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CGH-based microarray detection of cryptic and novel copy number alterations and balanced translocations in cytogenetically abnormal cases of b-cell all

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ABSTRACT

Acute lymphoblastic leukemia (ALL) is the most common malignancy in children, with the majority of cases being of precursor B-cell phenoltype. Conventional cytogenetic analysis plays an important role in the diagnosis of B-cell ALL, identifying characteristic chromosomal abnormalities associated with a given prognosis therein facilitating optimized treatment. The more recent introduction of microarray technology to the analysis of B-cell ALL has afforded both higher resolution for the detection of known abnormalities and an ability to identify novel copy number abnormalities (CNAs) with potential clinical relevance. In the current study, microarray analysis was performed on 20 cytogenetically abnormal B-cell ALL cases (10 pediatric and 10 adult), while a novel microarray-based balanced-translocation detection methodology (translocation CGH or tCGH) was applied to that subset of cases with a known or suspected recurrent balanced translocation. Standard microarray analysis identified that CNAs was not detected by previous conventional cytogenetics in 75% (15/20) cases. tCGH identified 9/9 (100%) balanced translocations defining *BCR/ABL*1 (x4), *ETV6/RUNX*1 (x3), and *MLL/AFF*1 (x2) breakpoints with high resolution. The results illustrate the improved molecular detail afforded by these technologies and a comparison of translocation breakpoints, CNAs and patient age offers new insights into tumor biology with potential prognostic significance.

Keywords: Acute Lymphoblastic Leukemia; B-Cell ALL; Microarray; Balanced Translocation; Translocation CGH; Hematologic Malignancies

1. INTRODUCTION

B-cell acute lymphoblastic leukemia (B-cell ALL) is the most common pediatric malignancy. Despite significant advances in treatment resulting in a cure rate of nearly 80% in the pediatric population [1-3], approximately 20% to 25% of children and more than half of adult patients experience relapse [4,5]. Conventional cytogenetic analysis has been an integral part of the evaluation of B-cell ALL, influencing prognosis and treatment decisions [6,7]. Specific cytogenetic findings, such as the t (9;22) (q34;q11.2) resulting in *BCR/ABL*1 fusion, *MLL*

rearrangements, and hypodiploidy are associated with a poor prognosis [8]. In contrast, hyperdiploidy, particularly involving gains of chromosomes 4 and 10, and the t (12;21) (p13;q22) resulting in *ETV6/RUNX*1 fusion, are associated with a good prognosis [8]. However, some cases of B-cell ALL lack alterations detectable by conventional cytogenetics and, importantly, some patients with favorable cytogenetic alterations still relapse, which may indicate that that there are additional undetected chromosome aberrations [9].

In recent years, genomic profiling of B-cell ALL using newer technologies has uncovered additional genetic alterations that cannot be detected by conventional cytogenetics [10-12]. Common alterations include genes involved in the regulation of B-cell development and differentiation (*PAX*5, *IKZF*1, *EBF*1, *LEF*1 and *TCF*3), cell cycle regulation and tumor suppression (*CDKN*2*A*, *RB*1 and *PTEN*), and lymphoid signaling (*CD*200, *BTLA*, and *CRLF*2) [10-16]. Many of these alterations are submitcroscopic deletions that can be identified using high-density oligonucleotide comparative genomic hybridization (CGH)-based microarrays (aCGH) and single nucleotide polymorphism (SNP) microarrays. These novel findings have not only led to insights concerning the boilogy of B-cell ALL, but some have also been shown to be predictors of high-risk disease and have potential as future therapeutic targets. For example, deletions of *IKZF*1, often too small to be detected by fluorescence *in situ* hybridization (FISH), have been found to be a strong predictor of relapse [13,17]. Thus, it will be important in the clinical laboratory to employ technology with the capacity to identify these submicroscopic alterations that are below the resolution level of conventional cytogenetics.

Microarray-based detection of copy number alterations (CNAs) has become standard of care for the diagnosis of most constitutional chromosomal imbalances in children with developmental disabilities [18], but its potential for the evaluation of neoplasia in the clinical laboratory has been compromised by the inability to detect balanced rearrangements, which are important recurrent diagnostic and prognostic markers. To circumvent the limitation of balanced translocation detection, traditional microarray may be supplemented with routine karyotyping, selected FISH studies, targeted rtPCR analysis when appropriate, or a broader and more recently described technique of translocation array (tCGH)] [19,20]. This novel technique couples linear amplification of genomic DNA using multiplexed primer sets targeted to a set of translocation breakpoint intervals with microarray analysis, permitting diagnostic detection of balanced translocations with unprecedented genomic resolution of the breakpoints. In the study presented here we characterize 20 cases of cytogenetically abnormal B-cell ALL by microarray analysis, including microarray analysis of putative translocations in 10 cases using a single tCGH assay designed to interrogate genomic DNA for the presence of 8 distinct translocations common in B-cell ALL. Joint analysis of aCGH and tCGH results provides detailed resolution of CNAs and genomic breakpoints offering potential new insights into tumor biology and prognostic significance.

2. MATERIALS AND METHODS

2.1. Specimen Ascertainment

Residual bone marrow or leukemic peripheral blood was obtained after routine testing from seven clinical cytogenetic laboratories for DNA extraction. Routine G-banded chromosome analysis was performed in the referring laboratories on all samples, and many cases were also analyzed by FISH as directed by standard clinical practice. The cases consisted of 13 bone marrow and four leukemic peripheral blood specimens at the time of initial diagnosis, one bone marrow three weeks after initial diagnosis, and two bone marrow specimens at relapse. Ten cases were from pediatric patients (1 - 18 years old), and ten cases were from adults (25 - 73 years old). Samples were de-identified for all information other than B-cell ALL indication for study, age, sex and prior cytogenetic findings. Except as otherwise indicated, additional specimen was not available for any post-analysis confirmatory studies. This study was conducted in accordance to Signature Genomics' Spokane-IRB approved protocol.

2.2. DNA Extraction

Genomic DNA was extracted from unenriched blood and bone marrow specimens using the Gentra Puregene Blood Kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. Two million cells or 150 µl (if cell counts were unavailable) of blood or bone marrow were used as starting material. Additional Cell Lysis Solution (Gentra Puregene Blood Kit) was added to samples with high viscosity to ensure complete lysis. Samples were stabilized in Cell Lysis Solution within 24 to 48 hours of specimen receipt, when possible, to ensure high-quality DNA for use on the microarray.

DNA quality was assessed by measuring DNA concentration, 260/280 nm and 260/230 nm readings on a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA). The DNA was also run on a 1% agarose gel with ethidium bromide to detect degradation. To be included in the study, samples had to have minimal degradation with 260/280 nm values near 1.8 and 260/230 nm readings greater than 1.35.

2.3. Oligonucleotide Microarray Labeling Hybridization and Analysis

Array CGH for copy number analysis was performed using a 135K-feature whole-genome oligonucleotide microarray (aCGH) (Signature OncoChip®, designed by Signature Genomics, Spokane, WA; manufactured by Roche NimbleGen, Madison, WI). When compared to probe coverage over the rest of the genome, this microarray has denser oligonucleotide coverage over 1893 cancer features, including genes with known roles in hematologic malignancies or solid tumors in which deletions or mutations had been previously reported, genes with suspected roles in cancer based on prior expression studies without specific evidence of genomic copy changes, genes with previously speculated roles based solely on association with a biological pathway or gene family, and genes involved in protein and microRNA (miRNA) coding. The microarray has an average coverage of one oligonucleotide per 0.2 - 7.0 kb for the targeted cancer features and genomic backbone coverage of one oligonucleotide per 35 kb. Labeling, hybridization, and washing were performed using previously published methods [21]. Data were analyzed and displayed using custom oligonucleotide aCGH data analysis and visualization software (Oncoglyphix®; Signature Genomics) as previously described [22]. Variants were identified based on the number of consecutive oligonucleotides involved and the magnitude in shift for the log2 ratio, with 5 - 99 oligonucleotides requiring a shift ≥ 0.300, 100 - 999 a shift ≥ 0.200, and a ≥ 1000 a shift ≥ 0.100.

2.4. Translocation CGH

For 10 of the 20 cases, prior chromosome and/or FISH analysis revealed evidence of a clinically relevant balanced translocation. For eight of those cases, translocations were identified by karyotypes only, for one case by both FISH and karyotype, and for one case by FISH only. Nine of those 10 cases had sufficient DNA to be assessed by both aCGH and the translocation CGH array (tCGH). For one additional case (Case 10), aCGH revealed a breakpoint for a genomic copy gain within the *MLL* gene, raising suspicion of a putative translocation. Thus, case 10 was also evaluated by the tCGH technique, a microarray strategy is based on linear amplification of the junction between partner genes involved in balanced translocations [19,20]. For tCGH, cases were analyzed using a single multiplexed assay designed to detect eight possible translocations (**Table 1**) that are all recurrent in B-cell

Table 1. Primer coverage in the multiplex tCGH assay designed to simultaneously detect eight different balanced translocations associated with B-cell ALL.

