Pharmacological targeting of β-catenin in normal karyotype acute myeloid leukemia blasts

Over 11,000 people in the US are diagnosed with acute myeloid leukemia (AML) each year, and despite attempts to improve therapy, the overall 5-year survival remains 26%, largely due to a high rate of relapse. Defining the mechanisms responsible for persistence of the malignant clone is essential to improve outcome in AML.

Activation of WNT signaling pathways through nuclear localization of β-catenin has been linked to development and progression of AML.[2,3] It is well known that nuclear translocation of β-catenin in this manner induces target gene transcription through physical interaction with members of the TCF/LEF family of transcription factors. While there are conflicting data on the role of WNT signaling in normal hematopoietic stem cell self-renewal, mouse models have demonstrated that β-catenin is critical for AML stem cell self-renewal.[2-4] These data, combined with the clinical observation that increased canonical WNT signaling in AML blasts at diagnosis is associated with decreased rates of relapse-free and overall survival, suggested a potential clinical benefit for WNT suppression.[5,6] While this approach has shown promise in the treatment of AML,

Table 1. Clinical characteristics of samples from patients with normal karyotype AML.

Sample ID	Age	WBC (x 10^9/mL) (diagnosis)	BM Blast % (diagnosis)	Karyotype/ mutation status (diagnosis)	WBC (x 10^9/mL) (relapse)	BM Blast % (relapse)	Karyotype/ mutation status (relapse)
1	67	11.2	80	46, XY NPM1 Mut	14.40	66.5	NK FLT3-ITD
2	62	40.99	91.5	46, XX NPM1 Mut	1.31	70.5	NK
3	71	17.6	82.5	46, XY NPM1 Mut	NA	NA	NA
4	71	3.37	33	46, XY NPM1 Mut	163.7	83.5	t(7;14)(q36;q11.2){2} t(7;15)(q22;q22),add(22)(q13){2} FLT3-ITD
5	83	42.61	81	46, XX NPM1 Mut FLT3-ITD	4	11	NK
6	57	29.05	73.5	46, XY NPM1 Mut	NA	NA	NA
7	54	19.57	47	46, XX	NA	NA	NA
8	72	62.71	93	46, XX NPM1 Mut	NA	NA	NA
9	81	50.46	87.5	46, XY NPM1 Mut	NA	NA	NA
10	82	45.78	95.5	46, XY NPM1 Mut	NA	NA	NA
11	40	55.17	73.6	46, XX NPM1 Mut	NA	NA	NA
12	61	32	74	46, XX NPM1 Mut	2.41	56.5	ND
13	79	122.8	52.5	46, XX NPM1 Mut	NA	NA	NA
14	65	117.3	31	46, XY	NA	NA	NA
15	58	55.33	96	46, XX	3.71	23	47,48,XX,+12mar{cp3}
16	43	216	50.4	46, XY NPM1 Mut FLT3-ITD	NA	NA	NA
17	25	555	90.6	46, XY	NA	NA	NA
18	72	33.04	43.5	46, XY	NA	NA	NA
19	80	17.89	39.8	46, XX	3.87	49	NK
20	65	39.16	15.4	46, XY	NA	NA	NA
21	86	5.19	92	46, XY	NA	NA	NA

Samples are negative for NPM1 and FLT3-ITD except where noted as positive. Sample ID numbers correspond to those values shown in Figure 1. Characteristics of samples from patients who relapsed and who were available for analysis in Figure 1 are also shown. BM: bone marrow; NA: sample not available for analysis (either due to unavailability of sample or that the patient did not relapse); ND: not done.

questions remain as to which subtypes of AML will benefit.[7,8] In this study, we characterize activation of the canonical Wnt pathway in a cohort of patients with AML and found that while a majority of patients at diagnosis showed predominantly nuclear β-catenin, treatment of primary samples with a next generation WNT inhibitor had variable effects on blast survival and phenotype. Together, these data highlight the inherent challenges in targeting the WNT pathway in AML and emphasize that this strategy will require identification of subtypes of AML, or even individual patients, that will most benefit from this approach.

