GLOBAL DNA METHYLATION ANALYSIS IN RELAPSED PRE-B CELL ACUTE LYMPHOBLASTIC LEUKEMIA

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Doctor of Philosophy

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GLOBAL DNA METHYLATION ANALYSIS IN RELAPSED PRE-B CELL ACUTE
LYMPHOBLASTIC LEUKEMIA

Presented by Alexei Jay Stuckel

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Doctor of Philosophy

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DEDICATION

This work is dedicated to my parents, Jay & Christine Stuckel. I would also like to dedicate this work to former committee member, colleague, and friend Jerry Arthur who passed away last year.

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LIST OF ABBREVIATIONS

ALL= Acute lymphoblastic leukemia

cAL= canine acute leukemia

CD= Cluster of differentiation

CpG= CG dinucleotide

CGI= CpG island

DMR= Differentially methylated region

DNMT= DNA methyltransferase

FDR= False discovery rate

UCB= Human umbilical cord blood

H3K4me1= Histone H3 lysine 4 monomethylation

H3K27ac= Histone H3 lysine 27 acetylation

MBD= Methyl CpG binding domains

MIRA-seq= Methylated CpG island recovery assay (MIRA) followed by next generation sequencing

miRNA= Micro-RNA

Pre-B= Precursor B-cell

TF= Transcription factor

ABSTRACT

Reliable biomarkers for relapsed acute lymphoblastic leukemia are scarce. Currently, minimal residual disease (MRD) is the best method in predicting a relapse event, but is invasive to the patient. In addition, high quantity amount of cells are needed for flow-MRD and PCR-MRD requires stable IG-TCR rearrangements. An alternative MRD strategy may involve DNA based technologies involving mass spectrometry that have the potential to monitor the progression of ALL. There are also many recurrent karyotypes that stratify patients into either low-standard risk or high-risk of relapsing. However, many patients with favorable karyotypes (e.g. high-hyperdiploidy, ETV6/RUNX1) still experience one or more relapse events. DNA methylation has the potential to serve as a biomarker throughout the course of the disease in several aspects. DNA methylation associated microarrays have demonstrated the ability to stratify patients into their recurrent cytogenetic subtype. Further, Others have identified CpG loci that have the potential to stratify patients at diagnosis that are at risk of relapsing. This dissertation identified differential DNA methylation between matched diagnosis and relapsed patients by creating a methylome profile (MIRA-seq) for each patient. We are the first to report global hypomethylation that occurs at relapse, predominately within retrotransposable elements. In addition, genes that exhibited 5' regulatory aberrant methylation from diagnosis to relapse were identified. Some of the genes harboring epigenetic lesions may be considered an "epidriver" of ALL. A "driver" mutation can be defined as a mutation that can directly or indirectly offer a Darwinian advantage in terms of growth for leukemic blasts. The "epi" component refers to the nature of the mutation as epigenetic in origin and in the context of this study is synonymous with deviant or aberrant methylation. Further, our MIRA-seq study in canine acute leukemia observed epigenetic lesions of epidriver genes that were also present at

diagnosis and relapse in human ALL. Lastly, MIRA-seq profiles from ALL patients at diagnosis who either did or did not go on to relapse were compared against each other in order to identify individual CpGs using pyrosequencing that were associated with relapse (prognostic). These combined efforts were done with the foresight of identifying potential novel targets that exist as epidrivers of ALL or loci that hold prognostic power at diagnosis. This dissertation builds upon others who have previously identified relapse-associated biomarkers with implications of improved patient care and risk stratification.

CHAPTER 1: BACKGROUND

1.1 RELAPSED ALL

Acute lymphoblastic leukemia (ALL) is a heterogeneous disease that accounts for 25% of all childhood cancers and constitutes approximately 72% of childhood leukemia. Each year, around 4,900 children are diagnosed with ALL with a peak incidence occurring between 2 and 5 years of age. With an optimistic 90% cure rate at diagnosis, the outlook for relapsed ALL patients is dismal and accounts for more deaths than any other childhood cancer. Relapse events can be attributed to a number of factors, including incorrect risk stratification of patients at diagnosis and/or after initial treatment. This can result in high-risk patients subjected to treatment under low to standard risk protocols or low to standard risk patients treated under a more potent and potentially fatal regimen. In addition, there is a lack of risk stratification power for patients at relapse who are also subjected to limited treatment options. ALL treatment protocols for low to standard risk patients at diagnosis primarily consist of 3 phases (induction, consolidation, and maintenance). High-risk candidates determined at either diagnosis or after initial treatment, undergo additional treatment phases (interim maintenance and delayed intensification phase) with added chemo agents coupled with increased dosage.¹

Current prognostic factors used to stratify patients into risk groups at diagnosis and/or after induction treatment combine the utility of clinical and genetic features. These include a patient's age, white blood cell count (WBC), gender,

race, immunophenotype, and cytogenetic subtype. Specifically, age and leukocyte count are independent predictors of prognosis, where a linear relationship exists between leukocyte number and outcome.¹ In addition, karyotyping is a crucial determinant factor of prognosis and treatment response. However, several limitations of karyotyping exist, resulting in false patient subtypes. Minimal residual disease (MRD) diagnostics occur after initial induction treatment and remain the gold standard in terms of the most reliable prognostic factor for an ALL patient relapsing. Studies have determined that post induction treatment days 33 and 78 offered the most reliable and valuable prognostic knowledge.²

1.1.1 Future treatment of relapsed ALL

In the case of a bone marrow relapse, chemotherapy and hematopoietic stem cell transplantation (HSCT) remain the two primary forms of treatment.¹

Complete remission can be attained for the majority of relapsed ALL patients where treatment may remain consistent as before or additional drugs may be added such as anthracycline derivatives like daunorubicin or mitoxantrone.

Moreover, HSCT is widely considered a last resort option for relapse patients having a positive minimal residual disease (MRD+) outcome after chemotherapy treatment. Central nervous system (CNS) relapse constitutes less than 10% of relapsed ALL patients, where 90% will experience CNS remission.³ Successful treatment of CNS relapse includes cranial radiotherapy (CRT) coupled with intrathecal (IT) treatment of either methotrexate or triple IT (methotrexate,

hydrocortisone, cytarabine). Testicular relapse has decreased from around 10-15% to around 2% currently. Testicular relapse is frequently associated with systemic relapse, therefore chemotherapy intensification and testicular irradiation are the primary treatment options with an event free survival (EFS) > 90% for ALL patients with late testicular relapse.⁴

Promising new chemotherapy and immunotherapy options are on the horizon for relapse patients. This includes the proteasome inhibitor bortezomib which has shown promising results for relapsed ALL patients in phase I and II trial studies.⁵ In addition, monoclonal antibodies (mAB) conjugated with chemo agents or immunotoxins (ricin) have demonstrated efficacy when administered alongside conventional therapy. These mABs would target antigens displayed on leukemic blasts of B-cells, which would include CD19, CD20, and CD22.

Immunotherapeutic approaches include novel drugs that can bind B cell and T cell antibodies which enables T-effector cells to come within proximity of leukemic blasts, termed bispecific T-cell engaging antibodies (BITes).⁶ In addition, the highly anticipated CAR T-cell therapy has shown promising results in phase I/II trials of relapsed ALL patients. This process involves extracting and then adding an artificial receptor to a patient's T cells that are genetically programmed to bind leukemic blasts, thereby promoting other T cells to eliminate blasts at the site.^{7,8}

1.2 EPIGENETICS

Conrad Waddington termed the word “epigenetics” in 1942 where he defined it as “the branch of biology which studies the casual interactions between genes and their products, which bring the phenotype into being.”⁹ A modernized definition may describe ‘epigenetics’ as the study of mitotically or meiotically heritable changes in gene expression that do not involve a change in DNA sequence. Mechanisms by which epigenetic regulation occurs include:

Methylation of cytosines in DNA, post-transcriptional modification of histones (changes in chromatin conformation), microRNA (miRNA) and long non-coding RNAs (lncRNA) targeted interaction and subsequent degradation of mRNA transcripts. Epigenetic regulations like DNA methylation and histone modifications play a pivotal role in all cellular differentiation events that start as early as the blastocyst stage in human development and in later somatic events that include lymphopoiesis and B cell maturation.

