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ORIGINAL ARTICLE

Chromosomal aberrations in congenital bone marrow failure disorders—an early indicator for leukemogenesis?

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Abstract As chromosomal instability may contribute to leukemogenesis in patients with congenital bone marrow failure (CBMF) disorders, it was the aim of this study to characterize chromosomally aberrant clones that arise during the clinical course of disease by means of R-banding and fluorescence in situ hybridization (FISH) analyses. In addition, multicolor-FISH and array-comparative genomic hybridization (CGH) were applied to characterize clonal chromosome aberrations in more detail. Between January 2004 and December 2005, we prospectively analyzed 90 samples of 73 patients with proven or suspected CBMF disorders enrolled in a German Study Network of CBMF diseases. Clonal aberrations could be identified in four of 73 patients examined. In one child with congenital thrombocytopenia, Jacobsen syndrome [del(11)(q24)c] was diagnosed, and thus a CBMF could be excluded. In a girl with Shwachman–Diamond syndrome, two independent clones, one with an isochromosome i(7)(q10), another with a complex aberrant karyotype, were identified. Simultaneously, transition into a myelodysplastic syndrome (MDS) occurred. The brother, who was also afflicted with Shwachman-Diamond syndrome, showed an isochromosome i(7q) as a single aberration. In the fourth patient with severe congenital neutropenia, an add(21)(q22) marker containing a low-level amplification of the AML1 gene was identified at the time point of transition into acute myelogenous leukemia (AML). In summary, we suggest that follow-up of patients with CBMF using chromosome and FISH analyses will be helpful for the early detection of transition into MDS or AML and thus should be an integral part of the clinical management of these patients.

Keywords Inherited bone marrow failure syndromes · Congenital neutropenia · Chromosomal aberration · Chromosomal instability · Leukemogenesis

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Introduction

Congenital bone marrow failure (CBMF) disorders are rare diseases characterized by peripheral blood (PB) cytopenia and hypoproliferation of one or more cell lineages in the bone marrow (BM). The most common CBMF disorders are severe congenital neutropenia (SCN), Shwachman–Diamond syndrome (SDS), Diamond–Blackfan anemia (DBA), Fanconi anemia (FA), and Dyskeratosis congenita (DC) (Table 1). It is well-known that patients with CBMF disorders are at increased risk for cancer. While solid tumors are uncommon in SCN and SDS, DC is associated

Table 1 Overview of congenital bone marrow failure disorders

Affected cell lineage	Name	Inheritance	Genes involved	Risk of developing leukemia	Typical chromosomal aberrations in malignant clone
All three lineages	FA	AR	11 FANC genes	10% (75%[a])	add(3q)
	SDS	AR	SBDS (7q11)	5–44%	i(7q)/−7/del(7q)
	DC	AD/AR + X	hTERC, DKC1 (Xq28)	(9%[b])	Unbalanced translocations
	CAMT	AR	c-MPL (1p34)	Unknown	Unknown
Red cells	DBA	AD/AR	RPS19 (19q13.2), unidentified gene on 8p23	2.5%	Involve chromosomes 1 and 19
Granulocytes	SCN	AD/AR	ELA2 (19q)/unidentified gene	>20%	−7/+8/+21
Platelets	TAR	AR	Unknown	Unknown	Unknown

AD autosomal dominant, *AR* autosomal recessive, *CAMT* congenital amegakaryocytic thrombocytopenia, *DBA* Diamond–Blackfan anemia, *DC* Dyskeratosis congenita, *FA* Fanconi anemia, *SCN* severe congenital neutropenia, *SDS* Shwachman–Diamond syndrome, *TAR* thrombocytopenia with absent radii, *X* X-linked

[a] Risk of developing cancer at the age of 45

[b] lifetime risk of developing cancer

with a 9% lifetime risk of solid neoplasia, specifically esophageal and pancreatic cancer [1], and in FA the risk of solid tumors is 9% [2].

