Extra Signal Fluorescence in Situ Hybridization for Detection of Typical and Atypical BCR/ABL Gene Rearrangements in Egyptian Chronic Myeloid Leukemia Patients

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Abstract: Background: For many years, conventional karyotyping has been used as the golden diagnostic tool for t (9; 22) (BCR/ABL) in chronic myeloid leukemia (CML). Recently, there have been an emerging generation of complex translocations and submicroscopic deletions involving BCR & ABL genes in addition to the classic t(9;22), which have a prognostic impact on the course of the disease, and require sensitive and specific molecular techniques for their detection. Objective: The present study aimed to explore the utility of extra signal fluorescence in situ hybridization (ES-FISH) compared to double fusion FISH(DF-FISH) and conventional karyotyping (CK); for detecting the incidence of typical and atypical patterns of *BCR/ABL* gene rearrangements and clarify their prognostic significance in CML. Subject and Methods: A series of 64 consecutive *BCR/ABL*$^+$ Egyptian CML patients (42 chronic phase, 9 accelerated phase, 13 blastic crisis),were investigated for typical and atypical BCR/ABL rearrangements using extra signal and double fusion FISH probes. Results: ES-FISH and DF-FISH showed higher sensitivity for detection of Philadelphia chromosome (Ph) as a sole anomaly when compared to karyotyping in all phases of CML. ES-FISH was the most sensitive method for detection of ABL deletion (14.2% in chronic phase, 33.3% in accelerated phase, 30.8% in blastic crisis) when compared to DF-FISH and karyotyping. Interestingly, ES-FISH, was the only method capable for detection of minor BCR/ABL rearrangement in 1 patient in blastic crisis phase. On the other hand, DF-FISH showed superiority for detection of BCR deletion. Both DF-FISH and karyotyping were capable of detection of trisomy 9 and variant translocation, while ES-FISH yielded confusing atypical signals regarding them. There was a moderate agreement between D-FISH & ES-FISH ($P<0.01$), a strong agreement between D-FISH and CK, while no agreement was found between the results of ES-FISH and CK and ($P>0.05$). In conclusion, karyotyping is mandatory to be applied at diagnosis of CML. ES-FISH is the method of choice for detection of ABL deletions, despite it cannot detect neither BCR deletions nor variant translocations. Karyotyping coupled with ES-FISH are adequate for the diagnosis and therapeutic monitoring of CML with the classical t(9;22) and for cases with ABL deletion.

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1. Introduction:

For many years, conventional karyotyping has been used as the sole diagnostic tool for t(9;22) (BCR/ABL). However, it has several limitations that may lead to failure for detecting BCR/ABL gene rearrangements in around 5% of all chronic myeloid leukemia (CML) patients *(Deininger et al., 2000).* In addition, about 5% of CML cases carry 'masked' translocations that can only be detected by molecular techniques such as fluorescence in situ hybridization (FISH) or reverse transcriptase polymerase chain reaction (RT-PCR) *(Druker and Lee, 2005).*

The first generation of BCR/ABL single fusion FISH probes detected the fusion gene with high specificity but with a low sensitivity, with a cut off value ≥6%. A new generation of FISH probes has been developed known as double fusion (DF-FISH). The developed probe is characterized by high sensitivity and specificity with a cut off value ≥ 1.3%. The probe is characterized by spanning wide area, about 450 kb proximal to the ABL gene on 9q34 and in some probes also with distant coverage on 22q (covering both major and minor breakpoint region) *(Primo et al., 2003).* Despite that, the double fusion FISH probe design does not distinguish between major and minor break point region. Therefore, unable to differentiate between de novo acute lymphoblastic leukemia (ALL) and ALL on top of CML, necessitating the application of polymerase chain reaction (PCR) to overcome this defect *(Hirose et al., 2002).*

More recently, an extra signal locus specific identifier (LSI) probe was developed to confirm the location of *BCR/ABL* other than 22q1 and to detect both typical and atypical BCR/ABL rearrangement, being capable of distinguishing between major (M) and minor (m) break point cluster region. This probe mixture contained directly labeled Spectrum Orange probe that spanned the *ABL* locus at 9q34 and directly labeled spectrum green probe that spanned the *BCR*

locus at 22q11.2.The most frequently detected patterns with the extra signal probe corresponded to typical BCR/ABL gene rearrangements involving the M*BCR* (one fusion, one green (BCR probe) and two red (ABL probe) signals) and the mBCR (two fusion, one green and one red signals).The extra signal LSI probe can also detect other atypical interphase FISH (iFISH) patterns as supernumerary Philadelphia (Ph), gain or loss of chromosomes 9 and 22, as well as deletions of 9q and 22q that can occur in BCR/ABL^{+} CML, ALL and acute myeloid leukemia (AML) cases *(Huntly et al., 2001,* Manisha et al., 2010).

