NOTCH2 and FLT3 gene mis-splicing are common events in patients with acute myeloid leukemia (AML): new potential targets in AML


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NOTCH2 and FLT3 gene mis-splicing are common events in patients with acute myeloid leukemia (AML): new potential targets in AML


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**Key points:**

1) Overall, our results suggest that aberrant splicing of NOTCH2 and FLT3 is a common event in AML that correlates with disease status and may correlate with disease outcomes.

2) Selected variants of NOTCH2 and FLT3 transcripts were detected in a significant number of AML patients and could be useful as disease markers.

**Abstract**

Our previous studies revealed an increase in alternative splicing (AS) of multiple RNAs in leukemic cells from patients with acute myeloid leukemia (AML) compared to CD34+ bone marrow cells from normal donors (NDs). Aberrantly spliced genes included a number of oncogenes, tumor suppressor genes, and genes involved in regulation of apoptosis, cell cycle, and cell differentiation. Among the most commonly mis-spliced genes (> 70% of AML patients) were two, NOTCH2 and FLT3, genes that encode myeloid cell surface proteins. The splice-variants of NOTCH2 and FLT3 resulted from complete or partial exon skipping and utilization of cryptic splice sites. Longitudinal analyses suggested that aberrant splicing of NOTCH2 and FLT3 correlated with disease status. Correlation analyses between splice-variants of these genes and clinical features of patients showed an association between NOTCH2 splice-variants and overall survival of patients. Our results suggest that NOTCH2 and FLT3 mis-splicing is a common characteristic of AML and has the potential to generate transcripts encoding proteins with altered function. Thus, splice-variants of these genes might provide disease markers and targets for novel therapeutics.
Introduction

In AML, the leukemic blasts are arrested at various stages of granulocytic and monocytic differentiation. Most patients with this incurable disease have chromosome translocations or mutations that promote self-renewal of leukemic stem cells, block differentiation, enhance growth, and block apoptosis. In addition, epigenetic abnormalities are very common in AML. Recently discovered gene mutations in AML, such as in TET2, DNMT3A, and EZH2, can potentially alter global gene expression by modulating epigenetic regulatory mechanisms. Understanding the epigenetic abnormalities in AML is of great interest, particularly given their potential role as targets for therapy.

Alternative splicing (AS) is a normal epigenetic phenomenon and a key regulator of gene expression. AS produces multiple transcripts and consequently multiple proteins from a single gene, thereby increasing the use of genetic information in any given cell. Even though AS is a normal process, mistakes are inevitable. Aberrant splicing events have been linked to malignant transformation, particularly in genes associated with susceptibility and/or progression of cancer. For some cancers, these splicing alterations create functionally significant biomarkers.

Using exome sequencing, frequent mutations in genes involved in regulating splicing were identified in myelodysplasia (MDS) and in some AML patients. Recently, using a SNP array and targeted sequencing of 1000 genes, novel somatic mutations of splicing factor SFPQ and splicing regulator CTCF were identified in 10% of AML patients. Graubert et al. reported that mutations in the U2AF1 gene contribute to progression from MDS to secondary AML. Prior studies of AS identified several genes mis-spliced in AML patients, including AML1, Gfi1b, CD96, survivin-2B, and GSK3beta. In our previous study we used exon arrays to compare genome-wide gene-splicing events in AML versus normal CD34+ cells, and observed widespread differences (Adamia et al., Clin Cancer Res. in press). Among the most frequently mis-spliced genes in AML patients were NOTCH2 and FLT3 and here we describe these events in detail.
Methods and Materials

Tissue and cell preparation
Diagnostic samples from 193 AML patients were obtained after IRB-approved informed consent. This study was conducted in accordance with the Declaration of Helsinki. Supplement-Table 1 describes the demographic and clinical characteristics of the patients with AML that were included in our studies. Details of sample preparation are described in Supplement-S1.

Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)
cDNA for the RT-PCR and PCR reactions was prepared as described previously. The NOTCH2 and FLT3 gene primer sequences are provided in Supplement-Table 2. Obtained PCR products were detected using gel electrophoresis or capillary electrophoresis and DNA fragment analysis on the ABI31/30XL DNA genetic analyzer (Life Technologies). Capillary electrophoresis and DNA fragment analyses were carried out as described previously.

Cloning and sequencing experiments
cDNAs for cloning and sequencing were prepared from 1ug RNA isolated from the blasts of 8 AML patients all expressing NOTCH2 and FLT3 splice-variants. TOPO-TA cloning and PCR reactions for sequencing were performed as described previously. Positive colonies containing NOTCH2 and FLT3 plasmids were identified through testing individual bacterial colonies by colony PCR using gene-specific primer sets (Supplement-Table 2). Sequencing was performed at the DFCI-MBL facility on the ABI3730 DNA Genetic Analyzer.