Translocation		Array 1 primer pool				Array 2 primer pool				Conditions and frequency
Gene fusion	Rearrangement	Gene	Strand	No. of primers	Mean spacing (kb)	Gene	Strand	No. of primers	Mean spacing (kb)	
*BCR/ABL*1	t (9;22) (q34;q11.2)	*ABL*1	−	47	3.3	*BCR*	+	55	2.5	5% of pediatric and 30% of adult ALL, 1% of AML, and nearly 100% of CML with only 95% observed by cytogenetics [48]
*MLL/AFF*1	t (4;11) (q21;q23)	*AFF*1	−	35	2.9	*MLL*	+	11	2.1	2% of childhood ALL [49], <1% of AML [50-52], 3% of t (4;11) seen in AML [53]
*MLL/MLLT*3	t (9;11) (p22;q23)	*MLLT*3	+	30	3.2	*MLL*	+	11	2.1	Reported rarely in ALL [54], 7% of de novo AML, 46% of secondary AML, seen exclusively in M5 in de novo cases, M0 and M4 seen in secondary cases [55]
*PBX*1/*TCF*3	t (1;19) (q23;p13)	*TCF*3	−	7	2.4	*PBX*1	−	41	3.2	5% of childhood ALL, 20% of childhood pre B-cell ALL, also seen in T-ALL and AML [56]
*ETV6/RUNX*1	t (12;21) (p13;q22)	*RUNX*1	+	54	3.2	*ETV*6	+	83	3.0	25% of pediatric B-cell ALL, 3% of adult B-cell ALL [48]
*MLL/EPS*15	t (1;11) (p32;q23)	*EPS*15	+	27	3.2	*MLL*	+	11	2.1	Reported rarely in ALL [54], <1% of AML [50]
*HLF/TCF*3	t (17;19) (q22;p13)	*TCF*3	−	7	2.4	*HLF*	−	6	2.4	1% of pediatric B-cell ALL [56,57]
*MLL/MLLT*1	t (11;19) (q23;p13.3)	*MLLT*1	+	23	3.1	*MLL*	+	11	2.1	2% of childhood AML [51], seen frequently in ALL [54,58]
Total primers				223				196		

ALL. For each specimen tested, two separate linear amplification reactions were performed using multiplex primer pools designed to amplify one partner of each of the eight targeted balanced translocations (**Table 1**).

Unique DNA primers for genes of interest were designed using MacVector V11.1 (MacVector, Inc., Cary, NC) software with an average spacing of one primer every 2000 to 3500 bp. Primers were mixed into gene-specific and multiplexed reactions at a final concentration of 200 nM for each primer. Linear amplification reactions using 600 ng genomic DNA were performed using the FailSafe PCR System with PreMix Choice using a single buffer (FailSafe PCR 2X PreMix D, Epicentre, Madison, WI) as described by the manufacturer, with 5% DMSO and a final reaction volume of 50 µl. After an initial denaturation at 94˚C for 2 minutes, reactions were amplified for 10 cycles with denaturation at 94˚C for 10 seconds, annealing at 62˚C for 30 seconds, and elongation at 68˚C for 20 minutes. This was followed by another 10 cycles with the same conditions except the denaturation time was increased to 15 seconds and the elongation time was extended by an additional 20 seconds on each successive cycle. The QuickStep 2 PCR Purification Kit (EdgeBio, Gaithersburg, MD) was used according to manufacturer's specifications to purify samples after the linear amplification reactions and prior to labeling.

Microarray analysis was performed using the following methods. Amplified DNA (40 µl of the purified, linear amplification product) and 600 ng of non-amplified, genomic control DNA were labeled with Cyanine dyes (Cy5 for the specimen and Cy3 for the control) using the NimbleGen Dual-Color DNA Labeling Kit (Roche NimbleGen) and purified as described by the manufacturer. Labeled specimen and control DNA were quantified using the NanoDrop 8000 Spectrophotometer (Thermo Scientific), combined (31 µg each), and coprecipitated with 50 µg of Human Cot-1 DNA (Invitrogen, Carlsbad, CA) in isopropanol. Co-precipitated DNAs were hybridized to the arrays at 42˚C for 40 - 72 hours and then washed as described by the manufacturer (Roche NimbleGen). Arrays were scanned at 2 µm using a Roche NimbleGen MS 200 Microarray Scanner and the data were analyzed with NimbleScan 2.6 software. Results were displayed and analyzed with Oncoglyphix®.

2.5. Affymetrix SNP 6.0 Array Analysis

For Case 2, SNP microarray analysis was performed using the Affymetrix SNP Array 6.0 (Affymetrix, Santa Clara, CA) according to manufacturer's instructions. Data were analyzed using Affymetrix Chromosome Analysis Suite (ChAS) software also in accordance with the manufacturer's instructions and visualized with Oncoglyphix®.

3. RESULTS

3.1. Detection of Balanced Translocations by TCGH

Prior chromosome analysis and/or FISH indicated the presence of a clinically relevant balanced translocation in 10 of 20 cases (**Tables 2** and **3**) and aCGH results identified a potential additional case (case 10) with a CNA within the *MLL* gene indicative of a possible translocation. Of the 11 cases of interest, 10 had sufficient DNA to perform the tCGH assay (**Table 3**). Of these, all nine cases that revealed evidence of a translocation by karyotypes or FISH analysis demonstrate translocations by tCGH. These included four cases of t (9;22), three cases of t (12;21), and two cases of t (4;11) (**Figure 1**). Case 10, which revealed a breakpoint for a genomic copy gain by aCGH, did not exhibit any translocation when analyzed with the tCGH assay. Results provided high resolution mapping of the breakpoints, with precise definition dependent on the oligonucleotide coverage present on the tCGH arrays (**Figure 1**, **Table 3**). All translocation breakpoints were confirmed by PCR using individual primers flanking breakpoints defined by tCGH and standard PCR techniques (data not shown).

3.2. Detection of CNAs

In 15 of the 20 cases (75%), CNAs that were not identified by G-banded analysis or FISH were detected by microarray (**Table 2**). These alterations ranged from focal intragenic imbalances to gains or losses of many megabases of DNA. Focal CNAs, predominantly deletions, involving genes that have previously been described in ALL included *PAX*5 (6 cases), *BTG*1 (4 cases), *IKZF*1 (6 cases), *TOX* (3 cases), *EBF*1 (2 cases), *LEF*1 (1 case), *BTLA* (1 case), *NR*3*C*1 (1 case), *FOXP*1 (1 case), and *TBL*1*XR*1 (1 case). Of the six cases with *PAX*5 CNAs, four consisted of heterozygous deletions (~200 to 250 kb) involving one or more exons at the 5' end of the gene (Cases 6, 9, 12, and 19; **Table 2** and **Figure 2(a)**), one case showed both a focal intronic loss and a multicopy gain involving exons 2 - 5 in *PAX*5 (Case 13; **Table 2** and **Figure 2(b)**) and another case demonstrated amplification within *PAX*5 that included exons 2 - 5 (Case 1, **Table 2** and **Figure 2(b)**). In addition to the six cases with focal *PAX*5 CNAs, one additional case (Case 5; **Table 2**) had heterozygous loss of *PAX*5 as the result of loss of 9p from a dic (9;20). Note that for *TBL*1*XR*1, the single observed deletion (Case 8) ends 37 kb distal to the 5'-end of the gene and may not impact expression.

All four cases with t (9;22) had loss of *IKZF*1 including deletions of varying sizes, and one t (9;22) positive case had monosomy 7 (Case 3). One case showed biallelic loss involving *IKZF*1 due to loss of 7p from an i (7q),

Table 2. Summary of microarray and other cytogenetic results for 20 B-cell ALL cases.

Case	Age	Cytogenetic Results	FISH Results	Microarray (hg18)	Significant microarray findings	Unclear microarray findings
1	18	47, XY, add (3) (p?12), + 5, ? add (5) (q?31), add (7) (q32), add (9) (p?13), der (9) t (3;9) (p21; p?21), add (11) (q?23) [16]/46, XY [6]	nuc ish (CDKN2Ax0, CEP9x2) [15/200], (MLLx2) [200]	arr 2p11.2 (89,257,482-89,276,547) x1, 2p11.2 (89,315,265-89,325,297) x1, 2p11.2 (89,613,887-89,624,078) x1, 2p11.2 (89,663,137-89,680,741) x1, 3p22.1p21.31 (40,634,611-49,200,205) x1, 5p15.33q35.3 (129,331-180,857,866) x3, 8q12.1q12.3 (58,374,940-62,633,890) x1, 9p23p21.1 (12,907,152-30,034,167) x0, 9p22.1p21.3 (19,729,146-24,769,648) x0, 9p13.2 (36,984,750-37,014,347) x4, 11q22.3 (107,132,679-109,709,712) x1, 11q23.2q23.3 (114,153,819-116,706,955) x1, 12q21.33 (90,847,497-91,060,832) x1, 14q11.2 (21,989,192-22,070,331) x1, 14q32.33 (105,399,628-105,511,549) x1, 22q11.22 (20,716,186-20,930,051) x1, 22q11.23 (22,674,846-22,723,991) x1 Abnormal Male	Trisomy 5 9p deletion with biallelic deletion of *CDKN2A* and *CDKN2B* Deletion of *ATM* Intragenic *PAX5* amplification	*IGK*, *IGH*, *GSTT*1 deletions 3p deletion including several genes (*CTNNB*1 involved in wnt-catenin signaling)
2	65	46, XX, add (4) (p11.2) [4]/46, XX [16]	NA	arr 1p36.33q44 (856,951-247,148,324) x2 ~ 3, 6p25.3q27 (128,203-170,736,131) x3 ~ 4, 8p23.3q24.3 (1-146,274,826) x3 ~ 4, 9p21.3 (21,909,765-22,054,630) x0 ~ 1, 10p15.3q26.3 (143,762-135,253,240) x4, 11p15.5q25 (188,204-134,425,038) x2 ~ 3, 12p13.33q24.33 (60,861-132,267,241) x2 ~ 3, 13q12.11q34 (18,454,945-114,103,644) x3, 14q11.2q32.33 (19,528,022-106,340,244) x3, 14q32.33 (105,402,089-105,421,752) x1, 19p13.3q13.43 (1-63,811,651) x4, 21q11.2q22.3 (14,406,100-46,915,771) x4, 22q11.1q13.33 (15,912,798-49,691,432) x4 Abnormal Female	Mosaic or non-mosaic tetrasomy for 1,6,8,10,11,12,13,14, 19,21,22 Deletion of *CDKN2A* and *CDKN2B*	Deletion in *IGH*
3	65	45, XX, -7, t (9;22) (q34; q11.2) [12]/46, XX [8]	NA	arr 1p33 (51,174,821-51,208,318) x1, 2p11.2 (88,696,337-89,376,097) x1, 2p11.2 (89,561,572-89,912,623) x1, 7p22.3q36.3 (1-158,821,424) x1, 14q32.33 (105,401,418-105,602,516) x1, 22q11.22 (20,634,021-20,930,168) x1 Abnormal female	Monosomy 7 (*IKZF*1)	Deletion involving *CDKN2C* Ig deletions
4	73	45, XX, der (3;7) (p10; q10) [6]/46, XX [14]	NA	arr Xq27.3q28 (144,670,466-154,876,029) x3, 2p11.2 (89,014,482-89,348,801) x1, 2p11.2 (89,590,599-89,912,623) x1, 3q11.2q21.1 (95,167,813-124,286,166) x1, 3q21.3q26.2 (130,654,972-169,688,965) x1, 7p22.3p11.1 (130,978-57,515,054) x1, 7q31.33q36.3 (124,339,888-158,804,322) x1, 9p21.3 (21,005,360-22,356,302) x1, 12p13.31p12.3 (9,155,209-17,104,302) x1, 14q32.33 (105,345,270-105,612,992) x1, 22q11.22 (20,643,480-20,930,051) x1 Abnormal female	Large deletions involving 3q Deletions of 7p (*IKZF*1) and 7qter Deletion of *CDKN2A* and *CDKN2B* Gain of Xqter	Ig deletions Large 3q deletion includes *EPHA*6, *LNP*1, *TFG*, *ALCAM*, *CBLB*, *CD*200, *BTLA*, *DRD*3, *ZBTB*20, *GAP*43, *GSK3B*, *MIR*198, *HSPBAP*1, *DIRC*2
5	1	45, XY, dic (9;20) (p13.2; q11.2) [15]/46, XY [5]	nuc ish (CDKN2Ax0, CEP9x2) [39/200], (CEP4, ABL1, CEP10, MLL, ETV6,D17Z1, BCR) x2 [200]	arr 2p11.2 (88,932,826-89,325,297) x0~1, 2p11.2 (89,613,887-89,912,623) x0~1, 9p24.3p13.2 (188,160-36,846,193) x1~2, 9p21.3 (21,132,703-22,868,081) x0, 14q11.2 (21,937,493-22,074,385) x1, 14q32.33 (105,401,418-105,597,823) x1 20q11.21q13.33 (30,646,941-62,359,694) x1 Abnormal female	9p deletion with biallelic deletion of *CDKN2A* and *CDKN2B* Large deletion involving most of 20q	*IGK* and *IGH* deletions

