Original Article

BCR/ABL Fusion Gene by FISH Technique: What is the Appropriate Cut Off for Diagnosis and Monitoring Response to Treatment?

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Objective: Fluorescence in situ Hybridisation (FISH) is a widely used and useful cytogenetic technique for diagnosis and monitoring treatment responses in chronic myeloid leukaemia (CML). The positive or negative FISH result is interpreted based on individual standard normal cut off percentage, but universal cut off is unaddressed. We aimed to determine the performance of the FISH technique based on our routine normal cut off.

Materials and Methods: A retrospective descriptive and analytical study was conducted on CML patients followed-up at Srinagarind Hospital. Data has been collected from laboratory records over the past ten years. We excluded patients who were not completely tested FISH and chromosome or RQ-PCR.

Results: 675 FISH tests from 255 CML patients were analyzed. Specimens were mainly from bone marrow (98.2%) Chromosome analysis (G-banding) showed no metaphase in 31.9%. FISH test was positive in 99 samples with normal cut off at 9.7%. The false-negative rate of FISH was 2.6% (0.65% by using the standard cut off at 1%) and the false positive rate was 3%.

Conclusion: The FISH results should be interpreted carefully. Undue high level of normal cut off causes a high false-negative rate of FISH. The standard normal cut off for double signal FISH (D-FISH) was 1%. However, the cutoff FISH interpretation should be individually set and regularly validated. The FISH result should also be construed with G-banding, and RQ-PCR, which will improve the accuracy and will prevent misdiagnosed of CML.

Keywords: Fluorescence in situ Hybridisation (FISH); Chronic Myeloid Leukemia (CML); Cut off; False positive; False negative

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The Chronic Myeloid Leukemia (CML) is a unique myeloproliferative neoplasm (MPNs). The CML accounts for 15% of adult leukemias[1]. The CML has the pathognomonic cytogenetic abnormality. It is diagnosed by the positive Philadelphia chromosome [t (9; 22)] or the positive BCR/ABL fusion gene by Fluorescence in situ Hybridisation (FISH)[2-4] or Real-Time Quantitative Polymerase Chain Reaction (RQ/PCR) technique. The discover of targeted therapy called Tyrosine Kinase Inhibitors (TKIs) overcome the CML pathogenesis, resulting in an evolution of CML treatment undoubtedly beyond expectations[5]. TKIs block ATP-binding site of BCR/ABL fusion protein and later induce molecular remission[6]. TKIs become the gold standard to treat the CML and to change CML to a nonviolent chronic disease. The ten year-overall survival (OS) of CML in TKIs era was 83.3%[7]. To achieve this outcome, a disease burden should be regularly monitored based on a standard timeline[8]. Three cytogenetic techniques are widely used for diagnosis and treatment response monitoring, including banding karyotype, FISH and RQ-PCR. Combinations of these three tests dramatically improve sensitivity, specificity and also accuracy. The FISH technique is one of a useful technique in CML. The positive or negative FISH result is interpreted based on an individual standard cut off percentage. Samples that produce abnormal signal higher than a standard cut off will be labelled as positive. Contrarily, samples that provide abnormal signal lower than a standard cut off will be labelled as negative. However, to date, there is no universal cut off for FISH interpretation.

This study aims to determine the performance of FISH technique based on our routine cut off. Findings of this study will be evidence to support setting up of universal cut off in the future.

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Materials and Methods

Banding karyotype combined with RQ-PCR was used as reference standard and FISH for BCR/ABL was an index test of this study

Operational definitions

The cutoff refers to the maximum percentage of scorable cells with false-positive signals for BCR/ABL fusion.

FISH signal pattern designation. The Vysis BCR probe has a green signal (G), and the ABL probe has a red signal (R). The background chromatin is blue with both of these products. The BCR/ABL fusion is observed as touching red and green signals or as a yellow signal. The probe letter is used to designate signal patterns; for example, 1R1G2F indicates one red, one green and two fusion signals.

A signal pattern produced by the FISH in cells with a t (9; 22) (q34; q11.2): There are two patterns including typical pattern (most common signal) and atypical pattern (unusual signal).

