

# Epigenetic regulation of the non-canonical Wnt pathway in acute myeloid leukemia

Vanesa Martín,<sup>1</sup> Ana Valencia,<sup>2</sup> Xabier Agirre,<sup>3</sup> Jose Cervera,<sup>2</sup> Edurne San Jose-Eneriz,<sup>3</sup> Amaia Vilas-Zornoza,<sup>3</sup> Paula Rodriguez-Otero,<sup>3</sup> Miguel A. Sanz,<sup>2</sup> Concepción Herrera,<sup>1</sup> Antonio Torres,<sup>1</sup> Felipe Prosper<sup>3</sup> and Jose Román-Gómez<sup>1,4</sup>

<sup>1</sup>Hematology Department, Cellular Therapy Area, Reina Sofia Hospital, Maimonides Institute for Biomedical Research, Cordoba; <sup>2</sup>Hematology Department, La Fe Hospital, Valencia; <sup>3</sup>Hematology Department, Cellular Therapy Area, Clinica Universitaria/School of Medicine, Foundation for Applied Medical Research, University of Navarra, Pamplona, Spain

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Wnt5a is a member of the Wnt family of proteins that signals through the non-canonical Wnt/Ca<sup>2+</sup> pathway to suppress cyclin D1. Deregulation of this pathway has been found in animal models suggesting that it acts as tumour suppressor in acute myeloid leukemia (AML). Although DNA methylation is the main mechanism of regulation of the canonical Wnt pathway in AML, the role of WNT5A abnormalities has never been evaluated in this clinical setting. The methylation status of WNT5A promoter–exon 1 was analyzed by methylation-specific PCR and sequencing in eleven AML-derived cell lines and 252 AML patients. We observed WNT5A hypermethylation in seven cell lines and in 43% (107/252) of AML patients. WNT5A methylation was associated with decreased WNT5A expression ( $P < 0.001$ ) that was restored after exposure to 5-Aza-2'-deoxycytidine. Moreover, WNT5A hypermethylation correlated with upregulation of CYCLIN D1 expression ( $P < 0.001$ ). Relapse (15% vs 37%,  $P < 0.001$ ) and mortality (61% vs 79%,  $P = 0.004$ ) rates were lower for patients in the non-methylated group. Disease-free survival and overall survival at 6 and 7 years, respectively, were 60% and 27% for unmethylated patients and 20% and 0% for hypermethylated patients ( $P = 0.0001$  and  $P = 0.04$ , respectively). Interestingly, significant differences were also observed when the analysis was carried out according to cytogenetic risk groups. We demonstrate that WNT5A, a putative tumor suppressor gene in AML, is silenced by methylation in this disease and that this epigenetic event is associated with upregulation of CYCLIN D1 expression and confers poor prognosis in patients with AML. (*Cancer Sci* 2010; 101: 425–432)

Wnt proteins form a large family of secreted glycoproteins that activate the highly conserved Wnt pathway through their receptor Frizzled. Wnt–Frizzled signaling controls a wide variety of processes, including proliferation, differentiation, migration, and cell–cell adhesion, playing a crucial role not only during development but also in hematopoietic stem cell maintenance and oncogenesis.<sup>(1–4)</sup>

Wnt signaling occurs through the canonical and non-canonical pathways. The canonical pathway has a central mediator,  $\beta$ -catenin. In the absence of Wnt ligand,  $\beta$ -catenin is phosphorylated and ubiquitinated by the activity of a multiprotein destruction complex that contains axin, adenomatous polyposis coli, glycogen-synthase kinase 3 $\beta$ , and casein kinase 1, leading to proteasome-mediated degradation. On the other hand, binding of Wnt to the Frizzled receptor leads to inhibition of the destruction complex through activation of the protein Dishevelled. Then,  $\beta$ -catenin is stabilized and translocated into the nucleus. This allows binding of  $\beta$ -catenin to transcription factors such as lymphoid enhancing factor-1 and T-cell factor, and leads to transcriptional activation of multiple target genes like CYCLIN D1 and C-MYC.<sup>(1–4)</sup>

The non-canonical signaling pathway, although not completely understood, is independent of  $\beta$ -catenin and includes two defined pathways: the planar cell polarity and Wnt/Ca<sup>2+</sup> pathways.<sup>(5)</sup> Non-canonical signaling is often initiated by Wnt5a, Wnt4, and Wnt11 through Frizzled and Dishevelled and might involve G proteins.<sup>(6)</sup>

Deregulation of Wnt signaling has become a hallmark of various types of tumours, including leukemias.<sup>(1–4,7–16)</sup> Several lines of evidence have shown that aberrant canonical Wnt signaling contributes to the pathogenesis of acute myeloid leukemia (AML): (i) the Wnt pathway is constitutively activated in AML with normal karyotype or low-risk cytogenetics<sup>(17,18)</sup> and also in cases with FLT3 internal tandem duplication;<sup>(19)</sup> (ii)  $\beta$ -catenin is upregulated in AML, representing an independent prognostic factor associated with relapse and shorter survival;<sup>(20)</sup> (iii) comparative expression analysis between human hematopoietic stem cells and leukemic stem cells from AML patients identified 3005 differentially expressed genes, most of them implicated in Wnt signaling;<sup>(21)</sup> and (iv) recent studies have shown that impaired regulation of Wnt antagonists such as secreted Frizzled-related protein (sFRP) genes by promoter hypermethylation contributes to Wnt pathway activation in AML.<sup>(22,23)</sup>

Wnt5a is a member of the Wnt family of proteins that signals through the non-canonical Wnt/Ca<sup>2+</sup> pathway to suppress cyclin D1 expression. WNT5A hemizygous mice develop myeloid leukemias and display loss of Wnt5a function in tumor tissues. Furthermore, analyses of human AML reveal loss of Wnt5a expression in the majority of patient samples, including those patients who retain WNT5A coding sequences.<sup>(6)</sup> Taken together, these data suggest that WNT5A could function as a tumor suppressor gene in myeloid leukemogenesis and also that downregulation of this gene in AML might be dependent on epigenetic mechanisms similar to what we and others have previously demonstrated in acute lymphoblastic leukemia and other malignancies.<sup>(24,25)</sup> In the present study, we demonstrate that epigenetic regulation of WNT5A by promoter methylation in AML is associated with the outcome of this group of patients.

