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Research paper

High resolution Chromosomal Microarray Analysis (CMA) enhances the genetic profile of pediatric B-cell Acute Lymphoblastic Leukemia patients

Anastasios Mitrakos[a,*], Antonis Kattamis[b], Katerina Katsibardi[b], Stefanos Papadhimitriou[c], Sophia Kitsiou-Tzeli[a], Emmanuel Kanavakis[a], Maria Tzetis[a]

[a] Department of Medical Genetics, Medical School, National and Kapodistrian University of Athens, Greece
[b] Hematology-Oncology Unit, First Department of Pediatrics, National and Kapodistrian University of Athens, "Aghia Sofia" Children's Hospital, Athens, Greece
[c] Department of Laboratory Hematology, Athens Regional General Hospital "G. Gennimatas", Athens, Greece

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ABSTRACT

Acute Lymphoblastic Leukemia (ALL) is a malignancy of the immature lymphoid cells mainly associated with numerical and structural chromosomal aberrations. The current standard for profiling the diverse genetic background comprises a combination of conventional karyotype and FISH analysis for the most common translocations, albeit with many limitations. Chromosomal Microarray Analysis (CMA) is a high throughput whole genome method that is gradually implemented in routine clinical practice, but not many studies have compared the two methods. Here we aim to investigate the added benefits of utilizing the high resolution 2 x 400 K G3 CGH + SNP CMA platform in routine diagnostics of pediatric ALL. From the 29 bone marrow samples that were analyzed, CMA identified clinically relevant findings in 83%, while detecting chromosomal aberrations in 75% of the patients with normal conventional karyotype. The most common finding was hyperdiploidy (20%), and the most common submicroscopic aberration involved *CDKN2A/B* genes. The smallest aberration detected was a 9 kb partial *NF1* gene duplication. The prognosis of the patients when combining conventional cytogenetics and CMA was either changed or enhanced in 66% of the cases. A rare duplication possibly indicative of a cryptic *ABL1-NUP214* fusion gene was found in one patient. We conclude that CMA, when combined with conventional cytogenetic analysis, can significantly enhance the genetic profiling of patients with pediatric ALL in a routine clinical setting.

1. Introduction

Acute Lymphoblastic Leukemia (ALL) is a malignancy of the immature lymphoid progenitor cells, resulting in abnormal differentiation and proliferation with detrimental consequences [1]. ALL is characterized by a high degree of heterogeneity in regards to severity, response to therapeutic schemes, as well as the overall survival rates, owing to the diverse genetic alterations that accumulate over time. These include both numerical and structural chromosomal rearrangements, gains and deletions of genomic regions (Copy Number Variations, CNVs) and, less commonly, single nucleotide alterations [2,3].

Much of our current understanding of the genetic landscape of B-cell ALL is due to G-banding karyotype and Fluorescent in Situ Hybridization (FISH). However, conventional karyotypic analysis can be hampered by a number of issues, including the need for culture of malignant cells, poor chromosome morphology and low resolution. FISH analysis provides sensitive detection of structural rearrangements but requires a large number of unique probes and is mostly aberration specific.

The combination of conventional cytogenetics with Chromosomal Microarray Analysis (CMA) based on high resolution DNA microarrays that provide genome wide screening for chromosomal aberrations at the exon level, is necessary for accurate prognosis and risk stratification of B-ALL patients. CMA is already considered a first-tier clinical diagnostic test for individuals with developmental disabilities and congenital anomalies of unknown etiology [4]. The higher resolution provided can refine conventional cytogenetic results, identify marker chromosomes and detect submicroscopic aberrations, while overcoming most of the limitations of conventional cytogenetics. At the same time, it can detect novel alterations which can serve as future biomarkers for risk stratification and prognosis. A number of studies on various hematological malignancies have suggested that it can improve

* Corresponding author at: Department of Medical Genetics, Medical School, National and Kapodistrian University of Athens, Choremio Research Laboratory, Thivon & Levadeias, Goudi, Athens, Greece.
E-mail address: amitrakos@med.uoa.gr (A. Mitrakos).

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Table 1
Clinical information, risk stratification and therapy outcome status of the patients in our cohort (SR: Standard Risk, IR: Intermediate Risk, HR: High Risk, CR: Complete Remission, N/A: Information not available).

Patient code	Age at diagnosis	Sex	Risk Group	Outcome
L1	2	M	IR	CR
L2	7	M	IR	CR
L6	12	F	IR	CR
L10	5	F	SR	CR
L11	1	M	SR	CR
L12	5	M	IR	CR
L13	3	F	IR	CR
L14	7	M	IR	CR
L15	10	M	Deceased	–
L16	10	F	IR	CR
L17	6	F	IR	CR
L18	4	M	IR	CR
L19	4	M	SR	CR
L20	2	F	SR	CR
L21	14	M	IR	CR
L22	4	F	SR	CR
L23	4	F	IR	CR
L25	5	M	SR	Relapse
L26	6	M	N/A	N/A
L28	7	M	N/A	N/A
L31	8	M	IR	CR
L32	7	M	IR	CR
L36	11	M	HR	Maintenance
L37	11	F	IR	Maintenance
L40	13	F	Deceased	–
L41	5	F	HR	Maintenance
L42	6	M	SR	Maintenance
L43	10	M	HR	Maintenance
L44	4	F	SR	Maintenance

the cytogenetic characterization [5–7], but not many studies have focused on pediatric ALL [8].

This manuscript discusses the additional findings obtained by high resolution CMA in a cohort of pediatric B-ALL patients and provides a comparison to conventional cytogenetic analysis, with a final aim to evaluate the added benefit in the detection rate of chromosomal aberrations when using a combined approach, as well as the feasibility of applying the method in routine diagnostics of ALL in the clinical setting.

2. Materials – methods

2.1. Patients

A total of 29 pediatric patients (median age: 6.6 years, range: 1–14 years, 17 male, 12 female), diagnosed with B cell Precursor (BCP) ALL from 2014 to 2017 were included in the study. The patients were treated at the Hematology and Oncology Unit of the First Pediatric Department of the Medical School of the National & Kapodistrian University of Athens, according to the BFM-ALLIC 2009 protocol, after being stratified to either high, intermediate or standard risk groups (Table 1).