Real-time quantitative RT-PCR (qRT-PCR). cDNAs for qRT-PCR were prepared as above. qRT-PCR was performed using inventoried Taq-man assays for HES1, DTX1, and HEY1 with the TaqMan Universal PCR Master Mix from Life Technologies. All samples were run in triplicate on a 7500Real-Time PCR system. Results were analyzed using the relative standard curve method. In AML patients, the expression level of each gene was determined by comparing it to the gene level in CD34+ cells obtained from three normal donor bone marrow aspirates (AllCells).

Confocal laser scanning microscopy. A NOTCH2-FL open reading frame was obtained from OreGene Inc. NOTCH2-Va and NOTCH2-Vb transcripts were engineered according to the splicing
modes detected by cloning and sequencing analysis of 8 AML patient samples. NOTCH2 splice-variants were subcloned into a pCMV6-AN-GFP plasmid (OriGene). FLT3-FL and splice-variants were generated by a gene synthesis approach (GeneArt services, Life Technologies) followed by cloning them into a pDONR221 vector and subcloning into a pcDNA6.2_C-EmGFP destination vector according to the manufacturer’s recommendations (Life Technologies). Details of confocal microscopy experiments are described in Supplement-S1.

Western Blotting
HEK293T cells expressing NOTCH2-FL-GFP, NOTCH2Va-GFP, and NOTCH2Va-GFP were co-cultured 24 hours with 3T3 cells or 3T3 cells expressing Jagged2 previously generated in our laboratory. After incubation, cells were scraped, washed, and GFP positive cells were sorted on the flow sorter. From sorted cells, whole cell lysates and nuclear fractions were obtained using the Nuclear Extract kit (Active Motif) according to the manufacturer’s recommendations. In other experiments HEK293T cells expressing FLT3-FL, FLT3-Va and FLT3Vb splice variants were serum-deprived, 24 hours later cells were stimulated for 10 min with FLT3L. After stimulation whole cell lysates were obtained using the RIPA lyses buffer from Cell Signaling Technology. Cell lysates were separated by gradient (4–12%) SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. Membranes were probed with the appropriate antibodies and visualized using standard techniques. As loading controls we used anti-actin or anti-TBP (TATA box binding protein) antibodies. For western blotting anti-NOTCH2, anti-Hes1, anti-actin and anti-TBP were purchased from Cell Signaling Technology, anti-pTyr antibody (Clone 4G10) from Millipore, and anti-FLT3 antibodies from eBioscience.

Statistical analysis
Statistical analysis is described in Supplement-S1. Concordance index, confidence interval and corresponding p-value were computed with use of the survcomp package (version 1.6.0) of the R statistical software (version 2.15.2). Associations between splice-variant expression and OS of AML patients were assessed using univariate Cox regression analyses, followed by the Kaplan-Meier analyses with the R survival package (version 2.36-14). All p-values were two-sided and if necessary, nominal p-values were corrected for multiple testing by estimating the false discovery
Results

Identification of splicing patterns in NOTCH2 and FLT3 in patients with AML

In preliminary studies, we looked for splicing abnormalities in 66 AML patients and 10 normal donors using exon arrays (Adamia et al., Clin Cancer Res. in press). In addition, RNA-seq analysis was performed in 12 of these AML patients and 3 of the normal donors (unpublished data) to validate exon array studies. We identified widespread RNA splicing abnormalities in AML patients compared to NDs (Supplement-Table 3a). For the current study, we focused on genes encoding myeloid cell surface proteins, NOTCH2 and FLT3 that were most frequently spliced in AML patients.

To determine precise splicing pattern for NOTCH2 and FLT3, we performed RT-PCR followed by cloning and sequencing of the cDNAs of these transcripts from the blasts of 8 AML patients and 12 NDs (Figure 1a). Two novel variants of NOTCH2, and three novel variants of FLT3, referred to as NOTCH2-Va and NOTCH2-Vb, and FLT3-Va, FLT3-Vb, and FLT3-Vc, respectively, were identified (Figure 1a). Except for NOTCH2-Vb, NOTCH2 and FLT3 splice-variants were absent from NDs. The levels of NOTCH2-Vb were elevated in some AML samples compared to the ND’s (Figure 1a).

Sequence alignment indicated that splice-variants of NOTCH2 and FLT3 derived from exon skipping and/or partial deletions of some exons (Figure 1b). Alignment followed by bioinformatic analysis showed that some of these splicing events avoided frame-shifts (NOTCH2-Va, NOTCH2-Vb, FLT3-Va, FLT3-Vb), while other combinations of alternative splicing led to frame-shifted transcripts (e.g., for FLT3-Vc) (Figure 1b). To determine whether splice-variants of NOTCH2 and FLT3 encode proteins, we generated NOTCH2 and FLT3 splice-variant plasmids tagged with GFP and expressed them in HEK293T cells. Transfected cells were harvested and examined by laser-scanning confocal microscopy and by flow cytometry (Figure 1c and Supplement-Figure 1a). These analyses demonstrated that Notch2-Va-GFP, Notch2-Vb-GFP, Flt3-Va-GFP, and Flt3-Vb-GFP proteins were readily expressed in HEK293T cells. Further, flow cytometric analyses with anti-NOTCH2 conjugated to phycoerythrin (PE) and anti-FLT3 conjugated to allophycocyanin. 

