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COMMUNICATION

Six Cases of Rare Gene Amplifications and Multiple Copy of Fusion Gene in Childhood Acute Lymphoblastic Leukemia

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Abstract Cytogenetic aberrations are very important factors in risk assessment of childhood hematological malignancies. We report six childhood acute lymphoid leukemia (ALL) cases with rare cytogenetic aberrations: five with RUNX1, ABL1 or MLL proto-oncogene amplification and one case of multiple copies of ETV6/RUNX1 fusion genes. The simultaneous presence of two adverse genetic aberrations is of special interest: ETV6-RUNX1 fusion gene is associated with good prognosis and intrachromosomal amplification of the homologue RUNX1 gene is associated with poor prognosis. We also report a patient with MLL amplification, a unique finding in childhood T-ALL. Report of these subtle rearrangements contributes to our understanding of diagnostic and prognostic significance of these rare cytogenetic abnormalities.

Keywords Childhood ALL · Gene amplification · FISH · Subtle rearrangement

Introduction

Accurate risk classification is a tipping point in childhood acute leukemia treatment. Cytogenetic aberrations, together with other factors, such as initial white blood cell count (WBC), immunophenotype, age and steroid response are major factors in risk assessment. However, some cytogenetic abnormalities are very rare and their proper evaluation is even more challenging when present in combination.

Gene fusions induced by chromosomal translocations and gene amplifications lead to proto-oncogene activation. Chromosomal translocations are more typical to hematological malignancies, while amplifications are usually detected in solid tumors. Reciprocal translocations and their roles in leukemogenesis have been extensively studied. Amplifications are mutations that result in multiple copies of specific genes (amplicons) and lead to overexpression in cancer cells. Cytogenetic manifestations of gene amplifications are "homogeneously staining regions" (HSRs), "double minute" (dmin) chromosomes and micronuclei [1]. Amplifications have been associated with poor prognosis when found occasionally in hematopoietic neoplasia. The MYC oncogene is the most thoroughly studied amplification described in about 40 hematological cases, mostly in acute myeloid leukemia (AML). The presence of amplifications in acute lymphoid leukemia (ALL) is a rare finding and its pathological implication is still elusive [2]. We report five childhood leukemia patients with gene amplifications (RUNX1, runt related transcription factor 1, also known as AML1 or core-binding factor, alpha subunit 2, CBFA2; MLL-myeloid/lymphoid or mixed-lineage leukemia; ABL1, Abelson murine leukemia viral oncogene homolog 1) and one case of copy number increase of ETV6/RUNX1 fusion gene in childhood ALL. All these amplifications were identified by fluorescence in situ hybridization (FISH) cytogenetic method.

Methods and Patients

Cytogenetic analysis was performed in all cases at the time of diagnosis on bone marrow cultures by trypsin-G-banding

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following the recommendations of the International System for Cytogenetic Nomenclature 2005. With one exception successful G-band karyotypes were made based on 20–25 metaphases. FISH was performed on methanol/acetic acid-fixed suspensions as part of a routine screen on 86 consecutive childhood ALL diagnostic bone marrow samples between 2006–2008 using of LSI BCR/ABL (dual fusion), LSI TEL/AML1 (extra signal) dual color translocation and LSI MLL dual color break apart rearrangement probes (Abbot Vysis, Wiesbaden, Germany). The analysis was done in at least 200 interphase nuclei and in few metaphases if they were present on hybridization area. Clinical characteristics of patients with gene and fusion gene amplifications are shown in Table 1. The group comprised of 5 male and 1 female patients ranged from 2 to 16 years (median 8.6 years), 4 with B- and 2 with T-cell immunophenotype. They were classified as standard (SR), intermediate (IR) and high risk (HR) patients according to the classification instructions of the corresponding treatment protocols based on their response to induction therapy, age, initial white blood cell count, immunophenotype and initial karyotype. Patient 1 was treated at the initial diagnosis by ALL-BFM'95 protocol. The primary treatment of all other ALL patients was carried out according to ALL-IC-BFM 2002 and ALL-REZ-BFM 2002 protocols respectively.