This work aimed to explore the utility of extra signal fluorescence in situ hybridization (ES-FISH) compared to double fusion FISH and conventional karyotyping (CK); for detecting the incidence of typical and atypical patterns of *BCR/ABL* gene rearrangements and clarify their prognostic significance in CML.

2. Materials and Methods:

Subjects and Methods

The study was approved by the committee of Medical Research Ethics, Medical School, Ain Shams University; an informed written consent was obtained from all studied subjects. This study was carried out on 64 CML patients who were attending the Hematology Oncology Clinics of Ain Shams University Hospitals.

The patients were classified according to the clinical phase at presentation into three groups:

Group I:

It comprised 42 patients in chronic phase (CP); they were 22 males and 20 females with male to female ratio of 1.1:1. Their ages ranged from 20 - 75 years, with mean age of 49.5 ± 16.2 years.

Group II:

It comprised 9 patients in accelerated phase (AP), they were 5 males and 4 females with male to female ratio of 1.2:1. Their ages ranged from 38 - 61 years, with mean age of 47.6 ± 6.9 years.

Group III:

It comprised 13 patients in blastic crisis (BC) acute myeloid leukemia, they were 5 males and 8 females with male to female ratio of 0.6:1. Their ages ranged from 28 - 51 years, with mean age of 41.2 ± 8.1 years.

Follow up for the patients was done over a period of 12 months. The patients' outcome was expressed according to The Italian Cooperative Study Group on Chronic Myeloid Leukemia (1994). Good prognosis was identified by complete hematological remission, complete cytogenetic remission and molecular remission. While good response in blastic crisis phase was identified by disappearance of all signs and symptoms of leukemia, bone marrow blast cells <5% and absence of abnormal cells in peripheral blood and cerebrospinal fluid (American Cancer Society, 2009).

Methods:

I. Samples:

One mL of bone marrow (BM) aspirate and 4 mL of venous blood were collected from each patient before initiation of treatment under complete aseptic conditions. Samples were divided as follows:

Two ml PB were collected in a tube containing ethylene diamine tetra acetic acid (EDTA) solution used for complete blood count (CBC) and Leishman stain (done for all cases), and myeloperoxidase stain (applied in BC only). One mL BM and/or 2 mL PB were collected in sterile Lithium heparin coated vacutainer tube for cytogenetic analysis, D-FISH and ES-FISH. Fresh drop of PB was used to prepare PB smear for Neutrophil Alkaline Phosphatase (NAP) score.

II.Conventional Karyotyping (CK):

The steps of conventional karyotyping were performed as previously described ***(Eberhard, 2001)***. Most of available metaphases were counted (at least 20), analyzed and karyotyped using Chromoscan Applied Imaging Cytovision 2.7. The banded chromosomes were interpreted according to ISCN 2009.

III. Fluorescent in Situ Hybridization (FISH):

Ø The used probes were:

A) LSI (dual color, dual fusion) probe for t(9;22) (q34;q11) supplied in 20 µL (vial) (Vysis).

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B) LSI dual color BCR/ABL ES probe for t(9;22) (q34;q11) supplied in 20 µL (vial) (Vysis).

The steps and interpretation were performed according to Primo et al.,2003

Cut off value for diagnosis of positive result was 1.3% for D-FISH and 3% for ES-FISH.

IV. Statistical analysis

Data were expressed as mean ± standard deviation (SD) (range) or as number (%) of cases. Comparison of proportions and means between two

groups was made by using the $\chi 2$ test and independent t-test, respectively. The Fisher's exact test was used when applicable. Analysis was performed by using the Statistical Package for the Social Sciences (SPSS, version15). The level $P < 0.05$ was considered the cut-off value for significance.