1.2.1 DNA methylation

DNA methylation is a well-studied epigenetic phenomenon that is primarily associated with an addition of a methyl group onto the 5' carbon of cytosine in the context of 5'CpG'3 dinucleotide sequence. The main players involved in DNA methylation include molecules that add the methyl group to DNA termed DNA methyltransferases (DNMTs), where S-adenosyl-L-methionine (SAM) donates the methyl group in an enzymatic fashion. In mammals, there are four

highly conserved DNA methyltransferases (DNMT1, DNMT3A, DNMT3B, DNMT3L).¹⁰ Three of the four DNMTs are biologically active, whereas DNMT3L is not and facilitates the catalytic activity of DNMT3A and DNMT3B. DNMT1 is considered a maintenance methyltransferase that is highly expressed during S phase of the cell cycle. During DNA replication, DNMT1 faithfully lays down methylation within naked CpG sites of the newly synthesized DNA strand that maintains the methylation pattern of the original strand in a palindromic like-fashion. The DNMTs involved in *de novo* methylation include DNMT3A and DNMT3B. This form of methylation is essential in establishing genomic imprinting in embryonic development where mutagenic defects of DNMT3A and DNMT3B result in latent death.¹¹ In contrast, DNMT3L mutations shown in mice can be non-lethal, but have been shown to impede normal spermatogenesis in males and leads to loss of imprinting in females; therefore contributing to infertility.¹²

The function of DNA methylation pertains to several essential molecular and biological processes. One prime example relates to embryonic development in which waves of non-random demethylation and remethylation dynamics occur within differentiating primordial germ cells.¹¹ Moreover, genomic imprinting occurs during this process through methylation-mediated gene silencing within the maternal or paternal allele. This results in monoallelic expression of signal transducer molecules like insulin-like growth factor 2 (*IGF2*) or tumor suppressor genes (*DIRAS3*).^{13,14} In humans there is also cross talk between DNA

methylation and histone regulation mediated by methyl-CpG-binding proteins (MBD1, MBD2, MBD3, MBD4, MECP2). Primarily, MBD proteins facilitate chromatin conformation by mobilizing components of molecules such as Mi-2-NuRD complex involved in histone deacetylation (chromatin condensation) leading to gene repression for example.¹⁵ Another example of an MBD derived process lays with the function of MBD4. MBD4 has demonstrated mismatch repair ability as a DNA glycosylase by removing aberrant thymine or uracil nucleotides from individual CpG sites.¹⁶ Other related processes involving DNA methylation include random X chromosome inactivation in females, promoter-mediated silencing of tissue specific genes, and overall genomic stability and protection against foreign molecules.

1.2.2 DNA methylation in cancer

Aberrant DNA methylation is found in most cancers, especially human leukemia. Many of the differentially methylated regions (DMRs) occur within a CpG island context. A CpG island can be described as a region that is at least 200bp with a 60% CpG observed-to-expected ratio.¹⁷ The majority of genes (70%) in humans, especially housekeeping genes that harbor CpG islands in their promoter and 5' region, remain unmethylated in healthy individuals.¹⁸ Some oncogenes are an exception to this observation and their malignant phenotype kept at bay due to promoter methylation-mediated silencing. A common observation in cancer is DNA hypermethylation within CpG islands of gene promoters that can lead to silencing of tumor suppressor, DNA mismatch/repair, apoptotic, miRNAs, and cell

cycle related genes (**Table 1.1**).¹⁹⁻³⁶ In conjunction with targeted promoter hypermethylation, global hypomethylation is also a trend that contributes to genomic instability, resulting in activation of retrotransposable and other repetitive elements or loss of genomic imprinting. In addition, promoter hypomethylation has shown to lead to oncogenic activation. For instance, upregulation of oncogenes such as PARP1 and FEN1 has been correlated with promoter hypomethylation in various cancers.^{37,38}

The mechanism(s) that characterizes aberrant methylation in cancer remains poorly understood. However, there is increasing evidence that supports the notion of mechanistic passive demethylation that can occur after many DNA replications and therefore may contribute to the global loss of methylation displayed in cancer.³⁹ In addition, the molecular players themselves that are involved in adding and removing DNA methylation can be compromised due to single nucleotide polymorphisms (SNPs). For example, our study detected decreased expression of TET proteins in ALL patients at diagnosis against healthy patients.²⁹ TET proteins are considered the “guardians of CpG islands” as they remove aberrant CpG methylation within promoter sequences.⁴⁰ Therefore, one may infer that the promoter hypermethylation signature is irreversible and unchecked, allowing for continued methylation to occur throughout the progression of some ALL individuals. Misregulation of DNA methyltransferases has also been identified in several cancers. Moreover, around 1/5th of AML patients had accumulated somatic mutations of *DNMT3A*,

which serves as another example of how epigenetic lesions may manifest on a genome-wide scale.⁴¹

1.3 DNA METHYLATION METHODS

Several techniques exist to study DNA methylation in a research and clinical based setting. Next-generation sequencing has broadened the utility of these techniques, which has incorporated a strong statistical/bioinformatics backbone to handle the high-throughput data generated. A widely used category of analyzing DNA methylation includes a bisulfite chemical conversion of DNA, which converts only unmethylated cytosines into uracils. This differentiation step is used for many downstream techniques that include combined bisulfite restriction digest analysis (COBRA), methylation specific PCR (MSP), MassARRAY EpiTYPER, microarray probe analysis (450K or 850K), pyrosequencing, and whole genome (WGSBS) or reduced representation (RRBS) bisulfite sequencing. Another category is not dependent upon chemical treatment of genomic DNA, but utilizes antibodies that bind 5-methylcytosine (MeDIP-seq) or methyl binding domain proteins (MIRA-seq/MBD-seq). The other category independent of bisulfite conversion includes HpaII enrichment by ligation-mediated PCR (HELP-seq) and methylation sensitive cut counting (MSCC) that use restriction enzyme digestion specific to methylated CpG sites. Bisulfite sequencing is considered the gold standard for methylation analysis as it provides individual CpG resolution and superior coverage to alternative methods. However, bisulfite sequencing is very expensive and unrealistic in studies

analyzing hundreds of patient samples in the interest of finding clinical significance. Therefore, affinity based assays (single-end) are monetarily attractive and can provide 25bp-100bp read count resolution for each CpG island, including many low CpG density regions containing at least 2 methylated CpGs within close proximity.⁴²

1.4 DNA METHYLATION BIOMARKERS IN CANCER

The sensitivity and reliability of DNA methylation based assays are attractive methods within a clinical setting as demonstrated by identifying chemo-resistant patients through analyzing the methylation status of *MGMT* and other genes. In addition, DNA methylation remains an attractive biomarker option in a clinical based setting for several reasons. One pertains to the robust nature of DNA methylation, which can be stored for long periods of time as shown in formalin-fixed paraffin-embedded (FFPE) samples.⁴³

DNA repair gene O⁶-methylguanine-DNA methyltransferase (*MGMT*) is a prime example of a DNA methylation based biomarker; one of the first DNA methylation predictive biomarkers identified. It is well established that glioblastoma patients fare better with Temozolomide (TMZ) when the promoter of *MGMT* is methylated and silenced. The hypothesis is that the absence of promoter methylation within *MGMT* leads to protein expression, thereby counteracting the chemotherapy effect of (TMZ) by removing alkyl adducts, resulting in chemo-resistant malignant cells. Multiple studies have concluded that the promoter

methylation status of *MGMT* is a predictive biomarker of patient response to alkylating agent-based chemotherapy.^{19,44,45} One study found that 95% of long-term survivor (>30 months) patients with glioblastoma had *MGMT* promoter methylation, while only 36% of patients with a shorter survival harbored *MGMT* promoter methylation.⁴⁴ Interestingly, one study's model suggests that after cell division, Temozolomide itself may have an inhibitory effect on maintaining the methylation status of *MGMT*.⁴⁶

Importantly, what could result from the DNA methylation studies of *MGMT* are potential changes in risk stratification and therapeutic strategies. For instance, recruitment of high risk glioblastoma patients for Phase II clinical trials from MD Anderson Cancer site are currently underway and estimated to be completed by June 2021 [NCT02717962]. This is a biomarker driven study assessing high-risk glioma patients classified as *MGMT* promoter unmethylated using an alternative drug. These patients will be tested using an alternative chemotherapy agent to alkylating agent Temozolomide, called VAL-083 or dianhydrogalactitol (DAG). Other examples of genes considered as promoter-hypermethylation derived putative biomarkers include *SEPT9* in colorectal cancer, *GSTP1* in prostate cancer, and *MLH1* in endometrial cancer.⁴⁷⁻⁴⁹

1.4.1 DNA methylation relapse-associated biomarkers in ALL

Multiple studies have identified a panel or individual CpG sites that are associated with relapse or poorer overall survival in ALL. For example, Borssen

and others identified a panel comprised of thousands of CpGs that could further stratify T-ALL patients based on a CpG island methylator phenotype (CIMP) profile, where patients exhibiting less methylation within the CpG panel (CIMP-) fare poorly compared against individuals with a more methylated profile (CIMP+).⁵⁰ Their later study examined 601 pre-B ALL patients at diagnosis and assigned each patient into CIMP+ or CIMP-.⁵¹ The CIMP+ cohort had a higher overall 5-year survival compared to CIMP- patients at diagnosis and at relapse, but was not a predictive indicator of relapse. This suggests a difference in treatment response at relapse between CIMP+ and CIMP- profiled patients. Importantly, CIMP status could stratify relapsed patients classified as and treated under the standard-intermediate risk protocol at diagnosis, where 69% survival was observed in CIMP+ patients compared to 45% in CIMP- patients. In the high-risk cohort, survival difference was even more pronounced (CIMP+= 56%) (CIMP-= 21%). Given that survival after relapse remains poor, this study demonstrates that even after relapse, CIMP profiling may be able to identify chemo-resistant individuals. This knowledge may provide clinicians the foresight needed to anticipate and strategize for additional relapse events.