To identify such patients, we utilized multispectral imaging flow cytometry technology to quantify nuclear localization of β-catenin, the biochemical hallmark of WNT pathway activation. Using this technique, we determined nuclear β-catenin in AML blasts obtained at diagnosis from

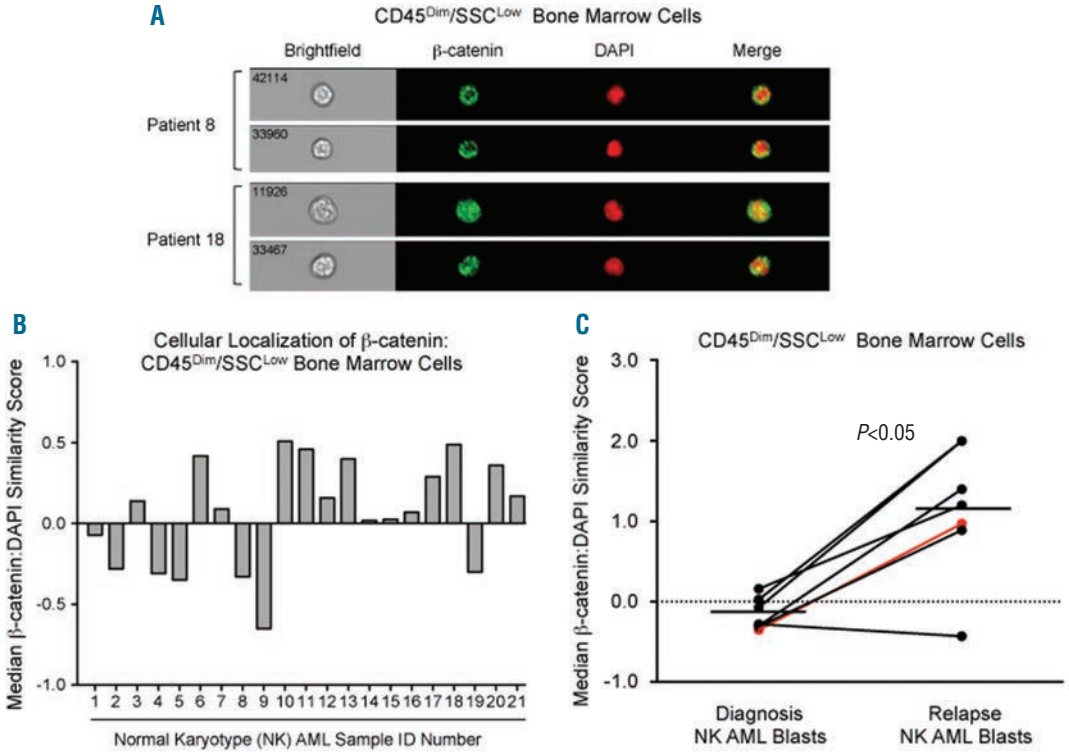

Figure 1. Analysis of nuclear β-catenin in primary AML samples. (A) Representative images obtained using an ImageStream imaging flow cytometer; primary AML samples from Patient 18 (top) and 8 (bottom). Images shown are brightfield, β-catenin (green), DAPI (red) and the merged β-catenin and DAPI images, and are representative of the median β-catenin similarity score for Patient 18 (0.49) and Patient 8 (-0.33), respectively. (B) Median β-catenin: DAPI similarity scores in CD45^{Dim}, Side Scatter (SSC)^{Low} bone marrow cells harvested from 21 patients with normal karyotype (NK) AML. (C) Dot plot of median β-catenin similarity scores of paired AML blasts harvested at diagnosis (left) *versus* relapse (right). Individual paired samples are connected by lines. *P* value calculated using Wilcoxon matched-pairs signed rank test.

