Epigenetic inactivation of *TWIST2* in acute lymphoblastic leukemia modulates proliferation, cell survival and chemosensitivity

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The online version of this article has a Supplementary Appendix.

ABSTRACT

Background

Altered regulation of many transcription factors has been shown to be important in the development of leukemia. *TWIST2* modulates the activity of a number of important transcription factors and is known to be a regulator of hematopoietic differentiation. Here, we investigated the significance of epigenetic regulation of *TWIST2* in the control of cell growth and survival and in response to cytotoxic agents in acute lymphoblastic leukemia.

Design and Methods

TWIST2 promoter methylation status was assessed quantitatively, by combined bisulfite and restriction analysis (COBRA) and pyrosequencing assays, in multiple types of leukemia and *TWIST2* expression was determined by quantitative reverse transcriptase polymerase chain reaction analysis. The functional role of *TWIST2* in cell proliferation, survival and response to chemotherapy was assessed in transient and stable expression systems.

Results

We found that *TWIST2* was inactivated in more than 50% of cases of childhood and adult acute lymphoblastic leukemia through promoter hypermethylation and that this epigenetic regulation was especially prevalent in *RUNX1-ETV6*-driven cases. Re-expression of *TWIST2* in cell lines resulted in a dramatic reduction in cell growth and induction of apoptosis in the Reh cell line. Furthermore, re-expression of *TWIST2* resulted in increased sensitivity to the chemotherapeutic agents etoposide, daunorubicin and dexamethasone and *TWIST2* hypermethylation was almost invariably found in relapsed adult acute lymphoblastic leukemia (91% of samples hypermethylated).

Conclusions

This study suggests a dual role for epigenetic inactivation of *TWIST2* in acute lymphoblastic leukemia, initially through altering cell growth and survival properties and subsequently by increasing resistance to chemotherapy.

Key words: *TWIST2*, acute lymphoblastic leukemia, DNA methylation, drug resistance, epigenetic.

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Introduction

Acute lymphoblastic leukemia (ALL) is the most common form of leukemia in childhood. While survival rates have improved dramatically this malignancy still accounts for nearly one quarter of all deaths from childhood cancers.[1] ALL in adults affects a comparatively young population and has proven difficult to treat, with 5-year survival rates of around 40%.[2]

Altered expression of key regulatory transcription factors has been shown to play a critical role in leukemia development.[3] TWIST2 is a basic helix-loop-helix protein and while it is not itself a transcription factor, it has been shown to regulate the activity of several well known families of transcription factors.[4-6] TWIST2 achieves this by binding to transcription factors and either sequestering them in the cytoplasm or functionally inactivating them.[4,6] This has been shown to function as a key differentiation switch in cells such as osteoblasts, myoblasts and adipocytes.[4,7,8] While a role in lymphoid development has not previously been shown, several lines of evidence suggest that TWIST2 may be functionally relevant in lymphoid cells and, potentially, in ALL. Firstly, its target proteins include the RUNX family of transcription factors and NF-κB, both of which have important roles in ALL biology. In addition *TWIST2* has been shown to be expressed in the B lymphocyte lineage and was found to exhibit differential promoter methylation and expression in chronic lymphocytic leukemia (CLL), which correlated with *IGHV* status.[9] Finally, it has recently been demonstrated that *TWIST2* can regulate differentiation of myeloid cells and also inhibits proliferation of granulocyte-macrophage progenitors, partly by inhibiting RUNX1 activity.[10]

It is now clear that epigenetic mechanisms are as important as genetic changes in the development of cancer.[11] Many well established tumor suppressor genes have been shown to be inactivated predominantly by promoter hypermethylation and many of the genes linked to leukemia development have themselves been shown to be epigenetic regulators, such as the histone methyltransferase *MLL*.[12] We. threfore, have investigated the functional relevance of epigenetic regulation of the *TWIST2* gene in ALL.

Design and Methods

Patients' samples

DNA was isolated from peripheral blood or bone marrow samples obtained from patients with clinically diagnosed leukemia. For childhood ALL 48 diagnostic samples were taken at diagnosis and 6 samples at relapse. For adult ALL 77 diagnostic samples were taken at diagnosis and 22 samples at relapse. For chronic myeloid leukemia (CML) 10 samples were taken at diagnosis and 10 from different patients following progression to blast crisis. For childhood acute myeloid leukemia (AML) 14 samples were taken at diagnosis and 14 were taken at relapse from separate patients. For CLL and adult AML all samples were taken at diagnosis. Further clinical details related to the ALL patients' samples are provided in Table 1. Peripheral blood samples were also obtained from anonymized healthy volunteers. Ethical approval for the collection of all samples and their analysis was obtained and the study was performed in accordance with the principals of the Declaration of Helsinki.

Both childhood ALL and AML samples were obtained from diagnostic bone marrow aspirates with more than 95% blasts by morphological assessment of bone marrow aspirate films. All chronic phase CML samples consisted of leukocytes derived from peripheral blood from patients undergoing leukapheresis. These samples were taken at diagnosis from patients with very high white cell counts and contained more than 95% *BCR/ABL*-positive cells. Blast crisis samples were obtained by isolation of leukocytes directly from peripheral blood samples. The blast crisis samples all had between 80 and 99% blasts. CLL samples were derived from peripheral blood mononuclear cells obtained by Ficoll-gradient centrifugation in order to concentrate blasts to more than 90% of the cell volume. Adult AML samples were obtained from either bone marrow or peripheral blood samples and had blast counts of greater than 80%.