## Materials and Methods

**Cell lines and patients.** Eleven AML-derived cell lines (MUTZ-3, F-39P, TF-1, HL-60, NOMO-1, KG-1, MV4-II, MOLM-13, KASUMI-1, EOL-1, and HEL) were purchased from the Deutsche Sammlung von Microorganismen und Zellkulturen (Braunschweig, Germany). Cells were grown at 37°C under 5% CO<sub>2</sub> in humidified air in RPMI medium (Gibco-BRL, Burlington, ON, Canada) supplemented with 20% fetal bovine

<sup>4</sup>To whom correspondence should be addressed.  
E-mail: peperosa@teletelne.es

serum (Biochrome, NY, USA), 1% penicillin/streptomycin, and 1% HEPES (Gibco-BRL).

The study population included 252 consecutive patients (137 men, 115 women) who were diagnosed with *de novo* non-M3 AML between September 1998 and September 2008 at the Hospital Universitario La Fe of Valencia and Hospital Reina Sofia of Cordoba, Spain. Approval was obtained from the Institutional Review Board. Informed consent was provided according to the Declaration of Helsinki. The diagnosis was made according to the morphological and cytochemical criteria of the French-American-British (FAB) classification.<sup>(26)</sup> The mean age at diagnosis was 57 years (range 8–92 years). The median follow-up of the series was 46 months. The main clinical characteristics of the patients are listed in Table 1. Fitted patients were enrolled into intensive chemotherapy trials in which induction therapy comprised standard combinations of anthracycline plus cytarabine. Patients achieving complete remission (CR) proceeded to consolidation therapy and eligible patients were selected for autologous or allogeneic stem cell transplantation. Seventy-seven patients received stem cell transplantation (38 autologous, 39 allogeneic). Sixty-two patients from the whole series relapsed, and 71 remain alive.

**Cytogenetic and molecular analysis.** Karyotype analysis was carried out using unstimulated short-term cultures according to the recommendations of the International System for Human Cytogenetics.<sup>(27)</sup> Whenever possible, at least 20 metaphases were evaluated. Cytogenetic risk groups were defined as follows: high risk,  $-5/\text{del}(5q)$ ,  $-7/\text{del}(7q)$ ,  $\text{abn } 3q$ , complex aberrations ( $\geq 3$  independent aberrations),  $t(9;22)$  and  $t(6;9)$ ; low risk,  $t(8;21)$  and  $\text{inv}(16)$ ; intermediate risk, all other karyotypic aberrations or a normal karyotype. FLT3 internal tandem duplications and nucleophosmin gene mutations (*NPM1*) were studied in cDNA samples following the methods described by Nakao *et al.*<sup>(28)</sup> and Schnittger *et al.*<sup>(29)</sup>, respectively.

**Table 1. Main clinical characteristics of the patients**

Characteristics	Non-methylated (%) (n = 145)	Methylated (%) (n = 107)	P-value
Age (years)			NS
≤60	70 (48)	62 (58)	
>60	75 (52)	45 (42)	
Sex			NS
Male	80 (55)	57 (53)	
Female	65 (45)	50 (47)	
WBC ( $\times 10^9/\text{L}$ )			NS
≤10	66 (46)	56 (52)	
>10	79 (54)	51 (48)	
FAB classification			NS
M0	10 (7)	6 (6)	
M1	35 (24)	27 (25)	
M2	38 (26)	28 (26)	
M4	26 (18)	10 (9)	
M5	22 (15)	15 (14)	
M6	9 (6)	16 (15)	
M7	1 (1)	1 (1)	
Unclassified	4 (3)	4 (4)	
Cytogenetic risk group			NS
Low	5 (3)	13 (12)	
Intermediate	78 (54)	63 (59)	
High	29 (20)	24 (22)	
Molecular alteration			NS
FLT3-ITD	20 (14)	18 (17)	
NPM1	25 (17)	12 (11)	

FLT3, Internal tandem duplication; NPM1, nucleophosmin; NS, not significant; WBC, white blood cells.

**Methylation-specific PCR.** Analysis of *WNT5A* (GenBank: NC\_000003) has revealed that *WNT5A* promoter and exon 1 possess a long 1246-bp CpG island located between nt48 and nt1294, showing >60% C + G content and an observed-over-expected CpG frequency of >0.6. The methylation status of the CpG island in the *WNT5A* gene promoter was analysed by genomic DNA bisulfite treatment followed by methylation-specific PCR (MSP) as reported by Herman *et al.*<sup>(30)</sup> Three different regions of this CpG island were analyzed for methylation (Fig. S1). The primers for the methylated (M) and unmethylated (U) promoter regions are reported in Figure S1. 'Hot start' PCR was carried out for 30 cycles consisting of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min, followed by a final 7-min extension for all primer sets. The products were separated by electrophoresis on a 2% agarose gel. Bone marrow mononuclear cell DNA from healthy donors was used as a negative control for methylation-specific assays. Human male genomic DNA universally methylated for all genes (Intergen Company, Purchase, NY, USA) was used as a positive control for methylated alleles. Water blanks were included with each assay. Results were always confirmed by repeat MSP assays after an independently performed bisulfite treatment.

