A Genome-Wide Aberrant RNA Splicing in Patients with Acute Myeloid Leukemia Identifies Novel Potential Disease Markers and Therapeutic Targets

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Abstract

Purpose: Despite new treatments, acute myeloid leukemia (AML) remains an incurable disease. More effective drug design requires an expanded view of the molecular complexity that underlies AML. Alternative splicing of RNA is used by normal cells to generate protein diversity. Growing evidence indicates that aberrant splicing of genes plays a key role in cancer. We investigated genome-wide splicing abnormalities in AML and based on these abnormalities, we aimed to identify novel potential biomarkers and therapeutic targets.

Experimental Design: We used genome-wide alternative splicing screening to investigate alternative splicing abnormalities in two independent AML patient cohorts [Dana-Farber Cancer Institute (DFCI) (Boston, MA) and University Hospital de Nantes (UHN) (Nantes, France)] and normal donors. Selected splicing events were confirmed through cloning and sequencing analysis, and than validated in 193 patients with AML.

Results: Our results show that approximately 29% of expressed genes genome-wide were differentially and recurrently spliced in patients with AML compared with normal donors bone marrow CD34+ cells. Results were reproducible in two independent AML cohorts. In both cohorts, annotation analyses indicated similar proportions of differentially spliced genes encoding several oncopgenes, tumor suppressor proteins, splicing factors, and heterogeneous-nuclear-ribonucleoproteins, proteins involved in apoptosis, cell proliferation, and spliceosome assembly. Our findings are consistent with reports for other malignances and indicate that AML-specific aberrations in splicing mechanisms are a hallmark of AML pathogenesis.

Conclusions: Overall, our results suggest that aberrant splicing is a common characteristic for AML. Our findings also suggest that splice variant transcripts that are the result of splicing aberrations create novel disease markers and provide potential targets for small molecules or antibody therapeutics for this disease.

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Introduction

Acute myeloid leukemia (AML) is a heterogeneous neoplasm characterized by the accumulation of myeloid blasts both in the bone marrow and peripheral blood of patients. The leukemic blasts are arrested at various stages of granulocytic and monocytic differentiation. Even though these blasts are blocked at different stages of differentiation, they are stem cells and have a natural ability to proliferate. During the proliferation process, AML stem cells accumulate various genetic and epigenetic abnormalities, including aberrations in pre-mRNA processing.

Pre-mRNA processing, referred to as alternative RNA splicing, is a critical determinant of protein diversity (1, 2). Alternative splicing produces multiple transcripts and,
Translational Relevance

Despite a century of medical advances in pharmaceutical drugs and diagnostic methods, acute myeloid leukemia (AML) remains an incurable disease. Standard treatment for AML is very toxic and includes intensive multiagent chemotherapy and/or hematopoietic stem cell transplantation. Neither of these therapies seems to be effective, as few patients are cured. To improve outcomes, there is a desperate need for new treatments specifically targeted to the genetic lesions that cause the disease. Also, more effective drug design requires an expanded view of the molecular complexity that underlies AML. To address this issue, we performed genome-wide splicing analysis in patients with AML and identified widespread alternative splicing changes in these patients compared with normal donors. Our results support that aberrant splicing is a common characteristic of AML. Our results also suggest that splice variant transcripts, which are results of aberrant splicing, can be used as selective disease markers. These markers provide potential targets for either therapeutic antibodies or for the delivery of cytotoxic drugs or toxins to leukemic cells.

Materials and Methods

Study design and patient cohort

This study includes a total of 228 samples from patients with AML; 193 samples were obtained from patients who were recruited at the Dana-Farber Cancer Institute (DFCI; Boston, MA) and 35 patients recruited at the University Hospital de Nantes (UHN; Nantes, France). From these samples, 66 were analyzed on the Affymetrix Human Exon 1.0ST Arrays (31 from DFCI and 35 from UHN). Of the 228 samples, 193 were used in validation studies and 29 in longitudinal study. All patient samples were taken at diagnosis after approval from the Institutional Review Boards, with written informed consent. Supplementary Table S1 describes demographic, clinical, and molecular characteristics of the patients with AML that were included in the exon array analysis; patient records were reviewed retrospectively to verify diagnosis and status. A control group of 12 normal donors of both genders was also included: 8 bone marrow samples of normal donors were obtained from AllCells (California) and 4 peripheral blood samples were obtained from healthy volunteers at the DFCI.