Continued

6	8	46, XY, del (1) (p34p36.1), del (6) (q13q21), del (9) (q13q22), add (10) (p11.2), t (12;15) (p13;q11.2), add (20) (q11.2) [8]/46,sl, del (1) (q32q42), t (6;21) (p21;q22) [cp7]/46, XY[5]	NA	arr Xp21.1 (32,857,314-33,252,699) x0, 1p36.32p35.1 (4,617,997-33,430,623) x1, 1q42.13q42.2 (226,166,028-231,338,987) x1, 2p11.2 (88,814,253-89,348,801) x1, 2p11.2 (89,590,599-89,912,416) x1, 4q21.3q22.1 (88,176,109-88,211,992) x1, 5q31.3 (142,709,231-142,776,040) x1, 5q35.1 (170,669,914-170,671,184) x1, 6p22.2p22.1 (25,011,637-26,167,410) x1, 6q16.3q21 (104,163,415-112,301,878) x1, 6q22.31 (125,873,889-126,139,853) x1, 6q23.3q25.1 (137,784,022-151,883,174) x1, 9p21.3p21.2 (20,101,054-26,709,187) x1, 9p21.3 (21,492,342-22,054,630) x0, 9p13.2 (37,018,557-37,223,691) x1, 9q22.2q33.2 (91,719,661-123,306,663) x1, 14q11.2 (21,943,554-22,052,134) x1, 14q32.33 (105,401,418-106,005,780) x1, 20p12.2 (10,369,721-10,400,575) x1, 21q22.3 (41,576,548-42,780,374) x1, 22q11.22 (20,716,186-20,852,537) x1 Abnormal male	Deletion of: *AFF*1, *NR*3*C*1, *TLX3*, *PAX*5, *C*20*orf*94 Biallelic deletion of *CDKN*2*A*	*DMD* deletion Ig and *TCR* deletions 1p deletion with large number of cancer features 1q deletion (*WNT*3*A* and *EGLN*1*)* 4q deletion (*AFF*1)
7	3	46, XX, del (12) (p11.2p13), der (12) (12pter- > 12p13::21q22- > 21q11.2::12p13- > 12qter), der (21) (21pter- > 21q11.2::12p13::21q22 - > 21qter) [4]/46, X [18]	NA	arr 2p11.2 (88,932,826-89,294,676) x1, 2p11.2 (89,653,265-89,740,401) x1, 3q26.2 (170,532,557-170,862,897) x1, 7q34 (141,848,384-142,230,606) x1, 12p13.31p11.1 (7,183,961-34,107,615) x1, 14q32.33 (105,402,089-105,592,918) x1, 22q11.22 (21,570,725-21,577,402) x1 Abnormal female	*MECOM* deletion *ETV*6 deletion	Ig and *TCR* deletions
8	3	46, XY, der (12) (21pter- > 1p12::12p13- > 12qter),der(19) (19pter-> 19q13.1::12p13::21q22 ->21qter), der(21)(19qter->19q13.1: :21p12-> 21q22::12p13-> 12pter)[8]/46, XY[12]	NA	arr 2p11.2 (88,932,826-89,912,901) x1, 3q26.32 (178,434,828-179,384,704) x0, 12p13.2 (11,789,624-11,810,529) x1, 12p13.2p12.1 (11,920,600-21,600,799) x1, 14q11.2 (21,978,139-22,054,108) x1, 14q32.33 (105,402,089-105,588,340) x1 Abnormal male	Biallelic deletion abutting 5'end of *TBL*1*XR1* *ETV*6 deletion	*IGK* and *IGH* deletions *TRA/D* deletion
9	52	46, XX, t (9;22) (q34; q11.2), add (20) (q11.2), del (21) (q21q22.3) [19]	NA	arr 2p11.2 (88,966,582-89,912,901) x1, 3p14.1 (71,174,477-71,536,728) x1, 3q13.2 (113,612,958-113,698,834) x1, 4q25 (109,187,792-109,298,195) x1, 6q21 (109,346,113-109,434,527) x1, 7p12.2 (50,214,532-50,444,361) x1, 9p21.3 (21,854,404-22,229,673) x1, 9p13.2 (37,003,771-37,259,921) x1, 14q11.2 (21,978,139-22,078,275) x1, 14q32.33 (105,346,465-105,550,411) x1, 14q32.33 (105,780,835-106,261,145) x1, 15q21.3 (55,091,960-55,138,547) x1, 17q21.1 (35,496,033-35,597,274) x1, 20q11.22q13.32 (33,293,965-56,987,158) x1, 22q11.22 (20,643,540-21,094,301) x1 Abnormal female	Deletions involving: *FOXP*1, *BTLA*, *IKZF*1, *CDKN*2*A/B*, and *PAX*5 Large deletion of 20q	Deletions of: *LEF*1, *SESN*1, *TCF*12 and *CASC*3 *IGH*, *IGL* and *TCRA/D* deletions
10	52	48, XY, + 19, + mar [1]/51, idem, + 6, + 13, + 21 [18]/46, XY [1]	NA	arr 3q27.3 (188,938,761-188,946,429) x1, 6p25.3q27 (1-170,899,992) x3, 8q24.21 (128,815,237-128,819,784) x1, 11q14.2q14.3 (85,873,769-89,596,519) x4, 11q22.1q22.3 (97,609,686-108,855,018) x4, 11q23.3 (116,666,706-117,858,516) x4, 11q23.3q24.1 (119,339,943-122,322,820) x4, 11q24.2q25 (125,708,527-134,425,038) x4, 13q12.11q34 (18,454,945-114,103,644) x3, 19p13.3q13.43 (1-63,811,651) x3, 21q11.2q22.3 (14,406,100-46,915,771) x3, 22q11.23 (21,888,154-21,893,301) x1 Abnormal male	Trisomy 6, 13, 19, 21	