Results

Table 1 summarizes the clinical and survival data as well as the results of G-banded karyotype analysis and FISH screening of the six childhood ALL cases in this study. Bone marrow was available for conventional cytogenetic analysis in 5 of 6 patients.

RUNX1 Amplifications (Patients 1, 2, 3)

Out of the 86 investigated childhood ALL cases RUNX1 amplification was detected in 3 cases (2.3 %): two at initial diagnosis and one at relapse (case 1). The overall incidence of RUNX1 amplification in newly diagnosed childhood ALL cases was 2,5 %. Clonal chromosomal abnormalities were present in two cases: a derivative 21 chromosome in patient 1 (Table 1, Fig. 1b); del(7)(q31) and 21 ring chromosome in patient 3 (Table 1, Fig. 1f). Patient 1 at the primary diagnosis had normal karyotype, FISH examination detected ETV6/RUNX1 fusion gene with loss of normal ETV6 homologue. He was treated according to the intermediate risk arm of the protocol ALL-BFM'95 based on high initial WBC count with neither testicular, nor central nervous system (CNS) infiltration, and reached complete remission on day 33 of treatment. He finished the therapy 2 years later and remained in remission over 6 months after ending the treatment. A new abnormality, over-representation of the RUNX1 gene (also known as

Table 1 Clinical features and cytogenetic analysis of the six reported patients

Patients	Age (yr)/sex	Immunophyenotype Risk category	WBC count x10^9L	Event-free survival (months)	Karyotypes	FISH results	Applied therapy protocol	Comments
1.[a]	7/M	PreB/relapsed	34,2	28	46,XY,der(21)add(21)(q22)[25]	RUNX1 × 4–5; ETV6 deletion ETV6-RUNX1fusion	ALL-BFM'95/ ALL-REZ-BFM 2002	deceased
2.	8/M	PreB/HR	9,4	4	No metaphases	RUNX1 × 8–10	ALL-IC-BFM ALL-REZ-BFM 2002	remission
3	15/M	PreB/IR	15,8	24	46,XY,del(7)(q31),der(21)r(21)(q?)dup(21)(q?)[20]	RUNX1 × 8	ALL-IC-BFM	remission
4.	2/M	PreB / SR	9,1	22	46,XY[20]	ETV6-RUNX1fusion × 1-5	ALL-IC-BFM	remission
5.	16/M	PreT / HR	261,0	10	47,XY,+mar[2]/46,XY[18]	ABL1 × 3 ~ 12	ALL-IC-BFM/ ALL-REZ-BFM 2002	deceased
6.	4/F	T-cell / IR	172,4	25	46,XX[20] Unusual 11q23 banding	MLL × 4–6	ALL-IC-BFM	remission

[a] Patient 1 presented normal karyotype and ETV6/RUNX1 fusion at the diagnosis (data not shown), he was included in this study 2,5 years later, when he relapsed. Abbreviations: *SR* Standard risk, *IR* Intermediate risk, *HR* High risk