Fresh bone marrow aspirates, obtained at first diagnosis and before treatment initiation, were used for the study.

2.2. Cytogenetic and FISH analysis

Conventional karyotype analysis was performed on fresh bone marrow aspirates after GTG chromosome banding with a 300bphs (bands per haploid set) resolution after 24, 48 and 72 h culture, as well as FISH analysis with probes for the most common alterations that appear in B-ALL (see Supplementary Materials). A karyotype was classified as non-diagnostic when < 10 normal metaphases could be studied for a specific sample, according to international guidelines [9].

2.3. CMA analysis

Genomic DNA was manually extracted using the QiAmp DNA Blood Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The quality and quantity of the DNA was determined using the NanoDrop 1000 UV-VIS spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Chromosomal Microarray Analysis (CMA) was performed using the high resolution 2 x 400 K G3 CGH + SNP microarray platform (G4842A, Design ID 028081, Agilent Technologies, Santa Clara, CA, USA). The specific platform features a total of 292,097 oligonucleotide CGH probes covering the whole genome, with a median CGH probe spacing of 7 kb, as well as 118,955 Single Nucleotide Polymorphism (SNP) probes for the detection of Copy-Neutral Loss of Heterozygosity (CNLOH), resulting in a resolution of 5-10Mb for CNLOH.

The laboratory protocol was carried out according to the manufacturer's instructions (Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis), and consisted of enzymatic digestion of each genomic DNA sample as well as a sex-matched reference DNA (Agilent Technologies, Santa Clara, CA, USA), followed by differential labeling with Cy3 and Cy5 fluorescent dyes for sample and reference respectively. The combined labeled DNA samples were applied to the microarray, left to hybridize for 40 h at 67 °C, washed and scanned at 3 μm resolution on the Agilent High-Resolution Microarray Scanner (G2505C, Agilent Technologies, Santa Clara, CA, USA). The images were extracted and analyzed using the Agilent Feature Extraction software and the CytoGenomics v.4.0.3 software suite. The ADM-1 aberration detection algorithm was utilized, with the minimum number of probes required for a call set to 4 and the log2 ratio threshold for duplications and deletions set to ± 0.25.

2.4. Data analysis

Recently, a set of guidelines for the implementation of CMA in acquired hematological malignancies was published [10]. Laboratories are advised to report aberrations larger than 5Mb and additionally microdeletions involving *BTG1*, *CDKN2A/B*, *EBF1*, *ERG*, *ETV6*, *IKZF1*, *PAX5*, *RB1* genes and the pseudoautosomal region 1 (PAR1) while the threshold for reporting regions of CN-LOH is suggested to be 10Mb, and only when these extend to the telomeres.

In accordance to these guidelines, all aberrant segments larger than 5Mb were classified as true aberrations and are reported. Aberrations smaller than 5Mb are only reported if they include known leukemia or other cancer related genes, including cell cycle regulators, oncogenes and tumor suppressor genes. Because paired control samples for the elimination of germline aberrations were unavailable, we excluded genomic segments that correspond to known common polymorphic CNVs (Copy Number Polymorphisms, CNPs), based on the Database of Genomic Variants (DGV dgv.tcag.ca/) as well as our internal control samples database. The findings were assessed in context to the literature and the following databases: UCSC (genome.ucsc.edu/), OMIM (omim.org/), Atlas of Genetics and Cytogenetics in Oncology and Hematology (atlasgeneticsoncology.org/) and the Catalog of Somatic Mutations In Cancer, COSMIC (cancer.sanger.ac.uk/cosmic). Regions with CN-LOH are to be considered as acquired only when extending to the telomeres and are larger than 10Mb. All aberrations were visually inspected in order to eliminate false positive calls. Findings are described according to ISCN 2016.

Full informed consent was obtained from the patient's legal guardians prior to their enrolment in the study.

The study was approved by the Bioethics Committee of the National and Kapodistrian University of Athens.

Table 2
G-banding Karyotype, FISH and Chromosomal Microarray Results (N/A: Information not available).