Past studies have also identified aberrant DNA methylation of specific loci within genes of patients at diagnosis that correlated with relapse. One study identified CpG sites that were also patient subtype specific, where the methylation intensity of individuals associated with relapse closely resembles healthy CD34+ and CD19+ stem-like cells. For example, Nordlund and colleagues identified 46

individual CpGs from 15 genes that corresponded with relapse among patients belonging to 3 different cytogenetic subtypes of ALL (ETV6/RUNX1, MLL/11q23, BCR/ABL1).⁵² The study highlighted 15 individual CpGs from three genes, where patients displaying the phenotype of high levels of methylation corresponded with a lesser likelihood of relapsing compared to patients of the same subtype exhibiting low levels of methylation. Similarly, another study found a different set of relapse associated CpG sites, where patients belonging to one of ten genetic subtypes and also displayed low levels of methylation in 21 out of 22 CpG sites were at greater risk of relapsing than those with a high methylation status.⁵³ The baseline methylation level for the given CpG sites in healthy control cells was not given in that study so a comparison could not be made to investigate whether or not the methylation pattern of the patients at higher risk of relapsing resembled healthy control cells as in Nordlund et al.⁵² Further, none of the CpG sites listed in the previously described studies were differentially methylated between ALL and healthy pre-B cells in our study.²⁹

The notion of CpGs displaying a stem-like methylation pattern is also consistent with Sandoval et al, where the methylation status of 20,661 individual CpG sites separated patients into two ALL groups at diagnosis (ALL-1, ALL-2), where the ALL-2 cohort correlated with a higher risk of relapse ($p < 0.01$).⁵⁴ Interestingly, all healthy control patients clustered with the ALL-2 group and shared a similar methylation pattern, where the majority of the CpG sites were lowly methylated in both groups. This is in contrast to the ALL-1 cohort, where 17,182 CpGs out of

20,661 were hypermethylated compared to ALL-2. This suggests that the previous studies may have identified regions where differential methylation between healthy controls is indeed a negative indicator of relapse.

These potential biomarkers differ from our regions of interest, which were predicated upon patients exhibiting hypermethylation at diagnosis, who later went on to relapse, compared to healthy patients. Similarly, another study found a correlation between the hypermethylated status of 31 CpG sites within the 5' regulatory CpG island of *PCDH17* and an increase risk of relapse and mortality ($P=0.001$, $P=0.017$) based on the notion of also displaying differential methylation against healthy control cells.⁵⁵ The study compared the methylation pattern of three cadherin genes: *CDH1*, *PCDH8*, and *PCDH17* and concluded that only *PCDH17* remained unmethylated among all ten control samples. This may also be supported by our data, where a 1.6kb hypermethylated region spanning the 5' regulatory region of the *PCDH17*'s CpG island at diagnosis and relapse was differentially methylated against pre-B control samples. In summary the previously described biomarker related studies have demonstrated the potential utility of DNA methylation as a risk stratification tool using either 1) a panel of CpGs (CIMP status) 2) individual CpGs that (a) resemble a control stem-like methylation pattern or (b) are differentially methylated against healthy control samples.

1.4.2 DNA methylation based cytogenetic subtyping in ALL

Cytogenetic subtyping is essential for stratifying ALL patients into low-standard and high-risk groups prior to treatment (**Table 1.2**).⁵⁶ As for childhood ALL, there have been multiple studies that have tested the utility of DNA methylation as an alternative molecular tool to karyotyping that can identify patients with various cytogenetic subtypes of ALL. Identifying a CpG panel in ALL patients may provide an additional safety net to act alongside standard karyotyping methods such as G-banding analysis and metaphase fluorescent *in situ* hybridization (FISH). This would also compensate for patients that are incorrectly karyotyped prior to treatment. For example, high-risk dic(9;20) ALL patients have been shown to escape G-band detection at diagnosis and some ALL patients labeled as having a normal karyotype in accordance to conventional karyotyping, did in fact harbor chromosomal abnormalities when tested using microarray CGH technology.⁵⁷

Multiple studies have conducted large-scale methylation analyses utilizing the Illumina 450k BeadChip array in an attempt to identify a panel of individual CpGs that sorts patients into their respective cytogenetic subtype. The latest study (Nordlund et al.) produced an accurate DNA methylation panel of 246 CpG sites that stratified patients into recurrent subtypes of ALL with high accuracy.⁵⁸ The subtypes of ALL patients included T-ALL, high-hyperdiploidy (HeH), ETV6/RUNX1, MLL, TCF3/PBX1, dic(9;20)+, BCR/ABL1, and iAMP21. The DNA

methylation panel was able to correctly assign 91% of patients (n=546) into their respective subtype as well as an additional 92% of newly diagnosed patients (n=39) in a blinded manner. This particular CpG panel has a high percentage of patient subtype accuracy from a large number of patient samples and should be considered for clinical use.

Interestingly, the panel was able to place around 47% of patients (n=105) with non-recurrent karyotypes into typical recurrent karyotypes, termed like-subtype. Patients placed into their like-subtypes had similar cytogenetic characteristics. For example, patients carrying 48-49 chromosomes, including those with Down's syndrome were assigned into the high-hyperdiploidy (HeH) subtype due to the similarity in methylation pattern. Moreover, the CpG panel grouped patients with RUNX1/ASXL1, ETV6/CBX3, and ETV6/AK125726 fusion genes into the ETV6/RUNX1 subtype. These observations bring up a key limitation for exclusively using a CpG derived panel for identifying patient subtypes. Due to the existence of like-subtypes, a clinician may not assume a patient's karyotype using the CpG panel alone. For instance, if a patient's CpG profile placed them into the recurrent ETV6/RUNX1 subtype, there is still the possibility (low) of a patient possessing a different gene fusion partner of ETV6 or RUNX1. Further, the risk factor pertaining to like-subtypes is not fully understood because of their infrequency in ALL.

Previous studies have attempted to subtype patients using DNA methylation based technology, but were not as accurate in doing so. However, they were important precursor studies that lead to the Nordlund study that identified bonafide CpG markers with the ability to differentiate ALL patients based on many cytogenetic subtypes. For example, Milani et al. subjected a list of 300 different CpG sites to unsupervised hierarchical clustering in an attempt to stratify patients (n=401) into their respective cytogenetic subtype: T-ALL, ETV6/RUNX1, MLL, TCF3/PBX1, HeH, BCR/ABL1, dic(9;20), iAMP21, hypodiploidy, and normal karyotype.⁵³ Clustering analysis was able to differentiate pre-B ALL from T-ALL. However, many of the pre-B cell patient subtypes were scattered, where 7 out of 9 dic(9,20) and 4 out of 6 iAMP21 patients clustered with other subtypes. The reason for the inaccurate clustering of patient subtypes may be attributed to the fact that the 300 CpG sites used to cluster patients were predetermined based on their association with genes displaying imbalanced allele-specific expression (ASE) coupled with having a high standard deviation cutoff (>0.16).⁵⁹ Two other previous studies stratifying patients based on genetic subtypes had comparable results to Milani et al. in that they too found individual CpGs that clustered ALL patients into their distinct genetic subtype with similar accuracy.^{60,61}

Interestingly, when identifying common individual CpGs to cluster patients into genetic subtypes from all four studies, only Nordlund et al. and Busche et al. shared 4 CpG sites in genes: *E2F6* (TSS), *ZDHHC3* (5'UTR), *CBFA2T3*

(5'UTR;Intron), and *DSC3* (TSS). Importantly, these individual CpG sites served as classifiers for patients harboring the ETV6/RUNX1 translocation in both studies and were hypomethylated compared to the other genetic subtypes. This observation is supported by the fact that both studies were able to cluster ETV6/RUNX1 carrying patients with high accuracy [Nordlund et al. (158/161 patients) 98%] [Busche et al. (12/13 patients) 92%]. Therefore these CpGs are sufficient classifiers for patients with ETV6/RUNX1 translocation and may also identify genes of potential functional interest related to ETV6/RUNX1 translocation.