Tissue and cell preparation

All samples from patients and normal donors were purified using Ficoll–Hypaque gradient centrifugation. Peripheral blood mononuclear cells and/or bone marrow cells obtained from patients with AML were enriched using the EasySep Human Progenitor Cell Enrichment Kit, which is designed to isolate hematopoietic progenitor cells by negative selection (Stem Cell Technologies). From normal donor bone marrow samples, CD34+ cells were obtained using the EasySep Human CD34 Positive Selection Kit (Stem Cell Technologies). After cell selection, the presence of CD34+CD38− and CD34+CD38+ cell populations were evaluated by flow cytometry, which is routinely done in our laboratory.

Monocytes and neutrophils were isolated from four peripheral blood samples obtained from healthy volunteers using the EasySep Human Monocyte or Neutrophil Enrichment Kits (Stem Cell Technologies). Cells were resuspended in RLT lysis buffer or in TRIzol followed by the total RNA isolation procedure using an AllPrep RNA/DNA Isolation Kit (Qiagen) or TRIzol (Life Technologies) reagent according to the manufacturer’s recommendations. Patient samples included in the longitudinal studies were not enriched.

Microarray hybridization

Before the Affymetrix GeneChip Whole Transcript Sense Target Labeling assay, total RNA samples were evaluated on the 2100 Bioanalyzer (Agilent) using the RNA 6000 Nano Kit (Agilent). Subsequently, samples were hybridized to the Affymetrix Human Exon 1.0ST Arrays. All procedures were carried out at the DFCI Microarray core facility according to the recommended protocols. Hybridized arrays were scanned on an Affymetrix GeneChip Scanner 3000 and visually inspected for hybridization artifacts.

As a result, multiple proteins from a single gene. Pre-mRNA splicing is executed in the nucleus by spliceosomes (1, 3, 4). The efficiency of this process is controlled by classical trans- and cis-splicing elements (2, 5, 6). Most studies of alternative splicing in patients with AML have focused on individual genes, including AML1, Gfi1b, CD96, survivin-2B, AML-ETO, and GSK3-β (7–13). Studies that report splice variants of these genes also document the role of some of these variants in disease biology, whereas others can be used as prognostic or diagnostic biomarkers (13–20). Recently, frequent splicing machinery pathway mutations in myelodysplasia, AML, and chronic lymphocytic leukemia were identified (21–30). Despite novel discoveries, genome-wide alternative splicing has not been extensively examined in patients with AML.

In present study, we evaluated genome-wide alternative splicing events in patients with AML. Alternative splicing was found to be a common event for AML involving many genes in patients with AML. Genome-wide, we found aberrant patterns of splicing in patients with AML compared with bone marrow CD34+ cells from normal donors in up to 29% of the annotated genes (P < 0.05). Some of these aberrant splicing events are highly recurrent in patients and affect genes encoding many oncogenes and tumor suppressor genes, or genes involved in regulation of apoptosis, the cell cycle, and cell differentiation. Overall, our study identifies widespread splicing abnormalities in AML, which points to a disruption in the mechanisms regulating in the splicing process. In the future, determining the underlying causes and consequences of aberrant splicing in AML could enhance our understanding of disease pathogenesis. Certain of the more common aberrant splice variants may generate new targets for the development of novel therapeutics for AML.
Exon array analysis
Data from the exon array experiments were analyzed with the XRay software package. On the exon array, there are approximately four probes per exon and roughly 40 probes per gene. Multiple probes per exon enable "exon-level" analysis and identification of putative splice variants. For each gene, exon (probe set) expression was analyzed using mixed model ANOVA according to the linear model:
\[ Y = M + D + E + ED + EC + err \]
where "M" is a global mean of expression for a gene, "D" is the probe set expression detected in AML sample versus normal donors, "E" is the expression attributed to each exon, and "C" is hybridization effect (random factor). Genes with significant "D" (AML vs. normal donors) effect are said to show significant AML differential gene expression. However, genes with significant "ED" values are said to display significant AML versus normal donors differential splicing. Computation was performed such that, in general, positive ED scores represent cases of higher exon inclusion (or expression) in AML versus normal donors and the opposite is true for negative ED terms. All P and significance values were corrected for multiple test effects using the Benjamini and Hochberg false discovery rate method.

