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REVIEW ARTICLE

Clinical implications of conventional cytogenetics, fluorescence in situ hybridization (FISH) and molecular testing in chronic myeloid leukaemia patients in the tyrosine kinase inhibitor era – A review

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Abstract

Chronic myeloid leukaemia (CML) provides an illustrative disease model for both molecular pathogenesis of cancer and rational drug therapy. Imatinib mesylate (IM), a *BCR-ABL1* targeted tyrosine kinase inhibitor (TKI) drug, is the first line gold standard drug for CML treatment. Conventional cytogenetic analysis (CCA) can identify the standard and variant Philadelphia (Ph) chromosome, and any additional complex chromosome abnormalities at diagnosis as well as during treatment course. Fluorescence in situ hybridization (FISH) is especially important for cells of CML patients with inadequate or inferior quality metaphases or those with variant Ph translocations. CCA in conjunction with FISH can serve as powerful tools in all phases of CML including the diagnosis, prognosis, risk stratification and monitoring of cytogenetic responses to treatment. Molecular techniques such as reverse transcriptase-polymerase chain reaction (RT-PCR) is used for the detection of *BCR-ABL1* transcripts at diagnosis whereas quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) is used at the time of diagnosis as well as during TKI therapy for the quantitation of *BCR-ABL1* transcripts to evaluate the molecular response and minimal residual disease (MRD). Despite the excellent treatment results obtained after the introduction of TKI drugs, especially Imatinib mesylate (IM), resistance to TKIs develops in approximately 35% - 40% of CML patients on TKI therapy. Since point mutations in *BCR-ABL1* are a common cause of IM resistance, mutation analysis is important in IM resistant patients. Mutations are reliably detected by nested PCR amplification of the translocated *ABL1* kinase domain followed by direct sequencing of the entire amplified kinase domain. The objective of this review is to highlight the importance of regular and timely CCA, FISH analysis and molecular testing in the diagnosis, prognosis, assessment of therapeutic efficacy, evaluation of MRD and in the detection of *BCR-ABL1* kinase mutations which cause therapeutic resistance in adult CML patients.

Keywords: CML, cytogenetics, FISH, molecular testing, tyrosine kinase inhibitors

INTRODUCTION

The myeloproliferative neoplasm, chronic myeloid leukaemia (CML) which originates in a pluripotent stem cell in the bone marrow[1], presents with leukocytosis, a left shift in the differential count and splenomegaly as the major clinical hallmarks.[2] CML is a triphasic disease which starts with an initial chronic phase (CP), an intermediate accelerated phase (AP), and a final, fatal blastic phase (BP). CP may last for several years; AP may have a variable duration from weeks to years whereas in BP, survival lasts for few months only.[2] CML ranks several first positions to its credit. It is the first haematological disease to be named leukaemia, first malignancy to be associated with a cytogenetic marker namely the Philadelphia (Ph) chromosome[3],

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first disease in which a *BCR-ABL1* fusion gene formed as a result of t(9;22) translocation was found to be responsible for its pathogenesis, and the first disease to be successfully treated with a molecularly targeted tyrosine kinase inhibitor (TKI) drug imatinib mesylate (IM).[4] It is no wonder that CML occupies the first place in the first chapter of WHO classification.

With an annual incidence of 0.7 - 1.8 per 100,000 population globally, the prevalence of CML is expected to plateau at 35 times its annual incidence by 2050.[5] This has been attributed to the substantial proportion of survival that is being achieved with the advent of targeted therapy. In Malaysia, CML accounts for 15% of approximately 740 new leukaemia cases diagnosed annually.[6] CML provide an illustrative disease model for both molecular pathogenesis of cancer and rational drug therapy. Additionally, CML also serves as a model for the disease where the knowledge as well as development of different new methods from molecular biology were successfully introduced into the clinical practice. For the estimated 1500 CML patients in Malaysia, lifelong treatment with TKIs remains the current standard of care.[7] Cytogenetic analysis, fluorescence in situ hybridization (FISH) and molecular tests especially involving polymerase chain reaction (PCR), have emerged as important tests that play important roles in the management of CML by helping to establish the diagnosis as well as predict prognosis, monitor response to treatment and disease progression. Hence, this review is aimed to give a conspectus of these laboratory approaches which are clinically useful and important in the management of adult CML patients.

Molecular anatomy of *BCR–ABL1* translocation and pathogenesis of CML

It was in 19th century that CML was first discovered as a clinical syndrome with characteristic features of myeloid hyperplasia in the bone marrow, leukocytosis and splenomegaly. Then, it was Nowell and Hungerford who first discovered a minute chromosome in the bone marrow cells of patients with CML and named it as Philadelphia (Ph) chromosome, after the name of the city in which it was first discovered.[3] Later in 1970, Janet Rowley (University of Chicago) demonstrated that Ph chromosome results from a reciprocal translocation between chromosomes 9 and 22.[8] Almost 95% of CML patients show the classical t(9;22)(q34;q11) translocation, which involves a reciprocal translocation between chromosomes 9 and 22, where the segments 9q34 of chromosome 9 and 22q11 of chromosome 22 are reciprocally exchanged. A karyotype showing the translocation t(9;22)(q34;q11) resulting in Ph chromosome is shown in Figure 1. Normally, the *ABL1* gene is located on chromosome 9q34 and encodes a tyrosine kinase protein with molecular weight 145 kilo dalton (kD). The *BCR* gene located at chromosome 22q11 encodes a 160 kD protein with serine/threonine kinase activity.[9] As a result of the t(9;22) translocation, the *ABL1* fuses with the *BCR* and a *BCR-ABL1* fusion gene is formed. The newly created *BCR-ABL1* forms a novel 8.5 kb mRNA which comprises about half of the *BCR* exons at the centromeric 5' end and *ABL1* exons 2 to 11 towards the 3' end. The breakpoints in the major breakpoint cluster region (M-bcr) are located either between exons b2 or b3 (exons 13 and 14 of the *BCR* gene) or between exons b3 and b4 (*BCR* exons 14 and 15). In the case of *ABL1*, breakpoints can occur 5' to exon 1b, as well as in introns 1,2 or 3. However, majority of patients demonstrate a chimeric transcript in which the *BCR* segment is spliced to exon a2 of the *ABL1* gene.[10] Accordingly, in most patients, the RNA transcripts consists of *ABL1* a2 spliced to *BCR* exons b2 or b3, which are denoted as e13a2 (b2a2) or e14a2 (b3a2). The *BCR-ABL1* fusion gene act as an oncogene which encodes *BCR-ABL1* transcripts, a chimeric fusion protein with molecular weight of 210 kDa and increased tyrosine kinase activity. This p210 BCR-ABL1 fusion protein possesses oncogenic dysregulated kinase activity and is involved in leukemogenesis through its interference with normal cell cycle events, such as signal transduction and regulation of apoptosis and cell proliferation.[11] It has been demonstrated that BCR-ABL1 activates main signal pathways such as *RAS/MAPK, PI-3* kinase, *c-ABL* pathways and *CRKL* pathways and *JAK-STAT* and the *Src* pathways which results in transformation and cell proliferation. Inhibition of apoptosis has been reported to result from activation of the *PI-3* kinase and *RAS* pathways with induction through *AKT* of *Myc* and *BCL-2*.[12,13] The knowledge of chromosomal and molecular abnormalities led to the elucidation of the etiopathogenetic mechanisms of CML and gave the basis for the development of gene-targeted therapy.