Patient code	Karyotype	FISH	CMA Results	CNV Size	Original profile / Profile after CMA	Genes Affected by CNVs
L1	52,XY,+X,+4,+6,-13,+14,+17,+18,+21[5]/46,XY[15]	(21)x3	arr[GRCh37] (4)x3,(6)x3,(14)x3,(17)x3,(18)x3, (21)x3,(X)x2		Hyperdiploidy/ Hyperdiploidy	
L2	46,XY[20]	*ETV6/RUNX1* (+), (9) x1	arr[GRCh37] 6q15q27(92780275_170900457)x1,	78Mb	*ETV6-RUNX1*/ *ETV6-RUNX1*	*CDKN2A/B, ETV6, MLLT3, TCTA, MYC*
			8q21.12q24.3(79208270_146294098)x3,	67Mb	9p21.3 x1	
			9p22.1p13.3(18941715_34851819)x1,	16Mb	12p13.2 x1	
			9p21.3(21994051_22146685)x1,	153Kb		
			9q22.31q22.33(95003110_100934951)x1,	6Mb		
			12p13.2(11833458_12135000)x1	300Kb		
L6	46,XX[25]	*ETV6/RUNX1* (+), DEL non translocated *ETV6*	arr[GRCh37] 6q15q21(89711213_112351032)x1,	23Mb	*ETV6-RUNX1* fusion gene/ *ETV6-RUNX1*	*CDKN2A/B, ETV6, RUNX1, NOTCH1*
			9p21.3(21902814_22006523)x0~1,	104Kb	9p21.3 x1	
			9q34.3(139405388_139422509)x2~3,	17Kb	12p13.2 x1	
			12p13.31p13.1(9875300_14315819)x1, (X)x1	4.4Mb	(X)x1	
L10	55,XX,+X,+4, +6,dic(7;9)(p11;p11),+8, +10,+14,+18, +18,+21,+21[3]/46,XX[17]	*BCR/ABL1* (-), *MLL* (-), t1;19 (-), *ETV6/RUNX1* (-), (21)x3	arr[GRCh37] (1-22,X)x2		Hyperdiploidy/ Hyperdiploidy	
L11	46,XY,der(19)t(1;19)(q23;p13)[20]	t(1;19) (+)	arr[GRCh37] 1q23.3q44(164717139_248702454) x3,	84Mb	*TCF3-PBX1 fusion gene/ TCF3/PBX1*	*TCF3, PBX1*
			19p13.3(281067_1616148)x1	1.3Mb	1q23.3q44 x3	
L12	46,XY[20]	*ETV6/RUNX1* (+), (21) x3, (X)x2, *MLL* (-), (11) x3, *BCR/ABL* (-), E2A (-)	arr[GRCh37] 4p16.3p11(45882_49076492)x3,	49Mb	*ETV6-RUNX1* fusion gene/ *ETV6-RUNX1*	*ETV6, RUNX1*
			12p13.2(11885201_11983834)x1~2, (21)x3~4, (X)x2	99Kb	12p13.2 x1 (21)x3 (X x2	
L13	46,XX[20]	*ETV6/RUNX1* (-), DEL non translocated *ETV6*, *MLL* (-), *BCR/ABL* (-)	arr[GRCh37] 5q33.3(157854558-158526514)x1,	670Kb	Normal/	*EBF1, ETV6*
			12p13.2p12.3(10772734_15747984)x1	5Mb	5q33.3 x1 12p13.2p12.3 x1	
L14	46,XY,t(1;16)(q11;q11)[5]	t(12;21) (-), t(9;22) (-), *MLL* (-), *PBX1* x3, *E2A/ PBX1* (-)	arr[GRCh37] 1q21.1q44(145021412_249211884) x3,	104Mb	t(1;16)(q11;q11), *PBX1*x3/ 1q21.1q44 x3	*TCF3, PBX1*
			19p13.3(281067_3338081)x1~2	3Mb	19p13.3 x1	
L15	N/A	N/A	arr[GRCh37] 9p21.3(20048412_22212612)x1,	2Mb	N/A/	*CDKN2A/B*
			9p21.3(21093730_22008485)x1,	915Kb	9p21.3 x1	
			Xp22.33(61529_2696179)x1	2.6Mb	Xp22.33 x1	
L16	62,XX,+X,+X, +2,+2,+4,+5, +6,+8,+10,+11,+12,+15, +18,+21,+21,+22[7]/46, XX[13]	N/A	arr[GRCh37] 1p33p12(48322411_119603431)x1,	71Mb	Hyperdiploidy/ Hypodiploidy	*VPREB1*
			(3)x1, (7)x1, (9)x1, (10)x3,			
			13q11q34(19438911_115092648)x1,	96Mb		
			15q11.2q26.3(22765628_102399819)x1,	82Mb		
			16q11.2q24.3(46441545_90148393)x1,	44Mb		
			(17)x1, (19)x1, (20)x1, (21)x3,			
			22q11.22(22242836_23012072)x1	719Kb		
L17	46,XX[20]	*ETV6/RUNX1* (+), *MLL* (-), *BCR/ABL* (-)	arr[GRCh37] 9q13q21.11(66710577_70984540) x1~2	4Mb	*ETV6-RUNX1* fusion gene/ *ETV6-RUNX1* 9q13q21.11 x1	

(*continued on next page*)

Table 2 (*continued*)

Patient code	Karyotype	FISH	CMA Results	CNV Size	Original profile / Profile after CMA	Genes Affected by CNVs
L18	58,XY, + Y,dup(1)(q21q25), + 4, + 6, + 10, + 14, + 15, + 17, + 18, + 19, + 21, + 21, + mar[10]/46,XY[10]	(9)x3, (21)X3, (X)x2, (Y)x2	arr[GRCh37] 1q21.2q32.2(149857649_208440690)x3,	58Mb	Hyperdiploidy/ Hyperdiploidy	*NOTCH2, MALL, TCF3*
			1q41q42.12(218968136_225939239)x3,	7Mb		
			2q13(110862477_110980401)x3~4, (4)x3, (9)x3, (10)x3,	102Kb		
			12p13.2(11822068_11905414)x1~2	83Kb		
			14q11.2q32.33(19728641_107278770)x3, (18)x3,	89Mb		
			19p13.3(281067_4934956)x1, (21)x3, (X)x2, (Y)x2	5Mb		
L19	55,XY, + X, + 4, + 6, + 10, + 17, + 18, + 21, + 21, + 22[1]/46,XY[19]	(21)x3, (17)x3, (X)x3, (Y)x3	arr[GRCh37] (4)x3, (6)x3, (8)x3, 14q11.1q32.33(19043189_107278770)x3,		Hyperdiploidy/ Hyperdiploidy	
			(17)x3, (18)x3, (21)x3, (X)x3, (Y)x3	88Mb		
L20	46,XXt(7;15)(q22;q15)[17]/46,XX[8]	N/A	arr[GRCh37] (1-22,X)x2		t(7;15)(q22;q15)	
L21	46,XY[15]	N/A	arr[GRCh37] 1q21.1q23.3(146542843_162569103)x3~4, (4)x3~4, (6)x3,	16Mb	Normal/ Hyperdiploidy	*ETV6*
			10p15.3p11.1(102539_38806838)x4,	39Mb		
			10q11.21q26.3(42409557_135434178)x3,	93Mb		
			10q11.22q23.1(49393577_87880430)x4,	38Mb		
			10q25.2q26.3(113377242_135106845)x4,	22Mb		
			12p13.2(11833458_12014829)x1, (17)x3~4, (18)x3~4, (21)x4, (X)x2	181Kb		
L22	46,XX[5]	*TCF3/PBX1* (-), 1q23 x3	arr[GRCh37] (1)x1, (3)x1, 9p24.3p12(115981_42416387)x1,		1q23x3/ Hypodiploidy	*PAX5,*
			9p13.2(36992705_37020741)x0~1	42Mb		*NF1*
			9q13q34.3(66341789_141018984)x1,	28Kb		
			(11)x1, (13)x1, (15)x1, (16)x1,	75Mb		
			17q11.2(29553704_29563355)x3, (19)x1, (20)x1, (21)x3	9.5Kb		
L23	Non diagnostic	*ETV6/RUNX1* (+), *BCR/ABL* (-), *MLL* (-), *TCF3* (-)	arr[GRCh37] 9p21.3(21837880_22006523)x1~2	169Kb	*ETV6-RUNX1*fusion gene/*ETV6/RUNX1,*	*CDKN2A/B*
			9p13.2(36934575_37020741)x1~2	86Kb	9p21.3 x1 9p13.2 x1	
L25	46,XY[20]	*ETV6/RUNX1* (-), 21q22 x5, *BCR/ABL* (-), *MLL* (-), 11q22 x1, *TCF3* (-), 9p21 x0,	arr[GRCh37] 4q13.1q13.2(62862052_68777117) x1,	6Mb	21q22 x5	
			9p24.3p13.2(115981_36980076)x1,	37Mb	11q22 x1	
			9p22.1p21.3(19726199_22246824)x0~1,	2.5Mb	9p21 x0/ Complex Karyotype – Possible iAMP21	
			11q21q22.3(97052031_104100454)x3,	7Mb		
			11q22.1q22.2(101793428_102826040)x4,	1Mb		
			11q22.3(104135318_105103561)x1,	1Mb		
			11q22.3q24.2(107832645_125707886)x1,	18Mb		
			11q24.2(125753780_126849275)x3,	1Mb		
			11q24.2q25(126856390_130809721)x4,	4Mb		
			11q25(131991056_134904063)x3,	3Mb		
			11q25(133045562_133656486)x1,	610Kb		
			13q13.3q22.2(38138626_76049699)x1,	38Mb		
			14q22.3q32.11(57895887_90035886)x1,	32Mb		
			15q14q21.1(35274708_45123335)x1,	10Mb		