Reverse transcriptase polymerase chain reaction
Reverse transcriptase PCR (RT-PCR) and capillary electrophoresis and DNA fragment analysis were performed as previously described by Adamia and colleagues (31). The CD13 gene-specific transcripts were amplified from cDNA obtained through an RT reaction using a reverse (CTTAAAGCACAGCTCCCTGAAT) and forward (ATGATTTTGCAGAAGGTCCA) primer set designed for the CD13 transcript. After the PCR reactions, samples were either stored at −20°C for later analysis or immediately processed for agarose gel or capillary electrophoresis and DNA fragment analysis on the ABI 31/30XL DNA genetic analyzer.

Cloning and sequencing
cDNAs for cloning and sequencing were obtained from the blasts of eight patients with AML all expressing CD13 splice variants. The CD13 transcripts were amplified in PCR reaction containing platinum Taq High Fidelity, cloned into the pCR4 TOPO TA cloning system and transformed into Mach1-T1 competent cells according to the manufacturer’s instructions. Positive colonies containing CD13 plasmids were identified through testing individual bacterial colonies by colony PCR. Positive CD13-TOPO TA colonies were grown overnight in Luria Broth (LB) medium. Plasmids were prepared using the QIAprep Miniprep Kit (Qiagen). Then subclones from each cloned RT-PCR product from individual patients were sequenced, in both reverse and forward directions.

Flow cytometry
CD13 full-length and splice variant transcripts were engineered according to the splicing modes detected by cloning and sequencing analysis of AML patient samples. Plasmids were subcloned into pCMV6-AC-GFP (OriGene) and transfected into HEK293T cells using DMRIE-C transfection reagent according the manufacturer’s recommendations (Life Technologies). Alternatively, cells were transfected via nucleofection using the Cell Line Nucleofector Kit V (Lonza). Three days after transfection, HEK293T cells were stained with 0.5 μg anti-CD13 antibody (My7, Beckman Coulter), followed by staining with Alexa Fluor 647-labeled anti-mouse immunoglobulin G (IgG). As a control, we used cells stained with anti-rabbit IgG-Alexa fluor 647 only or untransfected HEK293T cells. Stained cells were analyzed on a FACScanto II.

Results

Genome-wide splicing profiles for patients with AML
The Affymetrix GeneChip Human Exon 1.0 ST array was used to identify global changes in splicing profiles associated with AML. Two independent AML cohorts were studied, which included the DFCI cohort of 31 patients and the UHN cohort of 35 patients, for a total of 66 samples. No significant differences were found between these two cohorts with respect to any common clinical characteristics other than hemoglobin levels (Supplementary Table S1A).

Splicing events associated with the DFCI patient cohort
We compared splicing events in the DFCI cohort with those detected in normal donors. After normalization and filtering procedures, using XRay software, 101,792 probes identifying 7,771 transcripts were scored as having above background expression levels in the DFCI cohort or in normal donors. This analysis identified 29% (2,251 of 7,771) transcripts differentially spliced in patients compared with normal donors, with a significance expression level of nominal P < 0.05.

Next, we performed annotation analysis through NetAffx Analysis Center (affymetrix.com) to identify transcriptome regions of the DFCI cohort that were affected by aberrant splicing. This analysis showed that the 2,251 differentially spliced transcripts detected in the DFCI cohort correspond to 8,648 probe sets. Of these, 6,573 (76%) mapped to known translated regions of the genome, 1,785 (21%) mapped to untranslated regions (introns, 5′UTRs or 3′UTRs), and 290 (3%) mapped to partial transcripts/cDNAs (Table 1).

Using Ingenuity software, we identified gene categories affected by aberrant splicing in the DFCI cohort. We determined that 2,201 of the 2,251 differentially spliced transcripts mapped to the annotated genes in public databases. Mapped genes were categorized on the basis of their location as well as the functions of proteins they encode. Of the differentially spliced genes, 41% encoded cytoplasmic proteins, 31% encoded nuclear proteins, and 11% encoded plasma membrane proteins. Results of these analyses are reported in Supplementary Table S2.

