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Inferring a role for methylation of intergenic DNA in the regulation of genes aberrantly expressed in precursor B-cell acute lymphoblastic leukemia

Md Almamun¹, Olha Kholod¹, Alexei J. Stuckel¹, Benjamin T. Levinson¹, Nathan T. Johnson², Gerald L. Arthur¹, J. Wade Davis³, and Kristen H. Taylor¹

¹Department of Pathology and Anatomical Sciences, University of Missouri-Columbia, Columbia, Missouri, USA

²Bioinformatics and Computational Biology, Worcester Polytechnic Institute, Worcester, Massachusetts, USA

³Department of Health Management and Informatics, University of Missouri-Columbia, Columbia, Missouri, USA

Abstract

A complete understanding of the mechanisms involved in the development of pre-B ALL is lacking. In this study we integrated DNA methylation data and gene expression data to elucidate the impact of aberrant intergenic DNA methylation on gene expression in pre-B ALL. We found a subset of differentially methylated intergenic loci that were associated with altered gene expression in pre-B ALL patients. Notably, 84% of these regions were also bound by transcription factors known to play roles in differentiation and B-cell development in a lymphoblastoid cell line. Further, an overall down-regulation of eRNA transcripts was observed in pre-B ALL patients and these transcripts were associated with the down-regulation of putative target genes involved in B-cell migration, proliferation and apoptosis. The identification of novel putative regulatory regions highlights the significance of intergenic DNA sequences and may contribute to the identification of new therapeutic targets for the treatment of pre-B ALL.

Keywords

DNA methylation; enhancer; acute lymphoblastic leukemia; eRNA; transcription factor; precursor B-cell

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common cancer diagnosed in children under the age of 15 with a peak incidence between 2 and 5 years of age. ALL is

Corresponding Author: Kristen Hawkins Taylor, 1 Hospital Dr, Med Sci Bldg M214B, DC055.07, Columbia, MO 65212, Phone: 573-882-5523, FAX: 573-884-4612, taylorkh@health.missouri.edu.

POTENTIAL CONFLICTS OF INTEREST

The authors have no conflicts to disclose.

characterized by compromised early lymphoid development and can be classified as T or B cell ALL. ALLs represent 75% of all acute leukemias in children and there will be an estimated ~6,250 new cases and 1,450 deaths from ALL this year in the USA. Precursor B-cell leukemia (pre-B ALL) accounts for 85% of ALL cases. Many genetic aberrations are associated with pre-B ALL [1]; however, these do not explain all incidences and advancements in detecting other molecular mechanisms driving malignancy are currently lacking. Therefore, the identification of regulatory regions of the genome that may impact the development of pre-B ALL is critical to gaining a better understanding of the pathogenesis of the disease. In addition to genetic abnormalities, epigenetic modifications play a role in the regulation of gene expression and these modifications are known to be altered in cancer [2;3]. However, the functional consequences of changes in DNA methylation are far from understood. It is well established that DNA methylation in the promoters of genes and CpG islands are associated with abnormal gene expression in cancer (reviewed in [4]); however, these regions comprise only a very small portion of the genome.

Enhancers are regulatory sequences that can be located far away from the gene they regulate, and are the key element of cell type specificity. Noncoding transcripts or enhancer RNA (eRNA) are often associated with these sequences that also comprise multiple transcription factor binding sites which are utilized to orchestrate discrete cell type specific patterns of transcription factor binding that is required for the establishment/maintenance of long-range promoter–enhancer interactions [5–7]. Altered enhancer activity may contribute to the faulty regulation of target genes and ultimately lead to the pathogenesis of many diseases. Recently, genome-wide association studies have reported that a large number of disease susceptibility regions do not overlap with protein coding genes but rather map to putative “non-coding” regulatory intervals [8]. Furthermore, mutations, insertions and deletions in enhancer regions have been shown to disrupt the expression of respective target genes by altering the recruitment of transcription factors and other cofactors in many disease conditions [9].

