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Transcriptome profiling of patient derived xenograft models established from pediatric acute myeloid leukemia patients confirm maintenance of FLT3-ITD mutation

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Improvements in survival have been achieved for children and adolescents with acute myeloid leukemia (AML), with the 5-year survival rates increasing from less than 20% to more than 70% [1]. However, in the past decade, outcome has not improved and remains dismal for high-risk patients. Activating mutations of FLT3 are the most common somatic mutations observed in AML, occurring in approximately 15% of childhood cases, and are associated with a high risk of relapse [2–4]. To facilitate better outcomes for pediatric AML, development and implementation of preclinical *in vivo* models that faithfully recapitulate the human disease are imperative to enhance the predictive power of novel therapeutics and treatment strategies.

Successful xenotransplantation of primary AML cells from both adult and pediatric patients into immunodeficient mice have been reported [5–8]. However, a recent report revealed an unexpected shift in the clonal architecture, in which a distinct clone, not necessarily the major clone, demonstrated greater engraftment potential and became the predominate clone *in vivo* [5]. Despite reports suggesting more favorable engraftment rates for primary adult AML samples harboring FLT3 mutations, including internal tandem duplications (ITD) [8], presence of FLT3 mutations did not confer preferential engraftment and were often absent in the engrafting population [5]. These observations have major consequences in the utility of patient-derived xenograft (PDX) models to evaluate novel therapeutics, especially in regard

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to targeted agents, and supports a need to genetically evaluate the engrafting population to ensure faithful modeling of each human primary sample. We therefore performed xenotransplantation of unmanipulated pediatric leukemia samples (N=10) to address a lack of established PDX models from pediatric patients with FLT3-ITD-positive AML. Further, we sought to compare transcriptome profiles of the primary patient sample and PDX samples and determine the engraftment integrity of FLT3-ITD-positive clones.

Clinical characteristics of the primary patient samples and engraftment results are summarized in Supplementary Table S1. In this study, we defined engraftment as >5% human CD45+ cells in the bone marrow, whereas others have defined engraftment as >0.1—0.3% human CD45+/CD33+ cells [8, 9]. Of 10 FLT3-ITD-positive pediatric patient samples, 7 successfully engrafted (30 of 50 total mice). We did not observe preferential engraftment of human primary blast samples obtained from bone marrow (4 of 6) versus peripheral blood (3 of 4). Consistent with previous reports [5, 8], we found most samples had little peripheral blood involvement especially during the monitoring period (Supplementary Figure S1). Though, in samples that successfully engrafted we did observe a sharp spike in detectable CD45+/CD33+ cells near time of sacrifice (range, 25.9 – 56.8% hCD45+; mean, 36%), whereas higher infiltration of the bone marrow was noted (range, 20.8 – 97.4%; mean, 75.4%; Supplementary Table S1). Leukemic engraftment in the spleen was found to be minimal (range, 4.3 – 46.5%; mean, 18.1%) compared to engraftment in bone marrow, and therefore excluded from further analysis. Lack of overt peripheral blood involvement emphasizes a challenge in using PDX models for preclinical efficacy studies in regard to monitoring of tumor burden. Therefore, future efforts should consider utilizing retroviral systems to label primary cells with luciferase which would permit monitoring of engraftment through non-invasive imaging or performing serial bone marrow aspirates; both approaches have advantages and disadvantages. In the absence of these tools, studies will require additional mice that can be sacrificed for periodic assessment of tumor burden in the bone marrow compartment.

We performed RNA-seq on four sets of xenograft models generated from four human leukemia samples (7_32 diagnosis [D], 7_32 relapse [R], 7_33 D, and 7_29 TKI resistant [R2]) from three distinct patients. FLT3-ITD mutations were conserved in all xenograft samples evaluated; similarly the NUP98-NSD1 fusion was maintained in all xenograft samples from two primary patient samples (7_33 and 7_29) (Table 1). We compared the mutational frequency of patient specific FLT3-ITD sequences in the primary patient sample to the xenograft samples by calculating the percentage of FLT3-ITD reads compared to total FLT3 reads using RNAseq data which had >100x coverage for FLT3-ITD with the exception of one primary patient sample (7_33 D) where exome sequencing data was used with coverage >150x. Remarkably, we found the FLT3-ITD mutational frequency was maintained among primary and xenografted samples (Table 1). Using MiSeq (average coverage 70,000x) we evaluated the presence and conservation of clinically relevant tyrosine kinase domain point mutations occurring in FLT3 exons 17 and 20 (e.g. at amino acid position D835 and F691L) (Supplementary Table S2); for mutation call at each FLT3 loci, a cut-off of 0.1% total reads was implemented. We detected emergence of D835H (31.78%) in one xenograft from a primary sample where the mutation was not detectable. A D835H mutation acquired in a tyrosine kinase inhibitor resistant primary sample (42.6%) was maintained in 2