Continued

11	66	42, XY, del (3) (p13), del (4) (q21), add (5) (q10), -7, -9, -10, del (11) (q24), + der (11) ? dic r (11;11) (q10;q10) trp ~ qdp (11) (q23q23), -16,-17 [10]/46, XY[5]	NA	arr 3p22.2p12.1 (36,729,918-84,568,449) x1, 4q21.1q35.2 (77,873,417-191,152,793) x1, 5p13.2q35.3 (37,218,239-180,619,169) x1, 7p21.3q36.3 (8,233,377-158,804,322) x1, 9q13q31.3 (70,222,356-110,957,883) x1, 9q31.3q33.2 (112,622,698-123,571,177) x1, 10q11.21q23.31 (45,473,706-92,230,997) x1, 11p15.5 (188,204-674,923) x1, 11p15.5p15.1 (734,095-17,733,802) x3, 11p15.1p13 (19,677,938-31,108,639) x3, 11q13.2q14.1 (67,520,607-78,308,080) x3, 11q14.1q21 (80,148,837-93,138,032) x3, 11q21 (93,170,146-93,502,521) x1, 11q21 (94,786,412-95,754,049) x1, 11q21q23.3 (95,810,329-117,493,384) x3, 11q23.3 (117,516,396-118,565,878) x3~4, 11q23.3 (118,577,948-119,462,138) x1, 11q23.3q25 (119,499,554-134,425,038) x3, 16p13.3q24.3 (35,819-88,657,641) x1, 17p13.3p13.1 (1-7,574,347) x1 Abnormal male	Complex result Monosomy 16 Deletions including: *TP*53, *HRAS*, *IKZF*1 and many more Highly rearranged chromosome 11	
12	5	47, XY, + del (X) (q13q24), del (12) (p12p13) [4]/46, XY, + del (X) (q10), del (12) (p12p13), -13 [5]/46, XY [11]	nuc ish (ETV6x2, RUNX1x3) (ETV6 con RUNX1x1) [199/200], (CEP4, CEP10, CEP17)x2[500], (P16, ASS, ABL1,MLL, BCR)x2[200]	arr Xp22.33p11.21 (3,905,651-56,085,692) x2, Xq26.2q28 (130,503,588-154,584,236) x2, Xq28 (154,584,237-154,876,029) x2, 2p11.2 (88,932,826-89,188,304) x1, 9p13.2 (36,917,532-37,148,414) x1, 12p13.2p12.1 (11,924,656-23,155,752) x1, 13q11q34 (18,347,178-114,103,644) x1, 14q11.2 (21,937,493-22,074,385) x1, 14q32.33 (105,402,089-106,245,460) x1, 16p11.2 (30,798,095-31,840,945) x1, 20p12.2 (10,369,721-10,470,986) x1, 22q11.22 (20,848,203-21,608,981) x1 Abnormal male	Deletion of *PAX*5, *C*20*orf*94, *ETV*6 Monosomy 13 Large gain on Xp	Ig and *TCRA/D* deletions *FUS* and *MYST*1 deletion
13	6	46, XY, r (9) (p? 13q? 33) [12]/46, XY [18]	ish r (9) (CDKN2A-, CEP9+, ASS-, ABL1-). nuc ish (CEP4x2, CEP10x2, D17Z1x1) [24/200], CDKN2Ax0, CEP9x2) [120/200], (ASSx1, ABL1x1, BCRx2) [153/200], MLL,ETV6, RUNX1)x2	arr 7p14.1 (38,286,931-38,343,558) x1, 7q34 (141,693,456-141,719,136) x1, 8q12.1 (59,952,847-60,820,059) x1, 9p24.3p21.2 (188,160-27,486,736) x1, 9p21.3 (21,233,696-22,229,673) x0, 9p13.2 (36,965,324-36,969,492) x1, 9p13.2 (36,972,327-37,012,379) x3, 9q33.2q34.3 (123,131,618-140,130,559) x1, 12q21.33 (90,774,706-91,060,832) x1, 14q11.2 (21,309,211-22,080,207) x1, 14q32.33 (105,401,418-105,481,976) x1 Abnormal male	9p deletion with biallelic deletion of *CDKN2A* and *CDKN2B* and terminal loss of 9qter *PAX*5 deletion and duplication Deletion involving *BTG*1	Ig and *TCR* deletions *TOX* deletion
14	25	46, XY, t (2;12) (p15;q24.3), I (7) (q10), t (9;22) (q34;q11.2) [9]/47, sl, +8 [2]/48, sdl1, + der (22) t (9;22) [1]/46, XY[11]	NA	arr 2p11.2 (88,932,826-89,912,416) x1, 7p22.3p11.1 (171,273-57,515,054) x1, 7p12.2 (50,381,509-50,429,675) x0, 7q11.21q36.3 (62,030,364-158,821,424) x3, 9p21.3 (21,822,754-21,994,012) x1, 9p21.3 (21,964,305-21,994,012) x0, 12q21.33 (90,847,497-91,060,832) x1, 14q11.2 (21,989,192-22,099,342) x1, 14q32.33 (105,219,696-106,181,548) x1, 20p12.2 (10,369,721-10,400,575) x1, 22q11.22 (20,847,781-20,852,477) x1 Abnormal male	Gain of 7q/loss of 7p, consistent with i(7q) Homozygous *CDKN2A/B* deletion Deletions involving *IKZF*1, *BTG*1 and *C*20*orf*94	Ig and *TCR* deletions

Continued

15	7	58,XY,+X,+4,+5,+6,+9,+10,+11,+14,+16,+17,+18,+21	NA	arr Xp22.33q28 (1-154,913,754) x2, 2p11.2 (89,257,482-89,277,059) x1, 4p16.3q35.2 (45,627-191,152,793) x3, 5p15.33q35.3 (129,331-180,619,169) x3, 6p25.3q27 (128,203-170,736,131) x3, 7p14.1 (38,259,152-38,373,349) x1, 9p24.3q34.3 (188,160-140,130,559) x3, 10p15.3q26.3 (1-135,374,737) x3, 11p15.5q25 (1-134,452,384) x3, 14q11.2 (21,962,291-22,052,074) x1, 14q11.2q32.33 (19,528,022-106,340,244) x3, 14q32.33 (105,402,089-105,481,650) x1, 16p13.3q24.3 (35,819-88,657,641) x3, 17p13.3q25.3 (49,128-78,612,915) x3, 18p11.32q23 (123,388-76,100,854) x3, 21q11.2q22.3 (14,406,100-46,915,771) x3 Abnormal male	Trisomy 4, 5, 6, 9, 10, 11, 14, 16, 17, 18, 21, X	Ig and *TCR* deletions
16	52	46, XY, t (9;22) (q34;q11.2) [4]/46, idem, t (5;8) (q33;q13) [10]/46, XY [4]	NA	arr 5q33.3 (158,197,556-158,228,953) x1, 7p12.2 (50,381,509-50,444,361) x1, 8q12.1 (60,057,422-60,217,721) x1, 14q32.33 (105,400,678-105,481,976) x1 Abnormal male	Deletions of *EBF*1 and *IKZF*1	*TOX* deletion *IGH* deletion
17	37	46, XX, t (4;11) (q21;q23) [8]/46, XX [13]	nuc ish MLLx2) (5'MLLsep3' MLLx1) (181/248) nuc ish (ABL1, BCR) x2 [212]	arr 14q32.33 (105,400,678-105,466,992) x1, 19p13.2 (7,847,230-7,895,398) x1		Deletions involving *IGH* and *MAP2K7*
18	52	46, XY, t (4;11) (q21;q23) [16]/46, XY [2]	NA	arr 8q24.3 (145,486,837-145,741,796) x1, 9p21.3 (21,909,765-21,964,305) x1, 14q32.33 (105,387,659-105,481,976) x1 Abnormal female	Deletion involving *CDK2NA*	8q deletion involving several genes including *CYHR*1, *KIFC*2, *FOXH*1, *PPP*1*R*16*A*, *RECQL*4, *LRRC*14, *LRRC*24 *IGH* deletion
19	<18	NA	nuc ish (TELx2), (AML1x3), (TELconAML1x1) [186/200] or t (12; 21) (p13; q22)	arr Yq11.21q12 (12,808,314-57,443,437) x0, Yq12 (57,443,438-57,735,230) x0, 1p35.1 (32,264,901-32,617,308) x1, 2p11.2 (85,092,808-86,895,965) x1, 2p11.2 (88,932,826-89,233,635) x1, 2p11.2 (89,315,265-89,327,175) x0, 5q33.3 (158,373,309-158,465,259) x1, 6p22.1 (26,191,062-26,350,720) x1, 6q14.1q27 (81,894,370-170,736,131) x1 ~ 2, 8q24.21 (128,815,674-128,821,325) x1, 9p13.2 (36,917,532-37,020,544) x1, 10p15.3q26.3 (172,285-135,099,923) x3, 12p13.2 (11,696,155-11,820,208) x1, 12q21.33 (90,690,181-91,060,832) x1, 14q11.2 (21,945,491-22,064,305) x1, 14q32.33 (105,371,094-106,245,460) x1, 15q12q21.2 (24,919,738-48,645,080) x1, 19q13.31 (49,947,625-49,951,395) x1, 20p13p12.3 (16,653-8,652,488) x1 Abnormal male	Large deletions of 6qter, 15q12q21.2 and 20pter Deletions of *EBF*1, *MYC*, *PAX5*, *ETV6* Trisomy 10	Deletions of *LCK*, *HFE*, *BCL3* and Yqter Ig and *TCR* deletions
20	13	54, XY, + X, + 4, + 6, + 14, + 17, + 18, + 21, + 21 [9]	nuc ish (4p11-q11x3) [174/223], D17Z1x3) [188/223], (10p11.1-q11.1x2) nuc ish (ETV6x2, RUNX1x3-4) [223/225] nuc ish (ABL1, BCR) x2 nuc ish (MLLx2) [200]	arr Xp22.33q28 (1-154,913,754) x2, 4p16.3q35.2 (45,627-191,152,793) x3, 6p25.3q27 (1-170,899,992) x3, 14q11.2q32.33 (19,528,022-106,368,585) x3, arr 14q32.33 (105,402,089-105,481,770) x1, 17p13.3q25.3 (1-78,774,742) x3, 18p11.32q23 (123,388-76,100,854) x3, 21q11.2q22.3 (14,406,100-46,915,771) x4 Abnormal male	Trisomy 4, 6, 14, 17, 18, X; Tetrasomy 21	*IGH* deletion

Abbreviations: FISH, fluorescence *in situ* hybridization; NA, not applicable.

Table 3. Translocation microarray analysis (tCGH) for cases of B-cell ALL with known or suspected balanced translocations.

