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# WNT5A, a putative tumour suppressor of lymphoid malignancies, is inactivated by aberrant methylation in acute lymphoblastic leukaemia

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ABSTRACT

Wnt5a is a member of the Wnt family of proteins that signals through the non-canonical Wnt/$Ca^{2+}$ pathway to suppress cyclin D1 expression and negatively regulate B cell proliferation suggesting that it acts as an tumour suppressor for lymphoid leukemogenesis. Although canonical Wnt pathway is a 'hot spot' for methylation in acute lymphoblastic leukaemia (ALL), the role of *Wnt5a* abnormalities has never been evaluated in this clinical setting. The methylation status of the *WNT5A* promoter was analysed by methylation-specific PCR (MSP) and sequencing in six ALL-derived cell lines (TOM-1, NALM-20, MY, LOUCY, JURKAT and TANOUE) and in 307 ALL patients. *WNT5A* and *CYCLIN D1* expressions were assessed by quantitative RT-PCR. We observed *WNT5A* hypermethylation in all cell lines and in cells from 43% (132/307) of ALL patients. *WNT5A* methylation was associated with decreased *WNT5A* mRNA expression ($P < 0.001$) and this expression was restored after exposure to the demethylating agent 5-Aza-2′-deoxycytidine. Moreover, *WNT5A* hypermethylation correlated with upregulation of *CYCLIN D1* expression ($P = 0.002$). Disease-free survival (DFS) and overall survival (OS) at 13 and 14 years, respectively, were 59% and 53% for unmethylated patients and 28% and 31% for hypermethylated patients ($P = 0.0003$ and $P = 0.003$). Multivariate analysis demonstrated that *WNT5A* methylation was an independent prognostic factor predicting DFS ($P = 0.003$) and OS ($P = 0.04$). We have demonstrated that *WNT5A*, a putative tumour suppressor gene in ALL, 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 this group of patients.



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## 1. Introduction

Wnts are secreted proteins that exert their effects through the activation of distinct intracellular signalling pathways. Based on their biological activities in specific assays, vertebrate Wnt have been divided into canonical signalling with transforming activities in mammary epithelial cells and non-canonical pathways. Members of the canonical Wnt-signalling pathway act via Fz receptors to induce β-catenin stabilisation by prevention of its phosphorylation, which targets β-catenin for proteosomal degradation. Stabilised β-catenin in this canonical Wnt pathway enters the cell nucleus and associates with members of the T-cell factor and lymphoid enhancer factor (Tcf/Lef-1) family of transcription factors where it stimulates expression of Wnt/β-catenin target genes including regulators of cell growth and proliferation, modulators of cell death pathways and cell–cell communication.[1,2] This transforming activity of the Wnt/β-catenin pathway, which was initially delineated in the mammary epithelial cell line, C57MG, appears to be involved not only in the carcinogenesis of a number of human epithelial carcinomas[3–6] but also in leukaemogenesis. In fact, we have recently reported that epigenetic regulation is responsible at least in part for activation of the canonical Wnt signalling in acute lymphoblastic leukaemia (ALL).[7] We found that expression of the Wnt inhibitors *sFRP1*, *sFRP2*, *sFRP4*, *sFRP5*, *WIF1*, *DKK3* and *HDPR1* was downregulated due to the abnormal promoter methylation in ALL cell lines and this event was associated with constitutive activation of the Wnt-signalling pathway in ALL patients as demonstrated by the upregulation of the Wnt target genes *WNT16*, *FZ3*, *TCF1*, *LEF1* and *CYCLIN D1* and the nuclear localisation of β-catenin.

On the other hand, signalling and physiological function of the non-canonical group of Wnt-proteins is currently only incompletely understood. These Wnts do not transform C57MG cells. Indirect data in mammalian cells suggest that these non-transforming subclasses of Wnt-proteins, which include Wnt-4, -5a and -11, may trigger intracellular $Ca^{2+}$ release to activate $Ca^{2+}$-sensitive enzymes in a G-protein-dependent manner.[8,9] Wnt5a is a member of the Wnt family of secreted glycoproteins that play essential organising roles in development. Similar to other Wnt members, Wnt5a can upregulate cell proliferation and has been proposed to have oncogenic function.[10–14] However, it has been also reported that the Wnt5a signals through the non-canonical Wnt/$Ca^{2+}$ pathway to suppress CYCLIN D1 expression and negatively regulate B cell proliferation in a cell-autonomous manner.[15] Wnt5a hemizygous mice developed myeloid leukaemias and B cell lymphomas that were clonal in origin and displayed loss of Wnt5a function in tumour tissues. Furthermore, analysis of human primary leukaemias revealed loss of Wnt5a expression in a majority of the patient samples.[15] Interestingly, absence of Wnt5a expression was observed in ALL tumour specimens that retained *WNT5A* coding sequences. All these data suggest that *WNT5A* functions as a tumour suppressor gene in lymphoid leukemogenesis and also that downregulation of this gene in ALL might be due to epigenetic mechanisms that silence its promoter.

In this paper, we demonstrate that the putative tumour suppressor *WNT5A* gene is silenced by methylation in ALL and that this epigenetic event is associated with upregulation of *CYCLIN D1* expression and it is related to poor prognosis in this group of patients.

## 2. Materials and methods

### 2.1. Cell lines and patients

Six ALL-derived cell lines (TOM-1, NALM-20, MY, LOUCY, JURKAT and TANOUE) were purchased from the DSMZ (Deutche Sammlung von Microorganismen und Zellkulturen GmbH, Braunschweig, Germany). Cells were grown at 37 °C under 5% $CO_2$ in humidified air in an RPMI medium (Gibco-BRL, Burlington, Ontario, Canada) supplemented with 20 foetal bovine serum (Biochrome), 1% penicillin/streptomycin and 1% HEPES (Gibco-BRL, Burlington, Ontario, Canada).

