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Case Report

Fluorescence *in Situ* Hybridisation (FISH) is the First Tool to Identify Hypodiploidy in Paediatric Acute Lymphoblastic Leukaemia

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Abstract

Hypodiploidy has a low incidence in childhood acute lymphoblastic leukaemia (ALL). Patients are usually stratified into three subgroups, to allocate the correct treatment according to their ploidy level: high hypodiploidy (40-45 chromosomes), low hypodiploidy (33-39 chromosomes) and near haploidy (23-29 chromosomes). In this paper, a case is presented of near-haploid childhood ALL where fluorescence *in situ* hybridisation (FISH) provided an insight into the near-haploidy chromosomal aberration initially missed on routine karyotyping due to culture effect and analysis software constraints. FISH primary results followed by further confirmatory studies, inclusive of karyotyping and single nucleotide polymorphism (SNP) microarray, clinically placed the patient in the correct treatment stratification. The patient remained in morphological and cytogenetic remission 12 months after commencing the high risk arm of an international collaborative (*AIEOP-BFM-ALL [Associazione Italiana Ematologia Oncologia Pediatrica–Berlin-Franklin-Munste]) 2009 treatment protocol for children and adolescents with ALL.

Keywords
FISH; hypodiploidy; childhood ALL

1. Background

Acute lymphoblastic leukaemia (ALL) is the most common type of childhood blood disorder [1], and despite improvements in the outcome of therapy it remains the predominant cause of cancer-related demise in young children [2]. Hypodiploidy, or a chromosomal count of less than 45, is relatively rare and it is found in ~5% of adult and child ALL patients [3]. There are three distinct hypodiploidy subgroups with different genetic and clinical features:

1-High hypodiploidy [40-45 chromosomes]. Hypodiploidy with a count of 45 chromosomes is the most prevalent form of hypodiploid ALL [4].

2-Low hypodiploidy [33-39 chromosomes] occurs at all ages.

3-Near haploidy [23-29 chromosomes] is seen mainly in children and adolescents (age range 2-15) [5].

Near haploidy as well as low hypodiploidy are each detected in ~0.5% of children with ALL [4]. Doubled hypodiploidy (masked hypodiploidy), doubling of the hypodiploid chromosome content by endoreduplication, is often observed in near haploidy and low hypodiploid sub-groups and both are associated with an unfavourable outcome [6].

Patients with low hypodiploid ALL can express genetic changes targeting *TP53, RB1* and the *IKAROS* gene family member *IKZF2.* However, near haploid ALL cases can demonstrate changes within receptor tyrosine kinase- and Ras signalling as well as high incidences of *IKZF3* gene alterations [2]. High hypodiploid sub-group (44-45 chromosomes) can present some structural chromosomal rearrangement such as dicentric chromosomes including 9p, 12p or both or other known chromosomal rearrangements (e.g. *ETV6-RUNX1* fusion), but do not demonstrate as an unfavourable outcome compared with cases of more severe aneuploidy [7].

According to the Australian New Zealand Clinical Trial Registry (ANZCTR*), the standard/augmented BFM treatment protocol is adapted for children and adolescents with ALL. Hypodiploid subgrouping is crucial in order to stratify patients into the correct risk group for treatment. Cytogenetic studies using such techniques as G-band karyotyping, FISH, chromosome microarray (CMA) including SNP and next generation sequencing (NGS), are essential tools in categorising these patients.

Here we present a case of hypodiploid childhood ALL where FISH was the first tool that provided an insight into the near-haploidy chromosomal aberration. FISH primary results followed by further confirmatory studies, inclusive of karyotyping and microarray, clinically placed the patient in the correct treatment stratification.

2. Case History and Description

A four year old boy presented to the hospital with joint pain, fever, abdominal pain and maculopapular rash. On examination he had no palpable cervical or axillary lymph nodes. Liver and spleen were palpable just below the costal margin. He had a papular rash on his lower limbs

and his right ankle was tender to touch. An X-ray of the involved limbs showed no fractures and the bone scan demonstrated polyarthritis involving both elbows, right ankle and some small joints of the right foot. Abdominal ultrasound revealed a small amount of free fluid in the right iliac fossa with several lymph nodes (up to 7mm). The child's full blood count was normal and the blood film showed reactive lymphocytes, occasional lymphoid cells with large nucleoli and basophilic vacuolated cytoplasm. Bone marrow aspirates contained 70% blasts which were Periodic acid-Schiff (PAS) Stain positive. Flow cytometry showed the blasts to be positive for CD19, CD10 and negative for CD33, CD13 and CD117, consistent with diagnosis of precursor B acute lymphoblastic leukaemia.

