

## LETTERS TO THE EDITOR

## Novel splicing-factor mutations in juvenile myelomonocytic leukemia

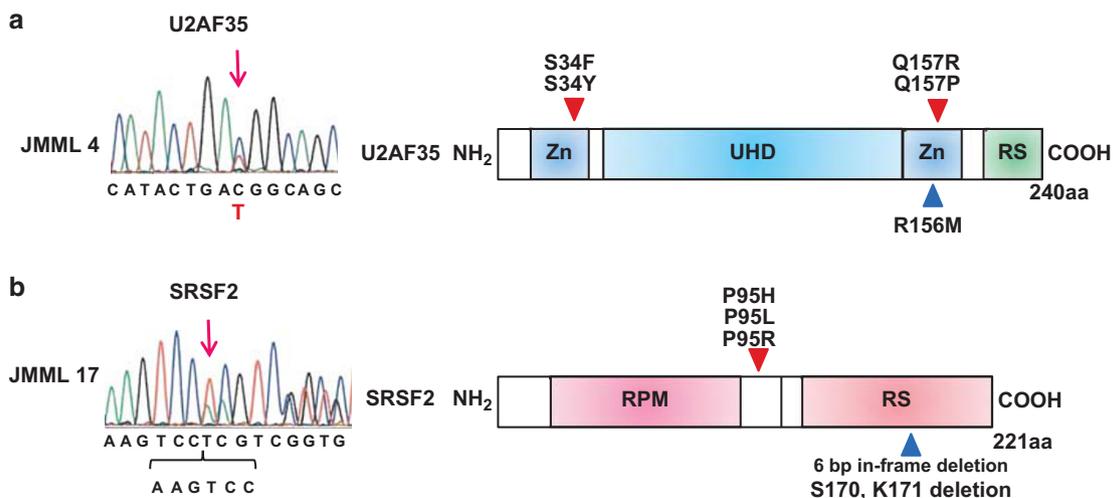
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Myelodysplastic syndromes (MDS) and myelodysplastic/myeloproliferative neoplasms (MDS/MPN) are heterogeneous groups of chronic myeloid neoplasms characterized by clonal hematopoiesis, varying degrees of cytopenia or myeloproliferative features with evidence of myelodysplasia and a propensity to acute myeloid leukemia (AML).<sup>1</sup> In recent years, a number of novel gene mutations, involving *TET2*, *ASXL1*, *DNMT3A*, *EZH2*, *IDH1/2*, and *c-CBL*, have been identified in adult cases of chronic myeloid neoplasms, which have contributed to our understanding of disease pathogenesis.<sup>2–7</sup> However, these mutations are rare in pediatric cases, with the exception of germline or somatic *c-CBL* mutations found in 10–15% of chronic myelomonocytic leukemia (CMML) and juvenile myelomonocytic leukemia (JMML),<sup>8</sup> highlighting the distinct pathogenesis of adult and pediatric neoplasms.<sup>9</sup>

Recently, we reported high frequencies of mutations, involving the RNA splicing machinery, that are largely specific to myeloid neoplasms, showing evidence of myeloid dysplasia in adult.<sup>10</sup> Affecting a total of eight components of the RNA splicing machinery (*U2AF35*, *U2AF65*, *SF3A1*, *SF3B1*, *SRSF2*, *ZRSR2*, *SF1* and *PRPF40B*) commonly involved in the 3' splice-site (3'SS) recognition, these pathway mutations are now implicated in the pathogenesis of myelodysplasia.<sup>10</sup> To investigate the role of the splicing-pathway mutations in the pathogenesis of pediatric myeloid malignancies, we have examined 165 pediatric cases with AML, MDS, chronic myeloid leukemia (CML) and JMML for

mutations in the four major splicing factors, *U2AF35*, *ZRSR2*, *SRSF2*, and *SF3B1*, commonly mutated in adult cases.

