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

Hyun-Woo Choi, M.D.,1 Hye-Ran Kim, Ph.D.,2,3 Hee-Jo Baek, M.D.,4,5 Hoon Kook, M.D.,4,5 Duck Cho, M.D.,1 Jong-Hee Shin, M.D.1, Soon-Pal Suh, M.D.1, Dong-Wook Ryang, M.D.1, and Myung-Geun Shin, M.D.1,2,5

Department of Laboratory Medicine1, Chonnam National University Hwasun Hospital, Hwasun; Brain Korea 21 Plus Project2, Chonnam National University Medical School, Gwangju, Korea; Laboratory of Metabolism3, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA; Department of Pediatrics4, Chonnam National University Hwasun Hospital, Hwasun; Environmental Health Center for Childhood Leukemia and Cancer5, Chonnam National University Hwasun Hospital, Hwasun, Korea

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

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 (CMMML), 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.

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 (Biogene, 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.html).

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.

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

| Characteristics          | Values |
|--------------------------|--------|
| Total number of patients | 53     |
| Age of diagnosis, median | 0-2    |
| WBC, × 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           | 4      |
| Cytogenetically abnormal | 37     |
| Favorable                | 24     |
| Intermediate             | 9      |
| Adverse                  | 4      |
| Cytogenetically normal   | 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.
Mutation analysis of the \textit{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 \textit{Nucleophosmin 1 (NPM1)} gene mutation was evaluated by using Ipsogen \textit{NPM1-A} and B/D Muta-Quant kit (QIAGEN) and the expression of \textit{Wilms tumor 1 (WT1)} and \textit{brain and acute leukemia cytoplasmic (BAALC)} genes was evaluated by using Ipsogen \textit{WT1} and \textit{BAALC} Profile-Quant kit (QIAGEN) following the manufacturer's instructions.

### RESULTS

Childhood AML patients did not carry any of the recurrent \textit{SETBP1} mutations that had previously been identified in aCML or other related hematologic malignancies. However, one patient in the group of AML with \textit{RUNX1/RUNX1T1} rearrangement displayed c.2903C>T (synonymous) alteration in the mutational hotspot of \textit{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 \textit{SETBP1} gene. On day 30 of the second induction chemotherapy, the \textit{SETBP1} gene alteration was undetectable coincident with the decrease of the \textit{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 \textit{SF3B1}, \textit{U2AF1}, and \textit{SRSF2} were identified in any of the 53 pediatric AML patients. However, single nuclear polymorphisms (SNPs) were detected, with a particularly high frequency in \textit{SF3B1} and \textit{SRSF2}. Forty-six (86.8%) patients showed SNP rs788018 in exon 18 of \textit{SF3B1} and all 53 (100%) patients showed SNP rs237057 in exon 1 of \textit{SRSF2}. The genotype frequencies of \textit{SF3B1} SNP rs788018 were TT 13.2%, TC 39.6%, and CC 47.2%. The genotype frequencies of \textit{SRSF2} SNP rs237057 were CT 7.6% and TT 92.4%.

### DISCUSSION

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

### Table 2. Primers used for PCR amplification and direct sequencing of \textit{SETBP1}, \textit{SF3B1}, \textit{U2AF1}, and \textit{SRSF2} genes

| Genes   | Exon     | Direction | Primers (5’ to 3’)\(^*\) |
|---------|----------|-----------|---------------------------|
| \textit{SETBP1} | exon 4   | forward   | CCACATTCAACACAGTTAGGTG   |
|          |          | reverse   | TCTCGTGTAGAAGGTGTAACCT   |
| \textit{SF3B1} | exon 14  | forward   | TAGAGTGGAAGCCGAGAAGA   |
|          |          | reverse   | TTCAAGAAGCCGACCAACC   |
|          | exon 15, 16 | forward | GTTGAATATGGAGGAAATC   |
|          |          | reverse   | TTTAAAATCTGTTAGAACC   |
|          | exon 18  | forward   | CGATTTTTGCTACCTTCTTCT   |
|          |          | reverse   | TGCTGTAACAATTAATGC   |
| \textit{U2AF1} | exon 2   | forward   | TGCTGTGACATATTCAGATGT   |
|          |          | reverse   | AGTCGATCACCTGCCTCACCT   |
|          | exon 6, 7 | forward  | ATAAAAGCGTGGATGGCAAG   |
|          |          | reverse   | TCCAAGAGGACATTGGAT   |
| \textit{SRSF2} | exon 1   | forward   | GTGGACAACCGGTACCTACCG   |
|          |          | reverse   | CCTCAAGCCGGCGTTACCT   |