3. Materials and Methods

3.1 G-Band Karyotype

Bone marrow was collected into a lithium heparin tube and sent to the cytogenetics laboratory. Two bone marrow cultures (24 and 48 hours) were established in 5mL Marrow Max Bone Marrow medium (GIBCO, Thermo Fisher Scientific, USA) at a final concentration of 1.0x106 cells/mL. Both cultures were synchronised using the combination of 5-fluoro-2'-deoxyuridine (FdU) and Uridine (Sigma-Aldrich, Australia) as S-phase blocking agents with 5-bromo-2'-deoxyuridine (BrdU) (Sigma-Aldrich, Australia) as the releasing agent [8]. Cultures were harvested and metaphase chromosome preparations GTL-banded according to our standard laboratory protocols. Metaphase images were captured using a fully automated slide scanning platform (Metafer MSearch), which automatically locates metaphases according to their size (i.e metaphases with low or high number of chromosomes would not be captured) and acquires high resolution images. Captured metaphase images were analysed using Ikaros karyotyping software module (MetaSystems, Germany).

3.2 Fluorescence in Situ *Hybridisation (FISH)*

FISH was performed on cultured (24hr) bone marrow interphases, according to our standard laboratory protocols. Briefly, slides were pre-treated with 2xSSC (10 min), 0.2% pepsin/0.01M HCl (30 sec), 1% paraformaldehyde (2 min) and dehydrated in a 70%, 90% and 100% ethanol series (2 min each). Co-denaturation (75°C, 2 min) was followed by hybridisation (37°C, 4-16 hours). Post hybridisation stringency wash using 0.4xSSC/0.3%NP40 (73°C, 2 min) was followed by 2xSSC/0.1% NP40 (room temperature, 1 min). FISH was performed using three probe sets (our common probes panel for ALL study IX): The tri-colour dual fusion BCR-ABL-ASS1 probe [for the t (9; 22) (q34; q11.2)] (Abbott Molecular, USA), the KMT2A break-apart probe [for KMT2A 11q23.3 rearrangement] (Abbott Molecular, USA) and the dual colour single fusion extra signal ETV6-RUNX1 probe [for the t (12; 21) (p13; q22)] (Abbott Molecular, USA). Further FISH testings to confirm CMA/SNP results were performed using IGH-MYC-CEP8 [three colour dual fusion] probe (for chromosomes 14q32, 8q24 and 8 centromere respectively) (Abbott Molecular, USA) and ELN probe (for chromosome 7q11.2) (Abbott Molecular, USA).

3.3 SNP Chromosome Microarray

Genomic DNA was extracted from bone marrow using the Chemagic 360i: DNA Blood Kit, as per the manufacturer's instructions (PerkinElmer, Australia). Chromosome SNP microarray was performed using the 850K Beadchip v1.2 (Illumina, USA). SNP microarray data was analysed using BlueFuse Multi v4.4 with a mean effective resolution of ~50 kb for copy number and 5 Mb for Loss of Heterozygosity (LOH).

4. Results

FISH showed no BCR-ABL1 [t (9; 22) (q34; q11.2)] or ETV6-RUNX1 [(t12; 21) (p13; q22)] fusion and no KMT2A (11q23.3) rearrangement. However, approximately 30% of the cells showed one copy each of the ASS1 (9q34.11), ABL1 (9q34.12), BCR (22q11.23), KMT2A (11q23.3), and ETV6 (12p13.2) probes on interphase cells examined (Figure 1, Figure 2 and Figure 3).

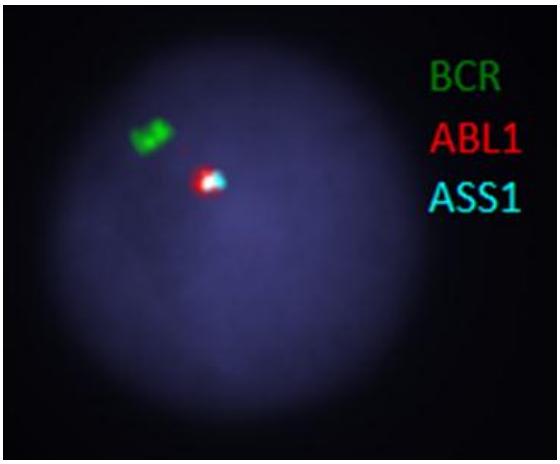

Figure 1 Interphase FISH result for the tri-colour dual fusion BCR(green)-ABL1(red)-ASS1(aqua) probe, showed a single copy of each probe with no evidence of fusion.