**Expression analyses.** Expression of *WNT5A* and the *WNT5A* target gene *CYCLIN D1* was analyzed by the RT-PCR technique. Total RNA was extracted from marrow samples with Ultraspec (Biotecx, Houston, TX, USA) following the manufacturer's instructions. Reverse transcription was carried out on 1 µg total RNA, after heating at 70°C for 5 min, with random hexamers as the reaction primer. The reaction was carried out at 42°C for 45 min in the presence of 12 U Avian Myeloblastosis virus reverse transcriptase. Quantitative real-time PCR (qRT-PCR) for gene expression was carried out with LightCycler technology, using 1 µL of cDNA in a 20-µL reaction, 0.5 µmol/L of each primer and 2 µL of 10 × LightCycler Fast-Star DNA Master SYBR Green I (Roche Molecular Biochemicals, Barcelona, Spain). The final  $\text{Mg}^{2+}$  concentration in the reaction mixture was adjusted to 3.5 mmol/L. Primers were: for *WNT5A* expression, forward 5'-CCACATGCAGTACATCGGAG-3' and reverse 5'-TGCCGGAAGTGTATGCG-3'; and for *CYCLIN D1* expression, forward 5'-CCCTCGGTGCTACTTCAAATGT-3' and reverse 5'-TGATCTGTTTGTCTCCTCCGCCT-3'. The following program conditions were applied for qRT-PCR running: denaturation program, one cycle at 95°C for 8 min; amplification program, 45 cycles at 95°C for 5 s, 60°C for 10 s, and 72°C for 15 s; melting program, one cycle at 95°C for 0 s, 40°C for 60 s, and 90°C for 0 s; and cooling program, one cycle at 40°C for 60 s. The temperature transition rate was 20°C/s, except in the melting program, which was 0.4°C/s between 40 and 90°C. The *Abelson* gene (*ABL1*) was used as a reference gene, and it was amplified in the same run and following the same procedure described above (forward 5'-CCCAACCTTTTCGTTGCACTGT-3'; reverse 5'-CGGCTCTCGGAGAGACGTAGA-3'). In order to reduce the variation between different assays and samples, a procedure based on the relative quantification of target genes versus their controls/calibrators in relation to the reference gene was used. Calculations were automatically carried out by LightCycler software (Real-Quant, version 1.0; Roche Molecular Biochemicals). The normalized ratios (N) for each gene were obtained from the next equation and expressed as a percentage of the control/calibrator:

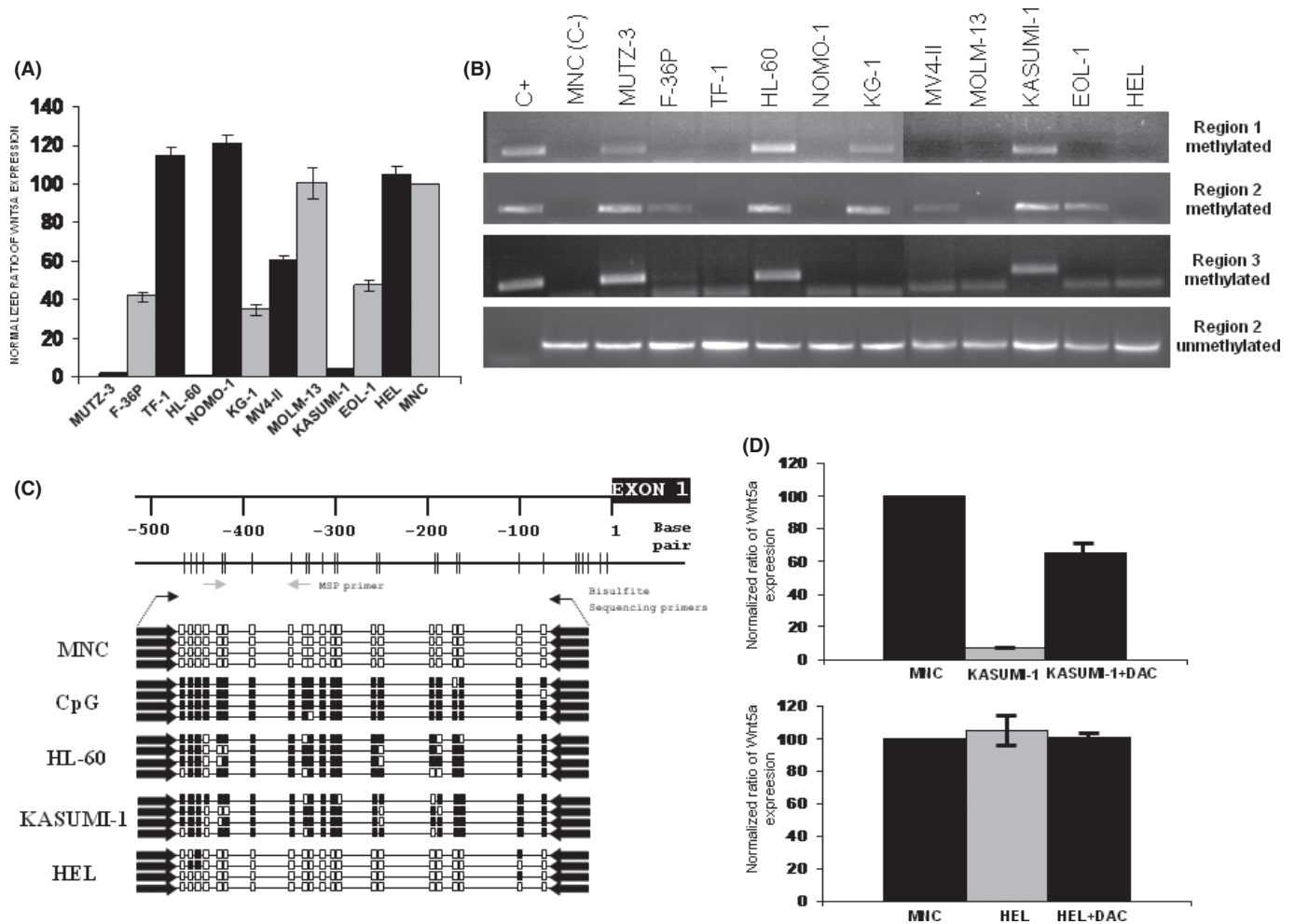
$$N = (E_{\text{target}})^{\Delta\text{Cp}_{\text{target}}(\text{control}-\text{sample})} \div (E_{\text{ref}})^{\Delta\text{Cp}_{\text{ref}}(\text{control}-\text{sample})}$$