\(^*\)Primer pairs modified from published protocols [2, 9].

### Fig. 1. Electropherograms of the patient with a synonymous \textit{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).
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.

Sympathetic 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 SF3B1, 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.

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REFERENCES

1. Hoischen A, van Bon BW, Gilissen C, Arts P, van Lier B, Steehouwer M, et al. De novo mutations of SETBP1 cause Schinzel-Giedion syndrome. Nat Genet 2010;42:483-5.

2. Piazza R, Valletta S, Winkelmann N, Redaelli S, Spinelli R, Pirola A, et al. Recurrent SETBP1 mutations in atypical chronic myeloid leukemia. Nat Genet 2013;45:18-24.

3. Damm F, Itzykson R, Kosmider O, Droin N, Renneville A, Chesnais V, et al. SETBP1 mutations in 658 patients with myelodysplastic syndromes, chronic myelomonocytic leukemia and secondary acute myeloid leukemias. Leukemia 2013;27:1401-3.

4. Minakuchi M, Kakazu N, Gorin-Rivas MJ, Abe T, Copeland TD, Ueda K, et al. Identification and characterization of SEB, a novel protein that binds to the acute undifferentiated leukemia-associated protein SET. Eur J Biochem 2001;268:1340-51.

5. Cristóbal I, Blanco FJ, Garcia-Orti L, Marcotegui N, Vicente C, Rifon J, et al. SETBP1 overexpression is a novel leukemogenic mechanism that predicts adverse outcome in elderly patients with acute myeloid leukemia. Blood 2010;115:615-25.

6. Yoshida K, Sanada M, Shiraiishi Y, Nowak D, Nagata Y, Yamamoto R, et al. Frequent pathway mutations of splicing machinery in myelodysplasia. Nature 2011;478:23-9.

7. Thol F, Sauna ZE, Ambudkar SV, Gottesman MM, Kimchi-Sarfaty C. Silent (synonymous) SNPs: should we care about them? Methods Mol Biol 2009;578:23-39.

8. Chamary JV, Parmley JL, Hurst LD. Hearing silence: non-neutral evolution at synonymous sites in mammals. Nat Rev Genet 2006;7:98-108.

9. Ho PA, Kuhn J, Gerbing RB, Pollard JA, Zeng R, Miller KL, et al. WT1 synonymous single nucleotide polymorphism rs16754 correlates with higher mRNA expression and predicts significantly improved outcome in favorable-risk pediatric acute myeloid leukemia: a report from the children's oncology group. Leukemia 2011;25:704-11.

10. Choi HJ, Kim HR, Shin MG, Kook H, Kim HJ, Shin JH, et al. Spectra of chromosomal aberrations in 325 leukemia patients and implications for the development of new molecular detection systems. J Korean Med Sci 2011;26:886-92.

11. Murati A, Brecqueville M, Devillier R, Mozziconacci MJ, Gelsi-Boyer V, Birnbaum D. Myeloid malignancies: mutations, models and management. BMC Cancer 2012;12:304.

12. Liang DC, Liu HC, Yang CP, Jang TH, Hung U, Yeh TC, et al. Cooperative gene mutations in childhood acute myeloid leukemia with special reference on mutations of ASXL1, TET2, IDH1, IDH2, and DNMT3A. Blood 2013;121:2988-95.