

Promoter Methylation-Mediated Inactivation of *PCDH10* in Acute Lymphoblastic Leukemia Contributes to Chemotherapy Resistance

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PCDH10 has been implicated as a tumor suppressor, since epigenetic alterations of this gene have been noted in multiple tumor types. However, to date, studies regarding its role in acute and chronic leukemias are lacking. Here, we have investigated the presence of promoter hypermethylation of two CpG islands of the *PCDH10* gene by methylation-specific PCR in 215 cases of various subsets of myeloid- and lymphoid-lineage leukemias. We found that *PCDH10* promoter hypermethylation was frequent in both B-cell (81.9%) and T-cell (80%) acute lymphoblastic leukemia (ALL), while it was present in low frequency in most subtypes of myeloid leukemias (25.9%) and rare in chronic myeloid leukemia (2.2%). *PCDH10* expression was downregulated via promoter hypermethylation in primary ALL samples ($N = 4$) and leukemia cell lines ($N = 11$). The transcriptional repression caused by *PCDH10* methylation could be restored by pharmacologic inhibition of DNA methyltransferases. ALL cell lines harboring methylation-mediated inactivation of *PCDH10* were less sensitive to commonly used leukemia-specific drugs suggesting that *PCDH10* methylation might serve as a biomarker of chemotherapy response. Our results demonstrate that *PCDH10* is a target of epigenetic silencing in ALL, a phenomenon that may impact lymphoid-lineage leukemogenesis, serve as an indicator of drug resistance and may also have potential implications for targeted epigenetic therapy. © 2011 Wiley Periodicals, Inc.

INTRODUCTION

The protocadherin subfamily of the cadherin superfamily of genes that encode cadherin-related neuronal receptors play a role in the establishment and function of specific cell–cell connections (Yagi, 2008). A number of protocadherin genes have been implicated as tumor suppressor genes (Waha et al., 2005; Imoto et al., 2006; Yu et al., 2008, 2009; Narayan et al., 2009; Cheung et al., 2010). Recently, it has been shown that *PCDH10* is epigenetically inactivated and functions as a tumor suppressor in multiple human cancer types, including nasopharyngeal, esophageal, gastric, breast, hepatocellular, and cervical carcinomas as well as in mature B-cell lymphomas (Ying et al., 2006, 2007; Narayan et al., 2009; Yu et al., 2009; Cheung et al., 2010). *PCDH10* methylation occurs early in precancerous stages of cervical and esophageal cancers suggesting an important role in tumorigenesis in these tumor types (Narayan et al., 2009; Yu et al., 2009).

PCDH10 methylation in adjacent normal tissue was associated with poor survival in gastric cancer patients and restoration of expression in cell line exhibits inhibition of tumor growth both in vitro and in vivo (Yu et al., 2009). Promoter CpG island hypermethylation-associated gene silencing has been reported as a frequent event in hematologic malignancies (Garcia-Manero et al., 2009). Currently, DNA methyltransferase (DNMT)

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

Supported partly by: The Lance Armstrong Foundation and the Department of Pathology & Cell Biology, Columbia University.

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Received 22 June 2011; Accepted 22 July 2011

DOI 10.1002/gcc.20922

Published online 24 August 2011 in

Wiley Online Library (wileyonlinelibrary.com).

inhibitors have been approved for the treatment of myelodysplastic syndrome (MDS) based on phase II and phase III studies and these agents have also shown a good response in acute myeloid leukemia (AML) (Oki et al., 2007). Epigenetic modifications, not only directly or indirectly affect the gene expression patterns, but specific genetic changes may also have an impact on epigenetic modifications, as in the case of *MLL* translocation-associated childhood acute lymphoblastic leukemia (ALL) (Krivtsov and Armstrong, 2007). As epigenetic mechanisms play critical roles in disease pathogenesis and serve as useful targets for epigenetic therapy, there has been intense focus in understanding the role of epigenetic modifications in leukemia.

Since epigenetic modifications have been implicated in leukemogenesis and in the therapy of leukemia, we have examined promoter hypermethylation of *PCDH10* in acute myeloid and lymphoblastic leukemias, MDS, and chronic myeloid leukemia (CML). We found differences in methylation patterns of *PCDH10* in a well-characterized cohort comprising various leukemia subtypes. High frequency of CpG island methylation was observed in the majority of primary B-cell and T-cell ALL samples, with a lower frequency in other leukemia subtypes. Promoter hypermethylation resulted in downregulated expression of *PCDH10* in ALL cases and cell lines, and ALL cell lines carrying *PCDH10* promoter methylation exhibited resistance to standard leukemia-specific drug treatment regimens.

MATERIALS AND METHODS

Patient Samples and Cell Lines

A total of 248 samples were analyzed, which represented 28 normal bone marrow ($N = 27$) or blood ($N = 1$) samples, 77 B-cell ALL, 10 T-cell ALL, 3 ambiguous lineage leukemia, 59 de novo AML, 21 MDS, 5 secondary MDS/AML, and 45 CML samples. All specimens used in this study had been submitted for pathologic and cytogenetic evaluation at our institute over a 12-year period (July 1997–June 2009). Left over tissue samples after completion of cytogenetic testing were stored frozen at -80°C and utilized in the present study. All specimens were evaluated by morphology, flow cytometry, and cytogenetic analyses (Supporting Information Table 1) and the cases were classified according to the current WHO criteria (Swerdlow et al., 2008). Data on clinical outcome and laboratory tests, including

karyotype and FISH findings, treatment information were obtained from our institutional laboratory information systems (Supporting Information Table 1). The T-ALL cell lines Jurkat, CUTLL1, HPB-ALL, MOLT-15, MOLT-16, P12-ICHIKAWA, KARPAS45, T-ALL1, and MOLT-4 (kindly provided by Riccardo Dalla-Favera, Columbia University, New York) and the B-ALL cell lines RS411 and REH (obtained from ATCC) were also utilized. All cell lines were grown in RPMI-1640 containing 10% fetal bovine serum. The study protocol was approved by our institutional review board. Frozen specimens and cell lines were utilized for isolation of high-molecular-weight DNA and RNA by standard methods. RNA quality and quantity were assessed by a bioanalyzer (Agilent Technologies, Foster City, CA).