Bone marrow or peripheral blood tumor specimens were obtained from 165 pediatric patients with various myeloid malignancies, including *de novo* AML ( $n=93$ ), MDS ( $n=28$ ), CML ( $n=17$ ) and JMML ( $n=27$ ), and the genomic DNA (gDNA) was subjected to mutation analysis (Supplementary Table 1). The status of the RAS pathway mutations for the current JMML series has been reported previously (Supplementary Table 2).<sup>11,12</sup> Nineteen leukemia cell lines derived from AML (YNH-1, ML-1, KASUMI-3, KG-1, HL60, inv-3, SN-1, NB4 and HEL), acute monocytic leukemia (THP-1, SCC-3, J-111, CTS, P31/FUJ, MOLM-13, IMS/MI and KOCL-48) and acute megakaryoblastic leukemia (CMS and CMY) were also analyzed for mutations. Peripheral blood gDNA from 60 healthy adult volunteers was used as controls. Informed consent was obtained from the patients and/or their parents and from the healthy volunteers. We previously showed that for *U2AF35*, *SRSF2* and *SF3B1*, most of the mutations in adult cases were observed in exons 2 and 7, exon 1, and exons 14 and 15, respectively.<sup>10</sup> Therefore, we confirmed mutation screening to these 'hot-spot' exons. In contrast, all the coding exons were examined for *ZRSR2*, because no mutational hot spots have been detected. Briefly, the relevant exons were amplified using PCR and mutations were examined by Sanger sequencing, as previously described.<sup>10</sup> The Fisher's exact test was used to evaluate the statistical significance of frequencies of mutations for *U2AF35*, *SF3B1*, *ZRSR2* or *SRSF2* in adult cases and pediatric cases. This study was approved by the Ethics Committee of the University of Tokyo (Approval number 948-7).



**Figure 1.** Novel *U2AF35* and *SRSF2* mutations detected in JMML cases. **(a)** Left panel: sequence chromatogram of a heterozygous mutation at R156 in N-terminal zinc-finger motifs of *U2AF35* detected in a JMML case (JMML 4) is shown. Mutated nucleotides are indicated by arrows. Right panel: illustration of functional domains and mutations of *U2AF35*. Red arrow heads indicate hot-spot mutations at S34 and Q157 detected in the adult cases.<sup>10</sup> Blue arrow head indicates the missense mutation at R156. **(b)** Left panel: sequence chromatogram of a 6-bp in-frame deletion (c.518-523delAAGTCC) in *SRSF2* detected in JMML 17 is shown. Mutated nucleotides are indicated by arrows. Right panel: illustration of functional domains and mutations of *SRSF2*. Red arrow head indicates hot-spot mutation at P95 frequently detected in the adult cases.<sup>10</sup> Blue arrow head indicates a 6-bp in-frame deletion leading to deletion of S170 and K171.

No mutations were identified in the 28 cases with pediatric MDS, which included 13 cases with refractory anemia with excess blasts, 5 with refractory cytopenia of childhood, 2 with Down syndrome-related MDS, 2 with Fanconi anemia-related MDS, 2 with secondary MDS and 4 with unclassified MDS. Similarly, no mutations were detected in 93 cases with *de novo* AML or in 17 with CML, as well as 19 leukemia-derived cell lines. Our previous study in adult patients showed the frequency of mutations in *U2AF35*, *SF3B1*, *ZRSR2* or *SRSF2* to be 60/155 cases with MDS without increased ring sideroblasts and 8/151 *de novo* AML patients, emphasizing the rarity of these mutations in pediatric MDS ( $P < 5.0 \times 10^{-9}$ ) and AML ( $P < 0.02$ ) compared with adult cases. We found mutations in two JMML cases, JMML 4 and JMML 17. JMML 4 carried a heterozygous *U2AF35* mutation (R156M), whereas JMML 17 had a 6-bp in-frame deletion (c.518-523delAAGTCC) in *SRSF2* that resulted in deletion of amino acids S170 and K171 (Figure 1). Both nucleotide changes found in *U2AF35* and *SRSF2* were neither identified in the 60 healthy volunteers nor registered in the dbSNP database (<http://www.ncbi.nlm.nih.gov/projects/SNP/>) or in the 1000 genomes project, indicating that they represent novel spliceosome mutations in pediatric cases.

*U2AF35* is the small subunit of the U2 auxiliary factor (*U2AF*), which binds an AG dinucleotide at the 3'SS, and has an essential role in RNA splicing.<sup>13</sup> With the exception of a single A26V mutation found in a case of refractory cytopenia with multilineage dysplasia, all the *U2AF35* mutations reported in adult myeloid malignancies involved one of the two hot spots within the two zinc-finger domains, S34 and Q157, which are highly conserved across species, suggesting the gain-of-function mutations.<sup>10</sup> In JMML 4, the R156M *U2AF35* mutation affects a conserved amino acid adjacent to Q157, suggesting it may also be a gain-of-function mutation, leading to aberrant pre-mRNA splicing possibly in a dominant fashion.