(APC) showed that a significant amount of Notch2 and Flt3 splice-variant proteins were expressed on the cell surface. Because the predicted difference between Notch2-FL or Flt3-FL and their splice-variants is small, our ability to detect splice-variant proteins in AML patients or cell lines using western blotting was felt to be unreliable. Antibody production against the novel Notch2 and Flt3 splice-variant proteins is underway.

**Correlation of NOTCH2 splice variants with NOTCH2 target gene expression**

We evaluated the expression of 3 known Notch target genes, HES1, HEY1, and DTX1, in 49 AML patients expressing the NOTCH2 splice-variant using Taq-man gene expression assays. As a control, CD34+ bone marrow (BM) cells from 3 NDs were used. Expression levels of HES1, HEY1, and DTX1 in AML patients were compared to expression levels of these genes in NDs. We observed down regulation of HES1 and DTX1 transcripts (an average 30-fold) in patients expressing both NOTCH2 splice-variants in combination with NOTCH2-FL, or expressing NOTCH2-Vb and NOTCH2-FL transcripts compared to expression levels in normal CD34+ cells (Figure 2a). We observed low expression of the HEY1 gene in all samples regardless of expression levels of NOTCH2-FL or splice-variants (Figure 2a).

In comparing expression levels of target genes across the AML samples, expression levels of HES1, HEY1, and DTX1 genes were generally inversely correlated with expression of NOTCH2 splice variants or NOTCH2-FL, and relatively higher in patient samples expressing only NOTCH2-FL or not expressing any NOTCH2 transcripts (Figure 2a). However these results were not statistically significant (p<0.1) as determined by the Spearman rank correlation coefficient test (analysis results not shown).

In an effort to determine if splice variants of NOTCH2 might be functional, HEK293T cells were transfected with NOTCH2-FL or a splice-variant transcripts tagged with GFP (Figure 2b) and co-cultured overnight with 3T3 cells stably expressing the Jagged2 Notch ligand. Cells expressing GFP were enriched by flow cytometry, seeded into dishes with glass coverslips, fixed-permeabilized, and stained with anti-Hes1 (Supplement-Figure 1b). Alternatively, after sorting, whole cell lysates and nuclear fractions were prepared and subjected to western blotting for Hes1 expression. Jagged2
modestly increased expression of Hes1 in cells transfected with NOTCH2-FL, but had no effect on Hes1 expression in cells expressing either NOTCH2-Va or NOTCH2-Vb (Figure 2b).

**FLT3 splice-variant activation in response to Flt3L**

We evaluated the functionality of the FLT3 splice-variant transcripts in HEK293T cells. The FLT3-Fl and splice-variants (FLT3-Va and FLT3-Vb) were stably expressed in HEK293T cells. Cells were serum-starved overnight and stimulated for 10 min with 100ng/ml Flt3 ligand (Flt3L). Flt3L increased the level of tyrosine phosphorylation in cells expressing either FLT3-FL or splice-variants (Figure 2c). We next examined the functionality of the FLT3 splice-variants in primary blasts from 4 patients by evaluating activation of important downstream targets of the activated FLT3 receptor, such as STAT5, MAP kinase (MAPK), and serine/threonine protein kinase AKT as markers for the activation of phosphatidylinositol 3-kinase (PI3K)-dependent pathways. Constitutive activation of STAT5 and MAPK/Erk was detected in AML patient cells expressing FLT3 splice-variants regardless of Flt3L activation (Figure 2d, Expression of the splice-variants are shown in the Table below of the Figure 2d). The FLT3 activation signature was not observed in AML patient cells lacking FLT3-Va and FLT3-Vb splice-variant expression (Figure 2d). More detailed studies regarding functional effects of splice variants are underway.

**NOTCH2 and FLT3 splice variant expression pattern in large cohort of AML patients**

We analyzed expression of the NOTCH2 and FLT3 novel variants by the RT-PCR capillary electrophoresis and DNA fragment analysis, in the peripheral blood (PB) or in BM samples from 193 AML patients, in CD34+ cells from BM of 8 NDs, and in PB monocytes and neutrophils from 4 NDs (Figure 3, Supplement-Figure 2-Representative example of DNA fragment analysis and capillary electrophoresis).

NOTCH2 and FLT3 splice-variants were expressed in the majority of AML patients. These variants, with the exception of NOTCH2-Vb, were not detectable in the NDs. DNA fragment analysis also documented overexpression of two novel splice-variants in AML, NOTCH2-Va and FLT3-Va respectively in 73% and 50% of the AML cases (Figure 3a-b and Table 2a). Because NOTCH2-Vb expression was seen in NDs (Figure 1a), this variant was excluded from validation studies performed within the 193 AML patient sample-set. However, we analyzed additional 49
AML patient samples and 10 normal donors for the expression of a NOTCH2-Vb splice-variant (Figure 3a). This analysis demonstrated that the NOTCH2-Vb/FL expression ratio in normal donors ranges from 2.1 to -3.7, while in AML patients from 5.9 to -2.4, thus NOTCH2-Vb is upregulated in AML patients as compared to NDs (Supplement-Figure 2b).