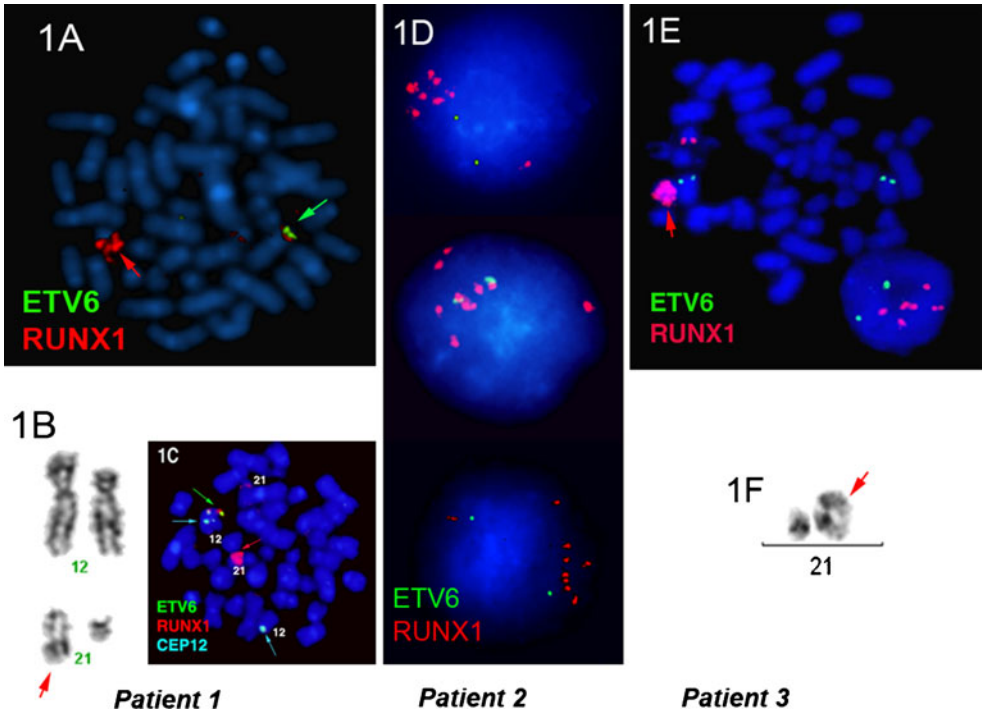

Fig. 1 RUNX1 gene amplifications (see Table 1). Patient 1: The red arrow shows the intrachromosomal amplification of RUNX1 gene using ETV6/RUNX1 specific green-red probe (**a**) and its G-banding correspondence on derivative 21 chromosome (**b**); the homologue RUNX1 participates in ETV6/RUNX1 fusion gene (green arrow) and the homologue ETV6 gene is deleted (absence of the second green signal). The aqua-blue arrowhead indicates the centromere signals of chromosome 12 and green arrow the ETV6/RUNX1 fusion signal unusually presented on derivative chromosome 12 (**c**). Patient 2 (**d**) presented on interphase nuclei multiple copy signals of RUNX1 (red) and two normal signals for ETV6 (green) genes. Patient 3: The red arrows show the G-banding configuration of the ring chromosome 21 (**f**) and its RUNX1 gene specific FISH pattern in metaphase and interphase cells (**e**)

iAMP21) was identified parallel with the original aberration at the time of the first bone marrow relapse, 2.5 years after the initial diagnosis. In relapse the initially normal karyotype has changed: on G-banding chromosome 21 the RUNX1amplification was visible as an additional material. Importantly, the ETV6/RUNX1 fusion gene was present as a variant of this well known subtle rearrangement being translocated from the regular place on chromosome 21q to the distal part of chromosome 12p (Fig. 1a, b, and c). In relapse he was treated according to ALL-REZ-BFM 2002 protocol. Stem cell transplantation (SCT) was not possible due to the lack of appropriate donor. One year after the first relapse treatment, a second bone marrow relapse occurred with the same cytogenetic aberrations, but it was biologically and clinically more aggressive. Remission could not be achieved even with FLAG-IDA (fludarabine, high dosage cytarabine, G-CSF and idarubicin), treatment and the patient died 3 weeks later.

In patient 2 bone marrow cell cultures didn't produce analyzable metaphases, but the FISH with ETV6-RUNX1 specific probe has shown 8–10 clusters of red RUNX signals in 70 % of interphase nuclei (Fig. 1d). Because of poor prednisone response chemotherapy was given according to the HR arm of the ALL-IC-BFM-2002 protocol. He completed the intensive part of the treatment and the maintenance therapy, he is currently in remission. According to the experience gathered using the BFM-protocols [3] BFM-type early reintensification (cyclophosphamide, cytarabine, mercapturine) therapy seems to be sufficient even for intrachromosomal amplification of chromosome 21 (iAMP21) HR group patients. An alternative treatment option (SCT) still remains for him in the case of relapse.

In patient 3 the FISH examination confirmed the result of the G-banding analysis: the aberrant chromosome 21 derived from the isochromosome of the long arm of chromosome 21 since the multiple RUNX1 signals covered the whole ring chromosome (Fig. 1e and f).

He had a good prednisolon-response and was treated in the IR group. Two and a half years after the initial diagnosis he had an isolated meningeal relapse. Chemotherapy was started according to the ALL REZ BFM-2002. After the first chemotherapy block he achieved remission and currently he is getting the last chemotherapy block and he is scheduled for cranial irradiation.