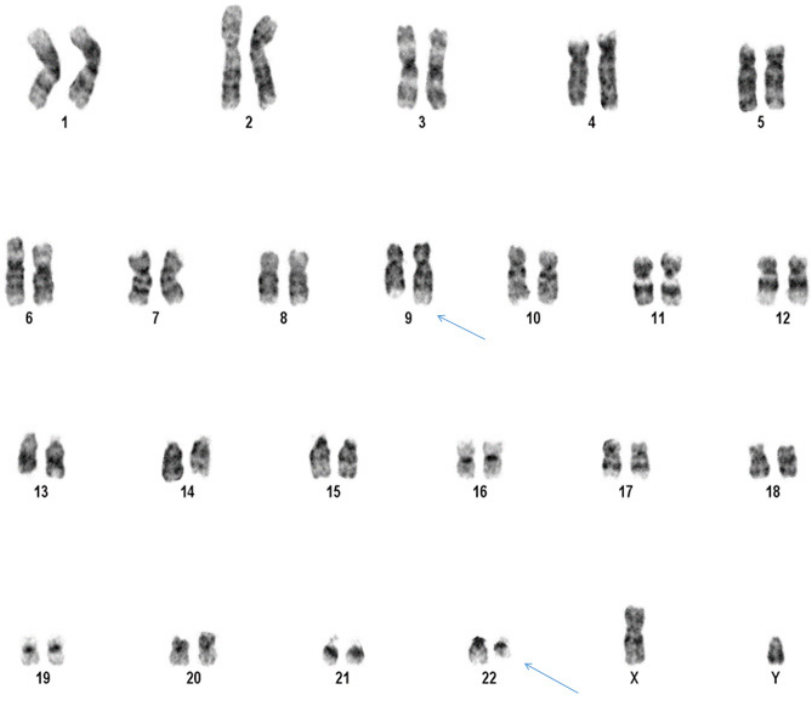

FIG. 1: 46, XY, t(9;22)(q34;q11) Karyotype of a male CML patient showing the Philadelphia chromosome formed as a result of reciprocal translocation between chromosome 9 and chromosome 22 (Courtesy of Cytogenetics Laboratory, Human Genome Centre, Universiti Sains Malaysia, Kelantan, Malaysia)

Initial work up at diagnosis

A straight forward diagnosis of most cases of CML is possible on the basis of a characteristic total and differential blood count (excessive granulocytosis with typical left shift of granulopoiesis) and marked splenomegaly. Confirmatory diagnosis of CML relies on bone marrow morphology, cytogenetics and molecular tests. For new CML patients, diagnostic assays are based on the standard tests of conventional cytogenetics, fluorescence in situ hybridization (FISH) and molecular testing based on polymerase chain reaction (PCR). Cytogenetic analysis can detect the Ph chromosome, FISH can detect the *BCR-ABL1* fusion gene signals whereas PCR based molecular tests can detect and quantitate the *BCR-ABL1* fusion mRNA transcripts.

Conventional cytogenetic analysis for Ph chromosome detection

Conventional Cytogenetic analysis (CCA) which involves karyotyping of the bone marrow metaphases is primarily used for detecting the Ph chromosome reciprocal translocation between chromosomes 9 and 22. Bone marrow cultures are preferred than blood cultures in order to get adequate numbers of metaphase cells. CCA uses light microscopy to analyse the mitotic cells arrested during metaphase in minimum of 20 GTG banded bone marrow metaphases. Karyotypes are described according to the International System for Human Cytogenomic Nomenclature.[14] Standard cytogenetic studies of the bone marrow show the Ph chromosome in approximately 95% of patients at diagnosis of CML. Ph chromosome is generally the only cytogenetic abnormality present in the chronic phase of the disease. Rarely variant Ph chromosome translocation and /or additional chromosome abnormalities (ACAs) also may be present. Only CCA enables the detection of ACAs in addition to the Ph chromosome, which when found at the time of diagnosis might predict a shorter duration of chronic phase or could be a component of defining an accelerated phase.[15,16] Furthermore, in interpreting subsequent clonal evolution, emergence of new cytogenetically abnormal Ph chromosome negative clones and cytogenetic monitoring of treatment effect, baseline karyotype information is important.[17] So CCA should be considered at the time of diagnosis in all patients.

Variant Philadelphia chromosome translocation

Ph translocation generated by variant rearrangement involving 9q34, 22q11 and one or several other genomic regions is defined as variant Ph chromosome translocation.[18] About 4 - 5% of patients with CML have variant Ph chromosome translocations, which involve chromosomes 9, 22 and another one or more chromosome(s). Sometimes the Ph chromosome morphology appears to be masked due to the involvement of an additional chromosome. If a variant Ph translocation is detected, the presence of *BCR-ABL1* rearrangement should be confirmed by *BCR-ABL* fusion signals by FISH or *BCR-ABL* fusion transcripts by molecular testing methods. CML patients with variant Ph translocations are treated with imatinib or 2nd generation TKIs and are considered to have a similar outcome to classical t(9;22) cases. These variant Ph chromosome translocations have been classified as complex when two, three or more chromosomes in addition to chromosomes 9 and 22 are involved.[19,20]

Despite the limitations such as low sensitivity (1:20 or 5%), labour intensive, time consuming, the need for invasive bone marrow aspiration as a source for obtaining and culturing proliferative cells for good quality metaphases, and the non-availability of sufficient or analysable good quality metaphases occasionally, CCA still remains the gold standard technique and standardized assay utilized globally.