Methylation-Specific PCR (MSP)

Two microgram of genomic DNA was converted using EpiTect 96 bisulfite kit (Qiagen, Valencia, CA). Placental DNA treated in vitro with *SssI* methyltransferase (New England BioLabs, Beverly, MA) and normal lymphocyte DNA converted with sodium bisulfite was used as methylated and unmethylated controls, respectively. Four sets of primers for amplification of methylated (M) DNA and two sets of primers for unmethylated (U) DNA spanning two CpG Islands (CGIs) of cDNA clone NM_032961 were designed (Fig. 1A). The four sets of primers spanning CGI-2 were described previously (Narayan et al., 2009). An additional set of methylated and unmethylated MSP primers spanning CGI-1 was also utilized in the present study (Supporting Information Table 2).

PCR was performed using standard conditions for 30 cycles with annealing temperatures varying between 56 and 62°C . PCR products were run on 2% agarose gels and visualized after ethidium bromide staining. All MSP experiments were performed in triplicate and promoter hypermethylation was considered positive when present in at least one of the regions in two independent experiments.

Sequenom EpiTyper Quantitative Methylation Analysis

Quantitative methylation analysis was performed using the Sequenom MALDI-TOF mass spectrometry platform as per manufacturer specifications (Sequenom). Briefly, 20 ng of bisulfite-treated genomic DNA was PCR amplified using T7-promoter tagged primers of three overlapping

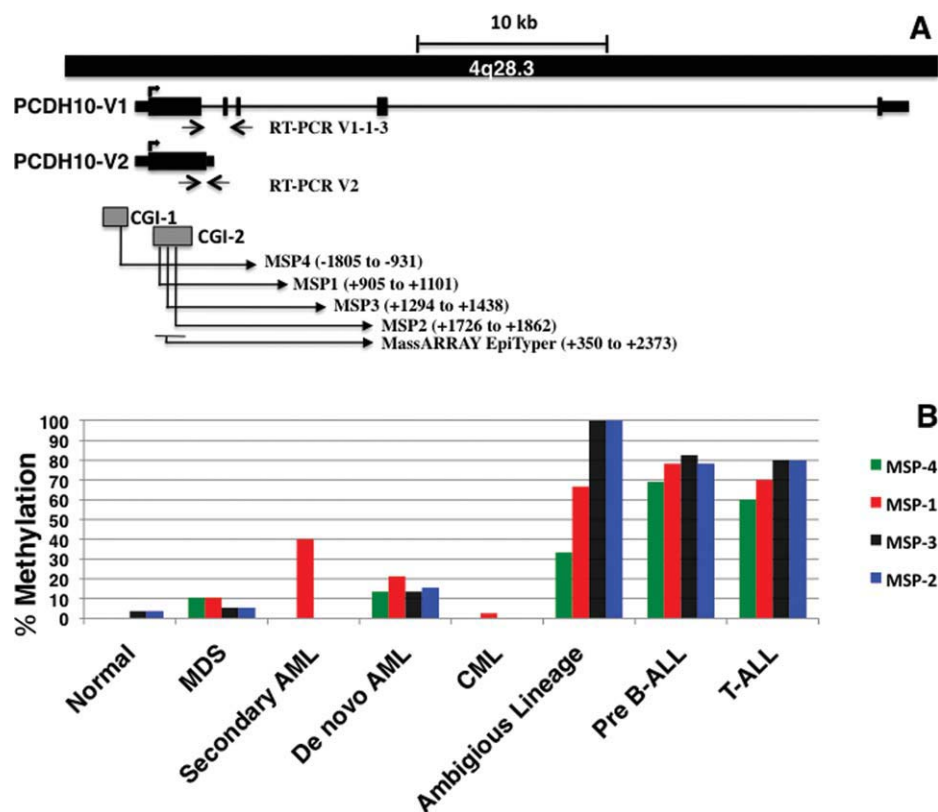


Figure 1. Methylation analysis of *PCDH10* in leukemia and myelodysplastic syndromes. (A) Genomic region of *PCDH10* at 4q28.3 is shown on top. Below, the coding (thick vertical rectangle), non-coding (thin vertical rectangle) regions, and the intronic regions (thin horizontal line) for two splice variants are shown. *PCDH10*-V1 is variant 1 and *PCDH10*-V2 is variant 2. Upward bent arrow indicates transcriptional start site. RT-PCR V1-3 and RT-PCR V2 primer positions are indicated by head to head arrows. Gray-filled rectangles indicate

CpG islands (CGI-1 and CGI-2). Downward bent arrows indicate position of methylation primers (MSP4, MSP1, MSP3, and MSP2) and EpiTyper sequenced region in CGI-2. (B) Patterns of MSP positivity of various regions in the CpG islands of *PCDH10* promoter in normal, MDS, and various types of leukemia. MSP, methylation specific primer; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; ALL, acute lymphoblastic leukemia.

regions spanning the CGI-2. The PCR primers designed using EpiDesigner software (Sequenom, Inc. San Diego, CA) for bisulfite converted DNA were shown in the Supporting Information Table 2.

