Published in final edited form as:
Leuk Lymphoma. 2010 September ; 51(9): 1711–1719. doi:10.3109/10428194.2010.496505.

Acute myeloid leukemia is characterized by Wnt pathway inhibitor promoter hypermethylation

ELIZABETH A. GRIFFITHS[1], **STEVEN D. GORE**[1], **CRAIG M. HOOKER**[1], **MICHAEL A. MCDEVITT**[2], **JUDITH E. KARP**[1], **B. DOUGLAS SMITH**[1], **HELAI P. MOHAMMAD**[1], **YING YE**[1], **JAMES G. HERMAN**[1], and **HETTY E. CARRAWAY**[1]

[1]Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, MD, USA

[2]Hematology Division, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Abstract

Nuclear localization of non-phosphorylated, active β-catenin is a measure of Wnt pathway activation and is associated with adverse outcome in patients with acute myeloid leukemia (AML). While genetic alterations of the Wnt pathway are infrequent in AML, inhibitors of this pathway are silenced by promoter methylation in other malignanices. Leukemia cell lines were examined for Wnt pathway inhibitor methylation and total β-catenin levels, and had frequent methylation of Wnt inhibitors and upregulated β-catenin by Western blot and immunofluorescence. One hundred sixty-nine AML samples were examined for methylation of Wnt inhibitor genes. Diagnostic samples from 72 patients with normal cytogenetics who received standard high-dose induction chemotherapy were evaluated for associations between methylation and event-free or overall survival. Extensive methylation of Wnt pathway inhibitor genes was observed in cell lines, and 89% of primary AML samples had at least one methylated gene: *DKK1* (16%), *DKK3* (8%), *RUNX3* (27%), *sFRP1* (34%), *sFRP2* (66%), *sFRP4* (9%), *sFRP5* (54%), *SOX17* (29%), and *WIF1* (32%). In contrast to epithelial tumors, methylation of *APC* (2%) and *RASSF1A* (0%) was rare. In patients with AML with normal cytogenetics, *sFRP2* and *sFRP5* methylation at the time of diagnosis was associated with an increased risk of relapse, and *sFRP2* methylation was associated with an increased risk for death. In patients with AML: (a) there is a high frequency of Wnt pathway inhibitor methylation; (b) Wnt pathway inhibitor methylation is distinct from that observed in epithelial malignancies; and (c) methylation of *sFRP2* and *sFRP5* may predict adverse clinical outcome in patients with normal karyotype AML.

Correspondence: Hetty E. Carraway, MD, MBA, Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, 1650 Orleans Street, Cancer Research Building 1, Room 290, Baltimore, Maryland 21231, USA. Tel: 410-502-5847. Fax: 410-614-3809. hcarraw1@jhmi.edu.

Declaration of interest: This work was supported by The Flight Attendant Medical Research Institute (FAMRI), grant number 032053. This work was also supported by a NCI Cancer Center Support Grant (CA06793), which helped the Specimen Acquisition Core (SAC) Laboratory with sample collection and storage.
Dr. James Herman is a consultant to and receives research support from OncoMethylome Sciences. The terms of this arrangement are being managed by the Johns Hopkins University in accordance with its conflict of interest policies.

Keywords

Cytogenetics; DNA methylation; epigenetics; leukemia; Wnt pathway

Introduction

Acute myeloid leukemia (AML) is characterized by the clonal proliferation of hematopoietic stem or progenitor cells and results in a failure of normal hematopoiesis [1]. Identification of cytogenetic and molecular events has provided insight into the pathogenesis of AML [2]. It is now understood that leukemic cells as a group are marked by the unique properties of self-renewal, resistance to apoptosis, and blocked differentiation, mediated in some cases by aberrant fusion proteins [PML–RARα, AML1–ETO, inv(16), t(1:14)] and in others by inappropriate activation of proliferation signals via mutation (*FLT3–ITD*, *NPM1*) [3]. Although these abnormalities are observed in many cases of AML, individual gene mutations have usually not been sufficient to result in the malignant phenotype in experimental models, suggesting that additional molecular abnormalities contribute to this process [4,5].

Recently, a critical role in adult stem cell biology has been identified for the Wnt pathway, an evolutionarily highly conserved signaling cascade critical for normal embryogenesis and hematopoiesis [6,7]. Wnt inhibitor proteins are lipid-modified glycoproteins that can bind cell surface receptors or interact with the pathway intracellularly [8,9]. They function through both canonical (β-catenin mediated) and non-canonical (Wnt Ca^{++} pathway, Wnt planar cell polarity pathway) signaling. These pathways are critical for cell fate determination, proliferation, migration, polarity, and gene expression [9–11].

Epigenetic abnormalities in Wnt pathway signaling are critical for the initiation of a variety of epithelial cancers, and it has been demonstrated that abnormalities of this pathway are common in hematopoietic malignancy [11–13]. The downstream effector of canonical Wnt signaling is nuclear non-phosphorylated β-catenin, which acts in association with the LEF/TCF (lymphoid enhancer factor/T-cell factor) family of transcription factors to mediate expression of several genes including *cyclin D1* and *c-Myc* [11]. In the absence of Wnt signaling, cytoplasmic levels of β-catenin remain low as a result of degradation of the phosphorylated form of the protein, which is targeted for ubiquitination and destruction [14]. In the presence of Wnt pathway activation, phosphorylation and degradation of β-catenin is inhibited, resulting in its accumulation in the cytoplasm and transport into the nucleus [14]. In normal cells, Wnt signaling and β-catenin localization are tightly controlled by a number of secreted and intracellular inhibitory proteins including Dickkopf 1, 2 (*DKK1*, *2*), serine/threonine kinase 11 (*LKB1*), Ras association (*RalGDS/AF-6*) domain family member 1 (*RASS-F1A*), runt-related transcription factor 3 (*RUNX3*), secreted frizzled related proteins 1, 2, 4, 5 (*sFRP1*, *2*, *4*, *5*), SRY-box containing gene 17 (*SOX17*), and WNT inhibitory factor 1 (*WIF1*) [11,15].