RT-CPR DNA fragment analyses show that the majority (141 of 193; 73%) of the AML patients express NOTCH2-Va transcripts, either alone or in combination with NOTCH2-FL (Figure 3a, Table 1a). However, three patients expressed only the NOTCH2-Va variant, and 31 did not express either transcript. Similar to NOTCH2-Va, splice-variants of FLT3 were also expressed in various combinations with FLT3FL transcripts, or with other FLT3 variants. Of 193 patients, 98 expressed one or more splice-variants in various combinations with other FLT3 variants (16 patients) or FLT3FL (82 patients), while 65 patients lacked any FLT3 variants, but expressed FLT3-FL at low level (Figure 3b). The Figure 3 shows, that expression of two or more splice-variants in various combinations was the most common in AML patients (Table 1a). The most frequently expressed splice-variant combinations were FLT3-FL/-Va/-Vb/-Vc and FLT3-FL/-Va/-Vc, expressed in 37 and 20 patients, respectively. We also investigated association between FLT3 gene-splicing and the presence of a FLT3 gene mutation. There was only a trend in the associations between certain FLT3 variant expressions with the FLT3-ITD/Asp835 mutations (Supplement-Table 3b).

**Aberrant splicing of NOTCH2 and FLT3 reflects disease status**

To evaluate whether aberrant splicing reflects disease status, we examined novel splice-variant expression over the course of disease in individual AML patients. NOTCH2-FL or FLT3-FL transcripts and their splice-variant expression levels were monitored longitudinally in paired samples of 17 AML patients; samples were taken at diagnosis and remission or relapse, or over the course of refractory disease. A total of 35 samples were collected.

This analysis showed specific expression patterns and levels of the novel splice-variants at different time points during disease development (Figure 3c-e); for example, at diagnosis, >79% of patients expressed two or three NOTCH2 and FLT3 novel splice-variants out of the four we have identified; at remission, expression frequencies of these splice-variants were markedly decreased (to 0%, or to 40%); and at relapse, expression of all splice-variants was sharply increased by which point >90%
of the patients expressed one of more variants in various combinations (Figure 3, Table 2);
Likewise, in a group of patients with persistent AML, novel splice-variant expression frequencies
remained high, such that >83% of patients expressed all splice-variants, alone or in various
combinations.

**Frequently occurring spliced transcripts may be associated with clinical outcome in AML**

To test whether splicing events are associated with clinical outcomes, we estimated the prognostic
value of NOTCH2-FL, FLT3-FL, and their splice-variant expression levels in the 193 patient
sample-set; NOTCH2-Va splice-variant expression was significantly associated with prognosis
(OS) (concordance index, CINDEX=0.60, 95%CI [0.53 to 0.67], nominal P-value=0.005,
FDR=5.8%) (Table 3). To further assess the clinical significance of the presence of NOTCH2 and
FLT3 splice-variants, patients were classified into two equal-sized groups divided by median
splice-variant transcript expression levels (Supplement-Figure 3a), and survival curves were
estimated using the Kaplan-Meier method. Amongst NOTCH2 and FLT3 splice-variants, patients
expressing higher-than-median levels of the NOTCH2-Va transcripts had significantly inferior
survival (HR=1.79, 95%CI [1.15 to 2.78], nominal P-value=0.0086, FDR=9.5%) (Supplement-
Figure 3b). Similar results were obtained in the group of patients <50 years (HR=3, 95%CI [1.28 to
7.09], nominal P-value=0.0084, FDR=9.2%; (Supplement-Figure 3b). First we tested the
significance of the association between FAB subtypes and expression status of NOTCH2/FLT3
splice-variants (low or high expression) we did not find any significant associations (data not
shown). To determine whether binary classification based on NOTCH2-Va expression has a
prognostic value that is independent of well-established clinical variables, we fitted multivariate
Cox models including FLT3-ITD, FLT3-D835 mutation, cytogenetic-risk groups, percentage of
blasts in BM, percentage of blasts in PB, and patient age (Supplement-Table 4). We conclude that
NOTCH2-Va binary classification may have prognostic value (significance at P<0.05).
Furthermore, a multivariate Cox model with selected clinical variables that were significantly
associated with survival (i.e., cytogenetic-risk group of patients and age of patients) revealed that
higher-than-median expression levels of NOTCH2-Va transcripts still correlate significantly with
OS (HR=1.81, 95%CI [1.06 to 3.10], P-value=0.029). The relevance of NOTCH2-Va expression
for clinical outcome was further illustrated by use of the Kaplan-Meier estimator in the subgroup of
patients within each cytogenetic risk group (Figure 4a). We observed that patients with an
intermediate cytogenetic-risk profile that express higher-than-median levels of NOTCH2-Va had a trend towards worse survival than patients in the group that express NOTCH2-Va at lower-than-median levels (HR=2.74, 95% CI [1.27 to 5.89], nominal P-value=0.0072, FDR=8%; Figure 4b). Interestingly, these patients exhibited survival similar to patients with an adverse-risk cytogenetic profile.

