Review Article

Comparison of Cytogenetic and Molecular Techniques Used for Philadelphia Chromosome Analysis: A Review Article

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ABSTRACT

Philadelphia chromosome is the characteristic hallmark of chronic myeloid leukemia and refers to the translocation between chromosome number 9 (ABL) and 22 (BCR) resulting in a shortened chromosome. The resulting ABL-BCR fusion gene encodes for tyrosine kinase activity leading to uncontrolled growth of cell. The coining of Philadelphia chromosome by Hungerford and Nowell went a long way in associating the genetic abnormality with cancer formation in humans. This discovery played a pivotal role in cancer research and proved to be a decisive episode in the arena of cancer cytogenetic. Their research was reported in Science journal in 1960 and was a first major identification of a chromosomal deviation in the neoplasm. A series of careful and repeated metaphase chromosomal observations led to the conclusion that one of the acrocentric chromosomes was seen to be significantly reduced in size and half of the long arm of this chromosome was missing. Different cytogenetic, molecular and hematological techniques are used to diagnose CML. Most lately, PCR based techniques have gained huge acceptance because of being sensitive and time efficient.

Keywords: Chronic myeloid leukemia, neoplasm, Philadelphia chromosome, Cytogenetic.

Introduction

The out-of-control growth of cells results in the formation of cancer. Leukemias or blood cancers can generally be divided into two main categories known as 1-Lymphoid and 2- Myeloid. Lymphoid is further classified as chronic lymphocytic leukemia and acute lymphocytic leukemia. Whereas, Myeloid can be classified further into Chronic myeloid leukemia (CML) and Acute myeloid leukemia (AML). To- date, different methods have been used to detect and determine the BCR-ABL +ve cells. These include but are not limited to traditional cytogenetics, reverse transcriptase PCR, real time PCR, nested PCR etc.[1] Different techniques have their applications and limitations, but PCR based methods for diagnostics have gained more acceptance because of being more sensitive and easy to use.

Molecular testing involving Real time RQ-PCR have become one of the basic pillars in CML identification. Other techniques also have their implications but being time consuming and requiring bone marrow limit their applications. Chronic myeloid leukemia generates from the bone marrow and is relatively slow in its progression. The ratio of occurrence of CML in males- to- females is 1.4:1 and the average age of its onset is 45 years. CML is the most widely occurring hematological abnormality in the Asian population and represents about 15-20% of the total adult lukemias. CML targets the myeloid cells- which are blood cell forming cells- including RBC's, platelets and various forms of the WBC's. The oncogene- BCR-ABL is known to be the earliest abnormality of the chromosomes which is linked to a type of human tumor, called as chronic myeloid leukemia and often coined as chronic myelogenous leukemia (CML).[2] It is the result of a reciprocal translocation t(9;22) which forms a shortened chromosome known as Philadelphia chromosome (Ph). It took nearly thirteen years to establish the reciprocal translocation involved in Philadelphia chromosome when Nowell and Hungerford discovered Ph chromosome and its link to CML. It took a further ten year period to identify the involvement of *ABL*-gene (proto-oncogene) found at chromosome 9, and a *BCR* gene (known for breakpoint cluster region) present on chromosome number 22 and this phenomena was reported by Rowley in 1973.[3] This particular translocation results in a shorter chromosome 9 and a relatively longer chromosome 22- known as Philadelphia chromosome (Figure 1) after the city it was discovered in. Numerous studies conducted at molecular level underline the ABL-BCR role in the disease pathogenesis. Chronic myeloid leukemia is perhaps one of the most widely studied and researched malignancy (tumor) and the earliest

of the abnormalities linked with cytogenetics and molecular anomaly. During 1960s, it was the work of Nowell and Hungerford which helped unearth Philadelphia chromosome and it's strong link to CML and this paved the way for understanding cancer biology. About 90-95% of CML cases prove to have a Ph chromosome (positive) and also have a BCR-ABL fused gene- a character of CML. The remaining 5-10% cases- although don't have Ph chromosome but they do tend to have composite translocations and nearly 50% of them show to have a BCR-ABL fusion gene. [4, 22]

It was during 1983 when Heisterkamp, Stephenson and Groffen cloned the genes at the point of translocations (breakpoints- known as BCR-ABL). Bone marrow for diagnostics makes cytogenetic methods more tedious and time consuming, while PCR based methods are more sensitive and more detective. [5]

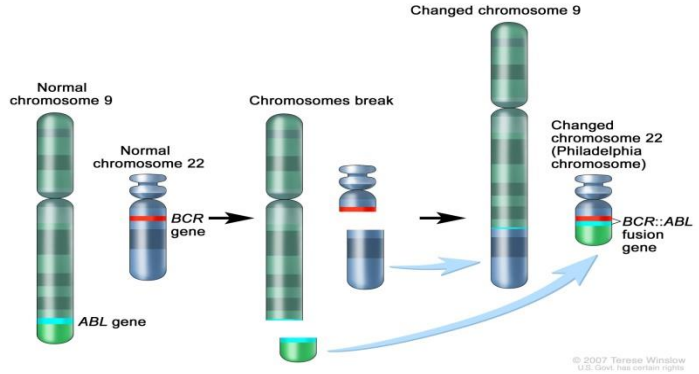

Figure 1. The translocation between chromosome 9 and 22 to form a BCR-ABL fusion gene.