In addition to solid tumors, all patients with CMBF are at increased risk for hematopoietic neoplasia. About 12% of patients with SCN [3, 4], up to 44% of patients with SDS [5], 2.5% of patients with DBA [6], 15% of patients with FA [7], and 3% of patients with DC [1] will develop myelodysplastic syndrome (MDS) or acute myeloid leukaemia (AML). In these secondary neoplasias, monosomy 7, deletion in 7q, trisomy 8, and trisomy 21 are the most common chromosomal abnormalities [1]. Approximately 50% of the SCN patients with MDS/AML and a chromosomal aberration carry a monosomy 7 [4], and about 40% of SDS with hematopoietic neoplasia have monosomy 7 or other structural aberrations of chromosome 7 [5].

Interestingly, the different CBMF disorders can also acquire specific chromosomal abnormalities. In SDS, 24% of patients with chromosomal aberrations show an isochromosome 7q [5, 8], and in FA, gains of the distal part of 3q are associated with an increased risk of developing leukemogenesis and again are often associated with monosomy 7 [9]. While in CBMF disorders all chromosomal abnormalities are acquired, there are some less common congenital hematopoietic conditions in which a constitutional chromosome aberration results in disturbed maturation of precursor cells and PB cytopenia. To investigate the development of chromosomal instability and chromosomally aberrant clones during the clinical course of CBMF disorder, we studied 90 samples from 73 patients using cytogenetic and molecular cytogenetic methods.

Materials and methods

Patients

Between January 2004 and December 2005, samples of 73 patients with proven or suspected CBMF disorders (Table 2) were analyzed in the reference laboratory. Median age was 10.5 years (range 1 month to 53 years). Sixteen patients were analyzed at two different time points, and one patient was analyzed at three time points.

Chromosome banding analysis

After a short-term culture of 12–72 h, metaphases of BM and PB were prepared according to standard procedures. Fluorescence R-banding using chromomycin A3 and

Table 2 Clinical characteristics of the patients with proven or suspected congenital bone marrow failure disorders

Disorder	No. of patients	Median age	Sex	No. of follow-up studies	No. of analyses
SCN	33	10 6/12	17 ♀/ 16 ♂	8	41
TAR? CAMT?	10	7 1/12	6 ♀/4 ♂	1	11
SDS	10	10 9/12	6 ♀/ 4 ♂	2	12
DBA	4	17 2/12	1 ♀/ 3 ♂	1	5
Suspected BMF disorder	16	9 10/12	7 ♀/ 9 ♂	5	21
Total	73	11 7/12	37 ♀/ 36 ♂	17	90

methyl green was performed as described in detail earlier [10]; 18 to 25 metaphases were analyzed in each case. Karyotypes were described according to the International System for Human Cytogenetic Nomenclature (ISCN) [11]. A complex karyotype was defined as three or more clonal aberrations.

FISH analysis

In all patients, fluorescence in situ hybridization analyses (FISH) on interphase nuclei were performed using probes for the centromeres of chromosome 7 and chromosome 8 and for the locus 7q31 as described earlier [12]. Depending on the cytogenetic aberrations detected, a break-apart probe for the MLL locus in 11q23, probes for the ATM locus in 11q22–q23, for the subtelomeric region of 11q, and for the AML1 gene in 21q22 were applied (all probes supplied by Abbott, Wiesbaden, Germany). At least 100 interphase nuclei were analyzed for each probe. Cut-off levels were evaluated by analyzing at least 1,000 cells from ten healthy donors and lay between 5 and 10% (data not shown).

M-FISH analysis

In three samples with clonal aberrations, multiplex-FISH (M-FISH) analysis was carried out using an M-FISH kit (MetaSystems, Altlussheim, Germany). The M-FISH procedure was performed according to the manufacturer's instructions. Fluorochromes were sequentially captured using specific single-band pass filters in a Zeiss Axioplan 2 microscope (Zeiss, Jena, Germany). M-FISH ISIS software (MetaSystems) was used for image analyses. At least five metaphase cells from each patient were analyzed.

Array-CGH

DNA-chips containing 6,251 individual BAC/PAC clones were used. They had a resolution of at least 1 Mb up to 100 kb for chromosome regions recurrently involved in human tumors as well as for regions containing known tumor suppressor genes and oncogenes [13]. Isolation and labeling of DNA, spotting of DNA probes and hybridization procedures were performed as described previously [13].