Additional chromosome abnormalities (ACAs)

Chromosome abnormalities observed in addition to the Ph chromosome in CML patients are known as additional chromosome abnormalities (ACAs). The incidence of ACAs is relatively low, around 2 - 15% of IM treated patients. Majority of CML patients develop ACAs in the Ph positive cells in advanced phases of the disease. In approximately 75 - 80% of CML patients in blast crisis, ACAs can be detected.[21,22] The emergence of ACAs which is also called clonal evolution, is thought to reflect genetic instability of the leukemic cells and as a sign of disease progression.[22] ACAs emerge during the course of treatment, even in Ph negative CML patients with CCyR or along in Ph positive CML patients who show no minimal/partial cytogenetic response. Sometimes ACAs can be seen at the time of diagnosis. The European Leukemia Net (ELN 2009)[23] recommendations suggest that presence of ACAs at diagnosis is a warning signal. Warning is defined as a characteristic of the disease which may affect the patient's response to TKI therapy adversely and may require more stringent and careful monitoring of the patient.[23,24] Hence it is important to establish whether ACAs are present at diagnosis. More details on emergence of ACAs during treatment are described during the later part of this review.

FISH analysis for *BCR-ABL1* fusion signals

FISH is a molecular cytogenetic method which has greatly enhanced the accuracy and efficiency of cytogenetic analysis. FISH involves the use of fluorochrome-labelled DNA probes that are hybridized to unstained interphase nuclei or metaphase on a microscopic glass slide.[25] Here firstly, the genomic DNA is denatured (converting the double strand DNA to single stand conformation) and then it is incubated with the specific FISH probes. FISH signals are detected by fluorescence microscopy. For CML, FISH strategies use a green (G) probe for *BCR* and a red (R) probe for *ABL1*. A yellow (Y) signal denotes *BCR-ABL1* fusion gene. The 1R IG 2Y is the characteristic pattern of CML. Sometimes atypical FISH patterns such 1R 2G 1Y, 2R 1G 1Y, and 1R 1G 1Y also may be observed which are indicative of sub-microscopic deletions on the derivative chromosome 9.[26] FISH can be applied to unstained glass slides of fresh or fixed specimens, can be performed even in non-dividing interphase cells from peripheral blood or bone marrow and the proportion of metaphases or interphase nuclei with the characteristic *BCR-ABL*1 fusion genes are measured. Usually 200 to 500 interphase nuclei need to be examined. Test sensitivity which ranges from 0.1 - 1% depends on the number of nuclei examined as well as the types of probes used.[26] Interphase FISH results may be available within 24 hours. FISH is especially important for detecting *BCR-ABL1* at diagnosis in CML patients with lack of adequate number of metaphases for CCA or those with inferior quality metaphases or those with variant Ph translocations when Ph chromosome cannot be detected in the usual pattern. However, it should be noted that FISH can have a false positive rate of 1% to 10% depending on the probes used. Ideally, usage of good quality commercial probes with a low false positive rate that can bring the sensitivity of FISH assays to 1 – 6 %, is recommended.[27]

Bone marrow karyotyping has few limitations. BM aspiration is an invasive procedure with

small but significant risks involved.[28] Around 5% of BM samples may not yield adequate number of suitable metaphases for CCA. Moreover, CCA requires highly trained staff and is labour-intensive.[29] In clinical settings, where BM sampling is not practical, FISH testing of peripheral blood can be an acceptable alternative for the diagnostic work up of CML. However, secondary or ACAs cannot be detected using dual colour FISH probes for *BCR-ABL* fusion gene.[22,30] As a result, periodic CCA is required even if FISH is used for regular monitoring. Therefore, metaphase karyotyping of BM samples remains the preferred choice for cytogenetic testing. Hence FISH testing should not be considered to be a replacement for conventional cytogenetic testing, but can be used as a complement to CCA.

CCA serves to identify Ph chromosome in only 95% of cases of CML. Some patients may have clinical and haematological profile of CML, but show normal karyotype without identifiable Ph chromosome. Molecular testing may be able to detect the *BCR-ABL1* fusion transcript in some of such cases who harbour a submicroscopic genetic fusion. In such patients, molecular testing serves as a primary tool in CML diagnosis. For the remaining patients who are negative for both the Ph chromosome and *BCR-ABL*1 fusion transcript, alternative diagnoses such as atypical CML (which represent a separate disease entity) need to be considered.[31,32]

Molecular detection of *BCR-ABL1* mRNA transcripts using qualitative and quantitative reverse transcriptase Polymerase Chain Reaction

More sensitive assays for detecting disease specific M-bcr and m-bcr transcripts involves polymerase chain reaction (PCR). PCR test essentially amplifies small amounts of specific pieces of either RNA or DNA to make them easier to detect. Chromosome translocations that result in fusion genes are especially suited for reverse transcriptase polymerase chain reaction (RT-PCR), a technique in which the disease specific fusion mRNA is reverse transcribed into complementary DNA (cDNA) and then, with appropriate oligo primers from each gene, the fusion gene is amplified by PCR. RT-PCR can be of qualitative or quantitative type. BCR-ABL1 fusion transcripts can be detected using qualitative RT-PCR or real time quantitative RT-PCR (qRT-PCR) where the later method can even quantitate the transcript levels. These PCR methods can detect abnormalities in marrow or blood samples at a level of 1 in 10^5 to 1 in 10^6 normal cells.[33]

Qualitative RT-PCR is indicated mainly to detect the *BCR-ABL1* fusion transcript to establish or confirm a diagnosis of CML. For qualitative RT-PCR, RNA is extracted from peripheral blood or bone marrow cells, disease specific mRNA is first converted to cDNA using an oligo (dT) primer and reverse transcriptase, and then it is subjected to standard PCR subsequently. In qualitative RT-PCR, the amplified product is assessed by gel electrophoresis after the entire PCR is completed. Qualitative RT-PCR can be performed with a simple nested or multiplex approach. However, due to significant risk of PCR contamination and consequent false positive results, it is advisable to avoid nested methods in the routine diagnostic laboratory. In the case of non-nested simplex RT-PCR, one assay is performed using a single pair of primers. The primers for *BCR* exon 13 (b2) and *ABL1* exon 2 (a2) identify both e13a2 (b2a2) and e14a2 (b3a2) fusion transcripts that differ in size by 75 bp. The atypical transcript variant, if present, can also be detected by this method. Molecular detection of CML in nearly all cases (approximately 99 %) is possible using PCR primers to these regions.[34]