Unpaired (student's) t test was used to test the difference about mean values of lab parameters, results were presented as mean and SD, non parametric data were analyzed using Mann-Whitney test (data presented as median and inter quartile range [IQR])

Kappa test: to measure the agreement between two observers. Strength of agreement (if present) is established according to k value as:

Mild agreement: k value is 0.2 – 0.4, Moderate agreement: k value is >0.4 – 0.6, Strong agreement: k value is >0.6 and <1, and Perfect agreement: k value is 1.

3. Results:

Cytogenetic analysis

Group I (Chronic phase)

Karyotyping was successful in 33/42 patients (78.5%), Out of them, Ph as a sole anomaly was detected in 26/33 (78.7%) patients, while it was associated with additional chromosomal aberrations in 7/33 patients (21.3%), in the form of Ph associated with +8 in 2 patients (6.1%), Ph associated with +9 in 3 patients (9.1%) and Ph associated with del 9q in 2 patients (6.1%). Re-analysis by *ES-FISH* showed Philadelphia chromosome as a sole anomaly in 36/42 (85.8%)(photos 1,2), while associated with ABL deletion in 6/42 patients (14.2%)(photo 3). Interphase FISH signals were atypical and confusing regarding detection of +9 and variant translocations. BCR deletion was not detected in any case. Using *D-FISH*, Ph was detected as a sole anomaly in 36/42 (85.8%), while it was associated with ABL deletion in 3/42 patients (7.1%)(photo 4), out of them both ABL and BCR deletions were detected in 2/42 patients (4.8%), +9 was detected in 3 patients (7.1%). No variant translocations were detected (Tables 2,3)

Group II (Acceletated phase)

Karyotyping was successfuly encountered in 7/9 cases (77.8%), of which, Ph as a sole anomaly was present in 4/7 patients (57.1%), and as complex aberration in the remaining 3/7 (42.9%); as Ph associated with 9q deletion in 1 patient (14.3%), Ph associated with +8 in 1 patient (14.3%) and Ph associated with +9 in 1 patient (14.3%). Re-evaluation by *ES-FISH* detected Ph chromosome as a sole anomaly in 6/9 (66.7%) patients. Ph associated with ABL deletion was detected in 3/9 patients (33.3%). No BCR deletion or variant translocations were detected. using *D-FISH* revealed the presence of Ph in 7 /9 (77.8%) patients. Ph associated with +9 was detected in 1/9 patient (11.1%) and associated with both ABL and BCR deletions in the remaining patient (11.1%). No variant translocations were detected (Tables 2,3)

Group III (Blastic crisis)

Out of the 13 patients, 9 (69.2%) showed successful mitosis, in which Ph chromosome was detected as the sole anomaly in 5/9 cases (55.6%), while complex aberrations were detected in the form of complex translocation t(3;9;22) in 1 case (11.1%), Ph associated with +9 in 1 case (11.1%), Ph associated with del 9q in 1 case (11.1%) and Ph associated with +8 in 1 case (11.1%). *ES-FISH* showed the presence of Ph chromosome as the sole anomaly in 8/13 cases (61.5%) (one of them showed minor BCR/ABL) (Photo….). Ph chromosome associated with ABL deletion was detected in 4/13 patients (30.8%) and in the remaining patient double Ph was detected (7.7%). No BCR deletion no variant translocations were detected.Re-evaluation using *D-FISH* detected Ph chromosome as the sole anomaly in 8/13 cases (61.5%). Ph was associated with additional anomalies in the form of Ph associated +9 was detected in 1 patient (7.7%), with ABL deletion in 2 patients (15.4%)(1 out of them showed combined ABL and BCR deletions [7.7%]), double Ph in 1 patient (7.7%) and variant translocation in 1 patient (7.7) (Tables 2,3).

The demographic, clinical features, prognosis and cytogenetic profile of all the studied groups are shown in table 1.