The efficiencies (E) of each gene were calculated from the slopes of crossover points (Cp) versus the DNA concentration plot, according to the formula  $E = 10^{(-1/\text{slope})}$ .  $\Delta\text{Cp}$  corresponded to the difference between control/calibrator Cp and

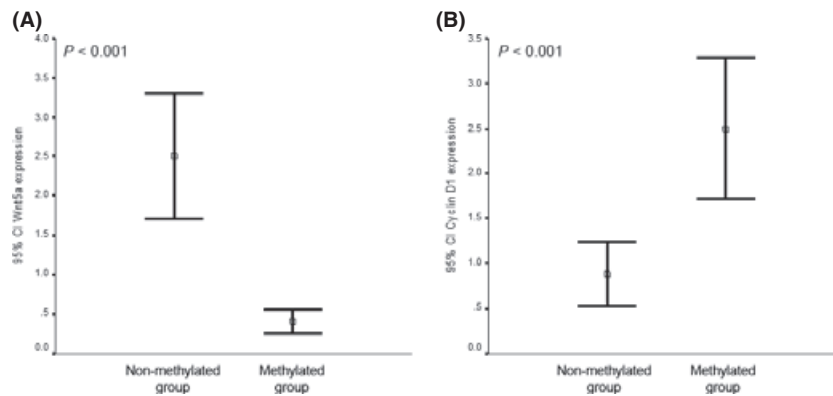
sample Cp, either for the target or for the reference sequences. The selected controls/calibrators were normal marrow mononuclear cells from healthy donors. They were considered as 100% expression.

**Wnt5a CpG island analysis by sequencing after sodium bisulfite modification.** The methylation status of *WNT5A* CpG islands was analysed by bisulfite genomic sequencing in HL-60, KASUMI-1, and HEL AML-derived cell lines and bone marrow mononuclear cells DNA from healthy donors. Genomic DNA (1 µg) was treated and modified using the CpGenomic DNA Modification Kit (Intergen Company). Human male genomic DNA universally methylated for all genes (Intergen Company) was used as a positive or methylated control. After bisulfite modification, region 2 from *Wnt5a* CpG island was amplified by PCR using 3 µL of modified DNA and Wnt5a-SB1 (5'-GGGGTTGATTTTTGTAGTTTATAGATG-3') and Wnt5a-SB2 (5'-TTAAAACCTTCCAACCCCAAATATA-3') primers under

the following conditions: 94°C for 10 min, 35 cycles at 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min, and a final elongation cycle at 72°C for 10 min. PCR were carried out in a total volume of 25 µL, with 1 U high-fidelity Platinum *Taq* DNA polymerase (Invitrogen Life Technologies, Paisley, UK), 1.5 mmol/L MgCl<sub>2</sub>, 0.2 mmol/L dNTPs, and 50 pmol of each primer. The PCR products were separated on a 2% agarose gel, stained with ethidium bromide, and visualized under UV light. Amplification products, containing 21 CpG dinucleotides, were subcloned into the pCR 4-TOPO plasmid using TOPO TA Cloning Kit for Sequencing (Invitrogen Life Technologies) and transformed into *Escherichia coli* according to the manufacturer's recommendations. Colonies with recombinant plasmids containing the described PCR products were screened by digestion with *EcoRI* (Amersham Biosciences, Buckinghamshire, UK). Candidate plasmid clones were sequenced using T7 and T3 universal forward and reverse primers.



**Fig. 1.** Expression and methylation analysis of the *WNT5A* gene in acute myeloid leukemia (AML)-derived cell lines. (A) Quantitative real-time PCR expression of *WNT5A* in AML-derived cell lines demonstrating downregulation of gene expression in seven of the 11 cell lines studied. Gene expression was normalized using the expression in bone marrow mononuclear cells (BM-MNC) from 30 healthy controls (normalized ratio = 100%). The figure represents the mean of three different studies in triplicate. (B) Methylation-specific PCR (MSP) analysis of *WNT5A* in the same AML-derived cell lines. Region 2 of *WNT5A* was methylated in all of the cell lines that had downregulated *WNT5A* expression. C+, positive methylated control; C-, BM-MNC cells from healthy donors. (C) Schematic description of the *WNT5A* CpG island region 2. Each vertical bar represents a CpG dinucleotide. The gray arrows show the location of the MSP primers and the black arrows show the location of bisulfite sequencing primers. The figure also represents the analysis of *WNT5A* CpG island methylation status by bisulfite sequencing in AML-derived cell lines. Each box indicates a CpG dinucleotide (white box, unmethylated; black box, methylated) and each line represents the analysis of 21 CpG dinucleotides of a single clone of the *WNT5A* analysed region. (D) Expression analysis of the *Wnt5a* gene in the methylated KASUMI-1 cell line and in the unmethylated HEL cell line, before and after treatment with the demethylating agent 5-Aza-2'-deoxycytidine (DAC) demonstrating upregulation of the gene after treatment in AML-derived cell line that was methylated. Gene expression was normalized with expression in BM-MNC from healthy controls (normalized ratio = 100%). The figure represents the mean of three different studies in triplicate.



**Fig. 2.** Expression of *WNT5A* and *CYCLIN D1* genes in samples from acute myeloid leukemia (AML) patients. (A) Expression analysis of the *WNT5A* gene in AML patients. Methylated patients showed downregulation of *WNT5A* expression. Bars represent the mean expression (95% CI) in AML patients in comparison with the expression in Bone marrow mononuclear cells (BM-MNC) from 30 healthy controls (normalized ratio = 100%). (B) Hypermethylation of the *WNT5A* gene is associated with upregulation of *CYCLIN D1* in AML patients. Bars represent the mean expression (95% CI) in AML patients in comparison with the expression in BM-MNC from 30 healthy controls (normalized ratio = 100%).