Diagnosis of Chronic Myeloid Leukemia

Broadly speaking, CML is identified in patients either having symptoms or other laboratory readings. Some of the key clinical aspects may include but are not limited to:

Clinical Aspects

1- Splenomegaly (enlarged spleen): This results in spleen elongation in size often leads to abdominal distention and discomfort. Irritable bowel syndrome is observed and weight gain is also reported.

2- *Fatigue due to anemia*: Since there is a decrease in RBC count so more often than not there is occurrence of anemia in CML patients.

3- *Bruising and Bleeding*: This is the result of low platelet count when the normal bone marrow cells get replaced by the normal cells and often results in bruising and bleedings. In patients having the above mentioned symptoms and laboratory abnormalities, there is a blood CP test recommended which evaluates the White blood cell, Red blood cell and platelet count.

Clinical findings linked to chronic myeloid leukemia have generally been well documented. Fatigue is most common in such patients irrespective of the fact that in which phase the patient is reported. [6]

Diagnostics Techniques- A comparative analysis:

Complete Blood Count:

The primary evaluation is made by a complete blood count. Patients reported to have CML show following features

1- Reduced hemoglobin levels

2- High to extremely high WBC count

3- Depending on the level of severity and stage of patient, the platelets can either be reduced or much higher than normal levels.

4- The blood cells of a patient can be stained for further examination under a microscope. These give a particular pattern of WBC's and small and large proportions of immature and mature white blood cells respectively.

Bone Marrow Aspiration- Biopsy

It's a very common and relatively simple test performed to study the marrow cells and see for general abnormalities. Once the patient's skin is numbed by giving a drug, the sample is drawn from the hip bone to study the marrow cells. A liquid sample is extracted by inserting a needle through the bone to the marrow. To do a biopsy analysis, Physicians generally use a particular kind of a needle to remove a bone piece carrying the marrow, this is followed by observing samples for chromosomal and cell deviations. This is generally more tedious as compared to some of the PCR based techniques. [7,8]

Cytogenetic Analysis:

As the name suggests, this method of diagnosis looks to see the numerical and structural changes of

the chromosomes. This allows seeing if there is presence of Philadelphia chromosome. A final diagnosis of CML is made once the presence of Ph chromosome, raised blood count and other bone and marrow tests reveal chromosomal abnormalities.[9]

Nearly 90% of patients having CML tend to have a Philadelphia chromosome. The remaining ten percent of patients although don't show Philadelphia chromosome but do have a ABL-BCR fusion gene which is confirmed by other tests.

Fluorescence In Situ Hybridization –(FISH):

A highly sensitive method to confirm the CML is FISH. It is comparatively more responsive as compared to the other cytogenetic tests available. This test is quantitative in nature which works by identifying the presence and quantity of the BCR-ABL fusion gene. How it Works: FISH works on the principle of labeling the BCR and ABL gene by certain chemicals which differ on the type of light they emit. The chromosome containing the gene gives a specific color (Figure 2) as in the case of chromosome 9 of ABL and chromosome number 22 for BCR. The probes show a fused (overlapping) color confirming the presence of fused ABL-BCR gene. [10] This test also detects BCr-ABL gene in cells- therefore it's a good way to see if there is decrease in the CML after the treatment is given.

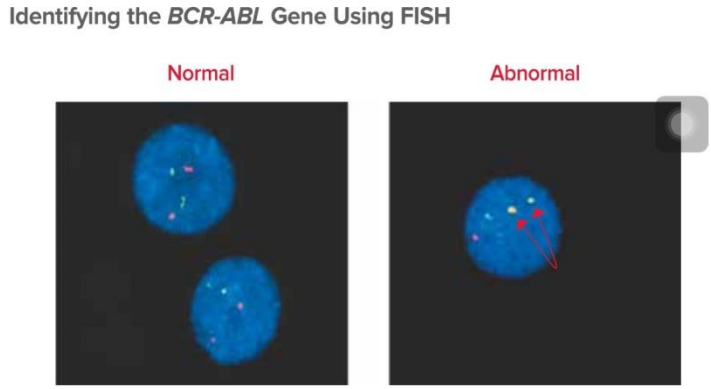

Figure 2. *FISH uses fluorescent dyes to locate BCR-ABL genes in CML. In first picture red and green spots indicate normal ABL and BCR gene while in second picture, the fused BCR-ABL gene genes are shown in yellow color.*

In a normal cell, the ABL gene is marked in red color while the BCR gene is displayed as green, whereas in an abnormal cell- having fused BCR-ABL gene between chromosome 9 and 22 the gene gives a yellowish color which is seen by the use of probes.

