Novel cryptic chromosomal rearrangements detected in acute lymphoblastic leukemia detected by application of new multicolor fluorescent *in situ* hybridization approaches

CONSTANZE KARST[1], MADELEINE GROSS[1], DETLEF HAASE[2], ULRICH WEDDING[3], KLAUS HÖFFKEN[3], THOMAS LIEHR[1] and HASMIK MKRTCHYAN[1,4]

[1]Institut für Humangenetik und Anthropologie, Kollegiengasse 10, D-07743 Jena; [2]Hämatologie/Onkologie, Zytogenetisches Labor 3D1.235, Georg-August-Universität, Robert-Koch-Str. 40, D-37075 Göttingen; [3]Klinik und Poliklinik für Innere Medizin II, Erlanger Allee 101, D-07747 Jena, Germany; [4]Department of Genetic and Laboratory of Cytogenetics, State University, 1 Alex Manoukian Street, Jerewan 375025, Armenia

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Abstract. Routine cytogenetic analysis provides important information on diagnostic and prognostic relevance for hematological malignancies. However, it is often difficult to obtain good karyotypes, especially of cells from cases with acute lymphoblastic leukemia (ALL) because of poor morphology and spreading. Thus, detailed karyotyping can be hampered and even in case of a 'normal karyotype' according to banding cytogenetics doubts remain if the result is reliable. In order to address this problem a series of 37 ALL cases without any detectable numerical or structural chromosomal defects was selected and studied by two recently developed multicolor fluorescence *in situ* hybridization (FISH) approaches: 1) multitude multicolor banding (mMCB) is a FISH-banding technique, which allows the analyses of inter- and intra-chromosomal rearrangements of the whole human karyotype in one single experiment; 2) chromosome-specific subcentromere/subtelomere-specific multicolor (subCTM-)FISH applies locus-specific subtelomeric and subcentromic probes and enables the characterization of the subtelomeric and peri-centric regions of the chromosomes, not analyzable by other FISH-approaches. Thus, we detected the following recurrent cryptic chromosomal aberrations: del(12)(pter) [8 cases], del(9)(qter) [3 cases], and del(11)(pter) [2 cases]. Moreover, cryptic changes in additional nine subtelomeric and in two subcentromeric regions were observed one time, each. In summary, mMCB and subCTM were proven to be powerful methods in the screening for new cryptic chromosomal aberrations, which considerably increased the accuracy of cytogenetic diagnosis.

Correspondence to: Dr Thomas Liehr, Institut für Humangenetik und Anthropologie, Postfach, D-07740 Jena, Germany
E-mail: i8lith@mti.uni-jena.de

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Introduction

The classification of acute leukemia into therapeutically relevant risk categories relies on clinical parameters, as well as on the tumor cell karyotypes. Based on such data, the current cure rate has made remarkable progress in children and in adults (1,2). However, the development of more reliable laboratory tests that predict patient prognosis should lead to an even more enhanced patient survival. This is especially valid and necessary in T-ALL, where no cytogenetically defined prognostic subgroups have been identified yet (3).

An integral part of the diagnosis of ALL is the cytogenetic screening for chromosomal abnormalities. In the past, routine cytogenetics identified many important karyotypic changes (4) and thus gave hints on the localization of oncogenes or tumor suppressor genes (reviewed in refs. 5 and 6). Even though the introduction of molecular cytogenetics in the last decades facilitated routine tumor cytogenetic analysis, high mitotic index and chromosomes of good quality still have importance, especially in the initial chromosome analysis. Nonetheless, the characterization of cryptic rearrangements remains problematic if standard techniques are applied, like GTG-banding combined with M-FISH (7) or SKY (8). This is mainly due to the fact that cell suspension of ALL-cases often present with only a few metaphase spreads per slide and bad chromosome morphology, i.e. short, bad shaped chromosomes with a low banding resolution. Thus, FISH-banding techniques (9) such as the multitude multicolor-banding (mMCB) (10) or approaches like the subcentromere/subtelomere-specific multicolor (subCTM-) FISH (11) were developed to overcome these difficulties.