**Treatment with the demethylating agent 5-Aza-2'-deoxycytidine.** AML-derived KAG-1 and HEL cell lines were grown at a density of  $1 \times 10^6$  cells/mL in 25-cm<sup>2</sup> flasks with 10 mL of RPMI-1640 medium supplemented with 20% fetal bovine serum and maintained at 37°C in a humid atmosphere containing 5% CO<sub>2</sub>. Cell lines were treated with 4 μmol/L of 5-Aza-2'-deoxycytidine (DAC; Sigma-Aldrich, Steinheim, Germany) for 4 days. After treatment, cells were washed with PBS, pelleted by centrifugation at 1500g for 5 min, and used for genomic DNA and RNA isolation. DNA was extracted using the QIAmp DNA Mini Kit (Qiagen, Hilden, Germany) and total RNA using RNeasy Mini Kit (Qiagen). Total RNA (1 μg) was used for cDNA synthesis using SuperScript II RNase H-RT (Invitrogen Life Technologies) with random hexamers.

**Statistical analysis.** For statistical purposes, AML patients were classified into two different groups according to the methylation status of the *Wnt5a* promoter (methylated and non-methylated groups). All descriptive statistics and tests (Mann-Whitney non-parametric *U*-test,  $\chi^2$  and Fisher's exact test) were calculated using the statistical package SPSS (Chicago, IL, USA) 11.0. A *P* < 0.05 was considered statistically significant. Unadjusted time-to-event analyses were carried out using the Kaplan–Meier method and log-rank tests for comparisons. Overall survival (OS) was measured from the time of diagnosis to the time of last follow-up or death from any cause. Disease-free survival (DFS) was measured from the date of CR. In the analysis of DFS, relapse and death were considered uncensored events, whichever occurred first. All *P*-values reported are two-sided. Multivariate analysis was carried out using the Cox proportional hazards model. The follow up of the patients was updated on January 2009 and all follow-up data were censored at that point.

## Results

**Expression of the *WNT5A* gene in AML-derived cell lines is regulated by promoter hypermethylation.** Quantitative expression of *WNT5A* transcripts was assessed by means of qrt-PCR using cDNA from healthy donors as a control (it was considered as 100% of  $N_{wnt5a}$  ratio). Low levels of *WNT5A* expression were observed in seven AML cell lines (Fig. 1A). Among all the possible mechanisms of transcription regulation, we first decided to study the possible hypermethylation of the *WNT5A* promoter as we have previously demonstrated that *WNT5A* is a target for epigenetic regulation in leukemia.<sup>(24)</sup> By MSP, region 2 of the *WNT5A* promoter was revealed to be methylated in

AML-derived cell lines with low *WNT5A* expression (Fig. 1B), which was clearly in contrast with the lack of *WNT5A* promoter methylation in this region observed in healthy MNC or AML cell lines with normal *WNT5A* expression (Fig. 1B). Methylation of regions 1 and 3 was observed in some cell lines but was always associated with region 2 methylation and never as an isolated event (Fig. 1B). Moreover, in order to confirm the results of the MSP, we screened bone marrow DNA from healthy donors and three AML-derived cell lines (HL-60, KASUMI-1, and HEL) using bisulfite genomic sequencing. As described in 'Materials and Methods', the amplification products of the *WNT5A* promoter, which are composed of 21 CpG dinucleotides, were subcloned and sequenced. Genomic sequencing after bisulfite modification revealed that DNA from healthy donors and the HEL cell line was unmethylated. In contrast, HL-60 and KASUMI-1 cell lines were heavily methylated in almost all analysed CpG dinucleotides and clones (Fig. 1C). Exposure of AML cell lines to the demethylating agent DAC restored *WNT5A* expression in KASUMI-1 cell lines that showed hypermethylation of the *WNT5A* promoter but it had not effect in the HEL cell line that showed an unmethylated promoter (Fig. 1D). These results indicate that *WNT5A* is downregulated in AML at least in part by hypermethylation and that region 2 of the *WNT5A* promoter is critical for this purpose.

**Table 2. Clinical outcome of *de novo* myeloid acute leukemia patients**

Characteristics	Non-methylated <i>n</i> (%)	Methylated <i>n</i> (%)	<i>P</i> -value
Induction response			NS
Complete remission	72 (50)	63 (59)	
Failure			
Resistance	21 (14)	18 (17)	
Death	19 (13)	8 (7)	
Not treated	33 (23)	18 (17)	
Post-induction therapy			NS
Chemotherapy only	66 (46)	56 (52)	
Autologous SCT	22 (15)	16 (15)	
Allogenic SCT	22 (15)	17 (16)	
Relapse	22 (15)	40 (37)	<0.001
Death	89 (61)	85 (79)	0.004

NS, not significant; SCT, stem cell transplantation.

**WNT5A hypermethylation in AML patients is associated with decreased expression of WNT5A and upregulation of CYCLIN D1.** To establish a more clinically relevant model of the role of the epigenetic regulation of *WNT5A*, we analyzed the methylation status of the three regions of the *WNT5A* CpG island by MSP in a group of 252 AML patients (Table 1). The methylation frequencies were as follows: 43% for region 2; 15% for region 3; and 11% for region 1. Unmethylated *WNT5A* was found in 145 of 252 patients (non-methylated group, 57%) whereas 107 (43%) of 252 AML had methylation of the *WNT5A* CpG island (methylated group). In line with the studies in cell lines, region 2 methylation was always present in methylated patients. Expression of *WNT5A* was downregulated in patients with methylation of *WNT5A* in comparison with non-methylated patients ( $41 \pm 40\%$  vs  $250 \pm 249\%$ ;  $P < 0.001$ ; Fig. 2A).