Image analysis was performed using a dual laser scanner and the GenePix Pro 4.0 imaging software (GenePix 4000 A; Axon Instruments, Union City, CA, USA). Two simultaneous scans of each array were obtained at a wavelength of 635 and 532 nm. DNA spots were automatically segmented, local background was subtracted and the intensity ratio of the two dyes was calculated for each spot using standard settings from GenePix Pro 4.0 software. Data normalization and analysis was performed using software packages marray and aCGH from R software3 (http://www.r-project.org). Raw fluorescence intensity values were normalized by applying the print-tip LOESS normalization function. Spot quality criteria were set as foreground to background >3.0 and SD of triplicates <0.2. For breakpoint calling, the software aCGHSmooth was used [14].

Results

In 88 of the 90 samples analyzed, metaphase cytogenetics was successful. In two patients with SCN, no metaphases were obtained, most probably due to a low cell number (less than 4 and 10×10^6 cells, respectively). Altogether, 83 samples showed a normal karyotype and normal signal constellations in FISH analyses. Clonal chromosomal aberrations were detected in four patients, including one with a constitutional aberration (Table 3).

Patient 1 was a mature newborn with petechiae, cerebral bleeding, cardiac defect, and facial dysmorphologies [15]. Platelet count was 26,000/μl. In the BM aspirate micromegakaryocytes were prominent. To prevent further bleeding, platelet transfusions were administered regularly. In the first chromosome analysis performed at the age of 2 weeks, a terminal deletion in chromosome 11 was detected cytogenetically (Fig. 1a,b). To narrow down the breakpoint, FISH probes specific for the ATM locus in 11q22–q23 and

Table 3 Patients with proven or suspected congenital bone marrow failure disorders and chromosomal aberrations

Patient	Age at analysis	Sex	Diagnosis	Hematological neoplasia	ISCN karyotype
1	2 weeks	♀	Jacobsen syndrome	–	46,XX,del(11)(q24)c[15]
2	6 years	♀	SDS	AML	46,XX,i(7)(q10)[2]/45~47,XX,+der(2)t(2;17)(p14;q11), der(3;12)(q10;q10), −5,+12,−13,−17,del(20)(q11)[cp10]/46,XX[6]
3	8 years	♂	SDS	–	46,XY,i(7)(q10) [4]/46,XY[11]
4	2 y	♂	CN	bilineage leukemia	46,XY,add(21)(q22) [19]/46,XY,+21 [1]