In the diagnostic setting, qualitative RT-PCR is advantageous for the following situations. CML can be distinguished from leukaemoid reactions or other myeloproliferative disorders based on the presence or absence of *BCR-ABL1* transcripts. For those patients in whom the Ph chromosome cannot be detected by routine karyotype analysis, a *BCR-ABL1* positive qualitative result will be highly beneficial for CML diagnosis. The determination of the type of *BCR-ABL1* transcripts not only validates the implementation of targeted therapy using TKIs such as imatinib mesylate, but also helps in subsequent treatment response monitoring based on the type of specific fusion transcript identified to the neoplastic clone. The attributes such as sensitivity, rapid turnaround time, and both blood and bone marrow constitute suitable specimens (thus obviating the need for invasive bone marrow procedures) make qualitative RT-PCR well suited for initial diagnosis. Qualitative RT-PCR assay is reported as being either positive or negative mainly at the time of diagnosis only. A limitation is that the starting amount of the target transcript cannot be quantitated.

On the other hand, real time quantitative PCR (qRT-PCR), which is based on the

measurement of fluorescence emission during the PCR[35] is a molecular technique by which *BCR-ABL1* fusion transcript levels can also be quantitated for clinical purposes. This method includes RNA extraction from a bone marrow or peripheral blood sample, conversion of disease specific mRNA to cDNA and an aliquot of the cDNA product is then used in real time fluorescence quantitative PCR to quantify both the target transcript (*BCR-ABL1*) and a control gene transcript. It has to be noted that variation in sample processing, storage temperature or transport duration can impact RNA stability and the integrity in *BCR-ABL1* qRT-PCR results. Therefore, issues such as initial sample handling and transportation to testing lab should be handled with utmost care.

In order to establish the baseline *BCR-ABL1* mRNA transcripts, qRT-PCR with either peripheral blood or bone marrow of CML patients should be performed before initiation of treatment with any TKIs. Currently, qRT-PCR based on TaqMan or Light Cycler technology[24,36] is the preferential test used. In the TaqMan assay system, an internal oligonucleotide probe bearing a 5' reporter fluorophore and 3' quencher fluorophore hybridizes to the target transcript first, and then it is hydrolyzed by the nuclear activity of the Taq polymerase during the primer extension phase of the PCR reaction. As a consequence, the reporter and quencher fluorochromes get separated resulting in emission of fluorescence. For fluorescence to be detected, obviously the specific transcript (*BCR-ABL1* mRNA in CML) must be present in the test sample. The detected fluorescence will be proportional to the amount of target present in the sample.[37]

The number of PCR cycles necessary to detect a signal above the threshold is called the cycle threshold which is directly proportional to the amount of target present in the beginning of the reaction.[35,37] From the serial dilutions of a positive control template, a standard curve is constructed and this is used to estimate, by inference, the starting amount of the target transcript in the test sample. By the normalisation of raw data against, that of an internal control (house-keeping gene), issues regarding PCR efficiency and RNA integrity are addressed.[35] In the TaqMan probe method, RNA or cDNA standards of known concentration are used to generate a standard curve [log (copy number) versus threshold cycle (CT)], from which the unknown sample quantity is determined and then normalised against any one of the internal references such as *ABL1, BCR,* or *GUSB* transcripts. The final result is usually reported as a percentage ratio of *BCR-ABL1* transcript numbers to the number of control gene transcripts (BCR-ABL1/ABL1).[38] Thus, qRT-PCR affords a more sensitive, rapid and reproducible quantification of the *BCR-ABL1* fusion transcripts that can be applied to either blood or bone marrow cells.

Treatment of CML patients

The discovery of *BCR-ABL1* mediated pathogenesis of CML and recognition of the BCR-ABL1 protein as the pivotal contributor involved in the pathogenesis and progression of CML provided the impetus for researchers to design inhibitory agents that target BCR-ABL1 kinase activity. This led to the search and development of a TKI drug imatinib mesylate (IM), as the first molecularly targeted drug for the treatment of Ph positive CP CML patients.[39] Since its approval by Food and Drug Administration (FDA) USA in 2001, IM has improved the prognosis of Ph positive CML patients[40,41], compared with previously available therapies.[42,43] With far fewer side effects, IM leads to durable cytogenetic response (CyR) and molecular response (MR) and drastically improves the five year survival rates, especially of CP CML patients. However, it was realised sooner that many CML patients on IM treatment had detectable levels of *BCR-ABL1* transcripts in their blood and bone marrow during the assessment of minimal residual disease. Furthermore, the development of resistance to IM in a significant proportion of CML patients on IM treatment also became a daunting problem in the management of CML patients. This fostered the development of more potent second generation TKIs such as dasatinib, nilotinib, bosutinib and third generation TKI such as ponatinib. These second and third generation TKI drugs are recommended and are available commercially as frontline and/or subsequent treatment options for CML patients.[24,40] The ELN[24] and European Society of Medical Oncology (ESMO)[44,45] guidelines recommend either imatinib 400 mg once daily, dasatinib 100 mg once daily or nilotinib 300 mg twice daily as the first line treatment of CP CML patients. The recently updated National Comprehensive Cancer Network (NCCN) guidelines[41] has upgraded their recommendation on bosutinib as an option for first line treatment along with the aforementioned TKIs recommended by the European bodies.[45] The search for novel TKIs

continues and few other newer TKIs with higher potency and activity are undergoing clinical development.

Monitoring of treatment response and assessment of minimal residual disease (MRD)

Once the initial diagnosis of CML is established, adequate monitoring of patient response to TKI therapy is a mandatory component of the successful management of CML. For CML patients undergoing TKI treatment, complete clinical remission is defined by the resolution of symptoms and signs of disease as well as the morphologic absence of leukaemic cells in the bone marrow. Regular monitoring through haematological, cytogenetic and molecular parameters is important for assessing the response to therapy, as well as for early identification of non-adherence, development of resistance to treatment or failure to treatment. Periodic monitoring of both the therapeutic response and the levels of minimal residual disease (MRD) are critical in therapeutic decision making. A haematologic response (HR) indicates improvement in peripheral blood cell counts and may be complete [CHR(normalised peripheral blood counts, white blood cell count below 10 x 10^9/L, platelets below 450 x 10^9/L, immature cells absent or normalised differential, no signs and symptoms of disease)] or partial (persistence of immature cells, platelets below 50% of pre-treatment levels but above 450 x10^9 /L).[24] Haematologic remission is achieved when the blood counts and spleen size have normalised. However, as per revised NCCN guidelines,[41] haematologic response is no longer considered to make treatment response/failure decision at 3 months.