The false-negative FISH test was defined by a negative FISH test with positive Philadelphia chromosome and RQ PCR.

The false-positive FISH test was demarcated by a positive FISH test with negative RQ PCR. Principally, the sensitivity of FISH is lower than RQ-PCR, therefore when the FISH test is positive, RQ-PCR should be positive as well.

Study design

This study is a retrospective descriptive and analytical study of all adult CML patients including newly diagnosed patients at Srinagarind Hospital, Thailand laboratory records. Data has been collected from laboratory medical records for ten years (January 1st 2010 to December 31st 2019). All files that contain entirely banding karyotype, RQ-PCR and especially FISH results were included and explored. Incomplete data defining by containing only RQ-PCR or FISH were excluded. Samples that came from the same patients but various treatment timing were counted and analyzed individually.

Sample size calculation

Sample size at the required absolute precision level for sensitivity and specificity was calculated by Buderer's formula[9].

Based on sensitivity: $n = Z^2_{1-\alpha/2} \times SN \times (1-SN)/L^2 \times Prevalence$

Based on specificity: $n = Z^2_{1-\alpha/2} \times SP \times (1-SP)/L^2 \times (1-Prevalence)$

n = required sample size,

SN = anticipated sensitivity,

SP = anticipated specificity,

α = size of the critical region ($1 - \alpha$ is the confidence level),

$Z1-\alpha/2$ = standard normal deviate corresponding to the specified size of the critical region (α)

L = absolute precision desired on either side (half-width of the confidence interval) of sensitivity or specificity.

According to previous study, anticipated sensitivity and specificity were 97.6% and 100%[10]. We expected α at 0.05 so $Z1-\alpha/2$ was 1.96. The L was 10% or accuracy was 90%. The prevalence of CML patients in our hematology unit was 3% with 10% drop out by estimation. The required sample size was 334 samples or tests from CML patients.

Methods

Technical specifications were described as below;

Chromosome preparation and karyotype banding from bone Marrow cells

Bone marrow (BM) samples (160 uL and 300 uL) were added into 5 mL of RPMI complete medium (RPMI1640 supplemented with 20% FBS and 1% penicillin-streptomycin solution) in cell culture flasks. The cells were incubated for 24 hours (h) in a CO_2 incubator (37°C, 5% CO_2). Then, 100 uL of 10 uM metrotrexate (MTX) solution was added into the BM cultures and incubated for 17 h in a CO_2 incubator. The cells then were transferred to centrifuge tubes and centrifuged at 3,000 rpm, room temperature for 5 minutes (min). The supernatants were removed. Following by, cell pellets were washed two times with 5 mL RPMI complete medium. After that, cell pellets were resuspended in 5 mL RPMI complete medium with 50 uL of 1 mM thymidine. Then, the cells were incubated in a CO_2 incubator for 5 h. After that, 1 mL of 1 ug/mL Cocemid was added and further incubated for 15 min in a CO_2 incubator. The cells then were centrifuged. The supernatants were removed. Cell pellets were resuspended in 10 mL of 75 millimolar (mM) KCl solution and incubated in 37°C for 15 min in a CO_2 incubator. The cell suspensions then next were centrifuged, and the supernatants were detached. Cell pellets were fixed with 5 mL of the fixative solution (3:1, methanol: acetic acid). The fixed-cells were centrifuged and the supernatants were removed. The fixations were repeated for two times. After that, the fixed-cells were resuspended in 1 mL fixative solution. Then, the fixed-cells were dropped onto the glass slides (4 drops/slide). The slides had been dried on the hotplate (60°C) overnight. The chromosomes slides were stained by Trypsin-Giemsa-banding to identify metaphase chromosomes. The representative chromosome sets had been imaged and captured. Lastly, the chromosome number was determined and representative description from twenty-five metaphase cells. The karyotype analysis was performed based on the criteria of the International System for Human Cytogenetic Nomenclature 2016[11].

Bone marrow interphase cell preparation and Fluorescence in situ hybridisation (FISH)

Bone marrow samples (160 uL) were added into 5 mL of RPMI complete medium (RPMI1640 supplemented with 20% FBS and 1% penicillin-streptomycin in a centrifuge tube. After that, the cells were centrifuged at 3,000 rpm, room temperature for 5 min. The supernatants were removed.