Methylation analysis of the TWIST2 promoter region

Combined bisulfite and restriction analysis (COBRA) was performed largely as described before:[13] 200 ng of genomic DNA were modified with sodium bisulfite using the Methylamp™ One-Step DNA Modification Kit (Epigentek, Brooklyn, NY, USA) according to the manufacturer's instructions. All samples were resuspended in 15 μL of TE and 1 μL of this suspension was used for subsequent polymerase chain reactions (PCR). The samples

Table 1. Frequency of *TWIST2* hypermethylation in leukemia.

Disease Category	Total	*TWIST2* Methylated*	*TWIST2* Unmethylated
Childhood ALL			
Total	54	30 (56%)	24 (44%)
Median age	5	5	4
Sex Ratio	27:25 (M:F)	14:15 (M:F)	13:10 (M:F)
Median WBC, $\times 10^9$/L	16	17.5	10.7
BCP-ALL	43	28 (65%)	15(35%)
T-ALL	7	2 (29%)	5(71%)
Diagnostic	48	26 (54%)	22 (46%)
Relapse	6	4 (67%)	2 (33%)
t(12;21) - positive†	14	11 (79%)	3(21%)
t(12;21) - negative†	36	16 (44%)	20(56%)
He H	14	5 (36%)	9(64%)
Adult ALL			
Total	77	52 (68%)	25 (32%)
Median age	39	38	42
Sex Ratio	38:35 (M:F)	26:23 (M:F)	12:12 (M:F)
Median WBC, $\times 10^9$/L	53.9	63.8	41
BCP-ALL	39	27(69%)	12(31%)
T-ALL	15	13(87%)	2(13%)
Relapsed adult ALL	22	20(91%)	2(9%)
CLL	110	5(5%)	105(95%)
CML	20	0(0%)	20(100%)
Adult AML	20	0(0%)	20(100%)
Childhood AML	28	4(14%)	24(86%)

**Samples were defined as methylated when the level of methylation was 50% or greater at the majority of sites assayed. †cytogenetic findings other than t(12;21) and HeH were present at only low numbers.*

were amplified in 25 μL volumes containing 1X manufacturer's buffer, 1 unit of FastStart taq polymerase (Roche, Welwyn Garden City, UK), 2 mM $MgCl_2$, 10 mM dNTP, and 75 ng of each primer. The PCR was performed with one cycle of 95°C for 6 min, 35 cycles of 95°C for 30 sec, 63°C for 30 sec and 72°C for 30 sec, followed by one cycle of 72°C for 5 min. Following amplification, the PCR products were digested with the appropriate restriction enzymes (TaqI and BsiEI, New England Biolabs, Hitchin, UK), specific for the methylated sequence after sodium bisulfite modification. Digested PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining. *In vitro* methylated DNA (Millipore, Watford, UK) was diluted into DNA extracted from normal peripheral blood to produce standards (100%, 66%, 33% and 0%) of known methylation status for all COBRA assays. The primers used were forward 5'- aacaactatttaacaacccaacccaac, and reverse 5'- gggygagttggagttttttttatgg which amplify a region of the *TWIST2* gene from -26 to +208 relative to the transcriptional start site.

Pyrosequencing analysis was carried out using the same initial PCR reaction as described above for COBRA, except that a biotin label was included on the reverse primer. Following amplification, sequencing was performed using a PSQ 96MA pyrosequencer (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The primers used for the initial PCR were identical to those used for the COBRA (*Online Supplementary Table S1*), with the addition of a 5' biotin label on the reverse primer. The sequencing primer was 5' – ctccraaaacrtatact – 3'.

TWIST2 expression analysis

Complementary DNA was synthesized using the SuperScript™ III First Strand Synthesis System (Invitrogen, Paisley, UK) according to the manufacturer's protocol. Quantitative reverse transcriptase PCR (qRT-PCR) analysis was performed on 10 μL volumes containing 1X master mix (SYBR® Green JumpStart™ Taq ReadyMix kit; (Sigma, Gillingham, UK), 37.5 ng of each primer and 0.5 μL cDNA. PCR was performed with one cycle of 94°C for 15 min, followed by cycles of 94°C for 30 sec, 55°C (*β2-microglobulin*) or 63°C (*TWIST2*) for 30 sec and 72°C for 30 sec, with plate reads carried out at 77°C (*β2-microglobulin*) or 82°C (TWIST2) at the end of each cycle. Each of the PCR assays was run in triplicate. β2-microglobulin was used as a control for normalizing relative expression levels in the different samples. Reactions were carried out on a TaqMan 7900HT (Applied Biosystems, Warrington, UK). Primer sequences were: forward primer 5'- ggacaataagatgaccagctg and reverse 5'- gttacagactcgaatgcatcc for *TWIST2* and forward primer 5'- gcattcagacttgtctttcagc and reverse 5'- atgcggcatcttcaaacctc for *β2-microglobulin.*