In the present study, we analyzed cytogenetic bone marrow preparations of 37 patients suffering from ALL; banding

Table I. Overview of the clinical diagnosis, the age of the patient, the molecular cytogenetic result and the techniques applied in the 37 studied patients.[a]

Case (age at diagnosis)	Result	Method
T-ALL		
1 (43)	38~45,XX[7]/38~43,XX,del(9)(q34.3)[3]/46,XX[2]*	**mMCB**; LSI MLL; **subCTM** #2, #3, #4, #5, #6, #7, **#9***, #11, #12, #13, #14, #16, #19, #22
2 (17)	46,XX,del(11)(p15.5)[3]/46,XX[2]* nuc ish 11p15.5[60/100]	**mMCB**; **subCTM #11***; LSI BCR/ABL-ES
3 (28)	46,XY,del(12)(p13)[3]/46,XY[4]*	**mMCB**; **subCTM #12***
4 (29)	46,XY,del(12)(p13)[3]/46,XY[2]*	**mMCB**; **subCTM** #5, #7, **#12***
5 (42)	46,XY,del(12)(p13),t(7;17)(p22;q22)[4]/46,XY[11]*	**mMCB***; M-FISH; **subCTM** #1, #2, #3, #4, #5, #6, **#7**, #9, #11, **#12**, #13, #14, #16, **#17**, #18, #21, #22
6 (30)	46,XY,del(13)(q34)[3]/46,XY[5]*nuc ish 13q34[14/100]	**mMCB***; LSI-MLL
7 (18)	45,XY,der(5)t(9;5;9)(9qter->9q13::5q13->5q23.1::5p13.1->5p15.2~15.3::5q23.1-> 5p15.2~15.3::5q13->5q23.1::5p13.1->5p15.2~15.3::5q13->5q23.1::9p21->9pter),-9,del(11)(q14),del(14)(q12)[9]/46,XY,del(4)(p13~14),der(5)t(18;5)(18pter->18p11.31: :5q13->5p15.2~15.3::5p12->5pter),-9,del(11)(q14),del(14)(q12),der(18)t(18;5;9)(18qter->18p11.31::5p15.3->5p12::9q13->9qter)[3]/46,XY,del(5)(q34)[3]/46,XY[37]*	**mMCB***; **SKY***
8 (16)	48,XX,der(4)?t(4;18),+mar1,+mar2 [3]/46,XX[12]*	mMCB; M-FISHM; LSI MLL; **subCTM** #1, #2, #3, **#4***, #5, #6, #7, #11, #12, #21, #22
9 (21)	45,XY,-19[8]/46,XY[12]*	**mMCB***; LSI MLL; **subCTM** #5, #7, #11, #18, **#19**
10 (8) pre-T-ALL	47,XY,+21[3/10]* 46,XY,ins(5;7)(q21;p14p22),t(3;7)(p21;q11.23)[5/10]** t(9;22)(q34;q11)[5/14]***	**mMCB; M-FISH***; LSI MLL; **LSI BCR/ABL-ES*****; **MCB #3/#5/#7****; **subCTM** #2, #3, #4, #9, #14, #17, #18, #19, **#21**, #22
11 (23)	46,XY	mMCB; SKY; M-FISH; M-TEL; WCP #5; LSI-MLL
12 (27)	46,XY	mMCB
13 (21)	46,XX	mMCB; subCTM #4, #7, #12, #19
B-ALL		
14 (65)	46,XX,del(11)(q25)[3]/46,XX[16]* 46,XX,del(12)(p13)[3]/46,XX[13]** 46,XX,del(17)(q25)[3]/46,XX[8]*** 46,XX,del(18)(p11.31),dup(18)(q11.2)[4]/46,XX[16]****	**mMCB**; LSI MLL; **subCTM** #1, #2, #4, #5, #7, #9, **#11***, **#12****, #13, #14, **#17*****, **#18******, #19, #21, #22
15 (54)	46,XY,del(12)(p13)[3]/46,XY[7]*	**mMCB**; **subCTM #12***
16 (23)	46,XX	mMCB; subCTM #1, #4, #5, #6, #9, #11, #12, #13, #14, #19, #21
17 (57) pre-B-ALL	45,XX,r(5)(p10q34),del(9)(p24),-21[3]/ 46,XX,del(4)(q31),del(5)(q21)[3]/46,XX[2]*	**mMCB***; **MCB #5**; LSI MLL; **WCP #5**; **M-TEL**
18 (64) pre-B-ALL	46,XX,del(9)(q34)[3]/46,XX[3]*	**mMCB***; LSI MLL
19 (19) pre-B-ALL	46,XX,del(11)(p15.5)[3]/46,XX[7]* 46,XX,del(22)(q13.33)[3]/46,XX,-22[3]/46,XX[4]**	**mMCB**; LSI MLL; **subCTM #11*, #22****