(*continued on next page*)

Table 2 (*continued*)

Patient code	Karyotype	FISH	CMA Results	CNV Size	Original profile / Profile after CMA	Genes Affected by CNVs
			20q11.21q11.22(31403522_32530213)x1,	1.1Mb		
			20q11.23q12(35406320_39480973)x1,	4Mb		
			20q12(39690107_40730817)x3,	1Mb		
			20q12q13.33(41653593_62904687)x1,	21Mb		
			21q11.2q21.3(15260897_29256563)x3,	14Mb		
			21q22.11q22.2(34974525_41447137)x4	6.5Mb		
L26	46,XY[20]	N/A	arr[GRCh37] (1-22)x1, (X,Y)x1		Normal/ Normal	
L28	N/A	N/A	arr[GRCh37] (14)x3, (21)x4, (X)x2		N/A/ Hyperdiploidy	
L31	55,XY, + X, + 1, + 4, + 5, + 6,i(7)(q10), + 10,i(17)(q10), + 21, + 21, + 22[16]/46,XY[4]	*ETV6/RUNX1* (-), *BCR/ABL* (-), *MLL* (-), *TCF3/PBX1* (-)	arr[GRCh37] (1-22)x1, (X,Y)x1		Hyperdiploidy/ Hyperdiploidy	
L32	46XY,t(6;9)(q13;p24),der(19)t(1;19)(q23;p13.3)[6]/46,XY[14]	*TCF3/PBX1* (+), 9p x1	arr[GRCh37] 1q21.1(142617943_145064610) x2~3,	2.5Mb	t(6;9)(q13;p24), der(19) t(1;19)(q23;p13.3)	
			1q21.1q44(145076126_249211884)x3,	104Mb	9p x1/ Complex Karyotype	
			1q21.1q23.3(145076126_163478885)x2~3,	18Mb		
			6p12.3p11.2(49273391_57337934)x1,	8Mb		
			6q12q15(69640437_90162511)x1,	20Mb		
			6q16.1q16.3(95852871_101705853)x1,	6Mb		
			6q22.1(115325664_117866764)x1,	2.5Mb		
			9p24.3p11.2(123950_43836428)x1,	44Mb		
			9q13q21.11(66341789_70964423)x1,	4.6Mb		
			19p13.3(281067_1616148)x1	1.3Mb		
L36	46,XY,del(17)(p12p13)[2]/ 46,XY[4]	*ETV6/RUNX1* (-), *BCR/ABL* (-), *MLL* (-), *TCF3* (-), DEL 9p21 (-), p53 x1, X/Y (-),	arr[GRCh37] 1p36.33p36.22(564424_9753179)x1,	9Mb	p53 x1	*TP53, VPREB1*
			13q12.11q13.3(20142055_38753788)x1,	19Mb	del 17p12p13/ Complex Karyotype	
			17p13.3p11.2(65183_18927334)x1,	19Mb		
			17p11.2(19160667_22154574)x3,	3Mb		
			20q11.21q13.33(31062497_62904687)x1,	32Mb		
			22q11.22(22569822_22600037)x1	30Kb		
L37	46,X,-X, + mar[20]	*ETV6/RUNX1* (+)	arr[GRCh37] (1-22)x1, (X)x1		*ETV6-RUNX1/ ETV6/ RUNX1*	
L40	46,XX[20]	(-)	arr[GRCh37] 7p12.2(50257460_50344541)x0~1	87Kb	Normal/ IKZF1 x1, 9q34.12q34.13x4	*IKZF1, CDKN2A/B, PAX5, ABL1, NUP214 NF1, MLLT3*
			9p21.3(20428098_20620723)x0~1	193Kb		
			9q34.12q34.13(133730203_134106126)x4	376Kb		
			17q11.2(29553704_29562744)x3~4	9Kb		
L41	54,XX, + X, + 4, + 6, + 10, + 14, + 17, + 18, + 21[20]	*ETV6/RUNX1* (-), *MLL* (-), BCR/ABL (-), *TCF3* (-), 9p (-), chr 17, 21 x3, XXX	arr[GRCh37] (4)x3, (6)x3,		Hyperdiploidy/ Hyperdiploidy	
			10p15.3p11.1(136361_39124316)x3,			
			10q11.21q26.3(42418957_135434178)x3,	39Mb		
			10q11.22(46984913_47691285)x4,	93Mb		
			14q11.2q32.33(19265142_107287505)x3,	706Kb		
			(17)x3, (18)x3, (21)x3, (X)x3	88Mb		
L42	N/A	N/A	arr[GRCh37] 4p16.3p11(72447_49505025)x4,	49Mb	NA/ Hyperdiploidy	
			4q11q35.2(52685197_190896674)x3,	138Mb		
			4q13.2q13.3(69080277_72423233)x3~4,	3.3Mb		
			4q31.21q31.23(146524353_149238648)x3~4,	2.7Mb		

