Original Article
Diagnostic Genetics

Ann Lab Med 2015;35:118-122
http://dx.doi.org/10.3343/alm.2015.35.1.118
ISSN 2234-3806 • eISSN 2234-3814

ANNALS OF LABORATORY MEDICINE

Alteration of the *SETBP1* Gene and Splicing Pathway Genes *SF3B1*, *U2AF1*, and *SRSF2* in Childhood Acute Myeloid Leukemia

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Background: Recurrent somatic *SET-binding protein* 1 (*SETBP1*) and splicing pathway gene mutations have recently been found in atypical chronic myeloid leukemia and other hematologic malignancies. These mutations have been comprehensively analyzed in adult AML, but not in childhood AML. We investigated possible alteration of the *SETBP1*, *splicing factor* 3B *subunit 1* (*SF3B1*), *U2 small nuclear RNA auxiliary factor 1* (*U2AF1*), and *serine/arginine-rich splicing factor 2* (*SRSF2*) genes in childhood AML.

Methods: Cytogenetic and molecular analyses were performed to reveal chromosomal and genetic alterations. Sequence alterations in the *SETBP1*, *SF3B1*, *U2AF1*, and *SRSF2* genes were examined by using direct sequencing in a cohort of 53 childhood AML patients.

Results: Childhood AML patients did not harbor any recurrent *SETBP1* gene mutations, although our study did identify a synonymous mutation in one patient. None of the previously reported aberrations in the mutational hotspot of *SF3B1*, *U2AF1*, and *SRSF2* were identified in any of the 53 patients.

Conclusions: Alterations of the *SETBP1* gene or *SF3B1*, *U2AF1*, and *SRSF2* genes are not common genetic events in childhood AML, implying that the mutations are unlikely to exert a driver effect in myeloid leukemogenesis during childhood.

Key Words: *SETBP1*, *SF3B1*, *U2AF1*, *SRSF2*, AML, Childhood

Received: March 24, 2014
Revision received: May 20, 2014
Accepted: September 25, 2014

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INTRODUCTION

Germline mutations in the *SET-binding protein 1* (*SETBP1*) gene (18q21.1) were first identified as causative genetic defects in children with Schinzel-Giedion syndrome (SGS), which is clinically characterized by severe mental retardation, distinctive facial features, and multiple congenital malformations [1]. In hematologic malignancies, recurrent somatic *SETBP1* mutations have been recently reported in atypical chronic myeloid leukemia (aCML), unclassified myelodysplastic/myeloproliferative neoplasm (MDS/MPN), chronic myelomonocytic leukemia (CMML), and secondary AML (sAML) [2, 3]. *SETBP1* encodes a protein that contains a homologous region to the SKI oncoprotein, a SET-binding region and three nuclear localization signals [4]. Piazza *et al*. [2] demonstrated that expression of a SETBP1 mutant showed a similar mechanism as SETBP1 overexpression. It has been suggested that overexpression of SETBP1 protects SET from protease cleavage [5]. Consequently, full length SET protein inhibits protein phosphatase 2 (PP2A) by forming a SETBP1-SET-PP2A protein complex, which leads to cell proliferation and

expansion of leukemic cells [5].

In a recent report on *SETBP1* mutations, the mutations in *splicing factor 3B subunit 1* (*SF3B1*), *U2 small nuclear RNA auxiliary factor 1* (*U2AF1*), and *serine/arginine-rich splicing factor 2* (*SRSF2*), which are highly recurrent splicing pathway gene mutations in myeloid neoplasms, were investigated and a strong association between *SETBP1* mutations and mutations in *SRSF2* was demonstrated [6, 7].

Childhood AML is a rare and heterogeneous disorder, which is reported to occur in 7 cases per million children under 15 yr of age, and possesses nonrandom gene mutations contributing to the heterogeneity of disease [8]. Compared with adult AML, there are fewer studies on gene mutations in childhood AML. Here, we investigated the *SETBP1* mutation and additional alterations of the splicing pathway genes *SF3B1*, *U2AF1*, and *SRSF2* in childhood AML patients.

METHODS

1. Patients and samples

Fifty-three childhoods AML patients who were diagnosed and treated at Chonnam National University Hwasun Hospital from March 2004 to July 2013, were enrolled after obtaining Institutional Review Board approval and informed consent. Patient ages varied from 11 months to 17 yr, and the average was 10 yr. One patient was diagnosed with sAML and the other 52 were diagnosed with *de novo* AML. Clinical, cytogenetic, and molecular characteristics are shown in Table 1. Prognostic grouping of cytogenetically abnormal patients was based on conventional cytogenetic analysis and multiplex RT-PCR results [8].