Splicing events associated with the UHN patient cohort
To ensure that the genome-wide splicing profiles identified in the DFCI cohort were reproducible, we used a "biologic repeats" approach for the exon array analyses. We performed identical exon array experiments for the independent UHN cohort. In this sample set, 91,673 probes
Aberrant RNA splicing patterns and their frequencies in patients with AML

Using annotation analysis, we observed three different patterns of splicing in patients with AML: Pattern 1—full or partial exon(s) skipping (sequence exclusions), pattern 2—full or partial cryptic exon or intron retentions (sequence inclusions), and pattern 3—various combinations of both sequence exclusions and inclusions (Fig. 1D). We evaluated the frequency of different patterns of gene splicing events in individual patients of the DFCI and UHN cohorts. We identified that collectively, in both cohorts, 486 transcripts were affected by full or partial exon skipping (pattern 1), whereas 1,148 transcripts were affected by full or partial intron retentions (pattern 1), and 2,531 transcripts were...
Reproducibility of the exon array results

29% (2,251) genes are differentially spliced ($P < 0.05$)

28% (2,004) genes are differentially spliced ($P < 0.05$)

Splicing patterns detected in AML patients

Pattern 1: sequence exclusion

Pattern 2: sequence inclusion

Table 2. Splicing event frequencies detected genome-wide in patients with AML

<table>
<thead>
<tr>
<th>Splicing events</th>
<th>DFCI cohort ($n = 31$)</th>
<th>UHN cohort ($n = 35$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average number of genes in each patient</td>
<td>350 of 2,251 (range 135–786)</td>
<td>506 of 2,004 (range 111–1152)</td>
</tr>
<tr>
<td>Any given gene is spliced in average number of patients</td>
<td>14 (range 1–29)</td>
<td>18 (range 1–34)</td>
</tr>
<tr>
<td>Splice variants previously reported</td>
<td>47% (1,062 of 2,251)</td>
<td>46% (922 of 2,004)</td>
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<tr>
<td>Novel splice variants</td>
<td>53% (1,189 of 2,251)</td>
<td>54% (1,082 of 2,004)</td>
</tr>
</tbody>
</table>

NOTE: Splicing event frequencies detected in the DFCI and UHN cohorts were evaluated as compared with normal donors ($P < 0.05$).
affected by patterns 1 and 2 in combinations. Also, on average, we identified seven transcripts per patient affected by pattern 1 splicing, 17 transcripts by pattern 2 splicing, and 38 transcripts were subjected to pattern 1 and 2 splicing in various combinations. Thus, complex combinations of splicing events are frequently detected in patients with AML.

Gene categories and pathways affected by aberrant splicing in AML

To identify deregulated pathways affected by aberrant splicing events in patients with AML, we performed a functional enrichment analysis using DAVID (the Database for Annotation, Visualization and Integrated Discovery). This enrichment analysis identified a number of signaling pathways with significance $P < 0.005$ affected by aberrant splicing (Table 3). In the DFCI cohort, we identified 26 signaling pathways affected by splicing events detected in patients with AML. Among the most affected pathways in AML are: FLT3, CD13, c-Kit, NOTCH, phosphoinositide 3-kinase, and mitogen-activated protein kinase (MAPK) signaling, pathways involved in regulation of cell cycle, apoptosis, cell transformation, and splicing (Table 3). Similar results were obtained from the analyses of significantly spliced genes in the UHN cohort. We detected 14 pathways affected by aberrant splicing in the UHN cohort, 12 of which were also detected in the DFCI cohort (Table 3). Also, for both cohorts for any given signaling pathway, this analysis identified multiple genes that were differentially spliced in AML as compared with normal donors (data not shown). These findings suggest that the effects of AML-specific splicing on signaling networks are widespread and that the splicing mechanism itself is compromised in AML.