(*continued on next page*)

Table 2 (*continued*)

Patient code	Karyotype	FISH	CMA Results	CNV Size	Original profile / Profile after CMA	Genes Affected by CNVs
			(5)x3, (6)x3, (8)x3,			
			14q11.2q32.33(20439005_107287505)x3,	87Mb		
			(17)x3, (21)x4, (22)x3, (X)x2			
L43	46,XY[15]	(-)	arr[GRCh37]	19Mb	Normal/	
			19q13.2q13.43(39856498_59065819)x1		19q13.2q13.43x1	
L44	46,XX[17]	(-)	arr[GRCh37] 22q11.22(22494963_23012072)x1	517Kb	Normal/ 22q11.22x1	*VPREB1*

3. Results

3.1. Karyotype

Karyotype results were available for 27/29 patients. Of these, 13 patients (48%) presented with normal or non-diagnostic karyotype and 7 patients (26%) presented with hyperdiploidy (> 50 chromosomes). The remaining 7 showed karyotypes of variable complexity, with findings ranging from single translocation events to multiple chromosomal rearrangements (Table 2).

3.2. FISH

FISH results were available for 25/29 patients. The most common finding was *TEL/AML1* (*ETV6/RUNX1*) t(12;21) translocation that was present in 6/25 patients (24%), followed by hemizygous and homozygous deletions of the 9p chromosomal region in 4 patients, that include *CDKN2A/2B* genes. Two patients carried a *TCF3* (*E2A*)*/PBX1* t (1;19) translocation, while one patient had 3 copies of 1q23 chromosomal region. 7/25 (28%) patients tested positive for various chromosomal translocations (Table 2). Other common findings include deletions of p53 gene on chromosome 17, as well as chromosomal duplications (Table 2). FISH analysis reported no clinically significant findings on 7/25 patients (28%).

3.3. CMA results

High resolution CMA yielded clinically relevant results in 24/29 patients (83%) detecting a total of 150 CNVs (Table 2), with an average of 7 alterations per sample. Chromosomal microarray analysis was able to identify 73% of all chromosomal rearrangements detected by conventional karyotype and also detected aberrations at the breakpoints of the translocations identified by FISH analysis in 71% of the cases.

3.4. Patients with normal karyotypes

Of the 16 patients in our cohort that had normal or non-diagnostic conventional karyotype, CMA managed to detect significant aberrations in 13 (81%), a result concordant with international literature, as it has already been shown that CMA can detect chromosomal abnormalities in as much as 80% of cases with normal karyotypes, in both pediatric and adult patients [11,12]. The findings ranged from complete ploidy changes, to cryptic CNVs (Table 2).

3.5. Patients with hyperdiploidy

Hyperdiploidy was detected by CMA in 6/29 samples studied (20%) (L1, L18, L19, L28, L41, L42). The chromosomes most commonly duplicated were 4, 6, 14, 17 and 21. Patient L18 harbored additionally a cryptic 39Kb deletion of 1p12 (*NOTCH2*), a 4.6Mb deletion of 19p13.3 (*TCF3*) and a duplication of 2q13 (118 kb, *MALL*) (Table 2).

Of the 7 patients identified with hyperdiploidy by conventional karyotype (L1, L10, L16, L18, L19, L31, L41), 5 were confirmed by CMA (L1, L16, L18, L19, L41). The discordance in the results can be attributed to a low percentage of clonal cells in the samples analyzed. Additionally, CMA detected hyperdiploidy in 4 patients that had normal or non-diagnostic conventional karyotype as a result of selective clonal amplification during cell culture.

3.6. Patients with hypodiploidy

Hypodiploidy was detected in 2/29 samples (7%), with patients L16 and L22 showing multiple chromosomal losses and gains as well as cryptic CNVs harboring important genes (*VPREB1, NF1, PAX5*) (Table 2).

3.7. Patients with chromosomal translocations

Patients L11, L14 and L32 had large duplications of the 1q chromosomal region accompanied by deletions of 19p that lead to impaired early lymphoid development [13]. Especially in patients L11 and L32 the findings were consistent with the unbalanced variant der(19)t(1;19)(q23;p13) as confirmed by G-banded karyotype, resulting in the *TCF3-PBX1* fusion gene, while in patient 14 a t(1;16) was detected in the karyotype, accompanied by deletion of 19p13.3 as observed by FISH, results that were confirmed by CMA. FISH for the *TCF3-PBX1* fusion gene was negative.

3.8. Complex karyotypes

There are reports that ALL patients with a complex karyotype with ≥ 5 aberrations who did not harbor an established translocation had significantly inferior Event Free Survival (EFS) and Overall Survival (OS) rates [14]. In our cohort 3/29 patients (L25, L32 and L36) had complex karyotypes, while not carrying recurrent alterations. Patient L25 in particular showed multiple regions with partial chromosomal deletions and ampifications, notably concentrated on chromosome 11 (Fig. 3), a possible indication of chromothripsis [15]. In total, 7/29 patients carried large aberrations of chromosome 1 (Table 2).

3.9. Submicroscopic findings

The most common finding in our cohort, observed in 5/29 samples (17%), was submicroscopic deletions of 9p21.3, that included *CDKN2A* and *CDKN2B* genes. Loss of 12p13.2 that includes *ETV6* gene was detected in 4 patients (L2, L12, L13, and L21). A microduplication of 17q11.2 that included exons 19–27 of the *NF1* gene was found in patients L22 and L40.