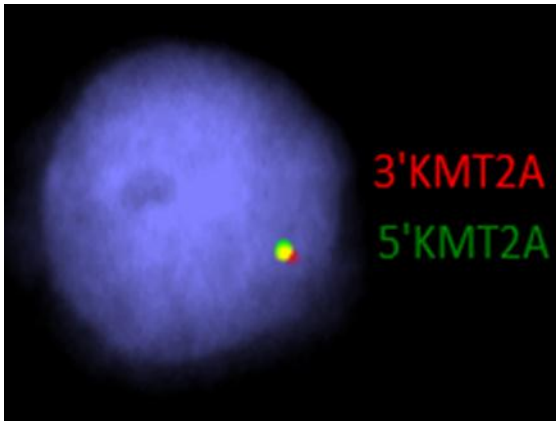

Figure 2 Interphase FISH result for the KMT2A break apart probe. A single connected copy of the KMT2A probe was observed with no evidence of a rearrangement.

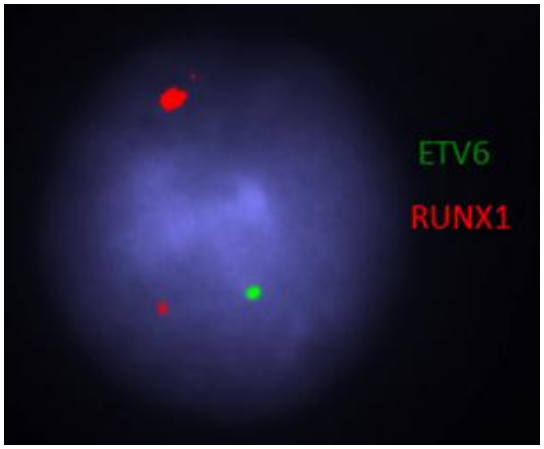

Figure 3 Interphase FISH result for the ETV6-RUNX1 dual colour single fusion–extra signal probe showed a single copy of the ETV6 probe (green) and two copies of the RUNX1 probe (red) with no evidence of a fusion.

According to the ISCN 2016 description, the interphase FISH results were: Nuc ish (ASS1, ABL1, BCR)x1 [60/200]; (KMT2Ax1) [32/100]; (ETV6x1, RUNX1x2) [48/200] (consistent with monosomy for chromosomes 9, 11, 12 and 22 in the abnormal clone).

Subsequent G-band karyotype analysis showed in 30 metaphase examined, a male karyotype 46, XY. Based on the FISH results that suggested the presence of a hypodiploid clone, additional metaphases were scanned to search for the hypodiploid clone. In total, four cells with near-haploid chromosome complement were found 27, XY, +8, +10, +21 [4] / 46, XY [30] (Figure 4). The missing chromosomes from the near haploid karyotype were consistent with the monosomy chromosomes found by FISH analysis.