Then, the cell pellets were washed two times with Hank balance salt. Cell pellets then were resuspended in 10 mL of 75 mM KCl solution and incubated in 37°C for 15 min in a water bath. The cell suspensions were centrifuged, and the supernatants were removed respectively. The cell pellets were fixed with 5 mL of the fixative solution (1: 3, acetic acid: methanol). The fixed cells were centrifuged and the supernatants were then removed. The fixations were repeated for two times. After that, the fixed-cells were resuspended in 1 mL fixative solution. Then, the fixed-cells were dropped onto the glass slides (4 drops/slide). The slides had been dried on the hotplate (60°C) for 3 h. FISH was carried to using Vysis probe kit (Abbott: USA), followed by the manufacture' recommendation. Briefly, co-denature of the specific probe with the test sample at 73°C for 5 minutes, followed by overnight hybridisation at 37°C was performed. After hybridisation, slides were washed and dehydrated in ethanol series before being mounted with the counter-stain DAPII (Abbott: USA). The interphase FISH slides were visualised and captured by fluorescence microscope. A minimum of one hundred interphase cells was scored for each case. The cutoff level for a positive value of BCR/ABL probe was 9.7%. The interphase FISH cells analysis was performed based on the criteria of the International System for Human Cytogenetic Nomenclature (2016)[11].

Determination of the appropriate cut off for FISH interpretation

Before 2018, the cut off was set at 0% to prevent false negative of FISH test and misdiagnose of Ph-positive hematologic disorders, especially CML. The False-positive of FISH was allowed because we interpret the FISH test with banding karyotype, RQ-PCR and clinical correlation.

At the end of 2018, The standard cut off was required for achieving the laboratory certification from Royal College of Pathologists of Thailand. Ten known negative FISH samples were prepared and blindly interpreted by our three cytotechnologists independently. There were three factors in determining the cutoff, including p-value from inter-observer variation (A), the maximum number of FISH positive cells (B) and a total number of analysed cells (C). The cut off was calculated by using the BETAINV formula on Excel software[12] as follow;

The cut off value (%) = BETAINV (A, B+1, C) x 100

In our lab, A, B and C was 0.95, 5 and 100. Then the final cut off was 9.75%.

Five bone marrow smears and five cytogenetic preparations obtained from normal healthy donors or patients with non-Hodgkin's lymphomas could possibly be the other method were analysed. Five hundred interphase nuclei were scored on each slide. The cutoff level was determined as mean + 2 standard deviations = 5.0%. For metaphase-FISH, five hypermetaphase preparations of normal healthy donors or lymphoma patients were scored. As no BCR-ABL-positive metaphases were observed, the cutoff level was set at 0%[13].

Statistical analysis

Laboratory result data was analyzed by statistical software STATA version 10.0 which includes categorical and continuous data. They were summarised separately and differently. For categorical data, the information was presented in a form of percentage, while two sets of statistical tools, the median performed with range and the mean shown with standard deviation (SD) were applied to interpreted continuous data. The magnitude of difference in prevalence had been presented as percentage, a p-value less than 0.05 is considered statistically significant.

Ethical consideration

This study protocol, including with the study information and case record form has been approved and accepted by the Ethics committee for Research in Human Subjected at Srinagarind Hospital (HE631435).

Results

There were 675 FISH tests from 255 CML patients. G-banding chromosomal analysis and FISH were performed periodically according to European LeukemiaNet recommendations to determine the depth of response after treatment. Specimens were mainly collected from bone marrow (663 samples, 98.2%), and the rest were obtained from peripheral blood (12 samples, 1.8%).

FISH was performed in 100 newly diagnosed patients, 575 tests during tyrosine kinase inhibitors (TKIs) treatment. 576 tests were performed in complete cytogenetic response (CCYR) and 575 tests were performed in major molecular response (MMR).