For assessing cytogenetic and molecular responses, data regarding the percentage of residual metaphase cells with the t(9;22) by CCA or FISH and/or the levels of *BCR-ABL1* transcripts measured by quantitative RT-PCR at various time points after initiation of treatment are incorporated. Cytogenetic response is quantified using CCA and graded based upon the percentage of residual Ph positive cells. Cytogenetic response (CyR) is defined by the reduction in percentage of metaphase cells carrying the Ph chromosome.[23] Complete cytogenetic response (CCyR) is defined as the absence of detectable Ph positive metaphase cells, which is a clinically important prognostic marker for CML patients. In those CML patients who lacks a CCyR, the degree of cytogenetic response is classified as partial [(PCyR 1% - 35 % Ph positive cells), minor (36% - 65% Ph positive cells) or minimal (66% - 95% Ph positive cells)]. A major CyR (MCyR) is defined as CCyR or PCyR. When an increase in Ph positive metaphases of 30% or more is observed, it is considered as loss of CyR.[24] For cytogenetic response monitoring by CCA, a minimum of 20 metaphase cells is recommended.[40] As CCA detects one abnormal metaphase out of only 20 metaphases scored, the limit of detection of this technique is relatively low (1:20 sensitivity of 5%). Nevertheless, CCA which has a resulting 5% level of analytical sensitivity is better than morphology alone. It serves as the gold standard for monitoring treatment and is an important predictor of patient survival. Compared to CCA, FISH can assess the *BCR-ABL1* fusion gene signals even in interphase nuclei of non-dividing cells. In general, it is ideal to examine 500 interphase nuclei. If results are normal with 500 nuclei, higher number of cells (preferably up to 6000 interphase nuclei) are examined. A normal result obtained with 6000 interphase nuclei (normal cut off 0.050%) is more reassuring than that obtained after evaluation of 500 interphase nuclei (normal cut off 0.60%). Applying FISH on interphase nuclei helps in analysing a higher number of cells. Because 100 to 500 interphase cells are usually assessed, FISH is more sensitive. Interphase FISH can detect even one abnormal cell out of 500 normal cells scored [1:500, sensitivity of 0.2% or one abnormal cell out of 200 normal cells scored (1:200, sensitivity of 0.5%) or one abnormal cell out of 100 normal cells (1:100, sensitivity of 1%)].[23] Therefore, compared to CCA, FISH is a more sensitive detection method for monitoring cytogenetic response of CML patients undergoing TKI treatment. Current guidelines[41] recommend that cytogenetic analysis be performed at 3 to 6 months intervals after treatment initiation for monitoring the cytogenetic response to treatment. However, residual disease below a threshold level will not be detected by karyotyping or FISH. Hence, NCCN[41] recommends more sensitive molecular methods to be used for monitoring TKI response.

Molecular Response monitoring

Molecular testing using qRT-PCR is indicated not only at the time of diagnosis of CML, but also for monitoring during and following TKI therapy. Quantitative changes in *BCR-ABL1* mRNA transcript levels are used clinically

to assess the molecular response to treatment and to detect minimal residual disease. Even though conventional haematologic remission and cytogenetic response have been achieved, molecular monitoring using qRT-PCR is more sensitive and reliable. This assay measures the levels (actual percentage) of *BCR-ABL1* mRNA transcripts in the peripheral blood or in the bone marrow and can detect one CML cell expressing *BCR-ABL1* mRNA in a background of 100,000 or greater normal cells. Thus the test sensitivity is estimated at 0.001% (1:10^5 which is equivalent to detecting one cell expressing *BCR-ABL1* mRNA in 100,000 - 1,000,000 normal cells).[30,46] Due to the strong correlation between the qRT-PCR results obtained from the peripheral blood and the bone marrow, it remains the most sensitive assay for molecular response monitoring.[46] The fact that peripheral blood samples can be used for this method, allows molecular monitoring without the necessity of obtaining bone marrow aspirations.[47] For molecular monitoring, majority of the laboratories use qRT-PCR, based on TaqMan or Light Cycler technology.[23,36] (details on the detection of base line *BCR-ABL1* transcript levels using qRT-PCR has already been described in the previous section on molecular analysis for detection of *BCR-ABL1* fusion transcripts).

qRT-PCR shows broad relevance to post-therapeutic monitoring, whether after transplantation or therapy with IM. Either the decrease or increase in the amount of *BCR-ABL1* mRNA is determined using qRT-PCR. With qRT-PCR analysis, four patterns may emerge: *BCR-ABL1* levels that (1) continue to decline, (2) are undetectable, (3) become stable/plateau or (4) rise. Rising levels of *BCR-ABL1* mRNA have been shown to precede disease recurrence and may signal a need for therapeutic intervention, while low, diminishing or stable fusion transcript levels identify patients in whom treatment has been effective. The results are expressed as a ratio of *BCR-ABL1* transcripts to the control transcripts, multiplied by 100 to give the result as a percentage, where 10%, 1%, 0.1%, 0.01% and 0.001% correspond to a reduction in tumour load of 1, 2, 3, 4 and 5 logs.[38] As qRT-PCR is the most sensitive test to identify and measure the *BCR-ABL1* mRNA transcripts, it has become the most used and relevant type of PCR test. About one abnormal cell in one million cells can be detected by qRT-PCR. It has a lower limit of detection of <0.01% and has the precision required in clinical diagnostic applications.