Patient L40, although appearing normal after conventional cytogenetic analysis, revealed several submicroscopic aberrations after CMA affecting critical genes, namely *IKZF1* (exons 1 & 2, 56Kb deletion) *MLLT3* (192Kb deletion), *NUP214* & *ABL1* (exons 4–12, 376Kb gain), *NF1* (exons 19–28, 9Kb gain) (Table 2, Fig. 1).

Patient L13 had a 670 kb microdeletion that included *EBF1*.

Deletion of the *MLLT3* gene was detected in patient L2, along with a number of other focal aberrations: 3p21.31 deletion (2.9Mb, *TCTA*), 8q21.12-q24.3 duplication (6.7Mb, *MYC, BAALC*), and deletions of 9p21.3 (152 kb, *CDKN2A/2B*), 9p22.31-q22.33 (5.9Mb) and 12p13.2 (301 kb, *ETV6*) (Fig. 2).

Three patients (L16, L36 and L44) carried a 170 kb microdeletion of 22q11.22 (*VPREB1*). A very small duplication (17 kb) that involved *NOTCH1* was detected in patient L6, while patient L18 had a deletion of 1p12 (39 kb, *NOTCH2)* together with a duplication of 2q13 (118 kb, *MALL*).

21q11.2q22 duplication was present in 6 patients in our cohort.

L2, L6, L25 and L40 showed multiple aberrations of relatively large size in many chromosomes. L25 in particular had partial deletions in chromosomes 9, 11, 13, 14, 15 and 20, as well as partial duplications in chromosome 21. This result, combined with the FISH results reporting 5 copies of *RUNX1* could potentially suggest intrachromosomal amplification of chromosome 21 (iAMP21). Additionally, in this patient chromosome 11 carried multiple deletions and duplications (Fig. 3).

3.10. Regions of CN-LOH

No regions of CN-LOH larger than 10Mb extending to the telomeres were identified in our cohort.

Most patients achieved complete remission. However, patients L15 and L40 presented resistance to treatment and died. Patient L25 relapsed. Patient L43 received bone marrow transplantation. No information on the outcome of treatment was available for 2 patients (Table 1).

4. Discussion

Acute Lymphoblastic Leukemia is characterized by recurrent genetic alterations that designate distinct entities and are used in clinical practice for prognostication and risk stratification, as they appear in 75% of children with ALL [16]. The gold standard for the genetic profiling has thus far been the combination of conventional G-banding karyotype and targeted FISH panels for the most common structural abnormalities. The inherent limitations (mainly the need for culture of malignant clones, low resolution, inability to identify marker chromosomes, need for locus specific FISH probes [17,18]) can hamper conventional cytogenetic analysis.

CMA is a high throughput molecular cytogenetic technique that enables detection of chromosomal aberrations at the exon level. CMA provides significantly higher resolution than conventional karyotype, accurate mapping of the genomic coordinates, and is not limited by poor development of malignant clones in cell culture, although it is not able to detect balanced rearrangements, nor clonal populations smaller than 15–20%. The turnaround time can be as low as 3 days, providing a fast and reliable tool for the genomic profiling of patients with ALL with the added ability to detect new alterations that can serve as potential biomarkers.

The American College of Medical Genetics and Genomics has recently released standards and guidelines for the study of chromosomal abnormalities in neoplastic disorders of the blood and bone marrow, acknowledging the benefits of CMA [19], and while it is being more regularly applied in routine clinical practice, few studies compare the detection rates with those of conventional cytogenetics, especially in regard to pediatric B-cell ALL.

In this study we attempt to evaluate the detection rate of a high resolution CMA platform and compare it to that of conventional cytogenetic techniques, in an effort to quantify the benefit of applying a combined approach to enhance the genetic profiling of ALL patients.

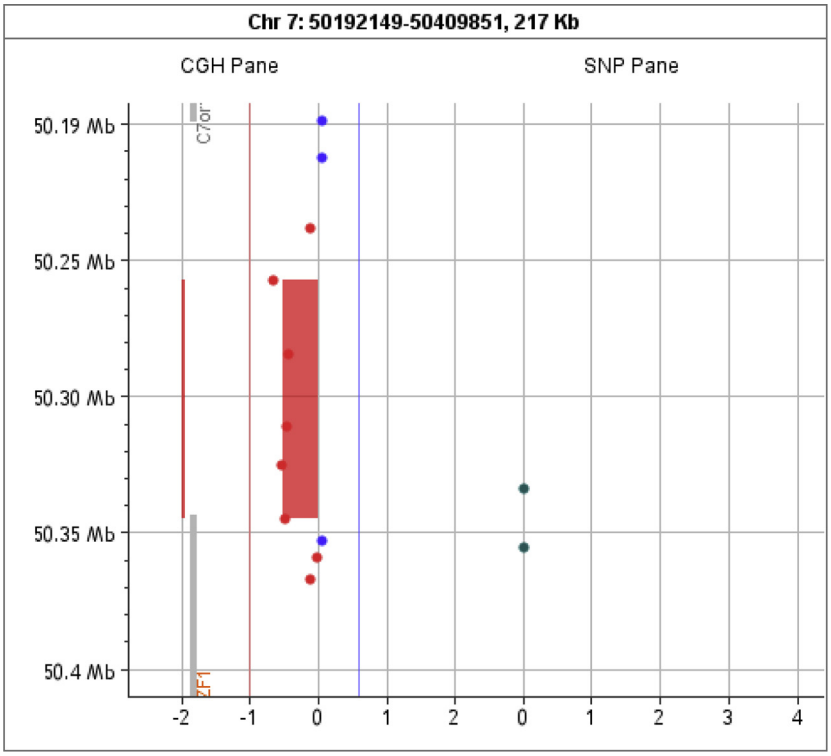
Overall, CMA managed to reveal cryptic aberrations of significance not detected by conventional cytogenetic analysis in 16/29 patients studied (55%), with 8 patients having aberrations above the theoretical detection threshold (> 10-15Mb) of conventional karyotype.