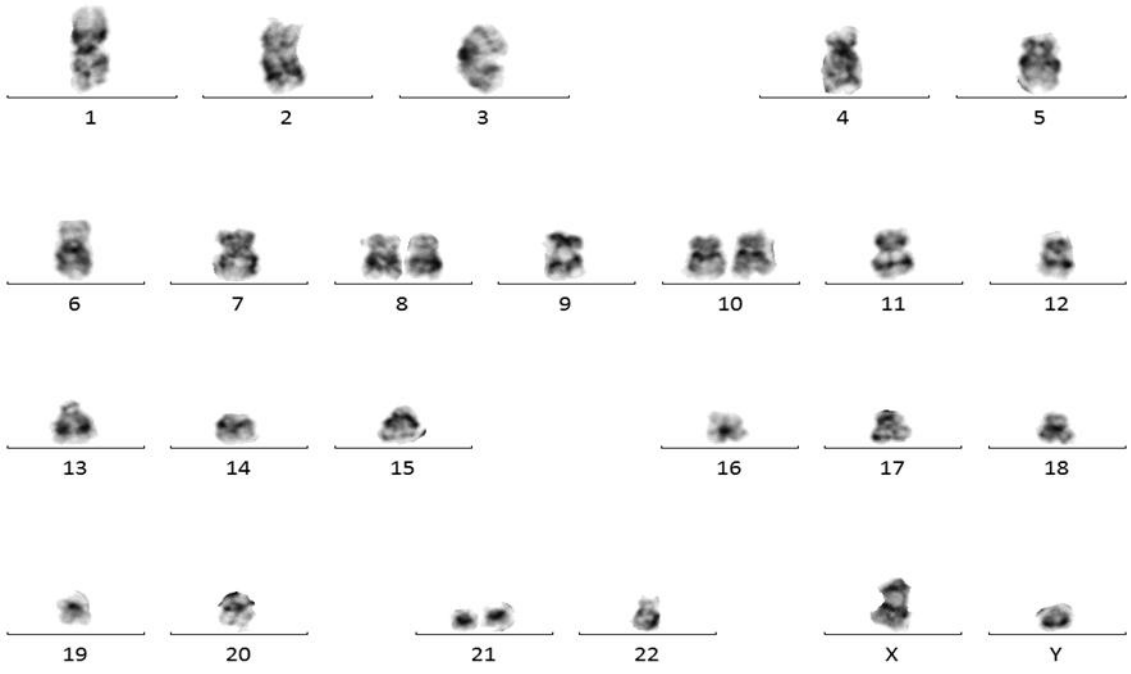

Figure 4 G-band karyotype of the near-haploid clone 27, XY, +8, +10, +21.

Chromosome microarray (Illumina CytoSNP 850K V1.1) testing indicated male (XY) genotype, with the SNP B-allele frequency distributions that showed 30%-40% mosaic deletion (i.e. B-allele frequency plot ratio of 0: 0.4: 0.6: 1) [9]. Mosaic deletion for whole chromosomes 1, 2, 3, 4, 5, 6, 7, 9, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 and 22 were observed, consistent with monosomy for these chromosomes. Chromosomes 8, 10 and 21 showed a normal heterozygous B-allele frequency distribution (0: 0.5: 1) consistent with disomy for these chromosomes. The SNP array result was consistent with the interphase FISH and G-banded karyotype findings of a near-haploid chromosome complement (Figure 5).