Case	Translocation seen by karyotype, FISH and/or aCGH	Detection by tCGH	Breakpoints (hg18)	Breakpoint Distinction
3	t (9;22) (q34;q11.2)	*BCR/ABL*1	chr9:132598080-132598270	5'-end *ABL*1
			chr22:21889002-21889168	Central m-BCR
7	del (12) (p11.2p13), der (12) (12pter- > 12p13::21q22- > 21q11.2::12p13- > 12qter), der (21) (21pter- > 21q11.2::12p13::21q22- > 21qter)	*ETV6/RUNX*1	chr12:11921539-11921655	3'-end *ETV*6
			chr21:35330287-35331177	5'-end of intron 1 in *RUNX*1
8	der (12) (21pter- > 21p12::12p13- > 12qter), der (19) (19pter- > 19q13.1::12p13::21q22- > 21qter), der (21) (19qter- > 19q13.1::21p12- > 21q22::12p13- > 12pter)	*ETV6/RUNX*1	chr12:11920600-11920834	near 3'-end *ETV*6
			chr21:35204437-35204739	3'-end of intron 1 in *RUNX*1
9	t (9;22) (q34;q11.2)	*BCR/ABL*1	chr9:132582114-132583554	5'-end *ABL*1
			chr22:21961745-21961958	Central M-BCR
10	Aneuploidy by karyotypes; Possible MLL gene breakpoint by aCGH	None Detected		
12	del (12) (p12p13) nuc ish(ETV6 × 2, RUNX1 × 3) (ETV6 con RUNX1x1) [199/200], (CEP4, CEP10, CEP17) × 2 [500], (P16, ASS, ABL1, MLL, BCR) × 2 [200]	*ETV6/RUNX*1	chr12:11924656-11924849	most 3-end *ETV*6
			chr21:35266695-35266968	center of intron 1 in *RUNX*1
14	t (9;22) (q34;q11.2)	*BCR/ABL*1	chr9:132635298-132635731	Central intron 1
			chr22:21922842-21923078	Terminal mBCR
16	t (9;22) (q34;q11.2)	*BCR/ABL*1	chr9:132651461-132651567	Central intron 1
			chr22:21908588-21908773	Central m-BCR
17	t (4;11) (q21;q23)	*MLL/AFF*1	chr4:88216921-88217006	Major brpt clust₸
			chr11:117860758-117860864	Major brpt clust₸
18	t (4;11) (q21;q23)	*MLL/AFF*1	chr4:88214869-88215163	Major brpt clust₸
			chr11:117860648-117860758	Major brpt clust₸
19	nuc ish (TEL × 2), (AML1 × 3), (TELconAML1 × 1) [186/200] or t (12;21) (p13;q22)	N/D†		

†N/D = Not determined due to insufficient DNA; ₸ = Major breakpoint cluster [59].

and an additional 48.2-kb deletion within the remaining *IKZF*1 allele (Case 14; **Table 2** and **Figure 3(a)**). Another case showed focal heterozygous loss of the entire *IKZF*1gene, but not flanking genes (Case 9; **Table 2** and **Figure 3(b)**), and a third case had a heterozygous deletion, approximately 60 kb in size, involving only the 3' end of *IKZF*1 (Case 16; **Table 2** and **Figure 3(b)**).

In addition to focal CNAs, gains and losses involving many megabases of DNA, which were only sometimes identified by conventional cytogenetic analysis, were detected by microarray (**Table 2**). Some of these alterations involved genes or regions previously known to be involved in ALL, such as deletions of 9p including *CDKN*2*A* and deletions of 12p including *ETV*6. Nine cases had losses involving *CDKN*2*A* that ranged from intragenic deletions to deletions of many megabases. Three of these cases had heterozygous deletions ranging from 145 kb to 1.4 Mb (Cases 4, 9, and 18; **Table 2**) and six had homozygous deletions (Cases 1, 2, 5, 6, 13, and 14; **Table 2** and **Figure 4**). In case 6, biallelic loss of *CDKN*2*A*, including a 6.6-Mb loss of 9p21.3p21.2, was not seen by conventional cytogenetics. Of note, for case 14 deletion of one of the *CDKN*2*A* alleles that was detected by microarray was below the level of resolution of FISH (~30 kb) (**Figure 4**). In Case 14, homozygous deletion of *CDKN*2*A* was likely to be detectable only by microarray as the lesion (~30 kb) on one allele was likely below the level of resolution of standard *CDKN*2*A* FISH probes (150 kb - 190 kb) (**Figure 4**).

Losses involving *ETV*6 were seen in all three *ETV6/RUNX*1 fusion cases (Cases 7, 8, and 12; **Table 2**, **Figure 1**) and in one case that did not have a t (12;21) (Case 19; **Table 2**). Case 8 demonstrated a loss in *ETV*6 of 21 kb that was only detectable by microarray. Case 10, which was found to have trisomies for chromosomes 6, 13, 19 and 21 but no abnormalities of chromosome 11 by G-banded analysis, also showed multiple two-copy gains throughout 11q by microarray; these included a 1.2-Mb

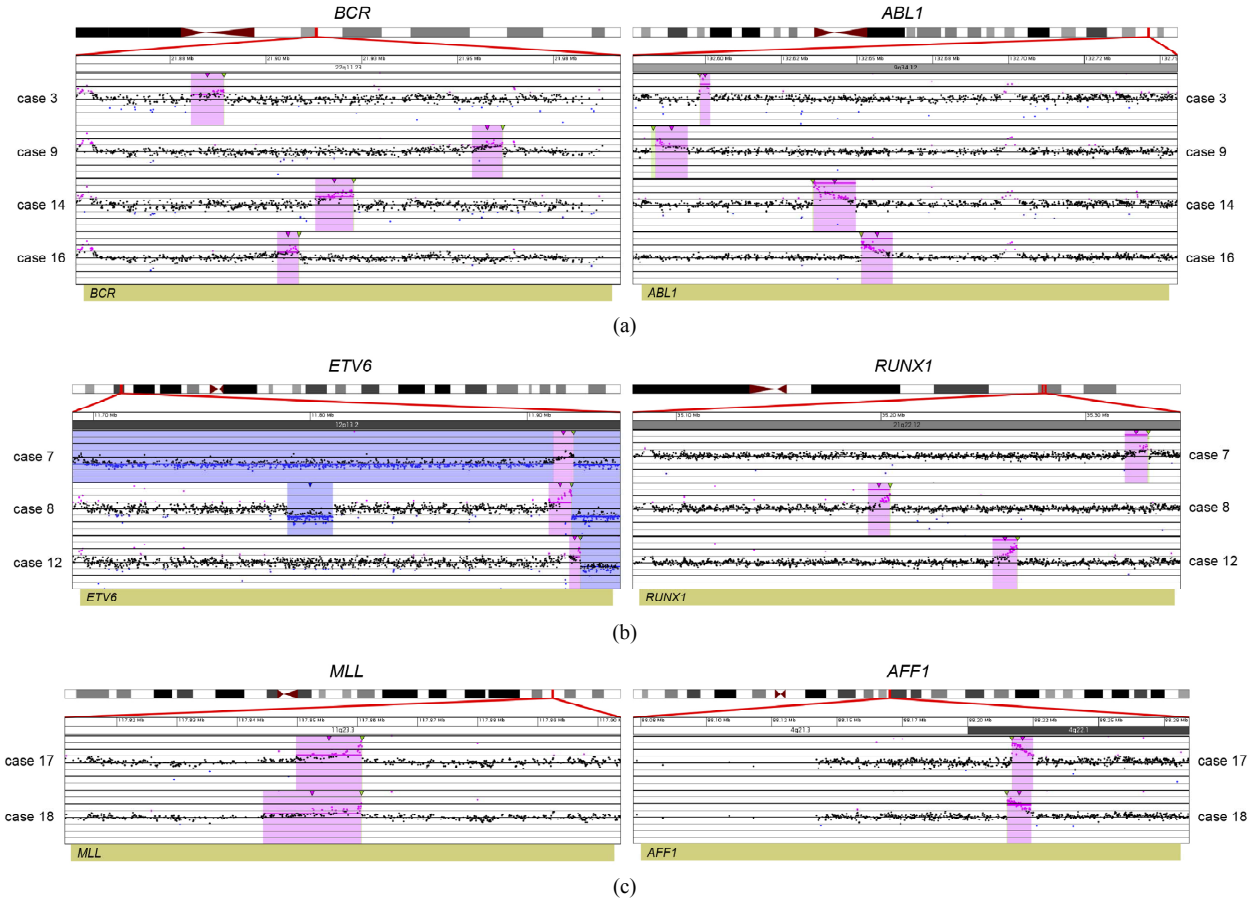

Figure 1. Balanced translocations detected by tCGH in B-cell ALL cases. The probes (pink dots) form a peak, indicating the amplification leading up to the breakpoint region (thin, green triangles). Copy gains (pink shaded regions) and copy losses (blue shaded regions) are also shown. (a) *BCR/ABL*1 translocations seen in cases 3, 9, 14, and 16. (b) *ETV6/RUNX*1 translocations seen in cases 7, 8, and 12. (c) *MLL/AFF*1 translocations seen in cases 17 and 18. For (a)-(c), probes are ordered on the *x*-axis according to physical mapping positions, and values along the *y*-axis represent $\log_2$ ratios of patient:control signal intensities. Results are visualized using Oncoglyphix (Signature Genomics).

gain in 11q 23.3 with a break in the *MLL* gene. Although one cell in this case was noted to have a marker chromosome by G-banding that could conceivably consist of 11q material, the presence of a single cell finding is not considered clonal. Even if the marker is part of the leukemic clone, the gains of 11q could not have easily been determined in this case, even by FISH. In cases that had chromosome abnormalities identified by G-banded analysis, microarray findings frequently resulted in more precise characterization of the genomic imbalances. For example, in case 11, abnormalities of chromosome 11 were noted by G-banded analysis; however, the complex pattern of chromosome 11 gains and losses detected by microarray could not be deduced from the karyotype.

Consistent with previous literature [23], cases with *MLL* gene rearrangements (Cases 17 and 18; **Table 2**) had only a few CNAs each. In contrast, cases with t (9;22) or t (12;21) frequently showed multiple CNAs. With the exception of case 3 that had a t (9;22) and only two CNAs, the remainder of cases with either a *BCR*/*ABL*1 (Cases 9, 14, 16, **Table 2**) or *ETV6*/*RUNX*1 (Cases 7, 8, and 12; **Table 2**) fusion had 4 to 17 CNAs.

3.3. Detection of Hypodiploidy/ Near-Haploidy

For Case 2, the presence of four copies of many chromosomes and two of others suggested that doubling of a hypodiploid/near-haploid clone had generated a hyperdiploid pattern that can be mistaken for high hyperdiploidy [24]. This is a classic cause for concern given the negative prognostic significance of the former and positive prognostic significance of an overlapping, but distinct, hyperdiploid pattern [25]. We, therefore, analyzed Case 2 by Affymetrix SNP 6.0 microarray analysis. The SNP data showed haplotypes consistent with the doubling of a hypodiploid clone with subsequent clonal evolution allowing the poor prognostic marker to be clearly distinguished from high hyperdiploidy in this case (**Figure 5**).