In AML, abnormal nuclear localization of non-phosphorylated β-catenin has been demonstrated both *in vitro* and in patient samples, and there is compelling evidence for the importance of Wnt pathway up-regulation in this disease [16–20]. Studies have shown a

correlation between nuclear β-catenin expression and survival in patients with myelodysplastic syndrome (MDS) and AML [19,21]. In addition, activation of this pathway been shown to interact with one of the most commonly recognized molecular events, the *FLT3–ITD* mutation, in AML [22]. Despite this convincing evidence for Wnt pathway dysregulation in myeloid malignancy, genetic mutations frequently observed in epithelial malignancy (involving *β-catenin* or *APC* [adenomatous polyposis coli] as seen in patients with colon cancer) have not been seen in AML, suggesting another mechanism of pathway activation.

Wnt pathway inhibitors possess CpG islands within their promoter regions, and methylation of these genes can contribute to the pathogenesis of cancers, including those of the lung, colon, and breast [15,23,24]. Additionally, it has been shown that methylation of six of the Wnt inhibitory genes, including *sFRP1*, *2*, *4*, *5* and *DKK1*, *3*, occurs in AML, and is associated with adverse prognosis within a subset of newly diagnosed, young patients with intermediate-risk karyotype AML [25]. Because of the role of the Wnt pathway in AML, we wanted to explore the association of the methylation of Wnt pathway inhibitors and clinical prognosis among patients with normal karyotype. Methylation was assessed by methylation-specific polymerase chain reaction (MSP), a technique which has previously been demonstrated as a reliable means of detecting promoter region methylation associated with alterations in gene expression [15]. Promoter methylation was evaluated for a number of Wnt pathway inhibitors including *APC*, *DKK1*, *DKK3*, *LKB1/STK11*, *RASSF1A*, *RUNX3*, *sFRP1*, *sFRP2*, *sFRP4*, *sFRP5*, *SOX17*, and *WIF1* in cell lines known to be without mutations in *APC* or *β-catenin* as well as samples from patients with primary AML.

Design and methods

Cell lines

Cell lines (HL60, K562, KG1, HNT34, KG1a, U937, and HCT116) were purchased from the American Type Culture Collection and grown in recommended media (ATCC, Manassas, VA). Pelleted cells from each line were used for protein analysis, and DNA was extracted and bisulfite-treated for analysis as described below. Freshly isolated cells were incubated with polylysine-coated glass slides and used for immunofluorescence as described below.

Patient samples

Freshly frozen bone marrow or peripheral blood tissue samples collected from 169 patients with a diagnosis of AML and one normal allogeneic bone marrow transplant donor were obtained from the Johns Hopkins Hospital Specimen Acquisition Laboratory. All leukemia samples contained at least 50% blast cells as determined by clinical flow cytometry. Of this cohort, 72 patients had normal cytogenetics. This clinical data set was enriched to include a greater number of samples from patients with AML with normal cytogenetics, since novel prognostic variables would be the most valuable in this group. The clinical characteristics known to be associated with prognosis were obtained: age, date of diagnosis, white blood cell (WBC) count at diagnosis, cytogenetics by karyotype and fluorescence *in situ* hybridization (FISH) analysis, and induction (cytarabine based) therapy. Table I details

demographic information for these patients. In addition, we collected clinical outcome information on all-cause mortality and disease-free status. A series of 54 stage I ductal breast adenocarcinoma samples procured for a previous study [26] were used as a basis for comparison between the Wnt inhibitor methylation profile of epithelial versus hematological malignancies. In accordance with HIPAA regulations (Health Insurance Portability and Accountability Act), all clinical samples were obtained as part of a protocol approved by the Institutional Review Board (IRB) at the Johns Hopkins Hospital. All patients signed informed consent according to Health and Human Services guidelines and the Declaration of Helsinki.

Protein extraction/Western blotting

Thirty milliliters of logarithmically growing cell lines were centrifuged and excess media removed. $CD34^+$ cells from a normal donor were thawed and assessed for viability using Trypan blue and then pelleted. The cell pellets were washed in 1 mL of phosphate buffered saline (PBS) containing 1 μL each of Halt^{TM} Phosphatase Inhibitor (Thermo Scientific, Rockford IL), AEBSF [4-(2-aminoethyl)benzenesul-fonylfluoride; EnzoLife Sciences, Plymouth Meeting PA], and Complete^{TM} Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN). Cells were incubated with standard radioimmunoprecipitation assay (RIPA) buffer on ice for 30 min with vortexing every 10 min. Cells were then lysed by passage through a QIAshredder Column (Qiagen, Valencia, CA), and the lysate incubated on ice for a further 30 min with vortexing every 10 min. Total protein extracts were run on a gel, transferred to a membrane, and probed for the presence of β-catenin using mouse anti-β-catenin antibody (BD Biosciences, San Jose, CA). Mouse anti-β-actin was used as a loading control.

Immunofluorescence

Cells were immunostained with mouse anti-β-catenin antibody (BD Biosciences, San Jose, CA) as previously described, and DAPI (4′,6-diamidino-2-phenylindole) was used as a nuclear counter-stain [27]. Images were acquired using a Nikon E800 microscope equipped with ×63 objective.

DNA extraction

Normal lymphocytes were isolated from freshly frozen bone marrow or peripheral blood samples. These samples were mixed with an equal volume of lysis buffer (50 mM Tris, 50 mM ethylenediamine-tetraacetic acid [EDTA], 2% sodium dodecyl sulfate, 10 mg/mL proteinase K [Invitrogen, Carlsbad, CA]) and incubated overnight at 55°C. One half volume of lysis buffer was added daily until the sample had been completely digested. Once digestion was complete, DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1), ethanol-precipitated, and re-suspended in 50–500 μL of double-distilled water (ddH_2O). DNA was quantified using the Nano-Drop® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE).

Bisulfite treatment

Bisulfite modification of 1–2 μg of DNA per sample was performed using the EZ DNA Methylation Kit™ (Zymo Research) as per the manufacturer's instructions. Bisulfite-treated DNA was resuspended in 40 μL of ddH_2O.