Cell lines and transfections

ALL cell lines were maintained in RPMI with 2 mM glutamine and 10% fetal calf serum in 95% air/5% CO_2 at 37°C. For *TWIST2* re-expression studies Nalm6 cells were treated with 1 μM 2'deoxy-5-azacytidine (Sigma) for 24 h on 2 consecutive days and then cells were collected for qRT-PCR analysis 5 days later. For transfections the *TWIST2* cDNA was cloned into the pIRES2-eGFP vector (Clontech, Mountain View, CA, USA) to produce the pIRES-*TWIST2*-eGFP vector. This allows expression of TWIST2 and eGFP from a single transcript, but the two proteins are translated separately due to the IRES sequence between *TWIST2* and *eGFP*. Transfections were carried out using the Nucleofector system (Amaxa, Koeln, Germany), according to the manufacturer's protocol and were performed using 5×10^6 cells and 2 μg of DNA. Cells were transfected with either pIRES-eGFP or pIRES-*TWIST2*-eGFP. Transfected cells were either used as transient transfections or were treated with 800 μg/mL G418 (Merk, Nottingham, UK) following transfection to allow for selection of stably transfected cells. Following out-growth of G418-resistant cells the level of GFP positivity was assessed using flow cytometry. Then GFP-positive cells were flow sorted using a FACSAria cell sorter (BD Biosciences, Oxford, UK), to produce a population of cells containing a high level of transfectants. *TWIST2* expression in this population was confirmed by qRT-PCR. These bulk cultures, as opposed to single clones, were used for subsequent experiments to avoid any potential influence of site of integration on downstream analysis.

Growth assays

The effect of *TWIST2* on growth of ALL cells was assessed using the flow sorted GFP-positive populations for either the Nalm6 or Reh cell lines. Stably transfected lines were grown for approximately 7 days after sorting to generate sufficient cell numbers. Cells were assessed for GFP levels and only lines which maintained high GFP positivity (>80% for Nalm6 and 70-80% for Reh) were used for downstream assays. Cells were counted using the Vi-CELL System (Beckman Coulter, High Wycombe, UK) to ensure highly accurate counts of viable cell populations. Twenty-thousand viable cells of parental origin, cells transfected by vector alone, or cells transfected by *TWIST2* were plated out in triplicate in 12-well plates. Samples were taken at 4 and 7 days for counting using the Vi-CELL. The results shown are the averages of four independent experiments.

Growth assays were also carried out after dexamethasone treatment. Following counting in the Vi-CELL, 30,000 transfected Nalm6 cells (transfected with either vector alone or with *TWIST2*) per well were plated out in triplicate in 12-well plates for each transfectant/dose point. These cells were treated with either 0, 1 or 5 nM dexamethasone. Samples were taken at 4 and 7 days for counting using the Vi-CELL. The results shown are the averages of three independent experiments.

Analysis of induction of apoptosis

Levels of apoptosis were measured using staining with annexin V. Assays were carried out using the Annexin V Apoptosis Detection kit I (BD Biosciences), adhering to the manufacturer's protocol. Phycoerythrin-conjugated annexin V was used for these experiments to allow differentiation from the green signal derived from GFP expression. For assessment of apoptosis in transiently transfected lines, Nalm6 or Reh cells transfected with either vector alone or vector expressing *TWIST2* were assessed 48 h post-transfection specifically in the GFP-positive (i.e. transfected) population. Background apoptosis due to the transfection procedure was determined by subtracting the apoptosis measured in the non-transfected GFP-negative cells from that of the GFP-positive cells. For assays using cytotoxic agents, transfected Nalm6 cells were treated with either daunorubicin or etoposide (Sigma) at 0, 0.1 and 0.3 μm. Twenty-four hours after initial treatment, cells were collected and assessed for apoptosis as described above. Apoptosis was only assessed in the GFP-positive fraction. The results shown are the averages of three or four independent experiments.

Statistical analyses

Methylation and cytogenetic data were compared using Fisher's exact test (a one-tailed test was used as the specific hypothesis being tested was that *TWIST2* methylation would correlate with the presence of the *RUNX1-ETV6* fusion gene). Comparison of methylation data with *TWIST2* expression levels was performed using the Mann-Whitney U test. For all cell culture analyses all experiments were conducted at least three times. Results are expressed as means (± SEM) and statistical analyses were carried out using the t-test.

Results

TWIST2 *is a frequent target for epigenetic inactivation in acute lymphoblastic leukemia, but not in other types of leukemia*

To determine the potential role of *TWIST2* promoter hypermethylation in leukemia, we quantitatively analyzed the methylation status of the gene in all common types of leukemia using the COBRA assay.[14] As shown in Figure 1, while methylation is not detectable in normal peripheral blood, COBRA identified that high levels of *TWIST2* methylation (>50% DNA methylated in sample) were frequently seen in ALL. This was true for both childhood and adult ALL (with 56% and 68%, respectively, of cases exhibiting >50% methylation, Table 1). To further analyze the methylation status of *TWIST2*, a second quantitative methylation assay, pyrosequencing, was used to confirm the methylation levels in a subset of the childhood and adult ALL samples. Confirming the results of COBRA, pyrosequencing demonstrated that high levels of *TWIST2* methylation (>50%) were frequently observed in childhood and adult ALL samples (*Online Supplementary Table S1*). There was strong agreement between the two techniques and all samples identified as exhibiting high levels of methylation using COBRA were similarly found to be highly methylated in the pyrosequencing assay (*Online Supplementary Table S1*).