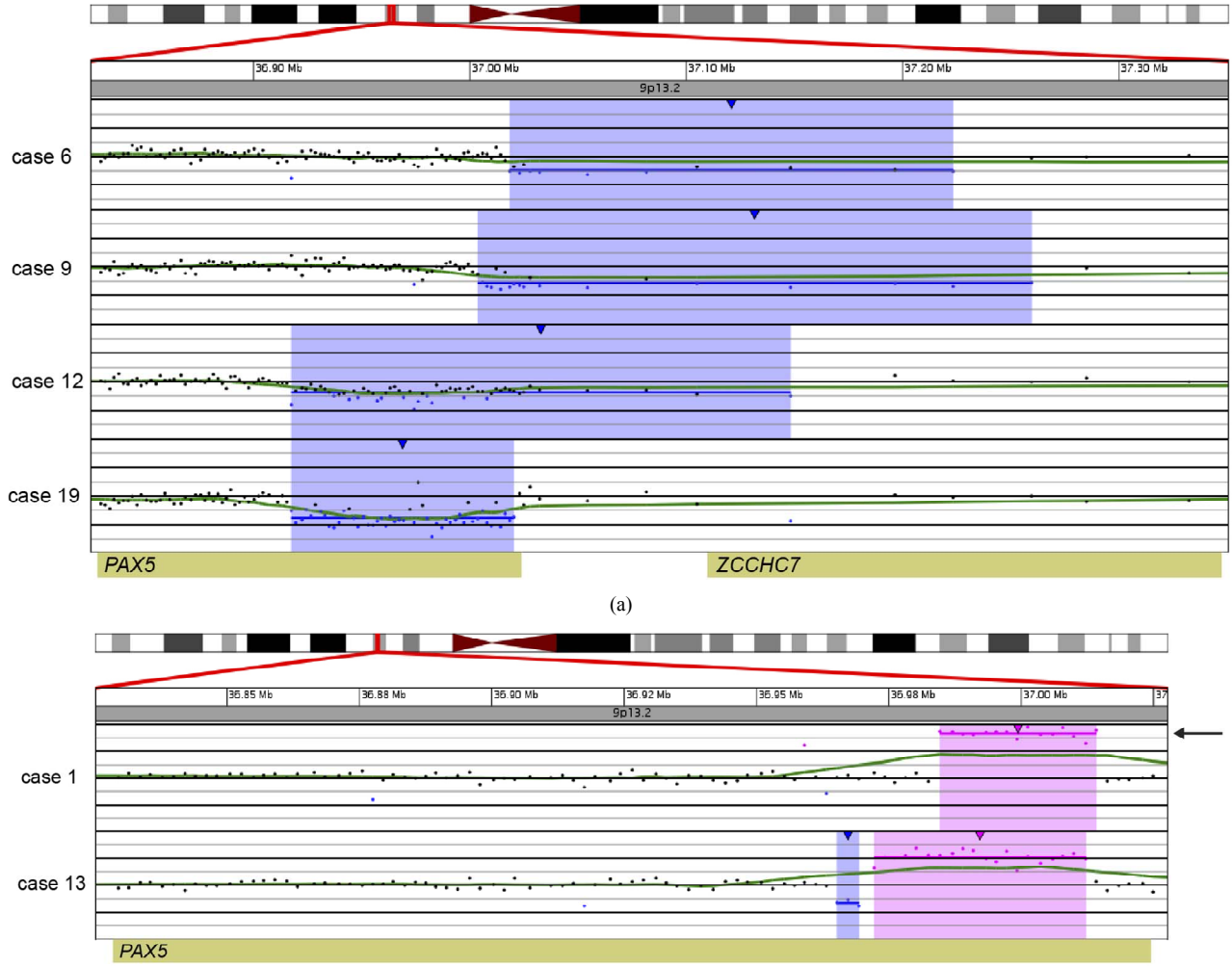

Figure 2. Copy number gains and losses of the gene *PAX5* at 9p13.2 detected by microarray analysis. *PAX5* is involved in the regulation of B-cell development and differentiation. (a) Cases with deletions including one or more exons at the 5' end of *PAX5*: Case 6 has a 205-kb loss, Case 9 has a 256-kb loss, Case 12 has a 231-kb loss, and Case 19 has a 103-kb loss. (b) Intragenic copy number alterations of *PAX5*: Case 1 has a two-copy gain of 30 kb within *PAX5* (arrow) and case 13 has a 4-kb loss and a 40-kb gain within *PAX5*. For A and B, probes are ordered on the *x*-axis according to physical mapping positions, and values along the *y*-axis represent $\log_2$ ratios of patient:control signal intensities. Results are visualized using Oncoglyphix (Signature Genomics).

3.4. Discrepancies between G-Banded Chromosome and Microarray Results

Although it was expected that many CNAs detected by microarray would be missed by G-banded analysis as noted above, there were a few cases in which alterations identified by G-banded analysis could not be substantiated by the microarray results. Some cases were noted to have chromosomes with additional material of unknown origin by G-banding (Cases 1, 2, 6, 9, and 11). If these were true "add" abnormalities, they would be expected to show a deletion distal to the breakpoint of the "add" chromosome and gain of the additional chromosomal material. These imbalances were not seen by microarray. It is unlikely that these abnormalities were missed by the microarray due to low-level mosaicism, because all of the "add" chromosomes were present in a high enough proportion of cells to be detected by microarray. It is possible that some of these "add" chromosomes represent balanced rearrangements, or that they are actually normal chromosomes with distortion due to poor morphology, which is a common problem in karyotyping ALL specimens. In addition, in Case 1, deletions of 3p and 9p were detected by microarray that may be the result of the add (3p) and the add (9p); however, there was no deletion of 7q that would be expected from the add (7q) reported in the same clone from this case. For Case 9, a del(21) (q21;q22.3) was reported by G-banding, but no 21q deletion was detected by microarray. Again, it may be that the 21q abnormality consists of a balanced rear-

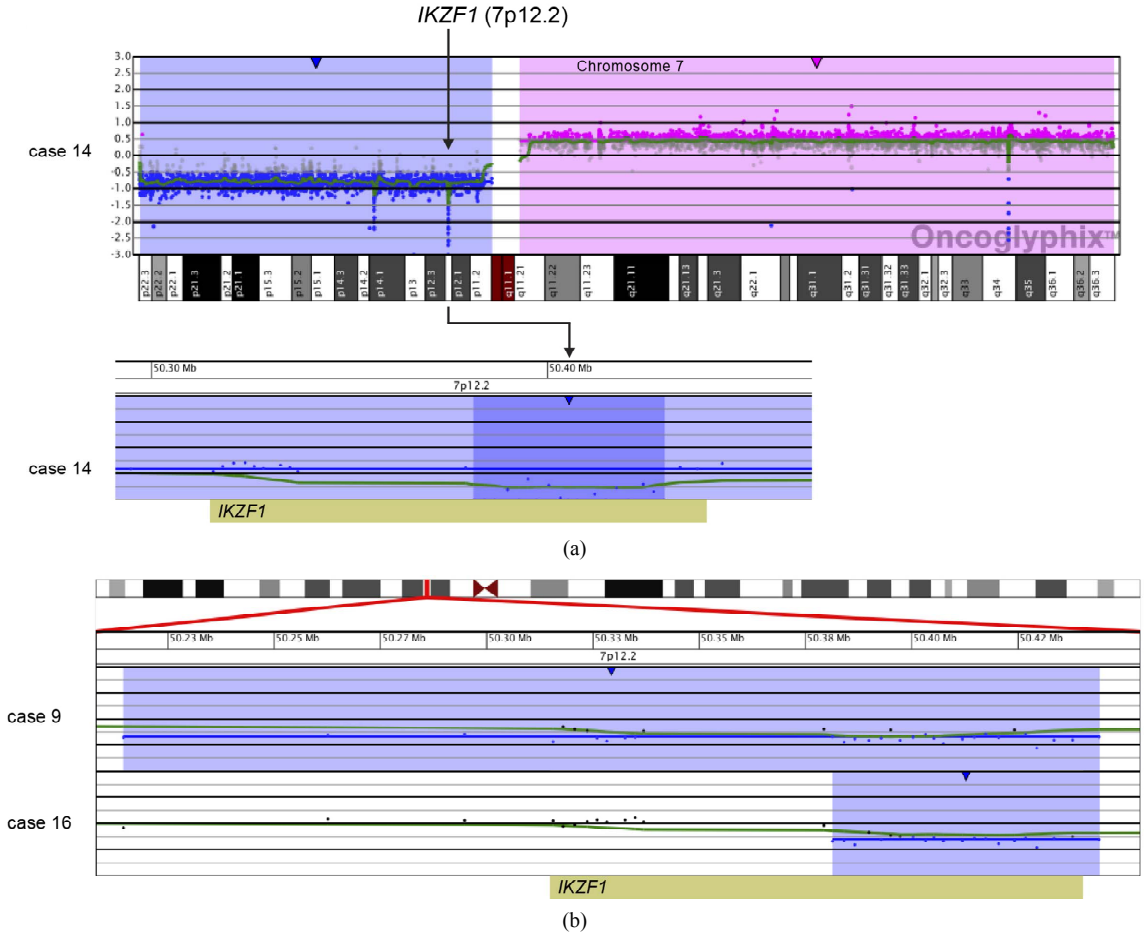

Figure 3. Copy number losses of *IKZF*1 at 7p12.2 detected by microarray analysis. *IKZF*1 is involved in the regulation of B-cell development and differentiation. (a) Microarray results for case 14, showing a copy number loss of the entire p-arm of chromosome 7 (shaded in blue) and a copy number gain of the entire q-arm (shaded in pink). The zoomed-in view of *IKZF*1 at 7p12.2 for case 14 shows an intragenic, biallelic 48.2 kb loss of part of *IKZF*1 (shorter arrow). (b) Microarray results for cases 9 and 16 with copy number losses (shaded in blue) of 7p12.2 that include *IKZF*1. Case 9 has a 320-kb deletion that includes the entire gene, and case 16 has a 62-kb deletion of the 3'-end of the gene. For a and b, probes are ordered on the *x*-axis according to physical mapping positions, and values along the *y*-axis represent $\log_2$ ratios of patient: control signal intensities. Results are visualized using Oncoglyphix (Signature Genomics).