Methylation-specific polymerase chain reaction

Methylation-specific polymerase chain reaction primers for *APC*, *DKK1*, *DKK3*, *LKB1*, *RASSF1A*, *RUNX3*, *sFRP1*, *sFRP2*, *sFRP4*, *sFRP5*, *SOX-17*, and *WIF1* have been published previously [15,28–30], and were purchased from Integrated DNA Technologies (Coralville, IA). All primers appear in Supplemental Table I. MSP was carried out in 25 μL reaction volumes containing 10 ×MSP buffer, 10 mM deoxynucleoside triphosphates, 0.5 μL of a 5 mM solution of each of the methylated or unmethylated primers, 0.5 μL of JumpStart™ REDtaq® DNA polymerase (Sigma-Aldrich, St. Louis, MO), and 2–4 μL of bisulfite-treated DNA [31]. DNA was amplified with the following protocol: 95°C for 5 min, followed by 35 cycles of 95 C for 30 s, 60 C for 30 s, 72 C for 30 s, and a final extension step of 72 C for 5 min. *In vitro* methylated DNA (IVD) was created by treating human cell-line DNA with SSI-1 methylase (New England Biolabs, Ipswich, MA) as directed, and used as a positive control following bisulfite treatment as described above. Normal lymphocytes collected from multiple healthy donors under an IRB approved protocol were used as a negative control. Seven-and-a-half micro-liters of each amplification product were resolved on 2% agarose gel containing GelStar® Nucleic Acid Gel Stain (Lonza, Rockland, ME) and visualized under ultraviolet light (UV).

Statistical analysis

Associations between individual gene markers and molecular factors such as *NPM1* mutations, *FLT3–ITD* mutations, antecedent disease status, and WBC count at diagnosis were assessed by means of logistic regression analysis. The primary end-points were time to all-cause mortality and time to relapse, measured from the date of diagnosis to death or relapse, respectively. Subjects were censored if no event was observed at the date of last clinic visit. Time to all-cause mortality and relapse was assessed using the Kaplan–Meier method. Differences between Kaplan–Meier survival curves were estimated using the log-rank test. Univariate and multivariable Cox proportional hazards regression models were utilized to estimate the independent effect of DNA methylation on mortality and relapse, respectively. The covariates included in the multivariable regression model were age at diagnosis (continuous), total WBC count at diagnosis (greater or less than 50 000/μL), antecedent cytopenias, *NPM1* mutations, and *FLT3–ITD* status. Results of all models are reported as hazard ratios with corresponding 95% confidence intervals. All statistical calculations were performed with use of the STATA 10 statistical package (College Station, TX). Two-sided *p*-values of less than 0.05 were considered to indicate statistical significance.

Results

Patient characteristics

Samples from a total of 169 patients with AML were studied, of which 106 had normal cytogenetics. Table I details demographic information for the patients. The median age was 61 years for both groups. Of the normal cytogenetics samples, 34 were procured at relapse and 72 were procured from patients at initial diagnosis. These 72 samples (from patients at initial AML diagnosis) were annotated for treatment received. All 72 patients received cytarabine-based induction therapy; the majority received combination therapy with cytarabine and daunorubicin. Of the 72 patients, there were 39 with *de novo* AML (four received FLAM [fludarabine, cytarabine, and mitoxantrone] [32], three received the Linker regimen [33], six received traditional '7 plus 3,' and 26 received ACDVP16 [cytarabine, daunorubicin and etoposide] [34,35]) and 33 patients with an antecedent MDS (five received FLAM and 28 received ACDVP16). None of the patients had received epigenetic therapy with 5-azacytidine or deoxy-azacytidine prior to sample analysis. These 72 patients treated with cytarabine-based high-dose induction regimens were assessed for event-free and overall survival and outcomes were correlated with methylation of individual genes or numbers of genes. The results were analyzed in a multivariate model including age, total WBC count at diagnosis (greater or less than 50 000/μL as a dichotomous variable), antecedent cytopenias, *NPM1* mutations, and *FLT3–ITD* status. Age was assessed as a continuous variable.

The patients with karyotypic abnormalities were not analyzed for survival, since there were not enough diagnostic samples in each subgroup to provide statistically meaningful results. Survival analysis was performed only on patients with normal karyotype treated with similar therapy for whom a pre-treatment diagnostic sample was available.

β-Catenin in cell lines

Figure 1 demonstrates total β-catenin levels in: (a) three leukemia cell lines known to be without mutations in genes which are commonly mutated in other cancers (*Axin*, *β-catenin*, and *APC)*, (b) normal $CD34^+$ bone marrow cells from a healthy donor, and (c) HCT116 colon cancer cells which harbor constitutively active, mutated *β-catenin*. In HL60, KG1, and K562, total β-catenin levels were higher than present in normal $CD34^+$ cells but less than present in HCT116 cells with a known activating *β-catenin* mutation. By indirect immunofluorescence, all three leukemia cell lines demonstrated increased levels of both cytoplasmic and nuclear β-catenin relative to $CD34^+$ normal bone marrow samples (Figure 2).

Methylation of Wnt pathway inhibitors in leukemia cell lines

MSP analysis of each of the 12 Wnt pathway inhibitors was performed in three cell lines, HL60, KG1, and K562 (Supplemental Figure 1A), with a final summary for a total of six leukemia cell lines (Supplemental Figure 1B). Methylation of each of multiple Wnt inhibitors was observed in all the leukemia cell lines, with the exception of *APC* and *LKB1*. *DKK1*, *DKK3*, *sFRP4*, *sFRP5*, and *RUNX3* were methylated in at least 50% of the cell lines tested, and methylation of s*FRP2*, *SOX17*, and *WIF1* was present in all six cell lines.

Methylation of Wnt inhibitors in primary AML samples

MSP analysis of primary AML samples (n = 169) was performed (Figure 3) and compared to samples with normal-karyotype AML (n = 106) and early stage primary breast cancer samples (n = 54), with the latter chosen because Wnt inhibitor methylation has been extensively studied in epithelial tumors. Normal peripheral blood monocytes from normal donors (n = 5) were unmethylated for these 12 genes (data not shown) as previously reported [15,23,26]. There were no significant differences in the frequency of methylation between the entire AML group (n = 169) and the patients with normal karyotype leukemia (n = 106). Gene methylation was common in AML samples, with Wnt inhibitors *sFRP1, 2*, and *5* being most frequent followed by *WIF1* and *SOX17*. These findings are consistent with the prevalence of methylation for each gene observed in our leukemia cell lines, and suggests that loss of *sFRP1, 2*, and *5*, *WIF1*, and *SOX17* signaling may be important in Wnt pathway dysregulation in AML. In contrast, breast cancer samples demonstrated a different pattern of Wnt inhibitor methylation, with frequent methylation of *APC1*, *RASSF1A*, and *sFRP4*, genes that were significantly less ($p < 0.05$) methylated in leukemia samples and cell lines. *DKK3* was also statistically significantly more methylated in AML than breast cancer, but the frequency of methylation (9%) was less than for the other genes.