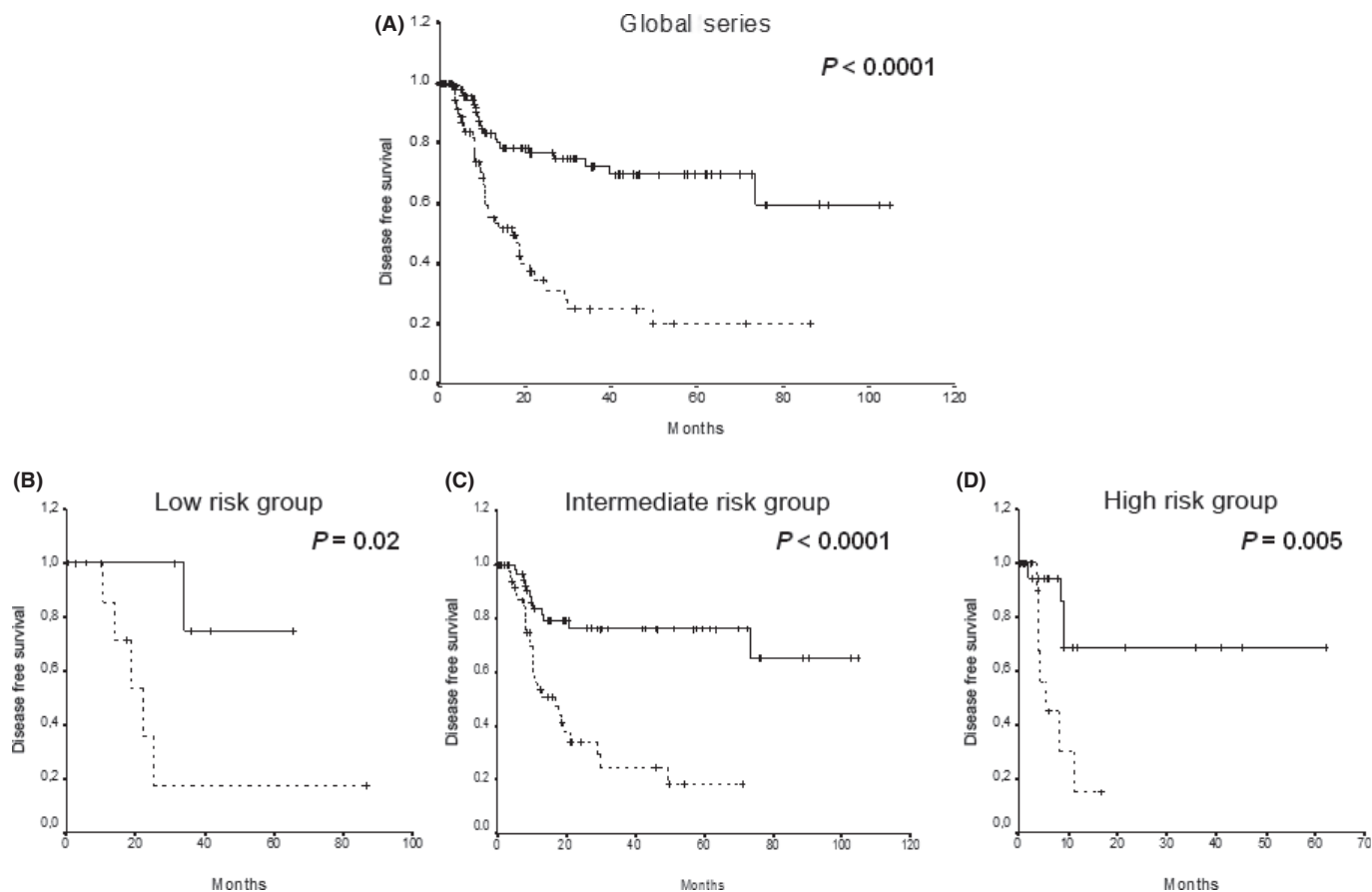
Recent studies have suggested that besides activating the canonical Wnt signaling, *WNT5A* may signal through the non-canonical Wnt/Ca<sup>2+</sup> pathway to suppress *CYCLIN D1* expression.<sup>(6)</sup> Consistent with this hypothesis, transcript levels of *CYCLIN D1* (mean normalized ratio:  $250 \pm 210\%$  vs  $88 \pm 114\%$ ,  $P < 0.001$ ) were significantly higher among *WNT5A* methylated patients compared with non-methylated patients (Fig. 2B).

**WNT5A methylation, clinical presentation, and outcome.** As shown in Table 1, aberrant *WNT5A* methylation was not associated with age, sex, white blood cell count, FAB classification, cytogenetic risk groups, or *FLT3* or *NPM1* mutations. Two hundred and one patients (80%) were eligible for intensive therapy. One hundred and thirty-five patients (54%) achieved CR (124