TWIST2 is known to bind to and inactivate RUNX1 in other cell types, through direct binding to the runt domain. Approximately 25% of childhood ALL samples are associated with the t(12;21) that fuses *RUNX1* to *ETV6*.[15] The resultant fusion protein retains the *TWIST2* binding runt domain. We therefore investigated *TWIST2* hypermethylation in this subgroup of patients. An additional six samples of *ETV6-RUNX1*-positive childhood ALL were obtained and assessed for *TWIST2* methylation status (Figure 1B) and cytogenetic data were obtained for the previously examined samples. *TWIST2* hypermethylation was found to be significantly more common in *ETV6-RUNX1-positive* childhood ALL than in childhood ALL cases lacking this fusion gene [79% (11/14) *versus* 44% (16/36), respectively, P=0.029 Fisher's exact test, Table 1]. *TWIST2* methylation status was not significantly correlated with age, white blood cell count, gender, immunophenotype or any other cytogenetic subgroup (Table 1).

To investigate the role of *TWIST2* methylation in ALL more thoroughly, 22 samples from adults with relapsed ALL were assessed for *TWIST2* methylation. This analysis showed that almost all relapsed patients (91%, 20/22) had hypermethylation of the *TWIST2* gene, consistent with the possibility that *TWIST2* could play a role in *in vivo* chemosensitivity, as was seen *in vitro* in the cell line models (see below). Subsequently, the corresponding diagnostic samples from the relapsed patients were also obtained to determine whether the high levels of *TWIST2* methylation were selected following treatment or were present at diagnosis. A direct pair-wise comparison of methylation levels determined by pyrosequencing demonstrated that the levels of *TWIST2* methylation were significantly increased in the relapse samples compared to the levels in the corresponding diagnostic samples (P=0.02, paired t-test, supplementary Figure 1A). However, the average increase in methylation was comparatively small (average methylation at diagnosis 67% *versus* 74% in paired relapse samples) and was restricted to samples with lower methylation levels at diagnosis [samples with <70% methylation at diagnosis (n=10) showed an average increase of 15% at relapse, P=0.0007, *Online Supplementary Figure S1B*). This suggests that a combination of high *TWIST2* methylation levels at diagnosis or increased methylation at relapse (in samples which lacked very high methylation at diagnosis) results in the extremely high level of *TWIST2* methylation seen in relapse samples.

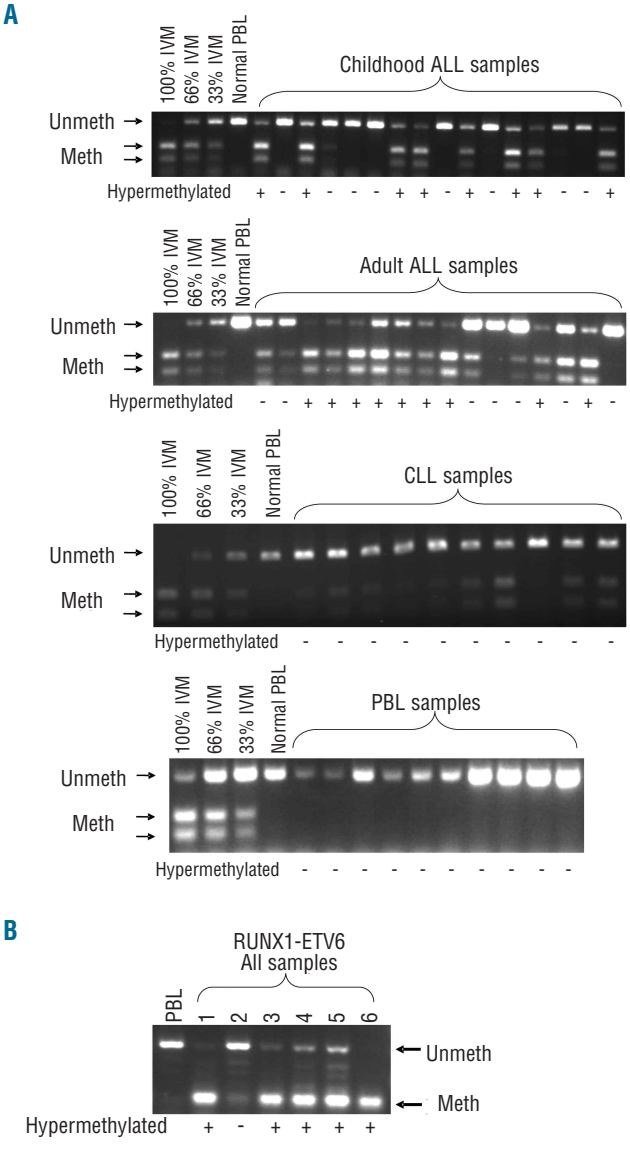

Figure 1. Examples of methylation analysis of the ***TWIST2*** promoter region in leukemia. (A) COBRA assays were used to quantify methylation levels at the ***TWIST2*** promoter in multiple types of leukemia. Examples of analysis in childhood ALL, adult ALL and CLL (BsiEI digest) are shown as indicated. The positions of bands representing methylated and unmethylated DNA are indicated by arrows and the presence or absence of hypermethylation in each sample is indicated by a + (hypermethylated) or a – (not hypermethylated) under each lane. 100%, 66%, 33% *In vitro* methylated (IVM) and peripheral blood leukocytes (PBL) were used as controls. This analysis identified frequent hypermethylation of ***TWIST2*** in ALL, but not in other types of leukemia. (B). Additional childhood ALL samples containing the ***RUNX1-ETV6*** fusion gene were also assayed by COBRA (TaqI digest). As indicated in the examples, hypermethylation was significantly more common in this subset of childhood ALL.