Wnt inhibitor methylation and prognosis

Mortality—In order to examine the influence of Wnt inhibitor methylation status on prognosis, diagnostic samples from a group (n = 72) of uniformly treated patients with normal-karyotype AML were obtained and analyzed for survival and the clinical end-point of relapse. These patients were annotated for prognostic factors including age, total WBC at diagnosis, antecedent cytopenias, *NPM1* mutations, and *FLT3–ITD* status. Table I demonstrates $NPM1^{mut}$ and *FLT3–ITD* frequencies, which are consistent with those in the reported literature. As expected, this group demonstrated prognostic effects for older age (hazard ratio [HR] 1.03, confidence interval [CI] 1.01–1.05), WBC greater than 50 000/μL (HR 2.15, CI 1.16–4.00), antecedent cytopenias/MDS (HR 1.22, CI 0.67–2.21), $NPM1^{mut}$ (HR 0.74, CI 0.33–1.66), and *FLT3–ITD* mutation (HR 1.86, CI 0.95–3.52).

Relapse—A univariate analysis was performed to assess the effect of each gene on relapse-free survival. The genes *APC1*, *LKB1*, and *RASSF1A* were excluded from the analysis as methylation of these three genes was rare (< 5%), and analysis would therefore be uninformative. Kaplan–Meier curves for methylation of *sFRP2* and *sFRP5* show a significant association with decreased relapse-free survival (log rank $p < 0.04$ and $p < 0.02$, respectively) (Figure 4). Furthermore, using the combined marker of concurrent methylation of *sFRP2* and *sFRP5*, methylation was also associated with a significant risk of AML relapse, hazard ratio 2.13 (CI 1.10–4.12), which persisted following multivariate analysis, that controlled for patient age at diagnosis, antecendent cytopenias, total white blood cell count at AML diagnosis, and *FLT3–ITD* and *NPM1* mutational status. Forest plots demonstrate the multivariate hazard ratios for relapse and death associated with methylation of each of the Wnt inhibitor genes [Figures 5(A) and 5(B)]. In our analysis, methylation of *sFRP2* and *sFRP5* separately were associated with a significant increase in the hazard ratio for relapse (*sFRP2* HR 2.04, CI 1.00–4.29; *sFRP5* HR 2.13, CI 1.04–4.38), while

methylation of *SOX17* demonstrated a trend toward increased risk of relapse (1.35, CI 0.69–2.65) [Figure 5(A)]. In multivariate analysis, only *sFRP2* methylation remained significantly associated with an increased risk of death (2.46, CI 1.13–5.38). Methylation of *sFRP4* and *SOX17* suggest an increased risk for death, but were not statistically significant (HR 1.50, 95% CI 0.62–3.66 and HR 1.51, 95% CI 0.77–2.97, respectively) [Figure 5(B)].

Discussion

There is accumulating evidence for the importance of Wnt pathway activation in AML. Several groups have demonstrated Wnt pathway activation in patients with AML, and others have shown that nuclear localization of β-catenin protein is associated with a worse prognosis in these patients [21,36]. Epigenetic inactivation of Wnt pathway inhibitors by CpG island methylation is a viable explanation for the Wnt pathway activation observed in these studies. Methylation of *sFRP1*, *2*, *3*, and *5* with corresponding transcriptional silencing has been shown in AML cell lines [37], and *sFRP2* methylation is associated with poor prognosis in adults with core binding factor leukemia ($n = 16$) [37] as well as in young patients (< 60 years old) with intermediate-karyotype leukemia [12]. Our data, obtained from patients with karyotypically normal AML of older age (median 61 years), support the previous studies' findings, confirming an association of Wnt pathway methylation (*sFRP2* and *5*) and a worse clinical prognosis (relapse and death).

Correlation of methylation data along with β-catenin levels in the primary patient samples would have been ideal. Unfortunately, due to the quality of the samples in our bank, protein and RNA expression analyses were not feasible.

The association of Wnt pathway inhibitor methylation with adverse outcome in patients with AML is of particular interest given the role of Wnt pathway signaling in hematopoietic stem cell self-renewal and progenitor development [11]. Since *sFRP2* methylation appears to correlate with adverse prognosis in patients with AML, its evaluation may be a useful addition to currently available prognostic markers such as *NPM1* mutation and *FLT3–ITD* status, each of which confer a prognostic significance similar in magnitude to that seen for *sFPR2* and *5* methylation in our study [38]. As such, *sFRP2* methylation may serve as a prognostic molecular marker that can potentially identify a subgroup of patients with AML with normal cytogenetics who should be approached differently from a therapeutic standpoint. Although commercial testing for protein and RNA remains challenging, the collection of DNA is routine, and simple, real-time-based, clinically relevant assays for methylation analysis are feasible.

Furthermore, although concerns remain with regard to the toxicity of global Wnt pathway inhibitors, novel small molecules targeting this pathway are under investigation and have demonstrated leukemia-specific efficacy *in vitro* [39]. It is possible that leukemias with the greatest silencing of Wnt inhibitors may be more dependent on Wnt activation and, therefore, more sensitive to inhibition of this pathway. The extensive nature of Wnt inhibitor methylation in leukemia may partly explain the activity of the hypomethylating agents, 5-azacytidine and deoxy-azacytidine. These agents, which are Food and Drug Administration (FDA) approved for the treatment of chronic myelomonocytic leukemias and

myelodysplastic syndrome, may restore the expression of Wnt inhibitors, thereby sensitizing leukemia stem cells to chemotherapy and apoptosis. Incorporation of these epigenetic agents into treatment paradigms as single agents or in combination with chemotherapy may be an attractive therapeutic option for patients with AML, particularly those with normal cytogenetics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