Five microliters PCR reactions were carried out in 384-well micro titer plates, treated with 2 μ l of Shrimp Alkaline Phosphatase at 0.5 units concentration followed by brief heat inactivation and verification of the amplification by agarose gel. Subsequently, 2 μ l of the PCR product was incubated for 3 hr at 37°C with 5 μ l of MascLEAVE™ mix for concurrent in vitro transcription and base-specific cleavage. Diluted and resin-cleaned samples were spotted onto the spectroCHIP® Arrays using the Samsung MassARRAY™ Nanodispenser and analyzed with the Sequenom MassARRAY Compact System following 4-point calibration with oligonucleotides of different masses provided in the Sequenom

kit. In silico fragmentation analysis of RNase A-digested target sequence for T-cleavage reaction, on the plus strand, was calculated for each predicted fragment and used to determine whether or not the corresponding MALDI-TOF peak occurs within the useable mass window (default is 1500–7000 Da). Additionally, fragments that share the same predicted mass were identified and flagged, corresponding to “duplicate or overlapping peaks” in the EpiTYPER software. Those fragments that met the defined criteria by the Sequenom software were flagged appropriately and are treated as CG-containing fragments. The quantitative fragment mass data generated by the MALDI-TOF MS and EpiTYPER software were further subjected to quality control analysis. We performed duplicate reactions for each primer set on all samples analyzed and found only small variation in methylation levels of *PCDH10* between repeat measurements, thus

excluding any possibility of experimental error. The final data analysis was performed on average methylation measurements of each informative CpG derived from duplicate experiments. The software estimates the relative methylation status by the sum of the signal-to-noise ratios of the methylated and unmethylated components. In each experiment, we included methylated DNA controls representing 100, 50, 25, and 0 ratios by combining fully methylated DNA with unmethylated DNA. The methylation fractions obtained were compatible with the methylated and unmethylated DNA ratios. The Sequenom methylation analysis software and R-Script were used for analysis of the quantitative measurements where multiple levels of quality control (QC) were applied. A Sequenom recommended uncertainty threshold of 0.01 was applied to the data. CpG units that yielded data in greater than 75% of the samples passed initial QC. These data were further filtered to exclude cases that yielded less than 80% for all informative CpG units within an amplicon/sample pair. Additionally, CpG units that had data available for less than 50% of all samples were excluded. Of the 78 CpGs analyzed, 58 CpGs fulfilled these criteria. The methylation data generated after QC were used in a one-dimensional hierarchical clustering analysis. Average methylation per sample per CpG of all informative CpGs was calculated after QC analysis. Using this value for each case, mean methylation index was calculated for the intended groups.

Drug Treatment

Cells in culture were treated with 5 μ M 5-aza-2'-deoxycytidine (5-aza-CdR) for 5 days by replacing the medium everyday, trichostatin (TSA) at a final concentration of 200 nM for 24 hr and a combination of both as previously described (Narayan et al., 2009). Cells collected from these experiments were used for isolating total RNA. We determined ICD50 values using 11 ALL cell lines for the following leukemic drugs: doxorubicin (5 ng/ml), dexamethasone (100 nM), bortezomib (2 ng/ml), methotrexate (50 nM), and L-asparaginase (2 U/ml). The ALL cell lines were treated with these drugs for 48 hr to assess cell viability and apoptosis.

RT-PCR Analysis

Total RNA isolated from normal samples, leukemia samples, and cell lines were reverse transcribed as described (Narayan et al., 2009). Semi-quantitative expression of *PCDH10* was performed in triplicate RT-PCR experiments using

the primers shown in Supporting Information Table 2 and standard thermal cycle conditions of initial denaturation at 5 min at 95°C; and 28 to 40 cycles at 30 sec 95°C, 30 sec 56°C, and 30 sec 72°C; and 7 min at 72°C of final extension. Primers for the β -actin gene were used to assess the relative intensity of gene expression.

Cell Viability and Apoptosis Assays

Cell viability and cytotoxicity due to each drug was assessed by standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Invitrogen, Carlsbad, CA) colorimetric assay. Briefly, 5000 cells were seeded in 96-well cell culture plates, grown overnight at 37°C in 5% CO₂ incubator, and treated with indicated concentrations of drugs for 48 hr. After incubation of cells in 5 mg/ml of MTT in phosphate buffer saline for 3.5 hr, the dye was dissolved in 150 μ l of MTT solvent and the optical density of solubilized formazan was assessed using a microplate reader (BioTek Quant). All treatments were performed in four replicate wells and repeated three times. To measure apoptosis, we used Pacific Blue™ Annexin V®SYTOX® AADvanced™ Apoptosis Kit (Invitrogen), which detects the externalization of phosphatidylserine in apoptotic cells using recombinant annexin V conjugated to violet-fluorescent Pacific Blue dye and dead cell using SYTOX AADvanced stain. After staining, apoptotic cells show violet fluorescence, dead cells red, and live cells no fluorescence. Briefly, one million cells were seeded in 6-well tissue culture plates, grown overnight at 37°C at 5% CO₂ incubator, and the indicated concentration of drugs was added. Cells collected after 48 hr of incubation were analyzed by a LSR II flow cytometer (BD Biosciences, San Jose, CA) using 405 and 488 nm excitation and 455 and 647 nm emission. A total of 20,000 events were analyzed using Flowjo software (Tree Star, OR) in duplicate experiments.

Statistical Analysis

ANOVA and *t*-test statistics were calculated using the GraphPad Prism software (LaJolla, CA).

RESULTS

MSP Analysis of *PCDH10* in Normal Bone Marrow, ALL, AML, MDS, and CML

The *PCDH10* gene promoter region contains two classical CpG islands; CGI-1 covering -2133 to -854 bp from the transcription start site (TSS) and CGI-2 mapped to +367 to +1972 bp from the TSS of the transcript (Accession No.

TABLE 1. Frequency of Promoter Hypermethylation of *PCDH10* in MDS and Leukemia

Type of specimen	No. studied	Methylated (%)
Normal	28	1 (3.6)
MDS	21	2 (9.5)
Secondary AML	5	2 (40.0)
de novo AML	59	18 (30.5)
CML	45	1 (2.2)
Ambiguous AL	3	3 (100.0)
pre B-ALL	72	59 (81.9)
T-ALL	10	8 (80.0)

MDS, myelodysplastic syndromes; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; B-ALL, pre B-cell acute lymphoblastic leukemia; T-ALL, T-cell acute lymphoblastic leukemia; Ambiguous AL, ambiguous lineage acute leukemia.