Standardisation of molecular testing using the international scale (IS)

A substantial effort has been made to standardise *BCR-ABL1* testing and reporting across academic and private laboratories.[48,49,50] In 2006, the National Institute of Health (NIH) consensus group[49] proposed the use of an international scale (IS) to standardise molecular monitoring with qRT-PCR across different laboratories. This group recommended the use of 1 of 3 control genes (*BCR, ABL, or GUSB*) and a qRT-PCR assay with a sensitivity of at least 4 log reduction from the standardised baseline. Ideally, control gene should have expression levels and degradation characteristics similar to *BCR-ABL1* and should be stable in expression, regardless of the disease state and treatment.[51] In the IS, the standardised *BCR-ABL1* mRNA transcript baseline is taken to represent 100%. Major molecular response (MMR), a 3 log reduction in the *BCR-ABL1* transcripts from this standardised baseline, is fixed at 0.1% (a 3 log reduction is a 1/1,000 or 1,000 fold reduction of the level of cells with the *BCR-ABL1* gene at the start of treatment). A 2-log reduction (*BCR-ABL1* transcripts 1 % IS) and 1 log reduction (*BCR-ABL1* transcripts 10 % IS) from the standardised baseline generally correlate with threshold responses indicative of complete cytogenetic response (CCyR) and major cytogenetic response (MCyR) respectively. Complete molecular response (CMR) is defined as undetectable *BCR-ABL1* transcripts as assessed by qRT-PCR with a sensitivity of 4.5 log reduction or more from the standardised baseline.[47] Hence, molecular response to TKI therapy is measured as the log reduction of *BCR-ABL1* mRNA from the standardised baseline and not a reduction from the actual baseline level in an individual patient.[50] If *BCR-ABL1* level rises, then it indicates disease progression or recurrence and it may also indicate that the patient has become resistant to TKI. In such cases, additional genetic testing needs to be performed to detect the development of *BCR-ABL1* kinase domain mutation associated with resistance to TKIs.

According to the ELN guidelines[24], molecular response to TKIs can be classified as optimal, failure and warning. An optimal response is defined by a 3-month post-TKI *BCR-ABL1* qRT-PCR value below 10% IS, a 6-month post TKI *BCR-ABL1* qRT-PCR value below 1 % IS and a 12-month post-TKI *BCR-ABL1* qRT-PCR value below 0.1% IS (MMR). A "failed" response is defined by a 6-month post –TKI *BCR-ABL1*

qRT-PCR value above 10% IS and a 12-month post-TKI *BCR-ABL1* qRT-PCR value above 1 % IS. Optimal response means that continuing treatment, survival is predicted to be normal or close to normal whereas failure means that treatment must be switched to an alternative TKI, or allogenic stem cell transplantation (allo-SCT) should be considered. Between optimal and failure lies the grey zone that is defined as "warning" which means that the response must be monitored more carefully and also that the patient may be eligible for potentially better treatments.[24] The failure to achieve optimal therapeutic landmarks as defined by *BCR-ABL1* mRNA transcript values (IS), implicates an increased risk of poor outcome and may indicate the need to switch to any alternate TKI or other treatment options.[24]

The NCCN guidelines[40] support molecular monitoring over bone marrow cytogenetics testing. Neverthless, IS standardised qRT-PCR may not be available in many laboratories. For those laboratories with no access to IS standardised qRT-PCR, O'Brien *et al*.[47] had suggested to establish their own laboratory specific standardised baseline based on a large number of pretreatment samples. However, NCCN[40] recommends bone marrow cytogenetic testing when access to IS standardised qRT-PCR is not available.

Since qRT-PCR affords sensitive, rapid and reproducible quantification of the *BCR-ABL1* fusion transcripts with a lower limit of detection of <0.01%, it has the precision required in clinical diagnostic applications. The NCCN[41] guidelines recommend molecular response monitoring with qRT-PCR (IS) with a sensitivity of 4.5 log reduction or more from the standardised baseline. The panel insists all institutions to use qRT-PCR (IS) for molecular response monitoring. But NCCN[41] do not recommend changes in the treatment based on molecular studies alone. In this era of targeted cancer therapy, *BCR-ABL1* mRNA qRT-PCR has become the paradigm of molecular monitoring for CML treatment response and also for detection of residual disease.

Molecular detection of minimal residual disease

Minimal residual disease (MRD) is defined as morphologically occult disease (in example, persistent leukaemia not appreciated by evaluation of bone marrow histology alone).[52] The tumour burden in a CML patient at the time of diagnosis is estimated to be 10^{12} leukaemic cells. At the time of complete clinical remission, some 10^{9} to 10^{10} residual leukaemic cells are estimated to be still present which might be a 2 to 3 log reduction from pretreatment levels.[53] Even after CCyR is achieved, the *BCR-ABL1* mRNA transcripts may remain detectable. Obviously, this demands more sensitive methods of detecting this morphologically "occult" tumour population which is important for prognosis and additional treatment decisions. qRT-PCR is the only sensitive tool that can detect these low levels of transcripts in patients who have achieved CCyR.[47] The high sensitivity of qRT-PCR serves it to be used for detecting MRD. It has been clearly shown that accurate monitoring for MRD could enable the detection of early relapse which in turn might allow early therapeutic interventions that are often more successful than those implemented at the time of overt relapse.[53,54] Usually molecular testing for MRD should be performed at specific time points after either TKI therapy or allogenic stem cell transplantation. Guidelines from NCCN[41] and ELN[24] recommend serial *BCR-ABL1* qRT-PCR assays at regular 3 to 6 months intervals for routine MRD monitoring of CML treatment. Periodic monitoring of both the molecular response to treatment and the level of MRD are critical in therapeutic decision making.

One drawback of molecular monitoring is that it cannot assess the ACAs which denote clonal evolution of disease. For this assessment, CCA is required and should be performed every six to twelve months throughout the monitoring process regardless of the therapeutic modality employed. The emergence of cytogenetically abnormal (but Ph negative) clones with the use of TKI, with an incidence of approximately 5%, further underscores the need for periodic CCA.

ACAs in the Ph positive clone post treatment

The appearance of ACAs during IM treatment is usually associated with reduced response to IM, increase in relapse of disease, clonal evolution and disease progression. Presence of ACAs is considered as a poor prognostic feature associated with reduction in overall survival. Most frequently occurring ACAs are +8, -Y chromosome, double Ph chromosome, +19, i (17q10), +21, -+17, -7, del(7q), 3q26.2 rearrangements and 11q23 rearrangements.[21,55] Earlier, during pre-imatinib era, all ACAs were classified as indicators of disease progression and with worse survival. But during the post-imatinib

era, ACAs are classified into two groups: Group 1 or major route ACAs include +8, loss of Y chromosome, +19 and double Ph chromosome.[55] Patients with these group 1 ACAs show relatively good prognosis, better treatment response and better survival. Whereas, patients with group 2 abnormalities (minor route ACAs) such as i(17q10), -7, del(7q), 3q26.2 rearrangements, 11q23 rearrangements show relatively poor prognosis and worse survival.

