Epigenetic Inactivation of Notch-Hes Pathway in Human B-Cell Acute Lymphoblastic Leukemia

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Abstract
The Notch pathway can have both oncogenic and tumor suppressor roles, depending on cell context. For example, Notch signaling promotes T cell differentiation and is leukemogenic in T cells, whereas it inhibits early B cell differentiation and acts as a tumor suppressor in B cell leukemia where it induces growth arrest and apoptosis. The regulatory mechanisms that contribute to these opposing roles are not understood. aberrant promoter DNA methylation and histone modifications are associated with silencing of tumor suppressor genes and have been implicated in leukemogenesis. Using methylated CpG island amplification (MCA)/DNA promoter microarray, we identified Notch3 and Hes5 as hypermethylated in human B cell acute lymphoblastic leukemia (ALL). We investigated the methylation status of other Notch pathway genes by bisulfite pyrosequencing. Notch3, Jag1, Hes2, Hes4 and Hes5 were frequently hypermethylated in B leukemia cell lines and primary B-ALL, in contrast to T-ALL cell lines and patient samples. aberrant methylation of Notch3 and Hes5 in B-ALL was associated with gene silencing and was accompanied by decrease of H3K4 trimethylation and H3K9 acetylation and gain of H3K9 trimethylation and H3K27 methylation. 5-aza-2'-deoxycytidine treatment restored Hes5 expression and decreased promoter hypermethylation in most leukemia cell lines and primary B-ALL samples. Restoration of Hes5 expression by lentiviral transduction resulted in growth arrest and apoptosis in Hes5 negative B-ALL cells but not in Hes5 expressing T-ALL cells. These data suggest that epigenetic modifications are implicated in silencing of tumor suppressor of Notch/Hes pathway in B-ALL.

Introduction
The Notch receptor signaling pathway has been implicated in regulating hematopoietic stem cell self-renewal, cell lineage commitment, differentiation, and maturation [1,2,3]. Human Notch family consists of four Notch receptors (Notch1, 2, 3 and 4) and five ligands (Jagged1/2, Delta-like ligand 1/3/4). Upon ligand binding, the receptors undergo cleavage and release of the intracellular domain, which translocates to the nucleus and associates with the CSL (also known as RBP-Jk) transcription factor. The Notch/CSL complex activates transcription of target genes containing CSL binding elements, most notably members of the Hairy/Enhancer of Split (HES) family (Hes1-6) of transcriptional repressors [4,5,6].

During lymphoid development, B- and T-lymphocytes make series of cell fate decisions [7,8]. Notch signaling has been shown to regulate T and B cell lineage commitment and direct the maturation of T cells at the expense of B cells [9]. Activation of the Notch signaling through point mutations and translocations of the Notch1 gene has been demonstrated in 50–70% of human T cell leukemia/lymphomas [6,7,10,11]. It has also been suggested that nearly all human T cell acute lymphoblastic leukemia (T-ALL) overexpress Notch3 [12]. Constitutive Notch signaling promotes T cell proliferation, results in neoplastic transformation of T lymphoid progenitors, and leads to T cell malignancy. On the other hand, Notch signaling can function as a tumor suppressor in a variety of tissue types [1,13]. For example, in human B-cell leukemia/lymphoma, constitutive expression of the active forms of the Notch receptors (ICN1-4) or the Notch downstream target gene Hes1 can induce growth arrest and apoptosis [14]. However, the molecular mechanisms underlying the oncogenic and tumor suppressive activities of Notch are not understood.

Appropriate cell lineage determination and differentiation are governed by epigenetic processes such as DNA methylation, histone modification which affect higher order chromatin structure [15]. Methylation of CpG islands in the promoter region of genes is known to correlate with repression of gene transcription [16]. Histone modifications can also act synergistically or antagonistically to define the transcription status of genes [17,18]. Aberrant promoter CpG island (CGI) methylation and its associated histone
modifications are widely accepted mechanisms in silencing tumor suppressor genes and both have been shown to be major contributors and an early events in leukemia pathogenesis [19]. Here we hypothesized that aberrant epigenetic regulation of the Notch-Hes pathway is involved in the pathogenesis of ALL.