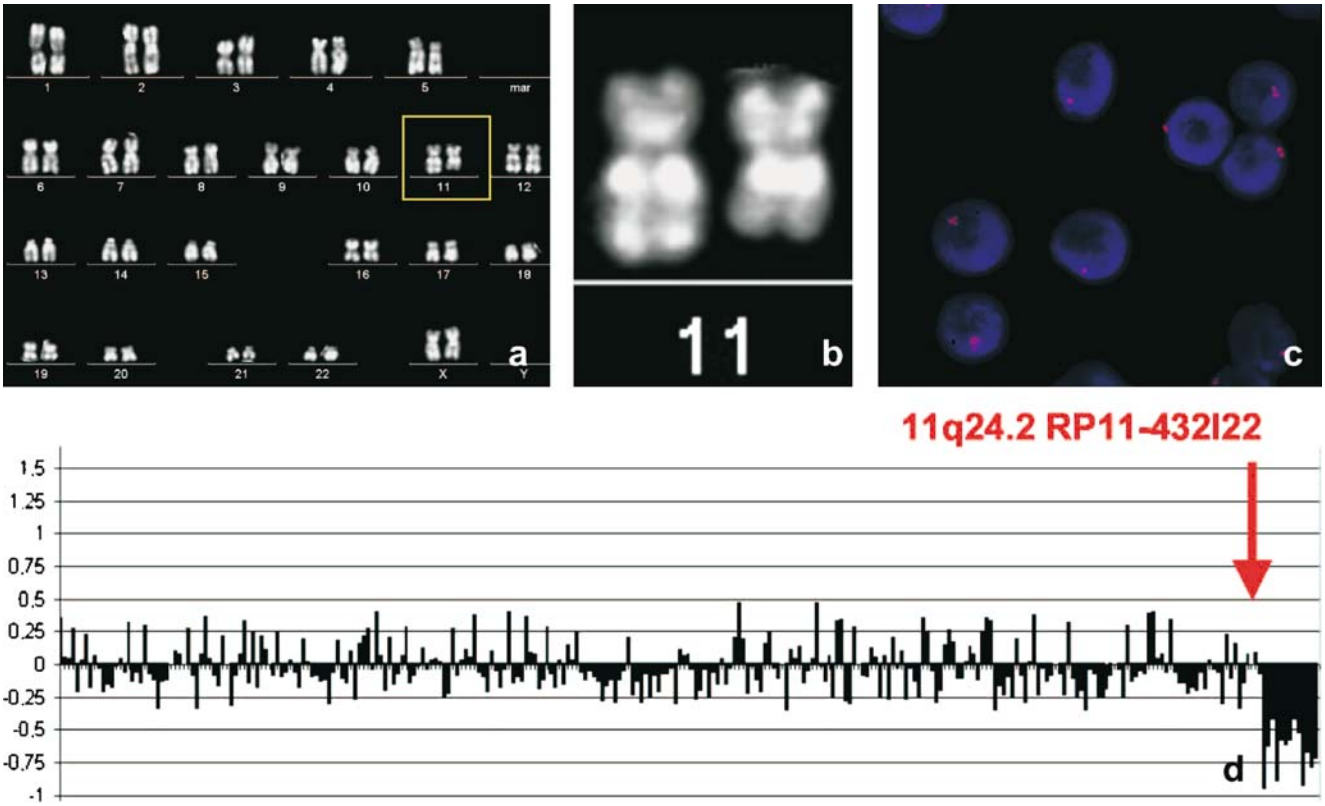

Fig. 1 Karyogram of patient 1 with Jacobsen syndrome (**a**). As shown in more detail, a terminal deletion in the long arm of a chromosome 11 was detectable (**b**). This deletion was confirmed by FISH using a telomere-specific probe revealing loss of one red signal in all nuclei analyzed (**c**). Plot of array-CGH for chromosome 11 demonstrates the log2 fluorescence intensity ratios of each BAC/PAC against the chromosomal position. An 8.2-Mb region 11q24.2 to qtel starting at PAC clone RP11-432I22 (Mb 125, 8) is deleted (*red arrow*) (**d**)

for the subtelomeric region in 11q were used (Fig. 1c). FISH analysis confirmed the terminal deletion and a breakpoint distal to the ATM locus. Array-CGH precisely determined the breakpoint to 11q24, 8.2 Mb (Fig. 1d). Based on these findings, the diagnosis of Jacobsen syndrome was made [15].

Patient 2 was a 7-year-old girl suffering from SDS. When her clinical condition deteriorated, pancytopenia was noted. Myelofibrosis, dyserythropoiesis, and dysmegakaryopoiesis in the BM biopsy indicated MDS. Until that time point, no therapy was received. Two independent chromosomally aberrant clones were identified, one clone with an isochromosome 7q [i(7)(q10)], another clone with a complex aberrant karyotype (Fig. 2). It contained an additional derivative chromosome 2 resulting from a translocation with a chromosome 17, a derivative chromosome resulting from a whole-arm translocation between the long arms of chromosomes 3 and 12, an additional chromosome 12, monosomies 5, 13 and 17, and a deletion in the long arm of chromosome 20. Thus, there were net losses of 3p, 5p, 5q, 17p, and 20q and net gains of 2q and 12q. An additional M-FISH analysis did not detect any further cryptic aberrations. An allogeneic hematopoietic stem cell transplantation (HSCT) was performed, and complete remission with complete donor chimerism (male into female) was demonstrated cytogenetically 4 months after HSCT.

Patient 3 is the 10-year-old brother of patient 2, also diagnosed with SCS. No treatment was given. Cytogenetic analysis demonstrated clonal isolated isochromosome 7q [i(7)(q10)]. Cytogenetic evaluation of parental PB excluded a constitutional aberration. Eight months after the first detection of the abnormal clone, there was no evidence of hematological neoplasia.