of 3 xenograft samples (45.9% and 35.5%), but only a minor clone was observed in 1 sample (0.83%) (Table 1). Other studies focused on adult AML, have demonstrated frequent loss of FLT3-ITD in xenografts [5]. We speculate that the observed differences may be due, in part, to the higher frequency of FLT3-ITD-positive AMLs in pediatric versus adult patients, which may ultimately reflect a different underlying biology of pediatric and adult AML. Technical differences, such as the number of cells injected per mouse and irradiation, may have also contributed to these differences.

Next, we compared transcriptome profiles of the primary patient samples and respective xenografts. In these analyses, we use normalized RNA-seq expression values obtained by positive quantile transformation (PQT) [10] of fragments per kilobase mapped (FPKM); overall coverage 20x. The PQT of a gene in a sample represents the expression of that gene relative to all genes expressed in that sample. Thus, PQT=0 indicates a gene is unexpressed in this sample (FPKM = 0, i.e., no mapped reads), PQT=0.5 indicates that the FPKM for this gene is the median among genes with FPKM > 0 in this sample, and PQT=1 indicates that this gene is the most highly expressed feature for this sample. Not surprisingly, hierarchical clustering show the diagnosis and relapse primary samples from patient 7_32 cluster together. At the next level, the patient samples cluster with the xenograft samples (Figure 1). The NUP98-NSD1 positive patient samples 7_33 D and 7_29 R2 cluster together, with their respective xenograft samples (Figure 1). The structure of the dendrogram based on all genes was robust across a leave-one-sample-out cross-validation analysis; where the general structure of the dendrogram described was retained across the analyses. Additionally, the clustering was consistent based on the 50, 100, 500, and 1000 most variable genes or when all genes were considered (Figure 1 and Supplementary Figure S2).

For each set of xenografts, the vast majority of genes had similar expression in the xenografts as in the primary samples (Figure 1, Table 1). The results clearly indicate that most genes do not exhibit extensive differential expression between the xenografts and the primary samples (correlation coefficient >0.9 for all paired samples). Loss of expression in the xenografts was the most common category of expression alteration (expressed only in primary: PQT primary > 0, PQT xenograft = 0); this was observed in 1.6 to 5.1% of all genes (Table 1). However, half of the genes that were expressed in the primary and not in the xenografts had expression less than PQT=0.1, indicating low expression. Although we did find some genes to show extensive variation among each set of xenografts from individual primary samples, there was a limited number of genes that showed substantial differences in expression across all samples. Our results are similar to a previous report using microarray analyses which observed a small number of genes (<5%) that differed in expression between primary adult AML patient cells as compared to AML cells obtained after xenotransplantation [11].

Genes that were altered in all xenografts were considered 'consensus genes' and categorized based on expressed only in the primary or xenograft and increased or decreased in the xenograft samples (Supplementary Table S3 and S4). Among the consensus genes that were expressed exclusively in either the xenograft or primary samples we observed differential expression, albeit at very low PQT values, of long noncoding (lnc) RNAs, which have emerged as important regulators of gene expression [12] and have recently been reported to

have a prognostic impact in AML [13]. *RN7SL1*, was found to be equally expressed among all xenograft and primary samples; this was also the most highly expressed gene in every sample (PQT = 1 for all samples). *RN7SL1* is an RNA molecule that interacts with polypeptides to form the signal recognition particle, a cytoplasmic ribonucleoprotein complex that mediates insertion of secretory proteins into the lumen of the endoplasmic reticulum [14]. Differential gene-set analysis demonstrated several gene-sets or pathways related to immune system processes, cell surface makers, transport, signal transduction, and apoptosis to be significantly decreased among all xenograft samples; whereas, gene-sets related to metabolic and biosynthetic processes were significantly associated with an increased expression among all xenograft samples compared to the primary samples (Supplementary Table S5 and S6).