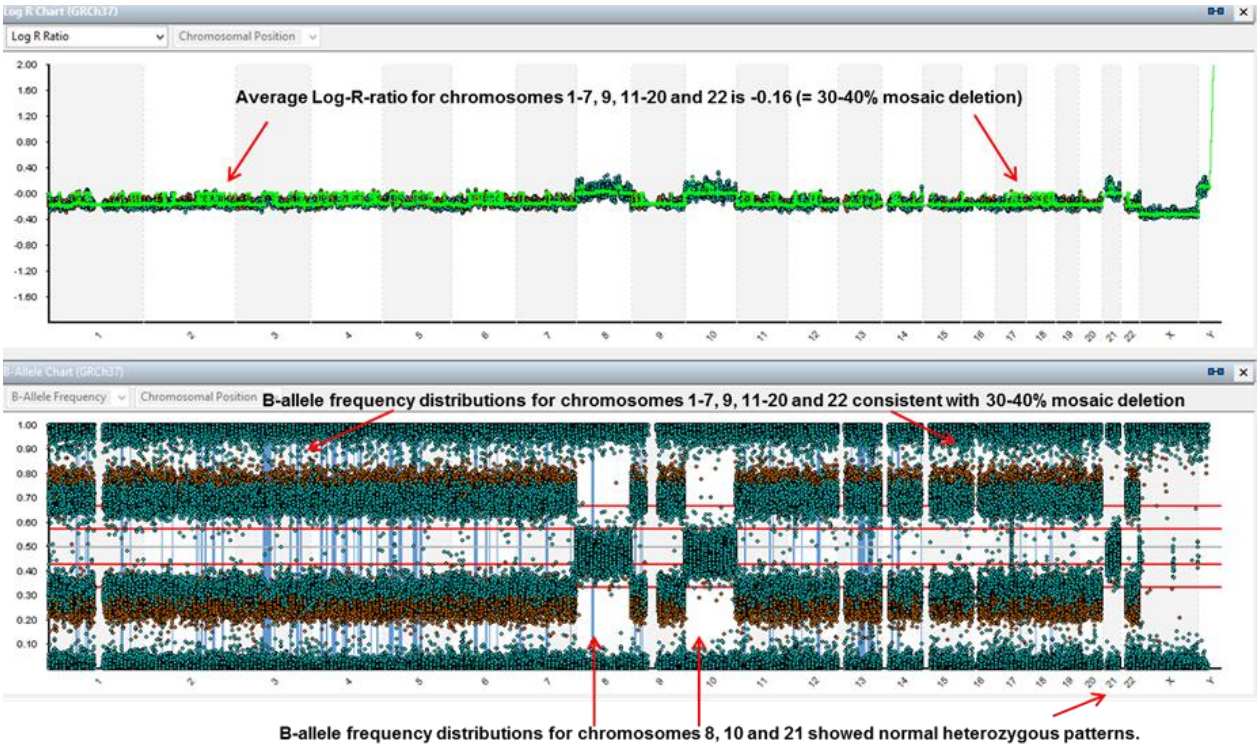

Figure 5 Illumina CytoSNP 850K v1.1 both Log-R ratio plot (top) and B-allele frequency distribution plot (bottom) showed 30%-40% mosaic deletion for chromosomes 1-7, 9, 11-20 and 22 consistent with monosomy for these chromosomes. The B-allele frequency distribution for chromosomes 8, 10 and 21 showed a normal heterozygous pattern consistent with disomy for these chromosomes. The SNP array result was consistent with the FISH and karyotype findings of a near-haploid chromosome complement.

Further FISH testing using the ELN and IGH/MYC/CEP8 probes showed a single copy of the ELN probe (in ~23% of cells examined), two copies of each the MYC and CEP8 probes and a single copy of the IGH probe (in 23% of cells examined). The FISH result was: nuc ish (ELNx1) [47/200]; (D8Z1x2, MYCx2, IGHx1) [46/200] consistent with the SNP array results and a disomy for chromosome 8 and a monosomy for chromosomes 7 and 14 (Figure 6 and Figure 7).

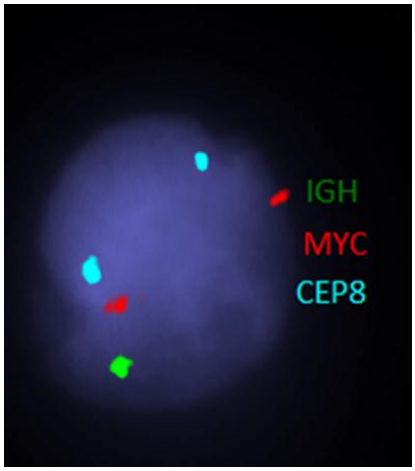

Figure 6 Interphase FISH result for the tri-colour dual fusion IGH-MYC-CEP8 probe, confirmed CMA finding of disomy for chromosome 8 [2 copies of each MYC (red) and CEP8 (aqua) probes] and monosomy for chromosome 14 [one copy of IGH (green) probe].

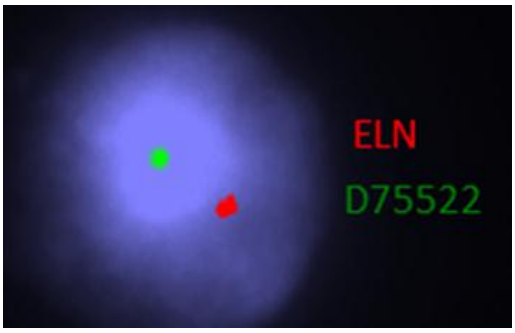

Figure 7 Interphase FISH result for the ELN/D75522 (locus specific) probe showed one copy of each the ELN (red) and D75522 (red) probes, consistent with CMA finding of monosomy 7.

5. Discussion

The detection of chromosomal abnormality is important for the diagnosis, treatment, management and prognosis of childhood ALL. FISH, karyotype and SNP microarray testing indicated that the patient in this case study fell within the near-haploid group, with a chromosome count of 27 chromosomes. ALL patients in the sub-group with 23-29 chromosomes represent a minor category with a particularly unfavourable outcome [10]. In near-haploid ALL, retained disomies primarily comprise chromosomes X/Y, 8, 10, 14, 18, and 21, whereas retention of disomies X/Y, 1, 5, 6, 8, 10, 11, 14, 18, 19, 21, and 22 is seen in low hypodiploid ALL [4]. The patient in the present case retained disomy for only chromosomes 8, 10, 21, X and Y and therefore was placed within this sub-group.

FISH detected an abnormal clone in 26.5±1.4% of the cells, whereas the SNP microarray detected ~30%-40% mosaic deletion of the cells affected. FISH could detect loss of certain chromosomes by using locus specific probes within the region of interest (localisation of specific DNA target sequences). These differences suggest that more than one type of investigation is necessary to determine a complete hypodiploidy profile. In the present case, by applying the

standard "study IX" B-ALL FISH panel (see the methods' section), monosomies for chromosomes 9, 11, 12, and 22 were suspected. The CEP4/CEP10 and CEP7/CEP17 probe sets (for chromosomes 4, 7, 10 and 17 centromeres) used to be included in our routine FISH testing to confirm hyperdiploid karyotype. The introduction of SNP microarray testing in our laboratory limited the use of additional FISH workup.