Validation of genome-wide splicing studies in patients with AML

To demonstrate that the exon array data accurately detected transcripts with altered splicing, validation studies were performed on the CD13 gene encoding a myeloid cell

<table>
<thead>
<tr>
<th>Term-pathways</th>
<th>DFCI cohort</th>
<th>UHN cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$P &lt; 0.005$</td>
<td>Fold enrichment</td>
</tr>
<tr>
<td>Lysine degradation</td>
<td>6.69E-05</td>
<td>2.7</td>
</tr>
<tr>
<td>AML-FLT3 and c-Kit</td>
<td>1.40E-04</td>
<td>2.4</td>
</tr>
<tr>
<td>Chronic myeloid leukemia</td>
<td>1.82E-05</td>
<td>2.3</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>5.63E-06</td>
<td>2.3</td>
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<tr>
<td>Lysosome</td>
<td>9.66E-08</td>
<td>2.3</td>
</tr>
<tr>
<td>Aminoacyl-tRNA biosynthesis</td>
<td>3.38E-03</td>
<td>2.3</td>
</tr>
<tr>
<td>Notch signaling</td>
<td>1.84E-03</td>
<td>2.3</td>
</tr>
<tr>
<td>B-cell receptor</td>
<td>5.50E-05</td>
<td>2.2</td>
</tr>
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<td>Colorectal cancer</td>
<td>5.71E-04</td>
<td>2.1</td>
</tr>
<tr>
<td>Pancreatic cancer</td>
<td>4.74E-03</td>
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<td>Phosphatidylinositol signaling</td>
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<tr>
<td>AML-FLT3 and c-Kit</td>
<td>6.30E-05</td>
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</tr>
<tr>
<td>Endocytosis</td>
<td>6.38E-06</td>
<td>1.8</td>
</tr>
<tr>
<td>Leukocyte transendothelial migration</td>
<td>6.44E-04</td>
<td>1.8</td>
</tr>
<tr>
<td>FcγR-mediated phagocytosis</td>
<td>3.25E-03</td>
<td>1.8</td>
</tr>
<tr>
<td>Neurotrophin signaling</td>
<td>8.58E-05</td>
<td>1.7</td>
</tr>
<tr>
<td>Cell cycle</td>
<td>1.89E-03</td>
<td>1.7</td>
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<tr>
<td>Regulation of actin cytoskeleton</td>
<td>6.33E-05</td>
<td>1.5</td>
</tr>
<tr>
<td>Focal adhesion</td>
<td>3.00E-03</td>
<td>1.4</td>
</tr>
<tr>
<td>Spliceosome</td>
<td>4.96E-03</td>
<td>1.7</td>
</tr>
</tbody>
</table>

NOTE: Pathways in bold letters have been identified as enriched in both DFCI and UHN cohorts.
Aberrant Splicing in AML: Novel Markers and Potential Drug Targets

Figure 2. Exon array validation studies. A, CD13 full length (FL) and splice variant transcript amplifications for cloning and sequencing analyses. CD13 novel splice variant transcripts were amplified from patients with AML and normal donor samples. After cycling, RT-PCR products were subjected to 1% agarose gel electrophoresis, and then bands representing full length (FL) and splice variants were cut out and cloned and subjected to 1% agarose gel electrophoresis. Bands representing novel splice variant transcripts were amplified by RT-PCR, separated by agarose gel electrophoresis, and then cloned and sequenced (Fig. 2A and B). The analysis identified four CD13 splice variant transcripts: CD13Va, CD13Vb, CD13Vc, and CD13Vd (Fig. 2B). These CD13 novel splice variant transcripts were not detected in normal CD34+ cells, peripheral blood monocytes, or neutrophils (Fig. 2A). Sequence alignment analyses showed that CD13 splice variants are results of exon skipping, partial deletion of an exon, and/or partial retention of an intron (Fig. 2B).

Alignment followed by bioinformatic analysis showed that some of these splicing events did not cause any frame shifts (CD13Vd), whereas other combinations of aberrant splicing led to frame-shifted transcripts (e.g., CD13Va, CD13Vb, and CD13Vc). All the CD13 splice variant transcripts retained their original start codons and conserved signal sequences. Also, alignment analysis showed that exons affected by aberrant splicing events mapped to extra-cellular domains of the CD13 protein sequences (data not shown).

CD13 splice variant proteins

To understand whether novel splice variant transcripts are expressed as proteins, we engineered CD13-Va-GFP and CD13-Vb-GFP, transfected into HEK293T cells, and confirmed their protein expressions by flow cytometry. The HEK293T cells transfected with CD13-Va or CD13-Vb splice variant transcripts were harvested 72 hours after transfection and stained with anti-CD13-PE antibodies that detect cell surface CD13 proteins. The CD13-Va-GFP or CD13-Vb-GFP–positive HEK293T cells showed high expression levels of CD13 compared with the untransfected cells or transfected with plasmid backbone (Supplementary Fig. S1) providing preliminary evidence that CD13-Va and CD13-Vb splice variants encode the proteins. More detailed analysis of splice variants at the protein level and their functional characteristics are underway.