Discussion
A genome-wide alternative splicing screen in AML patients identified NOTCH2 and FLT3 as two most frequently mis-spliced transcripts in patients compared to normal donors. FLT3 is already known as a frequently mutated oncogene in AML, while the role of NOTCH2 in this disease is not well understood, although decreased NOTCH2 expression has been linked to AML in two reports.34,35

Cloning and sequencing analysis identified several aberrant splice-variants of NOTCH2 and FLT3 that resulted from either skipping of one or more exons or activation of cryptic splicing sites. All these splicing alterations created transcripts that encoded proteins, as demonstrated through expression of NOTCH2-Va, NOTCH2-Vb, FLT3-Va, and FLT3-Vb splice-variants in HEK293T cells. These results were expected, because splicing alterations do not cause frame-shifts on NOTCH2 and FLT3 genes and because they occur on the gene segments that encode proteins in the extracellular domain, (spliced-out exons of NOTCH2 encode extracellular EGF-like domains, while FLT3 shows splicing aberrations in exons encoding extracellular Ig-like motifs).

The functionality of Notch2 splice-variant proteins was explored using primary blasts from AML patients. We observed an inverse correlation between the expression of NOTCH2 splice variants and Notch2 target genes HES1, TXL1, and HEY1 and in AML patients. Since splice-variants are expressed in various combinations with full-length NOTCH2 transcripts, it is intriguing to speculate that, similar to splice-variants of many other genes (including MDM2, CD44, and p53) NOTCH2 splice-variants may act in a dominant-negative manner that compromises the functions of NOTCH2 and are likely to block corresponding signaling pathways in AML cells.2,36-38 Based on these discoveries and the findings presented here, we suggest that silencing of the Notch2 signaling pathway could be a due to dominant-negative effects of Notch2 splice-variants on Notch2-FL. It is
thus possible to speculate that Notch2 pathway reactivation could be achieved by the using of blocking antibodies against Notch2-Va and Notch2-Vb splice-variants. Our observations indicate that NOTCH2 splice-variants are attractive targets for novel AML therapeutics. Also, based on our preliminary studies on FLT3 splice variants we speculate that some of these variants may be functional. Additional studies need to be done to determine the functional consequences of these variants.

Expression profiling of NOTCH2-FL, FLT3-FL and their splice-variants 193 AML patients identified NOTCH2-Va (73%) and FLT3-Va (50%) as the most frequently expressed variants in AML. These splice-variants were undetectable in plasma cells from 12 myeloma patients or in several lymphoma cell lines (data not shown). More detailed study regarding expression of these splice-variants in different cell populations in AML patients, as well as in patients with other hematological malignances is underway. Based on our studies we speculate that these splice-variants represent potential novel markers to differentiate AML cells from normal stem cells, offering the possibility for selective targeting of AML stem cells.

Recent reports of mutations in splicing factors in many different cancers, including MDS and AML, suggest that splicing events could contribute directly to disease initiation or progression. Aberrant splicing events have been linked to malignant transformation, particularly in genes associated with susceptibility and/or progression of cancer. For some cancers, these splicing alterations create functionally significant biomarkers. In light of these studies and the results presented here, we believe that NOTCH2 and FLT3 spicing events are associated with pathogenesis in AML and that identification of the causes and consequences of these splicing alterations will provide a better understanding of the biology of this disease. Since it is well known that splicing alterations can result from mutations and/or could be due to modulated expression of splicing factors, we evaluated whether expression of NOTCH2 and FLT3 splice-variants correlated with mutations detected on commonly mutated genes such as U2AF1 and SF3B1 in patients with AML (Supplement-Figure 4). We did not find any significant correlation from this preliminary study, possibly because of the small size of the cohort. It may also reflect the extensive complexity of the splicing machinery. Evaluation of a larger cohort of patients in these studies is underway. Additionally, to understand causes of altered splicing in AML we tested if expression levels of
splice-variants correlate with classical mutations such as NPM1, IDH1/2. We did not find any significant correlation from this preliminary study (Supplement-Figure 4).

In these studies we investigated clinical relevance of alternative splicing in AML patients. In longitudinal studies we detected significant differences in the frequency of expression of particular splice-variants of NOTCH2 and FLT3 at time points throughout the course of disease progression; for example, certain splicing events were elevated at diagnosis and relapse, and were “normalized” during remission. We also noted correlations between splicing events and the clinical outcome; NOTCH2-Va expression was associated with a poor outcome, particularly for patients with an intermediate-risk cytogenetic profile. Furthermore, patients who expressed NOTCH2-Va at higher-than-median levels had the same median OS as those with cytogenetically determined adverse-risk profiles. Thus, NOTCH2-Va expression has potential use for identifying a subgroup of patients within the larger group having cytogenetically determined intermediate-risk profiles. Additional studies with larger AML patient cohorts are needed to verify the clinical significance of splicing events of the NOTCH2 gene.