FISH analysis has been widely used with BAC probes to study the BCR-ABL fusion gene. In one of the studies, the fusion of the two genes and genomic realignment was observed in 9 patients studied which showed negative result for Ph but had a positive BCR-ABL translocation noticed. Even at the interphone stage, the FISH method can detect the chromosomal realignments. [10]

Polymerase Chain reaction

In terms of molecular testing, a quantitative Polymerase chain reaction is the most widely used method to detect BCR-ABL gene. Both blood and bone marrow cells can be used in such a method. Even very small pieces of RNA and/or DNA can be amplified using PCR which helps in their detection and measurement. This method helps in detection of BCR-ABL fusion gene even if it is present in fewer number of cells. One abnormal cell out of one million cells can easily be detected by using a PCR based molecular testing. [11]

In an Australian based trial, the researchers applied the genome DNA extraction and PCR method to measure the BCR-ABL gene at a lower level than the conventional mRNA based qRT-PCR based method. The method was able to detect the BCR-ABL gene at extremely lower levels before and after the imatinib mesylate therapy. [11]

Reverse Transcriptase Polymerase Chain Reaction

Often termed as RT-PCR, this technique amplifies the area where the BCR-ABL gene splicing junction is formed. This is extremely helpful in detecting the residual disease. The nature of PCR testing is either Qualitative- in which the presence is confirmed of the BCR-ABL while in Quantitative PCR- the amount of the fusion gene is measured. As with such reactions, false negative and false positive reaction s can take place. Normally false negative reaction occurs when mRNA is of poor quality while false positive results may occur when there is a contamination. Fluorogenic probes (Taqman) are used to quantify the probe. It is estimated that the Reverse transcriptase PCR method is nearly 4 t0 5 times more sensitive than the cytogenetic method.[12]

Benefits of Quantitative PCR:

Since Quantitative PCR detects even small amount of the disease and can be done with blood samples without the need of a bone marrow and a

biopsy process therefore Quantitative PCR holds many benefits over other techniques.

Differential Diagnosis

CML has to be distinguished from the leukemoid reactions, that produce WBC counts lesser then 50 X 10^9 /L. It is difficult to diagnose CML when patients report to have splenomegaly or leukocytosis but do not have the Philadelphia chromosome. The patients which are negative for Ph and also negative for ABL-BCR fusion gene are diagnosed as havng Ph negative CML.

Comparison of Cytogenetic, Hyper metaphase FISH, RT-PCR in CML- (W.r.t Trials)

A study was conducted by Cschoh et al. in which comparison was made between various techniques to see the efficiency of different techniques. Hyper-metaFISH gives high sensitivity at metaphase analysis as compared to cytogenetic analysis. Since chromosomal analysis only tells about the dividing cells whereas the RT-PCR and FISH analysis give information about the resting cells as well. In one of atrial conducted in {Pakistani population, Suhaib et al. found that there was 100% positivity of results with Teverse transcriptase PCR in ten patients. Anand et al. doing similar study in India also observed more or less the same results. All the patients were 100 percent BCR-ABL positive but about 96% showed CML positivity. Concluding these trials, we see the comparative advantages of PCR and FISH based methods based on their high sensitivity.[12,25]

Role of Diagnostic tests in treatment progress

The above-mentioned tests including CBC, Bone marrow aspiration, cytogenetic analysis, FISH and PCR are widely used in tracking the progress of CML patient after treatment has begun.

Management of CML

Treatment goals for CML include hematological remission (bringing CBC to normal levels), cytogenetic remission (ensuring 0% Ph-positives) and Molecular remission (ensuring negative PCR value for fusion gene (BCR-ABL).

Stages of Chronic Myeloid Leukemia

1- Chronic Phase: Patients in chronic phase generally tend to have less than 10% of cells having immature WBC's. Patients at this stage may/may not show any significant symptoms. They do tend to have raised WBC's and after the treatment is given, the symptoms fade away.

2- Accelerated Phase: The blast cells in this phase are more than the normal values.