The Chromosome analysis (G-banding) was done in 226 samples (33.5%) from 675 FISH tests. The Banding karyotype showed normal karyotype (131 specimens, 58%), no metaphase (72 specimens, 31.9%), Philadelphia chromosome (18 specimens, 7.9%) and abnormal karyotype without [t(9;22)] (5 specimens, 2.2 %) including 46X, +15, [t(1;9)] with +del(1), del(20), +11 and -9.

Performance of FISH for detection of BCR/ABL fusion gene was shown in Table 1. Sensitivity, specificity, positive predictive value, negative predictive value and accuracy were 96%, 99.5%, 97%, 99.3% and 99% respectively.

Ninety-nine samples from 675 FISH tests were positive for BCR/ABL fusion gene. Four specimens (2.6% or 4 of 154 satisfied banding karyotype specimens) revealed positive Philadelphia chromosome but negative FISH tests (defined as a false-negative FISH study). All false-negative FISH tests were rechecked, and the results were demonstrated in Table 2.

Although these samples came from the same patients, in different preparation has been performed. Banding karyotype samples were interpreted in metaphase cells after incubation; however, the FISH samples were interpreted in interphase cells from fresh specimens. Philadelphia chromosome was reconfirmed in all four false-negative FISH samples. Additionally, all of 4 samples were

Table 1. Diagnostic performance of FISH

FISH for BCR/ABL	Disease	
	Positive	Negative
Positive	96	3
Negative	4	572

Disease positive was defined by positive Philadelphia chromosome by banding karyotype (18 samples) or positive RQ-PCR for BCR/ABL gene (96 samples).
FISH positive defined by a number of BCR/ABL fusion gene-positive interphase cells per all interphase was higher than cut-off.

Table 2. Samples with false-negative FISH

No.	Number of Philadelphia chromosome, t (9; 22)	FISH results
1	1/50	Positive 2/100
2	6/50	Negative
3	18/47	Positive 4/100
4	2/20	Positive 6/100

Philadelphia chromosome was reported as a number of Philadelphia chromosome-positive metaphase cells per all metaphase cells.
The FISH result was reported as a number of BCR/ABL fusion gene-positive interphase cells per all interphase cells. Interphase cells were counted at least 100 cells.

positive RQ-PCR. Interestingly when we repeated FISH tests on G-banding specimens (cultured specimens), FISH was positive for all because our cut off for positive FISH was set at 9.7% according to the reference method described above. While BCR/ABL fusion genes were detected on 3 of false-negative FISH specimens, but FISH were reported as negative. A case of positive Philadelphia chromosome with the absence of a signal on FISH was explored, and clonal evolution was found in this sample.

Furthermore, when we compared FISH results with RT-PCR, the false-positive FISH tests were found in 3 samples (3%) from 99 FISH positive samples. The FISH signals were detected in 12, 13 and 14 interphase cells of 100 interphase cells, respectively.

Discussion

Our routine laboratory service agents conducted this study. It has reminded ourselves and other labs to recheck laboratory protocols and the way of result interpretation regularly. Any mistakes will be detected and adjusted to achieve the best accuracy tests. Numbers of specimens with no metaphase cell were high in our centre (31.9%) comparing with other laboratory centres (less than 18.2%)[14]. Our cell culture protocol should be explored, validated and modified to decrease the rate of no metaphase cell in banding karyotype. The FISH test is an essential technique to diagnose and to monitor disease response in CML. The prevalence of false-negative FISH should be zero or as low as possible in order to prevent misdiagnosis in CML patients. Metaphase FISH and Interphase FISH were widely used. Interphase FISH was advantageous to the rapid screening of many nuclei without the need for cell culture and metaphase chromosome preparation. It was also beneficial to the study of samples with a low mitotic index, especially in solid tumours. The critical limitations of interphase FISH were the ability to detect unknown structural chromosomal changes. Interchangeably, metaphase FISH analysis allowed a visual analysis of specific chromosome regions. Specific chromosome regions contain structural changes. Therefore, a metaphase FISH was useful in a variety of applications. Structural changes, including translocation, were often identified by metaphase FISH[15].