1. Jaffe ES. The 2008 WHO classification of lymphomas: implications for clinical practice and translational research. Hematology Am Soc Hematol Educ Program. 2009:523–531. [PubMed: 20008237]
2. Renneville A, Roumier C, Biggio V, et al. Cooperating gene mutations in acute myeloid leukemia: a review of the literature. Leukemia. 2008; 22:915–931. [PubMed: 18288131]
3. Steffen B, Muller-Tidow C, Schwable J, Berdel WE, Serve H. The molecular pathogenesis of acute myeloid leukemia. Crit Rev Oncol Hematol. 2005; 56:195–221. [PubMed: 16236521]
4. Scholl C, Gilliland DG, Frohling S. Deregulation of signaling pathways in acute myeloid leukemia. Semin Oncol. 2008; 35:336–345. [PubMed: 18692684]
5. Moore MA. Converging pathways in leukemogenesis and stem cell self-renewal. Exp Hematol. 2005; 33:719–737. [PubMed: 15963848]
6. Sato N, Meijer L, Skaltsounis L, Greengard P, Brivanlou AH. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. Nat Med. 2004; 10:55–63. [PubMed: 14702635]
7. Kielman MF, Rindapaa M, Gaspar C, et al. Apc modulates embryonic stem-cell differentiation by controlling the dosage of beta-catenin signaling. Nat Genet. 2002; 32:594–605. [PubMed: 12426568]
8. Bovolenta P, Esteve P, Ruiz JM, Cisneros E, Lopez-Rios J. Beyond Wnt inhibition: new functions of secreted Frizzled-related proteins in development and disease. J Cell Sci. 2008; 121:737–746. [PubMed: 18322270]
9. Katoh M, Katoh M. WNT signaling pathway and stem cell signaling network. Clin Cancer Res. 2007; 13:4042–4045. [PubMed: 17634527]
10. Moon RT, Bowerman B, Boutros M, Perrimon N. The promise and perils of Wnt signaling through beta-catenin. Science. 2002; 296:1644–1646. [PubMed: 12040179]
11. Khan NI, Bendall LJ. Role of WNT signaling in normal and malignant hematopoiesis. Histol Histopathol. 2006; 21:761–774. [PubMed: 16598675]
12. Valencia A, Roman-Gomez J, Cervera J, et al. Wnt signaling pathway is epigenetically regulated by methylation of Wnt antagonists in acute myeloid leukemia. Leukemia. 2009; 23:1658–1666. [PubMed: 19387464]
13. Figueroa ME, Skrabanek L, Li Y, et al. MDS and secondary AML display unique patterns and abundance of aberrant DNA methylation. Blood. 2009; 114:3448–3458. [PubMed: 19652201]
14. Giles RH, van Es JH, Clevers H. Caught up in a Wnt storm: Wnt signaling in cancer. Biochim Biophys Acta. 2003; 1653:1–24. [PubMed: 12781368]
15. Licchesi JD, Westra WH, Hooker CM, Machida EO, Baylin SB, Herman JG. Epigenetic alteration of Wnt pathway antagonists in progressive glandular neoplasia of the lung. Carcinogenesis. 2008; 29:895–904. [PubMed: 18308762]
16. Serinsoz E, Neusch M, Busche G, Wasielewski R, Kreipe H, Bock O. Aberrant expression of beta-catenin discriminates acute myeloid leukaemia from acute lymphoblastic leukaemia. Br J Haematol. 2004; 126:313–319. [PubMed: 15257703]

17. Simon M, Grandage VL, Linch DC, Khwaja A. Constitutive activation of the Wnt/beta-catenin signalling pathway in acute myeloid leukaemia. Oncogene. 2005; 24:2410–2420. [PubMed: 15735743]
18. Chung EJ, Hwang SG, Nguyen P, et al. Regulation of leukemic cell adhesion, proliferation, and survival by beta-catenin. Blood. 2002; 100:982–990. [PubMed: 12130512]
19. Ysebaert L, Chicanne G, Demur C, et al. Expression of beta-catenin by acute myeloid leukemia cells predicts enhanced clonogenic capacities and poor prognosis. Leukemia. 2006; 20:1211–1216. [PubMed: 16688229]
20. Martin V, Valencia A, Agirre X, et al. Epigenetic regulation of the non-canonical Wnt pathway in acute myeloid leukemia. Cancer Sci. 2010; 101:425–432. [PubMed: 19874313]
21. Xu J, Suzuki M, Niwa Y, et al. Clinical significance of nuclear non-phosphorylated beta-catenin in acute myeloid leukaemia and myelodysplastic syndrome. Br J Haematol. 2008; 140:394–401. [PubMed: 18217891]
22. Tickenbrock L, Schwable J, Wiedehage M, et al. Flt3 tandem duplication mutations cooperate with Wnt signaling in leukemic signal transduction. Blood. 2005; 105:3699–3706. [PubMed: 15650056]
23. Dhir M, Montgomery EA, Glockner SC, et al. Epigenetic regulation of WNT signaling pathway genes in inflammatory bowel disease (IBD) associated neoplasia. J Gastrointest Surg. 2008; 12:1745–1753. [PubMed: 18716850]
24. Klarmann GJ, Decker A, Farrar WL. Epigenetic gene silencing in the Wnt pathway in breast cancer. Epigenetics. 2008; 3:59–63. [PubMed: 18398311]
25. Tolwinski NS, Wieschaus E. A nuclear function for armadillo/beta-catenin. PLoS Biol. 2004; 2:E95. [PubMed: 15024404]
26. Carraway HE, Wang S, Blackford A, et al. Promoter hypermethylation in sentinel lymph nodes as a marker for breast cancer recurrence. Breast Cancer Res Treat. 2009; 114:315–325. [PubMed: 18404369]
27. Easwaran HP, Schermelleh L, Leonhardt H, Cardoso MC. Replication-independent chromatin loading of Dnmt1 during G2 and M phases. EMBO Rep. 2004; 5:1181–1186. [PubMed: 15550930]
28. Suzuki H, Watkins DN, Jair KW, et al. Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. Nat Genet. 2004; 36:417–422. [PubMed: 15034581]
29. Esteller M, Fraga MF, Guo M, et al. DNA methylation patterns in hereditary human cancers mimic sporadic tumor-igenesis. Hum Mol Genet. 2001; 10:3001–3007. [PubMed: 11751682]
30. Paz MF, Avila S, Fraga MF, et al. Germ-line variants in methyl-group metabolism genes and susceptibility to DNA methylation in normal tissues and human primary tumors. Cancer Res. 2002; 62:4519–4524. [PubMed: 12154064]
31. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA. 1996; 93:9821–9826. [PubMed: 8790415]
32. Karp JE, Smith BD, Levis MJ, et al. Sequential flavopiridol, cytosine arabinoside, and mitoxantrone: a phase II trial in adults with poor-risk acute myelogenous leukemia. Clin Cancer Res. 2007; 13:4467–4473. [PubMed: 17671131]
33. Linker CA, Ries CA, Damon LE, et al. Autologous stem cell transplantation for acute myeloid leukemia in first remission. Biol Blood Marrow Transplant. 2000; 6:50–57. [PubMed: 10707999]
34. Geller RB, Burke PJ, Karp JE, et al. A two-step timed sequential treatment for acute myelocytic leukemia. Blood. 1989; 74:1499–1506. [PubMed: 2676014]
35. Bolanos-Meade J, Karp JE, Guo C, et al. Timed sequential therapy of acute myelogenous leukemia in adults: a phase II study of retinoids in combination with the sequential administration of cytosine arabinoside, idarubicin and etoposide. Leuk Res. 2003; 27:313–321. [PubMed: 12531222]
36. Majeti R, Becker MW, Tian Q, et al. Dysregulated gene expression networks in human acute myelogenous leukemia stem cells. Proc Natl Acad Sci USA. 2009; 106:3396–3401. [PubMed: 19218430]