We studied 307 consecutive patients (188 male; 119 female) who were diagnosed with *de novo* ALL between January 1990 and December 2004. The median age at diagnosis in the study population as a whole was 14 years (range, 0.5–82 years). Of these patients, 148 were children (median age, 5 years; range, 0.5–14 years) and 159 presented adult ALL (median age, 29 years; range, 15–82 years). Informed consent was obtained from the patients or the patient's guardians. Diagnosis was established according to standard morphologic, cytochemical and immunophenotypic criteria. Patients were studied at the time of initial diagnosis; were risk-stratified according to the therapeutic protocol used, which was always based on recognised prognostic features (including cytogenetics); and were entered in ALL protocols of the *Programa para el estudio y tratamiento de las hemopatias malignas* (PETHEMA) Spanish study group. For statistical analyses, children were also grouped according to the National Cancer Institute (NCI) risk-classification criteria.[16] The specific PETHEMA ALL treatment protocols in which these patients entered included ALL-89 (between 1990 and 1993; $n = 51$) and ALL-93 (between 1993 and 2004; $n = 256$). The design and results of these studies have been previously reported .[17–20] One hundred and twenty-two patients relapsed. Fifty-three patients received stem cell transplantation (14 autologous, 39 allogeneic) in the first ($n = 20$) or second complete remission (CR) ($n = 33$). There are 143 patients currently alive. Clinical characteristics of the patients are listed in Table 1. Some of these patients ($n = 204$) were typed previously for methylation of seven inhibitors of the canonical Wnt-signalling pathway (*DKK3*, *sFRP1*, *sFRP2*, *sFRP4*, *sFRP5*, *WIF1* and *HDPR1*).[7]

### 2.2. Methylation-specific PCR (MSP)

Analysis of the *WNT5A* (GeneBank: NC_000003) has revealed that *WNT5A* possesses a 229 bp CpG island located between nt47 and nt225, showing >60% C + G content and an observed-over-expected CpG frequency of >0.6. Methylation status of the CpG islands in the *WNT5A* gene promoter was performed by genomic DNA bisulphite treatment followed by MSP as reported by Herman et al.[21] Primer sequences for the MSP amplification were Wnt5a-MD (5′-GTATTTTTCGGA-

**Table 1 – Clinical characteristics and outcome of 307 ALL patients according to WNT5A methylation status**

| Feature | Non-methylated ($n$ = 175) | Methylated ($n$ = 132) | $P$-value |
|---|---|---|---|
| *Age*, % | | | |
| <15 years | 65 | 35 | **0.01** |
| >15 years | 49 | 51 | |
| *Sex* (M/F), % | 58/42 | 60/40 | NS |
| WBC, % | | | |
| $<50 \times 10^9$/L | 55 | 45 | NS |
| $>50 \times 10^9$/L | 59 | 41 | |
| *FAB classification*, % | | | |
| L1 | 48 | 52 | NS |
| L2 | 50 | 50 | |
| L3 | 46 | 54 | |
| *Blast lineage*, % | | | |
| B cell | 55 | 45 | NS |
| T cell | 50 | 50 | |
| *NCI risk groups*, % | | | |
| Standard | 60 | 40 | NS |
| Poor | 55 | 45 | |
| *PETHEMA risk groups*, % | | | |
| Standard | 42 | 58 | NS |
| Poor | 45 | 55 | |
| *Treatment*, % | | | |
| PETHEMA 89 | 49 | 51 | NS |
| PETHEMA 93 | 52 | 48 | |
| BMT, % | 49 | 51 | NS |
| *Best response*, % | | | |
| CR | 92 | 90 | NS |
| *Cytogenetic/molecular abnormalities*, % | | | |
| BCR-ABL | 39 | 54 | **0.05** |
| t(1;19) | 12 | 15 | NS |
| 11q23 | 3 | 3 | NS |
| c-myc | 6 | 8 | NS |
| 7q35-14q11 | 10 | 9 | NS |
| Hyperdiploidy | 11 | 14 | NS |
| TEL-AML1 | 77 | 47 | **0.005** |
| Normal | 40 | 48 | NS |
| Others | 3 | 3 | NS |
| NT | 7 | 6 | NS |
| Relapse | 36 | 64 | **<0.001** |
| Death | 44 | 64 | **0.005** |

Data are expressed as percentages.
WBC indicates white blood count; FAB, French–American–British; NCI, National Cancer Institute; PETHEMA, *Programa para el estudio y tratamiento de las hemopatias malignas*; BMT, bone marrow transplantation; CR, complete remission. NT, non-tested.

GAAAAAGTTATGC-3′) and Wnt5a-MR (5′-ACAACCGCGAATTAATATAAACG-3′) for the methylated reaction and Wnt5a-UD (5′-GGTATTTTTTGGAGAAAAAGTTATGTG-3′) and Wnt5a-UR (5′-CTACAACCACAAATTAATATAAACATC-3′) for the unmethylated reaction. 'Hot start' PCR was performed 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 2% agarose gel. Bone marrow mononuclear cell and peripheral lymphocyte DNAs from healthy donors were used as negative control for methylation-specific assays. Human male genomic DNA universally methylated for all genes (Intergen Company, Purchase, NY) 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 bisulphite treatment.

### 2.3. *Expression analyses*

Expression of *WNT5A* and the *WNT5A* target gene *CYCLIN D1* was analysed by the RT-PCR technique. Total RNA was extracted from marrow samples with Ultraspec (Biotecx, Houston, TX) following the manufacturer's instructions. Reverse transcription was performed on 1 μg total RNA, after heating at 70 °C for 5 min, with random hexamers as 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 performed with the LightCycler technology using 1 μl of cDNA in 20 μl reaction volume with 0.4 μmol/l each primer and 2 μl of 10× LightCycler FastStar DNA Master SYBR Green I (Roche Molecular Biochemicals). The final $Mg^{2+}$ concentration in the reaction mixture was adjusted to 3.5 mmol/l. Primers for *WNT5A* expression were: forward, 5′-CCACATGCAGTACATCGGAG-3′ and reverse, 5′-TGCCGGAAGTGTATGCG-3′ and for *CYCLIN D1*: forward, 5′-CCCTCGGTGTCCTACTTCAAATGT-3′ and reverse, 5′-TGATCTGTTTGTTCTCCTCCGCCT-3′. The following programme conditions were applied for qrt-PCR running: denaturation programme, consisting of one cycle at 95 °C for 8 min; amplification programme, consisting of 45 cycles at 95 °C for 5 s, 60 °C for 10 s and 72 °C for 15 s; melting programme, one cycle at 95 °C for 0 s, 40 °C for 60 s and 90 °C for 0 s; and cooling programme, one cycle at 40 °C for 60 s. The temperature transition rate was 20 °C/s, except in the melting programme, which was 0.4 °C/s between 40 °C and 90 °C. *Abelson* gene (*ABL1*) was employed as reference gene, and it was amplified in the same run and following the same procedure described above (forward: 5′-CCCAACCTTTTCGTTGCACTGT-3′; reverse: 5′-CGGCTCTCGGAGGAGACGTAGA-3′). 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 performed by LightCycler software (RealQuant, version 1.0, Roche). The normalised ratios ($N$) for each gene were obtained from the next equation and expressed as percentage of the control/calibrator:

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

Efficiencies ($E$) of each gene were calculated from the slopes of crossover points (Cp) versus DNA concentration plot, according to the formula $E = 10^{(-1/\text{slope})}$. ΔCp 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 lymphocytes from healthy donors. They were considered as 100% expression.