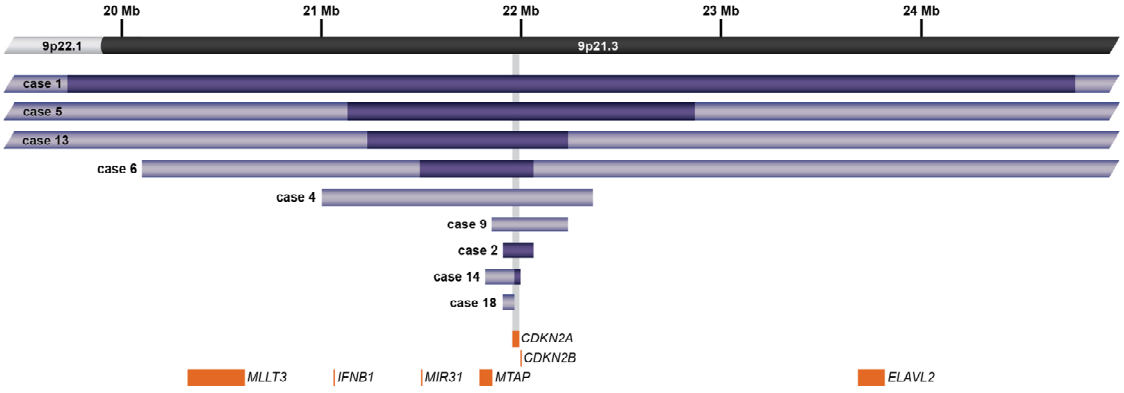

Figure 4. Schematic representation showing deletions of *CDKN2A* at 9p21.3 detected by microarray analysis in nine cases. *CDKN2A* is involved in cell cycle regulation and tumor suppression. Light purple bars represent deletion sizes for each case (based on UCSC 2006 hg 18 assembly); the deletions in Cases 1, 5, 6, and 13 are larger than the genomic region shown. Cases 1, 2, 5, 6, 13, and 14 had biallelic deletions represented by dark purple bars. Orange boxes represent cancer features of interest in the region.

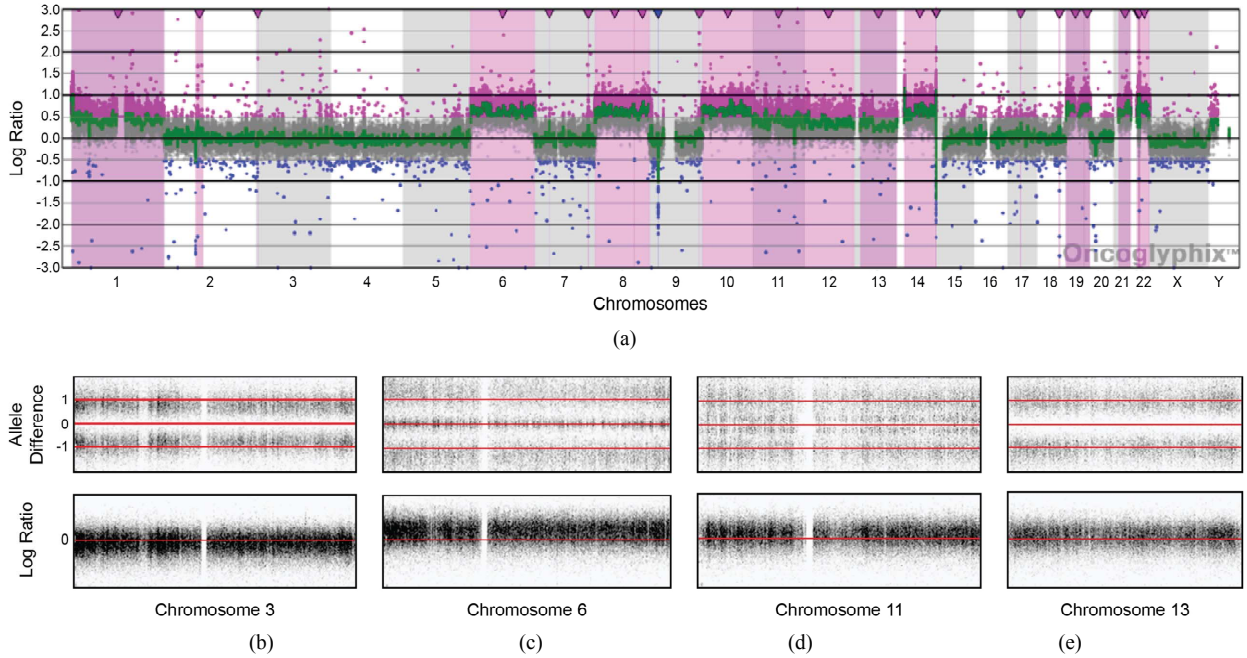

Figure 5. Copy number and SNP microarray analysis of Case 2. (a) aCGH microarray analysis of Case 2 with the Signature OncoChip. Microarray probes are ordered on the *x*-axis according to physical mapping positions, with data for chromosomes 1 - 22, X, and Y being displayed from left to right, and values along the *y*-axis represent $\log_2$ ratios of patient: control signal intensities. Copy gains are shown as pink shaded regions. Variations in $\log_2$ ratios suggest different clones are present in this patient sample. The presence of four copies of many chromosomes and two of others was suggestive of doubling of a hypodiploid clone. (b)-(e) Affymetrix SNP 6.0 analysis of Case 2. The allelic difference for the SNP probes is shown at the top of each panel with the log ratio for the copy number probes shown at the bottom. (b) Data for chromosome 3 are shown as a representative example for chromosomes X, 2, 3, 4, 5, 7, 9, 15, 16, 17, 18, and 20, which showed identical patterns. These data are consistent with a copy number of 2 and loss of heterozygosity with the presence of only two genotypes (AA and BB). (c) Data for chromosome 6 are shown as a representative example for chromosomes 6, 8, 10, 14, 19, 21, and 22, which showed identical patterns. These data are consistent with a copy number of 4 and no loss of heterozygosity with the presence of only three genotypes (AAAA, AABB, and BBBB). (d) Data for chromosome 11 are shown as a representative example for chromosomes 1, 11, and 12, which showed identical patterns. These data are consistent with a copy number of three chromosomes and no loss of heterozygosity with the presence of four genotypes (AAA, AAB, BBA, and BBB). (e) Data for chromosome 13 are shown, the only chromosome in this patient sample with this pattern. These data are consistent with a copy number of three chromosomes and loss of heterozygosity with the presence of only two genotypes (AAA and BBB). The presence of homozygosity in the chromosomes with two copies as well as the presence of heterozygosity in the chromosomes with four copies is consistent with doubling of a hypodiploid clone. For (a)-(e), results are visualized using Oncoglyphix (Signature Genomics).

rangement, with 21q material missing from one chromosome 21 homologue but inserted elsewhere in the genome. In case 11, monosomy 7, 9, and 10 were reported by G-banded analysis. The microarray results for this case showed loss of most of chromosome 7 but normal copy number from 7p21.3 to the terminus, an interstitial loss of 9q but normal copy number for the remainder of chromosome 9, and an interstitial deletion of 10q with normal copy number for the rest of chromosome 10. As this case had a complex karyotype, it is not surprising that the retained segments of chromosomes 7, 9, and 10 could be present in a rearranged form that was not identifiable by G-banding. These cases illustrate that microarray analysis can clarify the karyotype results and provide a more accurate and unbiased assessment of imbalances in the cancer genome.

4. DISCUSSION

Conventional cytogenetics has been the standard of care for the identification of diagnostically and prognostically significant genomic alterations in ALL, despite the inherent challenge posed by these cases due to poor chromosome morphology. Although FISH has allowed for the detection of known alterations regardless of the presence of metaphase cells or poor morphology, the number of FISH tests that can be performed on a given case is limited, and some of the recently detected deletions of known clinical significance are below the resolution level of FISH. Despite those limitations and in spite of the relative small size of the cohort studied, the cases examined showed an age-related pattern of abnormalities highly consistent with that previously reported ([26]).

Hyperdiploidy and *ETV*6/*RUNX*1 translocations were seen exclusively in pediatric cases, while *BCR*/*ABL*1 fusions were restricted to adults.

In this study, microarray analysis allowed for the identification of CNAs below the resolution of chromosome analysis and FISH in 75% of cases. Furthermore, a novel application of linear amplification prior to aCGH, termed tCGH, identified all known translocations in nine specimens. In addition, the translocation breakpoints were identified to within a few hundred base pairs, and in some cases submicroscopic deletions at the translocation breakpoints were evident by the microarray analysis.

Most cases in our study also had additional CNAs detected by microarray, some of which are known to be prognostically significant as summarized below. Similar to other recent studies, CNAs in genes that are involved in B-cell development and differentiation were frequently detected in ALL. Other less frequent deletions in genes involved in B-cell development and differentiation that we detected have been reported in other studies of ALL and include *LEF*1, *BTLA*, and *EBF*1 [27].

*PAX*5, one of the most frequent targets of alterations in ALL, encodes a transcription factor required for B-lineage commitment and maturation [28]. Consistent with recent array studies [10-12], *PAX*5 losses or gains were present in 33% of the ALL cases tested here (**Figure 3**). Not including a case with loss of the entire 9p due to a dicentric (9;20), all other cases with CNAs involving *PAX*5 were either intragenic or only a few hundred kilobases in size. For the current study, cases 9 and 12 showed *PAX*5 CNAs and were *BCR*/*ABl*1 and *ETV*6/*RUNX*1 positive, respectively, with the remaining 4 cases being translocation negative. The *PAX*5 CNAs consisted of both deletions and amplifications. Mutations in *PAX*5, including intragenic amplification as seen in case 1, have been previously described in B-cell ALL, although such alterations appear not to be correlated with a particular outcome [16,29].