DNA methylation regulates tissue specific gene expression and plays a significant role in hematopoiesis. Aberrant DNA methylation in vital regulatory regions of the genome, like enhancers, may cause initiation of many hematopoietic disease conditions including pre-B ALL. We have previously shown that aberrant methylation in enhancer regions was associated with the altered expression of neighboring genes involved in cell cycle processes, lymphocyte activation and apoptosis in pre-B ALL [10]. To further the understanding of the role that intergenic DNA methylation and/or differential eRNA expression plays in regulating the expression of target genes in pre-B ALL, the regulatory potential of putatively active enhancers has been investigated.

MATERIALS AND METHODS

***In Silico* Mapping of Enhancer Regions And Transcription Factors Bound In Normal Lymphoblasts**

This study was approved by the University of Missouri’s Institutional Review Board and conforms to the provisions of the Declaration of Helsinki. H3K4me1 (GSM733772) and H3K27ac (GSM733771) ChIP-seq data from the GM12878 lymphoblastoid cell line (LCL),

were obtained from publically available ENCODE Data Coordination Center at UCSC. Regions of the genome with a co-enrichment of both histone marks were identified and annotated using HOMERv4.3 to classify their genic properties [11]. Co-enriched, intergenic regions were extracted and extended 1000 bp in both directions to represent potential active enhancers in normal pre-B cells.

ChIP-seq data from the GM12878 LCL for 76 transcription factors (TF) was also utilized to identify TF that were bound to differentially methylated DNA sequence within the potentially active enhancer regions. The list of TF analyzed with accession numbers is included in Supplemental Table 1. To identify which of the potentially active enhancers in normal B-cells are associated with genomic regions that gained methylation in pre-B ALL, the potential enhancer regions were superimposed with previously identified differentially methylated regions (DMRs) in pre-B ALL [10] using a custom python script (Python 3.3.0). The potentially active enhancers concomitant with a DMR were surveyed for TF binding in the GM12878 LCL.

Gene Expression Analysis Of Putative Enhancer Derived RNA

Previously generated RNA-seq data from 20 pre-B ALL patients and 8 normal precursor B-cell samples was utilized [10]. Differential transcript expression was determined using Cufflinks with default parameters (version 2.2.1). Multiple testing correction was performed using Benjamini-Hochberg (q-value). Transcripts greater than 200 bp with a q-value of 0.05, that were located within intergenic regions in the pre-B ALL and normal precursor B-cells, and that were not associated with an annotated gene or pseudogene were considered as potential enhancer derived RNA (eRNA).

Identification Of Differentially Expressed Putative Target Genes And Enriched Functional Groups

The genomic regions enrichment of annotations tool (GREAT) was used to identify the two nearest genes within 1Mb upstream or downstream from each potential enhancer and to conduct gene ontology and disease ontology analyses to identify enriched biological processes associated with the potential target genes [12]. Differential expression data for each target gene was extracted from previously generated RNA-seq data [10].

Cell Line Treatments

The pre-B ALL cell line NALM6 was grown in RPMI 1640 medium (Gibco, ThermoFisher) supplemented with 10% fetal bovine serum, L-glutamine, and gentamicin. Cell culture treatments were conducted, as described previously with minor alterations [13]. Briefly, NALM6 cells were seeded at 3×10^6 cells/mL. Two separate treatments were conducted for 5-aza-2-deoxycytidine (5-aza) alone and in combination with trichostatin A (TSA). Based on prior practice, 5-aza was added at either a 0.3 or 0.4 $\mu\text{mol/L}$ final concentration with acetic acid as the vehicle and was incubated for 78 h, with new medium added every 24 h [13–15]. TSA was added at either a 0.3 or 0.4 $\mu\text{mol/L}$ final concentration after incubation with 5-aza and incubated for 6 h. Control cells were cultured with acetic acid alone. RNA from the cultured cells was extracted for use in next-generation sequencing, using the AllPrep DNA/RNA Mini Kit (Qiagen). High quality RNA was submitted to the University of

Missouri DNA Core Facility for library generation using the TruSeq mRNA stranded library preparation kit (Illumina).