### 2.4. WNT5A CpG island analysis by sequencing after sodium bisulphite modification

Methylation status of *WNT5A* CpG island was analysed by bisulphite genomic sequencing in TOM-1, NALM-20, JURKAT and LOUCY ALL-derived cell lines and bone lymphocyte DNA from healthy donors. Genomic DNA (1 μg) was treated and modified using the CpGenomic™ DNA Modification Kit (Intergen Company, Purchase, NY). Human male genomic DNA universally methylated for all genes (Intergen Company, Purchase, NY) was used as a positive or methylated control. After bisulphite modification, *WNT5A* CpG island was amplified by PCR using 3 μl of modified DNA and Wnt5a-SB1 (5′-GGGGTTGATTTTTGTAGTTTAGATG-3′) and Wnt5a-SB2 (5′-TTAAAACTTTCCAACCCCAAATATA-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 reaction was 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 mM $MgCl_2$, 0.2 mM dNTPs and 50 pmol of each primer. The PCR products were separated on a 2% agarose gel, stained with ethidium bromide and visualised under UV light. Amplification products, containing 21 CpG dinucleotides, were subcloned into pCR® 4-TOPO® plasmid using TOPO TA Cloning® Kit for Sequencing (Invitrogen Life Technologies, Paisley, UK) 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 EcoR I (Amersham Biosciences, Buckinghamshire, UK). Candidate plasmid clones were sequenced using T7 and T3 universal forward and reverse primers.

### 2.5. Treatment with the demethylating agent 5-Aza-2′-deoxycytidine (Aza)

ALL-derived TOM-1 and NALM-20 cell lines were grown at a density of 600,000 cells/ml in 25 $cm^2$ flasks with 10 ml of RPMI 1640 medium supplemented with 20% foetal bovine serum and maintained at 37 °C in a humid atmosphere containing 5% $CO_2$. Cell lines were treated with 4 μM of 5-Aza-2′-deoxycytidine (Sigma–Aldrich, Steinheim, Germany) for 4 days. After treatment, cells were washed in PBS, pelleted by centrifugation at 1500 rpm for 5 min and used for genomic DNA and RNA isolation. DNA was extracted using QIAmp DNA Mini Kit (Qiagen, Hilden, Germany) and total RNA using Rneasy® Mini Kit (Qiagen, Hilden, Germany). One microgram of total RNA was used for cDNA synthesis using SuperScript™ II RNase H-RT (Invitrogen Life Technologies, Paisley, UK) with random hexamers.

### 2.6. Statistical analysis

*P*-values for comparisons of continuous variables between groups of patients were two-tailed and based on the Wilcoxon rank sum test. *P*-values for dichotomous variables were based on the Fisher exact test. The remaining *P*-values were based on the Pearson $\chi^2$-test. Overall survival (OS) was measured from the day of diagnosis until death from any cause and was only censored for patients known to be alive at last contact. Disease-free survival (DFS) was measured from the day that complete response (CR) was established until either relapse or death without relapse, and it was only censored for patients who were alive without evidence of relapse at the last follow-up. Distributions of OS and DFS curves were estimated by the method of Kaplan and Meier with 95% confidence intervals calculated using Greenwood's formula. Comparisons of OS or DFS between groups were based on the log-rank test. Comparisons adjusted for significant prognostic factors were based on Cox regression models and hazard regression models. All relapse and survival data were updated on 31st December 2006 and all follow-up data were censored at this point.

## 3. Results

### 3.1. Expression of WNT5A gene in ALL-derived cell lines is regulated by promoter hypermethylation

Quantitative expression of *WNT5A* transcripts was assessed by means of qrt-PCR using cDNA from a healthy donor as control (it was considered as 100% of $N_{wnt5a}$ ratio). Low levels of *WNT5A* expression were observed in all the ALL cell lines studied (Fig. 1a). Amongst all the possible mechanisms of transcription regulation, we first decided to study the possible hypermethylation of *WNT5A* promoter because we have previously demonstrated that epigenetic events are important regulating mechanisms in ALL playing a role in its pathogenesis and clinical behaviour.[22–24] By MSP, the *WNT5A* promoter was revealed to be methylated in ALL-derived cell lines with low *WNT5A* expression (Fig. 1b) which was clearly in contrast with the lack of *WNT5A* promoter methylation observed in normal lymphocytes (Fig. 1b). Moreover, in order to confirm the results of the MSP, we screened bone marrow DNA from healthy donors and four ALL-derived cell lines (TOM-1, NALM-20, JURKAT and LOUCY) using bisulphite genomic sequencing. As described in Section 2 the amplification products of *WNT5A* promoter which are composed by 21 CpG dinucleotides, were subcloned and sequenced. Genomic sequencing after bisulphite modification revealed that lymphocyte DNA from healthy donors was completely unmethylated, showing the 21 analysed CpG dinucleotides unmethylated in all analysed clones. In contrast, ALL cell lines were heavily methylated in almost all analysed CpG dinucleotides and clones (Fig. 2). Exposure of ALL cell lines to the demethylating agent 5-Aza-2′-deoxycytidine (Fig. 3a) restored expression of *WNT5A* mRNA in two cell lines (TOM-1 and NALM-20) that showed hypermethylation of the *WNT5A* promoter (Fig. 3b). These results indicate that hypermethylation is an important mechanism through which *WNT5A* expression is downregulated in ALL cell lines.