Table (1) Demographic, clinical features, cytogenetic profile and prognosis of all the studied groups

	Chronic (42)		Accel (9)		Blastic (13)		Total (64)	
	N	%	N	%	N	%	N	%
Sex								
Male	22	52.4	5	55.6	5	38.5	32	50.0
Female	20	47.6	4	44.4	8	61.5	32	50.0
Splenomegaly								
Mild, moderate	34	81.0	4	44.4	7	53.8	45	70.3
Huge	8	19.0	5	55.6	6	46.2	19	29.7
CCA								
Ph alone	26	61.9	4	44.4	5	38.5	35	54.7

Complex aberration	7	16.7	3	33.3	4	30.8	14	21.9
Failed	9	21.4	2	22.2	4	30.8	15	23.4
ES FISH								
Ph alone	36	85.7	6	66.7	8	61.5	50	78.1
Complex aberration	6	14.3	3	33.3	5	38.5	14	21.9
D-FISH								
Ph alone	39	92.9	7	77.8	8	61.5	54	84.4
Complex aberration	3	7.1	2	22.2	5	38.5	10	15.6
Prognosis								
Good	33	78.6	5	55.6	4	30.8	42	65.6
Poor	9	21.4	4	44.4	9	69.2	22	34.4

Table (2)Karyotypic profile compared to ES-FISH and D-FISH in all patients

	Ph sole	ABL del 9q	BCR deletion	ABL/BCR deletion	+8	+9	Double Ph	Others
CCA	35	4	0	0	4	5	0	1: complex [t(8;9;22)]
ES FISH	50**	13	0	0	0	0	1	0
D-FISH	51	2	0	4	0	5	1	1: variant translocation

** One of them was presented as minor (mBCR)

Table (3): Statistical comparison between CCA, ES-FISH and D-FISH in the three phases

	Chronic (42)		Accel (9)		Blastic (13)		Total (64)		χ^2	P	Sig
	N	%	N	%	N	%	N	%			
CCA											
Ph alone	26	61.9%	4	44.4%	5	38.5%	35	54.7%	1.66	0.20	NS
Complex	7	16.7%	3	33.3%	4	30.8%	14	21.9%			
Failed	9	21.4%	2	22.2%	4	30.8%	15	23.4%			
ES FISH											
Ph alone	36	85.8%	6	66.7%	8	61.5%	50	78.1%	3.93	0.05	S
Complex	6	14.2%	3	33.3%	5	38.5%	14	21.9%			
D-FISH											
Ph alone	36	85.8	7	77.8%	8	61.5%	54	84.4%	7.61	0.006	S
Complex	6	14.2	2	22.2%	5	38.5%	10	15.6%			

Patients in blastic phase showed significant increase in percentages of complex aberrations by ES-FISH (P= 0.05) and highly significant increase by D-FISH (P<0.01) in comparison to other phases.

Table (4): Agreement between CCA, ES-FISH and D-FISH using Kappa test

	CCA				K	P	Sig
	Ph alone (35)		Complex aberration (14)				
	N	%	N	%			
ES FISH							
Ph alone (38)	29	59.2%	9	18.4%	0.20	0.16	NS
Complex aberration (11)	6	12.2%	5	10.2%			
D-FISH							
Ph alone (40)	33	67.3%	4	8.2%	0.69	<0.01	HS
Complex aberration (9)	2	4.1%	10	20.4%			

ES FISH: There was no agreement between CCA and ES-FISH (P>0.05)

D-FISH showed strong agreement with CCA; there was an agreement in 33 sole anomaly and 10 complex (88%) and disagreement 6 cases complex (12%), 2 cases diagnosed as complex by D-FISH but not by CCA ;1 Ph with ABL&BCR, and 1 double Ph. And 4 by CCA (as Ph with +8) not by D-FISH. Exclusion of 15 failed cases by Karyotyping; 9 in chronic phase, 4 in accelerated and 2 in blastic phase (Total 49/64 cases).