NM_032961.1) in GRCh37 assembly (Fig. 1A). For qualitative assessment of methylation status of *PCDH10*, we performed MSP using three sets of primers spanning the CGI-2 and one primer set covering the CGI-1 (Fig. 1A). Promoter hypermethylation was examined in 243 DNA samples derived from normal bone marrow, ALL, AML, MDS, and CML (Table 1). We identified hypermethylation in 1 (2.2%) of 45 CML, 2 (9.5%) of 21 MDS, 18 (30.5%) of 59 de novo AML, 2 (40%) of 5 secondary AML, 3 (100%) of 3 ambiguous lineage leukemias, 59 (81.9%) of 72 B-ALL, and 8 (80%) of 10 T-ALL cases (Table 1). Similar analysis on DNA isolated from 28 normal samples revealed hypermethylation of CGI-2 in one (3.6%) case. Therefore, these data provide evidence that a large majority of ALL and ambiguous lineage leukemias harbor *PCDH10* gene methylation suggesting that inactivation of this gene may play a role in the development of these leukemias. The lower frequencies of promoter methylation seen in myeloid-lineage disorders suggest that modifications of DNA methylation in *PCDH10* might not play a role in transformation or progression (Table 1). To examine if methylation is uniformly present across the two CGIs or is restricted to specific regions within a CGI, we compared the patterns of MSP of the four regions studied in each specific subtype of leukemia and MDS (Fig. 1B). This analysis revealed that methylation was relatively frequent in CGI-2 that spans exon 1 as compared with CGI-1 that maps 5' upstream to the promoter. Secondly, this analysis also revealed that the promoter methylation was restricted to a smaller region on CGI-2 in cases of MDS, CML and secondary AML compared with ALL (Fig. 1B).

Validation of Promoter Methylation Using Quantitative High-Throughput MALDI-TOF MS

To validate the MSP data and for quantitative assessment of methylation, we analyzed 96 samples (Normal, 12; MDS or AML, 18; CML, 11; B-ALL, 48; T-ALL, 4; and ambiguous lineage leukemia, 3), that were also analyzed by MSP, utilizing high-throughput MALDI-TOF MS methylation analysis (Sequenom). Since the quantitative values of CpG methylation assess the methylation of the total sample and thus can be diluted by normal hematopoietic cells present, we chose cases that had >50% tumor cells either by blast count or by FISH positivity for this analysis. As described above, CGI-2 was more frequently methylated; therefore, we chose this CpG Island for quantitative methylation covering 78 CpG sites in three amplicons. Of these, 47 CpG sites, which include single CpG sites and multiple CpGs that fall in one fragment, were considered informative after MALDI-TOF MS read out and quality control analysis (Fig. 2A). Methylation fraction per CpG calculated as described in Methods (mean + SD) was 0.115 + 0.03 for normal bone marrow specimens, 0.171 + 0.07 for MDS and AML cases, 0.111 + 0.017 for CML patients, 0.377 + 0.201 for pre B-ALL, and 0.321 + 0.259 for T-ALL and ambiguous lineage AL (Fig. 2B). The differences in methylation values between normal samples and CML were not statistically significant ($P = 0.70$), while MDS, AML, and T-ALL combined with ambiguous lineage AL samples showed marginally significant differences compared with normal samples ($P = 0.01$ for both). However, the levels of methylation were significantly higher in pre B-ALL compared with normal ($P = 0.0001$) (Figs. 2A and 2B). Supervised one-way hierarchical cluster analysis of normal samples showed only one specimen that was an outlier (Supporting Information Figure 1). This was the same specimen that also exhibited MSP positivity (Table 1). This sample showed a mean methylation fraction of 0.198 compared with 0.108 of the normal bone marrow specimens. Supervised analysis of normal and CML cases showed no distinct differences, a finding that confirms the MSP data that the promoter of *PCDH10* is not methylated in CML patients. A similar analysis between normal and MDS/AML samples showed a cluster containing four specimens with a higher level of methylation (Fig. 3, top panel). The comparison of normal with T-ALL samples and ambiguous lineage AL