Total DNA was extracted from bone marrow sample obtained at diagnosis by using a QIAamp DNA blood mini kit (QIAGEN, Hilden, Germany). Specimens were frozen in liquid nitrogen immediately after acquisition and were stored at -20°C for further evaluation.

Table 1. Clinical, cytogenetic, and molecular characteristics of the 53 patients with childhood AML

Characteristics	Values
Total number of patients, N	53
Age of diagnosis, median	
0-2	7
3-10	21
≥11	25
WBC, $\times 10^9$/L, median (range)	15 (0.9-166.5)
BM blast, %, median (range)	69.5 (2.7-95)
Male/female, N	25/28
Achieved CR, N	49
Relapse, N	6
FAB type, N	
M0	1
M1	2
M2	20
M3	12
M4	7
M5	3
M6	3
M7	3
Type uncertain	2
Cytogenetically abnormal, N	37
Favorable	24
Intermediate	9
Adverse	4
Cytogenetically normal, N	14
FLT3-ITD mutated	2
NPM1 mutated	2
WT1 highly expressed	10
BAALC highly expressed	3

Abbreviations: N, number; WBC, white blood cell; BM, bone marrow; CR, complete remission; FAB, French-American-British.

2. Direct sequencing of *SETBP1*, *SF3B1*, *U2AF1*, and *SRSF2* genes

PCR and sequencing reactions were performed by targeting the hot spots of *SETBP1* (exon 4, codon 778-979), *SF3B1* (exons 14, 15, 16, and 18), *U2AF1* (exons 2, 6, and 7), and *SRSF2* (exon 1) by using primer pairs based on a modification of a published protocol [2, 9] (Table 2). Each amplified gene product was purified by using an AccuPrep PCR Purification Kit (Bioneer, Daejeon, Korea) and sequenced with a BigDye Terminator v3.1 Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). The gene sequences were compared by using the Blast2 program (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.htlm).

3. Cytogenetic and molecular analyses

Conventional cytogenetic analysis was performed on G-banded preparations from 48-hr bone marrow cell cultures. Balanced translocations were identified by multiplex reverse transcription (RT)-PCR using the HemaVision multiplex RT-PCR Screen Test kit (DNA Technology, Aarhus, Denmark) as previously described

[10]. Mutation analysis of the *fms-like tyrosine kinase 3-internal tandem duplication* (*FLT3-ITD*) gene was performed by using PCR-restriction fragment length polymorphism (RFLP) and capillary electrophoresis for separation. The *Nucleophosmin 1* (*NPM1*) gene mutation was evaluated by using Ipsogen *NPM1*-A and B/D Muta-Quant kit (QIAGEN) and the expression of *Wilms tumor 1* (*WT1*) and *brain and acute leukemia cytoplasmic* (*BAALC*) genes was evaluated by using Ipsogen *WT1* and *BAALC* Profile-Quant kit (QIAGEN) following the manufacturer's instructions.

Table 2. Primers used for PCR amplification and direct sequencing of *SETBP1*, *SF3B1*, *U2AF1*, and *SRSF2* genes

Genes	Exon	Direction	Primers (5′ to 3′)*
SETBP1	exon 4	forward	CCACTTTCAACACAGTTAGGTG
		reverse	TCTCGTGGTAGAAGGTGTAACTC
SF3B1	exon 14	forward	TAGAGTGGAAGGCCGAGAGA
		reverse	TTCAAGAAAGCAGCCAAACC
	exon 15, 16	forward	GTTGATATATTGAGAGAATC
		reverse	TTTAAAATTCTGTTAGAACC
	exon 18	forward	CGATGTTTGGTCACTTTTCT
		reverse	TTGCTTGACAACTAATATGC
U2AF1	exon 2	forward	TGCTGCTGACATATTCCATGT
		reverse	AGTCGATCACCTGCCTCACT
	exon 6, 7	forward	ATTAAAGCGTGGATGGCAAG
		reverse	TCCAAAGAGGACATTTGGAT
SRSF2	exon 1	forward	GTGGACAACCTGACCTACCG
		reverse	CCTCAGCCCCGTTTACCT

*Primer pairs modified from published protocols [2, 9].