Patient 4 was a boy with SCN on G-CSF treatment. He was 1 year old when he had a control BM morphological examination performed, which did not show any evidence of MDS/AML. Cytogenetic analysis at the same time revealed a normal karyotype. Two years later, AML was suspected clinically. Cytogenetic analysis at this time showed additional genetic material of unknown origin in the long arm of chromosome 21 (Fig. 3a,b), and one

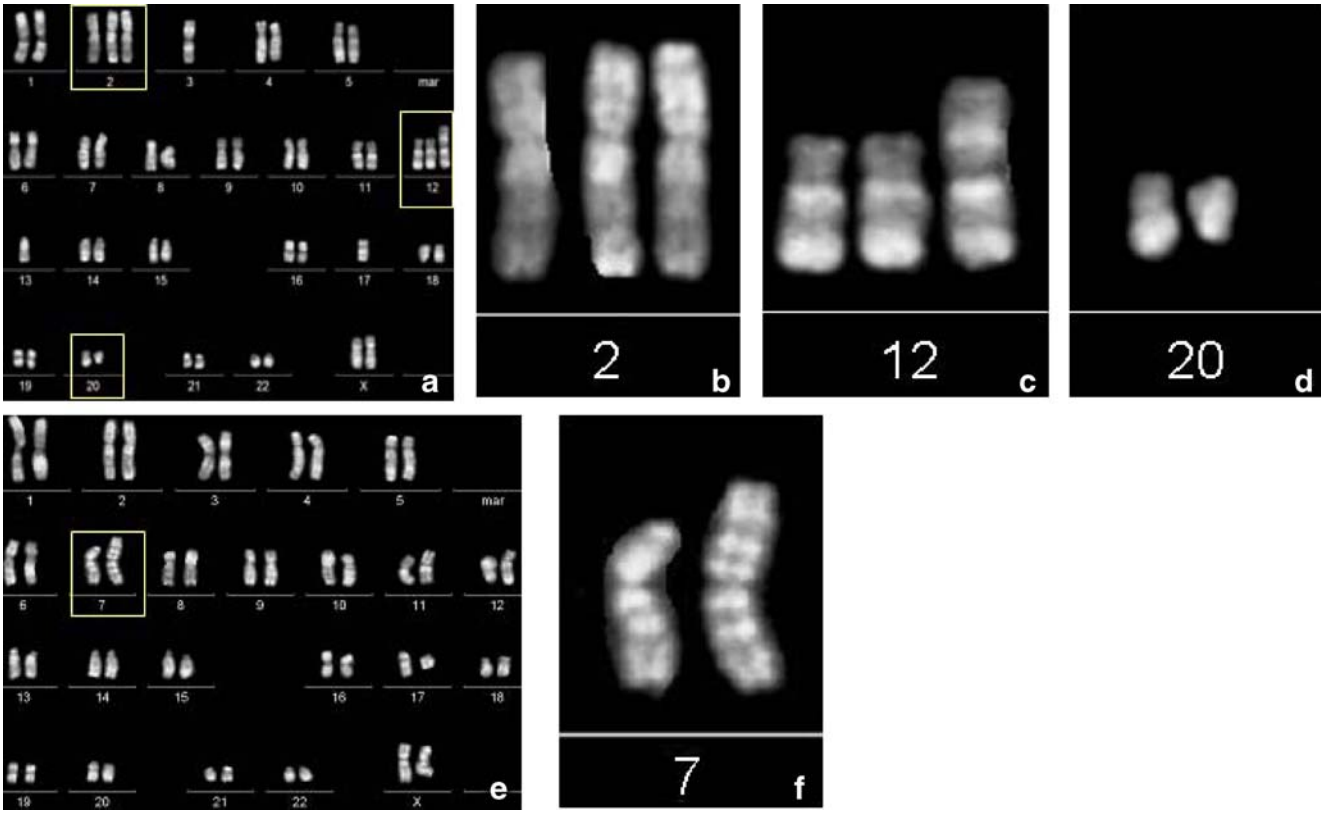

Fig. 2 Karyogram of the complex aberrant clone of patient 2 with Shwachman–Diamond syndrome (**a**) (for ISCN karyotype, see Table 3). The derivative chromosome 2 (**b**), the whole-arm translocation between 3q and 12q (**c**), and the deletion of 20q (**d**) are demonstrated. Monosomy 5 is not present in this metaphase; loss of chromosome 13 is due to a technical defect. Karyogram of the second independent clone of patient 2 (**e**). As shown in more detail, an isolated isochromosome of the long arm of chromosome 7 was detected (**f**)