Table I. Continued.

Case (age at diagnosis)	Result	Method
B-ALL		
20 (6) pre-B-ALL	46,XX	mMCB
21 (51) pre-pre-B-ALL	~89,XXXX[3]/46,XX[7]*	**mMCB***
22 (17) biphen. ALL/ B-ALL	47,XX,+8,del(4)(p16.3)[6/15]* 47,XX,+8,del(12)(p12)[6/17]** 47,XY,+8,del(5)(q34),t(5;7)(q31;p12)[8/20]***	**mMCB***; **M-FISH****; LSI MLL; **WCP #5**; **subCTM #4*, #5, #6, #7, #12****, #19, #21
C-ALL		
23 (49)	46,XY,der(1)dup(1pter-q44::q12qter)[3]/46,XY[7]*	**mMCB**; **M-FISH**; **WCP #1**; **MCB #1***
24 (22) FAB L3	45,XY,del(9)(q34),del(12)(p13),-22[3]/46,XY[3]*	**mMCB***; **subCTM #12**
25 (19)	46,XY,del(12)(p13.33)[3]/46,XY[3]* nuc ish 12p13.33 [9%]	**mMCB; subCTM** #4, #7, **#12***, #22
26 (40)	45,XX,del(5)(q32),-22[3]/46,XX[5]*	**mMCB***; **M-FISH**
27 (19)	46,XY	mMCB; M-TEL; MCB #5; WCP #5; WCP #9; LSI-MLL
28 (28)	46,XY	mMCB; SKY; LSI BCR/ABL-ES
29 (48)	46,XY	mMCB; subCTM #5, #7
30 (27)	46,XY	mMCB; LSI BCR/ABL-ES
ALL		
31 (67)	46,XX	mMCB
32 (55)	46,XX	mMCB
33 (14)	46,XY	mMCB; MCB12; subCTM#12
34 (3)	46,XX	mMCB; subCTM #9
35 (57)	46,XX	mMCB
36 (34)	46,XX	mMCB
37 (42)	46,XX	mMCB; LSI-MLL

[a]The probe sets leading to the identification of a previously cryptic rearrangement are in bold in the last column. The asterisks indicate which FISH-results was/were used for the karyotypic formula and determination of approximate clone sizes in the center column.

cytogenetics revealed no cytogenetic changes in these cases. After application of molecular cytogenetics, we detected cryptic aberrations in 21 of these cases (~57%).

Materials and methods

Studied cases and banding cytogenetics. Bone marrow cells of 37 patients were investigated for chromosomal changes in connection with an acute lymphoblastic leukemia (ALL). Cells were taken into short-term cell culture and chromosomes were prepared and GTG-banded according to standard protocols (12). Metaphase spreads (10 to 20) were available for cytogenetic evaluation on a banding level of 180-250 bands per haploid karyotype. Thirteen patients suffered from T-ALL, 9 had a B-ALL (4 including pre-B-ALL and 1 pre-pre-B-ALL case), 8 patients had a C-ALL and 7 presented a clinically not closer classified ALL (Table I). Apart from cases 10, 20 and 34, all were adult ALL cases.