ETV6/RUNX1 Fusion Gene Amplification (Patient 4)

In patient 4 with normal karyotype FISH examination revealed 4–5 copies of ETV6/RUNX1 fusion signals (Table 1, Fig. 2) in 80 % of cells.

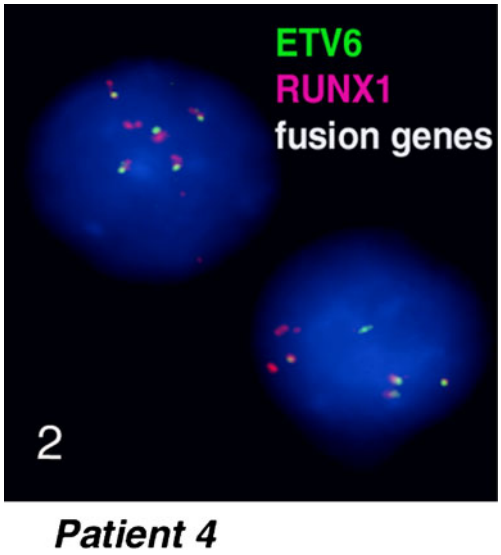

Fig. 2 (Patient 4): ETV6/RUNX1 fusion genes amplification. The interphase-FISH revealed 5–6 copies of ETV6/RUNX1 fusion signals

Patient 4 was treated according to the SR arm of the protocol. He responded well to therapy and currently he is in bone marrow remission 34 months after the treatment. However, a long-term follow-up is required to verify the possible prognostic effect of the ETV6-RUNX1 fusions amplification.

ABL1 Amplification (Patient 5)

Patient 5 presented with hyperleukocytosis (WBC of 261 × 10^9/L), liver, spleen and mediastinal enlargement and was diagnosed with T-ALL. G-banding analysis revealed only marker chromosomes in 2/20 cells. The t(9;22)(q34;q11) specific FISH probe did not show BCR-ABL1 fusion signals, but detected multiple uncountable ABL1 signals in nuclei indicating ABL1 amplification in 90 % of the cells (Fig. 3). In the metaphases found in the hybridization area the ABL1 signals were randomly distributed over the metaphases and the marker chromosomes were not involved in the gene amplification.

In addition to the high initial WBC count the prednisone response was poor and he had more than 1000/μL blasts after 7-day induction therapy. He was treated according to the HR arm of the ALL-IC-BFM-2002 protocol. The bone marrow aspirate on day 15 showed 78 % infiltration of blast cells, however, after 33 day of treatment he was in complete remission. One month after the end of the intensive part of the treatment, during the maintenance therapy he had a combined bone marrow and meningeal relapse. Amplified ABL1 signals were present in relapsed bone marrow blasts also. Chemotherapy was started according to ALL-BFM-REZ-2002 protocol, but he died due to infectious complications after the first treatment cycle.

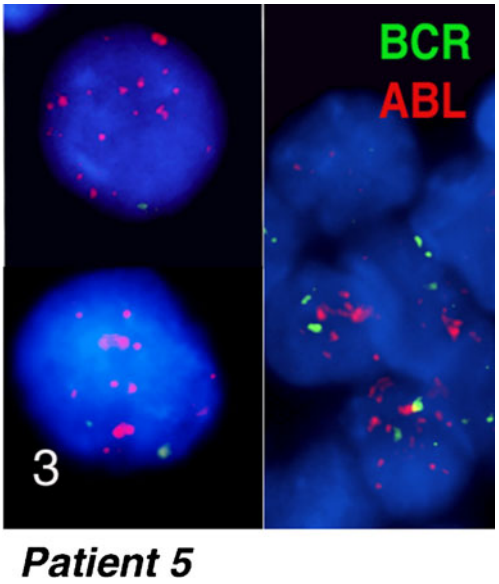

Fig. 3 (Patient 5): ABL1 gene amplification. The BCR-ABL1 specific probe identified two normal BCR green signals and multiple, uncountable ABL1 red signals

MLL Amplification (Patient 6)

In patient 6 the standard cytogenetic method showed two normal size chromosomes 11 with an unusual banding pattern of the long arm. The MLL specific FISH hybridization revealed 4–6 copies of red-green apparently unaltered gene signals. In interphase cells the double-color signals were located far from each other (Fig. 4a and e). However the metaphases showed poor morphology, the signal patterns (concentrated in the same place, on a single chromosome) and the G-banding results suggested that the multiple copies of MLL derived from a portion of 11q23 corresponding to intrachromosomal amplification of this gene (Fig. 4b and c).