In 5/27 patients (18%), conventional karyotype was false negative, as revealed by CMA. Futhermore, in 9 of 12 patients (75%) with normal conventional karyotypes, we identified one or multiple CNVs of varying sizes, including one patient with hyperdiploidy (64 chromosomes) and one hypodiploid (loss of 10 chromosomes and duplication of 2 chromosomes), leading to the conclusion that conventional karyotype may not reveal the complete picture in regards to the genetic profile of patients with hematological malignancies [5,17] (Table 2). In 2 patients (L10, L16) the results between conventional cytogenetics and CMA were discordant. In patient L10 the malignant clone was detected in 3/20 metaphases studied, and thus was below the detection threshold of CMA. In patient L16, hyperdiploidy was detected with conventional karyotype while CMA identified a hypodiploid karyotype with additional aberrations, which can be attributed to preferential amplification during cell culture. This example further stresses the fact that conventional cytogenetics alone may not provide an accurate profile of the genetic background of ALL patients.

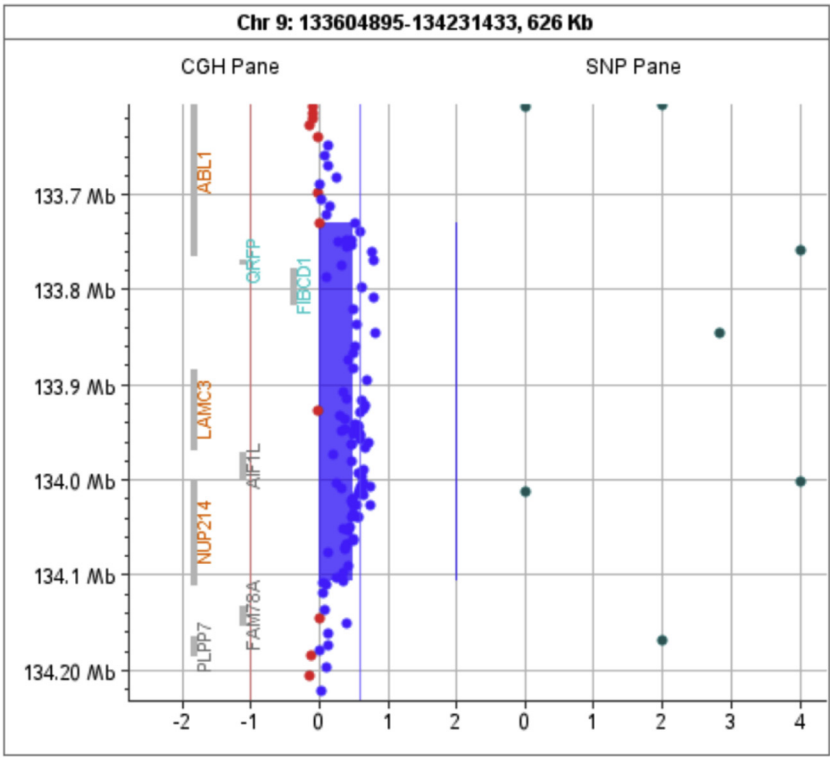
After combining the results from both conventional cytogenetics and CMA, the genetic profile remained the same in only 10/29 (34%) patients while in 19/29 (66%) it was either changed or was enhanced with additional findings.

Especially for patient L40 with negative conventional cytogenetics (G-banded karyotype & FISH), CMA detected several cryptic aberrations, involving key leukemia genes (namely *IKZF1, ABL1, NUP214, NF1*) (Table 2, Fig. 1).

Mutations or deletions of *IKZF1* are frequent events in ALL patients (especially in those with *BCR-ABL1* positive ALL) and are recognized as independent prognostic factors associated with a poor prognosis, mainly due to resistance to treatment conferred by Ikaros alterations



(a)



(b)

Fig. 1. Patient L40 carried 56Kb microdeletion affecting exons 1&2 of IKZF1 gene (a), as well as a 376Kb microduplication that included *NUP214* and *ABL1* genes (b). The deletions are highlighted in red in the image and the duplications highlighted in blue, next to the genes affected. OMIM morbid genes are depicted in orange color, OMIM genes in blue and all other genes in grey (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

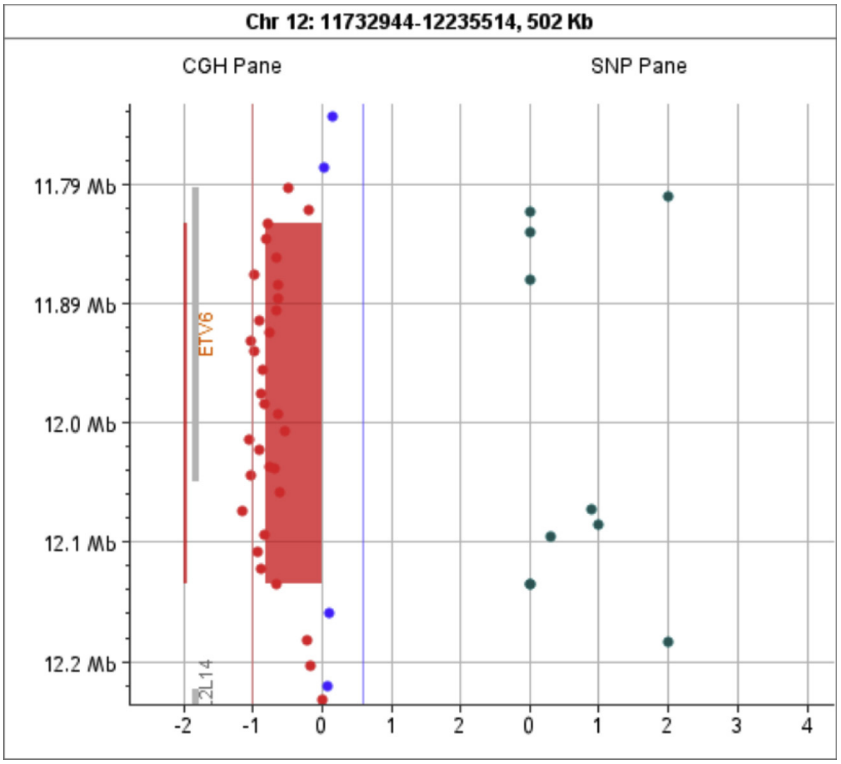


Fig. 2. A 300Kb deletion of the *ETV6* gene detected in patient L2 by Chromosomal Microarray Analysis.

