not switched or an unfitting TKI or inappropriate TKI dose.[35] NGS has also been extensively

used to detect other driving mutations, such as *ASXL1, RUNX,* and *IKZF1*, that might contribute to TKI resistance in CML patients.[36] Nevertheless, the application of NGS has been limited to certain laboratory settings. Currently, there is no well-established standard for NGS procedure and data interpretation regarding the application of NGS for the management of CML.

Conclusions

Monitoring of CML treatment by standardized genetic laboratory tests has essential role for management of CML patients undergoing with TKI therapy. Cytogenetic analysis is still the gold standard method for disease diagnosis and can be used to assess the additional chromosome abnormalities. In addition to the above, our laboratory setting currently performs molecular assays including RT-PCR, and qRT-PCR for identification *BCR*::*ABL1* transcript type and monitoring response using %IS level, respectively. Moreover, Sanger sequencing is also performed for TKD mutation detection. Nowadays, ddPCR has been introduced to monitor the %IS level with higher sensitivity than qRT-PCR. Likewise, NGS becomes widely used to identify *BCR*::*ABL1* TKD mutation because of the most sensitive tool to detect the low mutation and ability to find out the other important driving mutations.

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