Molecular cytogenetics. Fluorescence *in situ* hybridization (FISH) was performed according to standard protocols (13) using the commercially available probe LSI-MLL (11q23, Vysis) and/or LSI BCR-ABL-ES to exclude a cryptic 'Phildelphia-translocation' t(9;22)(q34;q11). M-FISH or

SKY (14), mMCB (10), MCB (15) and subCTM-FISH (11) or M-TEL-FISH (16) were done as previously described. The results were evaluated on a fluorescence microscope equipped with a CCD-camera and an image analysis system (Meta-Systems, Altlussheim, Germany). Metaphase spreads (5 to 10) were evaluated per case and probe (set) used. In some cases there was not enough cell suspension available to apply all probe sets (Table I).

Results

The routine banding cytogenetics detected no acquired chromosomal changes in the 37 ALL cases in the present study (Table I). The cases were studied by mMCB (10) at first. According to the mMCB results, additional studies were performed. M-FISH or SKY (14) were used when the mMCB result had to be verified for possible inter-chromosomal exchanges (7 cases). In case 10, it was necessary to confirm and substantiate mMCB also by single MCB experiments (15). If only sparse cell suspension was available, chromosome-specific subCTM-FISH probe sets (11) were selected according to the mMCB results; in cases with sufficient test specimens subCTM-FISH probe sets for (almost) all chromosomes and also the LSI MLL probe were applied (e.g. cases 1, 5, 8, 14, 16, and 22). Cases 11 and 27 were tested by M-TEL-FISH (16) for cryptic subtelomeric rearrangements.

In the aforementioned cytogenetic preparations of 37 patients suffering from ALL (Table I), molecular cytogenetics detected cryptic aberrations in 19 of the cases (~50%). They were divided into four groups: 1) such cases with cytogenetic changes detectable by banding cytogenetics, however, not detected in these cases, due to too few evaluated/evaluable metaphase spreads and/or too low banding resolution, i.e. in cases 7-10, 21, 23 and 26 (19% of 37 cases); 2) cases with by banding cytogenetics undetectable, i.e. real cryptic changes in cases 2-4, 6, 14-15, 18, and 25 (21%); 3) cases presenting a mixture of group 1 and group 2 aberrations: cases 1, 5, 17, 19, 22 and 24 (16%); and, 4) those cases without any detectable karyotypic changes after application of mMCB and/or the other molecular cytogenetic probes (cases 11-13, 16, 20, 27-37 = 44%).

The undetected clonal changes found during banding cytogenetics for group 1 were: hypodiploidy [case 1], hyperploidy [case 21], duplication of the long arm of chromosome 1 [case 23], translocation t(4;18) with two additional marker chromosomes, both changes detected in subCTM-FISH exclusively, and thus, not closer characterizable [case 8], trisomy 8 [case 22], monosomy 19 [case 9] and 22 [cases 19 and 24] and complex [case 17] and highly complex rearrangements [cases 7 and 10] (Fig. 1 and Fig. 2).

The following recurrent cryptic chromosomal aberrations were detected in group 2: del(12)(p13) [8 cases], del(9)(q34.3) [3 cases], and del(11)(p15.5) [2 cases]. Moreover, the following additional cryptic changes were detected for one case, each. However, they were at least 3 times present per case, which is the definition for clonality: del(4)(p16.3) [case 22], del(4)(q31) [case 17], del(5)(q11.2-q11.2) [case 17], del(5)(q34) [case 22], del(13)(q34) [case 6], del(17)(q25) [case 14], del(18)(p11.31) [case 14], del(22)(q13.33) [case 19], dup(18)(q11.2) [case 14], t(7;17)(p22;q22) [case 5]. In peripheral blood of 5 normal controls, similar changes were not observed with the applied subtelomeric and subcentromeric probes; the metaphase-FISH cutoff for artificial signal loss of the used probes was always 1 in 20 metaphases or below.

Discussion

In the present study, the mMCB technique (10) combined with subCTM-FISH (11) and the other aforementioned approaches (Table I) were applied to study chromosomes according to banding cytogenetics karyotypically normal bone-marrow probes of 37 ALL-patients. This is the first systematic study using this kind of methodology in leukemia research.