There were some limitations in Interphase FISH. However, high rates of no metaphase cells (31.9%) by continue using interphase FISH could reduce the inadequacy of FISH specimens. Moreover, with different preparations, Interphase FISH could be used as a marker in double-checking the performance of G-banding and RT-PCR.

In spite of the fact that the FISH result was interpreted based on cytogeneticist, the cut off for FISH was set individually. Based on the method mentioned above, in the centre that the probability of false-positive interpretation from cytogeneticist was high, the cut off will be set at a high percentage. On top of that, the cut off was varies depending on the type of signal production by FISH technique. There were three main types of FISH including single fusion FISH (S-FISH) produced single BCR/ABL fusion signal on Philadelphia chromosome, double fusion FISH (D-FISH) produced two fusion signals (one on the Ph-chromosome and one on the abnormal chromosome 9) and an extra signal FISH (ES-FISH) produced one fusion signal on Ph-chromosome and one additional signal on the abnormal chromosome 9[16]. D-FISH was chosen and routinely used in our lab because of D-FISH was highly sensitive to detect BCR/ABL fusion and consistent[17]. D-FISH could be mastered by most laboratories and could detect an atypical pattern of BCR/ABL[18,19]. The normal cut off for D-FISH was 1% when 500 nuclei were examined and 0.079% when 6,000 nuclei were studied[3,20].

The D-FISH probe was associated with low false-positive rates and could detect all variant translocations of Philadelphia chromosome[3]. Although the false-negative FISH test was 2.6% (4 samples) but a high percentage of no metaphase cell (31.9%) in our study caused lowering the prevalence of false-negative FISH test than actuality. Therefore, false-negative FISH cannot be ignored.

Interphase FISH from peripheral blood could be associated with a false-negative rate of 1 to 5% depending on

the specific probe used in the assay[21]. However, all of our false-negative FISH tests were performed from bone marrow specimens. False-negative FISH test in our lab could be explained by the inappropriate normal cut off. Three of 4 false-negative samples were turned to positive if our cut off was lower than 2%. To improve our D-FISH performance, the normal cut off should be regularly revalidated. Increase cell scores can reduce false-positive cell and the percentage of normal cut off. A virtuous way was to score only those cells that meet the scoring criteria for normal or neoplastic signal patterns. Strict scoring criteria should be used[2,18]. One of 4 false-negative FISH was a CML patient with clonal evolution and loss of molecular response. We acknowledge that clonal evolution produced complex BCR/ABL signal patterns and associated with poorer prognosis[22], but the details of the pattern were unrecognised. The signal pattern of this specimen should be reinvestigated cautiously. Finally, base on standard normal cut off at 1%, our false-negative FISH was only 0.65% (1 sample). Our false positive of FISH was higher than the standard range of 0.07 to 0.31% in D-FISH[23]. Accuracy of our RT-PCR should be reconnoitered to explain this situation.

Although FISH for BCR/ABL is useful and has therapeutic implication for CML patients but performing FISH test is quite complicated and requires experienced technician. On the other hand, the gold standard test (RQ-PCR for BCR/ABL) is widely available and has standard international unit (IS). In the near future, FISH may be replaced by RQ-PCR eventually.

Conclusion

Using FISH in CML also has limitations as other tests. It can also produce false-negative and false-positive results. Undue high level of normal cut off causes a high false-negative rate of the FISH. The standard normal cut off for D-FISH in CML was 1%. However, the cut off for interpretation of FISH result should be concerned, regularly validated and locally established in each lab. Interpretation of FISH result with G-banding and RQ-PCR will improve the accuracy and prevent misdiagnosed.

What is already known on this topic?

FISH is a useful test for diagnosis and monitoring treatment response of CML patients. Interpretation of FISH results as positive or negative is based on individual cut off percentage.

What this study adds?

FISH is a practical tool with good performance for CML patients. However, the cut off for interpretation FISH should be regulary validated to improve its accuracy.

Authorship

Contribution and Study design: Jureeporn Kampan (JK) and Chinadol Wanitpongpun (CW), Data collection: JK, Data validation: JK and CW, Data analysis and interpretation: CW, Manuscript preparation: CW.

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Potential conflicts of interest

The authors declare no conflict of interest.

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