Recently, major route ACAs at initial diagnosis and any ACAs in Ph+ cells acquired during therapy are considered as criteria for determining accelerated phase.[55] But according to Gong *et al*.[56], the frequency based stratification of ACAs into major- and minor- route ACAs may not necessarily reflect the underlying biology or their impact on disease progression and not all ACAs acquired during therapy have an equal impact on prognosis. Gong *et al*.[56], investigated the risk of blast phase based on ACA profiles in a cohort of 2326 CML patients treated with TKIs. Based on the frequency of and latency to BP associated with each ACA, Gong *et al*.[56] stratified patients into four cytogenetic risk groups: (1) standard risk (SR) group comprising of patients without ACAs, (2) high risk (HR) group which consisted of patients with isolated 3q26.2 rearrangements, -7/ del(7q) or i(17q) and patients with HR complex ACAs, (3) intermediate -1 (int-1) risk group comprising of patients with +8, +Ph or other single ACAs, (4) intermediate-2 (int-2) risk group consisting of patients with other complex ACAs. This classification was found to correlate well with patient survival and to provide a valuable alternative to the current CP vs AP binary classification. A group of patients who are at a HR of rapid disease progression can be identified by this classification, for which CCA is the best option and most appropriate method. As only CCA helps to identify ACAs present at diagnosis and also during treatment with TKIs, it is irreplaceable in detecting clonal evolution in Ph positive cells or other clonal abnormalities in Ph negative haematopoiesis.

Molecular testing for detection of *BCR-ABL1* mutations

Despite the excellent treatment results obtained, a significant proportion (35% to 40%) of CML patients on TKI therapy do not achieve a satisfactory response to first line TKI therapy. This is mostly due to development of resistance to TKI treatment. Once failure or sub-optimal response to TKI has been detected, a complete resistance works up should start with a thorough history to exclude non-compliance with medications as a trivial cause of resistance. Only after that, evaluate for other causes of resistance. Resistance could be due to a heterogeneous array of factors involving *BCR-ABL1* dependent and *BCR-ABL1* independent mechanisms.[57] *BCR-ABL1* dependent mechanisms involve point mutations in the *ABL1* tyrosine kinase domain (TKD) of *BCR-ABL1* and rarely amplification of the *BCR-ABL1* gene also.[57,58] A plethora of other *BCR-ABL1* independent mechanisms such as activation of *SRC, LYN* oncogenes, drug interactions, reduced drug influx, increased drug efflux, poor intestinal absorption, poor compliance, clonal evolution and quiescent stem cells, have also been reported to contribute to TKI resistance.[59,,60,61] Recently, it was reported that a deletion type of polymorphism in an intron of a gene coding for the apoptosis inducing molecule BIM[62] as well as polymorphism rs724710 in BIM[63] were also involved in poor response to TKIs. However, development of point mutations in *BCR-ABL*1 TKD is the most commonly identified cause for a substantial proportion of TKI resistance and have been detected in 45% of CML patients who develop resistance to TKIs.[64,65,66] Compared to primary resistance, *BCR-ABL1* TKD mutations are more common in CML patients who develop secondary resistance and are found in around two thirds of CML patients who develop secondary resistance. Although mutations are rarely seen in newly diagnosed CML patients, these are commonly seen in patients commencing TKI therapy in late CP and in AP.[64,67]

Detection of *BCR-ABL1* TKD mutations

Among all factors, mutation in the tyrosine kinase domain (TKD) of *BCR-ABL1* is the better characterised mechanism of resistance. Mutations in the *BCR-ABL1* TKD can (1) affect residue that make direct contact with TKI, rendering the active site inaccessible through steric hindrance, (2) prevent the structural rearrangements required for IM binding (for example; P-loop mutations that destabilise the inactive conformation) or (3) stabilise the active conformation of *BCR-ABL1* with reduced affinity to TKI.[68] The main cause of resistance is considered to be the expansion of subclone with acquired mutations encoding altered BCR-ABL1 protein sequences, which prevent BCR-ABL1 inhibitors from binding or favouring conformations with reduced affinity to TKI. These mutations affect amino acids

involved in IM/TKI binding or in regulating regions of the BCR-ABL1 kinase domain, resulting in decreased sensitivity to IM/TKI, while retaining aberrant kinase activity.

In order to detect mutations in the TKD of *BCR-ABL1*, RNA from either bone marrow or peripheral blood samples is reverse transcribed to cDNA and amplified by nested PCR using previously published primers[77] to generate an amplicon covering the entire *BCR-ABL1* TKD, followed by direct sequencing of the entire amplified kinase domain.[64] This method is suitable for detecting known and unknown mutations[64]. Few other more sensitive assays based on allele specific PCR (approximately 0.1% sensitivity), denaturing high performance liquid chromatography (dHPLC), matrix assisted laser desorption ionization time of–flight mass spectrometry (MALDI-TOF-MS), targeted micro array, PCR-based pyrosequencing and mutation-specific quantitative PCR are also used for *BCR-ABL1* TKD mutation screening.[67,68,69] But some of these methods are more suitable primarily for finding mutations already detected by sequencing and not suitable for screening the entire kinase domain for unknown mutations. And while using these methods, a final confirmation with direct sequencing is warranted for the abnormal cases. So, among the various methods available for detection of mutations, nested PCR amplification and direct sequencing of the TKD remains the most appropriate and widely used and preferred technique in most laboratories providing tests for the identification of *BCR-ABL1* TKD mutations. This method has been recommended by an international consensus panel.[49,70]

However, low level mutations (below the threshold level of 20%) can easily be missed in direct sequencing technique for which the established sensitivity is 15 – 25 %. Nevertheless, since the clinical significance of low-level mutations (below 20%) in development of resistance remains unknown, the above limitation is not considered to be a disadvantage.[71] Recently introduced more sensitive detection method such as next generation sequencing (NGS) may allow detection of *BCR-ABL1* mutations that are not evident through conventional sequencing.[72]