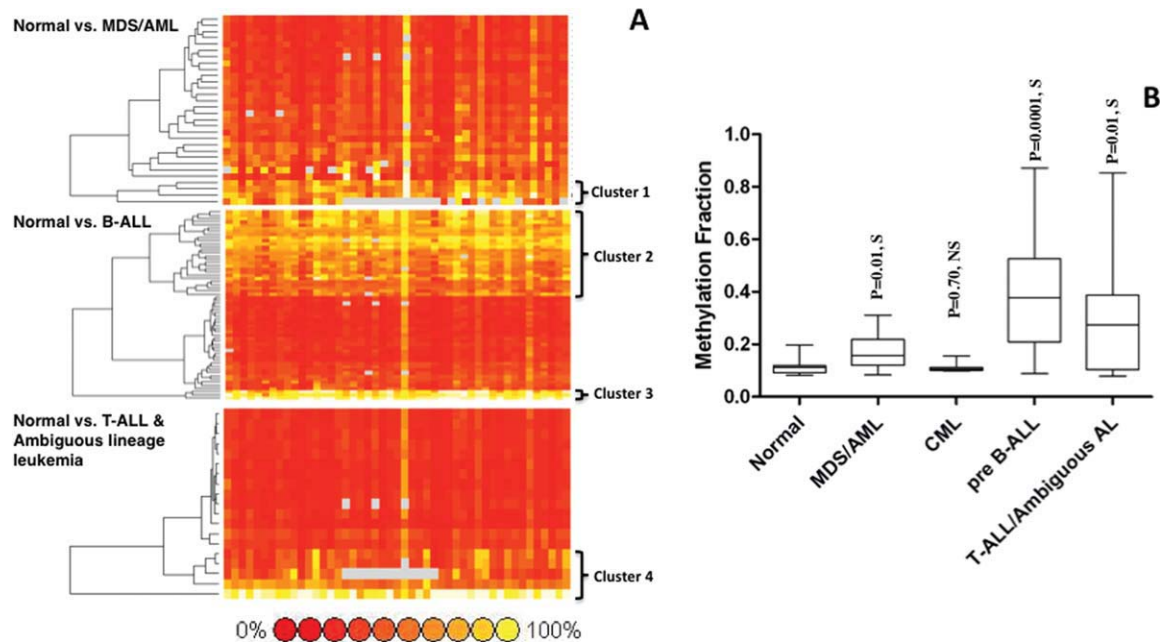


Figure 2. Quantitative analysis of methylation of *PCDH10* promoter region in normal bone marrow and in various subtypes of leukemia using Sequenom EpiTYPER. (A) Heat maps showing the one-dimensional cluster analysis of methylation data generated by Sequenom's MassArray and EpiTYPER. Top panel, normal versus MDS/AML, middle panel, normal versus B-ALL (middle) and bottom panel, normal versus T-ALL and ambiguous lineage leukemia. CpG Units are arranged along the x-axis and samples are on the y-axis. Clusters of methylated cases are indicated by right brackets. Missing data values

are annotated in gray. The red and yellow scale at the bottom represents the percentage methylation with dark red as lowest methylation and dark yellow as highest (~100%) methylation. (B) Methylation fraction per sample was calculated (see Methods) and plotted on box-plot. Significant differences between normal and MDS/AML, T-ALL/ambiguous lineage acute leukemia (AL), and pre B-ALL were observed. S, significant; NS, not significant. Box plot shows median, 25th and 75th percentile, minimum, and maximum values.

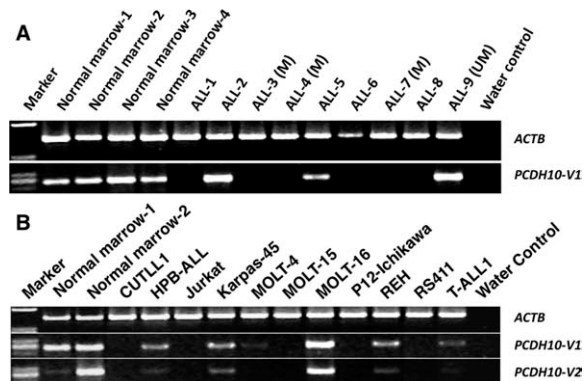


Figure 3. RT-PCR analysis of *PCDH10* expression in ALL. (A) Primary tumor samples. (B) ALL cell lines. ACTB, β actin; *PCDH10-V1*, variant 1; *PCDH10-V2*, variant 2. Bracketed M and UM indicates methylation and unmethylated promoter of *PCDH10*, respectively.

combined resulted in five cases (three T-ALL and two ambiguous AL) in a specific cluster (Fig. 2A, bottom panel). Analysis of B-ALL resulted in two distinct clusters (clusters 2 and 3 in Fig. 2A, middle panel) consisting of 41 (85.4%) of 48 cases compared with normal. All the samples that showed high methylation and were clustered by

supervised analysis also scored positive by MSP analysis. Therefore, the MALDI-TOF MS and MSP data showed a very close correlation of methylation between these methods (Supporting Information Tables 1 and 3). The MDS and leukemia cases that clustered with normal cases showed a methylation index closer to normal, while the cases that clustered separately from normal showed a higher methylation index (Supporting Information Table 3).

***PCDH10* Methylation in Relation to Clinical Parameters and Cytogenetic Subsets of ALL**

Since we observed a high frequency of promoter methylation of *PCDH10* promoter region in pre B- and T-ALL, we examined whether methylation of *PCDH10* correlates with various clinical and cytogenetic features (Supporting Information Table 1). We did not observe any correlation with clinical (time to relapse, overall survival, dead vs. alive) (Supporting Information Figure 3) or cytogenetic features (abnormal vs. normal karyotype, complex vs. simple karyotype, specific translocations, number of changes, ploidy, or

presence of any FISH detectable abnormality) by univariate analysis.

PCDH10 Promoter Hypermethylation Correlates with Downregulated Transcription in ALL Specimens and Cell Lines

Promoter hypermethylation of tumor suppressor genes generally results in transcriptional downregulation of the affected gene. Since a high frequency of promoter methylation was observed in both B-ALL and T-ALL cases, we wanted to examine if levels of *PCDH10* transcription were affected by promoter hypermethylation in these leukemias. To achieve this, we performed reverse transcription PCR analysis of the region between exons 1 and 3 of the *PCDH10* transcript variant 1 on a panel of four normal bone marrows, nine ALL specimens (eight B-ALL and one T-ALL) using 40 cycles of amplification and 29 cycles for the β -actin control. All four normal bone marrow specimens expressed high levels of *PCDH10* transcripts relative to control, whereas six (66.7%) of the nine ALL specimens showed a complete lack of expression and the remaining three showed expression levels similar to normal (Fig. 3A). Of the nine, ALL cases studied by expression, MSP analysis could be performed only on four cases, of which three were methylated and one unmethylated. The unmethylated case (ALL-9 in Fig. 3A) showed high levels of expression, while all the three methylated cases showed complete lack of expression (ALL-3, 4, and 7 in Fig. 3A). Overall, these data demonstrate that *PCDH10* is downregulated in the majority of ALL cases suggesting that this decreased expression may be a consequence of promoter methylation.

In addition, we examined the expression of both variants of *PCDH10* in 11 ALL cell lines (Figs. 1A and 3B). Since variant 2 is a single intronless exon, RNA isolated from these cell lines was treated with DNase to avoid DNA amplification. All the cell lines, except MOLT-16, showed downregulated expression of either variant 1 or variant 2, or both. These data further support the notion that *PCDH10* expression is downregulated in the majority of ALL cell lines.