Paired-end sequences (2×75) were generated by the University of Missouri DNA Core Facility using the Illumina NextSeq500 platform. Sequence files were generated in FASTQ format and processed using an in-house pipeline. Sequences were filtered and those with a quality score of 32 or higher were aligned to the *H. sapiens* reference genome (hg19) using TopHat v. 2.1.1 [16]. The aligned BAM files were congregated into transcripts and tests for differential expression were conducted using Cufflinks v. 2.2.1 [17]. After assembly with Cufflinks, the output files were processed in Cuffmerge in conjunction with a reference annotation file. To normalize multiple samples for differential expression analysis, the geometric method described in Anders and Huber was used [18]. For cross-replicate dispersion estimation, a “blind” method was utilized in which all samples are treated as replicates of a single global condition and used to build one model. The test for differential gene expression was conducted at the level of genes and transcripts and pairwise comparisons of expression between cells treated with 5-aza and controls, and also between cells treated with 5-aza + TSA and controls were employed. Genes with a q-value <0.05 were retained as significant.

RESULTS

Identification Of Putative Intergenic Regulatory Loci

In our earlier work, we have shown that much of the differential methylation associated with pre-B ALL patients is present within intergenic regions of the genome [10]. Because intergenic sequences are known to harbor regulatory elements, we sought to identify intergenic regions with regulatory potential. First, peak data was extracted from the UCSC genome browser for H3K4me1 and H3K27ac to identify potential enhancer regions in an LCL (Supplemental Table 2). These regions, while referred to as enhancers, have the ability to recruit molecules that may function as an activator or a repressor thereby increasing or decreasing the expression of target genes. Based on the co-enrichment of histone marks (H3K4me1 and H3K27ac), a total of 16,212 intergenic loci were identified to be putative active enhancer regions (Supplemental Table 3). Next, neighboring putative target genes were identified for each putative enhancer region followed by gene ontology analysis which demonstrated that the presumptive target genes play roles in immune response, B cell activation, leukocyte differentiation and other biological processes important to B-cell development (Figure 1). Because active enhancers are typically bound by TF that function during development and differentiation [19;20] and to provide further evidence in support of a potential regulatory role for these loci, ENCODE ChIP-seq data was utilized to determine the occupancy of 76 transcription factors within the putative intergenic enhancer sequences in an LCL. More than 75% of the putative active enhancers were occupied by TFs that play roles in normal development including B-cell development and differentiation (Supplemental Table 3).

Several studies have recently demonstrated that enhancers are transcribed to produce long non-coding RNA, and this enhancer-derived RNA (eRNA) plays a crucial role in regulating the expression of nearby genes, in normal development and in the pathogenesis of many

disease conditions [21–26]. Therefore, data generated in our previous work [10] was utilized to identify the presence of intergenic transcripts. A total of 2012 putative active enhancer regions concomitant with intergenic transcripts (eRNA transcripts) were identified (Supplemental Table 4). Of these, 381 were differentially expressed in pre-B ALL (q-value <0.05) and the majority of these were more highly expressed in the normal precursor B-cells than in the pre-B ALL cells demonstrating that these regulatory eRNA become transcriptionally inactive in pre-B ALL (Figure 2). The GREAT tool was utilized to extract neighboring genes within 1 Mb of each eRNA transcript and disease ontology analysis revealed that several of the presumptive eRNA target genes are also involved in hematological malignancies (Figure 3). To determine the potential effect of differentially expressed eRNA transcripts on neighboring target gene expression, RNA-seq data was used to compare the expression levels of the presumptive target genes in pre-B ALL and normal precursor B-cells. A total of 90 presumptive target genes were down-regulated in pre-B ALL, whereas 11 presumptive target genes were up-regulated in pre-B ALL (Table I).

Differential Methylation Of Intergenic Loci Is Associated With Differential Expression Of Putative Target Genes

Extending our previous work, the GREAT algorithm was used to identify potential target genes that were differentially expressed and located within 1Mb of each differentially methylated potential enhancer region followed by an *in silico* analysis to identify the loci concomitant with a bound transcription factor in an LCL. A total of 102 differentially methylated intergenic loci were associated with abnormal expression of 87 putative neighboring target genes in pre-B ALL patients versus normal precursor B-cell controls and more than 84% of these regions were also occupied by at least 1 transcription factor in an LCL (Supplemental Table 5; Figure 4). The 6 topmost bound TF include the B-cell related TFs ELF1 (n=49), RUNX3 (n=49), PAX5 (n=45), EBF1 (n=39), and SPI1 (n=38).