Clinical relevance of *BCR-ABL1* mutation testing

BCR-ABL1 TKD mutation analysis may be indicated in clinical setting where treatment failure is suspected, there is sub-optimal TKI response, rising levels of *BCR-ABL1* mRNA in patients with a prior good response or on patients who present with accelerated or blast phase crisis.[73] A literature search on the *BCR-ABL1* mutation analysis indicated a frequency ranging from 12 % to 63 % worldwide, in CML patients who developed resistance to TKI, mainly to IM.[75] Among IM resistant Ph positive CML patients from Malaysia, Elias *et al*.[76] reported *BCR-ABL1* mutation frequency of 22 %. Mutations were reported to be more frequent in CML patients who developed secondary resistance to IM, compared to patients showing primary resistance.[76] *BCR-ABL1* mutation frequency was reported to be between 21 % - 48 % in patients who developed primary resistance whereas the frequency was between 10 % - 68 % in CML patients who developed secondary resistance.[75]

More than 100 different types of *BCR-ABL1* TKD point mutations have been identified and around 50 different mutation hot spots are also known.[75] Among the *BCR-ABL1* mutations, there are 9 mutations (M244V, G250E, Y253F/H, E255K/V, T315I, M351T, F359V) that account for more than 85% of all mutations. Greatest degree of resistance has been associated with the T315I mutation and few other point mutations such as G250E, G252H, Y253E, E225K/V. T315I is the only mutation resistant to all the TKIs except to ponatinib. Hence T315I, the gatekeeper mutation, represents a major mechanism of resistance to TKI. CML patients with a *BCR-ABL1* T315I mutation have been reported to have a poor prognosis.[77] *BCR-ABL1* mutation analysis of Asian and White patients with CML by Kim *et al*.[78] found greater proportion of highly resistant B-loop mutations and the T315I mutation among the Asians. In Malaysian CML patients showing resistance to IM, Elias *et al*.[76] detected T315I mutation in 9 out of 125 patients (7.2%) whereas in another recent Malaysian study[79], T315I mutation was detected in 5.26% of the 285 CML patients screened.

Different types of mutations cause different strengths of resistance, affect the specific therapeutic response and dictate the selection of the appropriate TKI for optimal response.[46,64,74] Detection of mutations during course of IM therapy will aid in risk stratification as well as in determining therapeutic strategies. Some high-level resistance mutations such as Y253F/H, E255K/V and T315I confer a true resistant phenotype and suggest withdrawal of IM, in favour of second and/or third generation TKIs or alternative therapeutic strategies. Whereas for some other low-level resistance mutations

(M244V, F311L, and F359V), the biochemical resistance may be overcome by drug dose escalation.[45,75,77]

According to the sensitivity of the individual mutation, second line therapy could be selected.[45] It has been reported that some mutations are resistant or less sensitive to second generation TKIs.[77] For example, mutations Y253H, E255K/V, F359V/C are less sensitive or resistant to nilotinib. On the other hand, mutations F317L, and V299L are less sensitive or resistant to dasatinib but sensitive to nilotinib whereas T315I mutation is insensitive to IM, nilotinib and dasatinib.[80,81,82] Likewise, F317L, E255K, F359V, G250E and T315I mutations which are resistant to IM, dasatinib or nilotinib have demonstrated sensitivity to ponatinib.[44,81,82] Some of the recently reported mutations such as F317I/V/C and T315A are less sensitive or insensitive to dasatinib and Y299L is resistant to bosotinib.[45,78,80-82] All these are collectively referred to as second generation inhibitors (SGIs) clinically relevant mutations. So, for *BCR-ABL1* mutations, not only the presence of mutations, but also the amino acid change should also be characterised in CML patients displaying resistance to IM, in order to optimise therapeutic response. Hence, the NCCN guidelines[41] recommend dasatinib for mutations Y253H, E255K/V, (P-loop), and F359V/C/I (substrate–binding region). For those with V299L, T315A, and F317L/V/I/C (ATP binding region) mutations, nilotinib is recommended. Thus, *BCR-ABL1* mutation analysis in the appropriate clinical setting is pivotal in choosing the right second generation TKIs, instituting changes or modifications in therapy and thus in preventing disease progression. According to updated NCCN guidelines[41], mutation testing is recommended when the initial response is inadequate [no partial cytogenetic response or > 10% *BCR-ABL1* (IS) at 3 months, no CCyR at 12 or 18 months], when there is any sign of loss of response (haematologic or cytogenetic response or 1-log increase in *BCR-ABL1* and loss of MMR) or when CML progresses to advanced stages of disease.

FUTURE IMPLICATIONS AND CONCLUSIONS

The advent of TKI therapy has redefined the treatment of chronic phase of CML and also altered the natural course of the disease irrevocably in majority of patients. Detection of t(9;22)(q34;q11) by CCA or *BCR-ABL1* mRNA fusion/transcript by FISH, RT-PCR and/or qRT-PCR are essential for a confirmatory diagnosis of CML. Monitoring the status of t(9;22)(q34;q11) or the levels of *BCR-ABL1* transcripts by the above methods provide effective ways to assess the response to TKI therapy, detection of residual disease and for the early detection of emerging transformation to a more aggressive phase of the disease or those who fail to TKI therapy. Treatment response monitoring of CML patients is a continuum that begins at diagnosis and carries on serially throughout the entire course of treatment. Nested PCR amplification of the translocated ABL1 kinase domain followed by direct sequencing of the entire amplified kinase domain is used for reliable detection of known and unknown mutations of *BCR-ABL1* TKD which cause resistance in a significant percentage of CML patients undergoing TKI therapy. Clear, concise and accurate reporting of results using all these techniques is extremely important for effective clinical management of CML patients. Guidelines have been developed and updated timely to address the scenario of CML patients who fail TKI therapy. Through careful cytogenetics, FISH and molecular studies, CML has evolved from an incurable disease into a disorder amenable simply to lifelong oral medication and finally into a condition which can be managed and perhaps cured. It is presumed that novel methods such as digital PCR (RT-dPCR) and NGS which have distinct applications in the context of molecular monitoring of CML, may transform our approach to molecular monitoring of CML further in the coming years. It is also quite likely that new therapeutic strategies may emerge and the approaches to CML management continue to change in this dynamic field. Subsequently, laboratory guidelines and recommendations together with nascent molecular technologies may also evolve. These in turn may help in shifting CML treatment towards achieving faster and deeper responses that are considered surrogate for long term outcomes.

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recruitment and management. Rosline Hassan: *BCR-ABL1* molecular analysis.

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