**Materials and Methods**

**Cell lines and leukemia patient samples**

The following human leukemia cell lines were studied: of T cell origin: MOLT4, Jurkat, Peer, T-ALL1, CEM, J-TAG, SupT1 and Loucy; of B cell origin: B-LJAB, RS-11, ALL1, REH, RPMI8226, Raji and Ramos. T-ALL1 and Peer cell lines were obtained from the German Resource Center for Biological Material (DSMZ, Germany). The other cell lines, including 293T, were obtained from the American Type Culture Collection (ATCC). Cell lines were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) with 10% calf fetal serum (FCS, Gemini Bio-Products, Woodland, CA). Bone marrow (BM) aspiration specimens from patients with B-cell acute lymphoblastic leukemia (B-ALL) and T-ALL were obtained from established tissue banks at MD Anderson Cancer Center (MDACC) following institutional guidelines. This included approval by the MDACC Institutional Review Board (IRB) of both a tissue banking protocol and appropriate laboratory protocol for the proposed studies. Patients signed informed consent for those studies following MDACC IRB guidelines. All samples were collected using Ficoll-Paque density centrifugation. Normal CD19+ B cells were collected from 10 healthy volunteers. Consent was also obtained from volunteers. Normal CD19+ B cells were isolated using Human B Cell Isolation Kit (Miltenyi Biotec, Auburn, CA) as described. DNA was extracted using standard phenol-chloroform methods.

**DNA bisulfite treatment, Pyrosequencing and Bisulfite Sequencing**

Bisulfite induces deamination of unmethylated cytosines, converting unmethylated CpG sites to UpG without modifying methylated sites, as described [19]. For pyrosequencing, a two-step PCR reaction was performed [19]. Primer sequences and conditions are shown in Table S1. The final kit labeled PCR product was captured by Streptavidin Sepharose HP (Amersham Biosciences, Uppsala, Sweden). PCR products bound to the bead were purified and made single-stranded using a Pyrosequencing Vacuum Prep Tool (Biotage Inc, Uppsala, Sweden). The sequencing primer was annealed to the single stranded PCR product and pyrosequencing was done using the PSQ HS 96 Pyrosequencing System (Biotage Inc, Uppsala, Sweden). Quantification of cytosine methylation was performed using the PSQ HS96A 1.2 software package (Biotage Inc, Uppsala, Sweden). Bisulfite sequencing was performed as described to confirm pyrosequencing results in selected samples [19,20].

**RNA extraction, cDNA synthesis and Real-time PCR**

Total cellular RNA was extracted with Trizol (Invitrogen, Carlsbad, CA) and reverse transcribed with MMLV-RT kit (Invitrogen, Carlsbad, CA) and random hexamers. Whole bone marrow (BM) cDNA, CD34+ BM CDNA was purchased from Cell Systems (All Cells, LLC. Emeryville, CA). For real-time PCR analysis of mRNA expression, TaqMan probes were purchased from Applied Biosystems and analyzed using an Applied Biosystems Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA).

5-aza-2′-deoxycytidine and/or suberoylanilide hydroxamic acid treatment

To study the effect of epigenetic modulation, leukemia cell lines were cultured in media supplemented with 2 μmol/L of 5-aza-2′-deoxycytidine (DAC) (Sigma, St Louis, MO) for daily 4 days, 2 μmol/L of DAC for 4 days and then 1 μmol/L suberoylanilide hydroxamic acid (SAHA) (ICN Biomedicals) for the last 24 hours, or 1 μmol/L SAHA for 24 hours alone as described [19].

**Chromatin immunoprecipitation (ChiP) assays**

ChiP assays were performed with EZ ChiP™ kit (Millipore Upstate, Charlottesville, VA) according to the manufacturer protocol with modification. Briefly, cross-linked, sonicated DNA products were incubated with anti H3K9Ac, H3K4me3, H3K9me3, H3K27me3 or H3-Ac antibody (Millipore Upstate, Charlottesville, VA) and protein A agarose beads (Millipore Upstate, Charlottesville, VA). Controls were based on manufacturer recommendation. ChiP-real-time PCR analysis was analyzed using an ABI Prism 7900 (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol.