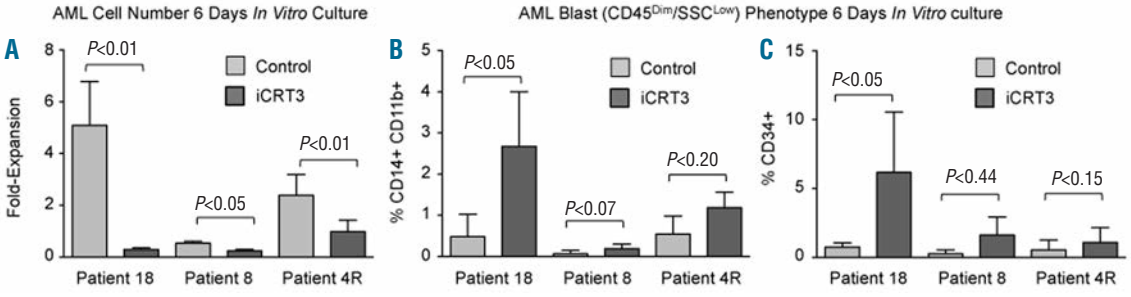

Figure 2. Pharmacological inhibition of WNT signaling in primary AML samples. (A) Average fold expansion of cell number of AML samples (n=4 biological replicates for each sample) following six days of treatment with vehicle control (light gray bar) *versus* 25 μM iCRT3 (dark gray bar). *In vitro* cultures of AML samples were performed as described in the Methods section. (B) Average percentage of CD14^{+}/CD11b^{+} AML blasts following culture with vehicle control *versus* iCRT3. (C) Average percentage of CD34^{+} AML blasts following culture with vehicle control *versus* iCRT3. For all panels, error bars represent standard deviation and *P* values were calculated using paired *t*-test.

a cohort of 21 patients exhibiting normal karyotype (Table 1). We selected this population since 50% of patients exhibit a normal karyotype, representing the largest group of patients with AML.[1] Ficoll-separated, unfractionated bone marrow mononuclear cells were obtained from patients with AML through the tissue repository at Roswell Park Cancer Institute (RPCI) under an IRB-approved protocol. To prepare the patient samples for analysis, they were first incubated with LIVE/DEAD Far Red stain according to the manufacturer's instructions (Invitrogen). Cells were stained with mouse anti-human CD45 (phycoerythrin (PE)-conjugated, clone HI30) and CD34 (allophycocyanin (APC) conjugated, clone 4H11, (eBioscience, San Diego, CA, USA) followed by fixation with 2% methanol-free paraformaldehyde (Polysciences, Inc., Warrington, PA, USA). Cells were permeabilized with 0.1% Triton-X and stained with 0.5 μg monoclonal mouse anti-β-catenin antibody (AlexaFluor 488-conjugated, clone L54E2, Cell Signaling Technology, Danvers, MA, USA) in 0.1% Triton-X for 30 min on ice. Prior to analysis on an ImageStream X (Amnis Corporation, Seattle, WA, USA), DAPI (Life Technologies, Grand Island, NY, USA) was added to the cells.

For all samples, single cells were identified for analysis based on focus and the blast gate was defined based on the $CD45^{Dim}$/side $scatter^{Low}$ (SSC^{Low}) immunophenotype. Among our patients, we observed blasts that exhibited predominantly cytoplasmic or nuclear β-catenin (Figure 1A). To quantify cellular distribution of β-catenin, we used the IDEAS v.6.0 software package (Amnis). Nuclear β-catenin was quantified through calculation of a similarity score, a log-transformed Pearson correlation coefficient between the pixel values of β-catenin and DAPI images.[9] A higher score indicates increased nuclear localization of β-catenin. Overall, we observed that 14 of 21 samples exhibited a median similarity score over 0, indicating that in these diagnostic samples the median cellular localization of β-catenin was biased towards the nucleus (Figure 1B).

Since previous studies have demonstrated a correlative association between nuclear β-catenin levels at the time of original diagnosis and increased risk of relapse,[5] we also quantified β-catenin localization in 7 paired samples obtained at diagnosis and at relapse (Figure 1C). In this selected cohort, we observed a significant increase in the β-catenin similarity score in relapsed samples compared to their paired diagnostic sample. With the caveat that this cohort is limited in terms of numbers, localization of β-catenin to the nucleus appeared to be greater in AML blasts at relapse compared to blasts at diagnosis.

