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DNA copy number changes in childhood acute lymphoblastic leukemia

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Abstract

Background and Objective. **Comparative genomic hybridization (CGH) allows the study of DNA copy number changes in a single hybridization from tumor DNA without any cell culture. Three reports of childhood acute lymphoblastic leukemia (ALL) studied by CGH have been published so far, with somewhat discrepant results. In the present study we performed CGH analysis on 36 patients with childhood ALL. The results were compared to those reported earlier on 157 cases.**

Design and Methods. **DNA was extracted from bone marrow specimens from 36 patients with childhood ALL. The tumor and reference DNAs were labeled with fluorescein-isothiocyanate conjugated dCTP and dUTP, and Texas red-conjugated dCTP and dUTP. The hybridizations were analyzed using the ISIS digital image analysis system.**

Results. **The most commonly gained chromosomes were X (42%), 4 (31%), 6 (31%), 10 (36%), 14 (28%) and 18 (33%), and the most common losses were at 9p22-pter (6%) and 12p13-pter (14%).**

Interpretation and Conclusions. **The pattern of gains of DNA sequences was very similar in the four reports, but the 9p and 12p deletions were observed only in the present study and one previous report. Our review of the results of 193 patients studied so far shows that the success rate using CGH was close to 100%, whereas cytogenetic analysis failed to reveal any information in 21 patients (11%). Furthermore, in 69 (36%) out of 193 patients CGH gave additional information to the banding analysis. CGH should, therefore, be used to supplement standard cytogenetics in the analysis of childhood ALL patients.**

Key words: comparative genomic hybridization, acute lymphoblastic leukemia, DNA copy number changes, gains, losses

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Conventional cytogenetic analysis of G-banded metaphase chromosomes is methodologically more demanding to perform in pediatric ALL than in most other types of leukemia. Comprehensive cytogenetic analysis of the malignant clone(s) in children with ALL remains problematic, despite the current progress in methodology.[1,2] The limitations and difficulties that are particularly critical in pediatric ALL are still present. Metaphases, which may not always represent the neoplastic clone, lead to false-negative findings (i.e. normal karyotype), and difficulties are also encountered in the interpretation of complex karyotypes, especially in poor-quality metaphase chromosomes. Consequently, the prognostic and/or diagnostic significance of many of the chromosomal aberrations detected in the lymphoid blasts, has not been reliably evaluated.

Comparative genomic hybridization (CGH) is a powerful technique for molecular cytogenetic analysis of cancer.[3] DNA copy number changes (gains, losses and high-level amplifications) can be analyzed in the entire tumor genome in a single hybridization and no metaphase cells are needed for the analysis. Clonal aberrations that are present in more than half of the cells can be detected. The resolution of the technique ranges from 10 to 20 megabases (Mb). Balanced translocations, inversions and ploidy changes are beyond the resolution of CGH.

CGH is based on fluorescence *in situ* hybridization which uses whole genomic DNA as probes. The normal DNA and tumor DNA are labeled with different fluorescent colors, equal amounts of the DNA are mixed and hybridized onto normal metaphase spreads prepared from the lymphocyte culture of a healthy individual. After the hybridization, intensities of two fluorescent colors are measured by a digital image analysis system and calculated for each chromosome from p- to q-telomere. If no changes in the tumor genome are detected, the ratio of the signal intensities of two fluorochromes is one. The chromosomal regions that are overrepresented (gains and high-level amplifications) or underrepresented (losses) in the

test genome are seen as a stronger or weaker signal intensity, respectively, than the reference color.

Very few CGH studies in pediatric ALL at the time of diagnosis have been reported so far, Karhu *et al.*[4] have presented results obtained from 13 patients, and Paszek-Vigier *et al.*[5] have reported data from 72 consecutive affected children. Recently, we analyzed 72 consecutive patients.[6] We present here data from 36 new patients with ALL, who were analyzed using CGH at the time of diagnosis, and compare the results with those from the previously reported 157 cases.