**Luciferase assay**

The Hes5 regulatory sequence from 436 bp upstream to 264 bp downstream of the Hes5 transcription start site (TSS) was cloned into luciferase reporter vector pGL3 (Promega Co., Madison, WI). For in vitro methylation, the Hes5 promoter was treated with SssI methylase (New England BioLabs, Beverly, MA). 293T (human renal epithelial cell line) cells were transiently transfected with the methylated and unmethylated Hes5–pGL3 constructs or pGL3-basic vector, together with 0.1 ng pRL-TK control vector, which encodes Renilla luciferase (Promega Co., Madison, WI). Luciferase activity was determined using a Dual Luciferase Assay System (Promega Co., Madison, WI). All experiments were carried out in triplicate.

**Lentivirus constructs and gene transduction**

Human Hes5 lentiviral construct was generated by inserting human full length Hes5 cDNA (OriGene Technologies, Rockville, MD) into an lentiviral-CMV LTR-Ubiquitin-IRES-GFP transfer vector (FUGW), as described previously [21,22].

**Statistical analysis**

Statistical analyses were performed using Prism 4 (GraphPad Software, Inc.) or Statistica 6 software (Statistica for Windows version 6.0, StatSoft, Tulsa, OK). The Fisher’s exact test and t-tests were used to compare gene methylation frequencies or expression levels in leukemia cell lines or leukemia patients and normal control groups. The Spearman non-parametric test was used to determine correlations. All reported p values were 2-sided and P<0.05 was considered statistically significant.

**Results**

**Identification of hypermethylated Notch3, JAG1, Hes2, Hes4 and Hes5 genes in leukemia cell lines**

Using MCA/DNA promoter microarray, we identified Notch3 and Hes5 as potential methylated targets in primary B-ALL samples [19]. We further investigated the methylation status of Notch pathway genes in a panel of B-ALL and T-ALL cell lines and normal PB cells. Multiple Notch pathway genes were found to contain CpG islands in their promoter regions as identified by using Human Blat program (http://www.genome.ucsc.edu), including receptors (Notch1, Notch2, Notch3), ligands (Jag1, Jag2, Notch-Hes Methylation in B Cell ALL
DLL1, DLL3, DLL4) and target genes in the Hes subfamily (Hes2, Hes4, Hes5, Hes6). Their methylation profiles are shown in Figure 1A. Notch3, Hes5, Hes2, Hes4 and JAG1 genes were found frequently hypermethylated in various leukemia cell lines but not in normal controls. Notch3, Hes5, Hes2, Hes4 were methylated more frequently and to a greater extent in B-ALL cell lines while Jag1 was methylated in T-ALL cell lines (Figure 1). Specifically, methylation frequencies of these genes in B-ALL vs. T-ALL were: 100 vs. 50% for Notch3 (P < 0.05), 86% vs. 50% for Hes5 (P < 0.05), 86% vs. 50% for Hes2/P < 0.05), 57% vs. 25% for Hes4 (P < 0.05, Figure 1A&B). Methylation density is shown in Figure 1C&D. Significant high density methylation of Notch3 and Hes5 was found in B-ALL cells. The mean methylation density of these two genes in B-ALL vs. T-ALL were 84% vs. 36% for Notch3 and 78% vs. 47% for Hes5. In contrast, Notch1 and Notch2 genes were un-methylated in any of the leukemia cell lines and normal controls, while DLL1, DLL3, DLL4 and Hes6 showed only low levels of methylation (9-27%) in these leukemia cell lines.

Differential DNA methylation of Notch3 and Hes5 genes in primary B cell leukemia compared to T-ALL

We subsequently evaluated the methylation status of Notch3, JAG1, Hes2, Hes4 and Hes5 genes in pretreatment patients with different types of ALL. These included 54 patients with B-ALL and 14 with T-ALL. Patient characteristics have been reported previously [19]. To exclude cell lineage specific methylation, we used 10 normal CD19+ B cells as controls. Methylation frequencies and densities are shown in Figure 2. As in ALL cell lines, hypermethylation of Notch3 and Hes5 was observed preferentially in primary B-ALL and was much lower in T-ALL (70 vs. 7% and 71 vs. 8% respectively, P < 0.05). Hypermethylation of Hes4 occurred more prominently in B-ALL (71 vs. 14%, P < 0.05), while Hes2 methylation was similar between groups (33 vs. 40%, P > 0.05). Interestingly, hypermethylation of JAG1 was seen to a greater degree in T-ALL than B-ALL patient samples (50 vs. 30%, P < 0.05), which is consistent with our findings in ALL cell lines (Figure 1).