We also assessed *TWIST2* methylation in other types of leukemia (CLL, AML, CML and childhood AML) (Table 1). Little or no *TWIST2* promoter methylation was seen in CML or adult AML, although a small number of childhood AML samples (14%, 4/28) did exhibit high levels of *TWIST2* methylation (Table 1). In agreement with a previous report[9] we found frequent methylation of *TWIST2* in CLL samples. However, methylation levels in individual CLL samples were lower than those seen in ALL and only rarely in excess of 50% (see examples in Figure 1A, *Online Supplementary Table S1* and Table 1). As all leukemia samples analyzed contained a high percentage of leukemia cells, the absence of high levels of *TWIST2* methylation in other types of leukemia was not due to high levels of contamination by normal cells. These results suggest that epigenetic inactivation of *TWIST2* may be of primary importance in ALL.

Hypermethylation of TWIST2 *results in loss of gene expression*

Expression of *TWIST2* in adult ALL was assessed using qRT-PCR in 22 samples (11 unmethylated and 11 hypermethylated samples). Expression was detected in almost all unmethylated samples, but was low or absent in methylated samples (10/11 unmethylated samples positive *versus* 2/11 methylated samples positive, P=0.002, Mann-Whitney U test) (Figure 2A). To further explore the importance of *TWIST2* methylation, expression was examined in ALL cell lines. All four cell lines examined (Nalm6, Reh, CCRF-CEM and Molt-4) exhibited hypermethylation of *TWIST2* and an absence of expression, including the Reh and Nalm6 cell lines which were used for subsequent functional assays. Treatment of one of these cell lines, Nalm6, with the DNA methyltransferase inhibitor 2'-deoxy-5-azacytidine resulted in reduced methylation of the *TWIST2* promoter and re-expression of *TWIST2* mRNA, demonstrating that DNA methylation of the gene was required for suppression of expression (Figure 2B).

Restoration of TWIST2 *expression in acute lymphoblastic leukemia cells inhibits cell growth and induces apoptosis in Reh cells*

To assess the functional significance of *TWIST2* in ALL cells, the gene was re-introduced into the Nalm6 cell line, in which *TWIST2* is epigenetically silenced. As transfection of leukemia cell lines is generally inefficient, this was done using the pIRES2-eGFP vector which also expresses eGFP from an internal ribosome entry site. Following selection in G418, eGFP-expressing cells (and thus *TWIST2*-expressing cells) were submitted to flow cytometry to allow isolation of a relatively pure population of eGFP/*TWIST2*-positive cells (>80% positive). *TWIST2* expression in this population was confirmed by qRT-PCR. The growth of this population of cells was then followed for 7 days. As shown in Figure 3 the *TWIST2*-expressing cells exhibited a dramatic defect in cell growth compared with either parental Nalm6 cells or cells transfected with vector alone. A similar inhibition of proliferation following *TWIST2* transfection was also seen in a second ALL cell line, Reh, which expresses the *RUNX1-ETV6* fusion gene (Figure 3A).

It was noted that continued growth in culture of both cell lines resulted in a decline in the fraction of *TWIST2*-positive cells, presumably because of their lower proliferation rates. This effect was much more dramatic in the Reh cell line than in the Nalm6 cell line (levels typically dropped from 70-80% to <40% within 7 days in Reh cells, whereas 3-4 weeks were required for a similar drop in Nalm6 cells). To determine whether the apparent increased selection against *TWIST2* expression was due to toxicity of *TWIST2* in Reh cells, levels of apoptosis were measured in Nalm6 and Reh cells following transient transfection. This was again done using the pIRES2-eGFP vector so that apoptosis could be specifically monitored in transfected (GFP-positive) cells. As shown in Figure 3B transfection of Nalm6 cells with *TWIST2* resulted in only a minor, non-significant increase in apoptosis compared to that caused by transfection with vector alone. In contrast