### 3.2. WNT5A hypermethylation in ALL patients is associated with under-expression of WNT5A transcripts

Amongst ALL patients, hypermethylation of *WNT5A* gene was observed in 43% (132/307) of samples obtained at diagnosis (Fig. 4a). In order to study the relationship between methylation and *WNT5A* expression, normalised ratios for *WNT5A* expression were determined in bone marrow specimens from 30 healthy individuals. $N_{wnt5a}$ ratios fell between 80% and

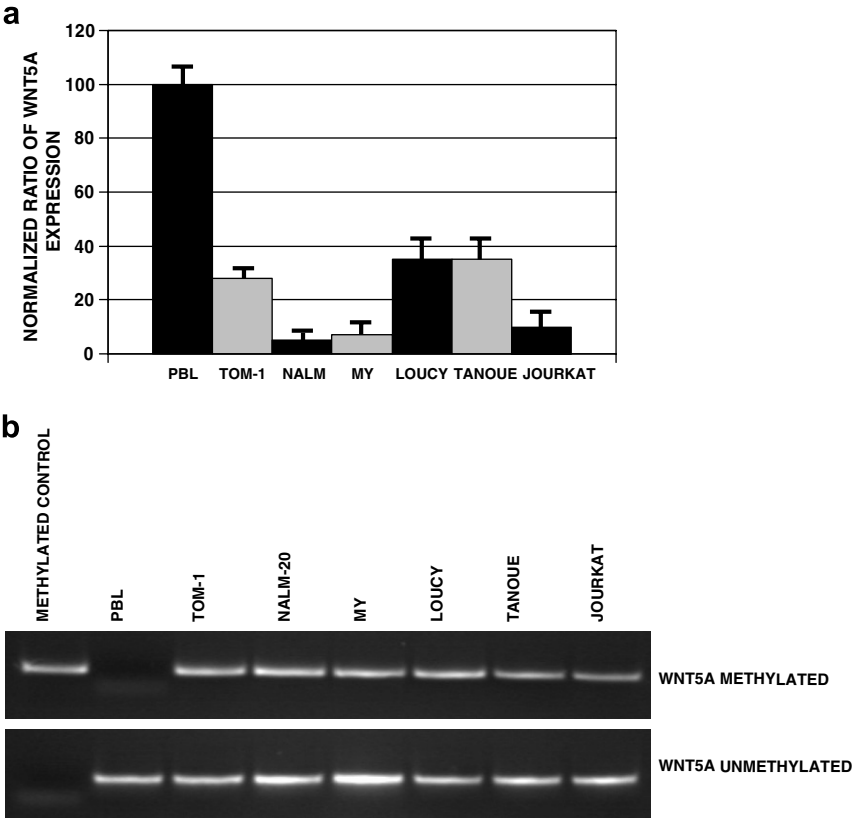


**Fig. 1 – Expression and methylation analysis of *WNT5A* gene in ALL-derived cell lines. (Panel a) Expression of WNT5A gene at mRNA level in ALL-derived cell lines demonstrating a downregulation of gene expression in all the cell lines. Gene expression was normalised with expression in peripheral blood lymphocytes from healthy donor (PBL, Normalised ratio = 100%). The mean ± SD of three different experiments is shown. (Panel b) MSP analysis of *WNT5A* gene in the same ALL-derived cell lines. *Wnt5a* was methylated in all the cell lines.**

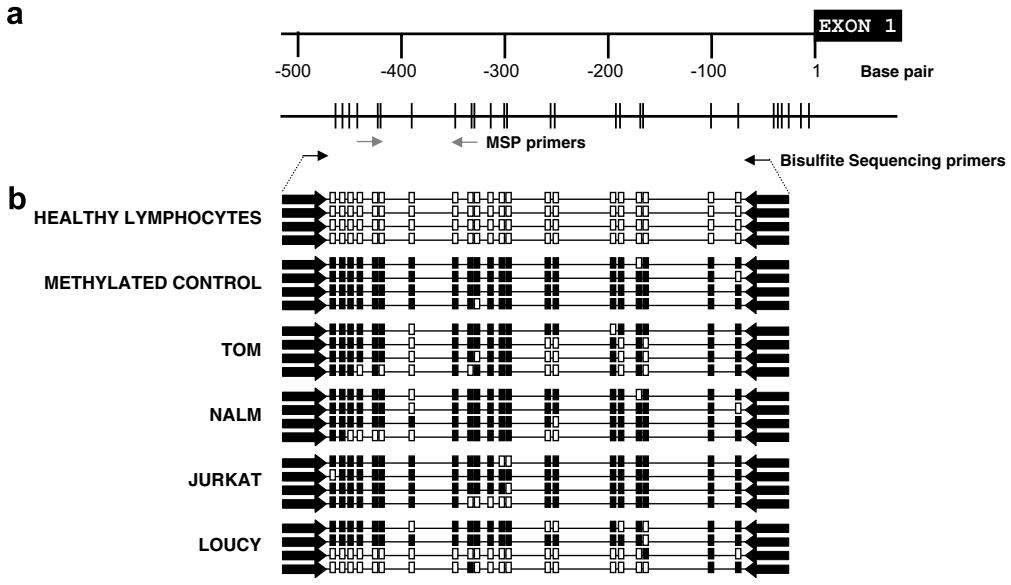


**Fig. 2 – Analysis of *WNT5A* CpG island methylation status by bisulphite sequencing in ALL-derived cell lines. (Panel a) Schematic description of the *WNT5A* CpG island. Each vertical bar represents a CpG dinucleotide. The grey arrows show the location of the MSP primers and the black arrows the location of bisulphite sequencing primers. (Panel b) Bisulphite sequencing of the *WNT5A* CpG island. 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 *WNT5A* analysed region.**