Distinct expression of Notch pathway genes in normal hematopoietic lineage cells

To investigate the role of DNA methylation in the regulation of gene expression, mRNA levels of Notch1-3, JAG1, Hes1, Hes2, Hes4 and Hes5 genes were analyzed by quantitative real-time-PCR in normal hematopoietic lineage cells, leukemia cell lines and patients primary bone marrow samples. Figure 3A shows the expression levels of these genes in healthy adult whole bone marrow (BM), CD34+ BM cells, whole peripheral blood (PB) cells, PB CD19+ B cells (PB-B) cells and PB-T cells. Notch2 and Hes5 transcripts were abundantly detected at all stages of human BM cell development, although the expression level of Hes5 was relatively lower than that of Notch2.
Dysregulation of Notch pathway gene expression by DNA methylation and histone modification in leukemia cells

We further examined the correlation between the expression levels and methylation status of Notch3, JAG1, Hes2, Hes4 and Hes5 genes in B- and T-ALL cell lines. Hes5, Notch3 and Hes4 genes were either not expressed or very weakly expressed in highly methylated B leukemia cell lines but were abundantly expressed in unmethylated T cell leukemia cell lines such as T-ALL1 and SupT1 (Figure 3B and Figure S1). Hypermethylation of Hes5 CpG islands correlated with down-regulated Hes5 expression as methylation density >15% was used as the cut off to determine a sample as methylated (Figure 3C). We also observed down-regulation of Hes5, Notch3 and Hes4 expression in unmethylated or partially methylated cell lines suggesting that histone deacetylation might be associated with silencing of these genes [18]. To determine the relationship between histone modifications and DNA methylation at the Hes5 locus, we performed ChIP assay in leukemia cell lines having different expression levels of Hes5. We observed that unmethylated and active Hes5 locus in T-ALL1 cells was enriched in H3K9Ac, H3K4me3, but lacked H3K9me3 and H3K27me3 (Figure 3D). In contrast, the hypermethylated and silent Hes5 locus in CEM and RS4;11 cells was hypoacetylated at H3K9Ac and lacked H3K4me3, but was enriched in H3K9me3 and H3K27me3 (Figure 3D). The unmethylated and down-regulated Hes5 locus in Mol4 cells was deacetylated at H3K9Ac and down-regulated H3K4me3, but lacked H3K9me3 and H3K27me3 (Figure 3D). These results indicate that distinct histone modification profiles correlate with Hes5 gene transcriptional activity. Specifically, histone deacetylation and H3K9me is also a feasible mechanism for the silencing of Hes5 in leukemia cells.

Decitabine treatment restores expression of Notch pathway genes

To explore the role of the DNA methylation in the silencing of gene expression, several cell lines were treated with the demethylation agent decitabine (5-aza-2'-deoxycytidine, DAC) and/or histone deacetylase inhibitor vorinostat (SAHA). In general, expression of Hes5, Hes4 and Notch3 was restored in methylated leukemia cell lines treated by DAC with or without SAHA, a phenomenon associated with gene demethylation (Figure 4A and Figure S2). We also observed an enhancement of Hes5, Hes4 and Notch3 expression in some unmethylated cell lines by SAHA treatment or the combination of DAC and SAHA, suggesting that histone deacetylation is associated with suppressed expression of these genes. We further analyzed Hes5 DNA methylation and histone acetylation status in Molt4, PEER, RS4;11 and REH cell lines before and after DAC treatment. DAC treatment for 5 days or DAC plus SAHA treatment resulted in hypomethylation of Hes5 promoter and hyperacetylation of histone H3 in these cell lines, as measured by bisulfite pyrosequencing and ChIP assay (Figure 4B/C). These data indicates that DNA methylation and histone deacetylation are associated with gene silencing.