Table (5): Agreement between ES-FISH and D-FISH in the whole studied group using kappa test

	ES FISH				k	P	Sig.
	Ph alone (50)		Complex aberration (14)				
	N	%	N	%			
D-FISH							
Ph alone (51)	45	70.3%	6	9.4%	0.48	<0.01	HS
Complex aberration (13)	5	7.8%	8	12.5%			

D-FISH showed moderate agreement with ES FISH; there was an agreement in 45 sole anomaly and 8 complex (83%) and disagreement in 11 cases (17%); 5 cases diagnosed as complex by D-FISH (Ph with +9) but missed by ES-FISH and 6 by ES-FISH (as Ph with ABL) but missed by D-FISH**.**

Table (6): Prognostic significance of Ph as sole anomaly and of complex aberrations (detected by CCA, ES-FISH and D-FISH)

	Prognosis (n)				P	Sig
	Good (42)		Poor (22)			
	N	%	N	%		
CCA						
Ph alone (35)	30	85.7%	5	14.3%	<0.0001	S
Complex aberration (14)	0	0%	14	100.0%		
Failed (15)	12	80.0%	3	20.0%		
ES FISH						
Ph alone (50)	39	78.0%	11	22.0%	<0.0001	S
Complex aberration (14)	3	21.4%	11	78.6%		
D-FISH						
Ph alone (54)	42	77.8%	12	22.2%	<0.0001	S
Complex aberration (10)	0	0%	10	100.0%		

Using Fisher's Exact Test cases showing complex aberrations by CCA, ES-FISH and D-FISH showed highly significant poor prognosis compared to those with Ph alone (P<0.0001)

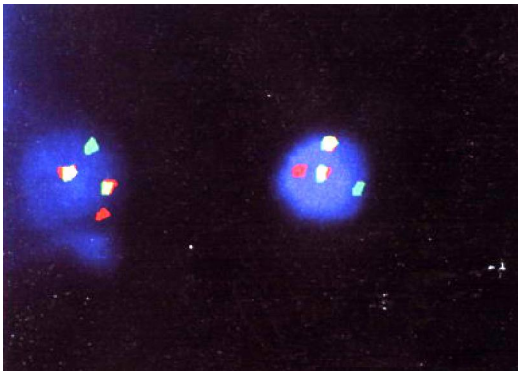

Photo (1): Interphase FISH analysis showing mBCR/ABL by ES-FISH (1R, 1G, 2Y signals)

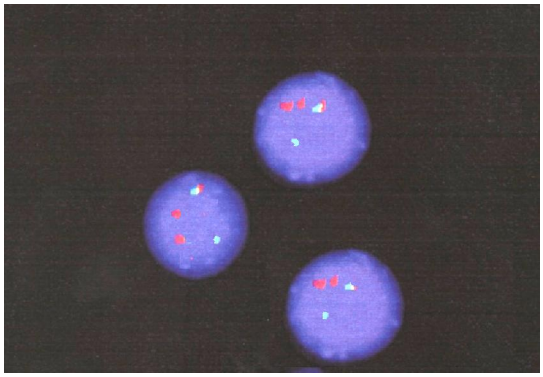

Photo (2): Interphase FISH analysis showing classical MBCR/ABL by ES-FISH (2R, 1G, 1Y signals)

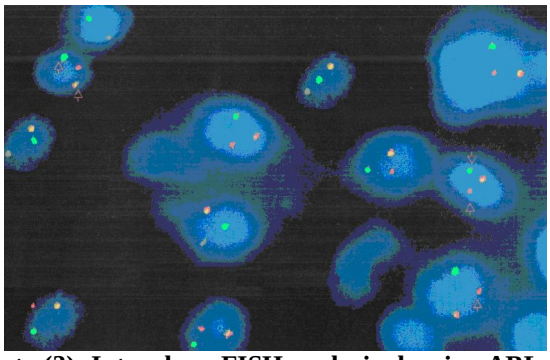

Photo (3): Interphase FISH analysis showing ABL deletion by ES-FISH (1R, 1G, 1Y) signals

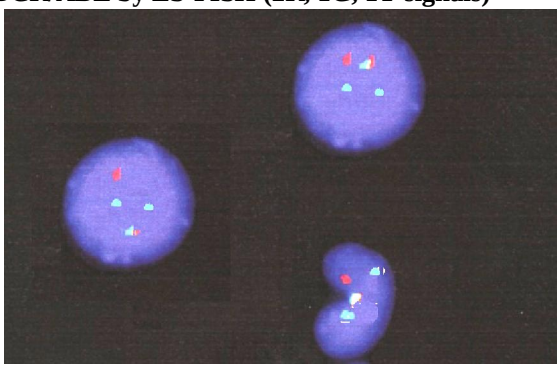

Photo (4): Interphase FISH analysis showing ABL deletion by D-FISH (1R, 2G, 1Y)