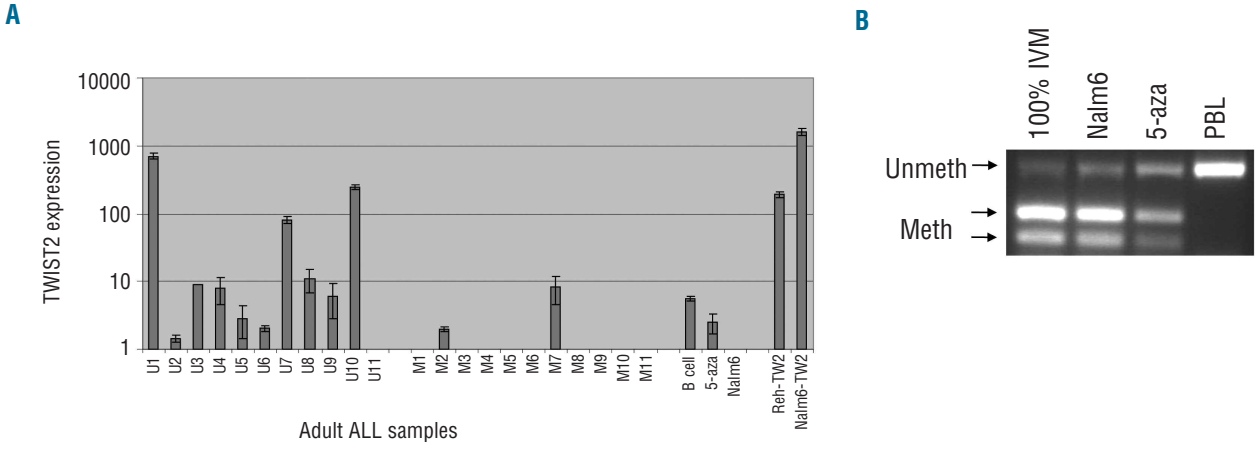

Figure 2. Hypermethylation of *TWIST2* is associated with loss of gene expression. (A) Gene expression was assessed in adult ALL samples using qRT-PCR. Relative expression (in arbitrary units) is shown in unmethylated (U) and hypermethylated (M) samples. Methylation was significantly associated with loss of gene expression (P=0.002, Mann Whitney U test). Nalm6 cells were either untreated (Nalm6) or treated with 1 μM 2'deoxy-5-azacytidine (5-aza) for 48 h and then assayed for TWIST2 expression by qRT-PCR 5 days later. Loss of *TWIST2* methylation was found to be associated with gene re-expression. (B) Nalm6 cells were either untreated (Nalm6) or treated with 1 μM 2'deoxy-5-azacytidine (5-aza) for 48 h and then assayed for *TWIST2* methylation 5 days later, using the COBRA assay. 100% *in vitro* methylation (IVM) and peripheral blood leukocytes (PBL) were included as controls.

re-expression of *TWIST2* in Reh cells resulted in very clear induction of apoptosis. This shows that in addition to negatively regulating cell growth, *TWIST2* can also negatively influence survival of ALL cells, but that this effect may be dependent on the genetic background. While this was not reflected in increased inhibition of proliferation in the data shown in Figure 3A, it was almost certainly due to an increased number of non-expressing cells in the transfected Reh population at the outset of this assay, because of the more rapid loss of *TWIST2*-positive cells from this population.

Re-expression of TWIST2 *in acute lymphoblastic leukemia cells is associated with increased sensitivity to chemotherapy*

In addition to its ability to inhibit RUNX1, *TWIST2* has also been shown to bind to and inactivate NF-κB, a known regulator of responses to chemotherapeutic agents, via binding to the p65 subunit.[6] This suggests that loss of *TWIST2* may also lead to increased drug resistance. Therefore, Nalm6 cells (with and without *TWIST2*) were assessed for apoptosis in response to etoposide and daunorubicin, both of which are commonly used in the treatment of ALL. Nalm6 cells were used for these assays rather than Reh cells, as *TWIST2* expression was lost very rapidly from the Reh cell population and also induced apoptosis even in the absence of cytotoxic agents. Despite the reduced proliferation of the *TWIST2*-expressing Nalm6 cells, which might be expected to reduce sensitivity to these agents, *TWIST2*-expressing Nalm6 cells exhibited increased levels of apoptosis at multiple concentrations of both drugs (Figure 4A, *Online Supplementary Figure S2*).

The glucocorticoid dexamethasone is a mainstay of treatment for childhood ALL. As significantly increased apoptosis was not observed in Nalm6 cells (with or without *TWIST2*), we assessed the effect of dexamethasone on proliferation of Nalm6 cells in the presence or absence of *TWIST2* expression. To account for the different proliferation rates of the cells due to *TWIST2* expression, results were calculated as a percentage of the growth observed in untreated cells transfected with either *TWIST2* or vector alone, as appropriate. Treatment with dexamethasone resulted in clear inhibition of cell growth in both the presence and absence of *TWIST2*, however the growth inhibition of *TWIST2*-expressing Nalm6 cells was significantly greater at both 1 nm dexamethasone (P=0.001) and 5 nm dexamethasone (P=0.01) doses (Figure 4).

Discussion

Epigenetic inactivation of genes is crucial in the development of leukemia and can have dramatic effects on the biological and clinical behavior of these diseases. Here we show that the *TWIST2* gene is hypermethylated in over half of childhood and adult cases of ALL. *TWIST2* has previously been shown to be expressed in normal B lymphocytes[9] and here we show that hypermethylation of the gene suppressed expression in both primary samples and in cell lines. Treatment with 2'-deoxy-5-azacytidine resulted in re-expression in Nalm6 cells, demonstrating that the DNA methylation was required for continued suppression of *TWIST2* expression. Functional studies indicate that *TWIST2* has multiple, important biological roles in ALL cells, including control of cell proliferation and survival and regulation of response to therapeutic agents.