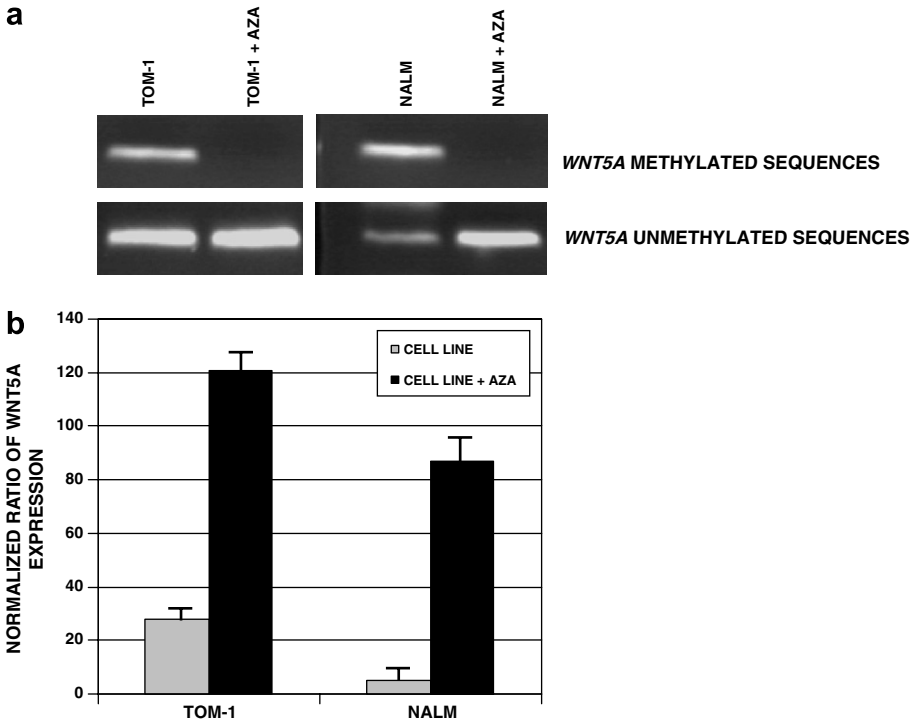


**Fig. 3 – MSP and expression analysis of *WNT5A* gene in methylated ALL-derived cell lines before and after treatment with 5-Aza-2′-deoxycytidine (Aza). (Panel a) MSP analysis of *WNT5A* gene in the TOM-1 and NALM-20 cell lines before and after treatment with the demethylating agent 5-Aza-2′-deoxycytidine demonstrating hypomethylation of the gene after treatment. (Panel b) Expression analysis of *WNT5A* gene in methylated TOM-1 and NALM-20 cell lines before and after treatment with the demethylating agent 5-Aza-2′-deoxycytidine demonstrating upregulation of the gene after treatment. Gene expression was normalised with expression in normal lymphocytes (Normalised ratio = 100%). The mean ± SD of three different experiments is shown.**

100% (mean $N_{wnt5a}$: 90% ± 7%). Amongst ALL patients, those with unmethylated *WNT5A* showed a mean $N_{wnt5a}$ of 81% ± 43%, similar to that found in healthy individuals, however, mean $N_{wnt5a}$ was significantly lower in those ALL patients who showed *WNT5A* hypermethylation (4% ± 20%, $P < 0.001$, Fig. 4b). Moreover, a $N_{wnt5a}$ value equal to or below 69% (determined as the mean $N_{wnt5a}$ from normal individuals minus 3 SD) was chosen to define under-expression of *WNT5A* in ALL samples. Using this cutoff value, downregulation of *WNT5A* was found in 85% of ALL patients with *WNT5A* hypermethylated and in only 7% of ALL patients with unmethylated *WNT5A* ($P < 0.001$).

The presence of promoter hypermethylation in seven antagonists of the canonical Wnt pathway (*DKK3*, *sFRP1*, *sFRP2*, *sFRP4*, *sFRP5*, *WIF1* and *HDPR1*) was determined previously for 204 of these cases.[7] There was a significant concordance between *WNT5A* methylation and the presence of methylation in at least one Wnt-antagonist; *WNT5A* methylation was present in 68 of 136 (50%) Wnt-antagonist methylated tumours versus 19 of 68 (29%) Wnt-antagonist unmethylated tumours ($P = 0.003$).

### 3.3. *WNT5A methylation is associated with upregulation of CYCLIN D1 in ALL*

Recent studies have suggested that *WNT5A* besides activating the canonical Wnt signalling may signal through the non-canonical Wnt/$Ca^{2+}$ pathway to suppress *CYCLIN D1* expression.[15] Consistent with this hypothesis, transcript levels of *CYCLIN D1* (mean *N*: 1770% versus 670%, $P = 0.002$) were significantly higher amongst *WNT5A* methylated patients compared with non-methylated patients. However, it is well established that *CYCLIN D1* expression not only depends on the non-canonical Wnt pathway but also the canonical pathway. For this reason, we analysed separately the role of the methylation of each pathway in the upregulation of *CYCLIN D1*. Significantly higher levels of *CYCLIN D1* were observed in both patients with only *WNT5A* methylation (554% versus 244%, $P = 0.026$) and patients with only methylation in the antagonists of the canonical Wnt pathway (1409% versus 244%, $P = 0.001$) compared with unmethylated patients in both pathways (Fig. 5). Interestingly, *CYCLIN D1* was further upregulated when both *WNT5A* and Wnt-antagonists were epigenetically inactivated (Fig. 5).

### 3.4. *WNT5A methylation, clinical presentation and outcome*

As shown in Table 1, aberrant *WNT5A* methylation was more frequently observed in adults (51%) than in children (35%, $P = 0.01$). In addition, lack of *TEL-AML1* fusion gene (51% versus 23%, $P = 0.005$) and presence of *BCR-ABL* rearrangement (54% versus 39%, $P = 0.05$) were significantly associated with the methylated group. However, other features including the

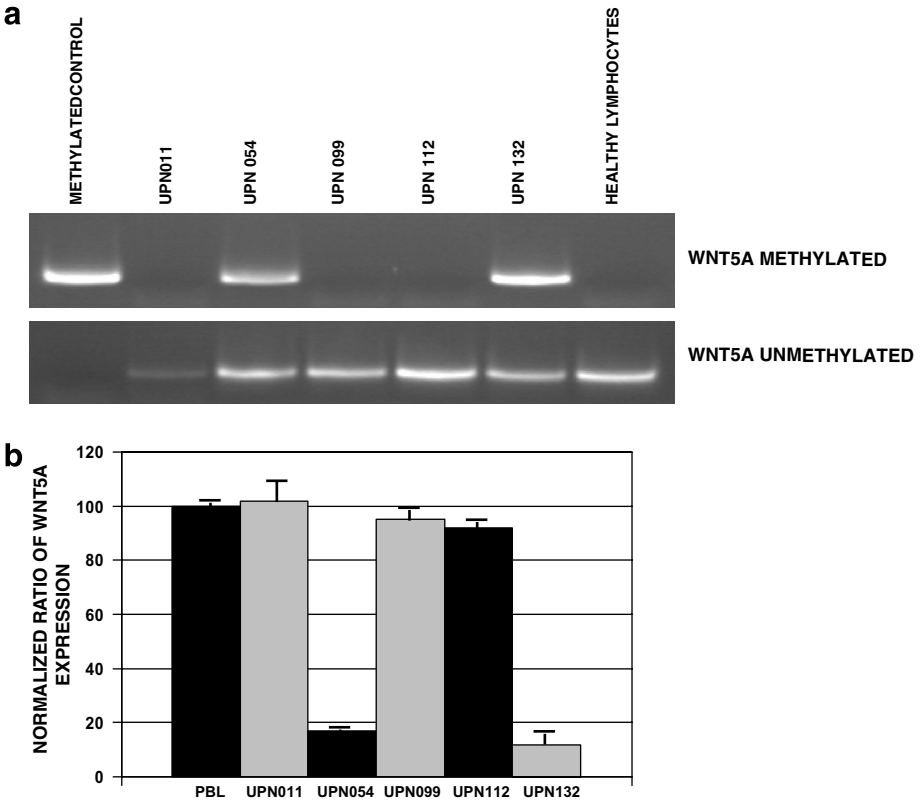


**Fig. 4 – Promoter hypermethylation and expression of *WNT5A* gene in samples from ALL patients. MSP (Panel a) and expression (Panel b) analyses of *WNT5A* gene in patients (UPN) with ALL. Methylated patients showed downregulation of *WNT5A* expression. Gene expression was normalised with expression in normal lymphocytes (Normalised ratio = 100%). The mean ± SD of three different experiments is shown.**

type of PETHEMA protocol administered, and the number of patients who received stem cell transplantation was similarly distributed amongst both methylation groups.