RESULTS

Childhood AML patients did not carry any of the recurrent *SETBP1* mutations that had previously been identified in aCML or other related hematologic malignancies. However, one patient in the group of AML with *RUNX1/RUNX1T1* rearrangement displayed c.2903C>T (synonymous) alteration in the mutational hotspot of *SETBP1* (Fig. 1A). This patient's bone marrow samples were available for analysis from the initial diagnosis to the last follow-up, and we performed serial direct sequencing for the *SETBP1* gene. On day 30 of the second induction chemotherapy, the *SETBP1* gene alteration was undetectable coincident with the decrease of the *RUNX1-RUNX1T1* transcript (Fig. 1B), but was present again during the consolidation therapy period (Fig. 1C).

None of the previously reported aberrations in the mutational hotspot of *SF3B1*, *U2AF1*, and *SRSF2* were identified in any of the 53 pediatric AML patients. However, single nuclear polymorphisms (SNPs) were detected, with a particularly high frequency in *SF3B1* and *SRSF2*. Forty-six (86.8%) patients showed SNP rs788018 in exon 18 of *SF3B1* and all 53 (100%) patients showed SNP rs237057 in exon 1 of *SRSF2*. The genotype frequencies of *SF3B1* SNP rs788018 were TT 13.2%, TC 39.6%, and CC 47.2%. The genotype frequencies of *SRSF2* SNP rs237057 were CT 7.6% and TT 92.4%.

DISCUSSION

None of the 53 childhood AML patients showed a recurrent *SETBP1* mutation found previously in MDS/MPN, MDS, sAML, or other related hematologic malignancies. Only one patient had

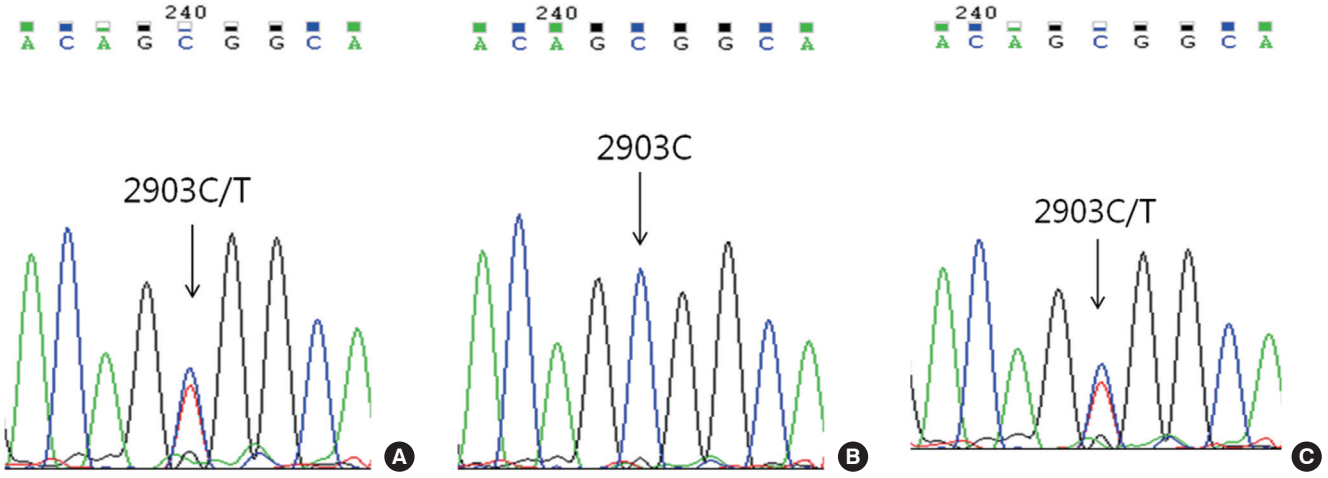

Fig. 1. Electropherograms of the patient with a synonymous *SETBP1* gene alteration. The synonymous alteration of c.2903C>T evident at diagnosis (A) disappeared on day 30 of the second induction of chemotherapy (B) and reappeared during consolidation therapy (C).

http://dx.doi.org/10.3343/alm.2015.35.1.118

a base-pair change in the hot spot of *SETBP1* gene alteration. Since the number of alteration-positive cases was limited, it was not possible to compare the biologic or clinical differences between *SETBP1*-alteration positive and negative patients. However, the patient with a synonymous *SETBP1* alteration showed the shortest survival time compared to the other patients with the same disease entity (median overall survival 4 months vs. 32 months, data not shown).