Next, to gain insight toward the impact of DNA methylation on eRNA transcription, differentially methylated intergenic enhancer loci were compared to eRNA transcripts. A total of 12 DMRs hypermethylated in pre-B ALL were associated with a concomitant decrease in eRNA transcript expression (FDR < 5%; Fold change ≥ 2; Figure 5). Altered gene expression was also observed for neighboring putative target genes, *ADRA2B*, *ASTL*, *ELOVL5*, *GCM1*, *GRB2*, *IFNGR2*, *IRF4*, *ISG20*, and *MS4A1* in pre-B ALL patients (bolded genes in Table I).

Treatment Of A Pre-B ALL Cell Line With A Demethylating Agent Reverses Aberrant Gene Expression *in Vitro*

A pre-B ALL cell line was treated with a demethylating agent (5-aza) alone and in combination with a histone deacetylase inhibitor (TSA) and RNA-seq was performed. Differential gene expression was calculated between control samples and treated samples using CuffDiff for each of the 87 abnormally expressed putative neighboring target genes identified above (Supplemental Table 6). Using a false discovery rate and p-value of < 0.05, the expression of 32 of the abnormally expressed neighboring putative target genes was reversed after treatment with 5-aza alone or in combination with TSA (Table II). Sixteen genes were down-regulated in pre-B ALL patients and up-regulated after treatment. The

genes identified play important functional roles in processes such as alternative splicing, acetylation, regulation of apoptosis, and regulation of transcription.

DISCUSSION

Potential regulatory sequences were detected by identifying regions of the genome in an LCL that harbor the two histone modifications most often associated with active enhancers [27–29]. Integration of methylation and gene expression data revealed that intergenic DNA hypermethylation in pre-B ALL was associated with the down-regulation of 49 potential target genes. Importantly, the observed down-regulation of functionally relevant genes associated with normal B-cell differentiation, such as *ICOSLG*, *IRF4* and *MSA1*, may lead to the pathogenesis of leukemia or contribute to poor treatment efficacy. For example, the transcription factor *IRF4* along with the apoptotic regulator *IRF8* is essential for the differentiation of precursor B-cells to immature B cells [30]. Therefore, the methylation mediated down regulation of *IRF4* may negatively regulate apoptotic genes leading to persistence and proliferation of lymphoid cells in pre-B ALL. Further, the surface antigen, *MSA1*, also known as CD20, is used as a target for monoclonal antibody treatment in pre-B ALL to improve treatment outcomes. Therefore, lower levels of CD20 may result in lower treatment efficiency and poor outcomes in pre-B ALL. In addition, down-regulation of genes in the BCR signaling pathway (*LYN*, *GRB2*, *NFATC1*), the Jak-STAT signaling pathway (*GRB2*, *IFNGR2*) and those with roles in tumor suppression (*SIK1*, *ERF*, *PTPN2*) was identified. These genes also have known roles in B-cell proliferation and cellular differentiation [31–34]. Therefore, the inhibition of these genes may block transformation and differentiation and result in an accumulation of precursor B-cells in pre-B ALL patients.

An overall down-regulation of eRNA transcripts was observed in pre-B ALL and these transcripts were associated with the down-regulation of putative target genes. Many of the potential target genes can be considered as functional candidate genes that may contribute to the development of pre-B ALL including genes involved in normal B-cell migration, proliferation, and apoptosis. Some of these loci appear to be regulated by DNA methylation which was associated with the down-regulation of eRNA transcripts in pre-B ALL. A one to one correlation between putative enhancers and the presence of eRNA transcripts was not observed. This may point to alternate modes of enhancer activity or could be the result of the inability to accurately detect all eRNA transcripts due to low levels of expression. Others, have established that targeted degradation of eRNA by RNA interference results in the diminished expression of target protein coding genes [35;36]. Therefore, targeting specific eRNA transcripts has great therapeutic potential in modifying disease specific gene expression in pre-B ALL.