Materials and Methods

Patients

The study was performed on a series of 36 diagnostic (i.e. prior to the initiation of chemotherapy) bone marrow samples from patients with childhood ALL. Patients were diagnosed and treated at the Kuopio University Central Hospital and the Hospital for Children and Adolescents, Helsinki University Central Hospital, Finland. The diagnosis was based on the morphologic evaluation of bone marrow aspirates and biopsies as well as on flow cytometric analysis of the marrow blast population. Some clinical parameters from the patients are summarized in Table 1. All the patients except two had pre-B phenotype. Three of the patients suffered from Down's syndrome. DNA from the bone marrow samples was extracted using conventional methods.

Comparative genomic hybridization

CGH was performed using direct fluorochrome-conjugated DNAs for all samples according to previously described methods with minor modifications.[3,7,8] Briefly, blast DNA and reference DNA (genomic DNA from peripheral blood lymphocytes from a normal donor) were labeled with fluorescein-isothiocyanate (FITC)-conjugated dCTP and dUTP (DuPont, Boston, MA, USA) and Texas-red-conjugated dCTP and dUTP (DuPont) by nick translation to obtain fragments ranging from 600 to 2000 base pairs as reported previously.[9] The hybridization mixture consisted of 400 ng of blast DNA, 400 ng of reference DNA, and 10 µg of unlabeled Cot-1 DNA (Gibco BRL, Life Technologies, Gaithersburg, MD, USA) dissolved in 10 µL of hybridization buffer (50% formamide, 1% dextran sulphate, 2× SSC). The hybridization mixture was denatured at 75°C for 5 min and hybridized to a slide preparation with normal metaphase spreads denatured in 70% formamide/2× SSC (pH 7) at 68°C for 2 min. Hybridization was performed at 37°C for 48 h. Then the slides were washed three times in 50% formamide/2× SSC (pH 7), twice in 2× SSC, and once in 0.1× SSC at 45°C, followed by 2× SSC, 0.1 M NaH_2PO_4- 0.1 M Na_2HPO_4- 0.1% NP40 (pH 8) and distilled water at room temperature, for 10 min each. After air-drying, the slides were counterstained with 4',6-diamidino-2-phenylindoledihydrochloride (DAPI) (Sigma Chemical Co., St. Louis, MO, USA) and mounted with an anti-fading medium (Vectashield® Vector Laboratories, Burlingame, CA, USA).

Digital image analysis

The hybridizations were analyzed using an Olympus fluorescence microscope and the ISIS digital image analysis system (MetaSystems, Altlussheim, Germany) based on an integrated high-sensitivity monochrome charge-coupled device (CCD) camera and automated CGH analysis software. Three-color images (red for reference DNA, green for blast DNA and blue for counterstaining) were acquired from 8-12 metaphases per sample. Chromosomal regions were interpreted as overrepresented when the corresponding ratio exceeded 1.17 (gains) or 1.5 (high-level amplification), and as underrepresented (losses) when the ratio was less than 0.85. When necessary, the threshold values were corrected according to the ploidy level as recommended elsewhere.[10] This resulted in lower and upper threshold values of 0.57 and 0.78 for pseudo-triploid blast cells and 0.43 and 0.58 for pseudo-tetraploid blast cells, respectively. The ploidy level was chosen as the closest round integral to the number of chromosomes revealed by the karyotype analysis (patients #21, 22, 34, and 36). In each CGH experiment, negative (peripheral blood DNA from a normal control) and positive (tumor DNA with known copy number changes) controls were included and run simultaneously with the blast samples. All results were confirmed using a 99% confidence interval. Briefly, intra-experiment standard deviations for all positions on the CGH ratio profile were calculated from the variation of the ratio values of all homologous chromosomes within the experiment. Confidence intervals for the ratio profiles were then computed by combining them with an empirical inter-experiment standard deviation and by estimating the error probability based on the t-distribution.

Results

The present 36 patients

Twenty-nine (81%) out of the 36 patients showed changes with a mean of 5.6 aberrations per patient (range, 1 to 19). Gains were more frequent than losses (gains:losses = 11:1). All chromosomal regions with an increased or decreased DNA sequence copy number are summarized in Figure 1.