Table 1 also details the relapse history, CR rates and mortality for patients who exhibited *WNT5A* methylation and the equivalent data for patients with the unmethylated *WNT5A* gene. CR rates of patients with unmethylated and methylated *WNT5A* gene were 90% and 92%, respectively, accounting for 91% of the overall CR rate. This suggests that methylation of the *WNT5A* gene did not correlate with the response to remission induction therapy. However, patients in the non-methylated group had a lower relapse rate than patients in the methylated group (36% versus 64%, $P < 0.001$). Mortality rate was also lower for non-methylated group compared with methylated group (44% versus 64%, $P = 0.005$). Similar results were obtained in the separate analyses of children (relapse rate, 22% for non-methylated group versus 58% for methylated group, $P = 0.001$; mortality rate, 17% for non-methylated group versus 38% for methylated group, $P = 0.02$) but not in adults (relapse rate, 56% for non-methylated group versus 67% for methylated group, $P$ = NS; mortality rate, 74% for non-methylated group versus 67% for methylated group, $P$ = NS).

We analysed the DFS amongst patients who achieved CR according to *WNT5A* methylation. Estimated DFS rates at 13 years were 59% and 28% for unmethylated and methylated patients, respectively, ($P = 0.0003$) (Fig. 6a). Amongst unmethy-

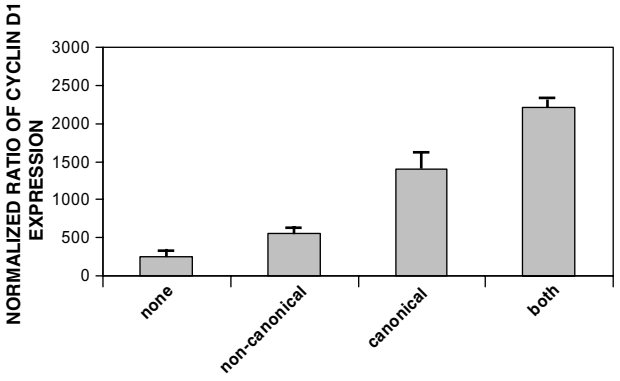


**Fig. 5 – Hypermethylation of *WNT5A* gene is associated with upregulation of *CYCLIN D1* in ALL patients. Expression of the *CYCLIN D1* gene in ALL patients was measured by Q-RT-PCR. Significantly higher levels of *CYCLIN D1* transcripts were detected in patients with only *WNT5A* methylation (non-canonical) and patients with only methylation of the canonical Wnt-antagonists (canonical) compared with unmethylated patients (none). Further upregulation was observed when both pathways were methylated. Bars represent the mean expression (95% CI) of three different experiments in patients with ALL in comparison with healthy lymphocytes (Normalised ratio = 100%).**

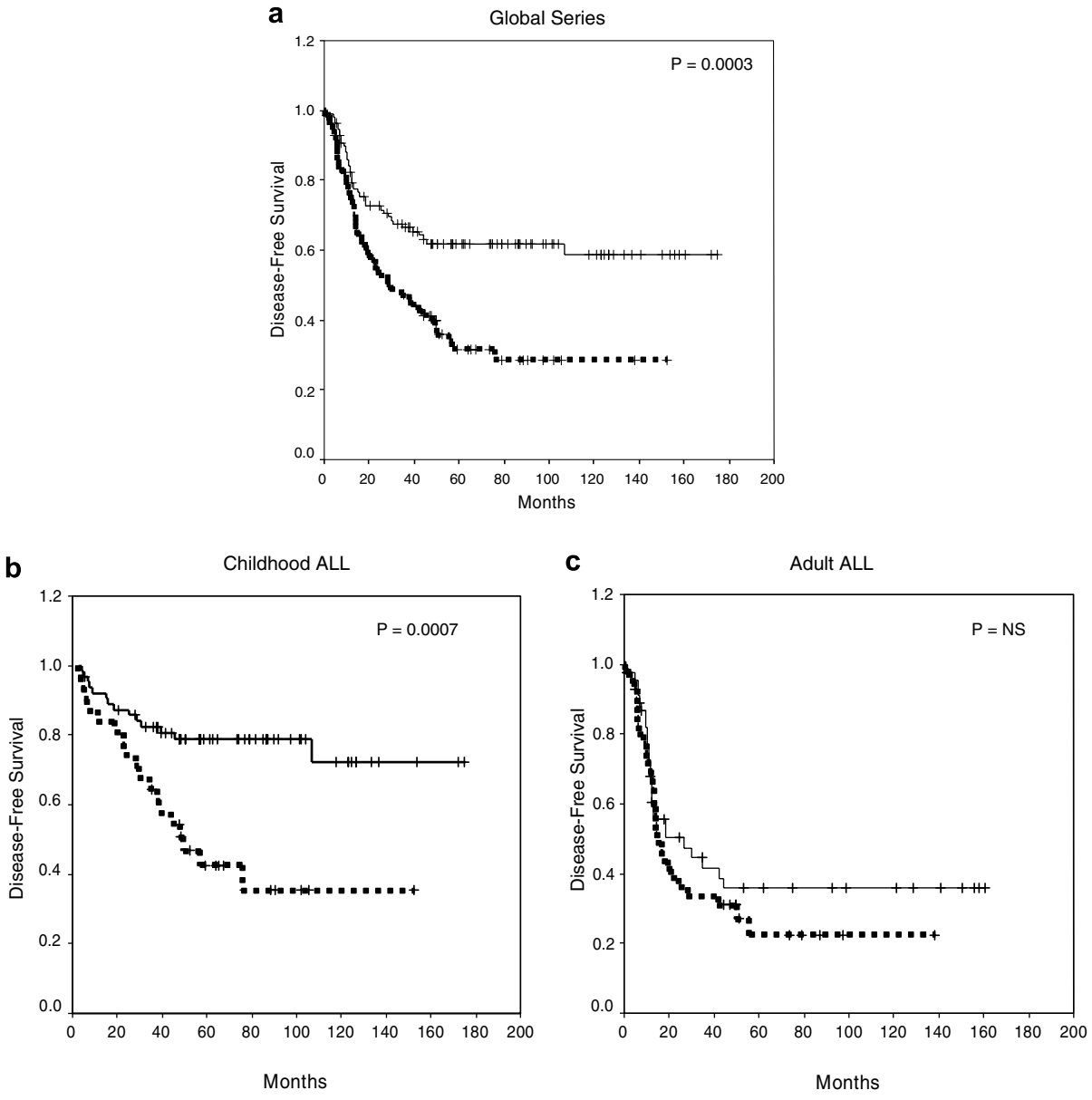


**Fig. 6 – Kaplan–Meier survivor function for ALL patients. DFS curves for all the patients enrolled in this study (Panel a), childhood ALL (Panel b) and adult ALL (Panel c) according to the *WNT5A* methylation status. Solid lines, *WNT5A* unmethylated patients. Dashed lines, *WNT5A* methylated patients.**