metaphase indicated trisomy 21. FISH using a locus-specific probe for AML1 gene in chromosome 21q22 (Fig. 3c) detected a low-level amplification with up to five signals in 93% of interphase nuclei. By morphological and cytochemical analyses, two population of blasts were identified. Within the bone marrow, 33% of the blasts showed lymphoid characteristics and 67% of the blasts showed myeloid characteristics. Based on these findings and additional clinical data, secondary bilineage leukemia was diagnosed after the EGIL classification (European

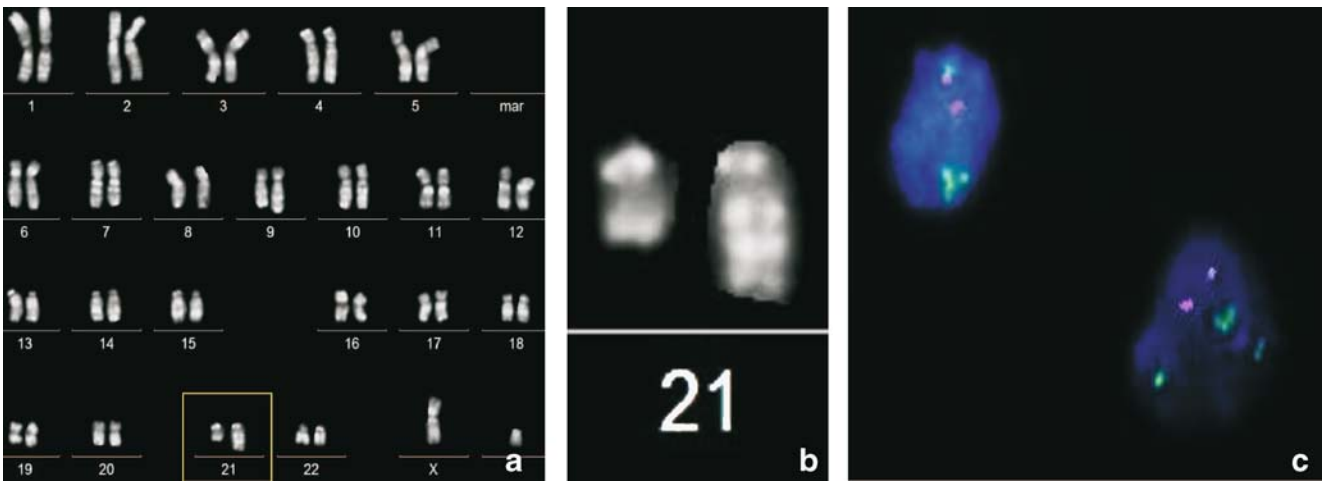

Fig. 3 Karyogram of patient 4 with severe congenital neutropenia (**a**). As shown in more detail, a derivative chromosome 21 was present (**b**). As demonstrated by FISH analysis (**c**) with a locus-specific probe for the AML1 gene in 21q22, there is a low-level amplification with up to five signals of this locus (*red signals*: 8q22 [ETO, used as an internal control], *green signals*: label 21q22 [AML1])

Group for the Immunological Characterization of Leukemia). The child achieved complete cytogenetic remission with intensive chemotherapy according to the AML-BFM-2004 protocol.

Discussion

Modern supportive therapy can enable young patients suffering from CBMF disorders to live a relatively normal life. However, CBMF patients, and specifically those with SCN, SDS, and FA, are at increased risk for hematopoietic neoplasia. Therefore, close monitoring of PB and BM is of the utmost importance. Although little is known about the mechanisms of leukemogenesis, it is evident that chromosome abnormalities play a major role in the development of hematological neoplasias. Currently, only a few data have been reported on the incidence of chromosomal abnormalities and their relation to leukemogenesis in patients with CBMF. In particular, there are no reports on longitudinal studies of defined cohorts of patients with CBMF disorders. In this paper we report on 73 patients with proven or suspected CBMF enrolled in a surveillance program to study chromosome banding and FISH analyses.