The differentiating factor between past microarray studies and the most recent study (Nordlund et al)⁵⁸ is the influence of informatics and statistical modeling that contributed to identifying robust classifiers from microarray data. Nordlund et al. took a predictive modeling approach using the nearest shrunken centroid (NSC) method to identify consensus CpG sites that accurately identified different patient genetic subtypes. With a total of ~28 million CpGs in the human genome, one could imagine an even more robust and accurate CpG panel of up to ~99% accuracy using the latest 850K microarray platform or even whole genome bisulfite sequencing with decreasing costs of sequencing in the near future.

1.4.3 DNA methylation in the context of minimal residual disease (MRD) in ALL

There are several challenges facing the instrumentation of MRD measurement. For example, flow cytometry MRD is the preferred method of choice in the United States and while this approach is relatively quick and inexpensive, achieving optimal sensitivity of leukemia cell detection ≤ 1 in 10,000 (MRD-negative) is less than perfect and a critical limitation for monitoring ALL. Further, MRD requires bone marrow aspiration, which is invasive and incredibly painful to the patient. In addition, the minimum cell requirement is demanding ($\geq 2 \times 10^6$) to even consider reaching a sensitivity of around 10^{-4} .⁶² Thus, new and resourceful biomarkers are needed to aid in the improvement of risk stratification before and during the induction phase of treatment.

MRD remains the most important prognostic factor in terms of the likelihood of a relapse event in ALL. However, the two standard measurements of MRD (flow-cytometry) and (Ig-TCR rearrangement PCR) have specific constraints that limit its applicability in ALL patients. For example, measuring MRD by amplification of IG-TR gene rearrangements has been shown to be unstable in many patients during disease progression.^{63,64} The EuroFlow 8-color antibody panel approach in ALL patients has a sensitivity now comparable to the Ig-TCR method with a limit of detection of (10^{-5}) and is faster and a more standardized method against conventional flow-cytometry.⁶⁵ However, very large quantities of cells are still needed ($> 4 \times 10^6$) to complement the accuracy of the EuroFlow and still require

the painful and invasive nature of bone marrow aspirations. Therefore, patients would benefit from other MRD based methods such as DNA methylation.

A DNA methylation based method could be a future viable option that could aid clinical institutions both financially and in time management. Several studies have addressed this vision, first by comparing DNA methylation differences between diagnosis and a combination of both remission (post-induction treatment) and healthy samples from the same patient. The initial objective was to find a DNA methylation signature that could distinguish between leukemic and non-leukemia samples. Hence, identifying suitable and stable loci that could be used for diagnostic purposes and disease monitoring.

For instance, Nordlund et al. identified 28 differentially methylated probes from 24 genes, while Wong et al. identified 15 separate probes in the promoter region of 15 genes that clearly distinguished between the diagnosis and remission stages of ALL and were predominately hypermethylated at diagnosis; independent of recurrent karyotypes conditions.^{66,67} Findings from Wong et al. was consistent with the increased methylation observed in our study which found that 13 of the 15 gene promoters (*FOXE3*, *TLX3*, *C1orf76*, *FBXO39*, *MYOD1*, *GUCY1A2*, *PKDREJ*, *PNMA2*, *PPARG*, *PRLHR*, *PTPRZ1*, *SCRN1*, *SOX17*) that were represented by the 15 probes, correlated with hypermethylation within 19 ALL patients compared to 10 healthy donor patients.²⁹ Only 3 promoters (*DBC1*, *EYA4*, *MYO3A*) among the 24 genes identified in Nordlund et al. were also

hypermethylated in ALL from our study.²⁹ Chatterton and colleagues went on to analyze the methylation status of 103 CpG sites within the 15 probes using the SEQUENOM EpiTYPER.⁶⁸ The methylation pattern for the 103 CpG sites was predominately hypermethylated in the leukemic group compared to the remission and control groups. Approximately 91% of the ALL patients (77/85) were correctly assigned into their respective leukemic or remission/control group. The remaining misclassified patients (8/85) were those with hypodiploidy status.

The same study also addressed the potential of DNA methylation used in disease monitoring using mass spectrometry. The methylation status of CpG 17 within *TLX3* was the focal point in comparing the sensitivity of the EpiTYPER and methylSABER chemistry in bone marrow patient samples from induction treatment through remission. The study found the primer extension technique of methylSABER having superior sensitivity over the EpiTYPER method in regards to detecting copies of methylated *TLX3* (10 in 100,000 total). A methylation extension (ME) threshold > 0.30 (corresponding with > 10 in 100,000 copies) was used to determine a positive residual presence of disease. This cutoff detected 2 out of the 3 patients at remission that would eventually have an early relapse event, but did not detect late relapse patients (n=2) at remission. Even though this assay did not detect patients that relapsed later, one could infer that patients presenting ME > 0.30 at remission would have a poorer prognosis. Moreover, a reduction of ME in all 10 patients can be seen throughout the course of treatment into remission, which provides a sense of disease monitoring in its infancy.

Consequently, this may allow for intervention and changes in therapy regimen for patients unresponsive to treatment.

This study would have been more impactful if they demonstrated that their methylSABER technique was as effective and sensitive in peripheral blood samples. Thus, entertaining the future direction of MRD as a noninvasive and accurate form of disease monitoring in patients throughout treatment. For example, Flow-MRD has been tested in peripheral blood samples of ALL patients. The study found that detection of MRD+ within the bone marrow was not as powerful alone in predicting relapse as detection of MRD+ within peripheral blood as well.⁶² Moreover, DNA quantity requirements for mass spectrometry based methods are significantly lower than flow cytometry, therefore peripheral blood should provide an adequate quantity of DNA for the methylSABER method. Molecular techniques that incorporate mass spectrometry based instruments may be considered among next generation biomarker applications that could monitor patients throughout treatment in a less laborious manner. Currently, the EpiTYPER assay is the method of choice in detecting methylation levels of *MGMT* in phase II clinical trials for relapsed or refractory small cell lung cancer patients [NCT01638546]. One limitation for mass spectrometry based methods includes the nature of polymorphisms present within the sequence of interest, resulting in a shift in the spectrum, which could lead to false results or interpretations. There is also a concern that DNA

fragment masses < 1700 Da may interrupt optimal calibration of the mass spectrometry appliance.⁶⁹

Another group demonstrated the ability to reach sensitivity comparable to the latest advancements of flow-MRD of up to 10^{-6} (1 leukemic cell in 1,000,000 healthy background blood cells) using methylation sensitive enzyme restriction PCR (MSR-PCR) within the CpG island of *DLC-1*.⁷⁰ The principal of this method relies on methylation sensitive enzymes prior to PCR, which are unable to cleave methylated CpG sites. PCR products can be visualized on an agarose gel from primers designed for methylated sequences exhibited in the promoter of *DLC-1* of ALL patients. However, unmethylated sequences within that region from healthy patients, would succumb to fragmentation, thus no PCR product would be seen. In order to reach a 10^{-6} sensitivity mark, a second primer set was designed for the previously amplified product of *DLC-1* (nested PCR). Like Wong et al, this study was also able to monitor disease progression using a different marker (*DLC-1*) in 4 ALL patients from both bone marrow aspirates and peripheral blood that was concordant with pathological examination, flow-cytometry, and clinical information.

The previously described studies have demonstrated the ability to monitor patient MRD using a DNA methylation based assay and have provided hope for a potentially new and innovative approach to MRD tracking for ALL patients in the future. Nonetheless, the stability in detecting a specific marker (e.g. *TLX3* and

DLC-1) for ALL patient monitoring remains a crucial limitation in both studies. For example, the MSR-PCR technique was only able to detect the presence *DLC-1* in 16/29 (55%) bone marrow samples and 15/28 (54%) peripheral blood samples.⁷⁰ As for *TLX3*, detection of CpG site 17 was not evaluated in any other patient cohort besides the 10 patients that were used for MRD tracking.⁶⁸ One might suspect a similar detection rate in a larger sample size as observed in patients tracked using the *DLC-1* marker. This notion may be supported by the several false negatives identified in 11% (10/95) of ALL patients that were incorrectly categorized into the clear bone marrow group predicated on 19 individual CpG sites from *TLX3* to quantitatively distinguish between ALL and healthy patients (i.e. between diseased and clear bone marrow) by mass spectrometry. Therefore, it is reasonable to assume that the detection rate may dramatically decrease when using only 1 of 19 CpG sites in *TLX3* (CpG 17) for patient MRD monitoring.