AML development is a multistep process and the leukemogenesis-altered proteins responsible for this clonal disease are presented as a "five-class model" [11]. This model comprises class I (signaling pathway components), class II (transcription factors), class III (epigenetic regulators), class IV (tumor suppressors), and class V (components of the spliceosome). Liang *et al*. [12] reported that the most frequent mutations in childhood AML were the class I mutations, and that epigenetic regulators (class III) mutations were much less frequent than they are in adult AML. In comparison of biologic properties and genetic abnormalities in childhood and adult AML, the frequency of *de novo* AML is higher (>95% vs. 83%) in childhood AML, but the frequency of sAML is lower (1% vs. 17%) in childhood AML [8]. In this study, similar results were observed. Among the 53 childhood AML patients, 52 patients (98%) had *de novo* AML, and we were unable to find any recurrent *SETBP1* mutations. An analysis of 944 adult patients with AML and MDS did not detect any *SETBP1* mutations in *de novo* AML patients and these results suggested that *SETBP1* mutations play a role in the setting of MDS and sAML, but not in *de novo* AML [7].

A recent report by Shiba *et al*. [13] also demonstrated that the *SETBP1* mutations are not recurrent in childhood AML. Considering the effect of a *SETBP1* mutation in leukemogenesis, Damm *et al*. [3] compared the timing of *SETBP1* mutations with other gene mutations during the evolution of CMML and sAML. Compared to spliceosomal gene mutations, *SETBP1* mutations occurred after the *SF3B1* and *SRSF2* mutations. Therefore, the authors suggested that the *SETBP1* mutations occur at later stages of disease evolution and might have more impact on the clinical course of the disease than on its initiation. Based on these reports, it is reasonable to propose that *SETBP1* mutations have little impact on leukemogenesis in childhood AML.

The splicing pathway gene mutations, included in class V from the aforementioned five-class model, were identified as frequent somatic mutations of genes encoding multiple components of the RNA splicing machinery. Mutations in these genes were frequently detected in MDS and MDS-related disorders and were also identified in *de novo* AML. Yoshida *et al*. [6] found the *SF3B1*, *U2AF1*, and *SRSF2* gene mutations in 4.6% of *de novo* adult AML, and Je *et al*. [14] in 5.6%. However, in this study, mutations were not found in *SF3B1*, *U2AF1*, and *SRSF2* genes, indicating little relationship with childhood AML. Earlier studies on splicing pathway gene mutations in childhood leukemia reported similar results. Kar *et al*. [15] investigated *SF3B1*, *U2AF1*, and *SRSF2* mutations in 49 patients with juvenile myelomonocytic leukemia, but none of the three splicing pathway genes was detected. Je *et al*. [14] showed that the *SRSF2* gene was mutated in childhood acute lymphoblastic leukemia (1.5%), but not in childhood AML.

Synonymous SNPs are thought to have no effect on coding sequences, and therefore are not expected to change the function of the protein, in which they occur. However, studies on the impact on gene functions and the association with diseases are continuously reported [16, 17]. Recently, the prognostic significance of synonymous SNPs of *WT1* (SNP rs16754) and *IDH1* (SNP rs11554137) was evaluated in adult and childhood AML, and the results suggested a different outcome between SNP-positive and SNP-negative patients [18-20]. According to the SNP database [21], SNP rs788018 of *SF3B1* appeared in 91.8% of the Japanese population and in 78.6% of the Han Chinese population. These results showed only a minor difference compared to the frequency (86.8%) of this study. SNP rs237057 of *SRSF2* was found in almost 100% of the Japanese and Han Chinese populations, which was consistent with the present result. Reports have not yet been published regarding the clinical impact of a splicing pathway gene SNP.

In conclusion, although we found a synonymous gene alteration in one patient, childhood AML patients did not harbor recurrent *SETBP1* mutations, and they did not harbor any mutations of *SF3B1*, *U2AF1*, and *SRSF2* genes. Alterations of the *SETBP1* gene or *SFS3B1*, *U2AF1*, and *SRSF2* genes were not common genetic events in childhood AML, implying that these may not exert a driver effect in myeloid leukemogenesis during childhood.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

Acknowledgments

This study was supported by the National Research Foundation

of Korea (NRF) and grants (No. 2011-0015304), the NRF Basic Science Research Program (grant 2010-0024326), the Leading Foreign Research Institute Recruitment Program (No. 2011-0030034) through the NRF funded by the Ministry of Education, Science and Technology (MEST), and a grant from the National R&D Program for Cancer Control, Ministry of Health & Welfare, Republic of Korea (No. 2013-1320070).

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http://dx.doi.org/10.3343/alm.2015.35.1.118