A high proportion of the gains (91%) were observed to affect entire chromosomes and less frequently chromosome arms (2.0%) or chromosomal bands (8%) (Table 1, Figure 1). Gains of DNA sequence copy number affected most commonly the whole of chromosomes X (42%), 4 (31%), 6 (31%), 10 (36%), 14 (28%), 17 (28%), 18 (33%) and 21 (44%, excluding patients with Down's syndrome). The most frequent minimal common regions of gain were narrowed down to 21cen-q21 (50%), 10q23-q25 (39%),

Table 1. Clinical data, chromosomal number and DNA copy number changes in the 36 new patients with pediatric ALL

Patient No. (sex/age)[a]	*Lab code*	*WBC (x 10^9/L)*	*Chromosomal number*[b]	*DNA copy number changes*	
				losses	*gains*
1 (M, 12.0)	KUH–21	35.7	46	–	–
2 (F, 1.4)[#]	KUH–4	92.5	46	–	–
3 (M, 14.4)	KUH–26	470	46	–	–
4 (M, 4.6)	KUH–27	3.9	NA	–	–
5 (M, 11.2)	KUH–20	5.8	46	12p13–pter	–
6 (F, 2.2)	KUH–24	21.6	46	12p13–pter	–
7 (F, 6.0)	KUH–23	8.1	46	12p	–
8 (M, 3.2)	KUH–33	1.9	46/47	12p	–
9 (F, 14.8)	KUH–18	15.1	46/53–57	9p22–pter, 12	13
10 (F, 8.6)	KUH–15	99.4	47	9p21–pter, 11p14–pter	21
11 (M, 12.1)	971074	4.3	46	18p	<u>21</u>
12 (M, 2.0)	KUH–11	2.6	46/55	12p	6, 10, 14, 15, 17, 18, 21, X
13 (F, 4.2)	KUH–12	5.6	46/49	7	9q, X
14 (M, 5.3) §	971023	1.1	45/46	9, 18	1q31–q42, 22q12–qter
15 (M, 4.6)	971207	40.5	46	–	21cen–q21
16 (F, 2.5)	KUH–2	211	46	–	9q22–qter
17 (M, 13.0)	KUH–3	3	46/56–57	–	9p13–p23, 21
18 (M, 6.0)	KUH–32	82.3	46/47	–	1q32–qter, 10
19 (M, 14.9)	KUH–22	1.7	46/55	–	4, 6, 10q22–q25, 15, 18, 21, X
20 (F, 10.6)	971081	1.7	58–60*	–	1q32, 4, 5, 6, 8, 10, 14, 17, 18, 21, X
21 (F, 8.3)	970633	60.2	46/60*	–	1q23–qter, 2, 3, 4, 5, 6, 7, 10, 14, 17, 18, 21, X
22 (F, 3.6)	KUH–7	10.3	46/65*	–	1, 2p22–pter, 2q, 3, 4, 5, 6, 9q22–qter, 10, 11, 12q21–q24.1, 14, 15, 16, 17, 18, 21, 22, X
23 (F, 4.5)	KUH–13	16.2	46/50	–	4, 10, 17q, 21
24 (M, 4.5) DS	KUH–1	77.6	47/47–48	–	21
25 (F, 9.7)	KUH–5	2.8	46/59–61	–	X
26 (F, 6.7) DS	KUH–6	19.5	47	–	21
27 (F, 12.1) DS	KUH–25	16.9	46/47	–	21
28 (M, 1.5)	KUH–29	6.2	46/54	–	X
29 (F, 12.0)	KUH–8	60.2	46/51	–	5, 21
30 (F, 2.9)	KUH–9	84.3	46/53	–	4, 8, 10, 18, 21, X
31 (M, 1.8)	KUH–10	27.9	46/54	–	4, 6, 10, 14, 17, 18, 21, X
32 (F,1.8)	KUH–17	2.1	46/55	–	4, 6, 10, 14, 17, 18, 21, X
33 (M, 1.8)	KUH–19	3.6	46/55	–	5, 6, 10, 14, 17, 18, 21, X
34 (F, 10.1)	KUH–16	1.3	46/58*	–	4, 5, 6, 10, 14, 17, 18, 21, 22, X
35 (F, 4.0)	KUH–31	2.8	46/57	–	4, 5, 6, 10, 14, 15, 17, 18, 21, X
36 (F, 10.5)	KUH–14	16.7	46/92*	–	1, 2, 3, 4, 5, 6, 8, 9, 10, 12, 13, 14, 16, 17, 18, 19, 21, 22, X

[a]*DS, Down's syndrome;* [b]*NA, data not available;* [c]*high-level specifications are underlined.* [#]*Phenotype pre-T;* [§]*Phenotype not known; *confidence intervals for ratio profiles corrected according to ploidy level for CGH analysis.*

17q (31%), 1q32 (17%) and 9q22-qter (11%) (Table 1, Figure 1). High-level amplifications were rare and observed only once, affecting chromosome 21 from patient 11 (3%) (Table 1, Figure 1).