B-cell related transcription factors were most commonly bound in unmethylated GM12878 cells within the intergenic DNA sequences that gained methylation in pre-B ALL patients and were associated with aberrant gene expression. The 6 topmost bound transcription factors within these 102 loci include 5 B-cell related transcription factors ELF1, RUNX3, PAX5, EBF1 and SPI1. These factors are known to play essential roles in early B-cell development therefore, if methylation blocks the ability of a B-cell related transcription factor to bind and function, one would expect to observe similar outcomes as with cases that

have defective transcription factors with the caveat that only a subset of genes would be affected. Because these transcription factors play major roles in early B-cell development and are commonly associated with hypermethylation in pre-B ALL, understanding the mechanisms by which their target genes are regulated is crucial. As a case in point, targeting a particular transcription factor may not be the best therapeutic strategy in cases that involve these factors because they play roles in regulating many genes and only a small number of target genes are affected. In these cases, the best targeting strategy may involve eRNA transcripts instead.

In general, whether DNA methylation is a cause or consequence of cancer is a point of contention. We and others have shown that treatment with the demethylating agent 5-aza, removes DNA methylation and restores the expression of particular genes including candidate epiDriver genes (genes that provide a selective growth advantage to malignant cells); however, the correlation between gene expression and promoter methylation is not consistent across all genes [13; 37]. Recent studies suggest that there is a stronger correlation between DNA methylation and gene expression when the methylation is present in an enhancer than when present within a promoter [38]. To elucidate the role of aberrant DNA methylation within intergenic putative regulatory regions, a pre-B ALL cell line was treated with 5-aza alone and in combination with TSA. In the NALM6 pre-B ALL cell line the expression of 32 potential target genes was reversed after treatment indicating that DNA methylation plays a regulatory role in the expression of these genes in this cell line. It is unclear whether experimentally removing DNA methylation in a particular region would have the same outcome on gene expression as observed between unmethylated healthy pre-B cells and methylated pre-B cells from individuals with ALL. It is possible, therefore, that DNA methylation plays a regulatory role in additional putative intergenic regulatory regions

Pediatric acute lymphoblastic leukemia has impressive cure rates but can be devastating to the families of children that relapse. Understanding the mechanisms that drive the misregulation of gene expression in pre-B ALL is vital to the development of novel therapeutic interventions to improve the outcomes for patients diagnosed with pre-B ALL and more specifically the outcome for patients that relapse. The precise role of DNA methylation in the pathogenesis of pre-B ALL is unclear. The integration of genome-wide DNA methylation data with gene expression data is the first step toward delineating “driver” DNA methylation events from “passenger” DNA methylation events. Towards this goal, this study has identified putative regulatory regions in normal lymphoblastoid cells that are occupied by transcription factors required for normal B-cell development. A large number of these regulatory regions are hypermethylated in pre-B ALL and are associated with errant expression of neighboring putative target genes with known and hypothesized roles in B-cell development and differentiation. Collectively, our results shed new light on the DNA methylation mediated regulation of enhancers that may play vital roles in the selective growth advantage and survival of leukemic cells in pre-B ALL patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Target genes associated with putative intergenic enhancers play roles in immune processes and hematopoiesis. GO biological processes enriched in the potential target gene “gene set” ordered by $-\log_{10}$ binomial p-values. The last 3 enriched processes have a p-value of 0.0.

Figure 2. Differentially expressed eRNA between normal precursor B-cell and pre-B ALL samples (q-value 0.05). Positive fold change: higher expression in normal precursor B-cells, negative fold change: higher expression in pre-B ALL samples.

Figure 3.
Disease ontology analysis of putative target genes associated with differentially expressed eRNA.

Figure 4.

Number of transcription factors bound in intergenic enhancers, hypermethylated in pre-B ALL, and associated with differentially expressed putative target genes.

Figure 5. Methylation is associated with the expression of select eRNA transcripts. Each bar represents the expression level associated with a DMR hypermethylated in pre-B ALL. DMR are identified by chromosomal position. Chromosome number followed by the starting and ending position for the DMR.

Table 1
Down-regulated target genes associated with down-regulated eRNA transcripts in pre-B ALL.