The FISH-banding technique (9) multitude multicolor banding (mMCB) was chosen as it has proven its ability and reliability in previous studies and is the only one which has been systematically adjusted with other FISH-techniques (17-25). However, as all (multicolor) FISH techniques based on whole or partial chromosome painting probes, also mMCB is not completely reliable concerning the subtelomeric and subcentromeric chromosomal regions. As this study was aimed to examine as comprehensively as possible all chromosomal sub-regions, subCTM-FISH was applied additionally. However, as subCTM can presently only be used chromosome-wise, a large amount of cell suspension is necessary for this kind of analysis. One has to admit that sufficient test specimens were not available in all cases and thus, the comprehensiveness of the present analysis was hampered. However, this problem is a well-known one in tumor cytogenetic studies (26).

Group 1/group 3: cases with cytogenetic changes detectable by banding cytogenetics. Twenty-one of 37 of the studied cases (~57%) presented cryptic aberrations. However, in 12 of the aberrant cases (32%; cases of groups 1 and 3) the cytogenetic aberrations could have been detected in banding cytogenetics, if more and/or better metaphase spreads would have been available for evaluation.

Hypodiploidy or hyperploidy as detected in cases 1 and 21, respectively, are well-known findings in two different small subgroups of adult ALL (27,28). The translocation t(4;18) in case 8 could not be described in more detail and a translocation event in which both chromosomes would have been involved has not been previously described in ALL (4). Trisomy 8 [case 22] and gain of the long arm of chromosome 1 [case 23] were previously reported in ALL (4). Monosomies of chromosomes 19 [case 9] or 22 [cases 19 and 24] were also not previously reported in acute leukemia, while complex [case 17] and highly complex rearrangements [cases 7 and 10] are known to appear and to be associated with an adverse prognosis in ALL (4,29,30).

Thus, a substantial part of cases diagnosed as karyotypical normal seem to have detectable chromosomal rearrangements when reanalyzed by molecular cytogenetics. This seems to be a more general problem, as our cases were recruited from two different German centers (Jena, Göttingen), both headed by well-experienced cytogenetists.

Group 2/group 3: cases with cryptic cytogenetic changes. In a control study using all 24 subCTM-probe sets on peripheral blood of 5 normal controls artificial deletion of the applied