Chromosomal abnormalities were noted in 4/73 (5.5%) patients studied. In the first patient, a neonate suspected of suffering from CBMF disorder, an 8.2-Mb terminal deletion of 11q24 was detectable by cytogenetics, array-CGH, and FISH analysis. Loss of this region is known to be associated with growth and mental retardation, dysmorphia, and with isoimmune thrombocytopenia [16]. This syndrome was first described by Jacobsen et al. [22]. The responsible gene or genes located in 11q23 have not been identified so far. Because in most of these cases thrombocytopenia disappears during childhood, patients achieve a normal life span. In summary, in this patient, cytogenetic studies were important to exclude a CBMF disorder. Because Jacobsen syndrome is not associated with leukemia or MDS, follow-up BM examinations could be avoided.

Most likely due to small numbers, none of the patients with chromosomal aberrations showed monosomy 7. However, in the SCN patient, the second most frequent chromosome aberration in SCN, trisomy 21, was noted in one metaphase. The majority of metaphases showed a structurally aberrant chromosome 21 with a low-level amplification of AML1, which has not been reported in CBMF disorder-associated leukemia yet. AML1 amplifications are known to rarely occur in childhood acute lymphoblastic leukemia and in AML in adulthood and are associated with poor prognosis, in agreement with the diagnosis with a bilineage leukemia in this patient [9, 17–19].

In SDS, isochromosome 7q resulting in a trisomy 7q and a monosomy 7p can be noted in approximately 24% of patients [8]. According to these findings we found an isochromosome 7q in 2/10 (20%) patients with a SDS. In the ten SDS patients analyzed in this study, we found a pair of siblings both harboring the same aberration. Moreover, the girl revealed a second and independent clone without [i(7)(q10)] but with a complex aberrant karyotype. In myeloid neoplasia, aberrations on chromosome 7 like monosomy 7 or deletions in 7q are generally associated with a poor prognosis. Interestingly, in SDS patients, the presence of an isochromosome 7q appears to have an inhibiting effect on leukemogenesis [20, 21]. These patients do not show classical myelodysplastic features, seem not to transform to AML and rarely develop secondary aberrations [20]. Isochromosomes are formed by misdivision of the centromere, which is interesting because the SDS gene is mapped to the pericentric region of chromosome 7 (7p12–q11). Mutations in the SDS gene may predispose to isochromosome formation or isochromosome 7q may disrupt the gene [20].

Myeloid neoplasia in children with CBMF disorders can be cured by HSCT when treated early in the clinical course. By morphology alone, the early stages of neoplasia can be difficult to diagnose because trilineage dysplasia is not only a feature of MDS but also noted in most CBMF disorders. Therefore, the detection of clonal chromosome aberrations, although not necessarily proof of the malignant nature of the disease, may be very helpful for clinical decision making. Interestingly, cytogenetic aberrations were noted with standard metaphase analysis in all four positive cases without the need for additional cytogenetic studies. However, an additional FISH analysis for the most frequent aberrations found in patients with CBMF disorders like monosomy 7, deletion in 7q, and trisomy 8 is recommended to detect small clones as well as having a baseline for quantitative measurements during follow up. Possibly with the exception of children with SDS and isochromosome 7q, all other patients with CBMF disorders and chromosomal abnormalities will require frequent follow-up investigations and the initiation of a donor search for prompt HSCT.

For surveillance of all children with CBMF disorders we currently recommend cytogenetic analysis as soon as symptoms or morphological features indicative of a hematological neoplasia are detected. Prospective studies are under way to clarify whether cytogenetic analyses once a year provide an advantage for CBMF patients. Cytomorphology, blood smears, and clinical investigation remain the essential diagnostics. In summary, chromosome and FISH analyses in CBMF disorders may be helpful for the early detection of myeloid neoplasia and should be an integral part of the diagnosis of hematological neoplasias in these patients.

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