Based on the FISH results, high resolution SNP microarray was performed to investigate the entire genomic profile of the abnormal clone. Mosaic deletions for whole chromosomes 1, 2, 3, 4, 5, 6, 7, 9, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 and 22 were revealed. Interphase FISH provided the most accurate estimate for the proportion of abnormal clone (26.5%) as it was performed objectively by scoring interphase cells (with no culture bias), whereas karyotyping only identified four near-haploid metaphases in 34 cells examined (11.7%). Karyotyping can be predisposed to culture bias as the abnormal clone may not divide, therefore analysis of metaphases might provide a biased view of the true proportion of the aberrant clone. SNP microarray allowed for simultaneous examination of DNA extracted from a large number of heterogeneous cell populations within the bone marrow sample, thus removing the culture bias element from the metaphase chromosome analysis. SNP microarray can also examine the whole genome at a higher resolution than karyotyping and FISH analysis. However, the ability of SNP microarray in the detection of mosaicism may be influenced by probe coverage, the signal to noise ratio, as well as the analysis algorithm of the software. Hence, the percentage of abnormal clone suggested by the Log-R ratio and B-allele frequency distribution are estimated figures based on the expected theoretical values. In the absence of the hypodiploid founded clone (masked hypodiploidy), SNP microarray is the primary test to reveal hypodiploidy based on loss of heterozygosity. Whereas, FISH has limitation of detection in these cases but might provide a suggestion that the genome has doubled because of the tetrasomy of some chromosomes that have retained their disomy.

The automated Metafer MSearch technology used to locate G-banded metaphases failed to identify the abnormal metaphase cell line for karyotyping. This innovative technology uses a specific classifier (object count) and algorithms to detect objects and automatically sort them by quality score, only acquiring metaphases within the pre-defined parameters. Metaphases with chromosome numbers slightly more or less than 46 can be captured with this device, but metaphases with half or double this number are not detected. However, Metafer classifier software training to detect and capture hypodiploid and hyperdiploid cells is also possible. Trained classifiers can demonstrate improved pick up rate of cells from these categories.

The karyotype was not useful in this case, as the abnormal clone was originally missed due to culture bias. Identification of lower levels of mosaicism can be challenging. It has been estimated that analysis of 20 cells (standard for routine chromosome analysis) will detect ~14% mosaicism (in the tissue being studied) with 95% confidence [11]. In this case we have examined 34 cells, then the lower level of mosaicism excluded with 95% of confidence is 9%. However, interphase FISH analysis identified the near-haploid cell line in ~30% of the cells examined, and then accordingly G-banded slides were scanned manually using light microscopy. Metaphases are arduous to obtain from ALL patients, and these abnormal metaphases often show poor chromosome morphology with limited resolution. FISH, on the other hand, does not require cell culture and can be performed on interphase cells. Furthermore, karyotype has limited application to the detection of cryptic or subtle chromosomal rearrangements, whereas interphase FISH can reveal these types of aberrations.

Microarrays and next generation sequencing are advanced, lengthy and costly techniques, which are incorporated into routine testing to detect genome aberrations. FISH is a relatively old technique developed in the early 1980s [12-14], but remains an essential tool for the detection of specific biomarkers such as BCR-ABL1 gene fusion, HER2 and ALK gene rearrangements, and has become crucial in the development of personalised medicine [13]. Nevertheless, it remains a rapid, economical and reliable technique in the detection of balanced rearrangement and more importantly in the detection of minimal residual disease (MRD).

FISH together with karyotyping and CMA, offered a flexible approach to the diagnosis of this ALL case, allowing the correct therapy to be chosen. The patient was treated according to the high-risk arm of the *AIEOP-BFM-ALL 2009 protocol and remained in complete cytogenetic and morphological remission 12 months after treatment.

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Author Contributions

Racha El-Hajj Ghaoui carried out the FISH studies and drafted the manuscript. Dorothy Hung carried out the G-band karyotype and chromosome microarray testing and helped in drafting the manuscript. Bhavna Padhye provided inputs for the clinical aspects. All authors read and approved the final manuscript.

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Conflicts of Interest:

The authors declare they have no conflicts of interest.

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