Role of DNA methylation in the transcriptional silencing of Hes5 gene

To test whether the CGI within Hes5 promoter is important for transcription of Hes5, we performed bisulfite sequencing using 159 and 141 bp PCR fragments from −191 to −290, +141 to +203 encompassing 24 CpG sites. Methylation mapping revealed that Hes5 was methylated over the CGI in Hes5 negative RS4;11 cells (Figure 4D). To investigate the role of DNA methylation in regulating Hes5 expression, we tested methylated and unmethylated versions of a Hes5 promoter-driven luciferase reporter and found that the promoter activity of the methylated pHes5-pGL3
Figure 3. Expression and histone modification analysis of Hes5 genes in leukemia cell lines and normal hematopoietic lineages. A. Expression levels of Notch1, Notch2, Notch3, JAG1, Hes1 and Hes5 in normal whole bone marrow (BM), CD34+BM, peripheral blood (PB), CD19+ B cells (PB-B) and CD3+ T cells (PB-T). The relative gene expression was determined by real-time PCR assays and normalized to GAPDH. B. Hes5 mRNA expression levels in leukemia cell lines by RT-PCR. Hes5 methylation levels from Figure 1A are shown under each cell lines. C. left. Hes5 expression is significantly lower in Hes5 methylated cell lines when compared with unmethylated cell lines. C. right. Hes5 mRNA expression inversely correlated to methylation level. The solid line represents the regression of the degree of methylation on the Hes5 expression level. D. ChIP assay of the Hes5 CpG islands. Chromatin DNA was immunoprecipitated with antibodies against the indicated histone modifications. Immunoprecipitated DNA was amplified by real-time PCR. Percent input was determined as the amount of immunoprecipitated DNA relative to input DNA. Experiments were performed in duplicate. Bars, SD.

doi:10.1371/journal.pone.0061807.g003
construct was 40 times lower (and virtually silenced) than that of the unmethylated construct (Figure 4D). Taken together, these results suggested that promoter hypermethylation of Hes5 gene silences its transcription.

Distinct expression patterns of Hes5 and Notch3 in primary B cell leukemia compared to T-ALL and their response to 5aza-dC treatment

To examine Hes5 and Notch3 expression during leukemogenesis, we performed real-time PCR analysis in BM samples from patients with B-ALL and T-ALL. Hes5 and Notch3 were highly expressed in T-ALL, but were significantly decreased or absent in B-ALL samples (Figure 5A and Figure S1). The down-regulation of Hes5 and Notch3 expression correlated with hypermethylation of their CpG islands (Figure 5B and data not shown). We further analyzed Hes5 methylation status in 17 B-ALL patients who received DAC 75 mg/m² daily for 7 days on an investigational clinical trial (protocol NCT00349596; Garcia-Manero, in preparation). We found substantial reduction in methylation of Hes5 promoter as measured by pyrosequencing in 7 of 14 patients.

Figure 4. Restoration of Hes5 expression by treatment with 5-aza-2’-deoxycytidine (DAC) and suberoylanilide hydroxamic acid (SAHA) in leukemia cell lines. A. Hes5 expression and the effect of demethylating treatment. The leukemia cells were either untreated (C), or treated with DAC only, SAHA only or both (D+S) as described in material and methods. Quantitative RT-PCR was used to measure Hes5 mRNA expression. B. Effect of epigenetic modulation on Hes5 gene methylation. Pyrosequencing was performed to determine methylation level. C. DAC and SAHA treatment increase acetylated histone H3 as detected by ChIP-real time PCR. D. Promoter hypermethylation silences expression of Hes5. Top. Diagram of the human Hes5 promoter region studied. CpG sites are indicated by short vertical bars. Arrows point to transcription start site (TSS). The region for bisulfite sequencing, ChIP PCR and promoter activity assay are indicated. Left bottom: Methylation analysis of Hes5 gene promoter region by bisulfite sequencing. Each row of circles represents the sequence of an individual clone. Open circles, unmethylated CpG sites; filled circles, methylated CpG sites. Right bottom: Promoter activity of the Hes5 CpG islands. The relative luciferase activities of the unmethylated and methylated pGL3-Hes5 constructs in 293T cells.

doi:10.1371/journal.pone.0061807.g004
Methylation analysis for one of them is shown in Figure 5C. For these 7 patients, hypermethylation of Hes5 was confirmed by bisulfite sequencing and the difference on day 30 vs day 1 DAC treatment was statistically significant (p<0.05) (Figure 5D). We also analyzed LINE methylation (a surrogate marker of global DNA methylation) dynamics during DAC treatment by pyrosequencing. We found a significant decrease in global methylation by day 12 (Figure 5C bottom), which was similar to the Hes5 methylation pattern (Figure 5C top).