Pharmacologic Inhibition of DNMTs Reactivates PCDH10 Expression in ALL Cell Lines

Since downregulated expression of *PCDH10* was found in 10 (90.9%) of 11 ALL cell lines, we wanted to examine its relationship with promoter methylation utilizing the MSP approach outlined above and in Figure 1. All the 11 cell lines exhib-

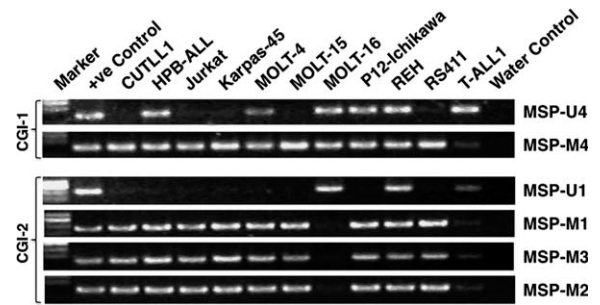


Figure 4. Methylation-specific PCR analysis of *PCDH10* in ALL cell lines. Top panel, CGI-1; Bottom panel, CGI-2. MSP, methylation specific PCR; U, unmethylated primer; M, methylated primer.

ited promoter methylation of either one or both the CGIs. Five of the cell lines (CUTLL1, Jurkat, Karpas-45, MOLT-15, and RS411) showed no unmethylated allele in CGI-1 and CGI-2. In addition, three other cell lines (HPB-ALL, MOLT-4, and P12-Ichikawa) lacked an unmethylated allele in CGI-2, while they were present in CGI-1. Two other cell lines (REH and T-ALL1) showed methylated and unmethylated alleles in both CGI-1 and CGI-2. The only cell line that was exclusively positive for methylation of CGI-1 but not CGI-2 was MOLT-16 (Fig. 4). This cell line showed no downregulated expression of either variant 1 or 2 (Figs. 3B and 5). Taken together these results suggest that methylation of CGI-2 is critical for transcriptional downregulation of *PCDH10*. These findings also demonstrate that the cell line data are concordant with those of the primary ALL specimens regarding the frequency of promoter hypermethylation and its correlation with downregulated expression of *PCDH10*.

Because DNA hypermethylation-mediated gene silencing is associated with histone modifications, which can be reactivated by the DNA demethylating agent 5-aza-CdR and the HDAC inhibitor trichostatin A (TSA), we tested whether the promoter hypermethylation-mediated downregulated *PCDH10* expression could be reversed after treatment with these drugs on ALL cell lines. We performed varying cycles (28, 30, and 40 cycles for variant 1; 28 and 30 cycles for variant 2) of semi-quantitative RT-PCR analysis of DNase-treated total RNA. Of the 11 cell lines studied, 9 (T-ALL1, MOLT-4, MOLT-15, REH, P12-Ichikawa, CUTLL1, HPB-ALL, Jurkat, and RS411) exhibited reactivation of the *PCDH10* gene after 5-aza-CdR alone or in combination with TSA (Fig. 5). Of these nine cell lines, four

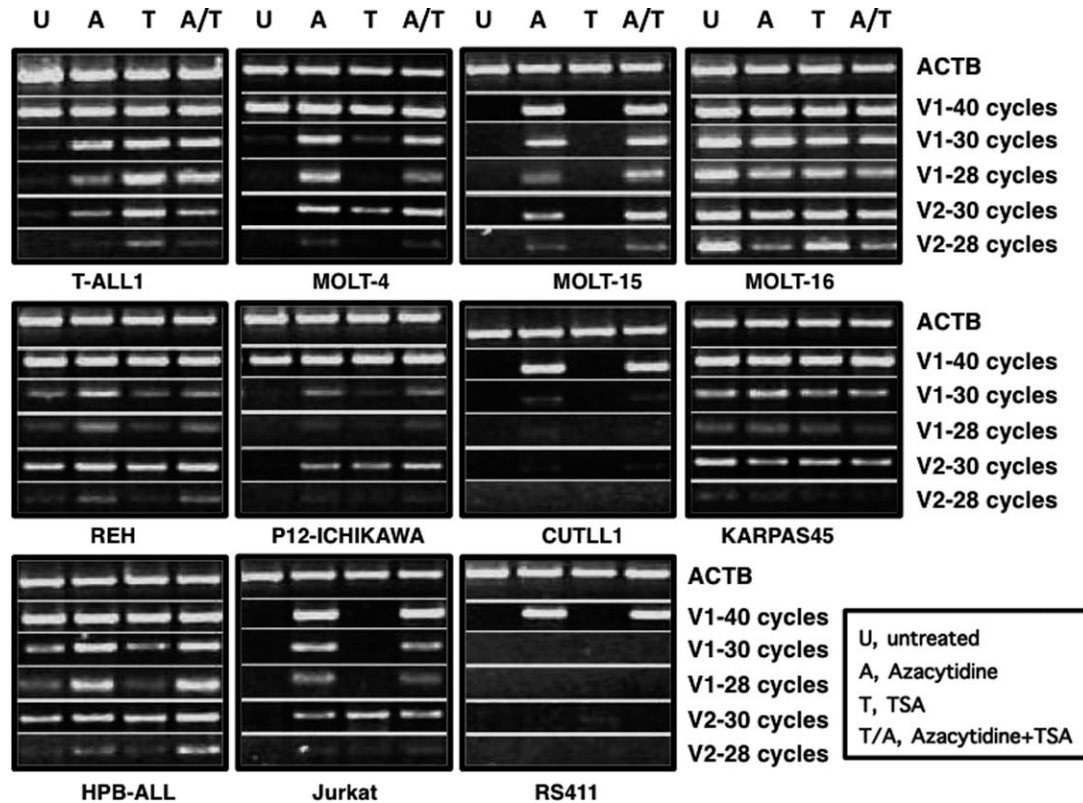


Figure 5. Effect of drug treatment using inhibitors of methylation and HDAC on gene reactivation by RT-PCR analysis in ALL cell lines. Varying cycles of PCR were performed to exclude the possibility of saturated or undetectable levels of transcript amplification. V1, variant 1; V2, variant 2.