Our results suggest that alternative splicing of NOTCH2 and FLT3 is a common event in AML. Because NOTCH2 and FLT3 variants are detected in a significant number of patients with AML, they can be used as novel disease markers and as selective targets for either therapeutic antibodies or for the delivery of cytotoxic drugs, toxins, or radionucleotides to leukemic cells. More extended functional studies are underway to clarify the oncogenic potential of NOTCH2 and FLT3 splice-variants in AML.
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Authorship contributions and discloser conflict of interests
Contributions: SA conceived the idea, executed experiments, coordinated and directed the study, and wrote the manuscript; MBN collected and processed patients’ clinical data; BHK performed statistical and bioinformatics analyses and wrote the manuscript, PMP and SP performed bioinformatics analyses; CB helped with the sequencing experiments; TC performed microscopy experiments; HAL and LL provided UHN samples and clinical data; SV and EAF collected patient samples; IG, DPS, GM, DJD provided DFCI patient samples; MW, SM and IDJ collected patient clinical data; JQ provided discussions and advice, RMS provided DFCI patient samples, assisted in writing the manuscript; JDG directed the study and edited the manuscript.

Discloser conflict of interests: JDG receives research support and is a consultant for Novartis Pharma AG.
References

Table 1a. NOTCH2FL, FLT3FL and their splice-variants expression patterns were examined in 193 patients with AML.

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<th>Patient (%)</th>
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Table 2. NOTCH2FL, FLT3FL and their splice-variant expression profiles reflect disease status.

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<th>FLT3 variants</th>
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<td>-FL (n (%))</td>
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</tr>
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<td>Remission (n=5)</td>
<td>5 (100%)</td>
<td>5 (100%)</td>
</tr>
<tr>
<td>Relapse (n=10)</td>
<td>8 (80%)</td>
<td>8 (80%)</td>
</tr>
<tr>
<td>Persistent AML (n=6)</td>
<td>6 (100%)</td>
<td>6 (100%)</td>
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Table 3. Concordance indexes of each splice-variants in the dataset of 193 patients

<table>
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<tr>
<th>Splice variants</th>
<th>CINDEX</th>
<th>Lower 95% CI</th>
<th>Upper 95% CI</th>
<th>Nominal P-value</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOTCH2-FL</td>
<td>0.564</td>
<td>0.494</td>
<td>0.634</td>
<td>0.074</td>
<td>0.406</td>
</tr>
<tr>
<td>NOTCH2-Va</td>
<td>0.600</td>
<td>0.530</td>
<td>0.670</td>
<td>0.005</td>
<td>0.058</td>
</tr>
<tr>
<td>FLT3-FL</td>
<td>0.559</td>
<td>0.478</td>
<td>0.641</td>
<td>0.152</td>
<td>0.419</td>
</tr>
<tr>
<td>FLT3-Va</td>
<td>0.538</td>
<td>0.460</td>
<td>0.615</td>
<td>0.339</td>
<td>0.600</td>
</tr>
<tr>
<td>FLT3-Vb</td>
<td>0.482</td>
<td>0.378</td>
<td>0.587</td>
<td>0.740</td>
<td>0.904</td>
</tr>
<tr>
<td>FLT3-Vc</td>
<td>0.545</td>
<td>0.459</td>
<td>0.632</td>
<td>0.304</td>
<td>0.600</td>
</tr>
</tbody>
</table>

For each concordance index the lower and upper bound 95% confidence interval (CI) is provided along with the corresponding p-value and false discovery rate (FDR)
Figure Legend

Figure 1. Identification novel splice-variants of NOTCH2 and FLT3

a. This figure displays NOTCH2 and FLT3 RT-PCR agarose gel electrophoresis results.

b. This figure displays AML-associated splicing patterns of NOTCH2 and FLT3 splice-variants. After cloning and sequencing experiments novel splice-variant sequences were identified through alignment with published sequences for human NOTCH2 and FLT3 mRNA (gi:317008612; gi:178535 NCBI).

NOTCH2Va and NOTCH2Vb are the result of complete deletions of exon 12 (111 bp) or exons 17 (120bp) and 18 (153bp) respectively. These splicing aberrations did not cause any frame-shifts.

FLT3Va is a result of skipping exon 7 (140bp) and a 76bp deletion of exon 8 at the 5’ end, while FLT3Vb was due to skipping exons 5 (130bp) and 7 (140bp), with partial deletions of exons 6 (33bp deletion at the 3’ end of exon 6) and 8 (48bp deletion at the 5’ end of exon 8). FLT3Vc appears to be most severely affected by splicing events when compared to other FLT3 splice-variants. FLT3Vc is a result of skipping of exons 5 (130bp), 6 (128bp) and 7 (140bp) and a 26 bp deletion of exon 8 at the 5’ end. As results of these aberrations 240bp are spliced out from FLT3Va, 351bp from FLT3Vb and 398bp from FLT3Vc. Thus, these aberrations did not cause a frame-shift on FLT3Va and FLT3Vb transcripts, while FLT3Vc transcripts were subjected to a frame-shift.