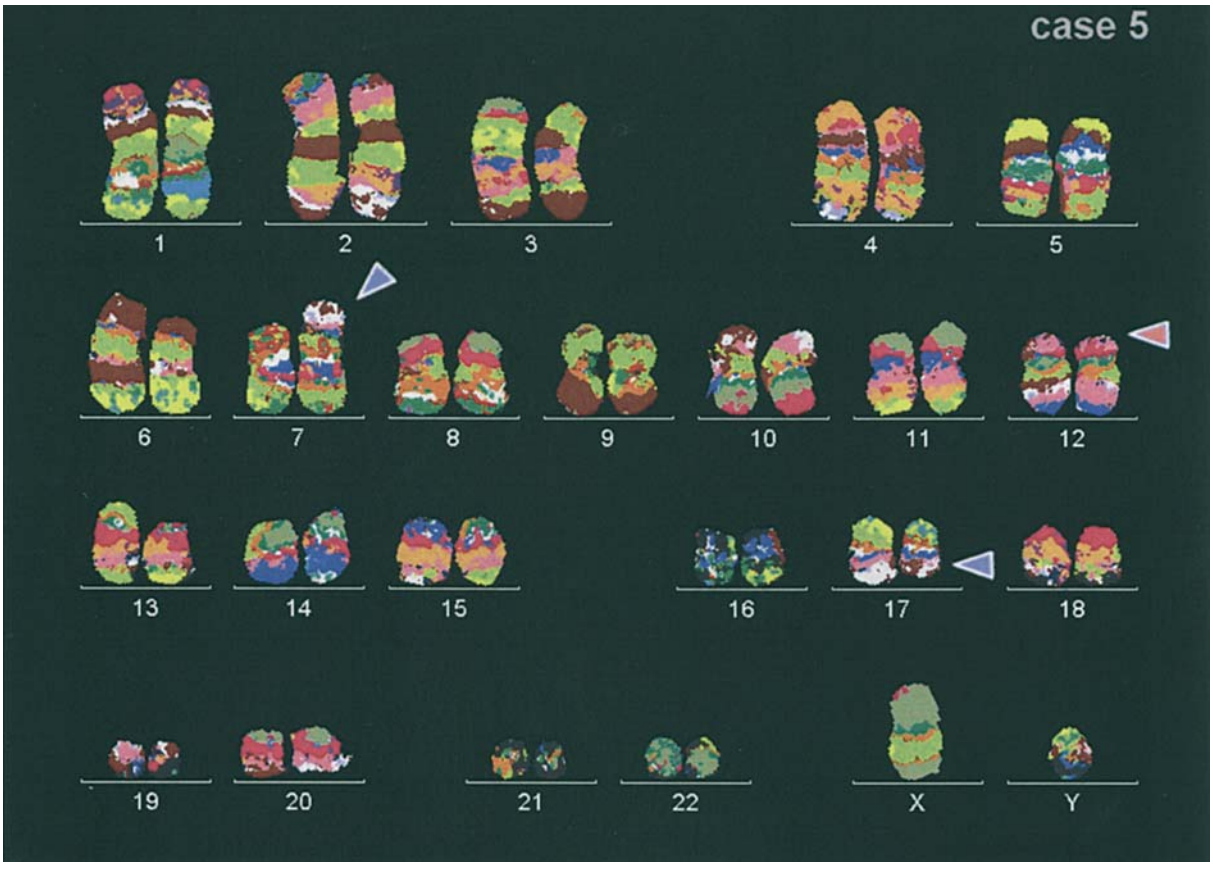

Figure 1. Representative multitude multicolor banding (mMCB) result of case 5 in pseudocolor depiction. The del(12)(p13) is marked with a red arrowhead, the t(7;17) with two blue arrowheads.

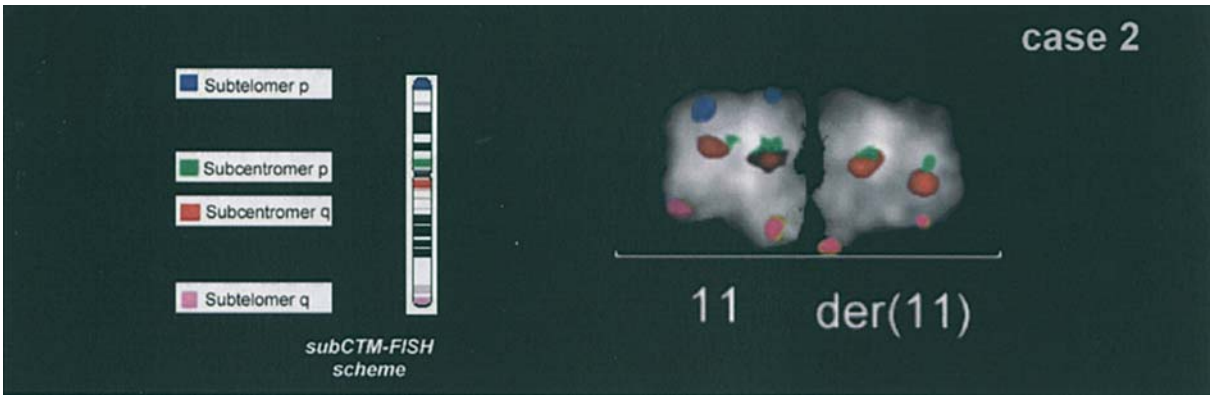

Figure 2. Results of subCTM FISH probe set 11 applied in case 2. On the left, chromosome 11 is depicted as an ideogram with the applied color code for subCTM-FISH given beside it. The color channel for the whole chromosome painting probe is not depicted here, however, both chromosome 11 and derivative chromosome 11 were stained completely.

subtelomeric and subcentromeric probes was observed in 0 to 1 out of 20 metaphases, each. Thus, the cut-off for signal loss was determined at 2/20 mitosis. According to this definition, recurrent cryptic chromosomal aberrations were detected as terminal deletions in 12p13 [cases 3, 4, 5, 14, 15, 22, 24 and 25], 9q34.3 [cases 1, 18 and 24], and 11p15.5 [cases 2 and 19] in 12 of the 37 studied cases (32%).