4. Discussion:

Several studies have demonstrated that a submicroscopic gene deletion in Ph positive CML is associated with a poor prognosis and reduced response to treatment *(Kim et al., 2005).* These deletions are proved to be a powerful and independent prognostic factor more potent than the scoring systems of Sokal et al. or Hasford et al. *(Xinh et al., 2006).* Molecular techniques are required to demonstrate the presence of these atypical rearrangements in a sensitive and specific manner *(Aoun et al., 2004).*

In this work, Ph was detected in all cases with successful mitosis. Ph was detected as a sole anomaly in 35/49 (71.4%) cases, distributed as 26/33 (78.7%) in CP, 4/7 (57.1%) in AP and 5/9 (55.6%) in BP, being highest in CP. The total percentage of Ph chromosome detected as a sole anomaly was midway between that detected by *Anoun et al., (2004)* and *Xinh et al., (2006)*, where both showed it to be about

83%, and that found by *Reena et al., (2006),* and *Patel et al., (2009),* which were 50% and 53.8% respectively. While complex aberration was detected in this study in 14/49 cases (28.6%) as 7/33 (21.3%) in CP, 3/7 (42.9%) in AP and 4/9 (44.4 %) in BP. Complex aberrations detected in the total cases were like that found by *Xinh et al., (2006)* (20%), but discrepant from that detected by *Anoun et al., (2004)* and *Reena et al., (2006)* (11% for both). Having higher percentages of complex aberrations in AP and BP than CP was previously documented by the well established role of additional anomalies in clonal evolution and acceleration of CML *(Reichard et al., 2009).*

Re-evaluation by ES-FISH improved the ability of detection of genetic aberrations, detecting Ph in 100% of the cases. Ph chromosome was detected as the sole anomaly in 50/64 cases (78.1%), with the peak incidence of 85.7% in CP as expected (36/42 cases). This was consistent with *Moon et al., (2007),* who found that the incidence of Ph as sole anomaly in CML by ES-FISH is 81%, and close to that found by *Primo et al., (2003)* (83.3%). This improved ability of detection was evident regarding detection of complex aberrations too, where complex aberrations were detected in 14/64 cases (21.9%), with peaks of incidence in the AP and BP (33.3% and 38.5% respectively). This was consistent with the findings of *Primo et al., (2003)* and *Moon et al., (2007)* who found the incidence of complex aberration in the patients of CML studied by ES-FISH to be 16.7% and 19% respectively. This low incidence is due to the minor role of complex aberrations in the pathogenesis of CP of CML, and playing the major role in acceleration and transformation only *(Reichard et al., 2009).*

The high ability of FISH in detection of specific chromosomal anomalies is that it can be done on interphase nuclei, poorly spread metaphases and well spread metaphases, allowing it to play an important role in such conditions for diagnosis and evaluation of MRD *(Kantarjian et al., 1990).* Several reports strongly suggest that all FISH data correlate very significantly with chromosme banding data *(Baccarani et al., 2008).*

The LSI ES-FISH increased this ability by its wide spanning area of 1.5 megabase *(Lim et al., 2005).* The use of the ES probe reduces the interpretation problems resulting from random juxtapositioning of differently labeled genes producing co-localization signals; and this is the role of the extra signal, it confirms the true positivity of the translocation detected *(Primo et al.,, 2003).*

The improved ability of detecting chromosomal anomalies was evident also by using DF-FISH, as Ph chromosome was detected in 100% of cases, as a sole anomaly in 54/64 cases (84.4%), again with the highest incidence of 92.9% in CP (39/42 cases). The percentage of sole anomaly in our study was the highest detected, despite wide variability among researchers, as the 70% found by *Huntley et al., (2001)* and *Lim et al., (2005),* the highly deviant 12.5% by *Loncarevic et al., (2002),* 81.6% found by *Kim et al., (2005)* and 77% found by *Siu et al., (2009).* Complex chromosomal aberrations were detected in 10/64 cases (15.6%), again with the peaks in AP and BP (22.2% and 38.5% respectively). This is matching with the result obtained by *Kim et al., (2005)* of 18.4%, and with that of *Siu et al., (2009)* of 22.3%.