*SRSF2*, better known as SC35, is a member of the serine/arginine-rich (SR) family of proteins.<sup>14</sup> *SRSF2* binds to a splicing-enhancer element in pre-mRNA and has a crucial role not only in constitutive and alternative pre-mRNA splicing but also in transcription elongation and genomic stability.<sup>14</sup> All mutations thus far identified in adult cases exclusively involved P95 within the intervening sequence between the N-terminal RNA-binding domain and the C-terminal RS domain.<sup>10</sup> This region interacts with other SR proteins, again suggesting that the P95 mutation may result in gain-of-function.<sup>10</sup> This proline residue is thought to determine the relative orientation of the two flanking domains of *SRSF2*, and a substitution at this position could compromise critical interactions with other splicing factors necessary for RNA splicing to take place. In contrast, the newly identified 6-bp in-frame deletion in JMML 17 results in two conserved amino acids, S170 and K171, within the RS domain. Although it may affect protein-protein interactions, the functional significance of this deletion remains elusive.

JMML is a unique form of pediatric MDS/MPN characterized by activation of the RAS/mitogen-activated protein kinase signaling pathway; in 90% of cases, there are germ line and/or somatic mutations of *NF1*, *NRAS*, *KRAS*, *PTPN11* and *CBL*.<sup>8</sup> Although JMML shares some clinical and molecular features with CMML, its spectrum of gene mutations suggests that it is a neoplasm distinct from CMML.<sup>15</sup> This was also confirmed by the current results that the splicing-pathway mutations are rare in JMML, whereas they are extremely frequent (~60%) in CMML.<sup>10</sup> Although the two JMML cases carrying the splicing-pathway mutations had no known RAS-pathway mutations, both the pathway mutations frequently coexisted in CMML.<sup>8</sup>

To summarize, no mutations of *SF3B1*, *U2AF35*, *ZRSR2* or *SRSF2* are found in pediatric MDS and AML. In our study, except for *ZRSR2*, mutations were examined focusing on the reported hot spots in adult studies, raising a possibility that we may have missed some mutations occurring in other regions. However,

these hot spots represent evolutionally conserved amino acids and have functional relevance, it is unlikely that the distribution of hot spots in children significantly differs from adult cases and as such, we could safely conclude that mutations of *SF3B1*, *U2AF35*, *ZRSR2* and *SRSF2* are rare in myeloid neoplasms in children. Finally, mutations of *U2AF35* and *SRSF2* may have some role in the pathogenesis of JMML, although further evaluations are required.

## CONFLICT OF INTEREST

The authors declare no conflict interest.

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Supplementary Information accompanies the paper on the Leukemia website (<http://www.nature.com/leu>)

## Sequencing histone-modifying enzymes identifies UTX mutations in acute lymphoblastic leukemia

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Mutations affecting epigenetic regulators have long been known to have a crucial role in cancer and, in particular, hematological malignancies.<sup>1,2</sup> One of the earliest epigenetic factors described altered in leukemia was the mixed lineage leukemia (*MLL*) protein which is found translocated in 10% of adult acute myeloid leukemia (AML), 30% of secondary AML and >75% of infants with both AML and acute lymphocytic leukemia (ALL). *MLL* is a SET domain-containing protein, which is recruited to many promoters and mediates histone 3 lysine 4 (H3K4) methyltransferase activity, thought to promote gene expression.<sup>3</sup>