The establishment of PDX models that faithfully recapitulate human disease are a desirable tool in the preclinical evaluation of new chemotherapeutics, targeted agents, and novel combinatorial treatment strategies. Our data indicate that minimal transcriptome alterations occur in pediatric PDX models which is emphasized by maintenance of FLT3-ITD mutations at frequencies similar to the primary patient sample. As multiple passages of xenografts are established, alterations in expression should continually be evaluated to ensure retention of specific lesions, like FLT3-ITD, but also in regard to total genetic landscape. The persistence of this lesion has implications for the utility of PDX models in preclinical drug development and suggest PDXs established from pediatric FLT3-ITD-positive AML patients would be reliable in predicting response to novel targeted agents or combinations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Gene expression signatures of patient derived xenograft samples are comparable to primary pediatric FLT3-ITD-positive AML blasts

Heat map indicating expression levels of the 50 genes most variably expressed across the primary patient samples and their respective xenografts. Rows represent a gene and columns, individual samples. Samples are ordered based on hierarchical cluster analysis, illustrated by dendrogram at top. Expression values are represented by color: red, PQT value of 0 and is not expressed; yellow, PQT value of 1 and is highly expressed. Color bar at top indicates the patient samples and corresponding xenograft samples; red, 7_33 D; gold, 7_29 R2; teal, 7_32 D; blue, 7_32 R. D, diagnosis; R, relapse; R2, TKI resistant; X, xenograft

Table 1

Minimal transcriptome alterations and maintenance of FLT3-ITD mutation and NUP98-NSD1 fusions gene in xenograft samples

Patient ID	Sample ID	FLT3-ITD	FLT3-ITD reads (RNA-seq)	TKD status (frequency)	NUP98-NSD1	Gene expression category					
						1 # genes (% total)	2 # genes (% total)	3 # genes (% total)	4 # genes (% total)	5 # genes (% total)	6 # genes (% total)
7_32 Diagnosis	Primary	+	47.0	WT	-						
	EA4432	+	37.36	WT	NE						
	EA4433	+	35.04	WT	NE	1114 (3.5%)	1 (0.003%)	2 (0.006%)	6 (0.019%)	0 (0.0%)	0 (0.0%)
	EA4434	+	28.14	WT	NE						
	EA4435	+	42.86	D ₈₃₅ H (31.80%)	NE						
7_32 Relapse	Primary	+	32.0	WT	-						
	EA4438	+	44.05	WT	NE						
	EA4439	+	47.54	WT	NE	1066 (3.4%)	0 (0.0%)	0 (0.0%)	1 (0.003%)	0 (0.0%)	0 (0.0%)
	EA4442	+	46.03	WT	NE						
7_33 Diagnosis	Primary	+	36.0*	WT	+						
	EA4444	+	22.76	WT	+	1620 (5.1%)	0 (0.0%)	0 (0.0%)	2 (0.006%)	0 (0.0%)	0 (0.0%)
	EA4447	+	30.65	WT	+						
7_29 TKI resistant	Primary	+	27.0	D ₈₃₅ H (44.0%)	+						
	EA4458	+	21.18	D ₈₃₅ H (46.89%)	+						
	EA4462	+	31.91	D ₈₃₅ H (0.83%)	+	500 (1.6%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
	EA4463	+	23.24	D ₈₃₅ H (35.30%)	+						
Consensus genes						8 (0.03%)	96 (0.3%)	2501 (7.9%)	1340 (4.2%)	23608 (74.7%)	4052 (12.8%)

NE, not evaluated; TKD, tyrosine kinase domain; TKI tyrosine kinase inhibitor;

* , % FLT3-ITD reads based on exome sequencing;

Consensus genes, genes that were altered in all xenografts. Gene expression category: 1, Expressed only in primary; PQT primary > 0, PQT xeno = 0; 2, Expressed only in xenograft(s); PQT primary = 0, PQT xeno > 0; 3, Expression decreased in xenograft(s); PQT primary > mean PQT xeno; 4, Expression increased in xenograft(s); PQT primary < mean PQT xeno; 5, Expression in primary equals expression in xenograft(s); PQT primary = mean PQT xeno; 6, Unexpressed in both primary and xenograft(s); PQT primary = 0, mean PQT xeno = 0