1.4.4 Differential DNA methylation and gene expression provides insight into gene-targeted therapy at relapse

Novel targeted therapies are needed to help to decrease the fatality rate of relapse patients. Patients that relapse have a poor chance in survival and unfortunately, treatment outcomes have not significantly improved over the past few decades. Combining the power of gene expression and DNA methylation studies between diagnosis and relapse patients may identify potential novel targets. Hence, the selection process should also include targeting genes that

are also epigenetically altered at relapse by aberrant DNA methylation events that leads to gene misregulation, termed epidriver genes.

Contrary to previous studies, our MIRA-seq results demonstrated an overwhelming pattern of hypomethylation that occurs at relapse compared to 10 matched diagnosis patients. Hogan et al. and Nordlund et al. identified a combined total of 109 genes, while our study classified 2,777 genes exhibiting hypomethylation within the promoter, 5' UTR, or first exon regulatory region at relapse.^{52,71} Commonalities of hypomethylation between all 3 studies were sparse, where only 10 genes overlapped from no more than two of the studies. Interestingly, our set of hypomethylated genes included potential oncogenes involved in promoting DNA replication, MAP-kinase or Wnt/ β -catenin signaling pathways. Hence, hypomethylation may result in the upregulation of oncogenes that contribute to leukemogenesis that may also serve as novel therapeutic targets for relapsed ALL in the future.

Three separate studies have evaluated gene expression differences between diagnosis and relapse matched patient samples using microarray technology (n=3) as well as a meta-analysis study that synthesized the data from those studies in order to identify a consensus set of differentially expressed genes.⁷¹⁻⁷⁴ Our hypomethylated list of genes correlated with genes upregulated from the previous studies suggests there may be potential relapse-specific oncogene targets. When overlapping genes that were upregulated in each of the 4 studies

and were concomitant with hypomethylation at relapse from our study, a sum of 215 genes were found to be upregulated and hypomethylated in our relapse cohort (**Figure 1.1**). Strikingly, *TOP2A* was the only gene that correlated with hypomethylation in our study and was upregulated in each study previously reported. It therefore stands out as a potential candidate for targeted therapy. *TOP2A* is a topoisomerase gene involved in transcription and DNA replication. Further, overexpression of *TOP2A* has been correlated with several malignancies, including adrenocortical carcinoma (ACC) and prostate cancer.⁷⁵ In addition, knockdown (siRNA) of *TOP2A* in ACC cell lines significantly reduced proliferation, suggesting that *TOP2A* may play a role in oncogenesis and warrants further investigation in relapsed ALL.⁷⁶

1.5 DNA METHYLATION INHIBITORS IN RELAPSED ALL TREATMENT

Aberrant DNA methylation has been identified at relapse in two separate 450K microarray studies that found predominate increases of methylation at relapse.^{52,71} Hogan and colleagues identified target genes of interest that was epigenetically misregulated, which lead to subsequent studies administering demethylating agent (5-aza-2'-deoxycytidine/decitabine) to relapsed ALL patients. Decitabine is a cytidine analog that is incorporated into DNA, resulting in a DNA methyltransferases-cytidine analog irreversible covalent bond. First, several potential tumor suppressors exhibiting hypermethylation at relapse were found. This observation led to the hypothesis that decitabine would induce expression of previously methylated and silenced tumor suppressor genes,

thereby promoting leukemic cells to undergo apoptosis. *In vitro* studies administering decitabine to leukemic cell lines, resulted in demethylation and upregulation of several tumor suppressor genes previously identified at relapse (*PTPRO*, *COL6A2*, *CDKN2A*).⁷⁷ Furthermore, cell viability assays on primary patient samples demonstrated decitabine's ability to induce chemosensitivity, warranting a clinical study using decitabine coupled with vorinostat (histone deacetylase inhibitor). The pilot study revealed encouraging results in relapsed patients with a 50% (n=8) complete remission rate, where every patient that underwent complete remission became MRD-negative [NCT00882206]. However, when decitabine and vorinostat were tested in a larger cohort (n=23), the study was terminated due to drug toxicity, where 18/23 patients died [NCT01483690]. The authors concluded that a future study with modulations to therapeutic dosage would be conducted. Thus, clinical use of decitabine in relapsed ALL treatment is unlikely in the near future.

1.6 ALL IN DOGS

The manifestation of ALL in dogs is the result of malignant hematopoietic progenitors accumulating in the bone marrow. Unfortunately, ALL diagnosis in dogs is fatal due to poor response to treatment and a general lack of clinical insight partially attributed to the scarcity of large sample size studies. In a review of 50 cases of ALL, only a few patients survived longer than six months and none reached the one-year milestone.⁷⁸ Another study assessing 30 dogs with ALL found an average of 4-months' survival.⁷⁹ The induction treatment in dogs is

analogous to human ALL, where vincristine, prednisone, and anthracyclines are the primary frontline chemoagents used. There is a large difference in remission rate between the two species, however. Induction treatment in humans results in over 90% of ALL patients to experience complete remission. This number is drastically smaller in dogs, where one study found only 23/36 dogs achieved either partial or complete remission.⁷⁸ B-cell origin predominates in human ALL, however the true prevalence of B or T-cell origin in dogs is conflicting. Based on immunophenotyping ALL, dogs with B-cell origin typically display CD20, CD21, and/or CD79a, whereas T-cell origin show CD3, CD4 or CD8 antigens on their cell surface.⁸⁰ In addition, there is speculation that CD34+ may be a negative prognostic marker in B-cell origin in dog, as evidence of correlation between CD34+ and poorer prognosis has been observed. For example, one study found 76% of patients expressed CD34.⁸¹

1.6.1 DNA methylation in canine ALL

In the context of CpG islands, the genetic makeup is quite different between human and dog. Although, the dog genome has approximately 1 billion fewer bps compared to human (~3.4 billion bps), they have over 20,000 more CpG islands.⁸² Interestingly, there are fewer promoter-associated CpG islands in dogs than human. However, the methylation landscape of dog ALL is currently unknown. There is evidence that promoter hypomethylation may be associated with chemoresistance as demonstrated in canine lymphoid cell lines. Smallwood and colleagues found increased resistance to L-asparaginase that was

associated with and could be induced by demethylating the promoter of asparagine synthetase (ASNS) via 5-azacytidine treatment.⁸³ In canine AML, one study quantitated methylation levels using a 5'mC ELISA assay and found higher methylation-heterogeneity in AML samples.⁸⁴ Because the DNA methylation pattern is subtype (cytogenetic) specific in human acute leukemia, this observation is likely attributed to the various cytogenetic lesions that exists in canine AML. For instance, BCR-ABL translocation has also been reported in canine acute leukemia.⁸⁵ Unfortunately, there is insufficient amount of cytogenetic data for dogs due to the laborious and extremely difficult task of karyotyping 76 acrocentric autosomal chromosomes of similar shape.

Gene Name	Methylation status	Cancer type
<i>MGMT</i>	Hypermethylation	Glioblastoma ¹⁹
<i>MLH1</i>	Hypermethylation	Colorectal ²⁰
<i>GSTP1</i>	Hypermethylation	Prostate ²¹
<i>SEPT9</i>	Hypermethylation	Head and neck ²²
<i>BRCA1</i>	Hypermethylation	Breast ²³
<i>ATM</i>	Hypermethylation	Breast cancer ²⁴
<i>NEIL1</i>	Hypermethylation	Head and neck ²⁵
<i>LIG4</i>	Hypermethylation	Colorectal ²⁶
<i>FANCB</i>	Hypermethylation	Head and neck ²⁷
<i>PTPRO</i>	Hypermethylation	Acute leukemia ²⁸
<i>MCOLN2</i>	Hypermethylation	Acute leukemia ²⁹
<i>miR-9-1</i>	Hypermethylation	Head and neck ³⁰
<i>miR-34a</i>	Hypermethylation	Colorectal ³¹
<i>miR-130b</i>	Hypermethylation	Ovarian ³²
<i>miR-124-2</i>	Hypermethylation	Breast ³³
<i>p16</i>	Hypermethylation	Acute leukemia ³⁴
<i>p15</i>	Hypermethylation	Acute leukemia ³⁴
<i>CDH1</i>	Hypermethylation	Cervical ³⁵
<i>p14</i>	Hypermethylation	Esophageal ³⁶
<i>RB</i>	Hypermethylation	Retinoblastoma ³⁴

Table 1.1 Cancer associated promoter-hypermethylated genes

Cytogenetic risk stratification of ALL	
Low-standard risk	High risk
Hyperdiploidy	Hypodiploidy
High-hyperdiploidy	BCR-ABL1
ETV6-RUNX1	MLL rearrangement
PBX1-TCF3	iAMP21
Normal/diploidy	dic(9;20)
	T-ALL

Table 1.2

Figure 1.1 Venn diagram containing genes that were upregulated at relapse in each of the four studies that also correlated with promoter hypomethylation at relapse in our study.