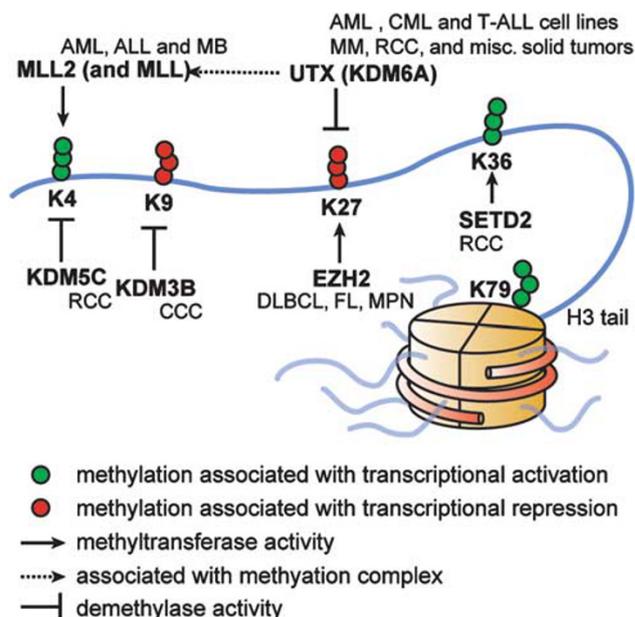
In addition to *MLL* fusions, recently, somatic mutations of *UTX* (also known as *KDM6A*), encoding an H3K27 demethylase, were described in multiple hematological malignancies, including multiple myeloma and many types of leukemia cell lines.<sup>4,5</sup> H3K27 methylation is generally thought to cause gene repression. Complementary to *UTX*, mutations of *EZH2*, a H3K27 methyltransferase, have been reported in both lymphoid and myeloid tumors (Figure 1).<sup>6,7</sup> These mutations lead to altered *EZH2* activity and influence H3K27 in tumor cells. Mutations in *EZH2*, *EED* and *SUZ12*, which all cooperate in Polycomb repressive complex 2 have been recently described in early T-cell precursor ALL.<sup>8</sup> Similarly, point mutations affecting the functional jumonji C (jmc) domain of *UTX* inactivates its H3K27 demethylase activity. In addition, *UTX* associates with *MLL2* in a multiprotein complex, which promotes H3K4 methylation, and recently *MLL2* has also been found mutated in cancer, further pointing to a common and complex epigenetic deregulation in cancer.<sup>9</sup> In line with the growing evidence for epigenetic regulators as important in tumorigenesis, additional mutations affecting epigenetic regulators such as *SETD2*, a H3K36 methyltransferase, *KDM3B*, a H3K9 demethylase, and *KDM5C*, a H3K4 demethylase, have been reported and are associated with distinct gene expression patterns (Figure 1).<sup>4</sup>

Though the clinical significance of these findings remains to be explored, it is evident that epigenetic deregulation is having an important role in both lymphoid and myeloid leukemogenesis. Furthermore, with novel drugs at hand, such as histone deacetylase inhibitors or demethylating agents that can target and reverse epigenetic alterations, understanding the underlying molecular aberrations is of growing interest.<sup>10</sup> We therefore undertook an effort to examine the prevalence of somatic mutations in genes encoding histone-modifying proteins, in particular, *KDM3B*, *KDM5C*, *UTX*, *MLL2*, *EZH2* and *SETD2*, which previously were reported mutated in cancer.<sup>4,5</sup>

For an initial screen, we analyzed banked diagnostic primary leukemia samples from 44 childhood B-cell ALL and 50 adult

AML patients, and, where available, used bone marrow samples obtained in complete remission to validate the somatic nature of the mutations. Samples had been collected with patient/parental informed consent from patients enrolled on Dana–Farber Cancer Institute protocols for childhood ALL (DFCI 00-001 (NCT00165178), DFCI 05-001 (NCT00400946)) or AML treatment protocols of the German–Austrian AML Study Group (AMLSG) for younger adults (AMLSG-HD98A (NCT00146120), AMLSG 07-04 (NCT00151242)), and the study was approved by the IRB of the participating centers.

Using conventional Sanger sequencing of primary leukemia sample-derived genomic DNA, we first screened all coding exons in which mutations have been reported previously.<sup>4,5</sup> Initially, we analyzed a total of 36 of 174 exons (*KDM3B* (2/24), *KDM5C* (9/26), *UTX* (7/29), *MLL2* (8/54), *EZH2* (1/20) and *SETD2* (9/21)) and found 7 non-synonymous tumor-specific aberrations. In AML, we found one *EZH2* mutation (p.G648E) in a t(8;21)-positive, and two *MLL2* missense mutations (p.R5153Q and p.Y5216S; Table 1) and one



**Figure 1.** Histone 3 methylation and selected histone demethylases and methyltransferases. Cancers are shown in italics next to the mutated protein they are associated with. MM, multiple myeloma; FL, follicular lymphoma; DLBCL, diffuse large B-cell lymphoma; RCC, renal cell carcinoma; CCC, clear cell carcinoma; MPN, myeloproliferative neoplasm; MB, medulloblastoma.