Among the documented consequences of β-catenin activation in myeloid leukemia are decreased apoptosis and differentiation and increased proliferation.[10,11] To test whether inhibiting WNT signaling affected survival or differentiation of primary AML blasts with different levels of nuclear β-catenin, we used a compound (iCRT3) that inhibits WNT signaling by blocking the interaction between β-catenin and TCF family transcription factors.[12] We tested the effects on 3 different samples; a diagnostic sample from Patient 18 (relatively high levels of nuclear β-catenin); a diagnostic sample from Patient 8 (predominantly cytoplasmic localization of β-catenin); and the relapse sample from Patient 4 (termed 4R), which exhibited increased nuclear β-catenin compared to its diagnostic sample.

AML samples were co-cultured with irradiated HS-27 feeder cells supplemented with human hematopoietic growth factors, as previously described.[13] For all 3 samples, treatment with iCRT3 resulted in a significant decrease in the expansion of viable cells compared to control ($P<0.05$) (Figure 2A). We then analyzed the immunophenotype of surviving blast cells ($CD45^{Dim}SSC^{Low}$) using standard multi-spectral flow cytometry. Treatment of blasts from Patient 18 with iCRT3 significantly increased the percentage of blasts expressing the mature CD14 and CD11b markers compared to vehicle control ($P<0.05$) (Figure 2B). Interestingly, treatment of this sample with iCRT3 also increased the percentage of $CD34^+$, $CD38^+$ blasts compared to control ($P<0.05$) (Figure 2C). Thus, while inhibition of WNT signaling may increase differentiation of some blasts, there may be other populations that are resistant to or even stimulated by blockade of the pathway. In contrast, neither the diagnostic sample with low levels of nuclear β-catenin or the relapse sample with high β-catenin exhibited any changes in blast immunophenotype after culturing with iCRT3 compared to vehicle.

In total, these observations that β-catenin has variable effects on AML blast immunophenotype and number support those of Gandillet *et al.*[7] While base-line nuclear β-catenin levels in primary AML blasts may not correlate with response to pharmacological inhibition of this pathway, samples with low base-line levels still responded to therapy, as shown by significantly decreased cell numbers. This suggests this approach may be more widely applicable than previously thought. Further separation of patients with normal karyotype AML based on mutation status or even age may refine the definition of the ideal target population. Sample availability precluded testing paired samples from diagnosis *versus* relapse to determine the relative efficacy of inhibiting β-catenin. However, a critical experiment will be to compare the relative sensitivity to β-catenin inhibition between paired samples obtained at diagnosis *versus* relapse. In addition, it is possible that these data are specific to the iCRT3 inhibitor, which targets the interaction between β-catenin and TCF/LEF proteins. Other WNT signaling inhibitors, such as XAV939 and C59, which target the pathway at alternative points (by enhancing degradation of β-catenin or reducing WNT ligand activity respectively) could produce different effects.[14,15]

In summary, our studies using a next generation pharmacological agent to target WNT signaling in primary AML show the clinical potential of targeting this pathway while confirming previous studies demonstrating that blockade of WNT signaling will likely have variable results.

Thus, these findings highlight the fact that additional work is necessary to identify those clinical subsets of AML patients most susceptible to anti-WNT therapy.

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Acknowledgments: the authors thank Brandon Martens and Linda Lutgen-Dunckley from the Leukemia Procurement Bank at Roswell Park Cancer Institute for providing primary AML samples and Dr. Hans Mindermann, Dr. Orla Maguire, and Kieran O'Loughlin from the Flow Cytometry Resource for assistance with imaging flow cytometry.

Funding: this work was supported by the Louis Sklarow Foundation (to EAG), Alliance Developmental Awards from the Roswell Park

Alliance Foundation (to EAG and MJN), the Jacqueline Hirsch Leukemia Research Fund (to ESW), the Cancer Clinical Investigator Team Leadership Award (CCITLA) awarded by the National Cancer Institute through a supplement to P30CA016056 (to ESW), the Szefel Foundation (MW), the Leonard S. LuVullo Endowment for Leukemia Research (MW), the Nancy C. Cully Endowment for Leukemia Research (MW), the Babcock Family Endowment (MW), the Heidi Leukemia Research Fund (MW) and NCI Cancer Center Support Grant CA016056.

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doi:10.3324/haematol.2014.113118

Key words: WNT signaling, acute myeloid leukemia, β-catenin, WNT inhibitors.

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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