CD13 novel splice variant expression frequencies in a larger AML patient cohort

To identify which of the CD13 splice variants were most frequently expressed in patients with AML, we analyzed expression of these novel variant transcripts by highly sensitive semiquantitative RT-PCR capillary electrophoresis and DNA fragment analysis in the peripheral blood or bone marrow samples from 193 patients with AML, in CD34+ cells from bone marrow of eight normal donors, and in peripheral blood monocytes and neutrophils from four normal donors. Examples of this assay are shown in Supplementary Fig. S2.

The CD13 novel splice variants were expressed in the majority of patients with AML (64.3%, 124 of 193) but were not detectable in the normal donors (CD34+ bone marrow cells, peripheral blood monocytes, and neutrophils; Fig. 2A). Four CD13 novel splice variants were expressed in patients with AML in various combinations with CD13 full-length transcripts, or with other CD13 variants (Fig. 2A). Of the 193 patients, 124 patients expressed one or more splice variants in various combinations with CD13 full length (117 patients) or with other splice variants of this gene (three patients), whereas 47 patients did not express any CD13 transcripts (variants or full length) and 22 expressed only CD13 full-length transcript. Only four patients...
Figure 3. CD13 splice variants are commonly expressed in patients with AML and are associated with disease status. This figure displays overall expression patterns of CD13 full length (FL) and their splice variants transcripts. On the figure, the x-axes display 193 patient samples and the y-axes relative fluorescent units (RFU). PCR product RFU = \log_{2}RFU. 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 to 800 units as recommended by the manufacturer. All calculations and instrument calibration were done according to Applied Biosystems recommendations. A, CD13 and splice variant expression profiles in 193 patients with AML. B–D, display overall expression patterns of CD13 full length and novel splice variants transcripts over the course of the disease in 14 patients. Patient samples were collected and expression profiles of CD13 full length and novel splice variant transcripts were evaluated. B, at diagnosis (Dx) and remission (REM), C at relapse (REL), diagnosis (Dx), and during persistent disease (PER), D at diagnosis (Dx) and relapse (REL). A total 29 AML patient samples were collected including: ten samples obtained at diagnosis (Dx 1–Dx 3 and Dx 8–Dx 14); three samples obtained at remission (REM 1–REM 3); six samples obtained during regular visits over the course of refractory disease (PER 4, 5, 7); nine samples obtained at relapse (REL 6, REL 8–REL 14). Samples taken at first and second relapse or visits are marked as -1 or -2.
expressed CD13 variants alone (three patients express CD13-Va and one patient CD13-Vb). As Fig. 3A and Supplementary Table S3 show, expression of two or more splice variants in various combinations was the most common pattern in patients with AML.

We also evaluated the expression consistency of novel splice variant transcripts over the course of disease in individual patients with AML (Fig. 3B–D). CD13 full length and their splice variant transcripts were examined in paired samples of 14 patients; samples were taken at diagnosis and remission (three patients), over the course of refractory disease (four patients) or diagnosis and relapse (seven patients). A total of 29 samples were collected. This analysis showed specific expression patterns and levels of the novel splice variants at different time points during disease development (Fig. 3B–D). At diagnosis, the majority (>80%) of patients expressed CD13 splice variants in various combinations; at remission, expression frequencies of these splice variants were decreased; and at relapse, expression of all splice variants was dramatically increased at which point each patient expressed two or more CD13 variants in various combinations (Fig. 3C and D). Similarly, in a group of patients with persistent AML, novel splice variant expression frequencies remained constantly high (Fig. 3C).

Discussion

On the basis of genome-wide alternative splicing analyses of patients with AML and normal donors, in this manuscript, we report the presence of widespread splicing changes in patients with AML compared with normal donors. These splicing events were considered aberrant and specific to AML because they were not detected in CD34<sup>+</sup> bone marrow cells, monocytes, or neutrophils from normal donors. Thus, these events may provide potential markers for leukemic stem cells.

