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Mutational Analysis of RNA Splicing Machinery Components in 206 Children with Myeloid Malignancies

Hirotochi Sakaguchi, M.D.¹, Hideki Makishima, M.D., Ph.D.², Hideki Muramatsu, M.D., Ph.D.¹, Valeria Visconte, Ph.D.², Andres Jerez, M.D.², Anna M Jankowska, Ph.D.², Ramon V. Tiu, M.D.³, Jaroslaw P. Maciejewski, M.D., Ph.D.², and Seiji Kojima, M.D., Ph.D.^{1,*}

¹Department of Pediatrics, Nagoya University Graduate School of Medicine, Nagoya, Japan

²Department of Translational Hematology and Oncology Research, Taussig Cancer Institute, Cleveland Clinic, Cleveland, OH, USA

³Department of Hematologic Oncology and Blood Disorders, Taussig Cancer Institute, Cleveland Clinic, Cleveland, OH, USA

Myeloid malignancies involve a clonal proliferation of abnormal stem cells and show various phenotypes consisting of acute myeloid leukemia (AML), myeloproliferative neoplasms (MPN) and myelodysplastic syndromes (MDS) including various overlap and transition forms of these diseases¹. Juvenile myelomonocytic leukemia (JMML) is an aggressive myeloid neoplasm of early childhood that is clinically characterized by overproduction of monocytic cells that can infiltrate organs, including the spleen, liver, gastrointestinal tract, and lung. JMML is categorized as an overlap MPN/MDS by the World Health Organization (WHO) and also shares some clinical and molecular features with chronic myelomonocytic leukemia (CMML), a pathomorphologically related disease in adults. Or both CMML and JMML have the following clinical characteristics: 1) persistent peripheral blood monocytois $>1 \times 10^6/\text{ml}$; 2) no Philadelphia chromosome; 3) no eosinophilia; 4) fewer than 20% blasts in the blood and bone marrow; and 5) dysplasia in one or more myeloid lineages.

Recently, we have applied whole exome sequencing to a group of MDS patients followed by a targeted screen of specific genes for putatively recurrent mutations identified in the initial whole exome sequencing screen. In a systematic analysis of somatic events tested for recurrence and potential functional significance, we noted several somatic mutations affecting genes involved in the splicing machinery, consisting of *SF3B1* (splicing factor 3b, subunit 1), *U2AF1* (U2 small nuclear RNA auxiliary factor 1) and *SRSF2* (serine/arginine-rich splicing factor 2)^{2,3}, which Yoshida K and Papaemmanuil E reported to be frequently

*Corresponding author: Seiji Kojima, M.D., Ph.D., Department of Pediatrics, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Japan, TEL: +81-52-744-2294, FAX: +81-52-744-2974, kojimas@med.nagoya-u.ac.jp.

Conflict of interest

The authors declare no conflict of interest.

Contributions

H.S. performed the research and wrote the paper; H.Ma. contributed to design the research study and essential data; H.Mu. and V.V. contributed essential data; A.J. and A.M.J. contributed essential samples; R.V.T. contributed to design the research study; J.P.M. and S.K. contributed to design the research study and reviewed the paper.

mutated in adult MDS, AML, and in particular chronic myelomonocytic leukemia (CMML) patients^{4, 5}. These findings suggested that mutations of the genes involved in splicing machinery were a common pathogenetic pathway in adult myeloid malignancies. However, while it is likely that spliceosomal genes are also involved in the pathogenesis of childhood myeloid malignancies, including JMML, to date such a screen has been performed by only one institute⁶. In the past, several similarities (e.g., *CBL* mutations in CMML and JMML) but also significant differences (e.g., *TET2* mutations in CMML and MDS but not in JMML) were found in the mutational spectrum of patients with seemingly related disorders of adults and children. Consequently, we have designed this study to establish whether spliceosomal mutations contribute to the pathogenesis of pediatric myeloid malignancies, in particular JMML.

We have studied 206 children, aged 0–18 years with myeloid malignancies, consisting of 102 with AML, 94 with JMML, and 10 with MDS. Patients with AML and MDS were classified according to the FAB classification. A total of 102 AML patients were studied [M0=1, M1=7, M2=16, M3 variant=1, M4=9, M5=14, M7=44 (including 21 patients with Down syndrome), and 10 with unknown subtypes]. All of the 10 children with MDS were classified as refractory anemia with excess blasts (RAEB) or RAEB in transformation (RAEB-T). JMML was defined as described in the 2008 WHO classification. Four of 94 JMML patients had clinical evidence of neurofibromatosis type 1. The results in children were contrasted with those obtained in an additional series of adult CMML tested for the presence of somatic mutations in the same set of genes.

Previously we performed mutation analyses of *PTPN11*, *NRAS*, *KRAS* and *CBL* genes in 94 JMML patients, and detect genetic mutations in 29 (31%) for *PTPN11*, 12 (13%) for *NRAS*, 11 (12%) for *KRAS* and 7 (7%) for *CBL* genes⁷. Moreover, *EZH2*, *RUNX1*, *ASXL1* and *TET2* mutations were found in 0%, 0%, 4% and 0% of patients with JMML, respectively^{7,8}. In contrast, our previous studies in adult CMML have shown the presence of *TET2*, *ASXL1*, *CBL*, *EZH2*, *NRAS* and *KRAS*, in 49%, 43%, 14%, 6%, 7% and 4% of patients, respectively⁶⁻⁸.