37. Jost E, Schmid J, Wilop S, et al. Epigenetic inactivation of secreted Frizzled-related proteins in acute myeloid leukaemia. Br J Haematol. 2008; 142:745–753. [PubMed: 18537968]

38. Schlenk RF, Dohner K, Krauter J, et al. Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. N Engl J Med. 2008; 358:1909–1918. [PubMed: 18450602]

39. Minke KS, Staib P, Puetter A, et al. Small molecule inhibitors of WNT signaling effectively induce apoptosis in acute myeloid leukemia cells. Eur J Haematol. 2009; 82:165–175. [PubMed: 19067737]

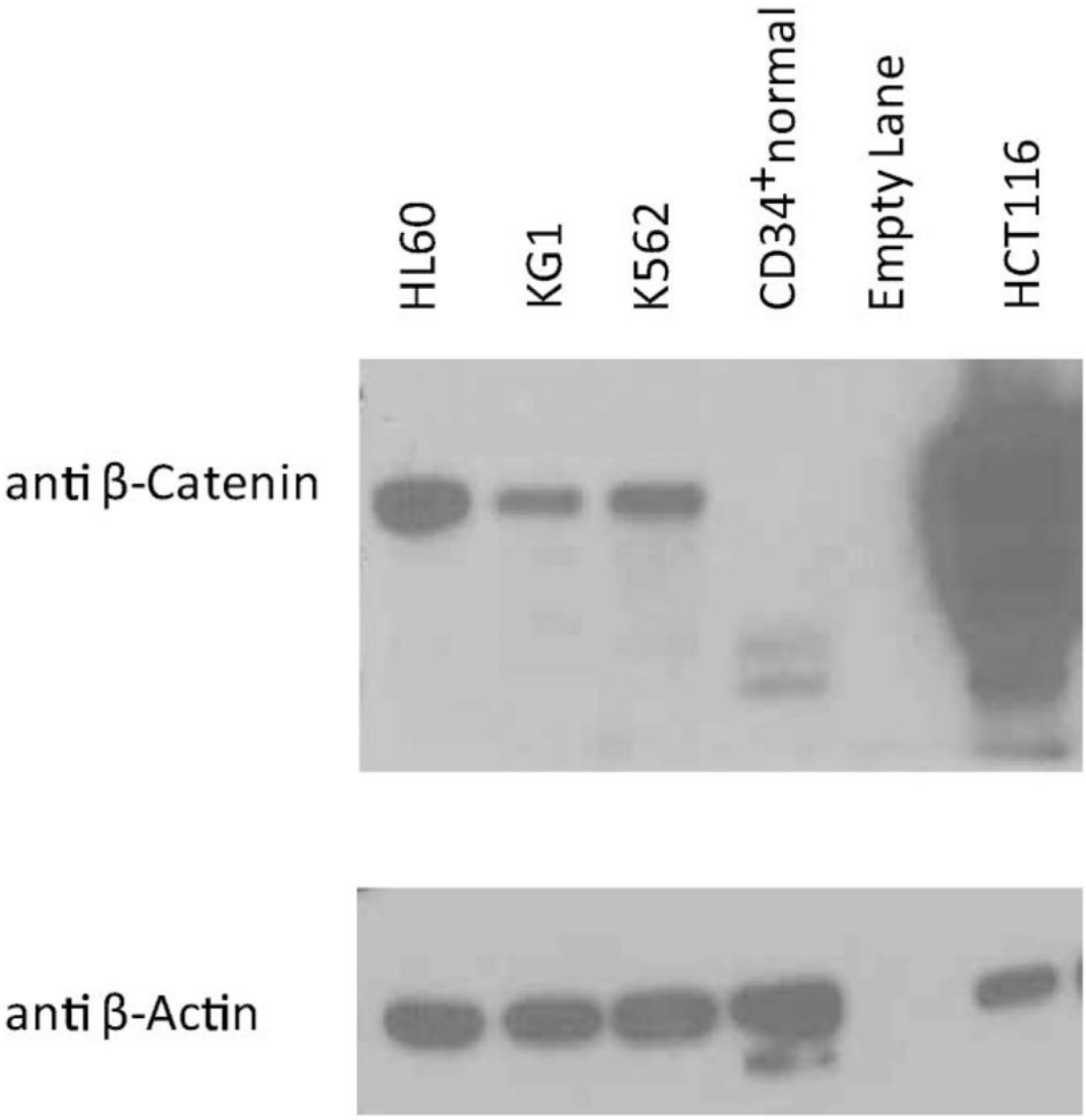

Figure 1.

Westen blot for total β-catenin in three leukemia cell lines (HL60, KG1, and K562) known to be without mutations in *APC*, *Axin*, and *β-catenin*, normal $CD34^+$ bone marrow cells, and HCT116 colon cancer cells harboring a constitutively active β-catenin protein. The leukemia cell lines have higher levels of β-catenin protein than are seen in normal $CD34^+$ bone marrow cells, but are lower than observed in HCT116 cells.