**Discussion**

Leukemia is both a genetic and epigenetic disease. Abnormal promoter DNA methylations and histone modifications have gained increasing recognition as an important mechanism for silencing of tumor suppressor genes and contribute to leukemogenesis along with genetic alterations [23]. Using MCA/microarray, we identified Notch pathway genes Notch3 and Hes5 as hypermethylated in human B-ALL samples. In this study, we investigated the methylation status of Notch pathway genes in leukemia cell lines and patient samples by pyrosequencing. Methylation verification revealed that Notch3, Hes5, Hes2, Hes4 and JAG1 genes were frequently hypermethylated in various leukemia cell lines but not in normal controls. Methylation analysis of these genes were different in various types of leukemias. JAG1, Hes2 and Hes4 were commonly methylated in various leukemia cell lines and primary B-ALL and T-ALL but not in normal CD19+ B cells. In contrast, Notch3 and Hes5 were found preferentially hypermethylated in B-lineage lymphoblastic cell lines and primary B-ALL, but methylated at very lower levels or unmethylated in T cell lines or primary T-ALL. In most cases, Notch3, Hes4 and Hes5 are found to be coordinately methylated.
The observation of concomitant methylation of several Notch pathway genes at different chromosomal loci suggests that epigenetic disruption of Notch signaling may be an important event in leukemia pathogenesis. The distinct methylation pattern of Notch3 and Hes5 genes in primary B cell leukemia compared to T-ALL further suggest that aberrant DNA methylation occur in a tumor specific and lineage-specific fashion.

In the present study, we also investigated the expression patterns of Notch pathway genes in normal hematopoietic lineage cells. We demonstrated that Notch2 and Hes5 were highly expressed in multiple lineages, whereas Notch3 was not expressed in mature lymphocytes, but was expressed on a subset of CD34+ stem/progenitor cells in BM. These expression patterns imply that the different Notch genes may have very distinct functions during hematopoiesis and that Notch3 could be a specific regulator of stem cell development.

We further examined the expression levels of Notch pathway genes on primary leukemia cell blasts and leukemia cell lines. Notch3 and Hes5 genes were predominantly expressed in primary T-ALL and some T cell lines but were silenced in majority of B cell leukemia and B cell lines, suggesting that Notch3 and Hes5 could be used as T cell lineage specific markers for leukemia diagnosis. We demonstrated a leukemia specific hypermethylation and aberrant histone modifications in transcriptional silencing Notch pathway gene expression. All normal CD19+ B cells were completely unmethylated at the Notch3, Hes2, Hes4 and Hes5 CpG islands, excluding the possibility that cell lineage specific methylation accounted for the observed methylation in B-ALL. Most importantly, hypermethylation and histone deacetylation of Notch pathway gene correlated with down-regulation of gene expression. The transcriptionally active Hes5 locus in T-ALL1 cells was unmethylated, hyperacetylated at H3K9 and hypermethylated at H3K4. In contrast, the silent Hes5 locus in CEM and RS4;11 cells was hypermethylated, hypoacetylated at H3K9Ac and hypomethylated at H3K4, but was hypermethylated at H3K9 and H3K27. We established a further link between Notch pathway gene CpG islands hypermethylation and their gene silencing by demethylation treatment.

Figure 6. Hes5 inhibits proliferation and induces apoptosis in B cells but not in T cells. A. REH, RS4;11 and T-ALL1 cell lines were transduced with lentivirus expressing Hes5 or empty vector. Cell numbers were measured on day using trypan blue exclusion assay. B. Cell cycle distributions measured 2 days after lentivirus infection using propidium iodide (PI) staining to measure DNA content. The percentage of cells in sub-G1 (<2N DNA) is presented. C. Analysis of apoptosis 3 days after lentivirus infection using flow cytometry, PI staining and annexinV staining.

doi:10.1371/journal.pone.0061807.g006
agent DAC and histone deacetylation inhibitor SAHA treatment restored the expression of the Notch pathway genes in several hypermethylated and silenced cell lines. In addition, the CpG sites around the Hes5 promoter region, whose methylation was associated with the silencing of this gene in B cell lines, showed clear promoter activity. Therefore, DNA hypermethylation, as well as histone deacetylation and methylation are potential mechanisms of inactivation of Notch pathway genes in leukemias. However, some cell lines showed reduced Hes5 expression without DNA methylation, and the effect of DAC alone or with SAHA increased Hes5 expression, suggesting that histone modification rather than DNA methylation contributed to the silencing of Hes5.