Regarding the complex aberrations detected, CCA detected 4/49 (8.2%) cases with ABL deletion (in the form of del9q), ES-FISH detected 13/64 (21.3%), and D-FISH detected 6/64 cases (9.3%) (4 of them were accompanied by BCR deletion). This magnifies the superiority of ES-FISH as a tool for detecting the ABL deletion.In the current work, result of ABL deletion by ES-FISH is midway between the results reported by *Sinclair et al., (2000), Huntly et al., (2001)* and *Lee et al., (2003)* (15 - 28.6%).

Primo et al., (2003) stated that ABL deletion is the most common atypical Ph chromosome signal detected, and this agrees with our result, by detecting 13 as ABL deletion from the 14 cases with complex aberration, and is consistent with *Huntly et al., (2001),* who reported it to be >30% of atypical signals, however, it is much higher than what's found by *Anoun et al., (2004)* (12%), who found no explanation to this discrepancy from others' results, except the probability of having a relatively low number of cases with an atypical signal in their study.

Results ABL deletion by D-FISH obtained in this work were similar to that found by *Kim et al., (2005)* and *Siu et al., (2009)* (6.6 and 9% respectively), higher than that found by *Sinclair et al., (2000), Huntley et al., (2001), Lim et al., (2005)* (3.6, 2.8, 3.1 respectively), and much less than that found by *Loncarevic et al., (2002)* (56.2%). This is in agreement with the previously stated wide range of ABL deletion detected. Regarding BCR deletion, D-FISH is the only technique that can detect BCR deletion. In the current study, no BCR deletion alone was detected, as was the situation with *Sinclair et al., (2000)* and *Kim et al., (2005).*

Huntley et al., (2001), Loncarevic et al., (2002), Lim et al., (2005) and *Siu et al., (2009)* reported a wide range of BCR deletion of 0.8, 31.3, 2.1, 3.3 respectively, which were of low values except for the 31.3% reported by *Loncarevic et al., (2002),* who seem to have a certain form of problem concerning interpretation of the signals, with a high false positive rate, as shown also from the extraordinarily high

value of ABL deletion, which was above the reported range of detection.

ABL and BCR deletions were detected in combination in 4/64 cases (6.2%), which was similar that found by *Kim et al., (2005)* (6.6%), despite the presence of wide range as found by *Sinclair et al., (2000), Huntley et al., (2001), Loncarevic et al., (2002), Lim et al., (2005)* and *Siu et al., (2009),* whose results were 25, 11.9 , Zero, 10.4 and 9% respectively. However, apart from the extreme values of Zero and 25, most of the results were close to that detected in our study.

Trisomy 8 was detected in 4/49 cases (8.2%), and detected by CCA only. This is due to the non directed nature of the CCA, which favors it as the gold standard for the genome wide screen *(Hochhaus et al., 2000*).Trisomy 9 was detected by CCA in 5/49 cases (10.2%), and by D-FISH in 5/64 (7.8%), and they were the same cases. No signal pattern was reported for +9 by D-FISH, but we interpreted the signal in conjunction with the results of CCA.

Supernumerary Ph was detected by ES and D-FISH in 1/64 case (1.5%) and was in BP. This is close to the reported percentage by *Lim et al., (2005)* of 3.1%. They reported one case in AP and one case in BP, which confers to the association made between additional Ph and disease progression, and being one of the major pathways of clonal evolution seen during blast crisis. One case of the 64 cases (1.5%) showed variant translocation by D-FISH, and was detected as complex translocation t(8;9;22) by CCA. However, this issue is not a subject of interest for many researchers at the moment, with well interpreted results obtained only by *Lim et al., (2005)*

Comparing ES-FISH to the CCA and D-FISH, ES-FISH is the only technique that can differentiate MBCR/ABL from mBCR/ABL rearrangements. ES-FISH detected one case (1.5%) of mBCR/ABL. This was compatible with *Primo et al., (2003)* who found mBCR/ABL in 1.5% of CML patients. However, it is important to note that ES-FISH failed to detect variant translocation, the BCR deletions and +9. This is a considerable pitfall of ES-FISH, since these aberrations have a prognostic impact, to be mentioned later.