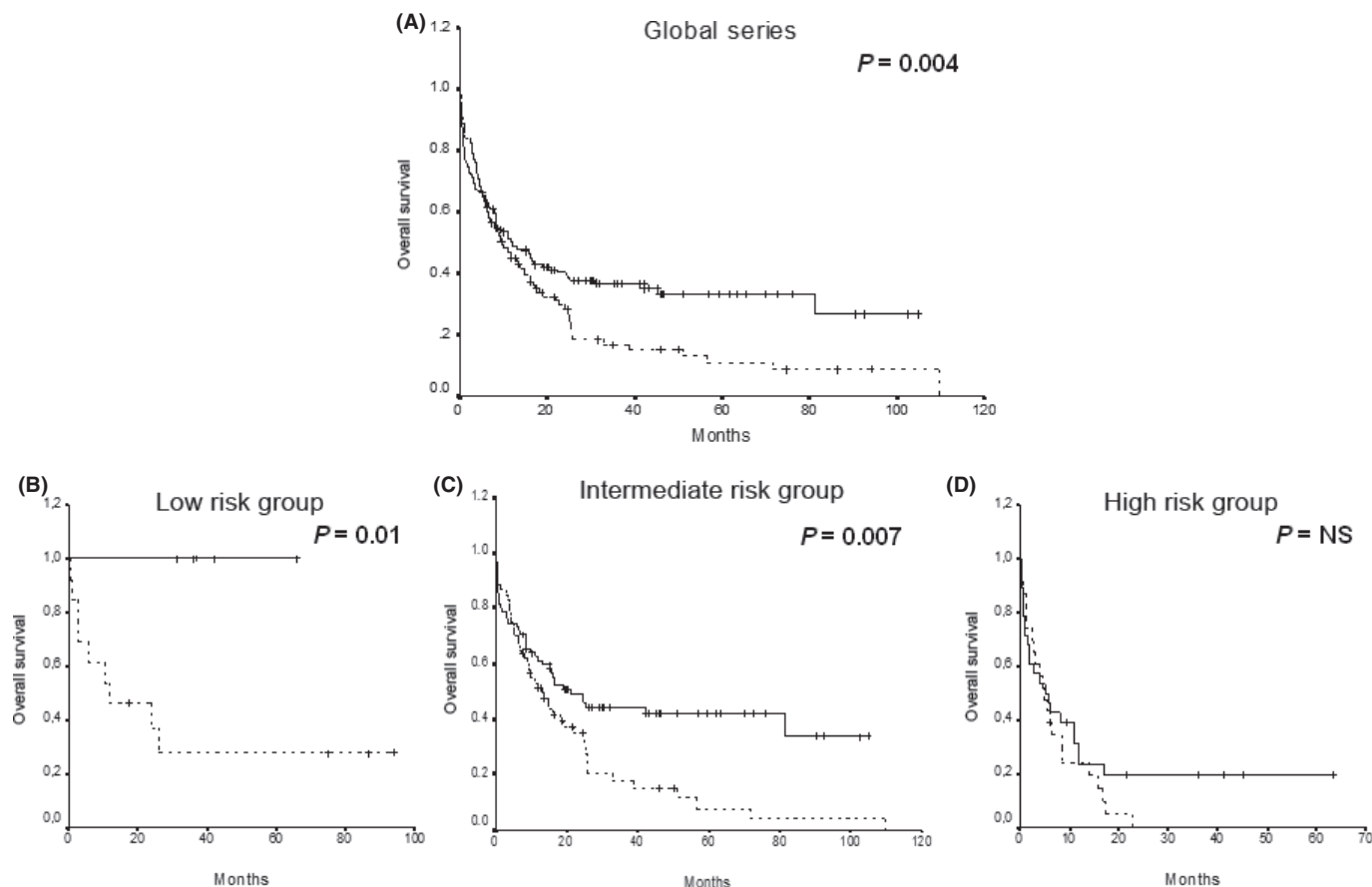
after one course of induction therapy and 11 after a second cycle). No effect of the *WNT5A* methylation status on the response to induction therapy was observed (Table 2). However, patients in the non-methylated group had a lower relapse rate than patients in the methylated group (15% vs 37%,  $P < 0.001$ ; Table 2). The mortality rate was also lower for the non-methylated group compared with the methylated group (61% vs 79%,  $P = 0.004$ ; Table 2).

We analyzed the DFS among patients who achieved CR according to *WNT5A* methylation. Estimated DFS rates at 7 years were 60% and 20% for normal and methylated patients, respectively ( $P < 0.0001$ ) (Fig. 3A). Among patients included in the cytogenetic low-risk group, 5-year DFS was 75% for unmethylated patients in contrast to 18% for methylated patients ( $P = 0.02$ ; Fig. 3B). Among intermediate-risk group patients, 6-year DFS was 18% for methylated cases and 65% for normal cases ( $P < 0.0001$ ; Fig. 3C). When the analysis was restricted to patients with high-risk cytogenetics, the methylated group had a significantly reduced DFS (15% vs 69%,  $P = 0.005$ ) at 1 year than the non-methylated group (Fig. 3D).

The actual OS calculated for all leukemic patients was 27% and 0% at 9 years for cases with normal and hypermethylated *WNT5A* gene, respectively ( $P = 0.04$ ; Fig. 4A). OS was also significantly different between normal and methylated patients in a separate analysis of the cytogenetic low-risk group (100% vs 28%,  $P = 0.01$ ; Fig. 4B) and intermediate-risk group (34% vs 0%,  $P = 0.007$ ; Fig. 4C) but not in the high-risk group (20% vs 0%,  $P =$  not significant; Fig. 4D).



**Fig. 3.** Kaplan–Meier disease-free survival (DFS) function for acute myeloid leukemia (AML) patients. DFS curves for (A) all of the patients included in this study, and for patients classified into cytogenetic (B) low-risk, (C) intermediate-risk, and (D) high-risk groups according to the *WNT5A* methylation status. Solid lines, *WNT5A* unmethylated patients. Dashed lines, *WNT5A* methylated patients.



**Fig. 4.** Kaplan–Meier overall survival (OS) function for acute myeloid leukemia (AML) patients. OS curves for (A) all of the patients included in this study, and for patients classified into cytogenetic (B) low-risk, (C) intermediate-risk, and (D) high-risk groups according to the *WNT5A* methylation status. Solid lines, *WNT5A* unmethylated patients. Dashed lines, *WNT5A* methylated patients.

Multivariate modelling of the global series took into account all prognostic variables including age, sex, FAB subtype, leukocytosis, cytogenetic risk group, and *FLT3* mutations, and the methylation profile showed that age over 60 years, cytogenetics, and *WNT5A* methylation status were independent prognostic factors for OS and DFS (Table S1).

## Discussion

In the present study, we show for the first time that the *WNT5A* promoter is subject to methylation silencing in AML cell lines. We analyzed its methylation by MSP and detected methylation in all cell lines with downregulated *WNT5A* expression. Detailed methylation analysis with bisulfite genomic sequencing confirmed the MSP analysis and revealed a high density of methylated CpG sites in these cell lines. After exposure to DAC, *WNT5A* expression was dramatically increased in silenced cell lines, indicating that epigenetic mechanisms seemed to deregulate the expression of *WNT5A* in AML. Importantly, *WNT5A* methylation was also observed in primary tumor samples from a large series of AML patients. Methylated *WNT5A* was observed in 43% of our patients at diagnosis, supporting the role of *WNT5A* methylation in the early phases of myeloid leukemogenesis.