Also consistent with recent literature, all cases with a t (9;22) in our study were found to have loss of *IKZF*1 [30]. In one of these cases (Case 16), the deletion was only 63 kb in size, which is below the resolution of FISH probes that are typically used by clinical laboratories. Deletions of *IKZF*1, which encodes the early lymphoid transcription factor IKAROS, have been found in 84% of *BCR*/*ABL*1-positive and 28% of *BCR*/*ABL*1-negative ALL [17,30]. *IKZF*1 alterations have been shown to be associated with poor outcome both in *BCR*/*ABL*1-positive and *BCR*/*ABL*1-negative ALL [13,27,31]. Thus, the ability to detect *IKZF*1 deletions is critical for appropriate risk stratification.

Deletions in 8q12, ranging from 160 kb to 4.3 Mb, were detected by microarray in three of our cases. All three of these deletions included or partially overlapped *TOX* (*KIAA0*80). This gene has been proposed as one of two candidate genes in recurring 8q12.1 deletions that are present in approximately 4% of ALL [32]. *TOX*, originally shown to play a role in T-cell development, has recently been found to be involved in the development of many cell lineages of the immune system [33]. In Case 16, the 160-kb deletion of 8q12.1 includes the first 5' exon of *TOX* and does not involve any other genes; therefore, our results further support *TOX* as a significant candidate gene within these 8q12.1 deletions.

The microarray results also identified deletions of genes involved in glucocorticoid resistance. Synthetic glucocorticoids are used in the treatment of pediatric ALL due to their ability to induce apoptosis in leukemic blasts; resistance to glucocorticoids is predictive of poor therapy outcome [34]. Focal deletions of *BTG*1 were present in four of our cases. Deletion of *BTG*1 is frequently seen in ALL, and loss of *BTG*1 expression has been shown to result in glucocorticoid resistance [35]. One case in our study demonstrated a deletion within *NR*3*C*1 (Case 6), and another had a biallelic loss within *GSTT*1 (Case 1). Germline mutations in *NR*3*C*1 have been found in patients with glucocorticoid resistance, and deletions of this gene have also been described in ALL [27]. Polymorphisms in the glutathione S-transferase (GST) genes have been implicated in glucocorticoid resistance. Inherited homozygous deletions of *GSTT*1 have been associated with an initial good response to prednisone, although the association between GST polymorphisms and relapse is controversial [36-39].

Other CNAs of interest detected in the cases studied here include frequent *CDKN*2*A* losses (9/20; 45%) ranging from small intragenic deletions to many megabases in size (**Figure 4**). The frequency of losses seen here was higher than that reported in some previous studies [40, 41], although our findings agreed with others [17,42]. Six of the *CDKN*2*A* deletions appeared homozygous by array, while the remaining cases may reflect either heterozygosity or homozygosity in a subclone of cells, given the inherent non-cellular limitations of array technology. In a previous study of childhood ALL, *CDKN*2*A* deletion was found more frequently in cases with t (9;22) (61%) than with t (12;21) (15%) or *MLL* gene rearrangements (13%) [40]. The present study showed similar results with *CDKN*2*A* deletion found in 2/4 cases with t (9;22), 1/2 cases with a *MLL* gene rearrangements (Case 18) and in none of the cases with t (12;21).

Cases 17 and 18 exhibited *MLL*/*AFF*1 translocations and represent cases with the least number of CNAs detected by aCGH. This is consistent with the notion that *MLL* gene rearrangement is a potent and sufficient oncogenic driver mutation. Of those cases exhibiting *CDKN*2*A* deletion, case 18 exhibited deletion of *CDKN* 2*A* at an apparently heterozygous level, the only clini-

cally significant finding in addition to the *MLL*/*AFF*1 translocations in the case. In contrast, all three cases with a t (12;21) cases showed intermediate levels of instability as measured by CNAs, including loss of or deletion in *ETV*6. In addition, one t (12;21) case showed biallelic loss at the 5'-end of *TBL*1*XR*1, which has also been found to be a recurrent event in this ALL subtype [43].

The cases showing the greatest number of CNAs were those that did not exhibit recurrent translocations, perhaps a consequence of cellular evolution in search of an oncogenic threshold (e.g., Cases 1, 6, 10, 11, and 13). Of these, Case 1 exhibited a substantial number of CNAs that included both homozygous deletion of *CDKN*2*A* and a two-copy gain within the *PAX*5 locus. As both abnormalities reside on chromosome 9p and are homozygous, the results are suspicious for copy neutral loss of heterozygosity event, although confirmatory SNP analysis was not performed. As shown in **Figure 2**, the *PAX*5 two-copy duplication occurs within the gene. Although duplications in *PAX*5 have previously been thought to result in loss-of-function alleles [12], this duplication involves exons 2-5 that encode the Paired box DNA binding domain that potentially produces an in-frame product. A similar duplication involving the same specific exons was seen in case 13 (**Figure 4**), a case that also exhibited homozygous deletion of *CDKN*2*A*. Thus, a novel PAX5 function related to paired box duplication may be related to greater instability, homozygous *CDKN*2*A* mutation and lack of translocation, features shared by these two cases.

In the current study, copy number variation of the immunoglobulin and T-cell receptor genes were noted due to high-density coverage within these genes (see **Table 2**). Such alterations may also be observed in normal individuals due to somatic rearrangement; however, in hematological malignancies, these copy number changes can be indicative of clonal cell populations (see **Table 2**). Clonality, if present, may be related to viral infection, immunodeficiency, or neoplasia [44]. Thus, gains and losses of these regions present a unique challenge in the analysis and interpretation of oncology aCGH cases. Further studies employing additional clinical information and a standardized statistical means of analysis will be required for interpreting copy number variation in these regions and ascertaining potential clinical significance of these alterations.

It is important to note that although the aCGH analysis in Case 2 was suggestive of the doubling of a hypodiploid/near-haploid clone, the confirmation of this abnormality was only made possible by subsequent SNP microarray analysis. This information is critical to this case in order to distinguish apparent hyperdiploidy that is associated with a good prognosis from true hypodiploidy that is associated with a poor prognosis.

The tCGH technology offers unprecedented resolution of translocation breakpoints affording a new opportunity to explore the potential clinical and biological value of such data. To this end, Case 9 is of potential interest. Of the *BCR*/*ABL*1 positive cases, Case 9 had the most CNAs detected by aCGH and represented newly diagnosed disease. The current data provide high-resolution analysis of the genomic breakpoints for each recurrent translocation. Relative to the other *BCR*/*ABL*1 positive cases in this study, for Case 9 those breakpoints map to the furthest 3'-end of intron 1 for *BCR* and the furthest 5'-end of intron 1 for *ABL*1, yielding a genomic fusion gene larger than for other *BCR*/*ABL*1 translocations. Although all four *BCR* and *ABL*1 breakpoints occurs within the first introns of those genes, it is interesting to speculate that regulatory and/or cryptic alternative splice sites might influence the oncogenic potential for the specific fusions. The additional genomic landscape included in the fusion for Case 9 may necessitate the acquisition of additional driver mutations (e.g., deletions of *CDKN*2*A*, *PAX*5, *IKZF*1) to produce disease. A similar albeit less dramatic example comes from data comparison for Cases 17 and 18. Cases 17 and 18 exhibited *MLL*/*AFF*1 translocations and represent cases with the least number of CNAs detected by aCGH. This is consistent with the notion that *MLL* gene rearrangement is a potent and sufficient oncogengic driver. Consistent with that assumption these cases show minimal CNAs. As determined by tCGH the cases share nearly identical *MLL* gene breakpoints within the common cluster region (Case 17 = chr11:117860758-117860864; Case 18 = chr11:1178606 48-117860758), with *AFF*1 breakpoints separated by only a few kb (case 17 = chr4:88216921-88217006; case 18 = chr4:88214869-88215163). Once again, one could speculate that the precise sequence context of the breakpoints or specific elements contained in the sequences of difference observed (several kb on chromosome 4) was a contributing factor for Case 18 acquiring the additional oncogenic lesion at *CDKN*2*A*. Similar proposals could be generated for the significance of *ETV6*/*RUNX*1 breakpoints. Obviously a much larger study would be required to discern to what degree breakpoints correlate with presence of additional oncogene CNAs.

The current study was completed with cases all previously analyzed by chromosome and/or FISH analysis. One potential limitation of microarray-based analysis for leukemia is that separate clones cannot be distinguished; however, current studies indicate that the presence or absence of certain CNAs at diagnosis, such as *IKZF*1 deletions, are important for determining relapse, regardless of whether they represent the predominant clone at diagnosis [45]. Also certain alterations may be inferred from microarray results, although the mechanism that leads to the alteration(s) may only be confirmed by con-

ventional cytogenetic methods that allow visualization of the chromosomes. For example, microarray results on case 5 showed loss of 9p and 20q that would suggest the presence of a dic (9;20), although confirmation of this finding requires visualization of the metaphase cells by G-banding or FISH. However, if the loss of genetic material is the prognostically significant finding, which it appears to be in the case of the dic (9;20) [46,47], then the specific mechanism of loss is probably not as important as the genetic content of the altered region. Another issue that clinical laboratories will need to address is the potential of uncovering germline CNAs during the course of testing neoplastic specimens for acquired alterations. For example, deletions in the *DMD* gene have been uncovered in studies of ALL [27] (see also our case 6). Other examples are given in the study performed by Dougherty *et al.*, 2011) [3]. A non-neoplastic source of DNA from the patient may be required to determine if these are acquired or germline alterations and families would ideally receive appropriate pre-test counseling for these scenarios.

Despite the aforementioned issues, the ability to detect diagnostically and prognostically significant translocations and CNAs by microarray has significant advantages over conventional cytogenetics, with the ability to detect deletions below the resolution of FISH. Thus, arrays will be an important adjunct to conventional cytogenetics, and may eventually become the standard for first-tier testing. The ability to detect both prognostically significant balanced rearrangements and genomic imbalances is a major step toward implementing arrays in the clinical laboratory for evaluation of ALL and other forms of neoplasia.

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