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CHAPTER 2: METHYLOME ANALYSIS OF RELAPSED PEDIATRIC PRE-B ACUTE LYMPHOBLASTIC LEUKEMIA

2.1 ABSTRACT

Background: The chance of survival for relapsed pediatric pre-B cell acute lymphoblastic leukemia (ALL) patients is poor. Epigenetic alterations, involving DNA methylation may contribute in driving relapse events and have the potential to serve as biomarkers at diagnosis. In order to identify aberrant methylation associated with potential oncogenes, epidrivers, and prognostic biomarkers in ALL, genome-wide methylation profiles were constructed for diagnosis and relapse matched patient samples using the methylated CpG island recovery assay (MIRA), followed by next-generation Illumina sequencing. **Results:** First, paired samples at diagnosis and at relapse were compared to identify methylation changes that occur in patients at relapse. Approximately 99% of regions differentially methylated between paired patient samples at diagnosis and relapse were hypomethylated in the patients at relapse. 61 of these regions were present with regulatory elements of oncogenes. This is in contrast to previous studies that observed predominate hypermethylation at relapse. Predominate hypomethylation may contribute to oncogenic activation and overall genomic instability in relapsed ALL patients. Next, to identify potential biomarkers that were aberrantly methylated in patient samples at both diagnosis and at relapse, differentially methylated regions (DMRs) were identified between patients at diagnosis and pre-B cells from healthy individuals and also between

patients at relapse and pre-B cells from healthy individuals. Common DMRs identified in the patients at diagnosis and at relapse included those that were present in the promoter, 5' UTR, and first exon of 796 genes. This included potential epigenetic driver genes associated with tumor suppression (*PRUNE2*, *PTPRO*, *FRMD5*, *MLF1*) and B-cell function (*MCOLN2*, *IL7*, *SOCS2*, *BID*). Lastly, to identify biomarkers present at diagnosis with the potential to identify patients that will relapse, DNA methylation profiles of patients that relapsed were compared to the methylation profiles of patients that did not relapse. A total of 130 hypermethylated loci sustained from diagnosis to relapse were identified. These loci were not among the hypermethylated regions found in the non-relapse cohort when compared to normal controls. **Conclusion:** The study's results suggest that oncogenic activation and genomic instability may be consequences of regulatory aberrant hypomethylation, which may contribute to the drug resistant phenotype observed in relapsed ALL patients. In addition, potential epigenetic driver genes consisting of tumor suppressors and oncogenes may provide a selective advantage for neoplastic pre-B cells at diagnosis and relapse. Lastly, the 130 loci identified as putative biomarkers represent regions with risk stratification potential, by which patients may be assigned to either low-moderate or high risk groups prior to treatment at diagnosis. A quantitative approach is necessary to further delineate the methylation disparities of individual CpG within these regions between patients at diagnosis who did or did not relapse.

2.2 INTRODUCTION

Although the survival rate of pediatric pre-B cell acute lymphoblastic leukemia (ALL) has reached approximately 90%, relapsed ALL is still the leading cause of cancer related deaths among children. According to the National Cancer Institute, approximately 3,100 children and adolescents under 20 are diagnosed each year in the United States.¹ Patients at a higher risk of relapse are primarily associated with certain clinical features (white blood cell count (WBC) > 50,000 cells/ μ L) and a few recurrent cytogenetic subtypes (Philadelphia translocation (BCR/ABL1), mixed-lineage leukemia (MLL), hypodiploidy).^{1,2} However, relapse also occurs in ALL patients belonging to favorable cytogenetic subtypes (hyperdiploidy [51> chromosomes > 46], normal [chromosomes = 46]), alluding to alternate contributing mechanisms, such as aberrant DNA methylation.

There have been two studies that have analyzed DNA methylation differences between diagnosis and relapsed matched patient samples using either the 27k or 450k methylation BeadChip platform.^{3,4} The two studies observed predominate gains of DNA methylation (hypermethylation) at relapse compared to diagnosis. Pathway analysis of hypermethylated genes from both studies found enrichment for Wnt/ β -catenin signaling. Hogan and colleagues confirmed several negative regulators, such as *PTPRO* and *sFRP2* of the Wnt/ β -catenin pathway that exhibited promoter hypermethylation and downregulation at relapse.⁴ Other enriched pathways observed in either study, include the MAPK cascade and transcriptional regulatory network in embryonic stem cells.

Unlike previous studies, the primary aim of this study was to better understand the ramifications of aberrant DNA methylation at relapse in individuals that relapsed despite a cytogenetic profile associated with a low risk for relapse. First, differentially methylated regions (DMRs) between 10 children with favorable cytogenetic subtypes (hyperdiploidy or normal) at diagnosis and upon relapse were identified utilizing the methylated CpG island recovery assay, followed by next-generation sequencing (MIRA-seq). Next, aberrant methylation within regulatory regions of oncogenes or tumor suppressors were identified at relapse, followed by describing their biological role in relation to cancer. In addition, aberrant methylation sustained from diagnosis to relapse was analyzed, which may be helpful in identifying driver genes of ALL that are regulated by epigenetic changes, termed epidrivers. Finally, a relapse associated methylation signature was sought by comparing hypermethylated loci found in a cohort of patients at diagnosis that did not relapse to hypermethylated loci sustained from diagnosis to relapse.

2.3 MATERIALS AND METHODS

2.3.1 Patient samples

Bone marrow aspirates containing >87% leukemic blasts from ALL patient samples were used under full ethical approval of the institution review board at the University of Missouri (**Table 2.1**). MIRA-seq libraries were created for A27, A59, A70, A101, and A121 patient samples at diagnosis that relapsed and for all

ten matched relapsed samples in this study. These MIRA-seq profiles are accessible through the NCBI Sequence Read Archive (Accession PRJNA470682). Previously generated MIRA-seq libraries for A4, A15, A18-A26, A28-A31, A33, A36, A37 diagnosis patient samples and ten normal human umbilical blood (HCB1-HCB10) were used in one or more of the analyses in this study.⁵ The previously generated MIRA-seq profiles can be found in the NCBI Sequence Read Archive (Accession SRP058314).

2.3.2 Genomic DNA isolation and MIRA-seq profile construction

Using the QIAamp DNA Micro Kit (Qiagen cat. no. 56304), genomic DNA was extracted from ALL patient samples. Approximately 1µg of DNA in was used as the starting concentration in construction of each MIRA-seq library. The DNA was sonicated with the Diagenode Bioruptor (Diagenode; cat. no. B01010002) at 4°C, creating a range of 200 to 700bp fragments. Sonicated DNA (~150ng) was run on 1% agarose gel at ~96V for 45 minutes to verify the 200 to 700bp fragmented range. The sonicated DNA was purified using the MinElute PCR purification kit (Qiagen; cat. no. 28004) and eluted with 12µL nuclease-free water. End repair and dA tailing of the fragments was performed using the NEBNext DNA Library Prep Master Mix Set for Illumina kit (New England BioLabs; cat. no. E6040S) with a DNA purification step using Agencourt AMPure XP Beads (Beckman Coulter; cat. no. A63881). After End repair and dA tailing, NEB-Next Adaptors were added following another DNA purification step with 1x the concentration AMPure XP Beads and 10µL nuclease-free water elution. For

methyated DNA fragment enrichment, methyated CpG island recovery assay MIRA was performed with the Methyl Collector Ultra™ kit (Active Motif; cat. no. 55005). This pull-down method involves a methyl binding protein complex (MBD2b/MBD3L1) conjugated with nickel beads that compartmentalize the methyated fragments towards the magnetic stand, where the supernatant is exposed and removed with subsequent wash steps in between using AMPure XP Beads. The MIRA product was run on 1% agarose gel for 45 minutes at ~96V and bands between 200 to 700bps were excised and purified using the MinElute Gel Extraction kit (Qiagen; cat no. 28604). These methyated fragments were PCR enriched with the NEBNext DNA Library Prep Master Mix Set for Illumina kit with multiplex oligos for 12 cycles. The DNA product was purified again with AMPure XP Beads and eluted with 15µL nuclease-free water. End-point PCR was performed for a known methyated region of gene (*SLC25A37*) and a known unmethyated region of gene (*APC1*) in the methyated DNA enriched library. After running the PCR samples on 1% agarose gel at ~96V for 45 minutes, there was a distinct band present at around 160bp representing the *SLC25A37* gene product, but a slim to no band seen at the 160bp ladder mark for the *APC1* gene product. *SLC25A37*: forward primer 5'-GGTGGCTCCCACTTTAAGGAA-3' and reverse primer 5'-ATCTCGGCTGTCCCAT-3', (amplicon size = 158bp). *APC1*: forwards primer 5'-ACTGCCATCAACTTCCTTGC-3' and reverse primer 5'-GCGGATTACACAGCTGCTTC-3' (amplicon size = 162bp). Fragment analysis and Qubit quantitation was performed for each library prior to sequencing. No more than 4 MIRA-seq libraries were multiplexed in 10nM concentrations with

100bp single-end sequencing by the DNA Core facility at the University of Missouri on the HiSeq 2000.