lated children, the 13 years DFS was 72% contrasting with 35% for hypermethylated children ($P = 0.0007$, Fig. 6b). Amongst adult ALL patients, the 12-years DFS was 22% for methylated cases and 36% for unmethylated cases ($P$ = NS, Fig. 6c). The actuarial OS calculated for all leukemic patients was 53% and 31% at 14 years for cases with unmethylated and hypermethylated *WNT5A* gene, respectively ($P = 0.003$, Fig. 7a). OS was only significantly different between unmethylated and methylated patients in the separate analysis of children (76% versus 57%, $P = 0.02$, Fig. 7b) but not in adults (25% versus 16%, $P$ = NS, Fig. 7c).

A multivariate analysis of potential prognostic factors (including the type of PETHEMA protocol applied) demonstrated that hypermethylation profile was an independent prognostic factor in predicting DFS in the global series ($P = 0.003$) as well as in childhood ALL ($P = 0.01$, Table 2). Methylation status was also independently associated with OS in the global series ($P = 0.04$) and childhood ALL ($P = 0.05$; Table 3).

## 4. Discussion

In this study, we describe for the first time that the *WNT5A* expression in ALL cells is epigenetically regulated by hypermethylation of its promoter. The causal relationship between hypermethylation and expression is further suggested by the fact that treatment of leukemic cells with 5-Aza-2-deoxycytidine induces demethylation of the *WNT5A* promoter region and results in mRNA expression of *WNT5A*. The finding that a significant percentage of patients with ALL have a downreg-

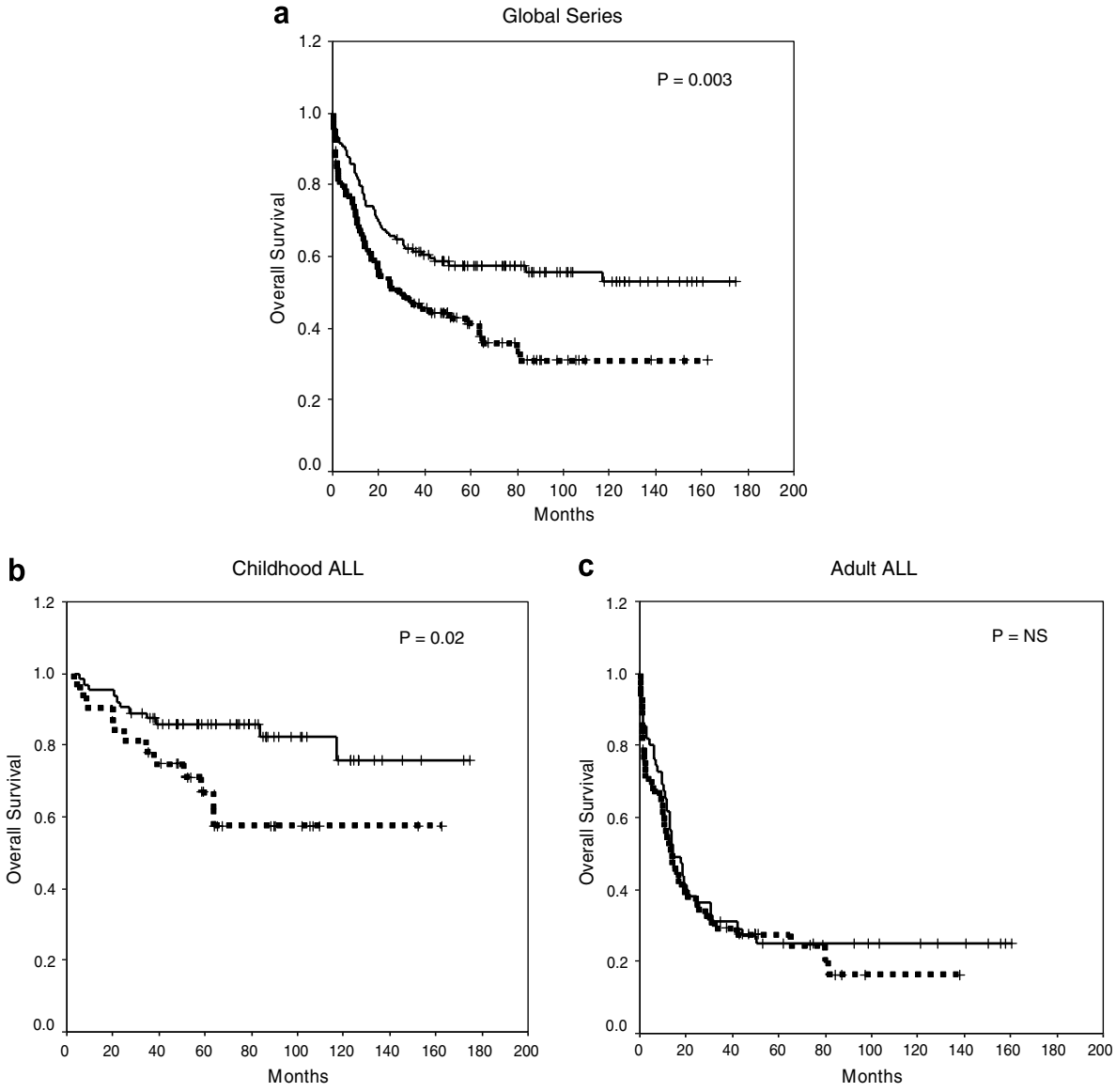


**Fig. 7 – Kaplan–Meier survivor function for ALL patients. OS curves for all the patients enrolled in this study (Panel a), childhood ALL (Panel b) and adult ALL (Panel c) according to the *WNT5A* methylation status. Solid lines, *WNT5A* unmethylated patients. Dashed lines, *WNT5A* methylated patients.**

ulation of *WNT5A* at diagnosis suggests that its inactivation is a frequent and early event in the process of leukemogenesis and probably has a potential role in the pathogenesis of the disease.