Re-expression of *TWIST2*, but not GFP alone, in ALL cell lines resulted in a dramatic inhibition of cell growth, indicating that *TWIST2* has functions compatible with a role in tumor suppression. The mechanism by which *TWIST2* inhibits cell growth is not yet clear, however RUNX1 would represent a potential candidate mediator of this effect. Several previous studies have demonstrated that *TWIST2* can bind to and inactivate RUNX1 in osteoblasts

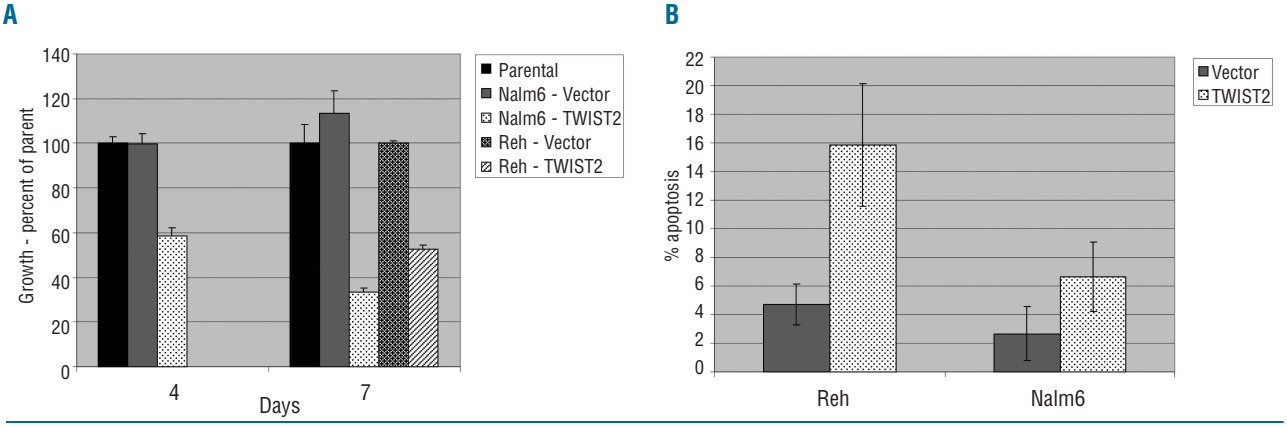

Figure 3. Re-expression of *TWIST2* in ALL cells is associated with reduced growth and increased apoptosis. (A) Nalm6 or Reh cells were either untransfected (parental), transfected with vector alone (vector) or transfected with a *TWIST2*-expressing vector (*TWIST2*). Parental cells or populations enriched for transfected cells [>80% (Nalm6) and 70-80% (Reh) GFP-positive transfectants, assessed by flow cytometry] were grown in identical conditions for 7 days. Relative growth was assessed by counting cells 4 and 7 days after initial plating. Expression of *TWIST2* was associated with a dramatic reduction in growth of Nalm6 cells (P=2.1x10^{-7}, t-test) and Reh cells (P=6.0X10^{-10}, t-test). Results shown are the averages of four (Nalm6) or six (Reh) independent experiments. (B) Apoptosis was assessed in Reh (*RUNX1-ETV6*-positive) and Nalm6 cells (*RUNX1-ETV6*-negative) transiently transfected with either vector alone or vector expressing *TWIST2* and assayed for apoptosis at 48 h post-transfection. Apoptosis, as judged by annexin V positivity, was significantly increased in Reh cells in the presence of *TWIST2* expression, but not in Nalm6 cells. Results shown are the averages of three independent experiments.