She was 4 years old at the time of diagnosis. She was diagnosed with T-cell ALL and was treated according to the IR arm of ALL-IC-BFM-2002 protocol. Currently she is in remission. The bone marrow FISH examination after therapy completion revealed normal MLL gene signals (Fig. 4d).

Discussion

Gene amplifications are frequent genetic abnormality in solid tumors, but they rarely occur in hematological malignancies. Gene amplifications may appear in the karyogram in the form of HSRs, dmin or micronuclei. FISH using specific probes is an easy and reliable method for identifying the amplified genes with or without cytogenetic signs. The best known amplifications, activated human protooncogenes in hematopoietic neoplasia, are MYC and MLL, detected mainly in myeloid and seldom in lymphoid malignancies [2, 4]. In this

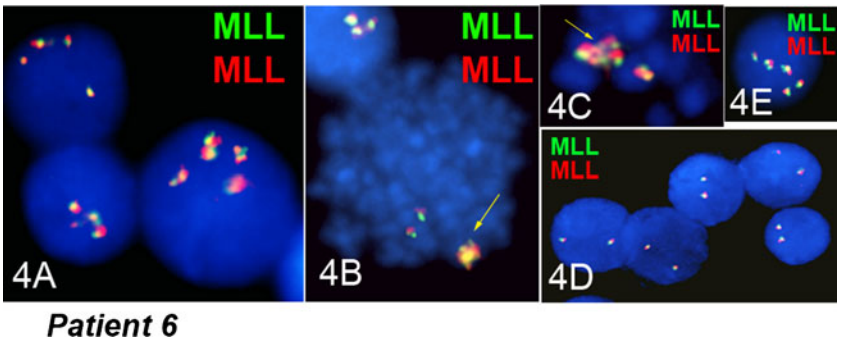

Fig. 4 (Patient 6): MLL gene amplification. MLL specific dual color probe identified in interphase nuclei 4–6 contiguous red and green signals without break apart spots suggestive of MLL rearrangement (**a**, **e**). Metaphase cells showed large block of signals concentrated on the long arm of chromosome 11 (**b** and **c** yellow arrow). On 4D one can see the normal MLL FISH pattern of this patient in remission

report we describe six childhood ALL cases: five with proto-oncogene amplification (RUNX1, ABL1 and MLL) and one case of multiple copies of ETV6-RUNX1 fusion genes.

The ETV6-RUNX1 is the most common fusion gene in childhood B-cell precursor ALL and has been associated with favorable outcome, 5-year event free survival being 89 % [5]. Secondary genetic changes such as ETV6 and RUNX1 gene copy number alterations as well as extra copies of ETV6-RUNX1 fusion gene negatively influence the prognosis [6–8]. The incidence of iAMP21 with RUNX1 (AML1) over-representation in childhood B-lineage acute lymphoblastic leukemia is about 2 % [3, 9]. We have found a 2,5 % occurrence in our series of 80 newly diagnosed ALL cases (patients 2 and 3). Patient 2 treated with the HR arm of the ALL-IC-BFM-2002 protocol is in remission for 1.5 years after the end of therapy. Patient 3 with an iAMP21 had RUNX1 amplification and 7q deletion at the time of diagnosis. Even though he had a good prednisolon-response and was treated in the IR group, two and a half years after the initial diagnosis he had an isolated meningeal relapse. Although the chemotherapy is still under way he is currently in remission.

In patient 1 an iAMP21 was identified in relapse 2.5 years after the initial diagnosis together with the primary aberrations (ETV6/RUNX1 fusion gene and ETV6 deletion). We are aware of only one previously reported case [10] in which RUNX1 gene homologues harbor prognostically contradictory abnormalities: ETV6-RUNX1 fusion with good prognosis effect and iAMP21 linked to a poor outcome and increased relapse risk. Some ETV6/RUNX1 cases with extra RUNX1 copies were described recently in a large cohort (619 cases) of ETV6-RUNX1 positive patients [11].