The clinical significance of *WNT5A* expression in human cancers is still unclear and also rather controversial. *WNT5A* was initially reported to belong to non-transforming members of *Wnts*, according to their ability to transform C57MG mammary epithelial cells.[8,9] However many studies have reported that *Wnt5a* also can induce cell proliferation in several cell lines and tissues.[10–14] For instance, Lejeune et al.[25] have found that, compared with normal breast tissue, benign and invasive breast tumours, respectively, show 10-fold and 4-fold higher levels of *WNT5A* mRNA. Up-regulation of the *WNT5A* mRNA has also been reported in several other types of cancer, such as malignant melanoma and lung, colon and prostate cancers.[10–14,26] On the other hand, evaluations of the *WNT5A* mRNA level in other human cancers contradict the notion that *WNT5A* is involved in tumour progression acting as oncogene and instead suggest that the human *WNT5A* gene displays tumour suppressor activity. In fact, It has been recently demonstrated that *WNT5A* may act as a tumour suppressor in haematopoietic tissue as it inhibits B-cell proliferation .[15] Our present finding that methylation of the *WNT5A* was present in 85% of ALL patients with downregulated *WNT5A* expression further supports the hypothesis that *WNT5A* functions as tumour suppressor gene in lymphoid leukemogenesis and also supports the notion that the function of *WNT5A* either as a suppressor or as a promoter of malignant progression is cell type specific.

Concerning the regulation of *WNT5A* expression, both gene amplification and gene rearrangement have been reported to

**Table 2 – Multivariate Cox model for disease free survival (DFS)**

| Feature | Univariate analysis | Multivariate analysis |
|---|---|---|
| | *P*-value | *P*-value |
| *Global series* | | |
| *WNT5A* Methylation status | <0.001 | 0.003 |
| WBC > $50 \times 10^9$/L | 0.015 | NS |
| BCR-ABL positivity | <0.001 | <0.001 |
| T-phenotype | 0.05 | 0.03 |
| Age > 15 years | <0.001 | 0.001 |
| PETHEMA poor risk | 0.05 | NS |
| *Chilhood ALL* | | |
| *WNT5A* Methylation status | 0.006 | 0.01 |
| NCI poor risk | 0.04 | NS |
| T-phenotype | 0.04 | NS |
| WBC > $50 \times 10^9$/L | 0.03 | NS |

**Table 3 – Multivariate Cox model for overall survival (OS)**

| Feature | Univariate analysis | Multivariate analysis |
|---|---|---|
| | *P*-value | *P*-value |
| *Global series* | | |
| *WNT5A* Methylation status | 0.01 | 0.04 |
| WBC > $50 \times 10^9$/L | 0.003 | 0.07 |
| BCR-ABL positivity | <0.001 | <0.001 |
| Age > 15 years | <0.001 | <0.001 |
| PETHEMA poor risk | 0.08 | NS |
| *Chilhood ALL* | | |
| *WNT5A* Methylation status | 0.01 | 0.05 |
| NCI poor risk | 0.09 | NS |
| T-phenotype | 0.05 | NS |
| WBC > $50 \times 10^9$/L | 0.07 | NS |

occur infrequently in human cancers.[26] In contrast, experimental studies using cell lines demonstrated the *WNT5A* expression to be regulated by various molecules, including hepatocyte growth factor, protein kinase C activity and MRP-1/CD9.[27–29] Such mechanisms could be implicated in the down-regulation of *WNT5A* observed in a minority of ALLs because we have found that only 7% of ALL patients showed under-expression of *WNT5A* gene despite the absence of promoter methylation. However, our work clearly demonstrates that hypermethylation is the main mechanism of transcription regulation of *WNT5A* in ALL. Moreover, epigenetics seems to be a general mechanism of *WNT5A* regulation not only in ALL but also in other types of tumours. For example, hypomethylation of *WNT5A* gene has been confirmed in prostate cancer cells by bisulphite sequencing.[30] Therefore, these data evidence that *WNT5A*, which have been implicated in cancer progression or suppression, is regulated at the transcriptional level by different epigenetic mechanisms, namely, hypermethylation or hypomethylation depending on the cellular context.

We have extensively demonstrated in patients with ALL that hypermethylation of gene promoters is a frequent mechanism of gene silencing and a finding associated with the prognosis of the disease and the response to therapy.[22–24] Epigenetic regulation of Wnt inhibitors is an important mechanism that significantly contributes to activation of the canonical Wnt signalling in ALL. In fact, we demonstrated that expression of the Wnt inhibitors *sFRP1*, *sFRP2*, *sFRP4*, *sFRP5*, *WIF1*, *DKK3* and *HDPR1* was downregulated due to abnormal promoter methylation in ALL samples and this event correlated with constitutive activation of the canonical Wnt-signalling pathway and upregulation of the *CYCLIN D1* target gene in ALL.[7] Our present work enhances our knowledge of the impacts of DNA methylation on the Wnt-signalling pathway and shows that the silencing of the *WNT5A* gene by inappropriate methylation has functional and clinical consequences upregulating *CYCLIN D1* and conferring a dismal prognosis in ALL specially amongst the paediatric population. However, one can speculate that the upregulation of the *CYCLIN D1* observed in our ALL patients depends only on the activation of the canonical Wnt signalling being the methylation of the *WNT5A* an innocent bystander of the more extensive methylation of the canonical Wnt-antagonists. To rule out this point, we have demonstrated that methylation of the *WNT5A* is able to upregulate *CYCLIN D1* regardless of the methylation status of the canonical Wnt pathway and also to potentiate the upregulation of the *CYCLIN D1* in those cases in which the canonical pathway is also constitutively activated. Following confirmation of the present findings in a more extensive prospective study, it may be possible to use *WNT5A* as a clinical marker of an aggressive tumour phenotype and disease recurrence. More speculatively, reconstitution of the *WNT5A* signalling pathway in ALL and modulation of the *CYCLIN D1* expression might provide therapeutic means of reducing the risk of relapse and death in this disease.

In conclusion, we have demonstrated that *WNT5A*, a putative tumour suppressor gene in ALL, is silenced by methylation in this disease and that this epigenetic event upregulates *CYCLIN D1* expression and confers poor prognosis in paediatric ALL.

## Conflict of interest statement

The authors declare no conflicts of commercial interest.

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