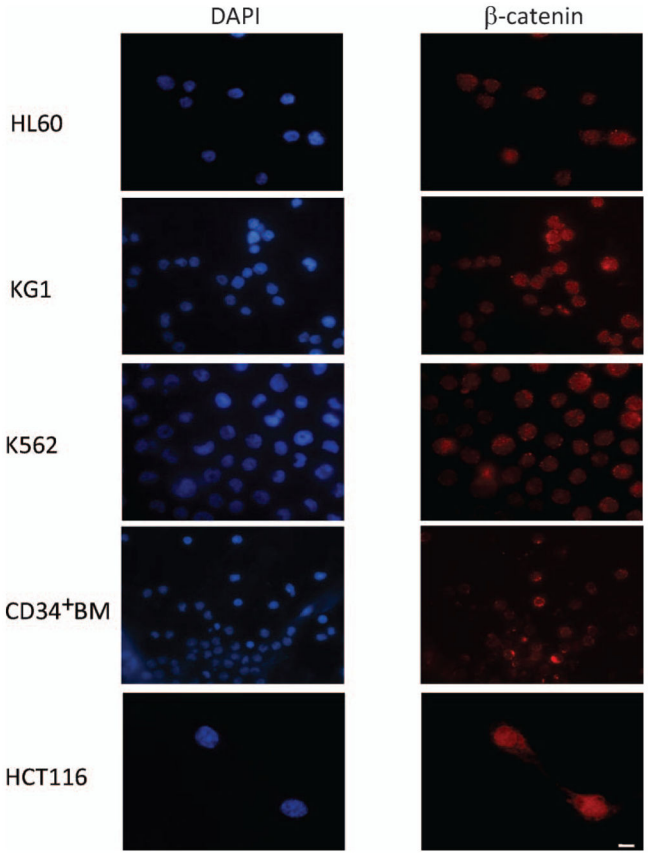

Figure 2.

Immunofluorescence for nuclei (blue) and β-catenin (red) demonstrating predominantly nuclear localization of β-catenin protein in leukemia cell lines. Low levels of β-catenin were observed in $CD34^+$ bone marrow samples, with most protein appearing to be cytoplasmic or membrane bound. HCT116 colon cancer cells are shown as a positive control with abundant β-catenin protein present. All images were taken at ×63, scale bar 10 μm.

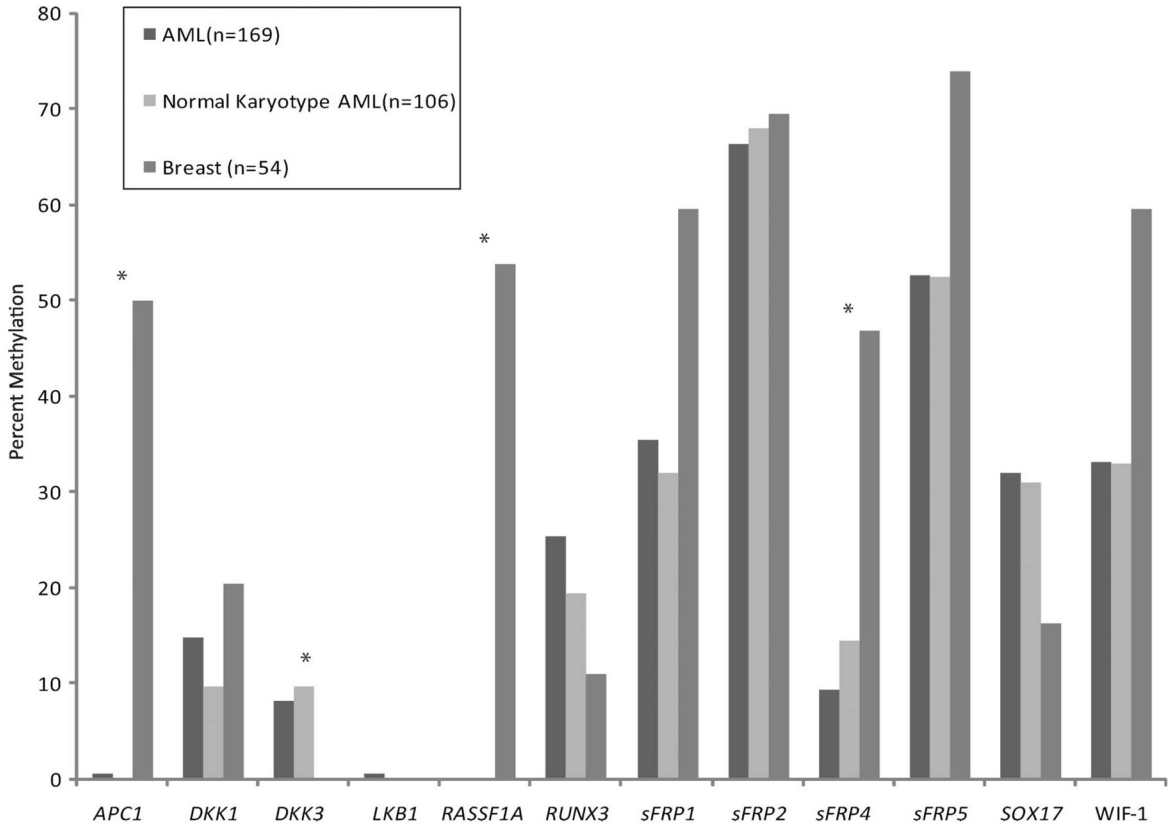

Figure 3.

Methylation frequency in patients with AML (n = 169), AML and normal karyotype (n = 106), and breast cancer (n = 54). Rates of methylation are similar in the AML group as a whole and in those with normal karyotype. Overall, 89% of the AML samples had at least one methylated Wnt inhibitor gene, and 70% had methylation of two or more genes. *APC*, *RASSF1A*, and *sFRP4* are frequently methylated in epithelial malignancies and rarely methylated in AML (*$p < 0.05$ for AML versus breast cancer).