Losses were observed to affect principally chromosomal bands (38%) and less frequently chromosome arms (31%) or entire chromosomes (31%). Losses most frequently occurred in 12p13-pter (14%) and 9p22-pter (6%) (Table 1, Figure 1).

Summary of reported patients

CGH findings from the 193 children with ALL are summarized in Figure 1. The most frequent gains and losses of DNA sequences are listed in Table 2. In 118/193 patients (61%) chromosomal gains and losses were identified using CGH analysis (range, 50%[5] to 62%[6]). Seventy-five (39%) patients showed normal results. Gains were 8.7 times more frequent than losses (487 gains vs 56 losses). The most common CGH findings were gains of entire chromosomes, mainly in chromosomes 21 (27%, excluding patients with Down's syndrome), X (26%), 18 (23%), 10 (21%), 14 (21%), 4 (19%), 6 (19%), 17 (16%) and 8 (12%) (Table 2). Other less frequently affected chromosomes are listed in Table 2. The most frequent interstitial gains were narrowed down to Xq25-

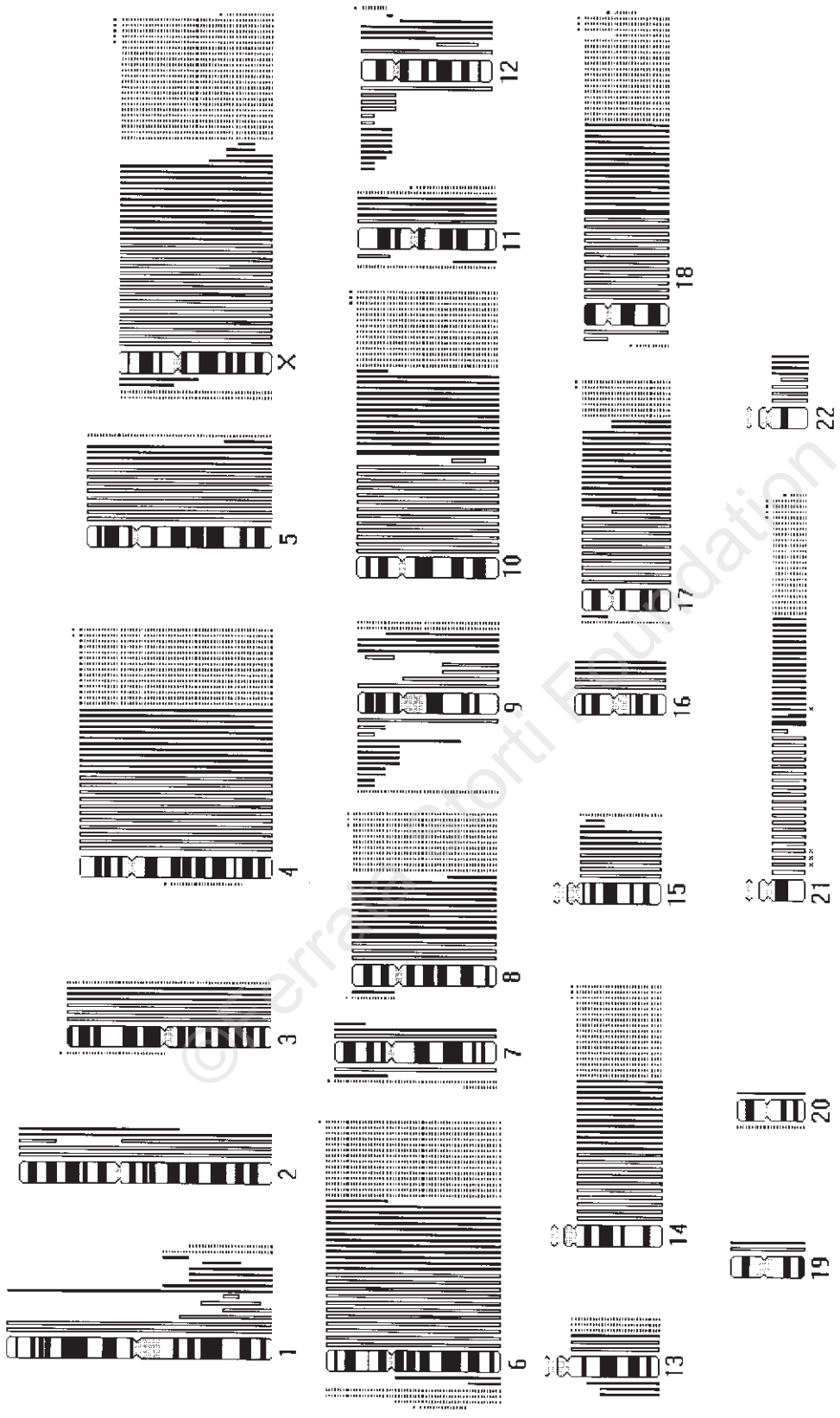

Figure 1. Summary of gains and losses of DNA sequence copy number in 193 cases of childhood acute lymphoblastic leukemia analyzed so far by CGH at diagnosis. Losses are shown on the left and gains on the right of each chromosome. Each bar or line represents a genetic alteration seen in one patient. High-level amplifications of small chromosomal regions are marked with thick bars and lines. An asterisk under a line or bar denotes a constitutional chromosomal alteration. Empty bars represent alterations observed in the 36 new ALL cases reported in this paper. Solid lines represent the alterations observed in the previously reported 72 cases of ALL.[6] Broken lines denote changes in 72 cases of ALL reported by Paszek-Vigier *et al.*[5] and dotted broken lines show the alterations found by Karhu *et al.*[4] in 13 ALL patients.