For both NOTCH2 and FLT3, the splice-variant transcripts retained their original start codons, and conserved signal sequences. Also, bioinformatics and alignment analysis showed that exons affected by aberrant splicing events mapped to extracellular domains of the Notch2 and Flt3 protein sequences. As a result of the splicing alterations, three EGF-like domains were entirely deleted from the Notch2 protein and two were partially affected. Splicing alterations caused deletion of the entire Ig-like C2 type domain on the Flt3 protein. In the figure, we zoomed out gene segments of NOTCH2 and FLT3 that we cloned and sequenced. In the figure, yellow-boxed exons are those that are affected by the splicing events.
c. HEK293T cells were transiently transfected with NOTCH2-FL-GFP, NOTCH2-Va-GFP, NOTCH2-Vb-GFP, FLT3FL-GFP, FLT3-Va-GFP, FLT3Vb-GFP, or GFP backbone plasmids; 72 h after transfection cells were stained using the live cell nuclear staining reagent (Life Technologies) that includes DAPI; cells were visualized under the Zeiss 710 confocal laser-scanning microscope. On the figure, GFP signal shown in green, and DAPI signal in blue. HEK293T cells transfected with NOTCH2 and FLT3 splice-variants were stained with anti-Notch2-PE (16F11, eBioscience) and anti-Flt3-APC (BV10A4H2, eBioscience) antibodies, then membrane expression of these variants was evaluated by flow cytometry. On the flow cytometry histograms, gray peaks represent HEK293T cells untrasfected and unstained, pink peaks represent HEK293T cells untransfected and stained with anti-Notch2-PE or anti-Flt3-APC antibodies, red peaks represent HEK293T cells transfected with NOTCH2-FL, FLT3-FL, or splice variants tagged with GFP. These cells were stained with anti-Notch2-PE or anti-Flt3-APC antibodies and staining was determined in GFP gated cells. IgG stained controls are provided in Supplement-Figure 1a.

Figure 2. Functional effects of NOTCH2 and FLT3 splice-variants

a. This figure displays unsupervised clustering analyses results obtained from the TaqMan gene expression assays of Notch2 target genes HES1, DXT1, and HEY1 carried out in 49 AML patients expressing NOTCH2-FL, FLT3-FL, and their splice-variants. Expression levels of NOTCH2-FL, FLT3-FL, and their splice-variants were determined by RT-PCR, and DNA fragment analysis described in this manuscript. On the heatmap, green color shades represent transcript relative upregulation and red color shades represent relative downregulation. In these studies relative transcript expressions are calculated as compare to the expression levels of the corresponding transcripts detected in normal donors.

b. This figure is a summary of co-culture experiments performed three times. HEK293T cells expressing NOTCH2-FL-GFP, NOTCH2Va-GFP, NOTCH2Va-GFP were co-cultured 24 hours with 3T3 cells (-Jagged2) or 3T3 cells expressing Jagged2 (+Jagged2). After incubation, cells were scraped, and GFP positive cells were sorted. Western blotting analysis for Notch2 and Hes 1 performed as described in methods. Western blots were quantified using the ImageJ software (http://rsb.info.nih.gov/ij). Densitometry measurements were normalized to loading control amount
c. FLT3-FL, FLT3-Va and FLT3Vb splice variants were stably expressed in HEK293T cells. Serum-deprived, transfected cells were stimulated for 10 min with FLT3L. Cellular tyrosine phosphorylation was analyzed by immunoblotting with a pTyr antibody (Clone 4G10 from Millipore). Elevation of tyrosine phosphorylation of bands at approximately 100 kDa was determined as compared to FLT3L un-stimulated cells. Additionally, in the samples Flt3 expression was determined by immunoblotting using anti-FLT3 antibodies from eBiosciences. As a loading control, the same membranes were re-probed with anti-actin antibody. As control MOLM14 and HEK293T-GFP stimulated and un-stimulated cell lysates were used for pTyr immunoblotting analyses.

d. Serum-starved blasts from patients were stimulated for 10 min with Flt3L 100 ng/ml, washed, and cell lysates were prepared to measure phosphorylation levels of STAT5, Akt, and Erk using InstantOne ELISA kits according to the manufacturer’s suggestions. Absorbance was measured at 450 nm using an automated ELISA plate reader. In the figure, results are presented as bar graphs. The X-axis shows the samples analyzed and the y-axis displays the phosphorylation level as an absorbance. Results obtained from positive and negative control samples are not displayed on the graph. Expression levels of FLT3-FL and its splice variants in patient samples are reported in a table included in this figure and presented as RFU = \log_2\text{RFU} (relative fluorescence units, described in the Figure 3 legend below).