Because these AML-specific splicing abnormalities were found not only in the coding region, but also in the 5' and 3' UTR of the AML transcriptome, we argue that such widespread aberrant splicing in AML leads to altered expression of the genes, which may be necessary to modulate epigenetic regulatory "circuits" in this disease. Those aberrant splicing events that occur in the 3'UTR of the AML transcriptome will compromise miRNA-dependent gene regulation, whereas splicing events in the 5'UTR will change the composition of translational regulatory elements, which in turn can alter protein expression. Furthermore, aberrant splicing events in the coding regions will modulate a core part of the AML proteome and generate altered proteins. Some of these proteins might act in a dominant negative manner and compromise normal functions of their wild-type counterparts. Similar effects have been reported for MDM2, p53, CD44, STAT3, and other genes (32–36).

Similar to our findings, several related studies have reported widespread defects in alternative splicing in different types of cancer, including lung, breast, colon, prostate, and brain using the Affymetrix Human Exon 1.0ST Arrays (37–40). This well-adopted platform seems to be more useful than direct high-throughput sequencing using RNA-Seq when limited amounts of tissue are available from patients. Exon array analysis requires only 50 ng of total RNA, whereas RNA-Seq requires 1.5 μg RNA (41). Also, even though the dynamic range of RNA-Seq analysis is unparalleled, evaluation of the technical reproducibility of the exon array and RNA-Seq platforms showed that the coefficient of variation for the microarray is lower than that of RNA-Seq (41, 42). Furthermore, human exon array analysis tools are well standardized, short analysis times are achieved, and few computational resources are required as compared with RNA-seq; thus use of exon arrays for screening large patient cohorts can be more practical. In addition to identifying novel transcripts, RNA-Seq analysis detects single-nucleotide genetic variants in screened samples, which is one of the powerful options of this technology; however, the tools for RNA-Seq data analysis are not fully mature and capturing low abundance transcripts is compromised. Thus, microarrays remain a more useful and accurate platform for screening large number of patient samples at this point. More details about the pros and cons of microarrays and RNA-Seq have been reported recently (41, 42). The combined use of both technologies holds much promise to identify genome-wide disease-related aberrations.

Our study identified that a majority of the transcripts in patients with AML are subject to complex splicing events that include partial exon deletions and partial or complete intron retentions in various combinations. These events are detected on single transcripts and are highly recurrent as demonstrated for the CD13 gene in this manuscript, and for the NOTCH2 and FLT3 genes elsewhere, in 193 patients with AML. Similar patterns of aberrant splicing have been observed for oncogenes or genes associated with other malignant phenotypes; in these malignancies, splicing alterations produced novel transcripts encoding functional proteins (17, 19, 31, 43, 44).

Our results suggest that splicing is a global phenomenon in AML, raising the question as to whether modulated splicing in AML is a sporadic process or is organized/associated with the disease status. In longitudinal studies carried on limited number of AML patient samples, we noted significant differences with respect to expression frequencies of the splice variants at different time points of disease progression. Our findings suggest that splicing events are increased at diagnosis and relapse and "normalized" during remission. Extended studies are in progress to confirm our findings, which well allow us identification novel biomarker reflecting disease status, and consequently identify selective druggable targets.

Finally, the splicing aberrations we report in this manuscript might alter mechanisms of splicing via modulation of splicing factors and/or proteins. In this context, it is noteworthy that we identified novel splice variants of multiple splicing factors and proteins in our study. Detailed studies are underway to elucidate functional consequences of these aberrations in patients with AML. Overall our findings are consistent with published studies and underscore a key role...
for "cancer-specific" alternative splicing in pathogenesis in AML.

Disclosure of Potential Conflicts of Interest

J.D. Griffin has commercial research grant from Jansen Pharmaceuticals and Novartis Pharmaceuticals and is a consultant/advisory board member of Curr Pharmaceuticals and Novartis Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Adamia, B. Haibe-Kains, J.D. Griffin
Writing, review, and/or revision of the manuscript: S. Adamia, B. Haibe-Kains, P.M. Pilarski, S. Pevzner, S. Verselis, J. Burke, D.J. Deangelo, J. Quackenbush, R. Stone, J.D. Griffin

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Avet-Loiseau, L. Lode, E.A. Fox, M. Wadleigh, G. Motyckova, J.D. Griffin, S. Adamia

Study supervision: S. Adamia

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