Table 2. Summary of the most recurrent DNA copy number gains and losses at diagnosis in 193 children with ALL

Gain	*A*	*B*	*C*	*D*	*Total n=193*	*Loss*	*A*	*B*	*C*	*D*	*Total n=193*
X	42	19	24	38	26	X	0	0	3	0	1
1q32	17	8	3	0	7	Xp	0	3	0	0	1
4	31	17	17	15	19	6	0	0	3	0	1
5	22	6	1	0	7	6q16-q23	0	3	1	8	2
6	31	17	18	8	19	6q24-qter	0	1	0	0	1
8	8	12 (1)	11	23	12 (0.5)	9p22-pter	6	12	0	0	6
10	36	18 (1)	15	23	21 (0.5)	12p13-pter	14	11	0	0	7
14	28	18	19	23	21	13q21-qter	0	4	0	0	2
15	11	6	1	0	5						
17	28	19	7	15	16						
18	33	21 (1)	21	23	23 (0.5)						
21*	44 (3)	22 (3)	24	31	27 (2)						

A) Present study, n=36; B) Larramendy et al. (1998), n=72; C) Paszek-Vigier et al. (1997), n=72; D) Karhu et al. (1997), n=13.
**Patients with Down's syndrome excluded. Frequency (%) of high-level amplifications in brackets.*

q26 (29%) and 1q32 (7%) (Table 2). Losses involving entire chromosomes were more frequently observed on chromosomes X (1%), 6 (1%), 7 (1%) and 9 (1%) (Table 2). Interstitial losses were found most often in 9p22-pter (6%), 12p13-pter (7%), 6q16-qter (3%) and 13q21-qter (2%) (Table 2). High-level amplifications were rare. They were only observed in chromosome 21 (2%) and in 8, 10, 12p and 18 (0.5% each) (Table 2).

Differences in DNA copy number changes between the four studies

The pattern of gains of DNA sequences was, in general, similar in the four reports.[4-6] Moreover, our new cohort of patients showed an increase in the frequency of gains of the whole of chromosomes X, 4, 5, 6, 10, 15, 17, 18 and 21, and of gains in 1q (Table 2, Figure 1). Comparison of the most common losses shows that 9p and 12p deletions were only observed in this study and in our previous study (Table 2, Figure 1).