(T-ALL1, MOLT-4, P12-Ichikawa, and Jurkat) also showed reactivation with TSA alone suggesting a role for chromatin modifications in suppressing *PCDH10* transcription. Of the two remaining cell lines, Karpas45, carrying methylated alleles of both CGIs, showed no evidence of reactivation on treatment with any of the drugs. This cell line showed a relative decrease in expression of both variants in untreated cells (Figs. 3B and 5). Thus, the mechanism for the lack of reactivation cannot be explained by DNA methylation alone and mutations in upstream regulators or the requirement for other transcriptional co-factors cannot be ruled out. The MOLT-16 cell line that showed methylation of only CGI-1 did not exhibit downregulated expression and we found no increase in expression on treatment with any of the drugs (Fig. 5). None of the treatments had any affect on the levels of expression of the control gene. Thus, these data indicate that demethylation of the *PCDH10* promoter effectively reactivates gene expression by reversing the effect of methylation in most cell lines.

ALL Cell Lines Harboring *PCDH10* Methylation and Downregulated Transcription Exhibit Resistance to Leukemia-Specific Chemotherapy Drugs

Since the DNA promoter methylation of specific genes plays a significant role in development, progression, and prediction of treatment responses, we examined the effect of promoter hypermethylation-associated silencing of *PCDH10* on treatment response on a panel of ALL cell lines after exposing to the standard leukemia-specific drugs. To test whether *PCDH10* promoter-associated inactivation plays a role in drug resistance in ALL, we analyzed the responses of six (RS411, Jurkat, CTLL1, p12-Ichikawa, T-ALL1, MOLT-16) ALL cell lines chosen based on their *PCDH10* promoter methylation status and expression to defined doses of doxorubicin, dexamethasone, bortezomib, methotrexate, and L-asparaginase. First group consisted of cell lines T-ALL1 and MOLT-16, which exhibited unmethylated promoter of the CGI-2 mapped within the exon 1 of *PCDH10* and expressed high level

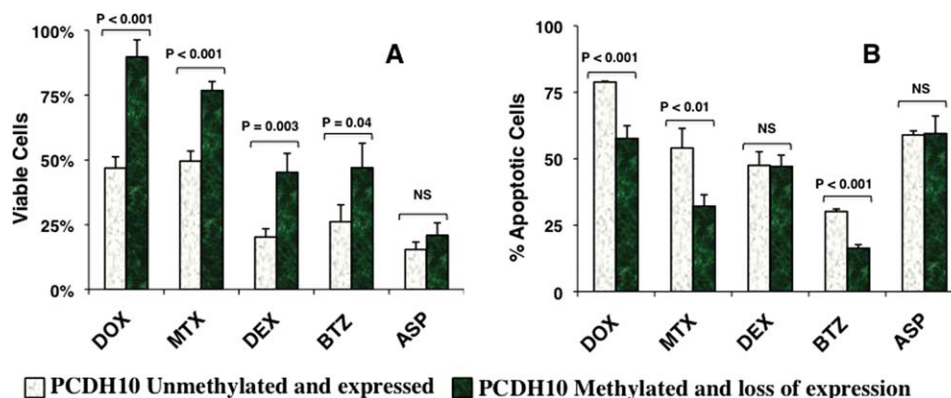


Figure 6. Analysis of cell viability and apoptosis in ALL cell lines exposed to leukemic drugs in relation to epigenetic silencing of *PCDH10* gene. Viability assay showing cell survival (A) and cell death by apoptosis assay (B) in cell lines with unmethylated promoter and normal expression (T-ALL1 and MOLT-16) and epigenetic silenced cell

lines (Jurkat, P12-Ichikawa, RS411, and CTULL1). Drugs used are L-asparaginase (ASP), bortezomib (BTZ), doxorubicin (DOX), dexamethasone (DEX), and methotrexate (MTX). NS, not significant. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

of transcripts of both variants (Figs. 4 and 5). The second group consisted of cell lines Jurkat, P12-Ichikawa, CUTLL1 and RS411 that were positive for promoter methylation of one or both CpG islands and a lack or downregulated expression of both variants (Figs. 4 and 5). The group containing Jurkat, P12-Ichikawa, CUTLL1, and RS411 cells exhibiting *PCDH10* promoter hypermethylation with inactivated transcription was highly resistant to doxorubicin, methotrexate, dexamethasone, and bortezomib showing significantly high cell viability by MTT assay, while L-asparaginase showed no significant difference in cell survival between the two groups (Fig. 6A). These data suggest that ALL cells with methylation-mediated inactivation of *PCDH10* are resistant to drug-induced response to cell lethality. We next asked if methylated cell lines also exhibit a similar resistance in apoptotic response to drug treatments. Utilizing the Pacific Blue Annexin V staining method, we showed a significantly reduced apoptosis in *PCDH10* inactivated cell lines after treatment with doxorubicin, methotrexate, and bortezomib compared to unmethylated cell lines (Fig. 6B and Supporting Information Figure 4). In contrast to the MTT data, dexamethasone treatment did not show any difference in apoptotic response between the methylated and unmethylated cell lines. Similar to the MTT data, L-asparaginase showed no difference in apoptosis between epigenetically silenced and unsilenced cell lines. Together, these results demonstrate that methylation-mediated inactivation of *PCDH10* negatively influences treatment responses in ALL cell lines.

DISCUSSION

Over the past three decades, the discovery of acquired karyotypic and genetic changes has played a crucial role in our understanding of the biology of hematologic malignancies and has led to the development of targeted therapies. However, the role of epigenetic modification and their interplay with genetic alterations remains poorly understood in hematologic malignancies. Recent studies have suggested that specific chromosomal translocations and gene mutations confer chromatin modifications at certain loci, which has led to the development of therapies influencing the epigenetic landscape of leukemia (Nowak et al., 2009). Although cancer cells frequently exhibit hypermethylation of gene promoters and coding regions, epigenetic changes that underlie the development of leukemias remain poorly understood.