Multivariate analysis of high number of patients showed that the presence of the iAMP21 chromosomal abnormality is an independent indicator of poor event free (26 %) and overall survival (69 %). Patients with this abnormality were treated according to the HR arm of UK IRC ALL 2003 trial and are considered for SCT in first remission [9]. The 6 year event free and overall survival rates of patients treated according to BFM-based regimens were higher (37 %) than in UK IRC/CLWP ALL 2003 trial studies, which might result from more intensive induction and early reintensification therapy in this protocol [3]. The minimal residual disease (MRD) values were the only reliable relapse predictor to discriminate between a low and a high risk of relapse. Our results are in agreement with the outcome of the ALL-BFM trial group. The two patients with an iAMP21 treated as SR and IR arm of BFM protocol relapsed. The patient treated with HR arm of BFM-protocol finished the maintenance therapy and is still in remission. The overall relapse rate is still high (50 %) even with BFM-treatment regiment. The current type of treatment is sufficient only for MRD low risk group and allogenic SCT remains a treatment option for iAMP21 relapsed patients [3].

Duplication of der(21)t(12;21) was found in 20–30 % of ETV6-RUNX1 positive patients, only one patient is described with double and quadruple fusions [6–8]. Double ETV6-RUNX1 patients tend to have slower therapy response and higher multiple relapse rate [8]. Presence of a single population with 4–5 ETV6-RUNX1 fusion gene copies at the time of diagnosis is a unique finding.

ABL1 amplification was recently found to be a cytogenetically hidden finding and is associated with poor outcome in immature T-cell ALL [12–14], identified in 2.3 % of children and 4–8 % of adult cases. Recent studies demonstrated that the amplified ABL1 gene could be involved in a cryptic translocation with NUP214, creating a microscopically invisible extrachromosomal episomal structure that contains the NUP214/ABL1 fusion gene which number varies from cell to cell due to unequal segregation [15, 16].

From a clinical standpoint our patient (patient 5) had similar findings to others described with this abnormality: hyperleukocytosis (WBC of 261×109/L), liver, spleen and mediastinal enlargement, refractory to induction chemotherapy. Presence of amplified ABL1 gene in leukemic blasts may influence the treatment strategy. The ABL1 fusion

proteins are sensitive to tyrosine kinase inhibitors, which offers a promising combination with polychemotherapeutic protocols in future [16, 17]. Since these patients have a poor outcome, prospective screening of this abnormality is required for targeted therapeutic approach. On the basis of FISH results ABL1 quantitative RT-PCR may be applied to monitor minimal residual disease in T-ALL patients with ABL1 amplification [14].

The split MLL gene and internal partial tandem duplication of MLL have been frequently described in a wide variety of leukemias. Amplification of the MLL gene in myeloid malignancy is also a documented phenomenon [5, 18], the number of reported cases being about 30. It is mainly associated with elderly patients, often dysplastic bone marrow, and complex karyotypic abnormalities. The short overall median survival (1 month) is possibly related to the older age and the unfavorable clinical manifestations. MLL amplifications in ALL are rare. There are only two reported cases of ALL with this amplification: a 7 years old male with common ALL and an 86 years old female with pre-B ALL [19, 20]. However, our 4 years old T-ALL patient and the other common-ALL childhood case are different from those reported in AML: they were detected at young age as the sole anomaly and the patients are in remission for more than 20 months. These data suggest that MLL amplification in ALL corresponds to a third variety of MLL gene abnormalities, which do not show the same clinico-pathological features as typical split MLL.

The development of cytogenetic methodology has led to the discovery of some unexpected gene amplification, which could represent a novel type of recurrent abnormalities in childhood ALL. FISH with probes directed towards the generally involved genes in pathogenesis of ALL (RUNX1, ABL1, MLL) is the only reliable detection method for these submicroscopic aberrations. Gene amplifications denote a new form of oncogene up-regulation in ALL, which might have diagnostic and prognostic significance. The ABL1 amplifications in T-ALL and iAMP21 in precursor B-cell ALL are associated with a poor prognosis and these recently discovered recurrent abnormalities may have a direct impact on treatment.

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