In the current study, we analyzed genomic DNA derived from patients' bone marrow or blood samples obtained before therapy according to appropriate protocol and consent approved by the institutional review board of the two academic research centers. According to our initial screen in 50 adult patients with CMML, we have detected 2/50 mutations in exon 14 and 15 of *SF3B1*, 8/50 in exon 2 and 6 of *U2AF1* and 18/50 in exon 1 of *SRSF2* genes. The screening of mutations in these exons were performed by polymerase chain reaction (PCR) and direct sequencing. These exons were amplified from genomic DNA using the Platinum® *Taq* DNA Polymerase (Life Technologies, Carlsbad, CA, USA) and the following forward and reverse primers: SF3B1_ex13-14-F (5'-TCCCTTGATTAACAAAAGTCCTG-3'), SF3B1_ex13-14-R (5'-TGAGTCCAGTCTGGGCAAC-3'), SF3B1_ex15-16-F (5'-GTTGATATATTGAGAGAATCTGGATG-3'), SF3B1_ex15-16-R (5'-TTTAAAATTCTGTTAGAACCATGAAAC-3'), U2AF1_ex2-F (5'-GCTGCTGACATATTCCATGTG-3'), U2AF1_ex2-R (5'-AAGTCGATCACCTGCCTCAC-3'), U2AF1_ex6-F (5'-

CATTTGGCAAAATCTTGGAC-3'), U2AF1_ex6-R (5'-TGGATAATGAAGTTACCTGTGTGC-3'), U2AF1_ex6-FS (5'-CGTGGATGGCAAGCACTTCTGTTT-3'), SRSF2_ex1-F (5'-AAGGCAACTGCCTGAGAGG-3'), SRSF2_ex1-R (5'-CGGACCTTTGTGAGGTCG-3'). In addition, we evaluated sequence analysis of spliceosome genes *ZRSR2* (zinc finger, RNA-binding motif and serine/arginine rich 2) and *LUC7L2* (LUC7-like 2) which we had identified as somatic mutations in adult MDS patients by whole exome sequencing³. The forward and reverse primers for *ZRSR2* and *LUC7L2* were summarized in supplemental table 1. The purified PCR products were directly sequenced using Big Dye Terminator V3.1 (Life Technologies) and analyzed using the ABI-3130 Genetic Analyzer. The most recurrent mutations in adult CMML cohort were S34F in U2AF1 and P95H in *SRSF2*. However, when children with myeloid malignancies including JMML were screened, no mutations in any of the genes were found in the 206 cases. Thus if present, spliceosomal mutations must occur at a frequency of <1% in pediatric leukemia.

Our results suggested that there are distinct differences in the molecular pathogenesis of adult and childhood myeloid malignancies. This is further supported by the study of Yoshida, et al. which showed that the mutations of splicing machinery genes were mainly found in CMML patients⁴ and not in JMML, which was consistent with the report by Hirabayashi, et al⁶. JMML shows very similar clinical features as CMML and is considered to be the pediatric equivalent of CMML. However, the striking differences in the genetic profiles of JMML and CMML have recently been revealed; as shown in figure 1, alterations in PTPN11 and NF1 are specific to JMML, while alterations in TET2, JAK2, and RUNX1 are specific to CMML, and alterations in RAS and CBL are common to both⁷⁻¹¹; while some CBL mutations are germline in JMML, they are acquired in CMML. The current study found that genetic mutations in RNA splicing machinery components were also specific to CMML but not to JMML, supported the notion of a distinct pathogenetic roots in JMML and CMML.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

The figure showed a scheme of difference of genetic mutations between CMML and JMML. Alterations in *PTPN11* and *NF1* are specific to JMML, while alterations in *TET2*, *JAK2*, *RUNX1* and splicing machinery are specific to CMML, and alterations in *RAS* and *CBL* are common to both.

Table 1

The results of genetic mutational analysis of genes associated in splicing machinery in children with myeloid malignancies.

Genes	Adult CMML (N=50)	Pediatric myeloid malignancies (N=206)		
		JMML (N=94)	MDS (N=10)	AML (N=102)
SF3B1	2 / 50	0 / 94	0 / 10	0 / 102
U2AF1	8 / 50	0 / 94	0 / 10	0 / 102
SRSF2	18 / 50	0 / 94	0 / 10	0 / 102
ZRSR2	1 / 50	0 / 94	0 / 10	0 / 102
LUC7L2	0 / 50	0 / 94	0 / 10	0 / 102

Abbreviations; AML, acute myeloid leukemia; CMML, chronic myelomonocytic leukaemia; JMML, Juvenile myelomonocytic leukaemia; LUC7L2, LUC7-like 2; MDS, myelodysplastic syndromes; SF3B1, splicing factor 3b, subunit 1; SRSF2, serine/arginine-rich splicing factor 2; U2AF1, U2 small nuclear RNA auxiliary factor 1; ZRSR2, zinc finger, RNA-binding motif and serine/arginine rich 2