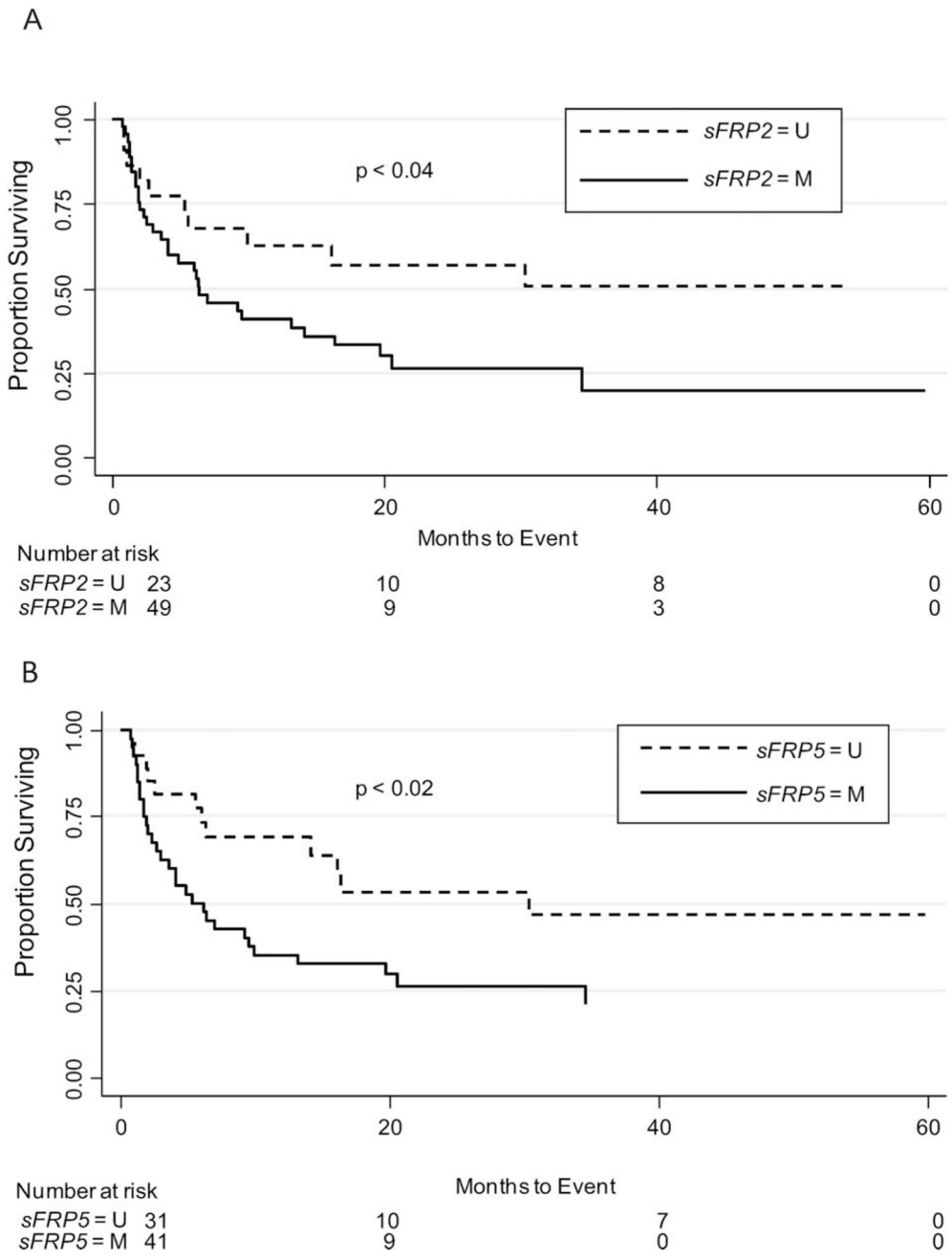

Figure 4.

Kaplan–Meier curves demonstrating the impact of *sFRP2* (A) and *sFRP5* (B) methylation on disease-free survival. In univariate analysis, *sFRP2* and *sFRP5* methylation was significantly associated with adverse prognosis ($p < 0.04$ and $p < 0.02$, respectively). ‘U’ designates unmethylated status and ‘M’ designates methylated status.

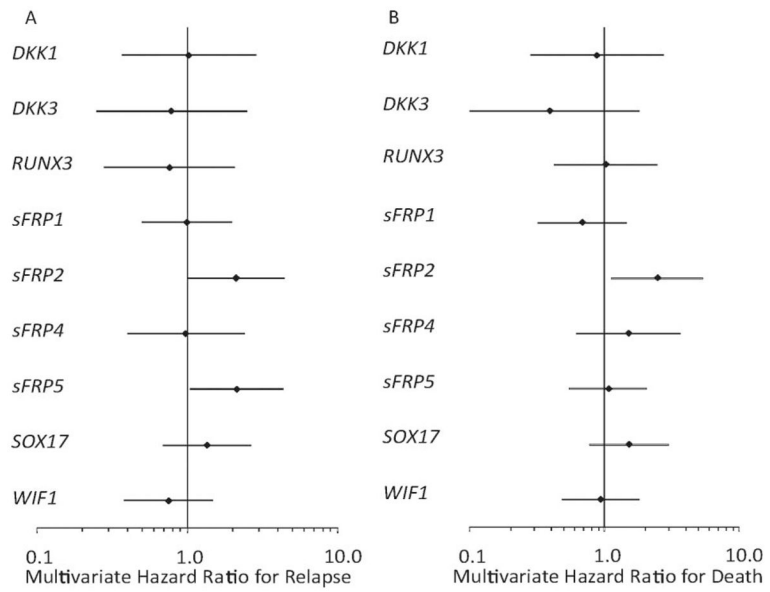

Figure 5.

(A) Multivariate analysis of disease-free survival for each of the Wnt genes in 72 uniformly treated patients with AML with normal cytogenetics. Methylation of *sFRP2* and *sFRP5* is associated with a significantly increased rate of relapse. (B) Multivariate analysis of the association between Wnt inhibitor methylation and all-cause mortality in this group. *sFRP2* methylation is associated with a significantly increased risk of death. The multivariate model includes patient age at diagnosis, history of antecedent cytopenias/MDS, total WBC at diagnosis, and *FLT3–ITD* and $NPM1^{mut}$ status.

Table I

Characteristics of the study population.

	All (*n* = 169)		Normal (*n* = 106)		
Characteristic	**Number**	**Percent**	**Number**	**Percent**	**HR for death (95% CI)**
Age (years), median (IQR)	61 (48–70)		61 (51–70)		1.03 (1.01–1.05)
Sex					
Female	78	46	53	50	
Male	91	54	53	50	
Cytogenetics					
Good	10	6			
Normal	106	63			
De novo AML			39	37	1.0
AML with antecedent MDS			33	31	1.22 (0.67–2.21)
Relapsed AML			34	31	NA
Intermediate	14	8			
Single adverse	9	5			
Complex	30	18			
Mean WBC	35 838		40 302		2.15 (1.16–4.00)
NPM1 mutation	26	15	23	22	0.74 (0.33–1.6)
FLT3–ITD mutation	47	28	32	30	1.86 (0.95–3.52)

HR, hazard ratio; CI, confidence interval for point estimate; IQR, interquartile range; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; WBC, total white blood cell count; NA, not applicable.

Leuk Lymphoma. Author manuscript; available in PMC 2014 April 25.