The protocadherin gene family members contain six extra cellular cadherin domains, a transmembrane domain and a cytoplasmic tail differing from those of the classical cadherins. *PCDH10*, encoding a cadherin-related neuronal receptor, is thought to play a role in the establishment and function of specific cellular connections in the brain (Frank and Kemler, 2002). Although the exact functions of protocadherins are not well understood, they are believed to play a role in signal transduction and growth control. Compelling evidence now exists that cadherins play an important role in tumor progression by functioning as suppressors of invasion and metastasis (Jeanes et al., 2008). The promoter region of the

PCDH10 gene has been shown to be methylated in a variety of tumors (Waha et al., 2005; Imoto et al., 2006; Yu et al., 2008, 2009; Narayan et al., 2009; Cheung et al., 2010). Demethylation or over expression of *PCDH10* significantly inhibits colony formation and proliferation of solid tumor cells in vitro (Ying et al., 2006). However, the role of *PCDH10* in leukemia has not been examined so far.

In the present study, we show that the promoter of *PCDH10* is hypermethylated and the expression of this gene is silenced in the vast majority of B-cell, T-cell, and ambiguous lineage ALL, while it is absent in CML and infrequent in other myeloid-lineage leukemia (MDS and AML). These data therefore suggest that epigenetic changes in the promoter of *PCDH10* differ between myeloid- and lymphoid-lineage leukemias. The identification of a high frequency of promoter hypermethylation of *PCDH10* in both B-ALL and T-ALL suggests that inactivation of this gene most likely plays a role during the neoplastic transformation of lymphoid-lineage cells to leukemic lymphoblasts.

B-ALL represents a highly heterogeneous disease consisting of different genetic subtypes that are characterized by specific chromosome translocations (Mrozek et al., 2004). Furthermore, these genetic subtypes exhibit distinct gene expression profiles and differ in their response to chemotherapy and prognosis (Kang et al. 2010). Similarly, T-ALL is a relatively heterogeneous disease, although its genetic sub-groups are less well defined and the patients exhibit overall poor prognosis (Marks et al., 2009). Thus, there is a need for better understanding of underlying genetic and epigenetic alterations in ALL to design novel therapeutic agents and to predict prognosis. Epigenetic changes DNA methylation and chromatin modifications dictate the biological behavior and response to therapy of cancer cells. In the present study, we analyzed the methylation status of *PCDH10* to examine its association with recurrent chromosomal changes such as *ETV6/RUNX1* [t(12;21)], *MLL* [11q23], *BCR/ABL1* [t(9;22)], and ploidy and found no correlation with specific genetic subtypes (Supporting Information Table 1). We also found no differences between the methylation status of *PCDH10* and overall survival or disease relapse. This observation reflects the fact that *PCDH10* methylation is common to most ALL cases, and is seen in a high percentage of patients; thus, this phenom-

enon might represent an early event in leukemogenesis for all subtypes of ALL.

Although the role of *PCDH10* in cancer has not been fully elucidated, evidence for the epigenetic silencing of this gene contributing to tumorigenesis has been noted (Ying et al., 2006). Most recently, *PCDH10* was reported to be methylated in early stages of gastric carcinogenesis and its methylation was associated with poor prognosis in gastric cancer patients (Yu et al., 2009). It has also been shown that silencing of *PCDH10* attenuates apoptosis in an imatinib-resistant CML cell line K562, suggesting that *PCDH10* might promote apoptosis. These data suggest that *PCDH10* expression is regulated by hBex1, where hBex1 over expression in leukemia cells results in increased expression of *PCDH10* (Ding et al., 2009). Thus, it is conceivable that deregulation of the hBex1/*PCDH10* pathway could play a role in drug resistance in leukemias. Our present work implicates a role for *PCDH10* methylation in ALL, since this gene was a target of epigenetic silencing in the majority of cases and in vitro resistance of ALL cells to leukemic drugs was attributable to epigenetic silencing. Although the mechanisms underlying cellular resistance to apoptosis in relation to *PCDH10* inactivation in ALL biology remains unclear, our findings provide a biomarker for chemotherapy resistance that could facilitate the design of epigenetic strategies to improve the outcome of treatment refractory ALL patients. Furthermore, additional mechanisms of inactivation of this gene cannot be excluded as tumor suppressor genes can be inactivated by multiple mechanisms including mutations.

Genetic alterations of both B-ALL and T-ALL have been well characterized illustrating the relationship between recurrent chromosome changes, mutations, and gene expression (Mullighan and Downing, 2009a, b). Although these advances have led to proper risk stratification and successful treatments, most adult ALL are not cured and a large proportion of childhood ALL experience relapse (Fielding et al., 2007; Nguyen et al., 2008). Identification of additional genetic and epigenetic pathways is therefore important in understanding the mechanisms involved in initiation and chemoresistance of ALL. Recent studies in cancer suggest that epigenetic lesions play a critical role in leukemogenesis and successful induction therapy using decitabine in myeloid leukemias. However, the role of DNMT inhibitors and their efficacy in ALL remains poorly

understood (Yanez et al., 2009). Dissecting the mechanisms of drug effect and designing suitable assays with clinical relevance or prognostic potential remain major challenges for therapy involving these agents.

In the present study, we describe the novel finding of epigenetic silencing of *PCDH10* in a high proportion of ALL and its role in resistance to standard leukemic drugs, suggesting a role for *PCDH10* in B- and T-cell lymphoblastic leukemogenesis. These findings add to the extant data regarding the role of epigenetic alterations in acute leukemias. Further investigations are warranted to explore the role of *PCDH10* in ALL biology and as a potential therapeutic target for this disease.

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