Figure 3. NOTCH2- and FLT3- FL and their novel splice-variant expression frequencies in AML patients and their association with disease status

a-b. This figure display overall expression patterns of NOTCH2 (a) and FLT3 (b) FL transcripts and their splice-variants. On the figures the x-axes display patient and normal donor samples, and the y-axes Relative Fluorescent units (RFU). PCR product RFU=Log _2RFU. RFU is a unit of measurement calculated relative to the size standards included in each reaction. For relative level determination, product levels were kept below 3500 RFU and size standard levels were within 500-800 units as recommended by the manufacturer. All calculations and an instrument calibration were done according to the Life Technologies recommendations. We note that in some patients FLT3-FL expression is lower than cut off and not displayed on the graph.
c-e. This figure display overall expression patterns of NOTCH2-FL and FLT3-FL transcripts and their splice-variants over the course of the disease in 17 patients. Patients are numbered from 1 to 17; patients samples were taken: (c) at diagnosis and relapse (patients 1-8), (d) at diagnosis and remission (patients 9-13), or (e) over the course of refractory disease (patients 14-17). A total 35 samples were collected including 14 samples obtained at diagnosis: Diag 1-13 and Diag 17; ten samples obtained at relapse: Rel 1-8, Rel-14-1 and Rel-14-2 (1st and 2nd relapse); five samples obtained at remission: Rem 9-13; 6 samples obtained during regular visits over the course of refractory disease: Pers 15-17 (samples take at 1st and 2nd visit are marked as -1 or -2). On the panels NOTCH2-FL and FLT3-FL and their splice-variant transcripts expression levels are presented as log2(PCR product RFU) and shown in a green color-coded scale.

**Figure 4. The relevance of NOTCH2-Va expression to clinical outcome of patients with AML**

a. The relevance of NOTCH2Va expression to clinical outcome was estimated in the subgroup of patients within each cytogenetic-risk group. As Fig (a) demonstrates a patient group expressing higher than median levels of NOTCH2Va have similar prognosis as adverse-risk group of patients. Fig b. shows the overall survival of AML patients with an intermediate-risk cytogenetic profile. When dividing patients into four categories based on NOTCH2-Va expression (divided by quartile) the resulting classification was borderline significant (P=5.9E-02) as expected due to the small number of patients in each group. However, separation trend remained the same.
Figure 1a. Identification novel splice variants NOTCH2 and FLT3

AML patients samples
NOTCH2-FL
NOTCH2-Va
NOTCH2-Fb

Normal donor samples

FLT3-FL
FLT3-Va
FLT3-Vb
FLT3-Vc

1 2 3 4 5 6 7 8
CD34+ cells  Neutrophils  Monocytes

b. Splicing modes of NOTCH2 and FLT3 identified in AML patients

Extracellular domains
EGF-like domains

Cytoplasmic domains

NOTCH2-Va

Extracellular domains
Ig-like C2 type domain

Cytoplasmic domains

intron
exon
Partially deleted exon
Partially retained intron

NOTCH2-Vb

FLT3-Va

FLT3-Vb

FLT3-Vc

Notch2-FL, Flt3-FL and splice-variant expression in HEK293T cells

GFP-plasmid  Notch2-FL-GFP  Notch2-Va-GFP  Notch2-Vb-GFP  Flt3-FL-GFP  Flt3-Va-GFP  Flt3-Vb-GFP

HEK293T transfected with NOTCH2-FL, FLT3-FL, or splice variants, stained with anti-Notch2-PE or anti-Flt3-APC Abs

HEK293T untransfected stained with anti-Notch2-PE or anti-Flt3-APC Abs

HEK293T untransfected, unstained
Figure 2. Functional effects of NOTCH2 and FLT3 splice-variants

a) NOTCH2 target gene expressions in AML patients expressing NOTCH2 splice variants

b) Hes1 expression in NEK293T cells transfected with Notch2-FL and splice variants after co-culturing with 3T3 (-Jagged2) or 3T3-Jagged2 (+ Jagged2) cells

c) Flt3-FL and splice variant stimulation by Flt3 ligand (Flt3L)

d) STAT5 activation in blasts from AML patients after stimulation by Flt3 ligand (Flt3L)

<table>
<thead>
<tr>
<th>Sample</th>
<th>FLT3-ITD</th>
<th>FLT3-FL</th>
<th>FLT3-Va</th>
<th>FLT3-Vb</th>
<th>FLT3-Vc</th>
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<td>AML-1</td>
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FLT3 and splice-variant expression (LRFU) in AML patients
Figure 3. NOTCH2- and FLT3-FL and their novel splice variant expression frequencies in AML patients and their association with disease status

a) NOTCH2-FL and splice variant expression profiles

b) FLT3-FL and splice variant expression profiles

c) Expression profiles at diagnosis (Diag) and relapse (Rel)

<table>
<thead>
<tr>
<th>FLT3-Vc</th>
<th>FLT3-Vb</th>
<th>FLT3-Va</th>
<th>FLT3-FL</th>
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<th>NOTCH2-FL</th>
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<td>Rel-8</td>
<td>Rem-9</td>
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</table>

d) Expression profiles at diagnosis (Diag) and remission (Rem)

e) Expression profiles at relapse (Rel), diagnosis (Diag) and during persistent (Pers) disease
Figure 4. Overall Survival among patients with AML

a) Patients groups with different cytogenetic profiles

b) Patients with an intermediate-risk cytogenetic