Discussion

Success rate of CGH vs standard chromosome analysis

Successful results were obtained using CGH for all 193 patients analyzed, but cytogenetic karyotyping failed to reveal any information in 11% of the cases, either because karyotyping was not performed (5 patients) or because the results were unsuccessful (16 patients).[4-6] Furthermore, in as many as 69 patients (36%) CGH supplemented the banding analysis, giving information about the chromosomes gained, lost or involved in the formation of marker chromosomes.[4-6] This study, as well as the three earlier ones on pediatric ALL[4-6] demonstrated that CGH analysis clearly supplements standard chromosome karyotyping.

Comparison of the DNA copy number changes and chromosome aneuploidy

Our summary of the DNA copy number changes seen in 193 patients shows that the most common gains were in chromosomes 21 and X (26-27%) and, at lower frequencies, in chromosomes 18, 10, 14, 4 and 6 (19-23%), whereas the most frequent losses were in 9p22-pter and 12p13-pter (6-7%) and, less frequently, in 6q16-qter and 13q21-qter (2-3%). These findings agree well with the cytogenetic results from four previous studies covering 2336 cases of ALL.[11-14] Trisomies were reported as follows: chromosome 21 (31%), X (24%), 18 (18%), 10 (16%), 14 (20%), 4 (20%) and 6 (22%). Deletions in 9p (9%), 12p (6%) and 6q (5-10%) were also reported.[11-14]

Even though copy number losses at 13q were observed in 2% of the cases, the cytogenetic studies did not confirm this finding.

Comparison of DNA copy number changes in the four CGH reports

The major discrepancy between our present and previous findings[6] and the results reported by Karhu *et al.*[4] and Paszek-Vigier *et al.*[5] lies in the higher frequencies we observed in both the most common chromosome gains and gains within 1q, and especially in the losses at 9p and 12p. Gains in 1q affecting the minimal common region of 1q31-q32 were the most frequent partial gains in our previous cohort of 72 children with ALL (8%)[6] as well as in the patients studied by Paszek-Vigier *et al.* (3%).[5] This gain is most probably due to an unbalanced translocation, t(1;19) (q24;p12), a well-known aberration detected in about 5% of the cases of ALL with clonal aberrations.[15,16] No study other than ours revealed losses at 9p and 12p.[4-6] Since the losses we found in 9p and 12q were confirmed by chromosome banding analysis (results from the present study

not shown), an overinterpretation of the losses can be ruled out.

In our studies, hybridizations of both positive control (with known copy number gains and losses) and normal negative control DNAs were included and run in parallel with the test DNAs. Only the hybridizations in which the positive control revealed the known changes and the negative control revealed normal CGH profiles were accepted for analysis.

Another important methodological aspect to consider when interpreting DNA copy number changes is that the calibration of the image analysis software is chosen according to the DNA ploidy level of the leukemic cells.[7,10] When triploid leukemic cells are studied using diploid threshold assessment, trisomic changes may not be detected. Standard chromosome banding analysis, interphase FISH or flow cytometry should be performed to determine the correct ploidy level of the leukemic cells. In addition to these methodological reasons, heterogeneity of leukemic cells may further explain the differences observed in the losses in 9p and 12p as well as in the frequencies of the most common gains.

Concluding remarks

Our summary of CGH studies in pediatric ALL shows that the CGH methodology produces successful results in all cases. It should, therefore, become a supporting technique for standard chromosome banding analysis, especially in cases of childhood ALL in which standard cytogenetics still give unsuccessful or inadequate results in almost half of the cases. CGH should not, however, replace standard karyotyping, because it does not reveal the acquired balanced translocations known to have an essential prognostic and biological significance in leukemogenesis. Great attention should also be paid to methodological controls when using the CGH technique.

Contributions and Acknowledgments

SK was responsible for the conception of the study, its design, ethical approval, funding, direct supervision, recruitment and contact with the participants. MLL, TH and KH performed CGH analyses and wrote the paper. The others took part in the study design. MLL and TH contributed equally to this work. Otherwise the criteria applied for the order of the authors was the degree of their contribution to the study. The last two authors are senior investigators. The authors thank Mrs. Vuokko Kumpulainen and Mrs. Ulla Korhonen from the Department of Special Chemistry at the Kuopio University Hospital for preparing the DNA samples.

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Disclosures

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