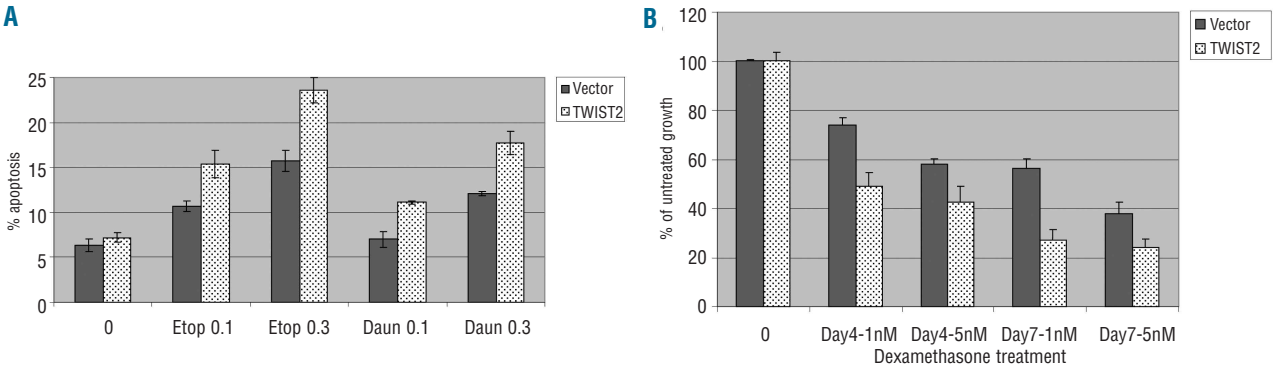

Figure 4. Re-expression of *TWIST2* is associated with increased sensitivity to chemotherapeutic agents. (A) Flow sorted Nalm6 cells, either with (*TWIST2*) or without (vector) re-expression of *TWIST2*, were assayed for sensitivity to etoposide- or daunorubicin-induced apoptosis (either at 0.1 μM or 0.3 μM as indicated). Levels of apoptosis (as assessed by annexin V positivity) were measured 24 h after the indicated treatment. Apoptosis levels were significantly higher in the presence of *TWIST2* expression (P=0.002 for etoposide and P=0.02 for daunorubicin, t-test). Results shown are the averages of four independent experiments. (B) *TWIST2* expression increases growth inhibition by dexamethasone. Growth of flow sorted Nalm6 cells, either with (*TWIST2*) or without (vector) re-expression of *TWIST2*, was assayed at 4 and 7 days after treatment with the indicated doses of dexamethasone. For *TWIST2*-expressing cells, the results are expressed as a percentage of the growth of untreated, *TWIST2*-expressing cells. Similarly, for cells transfected with vector alone, the results are expressed as a percentage of the growth of untreated, vector alone cells. This was necessary to account for the reduced rate of cell growth induced by *TWIST2* expression alone. A significantly greater reduction of cell growth was induced by dexamethasone treatment in *TWIST2*-expressing Nalm6 cells. Results shown are the averages of three independent experiments.

and in myeloid cells.[4,10] Furthermore RUNX1 is known to be able to drive proliferation of hematopoietic cells and enhance B-cell survival.[17,18] Consistent with the hypothesis that RUNX1 is a key target for *TWIST2* we found that loss of *TWIST2* expression in primary ALL samples was more common in patients with leukemia expressing the *RUNX1-ETV6* fusion gene. While it remains to be demonstrated that *TWIST2* binds to the product of the fusion gene, this appears likely as it retains the *TWIST2*-binding runt domain.[15] The ability of *TWIST2* to induce apoptosis in the *RUNX1-ETV6* positive Reh cell line but not in the Nalm6 cell line (which lacks the fusion gene, but does express high levels of wild-type RUNX1) may also suggest a greater role for *TWIST2* in RUNX1-ETV6-driven leukemia; however, there are likely to be multiple genetic differences between these cell lines and so the increased apoptosis in the Reh cell line cannot be linked directly to the presence of the RUNX1-ETV6 fusion. We also attempted to confirm the association between loss of *TWIST2* expression and presence of the RUNX1-ETV6 fusion in ALL by examining publically available gene expression data sets. However, *TWIST2* proved to be absent from most array formats used and so *TWIST2* expression could not be determined. In addition, a significant role for *TWIST2* in ALL lacking the *RUNX1-ETV6* fusion is also apparent: firstly, re-expression of *TWIST2* still produced a very clear inhibition of cell growth in Nalm6 cells and secondly, *TWIST2* hypermethylation was seen in over 40% of *RUNX1-ETV6*-negative cases of childhood ALL and in 68% of cases of adult ALL, in which the *RUNX1-ETV6* fusion is rare.[19] Further dissection of the molecular roles of *TWIST2* will be required to determine its comparative roles in *RUNX1-ETV6*-positive and *RUNX1-ETV6*-negative ALL.

The other well established protein target for TWIST2 is the p65 subunit of NFκB.[6] NF-κB has also been implicated as functionally relevant in ALL through its ability to regulate cellular responses to chemotherapy[20] and it has previously been suggested that around half of children with ALL have increased resistance to ionizing radiation due to increased levels of NF-κB activity.[16] Based on this we investigated the possibility that *TWIST2* expression may increase sensitivity to chemotherapeutic agents. This analysis determined that re-expression of *TWIST2* in Nalm6 cells resulted in increased levels of apoptosis in response to etoposide and daunorubicin treatment and reduced cell growth in response to dexamethasone treatment. This was demonstrated in wild-type *RUNX1* Nalm6 cells. Unfortunately it was not possible to assess chemosensitivity in the *RUNX1-ETV6*-positive Reh cell line, as *TWIST2*-expressing Reh cells were lost too rapidly from the population. The *in vitro* importance of *TWIST2* in determining chemosensitivity raises the possibility that altered *TWIST2* expression may be an important determinant of chemosensitivity in ALL patients. Consistent with this, hypermethylation of *TWIST2* was found to be extremely common in samples from adults with relapsed ALL (91% of samples hypermethylated), suggesting that exposure to treatment selects out either cells with increased CpG island methylation in general or cells with increased *TWIST2* methylation in particular.

Further studies will be required to fully elucidate the mechanisms by which *TWIST2* can control growth, survival and chemotherapeutic response of ALL cells. These effects may be due to loss of regulation of RUNX1 and NF-κB or through yet to be identified *TWIST2* target proteins. In particular, identifying the pathways regulated by *TWIST2* which modulate chemosensitivity would open up the possibility of targeting these pathways and potentially reversing the chemoresistance seen in *TWIST2*-deficient cells. Such an approach may be especially valuable in relapsed adult ALL, in which *TWIST2* hypermethylation is very frequent and outcome extremely poor.

Authorship and Disclosures

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