*WNT5A* is located at 3p14, a commonly deleted tumor suppressor locus in multiple tumors. *Wnt5a* has been classified as a non-canonical and non-transforming Wnt protein,<sup>(31)</sup> with its role in tumorigenesis still ambiguous. There is evidence indicating that increased *WNT5A* expression is important for cancer progression, and that *WNT5A* was initially proposed as a proto-

oncogene.<sup>(32)</sup> *WNT5A* has been shown to be a potent enhancer of cell motility and invasiveness of melanoma,<sup>(33)</sup> upregulated in cancers of the lung, breast, stomach, and prostate.<sup>(34)</sup> On the other hand, in other tumor models, including brain, breast, thyroid, and uroepithelial cancers, *WNT5A* has been shown to inhibit tumor cell proliferation,<sup>(6,35,36)</sup> with its expression being a good prognostic marker for patients with breast and colon cancer.<sup>(37,38)</sup> These results not only suggest that dysregulation of *WNT5A* expression is involved in tumor pathogenesis but also that the function of *WNT5A* either as a suppressor or as a promoter of malignant progression is cell type specific.

In hematopoietic tissues, we and others have shown that *WNT5A* is frequently silenced by methylation in lymphoid malignancies in a tumor-specific manner, including natural killer/T-cell lymphomas, acute lymphoblastic leukemia, and Burkitt lymphomas but not in normal lymph nodes and peripheral blood mononuclear cells.<sup>(24,39)</sup> Further functional studies showed that ectopic expression of *WNT5A* resulted in inhibition of tumor cell growth and clonogenicity in lymphoid cell lines, consistent with previous studies showing that *WNT5A* inhibits B-cell proliferation and *WNT5A* heterozygous mice develop B-cell lymphomas,<sup>(6)</sup> suggesting that *WNT5A* could serve as a tumor suppressor in lymphoid neoplasms. However, the role of non-canonical Wnt signaling in myeloid leukemias is unknown and still controversial. *WNT5A* hemizygous mice develop myeloid leukemias and deletion of the *WNT5A* gene, loss of *WNT5A* expression, and increased cyclin D1 expression is observed in some AML patient samples.<sup>(6)</sup> Moreover, it has been recently demonstrated that mRNA expression of some of the Wnt

antagonists is upregulated in response to apoptotic stimuli in myeloid leukemia cell lines. The correlation between the increased expression of non-canonical Wnt genes and apoptotic stimulation in the HL-60 AML-derived cell line is a novel observation suggesting the possible involvement of the non-canonical Wnt pathway in executing programmed cell death.<sup>(40)</sup> These data and our present finding that methylation of *WNT5A* was present in 43% of AML patients with downregulated *WNT5A* expression further supports the hypothesis that *WNT5A* also functions as tumor suppressor gene in myeloid leukemogenesis.

In AML, hypermethylation of CpG islands in the promoter region is an epigenetic pathway that appears to be common to several genes involved in cell-cycle control, apoptosis and signaling.<sup>(41)</sup> Among the genes hypermethylated in AML, those that antagonize canonical Wnt signaling seem to play an important role. In that sense, we have recently demonstrated that expression of the Wnt inhibitors *sFRP1*, *sFRP2*, *sFRP4*, *sFRP5*, *DKK-1*, and *DKK-3* is downregulated due to abnormal promoter methylation in AML samples and this event correlates with constitutive activation of the canonical Wnt signaling pathway and upregulation of the *CYCLIN D1* target gene in AML.<sup>(23)</sup> Our present work completes the epigenetic picture of the Wnt pathways and shows that silencing of the *WNT5A* gene by inappropriate methylation is associated with high levels of *CYCLIN D1* expression and has clinical consequences conferring a dismal prognosis in AML. However, one can speculate that the upregulation of *CYCLIN D1* observed in our AML patients depends only on the activation of canonical Wnt signaling being the methylation of *WNT5A* an innocent bystander of the more extensive methylation of the canonical Wnt-antagonists.<sup>(23)</sup> Further studies are ongoing in our laboratory in order to rule out this point. In the present study we show that methylation of *WNT5A* provides important prognostic information in AML. The presence in individual tumors of *WNT5A* epigenetic events is a factor of poor prognosis in all AML cytogenetic risk groups. Patients with *WNT5A* methylation had a poorer DFS and OS than unmethylated patients. Multivariate analysis confirmed that methylation profile was associated with a shorter DFS and OS.

AML is a heterogeneous disease with different clinical behaviours, of which cytogenetics is the most important prognostic factor. In the present paper, we demonstrate that methylation status is able to redefine the prognosis of selected AML groups with well-established cytogenetic features. Lack of *WNT5A* methylation improved the general poor outcome of patients pre-

senting high-risk cytogenetic abnormalities, whereas presence of methylation worsened the general good outcome of low-risk cytogenetic patients. However, the most important finding of our report appears in the so-called intermediate-risk group, which comprised a heterogeneous group of patients with apparently normal karyotypes or patients with a variety of other aberrations whose prognostic outcome was uncertain. In this group of patients, better identification of prognostic indicators has been of particular interest in the past few years. *FLT3* mutations have appeared as an important unfavorable prognostic factor in this subgroup, allowing the classification of this vast group of AML patients with normal karyotype into two categories with distinct responses to treatment.<sup>(42)</sup> Our data show that the methylation profile of *WNT5A* is a new poor prognostic factor in the subgroup of patients with intermediate-risk cytogenetics. Therefore, *WNT5A* methylation profiling in AML could have important clinical implications complementing standard cytogenetic and molecular studies for guiding the selection of therapy and providing a basis for developing novel therapies.

In conclusion, we have demonstrated that *WNT5A* is silenced by methylation in AML and that this epigenetic event is associated with high *CYCLIN D1* expression and confers poor prognosis in this group of patients regardless of the cytogenetic features.

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## Authorship and Disclosure Statement

JR-G, XA and FP contributed to the conception and design of the study, and to the analysis and interpretation of data; AV, JC, MAS, AT and CH provided the study materials and revised the article; JR-G, XA, VM, ES-E, PR-O and AV-Z carried out the majority of the experiments and drafted the article.

The authors report no potential conflicts of interest.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Sequence of the *WNT5A* promoter CpG island after bisulfite modification showing the three regions studied and the primers for methylation-specific PCR analyses.

**Table S1.** Multivariate analysis for overall survival (OS) and disease-free survival (DFS).

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