2.3.3 Sequence processing and annotation of patient sample libraries

Quality control check of FASTQ file reads were done using FastQC v0.11.4⁶, then adaptor sequences were removed, including reads < 50bps, using cutadapt v1.7.1.⁷ Read alignment was performed using Bowtie 2 v2.2.⁸ to the human reference genome (hg19) with default settings and allowing no more than two mismatches. The output SAM files were then converted to BAM files and sorted accordingly using “view” and “sort” options with samtools version 1.2.1⁹ Picard-tools version 1.92 under “MarkDuplicates” option, removed duplicate reads. The final BAM output files were then indexed with samtools “index” option. Genomic coordinate BED files containing the differentially methylated regions DMRs were annotated using HOMERv4.3¹⁰ (Hypergeometric Optimization of Motif EnRichment) with default settings. The X and Y-chromosomes were excluded from this analysis.

2.3.4 Identifying DMRs between diagnosis and relapse patients

Differentially methylated regions DMRs between diagnosis and relapse matched patients was based off a computational method used by Chavez and colleagues.¹¹ Sufficient coverage depth was observed in each sample file (BAM file) using a saturation analysis (saturation correlation > 0.90). The reads were accumulated in 200bp windows along with a 200bp extension (average fragment

size of ~400bp). These windows are regions of interest (ROIs). ROIs with a mean across all samples < 50 counts were excluded to avoid bias and enhancing power, while reducing false discoveries.¹² The remaining ROIs were tested with a modified version of MEDIPS package in R/Bioconductor, utilizing edgeR's generalized linear model function (appropriate for matched patient sample comparison) in determining if a ROI is a DMR, where the false discovery rate (Benjamini-Hochberg) is < 5% and a log₂ fold change ≥ 1. Normalized RPM values (Reads Per Million) were computed for each 200bp window and used to identify DMRs. DMRs were merged as separate entities in respect to hypomethylation and hypermethylation, if the adjacent 200bp window met the previously described criteria.

2.3.5 Identifying common DMRs between diagnosis and relapse patients

First, DMRs were identified between HCB and diagnosis, then HCB and relapse patients. ROIs of 200bp were tested using the MEDIPS package with default parameters (classic edgeR). ROIs with a mean across all samples < 50 counts were excluded and DMRs were selected with a false discovery rate (Benjamini-Hochberg) of < 5% and a log₂ fold change ≥ 1. DMRs were merged as hypermethylated or hypomethylated regions. Hypermethylated DMR coordinates at diagnosis were intersected with hypermethylated DMRs at relapse, likewise for hypomethylated regions. This was accomplished using the intersectBed feature from bedtools v2.24.0.¹³ The output from this analysis were DMRs that were sustained from diagnosis to relapse, termed common DMRs.

2.3.6 mirPath v3 software analysis

HOMER annotated microRNAs with common hypermethylation or hypomethylation were submitted to mirPath v3¹⁴ using the software's default parameters of a p-value < 0.05 and incorporating a false discovery rate correction option. The MicroT threshold default value was 0.8, which is the standard for human pathway predictions. The TarBase v7.0 was utilized as the miRNA gene interaction database in finding potential target pathways.

2.3.7 Identification of enriched biological processes of hypomethylated protein-coding genes

Hypomethylated protein-coding genes were submitted to Gene Ontology Consortium for biological processes enrichment analysis (p-value < 0.05).¹⁵

2.3.8 *In silico* analysis of 38 hypermethylated regions at relapse

Publically available ENCODE Data Coordination Center at UCSC, provided ChIP-seq coordinates from the GM12878 lymphoblastoid cell line (LCL).

Genomic coordinates were extracted into BED format and then intersected with the 38 hypermethylated regions at relapse. Putative enhancers were defined as overlapping genomic coordinates of H3K4me1 (GSM733772) and H3K27ac (GSM733771) histone marks. ChIP-seq coordinates from DNase hypersensitivity sites (GSM816665) and 76 available transcription factors were also utilized.

2.3.9 Identification of relapse associated regions in patients at original diagnosis

MEDIPS was utilized in finding 17,137 hypermethylated regions in 13 non-relapse ALL patients compared to 10 HCB normal control patients. Using intersectBed from bedtools v2.24.0, 130 regions were present within the 2,170 common hypermethylated regions found between diagnosis-relapse and absent among the 17,137 hypermethylated regions found within the 13 non-relapse patients. The 130 regions were then ranked based on odds ratios, where larger scores represented a greater presence of individuals with read counts > 50 in the relapsed cohort in correlation with the absence of read counts > 50 within the non-relapsed cohort. Odds ratio scores, including 95% confidence intervals, and their respective p-values were calculated using MEDCALC software v 12.4 (MedCalc Software, Ostend, Belgium) based on % of reads > 50 in the relapsed cohort = P2 and % of reads > 50 in the 13 non-relapsed cohort = P1. Regions where read count values were > 50 were labeled in red and regions < 50 read counts were labeled in yellow for a given patient. Haldane-Anscombe correction was applied to the data for each region, where a constant equal to 0.5 was added to each cell.

2.4 RESULTS

2.4.1 Patients with ALL lose methylation at relapse

High quality sequence reads were generated for each sample, resulting in approximately 100 million unique mapped reads for each patient at diagnosis and

140 million reads for each patient at relapse. The overall read alignment rate generated by Bowtie 2 ranged from 87.4 to 91.4%. Unique mapped reads were used to identify differentially methylated regions between diagnosis and relapse samples using a false discovery rate of $\leq 5\%$ and a $\log_2$ fold change ≥ 1 . Strikingly, 70,740 DMRs were identified with 99.9% of the DMRs losing methylation at relapse.

Importantly, a loss of methylation was observed in retrotransposable elements, satellite repeat sequences, and within the 5' regulatory region of protein coding genes, ncRNAs and pseudogenes (**Figure 2.1**). Further, among the top 20 DMRs with the largest $\log_2$ fold change, 12 resided within the 5' regulatory region of 8 genes. BedGraphs pertaining to genes exhibiting the largest $\log_2$ fold change within their 5' regulatory region, illustrate the distinct read count differences between diagnosis and relapsed patients (**Figure 2.2**).

In addition, two large stretches of hypomethylation were observed in patients with ALL at relapse. A 40kb hypomethylated region comprised of two 10.2kb DMRs and 12 additional DMRs was observed (**Figure 2.3**). This region is located on chromosome 1 and harbors the protein-coding gene *RHOU*, the pseudogene *DUSP5P1*, and 17 5S ribosomal RNA gene copies. The second region comprised of a large 692kb hypomethylated region encompassing 3 protocadherin gene clusters on chromosome 5 (**Figure 2.3**). A total of 111

DMRs were present within this region and correspond directly with CpG islands and the protocadherin genes.

To determine the biological processes associated with potential regulatory DMRs that lose methylation in patients with ALL at relapse, gene ontology (GO) enrichment analysis was performed. Enriched biological processes included cell adhesion, cell signaling, cell differentiation, and regulation of immune response. Next, DMRs associated with genes with oncogenic potential were identified and classified based on cellular function (**Table 2.2**). Genes with oncogenic potential were associated with processes and pathways pertaining to relapsed ALL, which include the MAP-kinase cascade, Wnt/ β -catenin signaling, and DNA replication.^{4,16}

Remarkably, only 38 regions of the total 70,740 DMRs found at relapse, gained methylation at relapse (**Table 2.3**). Three regions were located within the promoter or first exon of a protein coding gene and 4 regions were located within exon-intron junctions. To further determine the regulatory potential of the hypermethylated DMRs, publically available data for the GM12878 lymphoblastoid cell line was utilized to identify regions concomitant with active chromatin (DNase hypersensitivity), transcriptional enhancers (H3K4me1 and H3K27ac) and bound transcription factors. Half of the hypermethylated DMR were concomitant with at least one potential regulatory element.