   The platelets are decreased and chromosomal mutation can take place at this stage. Fatigue and pneumonia are common symptoms at this phase.

3- Blast Phase: Also known as crisis phase, patients at this stage have reduced RBC's and platelet counts. Fatigue, bone pain, and shortness of breath are common symptoms of this phase. [13]

Treatment Options and paradigm

Tyrosine kinase inhibitors were the first BCR-ABL inhibitor which was used on a large scale and gained approval from FDA. These essentially works by partly blocking the ATP attachment site of the BCR-ABL gene, and therefore inhibits a structural change of the oncogene to the active gene. This treatment was well tolerated and had an excellent response rate in younger population while the response and treatment rate were slightly lower in the adult population

Tyrosine Kinase Inhibitors are the class of drugs that work by targeting the fused ABL-BCR genes which result in the growth of CML cells. Tyrosine kinase is the protein made by BCR-ABL gene and lies close to the cell surface. These drugs (TKI's) stop the BCR-ABL gene from giving signals to the cells involved in growth of the cells. [14]

Novartis- a pharmaceutical giant developed one of the most fascinating breakthroughs in medical science for treatment of CML. Imatinib mesylate is a Tyrosine kinase inhibitor. It provides a significant control of total blood count and about 60-70 patients of patients in chronic phase show a molecular response in the shape of lowering BCR-ABL levels. [15]

Nilotinib (Tasigna) and Dasatinib (sprycel) are two other tyrosine kinase inhibitors that work more or less on the same principle as that of Imatinib. Patients who do not tolerate imatinib can beneft from the other TKI's. [23,24]

Discussion

Most of the cases of chronic myeloid leukemia are caused by translocation between chromosomes in the cells. Philadelphia chromosome (a shortened chromosome number 22) is a result of the shared translocation which occurs between Abelson- (ABL1) gene present at the chromosome 9 and Breakpoint cluster region gene (BCR) present at the chromosome. Philadelphia chromosome is by and large present in about 90% cases of the chronic myeloid leukemia. Various techniques have been used in diagnosis of Philadelphia translocation. These diagnosis techniques can be broadly divided into hematological, cytogenetic and molecular techniques. Karyotyping, FISH analysis, PCR, RT-PCR, RNA splice junction and Inter phase FISH techniques are widely used to detect the presence of Ph chromosome. [16]

Different techniques have their advantages and limitations. The results and analysis done on various molecular, cytogenetic and hematological techniques depicts that PCR based methods are highly sensitive, useful and can detect the BCR-ABL genes even when in extremely low quantity. Addition of PCR based methods to the currently used cytogenetic techniques has increased the reliability and credibility of the diagnostic procedures. We can only start the treatment- especially imatinib treatment once we have a positive BCR-ABL gene. In current diagnostic and treatment paradigm, we need to add PCR based methods to complement it with cytogenetic methods to contribute to the overall benefits of CML patients. Since BCR-ABL is one of a tyrosine kinase protein, therefore most of the molecular targeted based therapies work by inhibiting the tyrosine kinase activity. [17]

Cytogenetic, at times can be tedious and time consuming and this is the reason why most studies have focused on RT-PCR and FISH based analysis. [18]

Another key trial conducted by Kolialexi et al. also demonstrated the key effectiveness of RT-PCR and the results were in accordance with Ph positive cases. These patients were taking imatinib mesylate when the diagnosis was done. Conventional cytogenetics still remains in practice as not many laboratories have advanced PCR machines but as bone marrow and cytogenetic techniques remain tedious- more and more scope for PCR would be seen in coming days. PCR and FISH techniques do not require bone marrow sampling which make it a preferred choice. It can easily be conducted on peripheral blood and for that matter does not require excessive sampling. [19, 20, 21]

Conclusion

With the advent of FISH and PCR based molecular diagnostic methods, we can diagnose CML with more accuracy and ease. PCR based methods don't require bone marrow samples and are more detective as compared to cytogenetic and bone marrow biopsy. More studies need to be done and literature needs to be reviewed to cement the fact that PCR and FISH based techniques hold more applications in CML diagnosis. The point of translocation between chromosome 9 and 22 originates in the stem cell. Identifying the proto-oncogene causing this translocation is imperative in targeting it with proper treatment. Pathogenesis of CML is well understood now with the availability of Imatinib mesylate and similar therapies hold huge promise in such aspects.

Future Recommendation: Diagnostics and its proper implementation have great significance in properly identifying the BCR-ABL fusion gene. Cytogenetic techniques hold huge application in CML diagnosis, but since they can be time consuming so PCR and FISH based techniques hold more promise in diagnostics. RT-PCR should be added along with other techniques to solidify the diagnostic paradigm.

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