No agreement was found between the results of CCA and ES-FISH. A strong agreement was found between results of CCA and D-FISH for the results of the overall cases of the study (P <0.0001). This agreement study was done after exclusion of the 15 cases of failed mitosis, and this overestimated the diagnostic power and sensitivity of the CCA, whose main pitfall is the failure of mitosis, and led to the failure of agreement with the ES-FISH. This is consistent with *Patel et al., (2009)* who found a significant and positive correlation between results of CCA and D-FISH, also, *Baccarani et al., (2008)* stated that several reports strongly suggests that all FISH data correlate very significantly with chromosome banding data *(Schoch et al., 2002; Raanani et al., 2004).* There was a moderate agreement between ES-FISH and D-FISH as regards the overall patients (P<0.0001), which simulates the agreements between D-FISH and CCA but with reduced strength, so, despite the lack of direct agreement between CCA and ES-FISH, a trend is noticeable. Results of ABL deletion obtained by D-FISH and ES-FISH showed a moderate highly significant agreement (P <0.0001). This is in favor for ES-FISH in detection of ABL deletions, revealing it as the method of choice for detection of this aberration.

To determine the outcome of the patients, we assessed the hematological, cytogenetic and molecular responses at 1 year after therapy. This is because these responses at 1 year after therapy have been shown to correlate well with survival *(Li et al., 2005).*

As for cytogenetic results, there is an established strong association of major and complete cytogenetic response with improved long term survival with interferon-alpha therapy. These prognostic associations have also been confirmed with imatinib therapy, suggesting that the relationship between response and survival may be independent of the treatment that produced the response (Kasakyan et al.,2003). Cases showing complex aberrations by CCA, ES-FISH and D-FISH showed highly significant association with poor prognosis than cases with Ph alone (P<0.0001). This is appropriate with the poor prognostic impact of the chromosomal aberrations mentioned previously.

Detecting such significance by ES-FISH is a good point for it. This is due to the prognostic impact of the ABL deletion, which is the best anomaly detected by ES-FISH, and was the most frequent atypical signal detected in our study. Poor prognosis associated with ABL deletion has been reported by many researches as *Sinclair et al., (2000); Huntly et al., (2001); Lee et al., (2003)* suggested a favorable prognosis.

Regarding mBCR, ES-FISH detected it in case #1 in BP, and it had a moderate prognosis by achieving CHR and MCR, but NMR. Previous studies have shown that mBCR breakpoints develop blast crisis with monocytosis and this agrees with that our single case was in BP, despite failure of judgment by a single case. Adds to the power of ES-FISH is its detection of supernumerary Ph. Detecting supernumerary Ph in BP in our study, and in AP and BP by *Lim et al., (2005),* confers to the association made between additional Ph and disease progression,

and being one of the major pathways of clonal evolution seen during blast crisis.

Regarding D-FISH, its power is in detecting the BCR deletion, either alone or with ABL deletion. The statistical significance associated between complex aberrations it detected and poor prognosis can be attributed to the combined BCR and ABL deletions, since it was the most frequent atypical signal detected.

The relevance of BCR deletion on prognosis is still a matter of discussion. *Lee et al., 2006 stated that* BCR losses are accompanied by ABL deletion in 70-100% of cases, and might be involved in poor prognosis. Improved survival was once again reported only by *Kreil et al., (2007).*

Regarding variant translocation, it is inconsistent whether variant translocations confer the same clinical course and outcome as standard ones. Some studies concluded that a variant translocation showed no differences in the disease course of CML and had no effect on prognosis, compared with a standard translocation *(Valencia et al., 2009).* However, some suggested that variant translocations are associated with an adverse outcome *(Loncarevic et al., 2002).* Our study is in favor of the opinion of variant translocation's poor prognosis, despite the low number of cases in our study (one case, #3 in BP), but she was in BP and failed to achieve neither CR nor MR.

In conclusion, karyotyping is mandatory to be applied at diagnosis of CML. ES-FISH is the method of choice for detection of ABL deletions, despite it cannot detect neither BCR deletions nor variant translocations. Karyotyping coupled with ES-FISH are adequate for the diagnosis and therapeutic monitoring of CML with the classical t(9;22) and for cases with ABL deletion

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