[20,21]. The deletion that affected exons 1 and 2 of the *IKZF1* gene was not detected by conventional methods and would not have been detected by a lower resolution CMA platform.

The result involving duplication of *NUP214 & ABL1* genes has been reported before by Duployez et al. [22] in a female patient, accompanied by a partial deletion of IKZF1, as the one observed in our case. The authors suggested that this could be indicative of a *NUP214-ABL1* fusion, a constitutively activated tyrosine kinase similar to *BCR-ABL1*. The finding could not be confirmed, as our patient displayed resistance to the therapeutic scheme and died.

Patient L25 showed partial duplications of chromosome 21 by CMA and 5 copies of *RUNX1* by FISH, findings that could suggest the presence of iAMP21. Additional findings in this patient, including 11q and 13q deletions, have been associated with iAMP21 [23].

5/29 patients (17%) carried heterozygous deletions of *CDKN2A/B* genes, confirmed by FISH analysis (Table 2). Both of these genes are frequently involved in microdeletions in various neoplasms, including B-ALL [24]. Patient L15, with a deletion of 9p21.3 (*CDKN2A/B*) as its only CMA finding, did not respond to therapy and died. *PAX5* was found to be deleted in our cohort in 2 out of 29 patients, with both deletions being under 100 kb in size.

While gross chromosome aberrations are thought to be the primary initiating events of leukemogenesis, secondary alterations, mainly in the form of cryptic CNVs, are required for disease onset [25], are specific to each B-ALL cytogenetic subtype and can potentially serve as additional biomarkers for the stratification of intermediate risk patients [26].

In our cohort we identified a number of small CNVs affecting genes

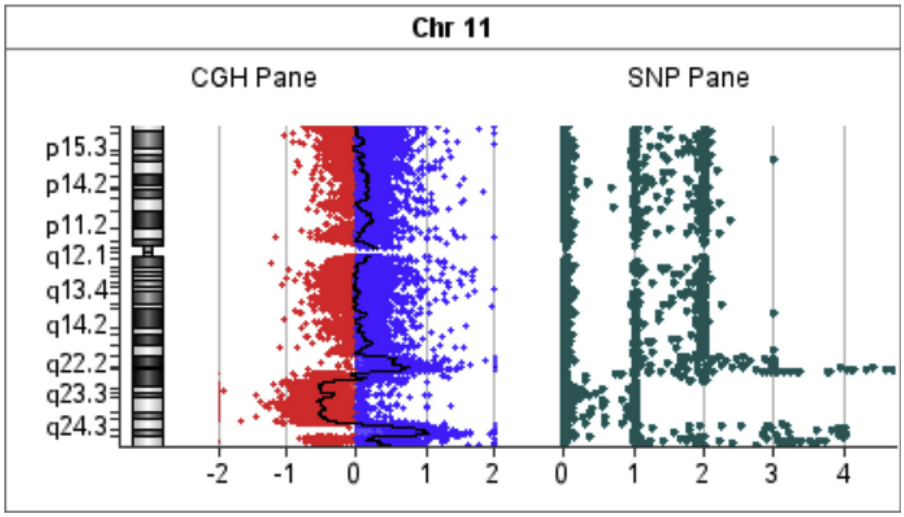


Fig. 3. Patient L25 carried multiple deletions and duplications on chromosome 11, as shown by both the CGH and the SNP probes, a possible indication of chromothripsis.

of interest that are not yet part of risk stratification criteria but can contribute to the overall profiling of the patients. Two patients carried submicroscopic partial *NF1* duplications of 9Kb, affecting exons 19-28. The *NF1* gene encodes neurofibromin 1, a protein that negatively regulates the Ras family of signaling proteins, hence it's loss of function is likely to be equivalent to an activating *RAS* gene mutation [27]. *NF1* microdeletions have been reported before in patients with ALL that had no clinical phenotype of neurofibromatosis [17]. Submicroscopic deletions of 22q11.22 encompassing *VPREB1* gene (a component of the surrogate light chain of the pre-B-cell receptor (pre-BCR) were detected in 3/29 (10%) of our patients. High risk patients that carry *VPREB1* focal deletions have been shown to have poorer overall survival [28]. Patient L6 showed a 17 kb duplication of 9q34.3, involving *NOTCH1* gene. Activating mutations of *NOTCH1* are implicated in almost 50% of T-ALL cases, possibly functioning through transcriptional activation of oncogenes such as *MYC* [29]. *MYC* gene has an established negative correlation with overall survival due to its involvement in the p53 pathway [30]. A duplication of *MYC* gene was observed in patient L2 along with other findings. A deletion of *TP53*, the main gene in the p53 pathway, was identified in patient L36 by both FISH and CMA analysis.

The CGH + SNP array design used in this study offers the added ability of copy number neutral or duplication associated Loss Of Heterozygosity (CNLOH) detection, a frequent event in hematological malignancies [31], that can provide insights to the underlying molecular causes of the disease as well as to the mechanism of clonal evolution responsible for the chromosomal state of patients [32], especially significant for cases with hypodiploidy that undergo chromosomal duplication and appear to be hyperdiploid (doubled hypodiploidy). While no such cases were identified in our cohort, the combined CGH + SNP design, that many platforms currently offer, could potentially provide valuable insight and thus it's use is recommended.

In conclusion, CMA, when combined with conventional cytogenetics, can significantly enhance the cytogenetic characterization of recurrent chromosomal aberrations in pediatric B-ALL patients, by detecting important cryptic submicroscopic variants. Futhermore, the growing application of Next Generation Sequencing technology (NGS) has provided a quick and accurate method of detecting single nucleotide variants in a large number of genes simultaneously. This in depth profiling of ALL patients will undoubtedly lead us closer to the ultimate goal of personalized medicine, where each patient will be able to receive individualized treatment, thus increasing efficiency and minimizing side effects.

Statement of ethics

This study has been approved by the Bioethics Committee of the National and Kapodistrian University of Athens. All patients/parents have provided signed informed consent prior to their enrollment in the study.

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