Target gene	Fold change ^d	Target gene	Fold change ^d	Target gene	Fold change ^d
ADAM28	-4.42	HLA-DOB	-3.36	RPL21	-1.50
ADRA2B	-2.90	HNRNPC	-1.00	RPS11	-1.44
ARHGEF1	-1.66	ICOSLG	-1.45	RPS14	-1.59
ARL5A	-0.88	IFNGR2	-2.48	RUNX3	-2.93
ASTL	-3.02	IL17C	-3.27	SCAF11	-1.92
B4GALT1	-2.88	IL21R	-4.16	SEC62	-1.32
BTLA	-4.24	IL4R	-4.00	SGMS1	-1.49
C11orf24	-1.97	IRF4	-2.46	SHOC2	-1.04
C17orf99	-2.27	IRF8	-4.30	SIK1	-3.59
CD48	-2.36	ISG20	-1.55	SLAMF1	-2.26
CDC42SE2	-1.74	ITPKB	-2.62	SLC38A1	-2.11
CDH22	-3.49	ITSN2	-1.79	SNX9	-5.00
CHD7	-0.72	JARID2	-2.18	SWAP70	-2.66
CLIC4	-2.18	LAPTM5	-1.77	SYPL1	-0.88
CTNNB1	-1.33	LPGAT1	-0.87	TAGAP	-2.37
CTSH	-1.82	LY9	-3.67	TMC8	-1.17
CTSK	-1.85	MALT1	-1.05	TMPRSS13	-2.77
CXXC5	-1.66	MAP3K8	-2.11	TNRC6B	-1.86
CYTH1	-2.65	MKNK2	-2.28	TOM1	-1.83
CYTH4	-0.83	MOB3A	-2.35	TPCN2	-2.18
ELOVL5	-1.89	MS4A1	-3.87	TRAF5	-1.74
EML4	-2.80	NEB	-1.46	TRIM69	-2.34
FAM159A	-2.89	OPA1	-1.29	TSC22D2	-1.67
GCM1	-5.08	PER1	-3.13	TSSK3	-4.12
GBBP1	-2.11	PLEKHG1	-1.75	USP12	-1.88
GPR160	-1.22	PPP1R2	-2.06	USP7	-2.32
GRB2	-1.31	PRDM2	-2.40	WBP11	-1.44
HCKTR1	-2.80	RALGPS2	-2.89	WIPF1	-1.62

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Target gene	Fold change ^a	Target gene	Fold change ^a	Target gene	Fold change ^a
HERPUD2	-0.99	RASL11A	-2.77	ZDHHC14	-2.49
HES1	-4.29	RGS2	-2.48	ZNF34	-2.66

Bolded genes are putative targets of differentially methylated eRNA transcripts.

^alog2 fold change

Table II

Potential target genes with reversed expression after treatment with 5-aza

Downregulated genes in pre-B ALL patients (Upregulated after treatment)		Upregulated genes in pre-B ALL patients (Downregulated after treatment)	
Gene	Functional Annotation	Gene	Functional Annotation
AAED1	antioxidant activity	ADCY9	nucleotide binding
AP3B1	alternative splicing	BRI3BP	acetylation
BIRC3	regulation of apoptosis	CBX4	regulation of apoptosis
CHD7	transcription regulation	CCND2	cell cycle
ERF	transcription regulation	CDC42BPA	metal binding
FASTKD5	acetylation	CXXC1	transcription regulation
GRB2	acetylation	DAD1	regulation of apoptosis
ISG20	alternative splicing	DNMT3A	chromatin regulator
PTPN2	acetylation	GCLC	regulation of apoptosis
RAD51B	regulation of apoptosis	GRIN1	regulation of apoptosis
RBM33	alternative splicing	LEPRE1	alternative splicing
RSPH1	meiosis	PCBP3	acetylation
TAGAP	alternative splicing	STK32C	alternative splicing
ZFP36L1	metal binding	TBC1D16	GTPase activation
ZMYM2	transcription regulation	TIA1	regulation of apoptosis
ZNHIT3	metal binding	TYRO3	nucleotide binding