Indications on involvement of 12p in ALL were previously found (31); interestingly in the present and in the previous study (31) no correlation with the ALL-subtype was detectable. Thus, the frequency of its appearance (~20%) may be interpreted as a hint on an early event during leukemogenesis.

While terminal deletions 9q are well-known in AML (acute myelogenous leukemia) (32), they are rarely described in ALL and then interpreted as interstitial deletions in 9q (4,33). However, the application of the subtelomeric probes in 9q encovered terminal deletions in 9q34 in one case of adult T-ALL, B-ALL and C-ALL, each. This observation was not due to 'Philadelphia-translocations' between chromosomes 9 and 22, as excluded by mMCB and subCTM-FISH.

The deletion 11p15.5 has not been described before in ALL - here, it was detected in a T-ALL (case 2) and a pre-B-ALL (case 19). For case 2, the subclone with a deletion 11pter was proven by interphase FISH to be present even in 60% of the cells. Deletions of 11p15 were previously discussed only in connection with treatment-related leukemia; however, cases 2 and 19 were not treated prior to cytogenetics (34).

For the additionally detected unique cryptic changes, the following data were available in the literature: involvement of the terminal region of 5q in rearrangements, as detected in case 22, were the first cryptic subtelomeric aberrations detected in leukemia (35). Not previously reported were a del(4)(p16.3) [case 22] a del(4)(q31) [case 17], a del(5)(q11.2-q11.2) [case 17], a del(13)(q34) [case 6], a del(18)(p11.31) [case 14], a del(17)(q25) [case 14], a del(22)(q13.33) [case 19], a dup(18)(q11.2) [case 14] or a t(7;17)(p22;q22) [case 5], however, an involvement of the p53 gene in the leukemogenesis of the latter case could not be excluded.

Some of the clonal cryptic rearrangements were observed concurrently with others [cases 5, 14, 17, 19, 22, and 24], or with larger rearrangements in the same patient (group 3). However, it is well-known that ALL tend to have complex chromosomal aberrations (see also case 7). The aberrations were detected by mMCB and/or by different subCTM-FISH experiments. Thus, it was not always possible to describe the aberrations and their frequencies in relation to each other; for cases 10, 14, 19, and 22 the aberrations were suspected after mMCB, but clearly visible only in subCTM-FISH. Thus, in these cases the clone sizes were estimated only by chromosome-specific subCTM-FISH experiments (Table I). Due to lack of material it was possible only in exceptional cases to prove the subclone presence in interphase-FISH [cases 2, 6, and 25].

Nonetheless, it can be stated that the application of up to now not available approaches for molecular cytogenetic characterization gave hints on possible new critical regions for ALL leukemogenesis and progression, which have to be confirmed and further studied in future. None of the new cryptic rearrangements of group 2 was detected either in the childhood ALL-cases of the present study [cases 10, 20, and 34] or in a recent study on childhood ALL (36).

Group 4: cases without detectable cryptic cytogenetic changes. In 16 of the 37 (44%) studied cases, no chromosomal aberrations were detected by mMCB or mMCB plus other approaches. However, in case 12, 20, 31, 32, 35 and 36 only mMCB could be performed as sufficient cell suspension for subCTM-studies was not available. Thus, one cannot be sure if aberrations would have been detected if more probes could have been applied.

In conclusion, the present study proved the usefulness of the mMCB-approach combined with subCTM-FISH in tumor cytogenetics for identifying chromosomal rearrangements that cannot be recognized by conventional GTG-banding. Up to present GTG-banding, often in combination with M-FISH or SKY, is the gold-standard in leukemia diagnostics. This and previous studies (26,35) showed that these methods alone cannot lead to comprehensive cytogenetic results. mMCB and subCTM-FISH could be the new molecular-cytogenetic standards for the determination of cytogenetic rearrangements.

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