To further confirm the importance of epigenetic mechanisms in down-modulation of negative growth regulatory genes and tumorigenesis [24], we re-expressed human Hes5 in leukemia cell lines with or without Hes5 methylation. Forced restoration of Hes5 resulted in cell growth inhibition and apoptosis only in Hes5 methylated and silenced B-ALL lines (REH and RS4;11) but not in Hes5 unmethylated and expressing T cell lines. These findings are of functional significance as epigenetic suppression of Notch pathway genes may be critical to disrupt their role in Notch signaling, allowing uncontrolled proliferation and apoptosis-resistance contributing to leukemia progression. It is also consistent with the model that activated Notch may function as either an oncogenic factor in T cell leukemia or a tumor suppressor in B cell leukemia/lymphoma [1,13]. It appears that the dual and opposing function of Notch signaling is cell lineage and cell context specific, and is possibly controlled by epigenetic regulation of Notch pathway gene expression in different cell types. Because several Notch pathway genes exhibit tumor suppressor function in B-ALL cells [14], the epigenetic silencing of the Notch signaling pathway may provide a selective growth advantage to leukemia cells. That said, one of the limitations of this study is that we have not elucidated the mechanisms for differential induction of apoptosis.

In summary, this is the first report that multiple members of the Notch pathway are commonly hypermethylated and down-regulated in human leukemia cell lines and primary B cell leukemias. We show distinct methylation and expression patterns of Notch3 and Hes5 in B cell leukemias compared with T-ALL. Treatment of leukemia cells with the demethylation and deacetylation agents induced expression of these pathway genes. Our study suggest that epigenetic regulation of Notch pathway gene expression correlated with their distinct function in human B versus T cell leukemias and strengthen the observation that some Notch pathway genes may function as tumor suppressors in B cell leukemias, being down-regulated by DNA methylation. This tumor suppressive properties are consistent with recently reported role of Notch pathway in myeloid leukemia [25] and confirm prior results on the tumor suppressive nature of Notch signaling, including Notch3, in B cell malignancies [14]. Our findings of coordinate down-regulation of multiple members of the Notch pathway through epigenetic remodeling may have major implications for the future understanding of leukemia initiation and progression. Further evaluation of epigenetic effects on the Notch pathway and other pathways of growth regulation may provide novel therapeutic approaches for the treatment of leukemias.

Supporting Information

Figure S1 Expression of Notch3, JAG1, Hes4 and Hes2 in normal bone marrow (BM), CD34+ BM, BMs from patients with T cell acute lymphoblastic leukemia (T-ALL), B-ALL, and various leukemia cell lines. The relative gene expression was determined by real-time PCR assays and normalized to that of GAPDH.

Figure S2 Notch3, Hes4, Hes2 and Hes6 expression levels in various leukemia cell lines. The leukemia cells were either untreated, or treated with 5-aza-2’-deoxycytidine (DAC) only, suberoylanilide hydroxamic acid (SAHA) only or both (D+S) as described in material and methods. Real-time PCR analysis. In general, expression of Notch3 and Hes4 was restored in some leukemia cell lines treated by DAC with or without SAHA. Hes2 was un-respond to any DAC, and SAHA treatment. In contrast, Hes6 was respond to DAC, and SAHA treatment.

Table S1 Primer sequences used for bisulfite pyro-sequencing, ChIP assay and Hes5 promoter cloning.

Acknowledgments

We thank Dr. Wang at Maine Medical Center Research Institution for providing FUGW-GFP lentiviral vector and Dr. Jelinek (MDACC) for normal T cell cDNA.

Author Contributions

Conceived and designed the experiments: SQK GGM. Performed the experiments: SQK ZHF HY YW EGC YB. Analyzed the data: SQK.

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