2.4.2 Identification of DMRs present at diagnosis and relapse

In order to identify putative regulatory DMRs that may contribute to the ALL phenotype, common DMRs observed in patients with ALL at diagnosis and relapse when compared to healthy precursor B-cells were identified using a false discovery rate of $\leq 5\%$ and a $\log_2$ fold change ≥ 1 . A total of 2,170 hypermethylated and 1,741 hypomethylated DMRs were present at both diagnosis and relapse (**Figure 2.4**). A predominance of hypermethylated DMRs were observed within promoters, 5' UTRs, and first exons of protein coding genes, non-coding RNAs, and within regions associated with CpG islands (CGI), whereas a predominance of hypomethylated DMRs were observed in retrotransposable elements and non-CGI regions of the genome (**Figure 2.4**).

Genes and miRNAs with aberrant regulatory methylation at diagnosis and at relapse were then characterized based on their potential to contribute to the overall pathogenesis of ALL. Common putative regulatory DMRs present in protein-coding genes and ncRNAs, included hypermethylation within genes involved in B-cell development (*IL7*, *MCOLN2*)^{17,18} and tumor suppression (*FRMD5*, *MLF1*, *PRUNE2*, *PTPRO*, *LOC389332*, *miR-124-3*, *miR-129-2*, *miR-375*, *LINC01018*)^{19,20,21,22,23,24,25,26,27}, whereas genes involved in hematopoietic stem cell (HSC) regulation (*SOCS2*, *BID*)^{28,29} exhibited hypomethylation (**Table 2.4**). Functional annotation clustering analysis of the 677 protein-coding genes that possessed a hypermethylated DMR within the 5'regulatory region revealed enrichment of genes associated with DNA binding homeodomain (Enrichment

Score: 20.16), fork head transcription factor (Enrichment Score: 7.53), peptidase M12B ADAM-TS (Enrichment Score: 5.04), myc-type, basic helix-loop-helix domain (Enrichment Score: 4.87), cadherin conserved site (Enrichment Score: 3.70), ionotropic glutamate receptor (Enrichment Score: 3.38), and core promoter proximal region sequence specific DNA binding (Enrichment Score: 2.39).

Functional annotation clustering of the 43 protein coding genes that possessed a hypomethylated DMR within the 5' regulatory region did not reach statistical significance.

In order to identify aberrantly methylated miRNAs associated with pathways and functional groups with undocumented but potential roles in the pathogenesis of ALL, miRNAs overlapping common DMRs were submitted to mirPath v3. Among the 14 miRNAs, the mirPath analysis identified an association between hypermethylated *miR-375* with Hippo signaling, proteoglycans in cancer, cell cycle, and others. Further, hypomethylated *miR-589* was associated with antigen processing/presentation, allograft rejection pathways, and others (p-value <0.05)

2.4.3 Identification of relapse associated DMRs present at diagnosis

To identify DMRs present at diagnosis exclusively in individuals that went on to relapse, data previously generated by Almamun and colleagues was utilized.⁵

When hypermethylated DMRs were sought in patients at diagnosis that had relapsed and patients that had not relapsed, compared to healthy patient

samples, 130 regions were identified in the patients that had relapsed. Odds ratios were calculated to identify the strongest candidate biomarkers and ranged from 0.70 to 57.9 (**Figure 2.5**). A significance level (p -value < 0.05) was met for 23 regions. These regions lay within introns ($n=3$) of protein-coding genes (*WDR60*, *LOC100506990*, *NBPF1*) and within intergenic sequences ($n=20$). DMRs within the intergenic regions did not overlap with any CpG island and varied in their distances to the nearest transcriptional start site (TSS) of a given gene (4.2kb to 1.7mb).

2.5 DISCUSSION

This is the first study to implement Illumina next-generation sequencing technology to create methylome profiles for matched diagnosis and relapsed ALL patients. The selected patients in this study contain a favorable cytogenetic profile, which enables a better understanding of how aberrant DNA methylation may contribute to relapsed ALL. This study is also the first to analyze sustained aberrant DNA methylation patterns that exist at both diagnosis and relapsed stage of ALL. This allowed for a comparative analysis against aberrantly methylated loci from patients at diagnosis who did not relapse.

This study did not incorporate input control samples when identifying DMRs between patient samples. When performing differential coverage between the different patient cohorts, local CpG density biases would affect read coverage in the same way. There may be impacts due to certain genetic components like

copy number alterations. However, these genetic alterations would need to occur within the same locus among many patients to have a sizable impact.

The differential methylation analysis between diagnosis and relapse patients revealed that losses of DNA methylation characterize the genomic landscape in patient samples at relapse. This finding may seemingly contradict past studies that have found predominate hypermethylation at relapse.^{3,4} There are two main factors that may contribute to the outcome disparities between this study and pasts. First, this study's smaller sample size relative to past studies has a direct effect on statistical power, potentially resulting in fewer regions of hypermethylation observed at relapse. This notion may be supported by the fact that 457 out of 740 genes that exhibited sustained 5' regulatory hypermethylation from diagnosis to relapse in this study corresponded to genes with 5' regulatory hypermethylation observed at relapse from diagnosis from the 450k array study done by Nordlund and colleagues.³ Therefore, a larger sample size most likely results in many of sustained hypermethylated DMR findings to actually have increases in DNA methylation at relapse compared to diagnosis.

The second factor pertains to the different methodological approach of regional differences (DMR) and individual CpG differences (DMC). This study sought out 200bp regions of differential methylation (DMR) by comparing read count differences counted across intervals as opposed to past microarray studies, which are predicated on differential methylation at the single CpG level (DMC) in

accordance to fluorescent signals (β -values) of individual probes. Therefore, individual CpGs that are differentially methylated within this study may go unaccounted for due to the influence of high read counts affiliated with adjacent CpGs within the same 200bp. For example, this effect may be more profound in CpG islands, where higher read counts are observed, which may mask methylation differences of individual CpGs.

A noticeable observation among the hypomethylated regions detected at relapse is that the majority lay within retrotransposable elements. Losses of methylation within these regions have well documented associations with malignant progression and overall genomic destabilization. DNA hypomethylation also occurred within the 5' regulatory regions of genes that may contribute to relapse. Due to the limitations of patient samples, gene expression differences between diagnosis and relapse were not evaluated. However, Chow and colleagues performed a meta-analysis incorporating the data from 3 gene expression studies that evaluated differential expression between individuals at diagnosis and relapse.³⁰ A total of 126 of the hypomethylated genes identified in this study were found to be upregulated at relapse in the meta-analysis. Functional annotation of these genes revealed an enrichment of genes involved in DNA replication and repair (*TOP2A*, *PCNA*, *MCM6*). One of the 126 hypomethylated genes that showed differential expression in the meta-study (*TOP2A*), showed differential expression in each of the individual studies.^{4,16,31} In general, increased expression of DNA repair genes has been associated with relapse,

where upregulation of *TOP2A* has been shown to be predictive of poor relapse free survival.³² While, others have also reported a role for TOP2A in relapsed ALL, this data supports a potential role for additional genes involved in DNA replication and repair including *PCNA* and *MCM6*.

Markedly large stretches of hypomethylation have been reported in cancer.^{33,34} In this study, two large stretches of hypomethylation were observed in patients with ALL at relapse. Interestingly, the 692kb region harboring the protocadherin gene clusters has been reported to be hypermethylated in numerous malignancies including pediatric ALL.^{5,35,36,37} This region lost methylation at relapse, thereby reverting to methylation profiles consistent with normal, healthy individuals. Therefore, it may be unlikely that methylation within the protocadherin clusters plays a causal role in the malignant phenotype. Conversely, the 40kb region spanned a large CpG island that is comprised of 17 5S ribosomal RNA gene copies. 5S ribosomal RNA has been shown to function by negatively regulating the p53 tumor suppressor signaling pathway.³⁸ Therefore, the loss of methylation in this region may have oncogenic potential in patients with ALL at relapse.

In this study, the identification of DMRs present in regulatory elements at diagnosis and at relapse unveiled promising candidate epigenetic loci that are potentially emblematic of ALL. For the purposes of this discussion, an epigenetic locus can be defined as a DMR that was present in individuals at both diagnosis

and relapse and that was also associated with the aberrant expression of a gene with a potential role in the pathogenesis of ALL identified in our previous work.⁵ Specifically, potential epidrivers of ALL that were identified are involved in tumor suppression, B-cell development, and hematopoiesis. The most promising candidates include hypermethylated DMR that were associated with the down regulation of tumor suppressor genes (*FRMD5*, *MLF1*, *PRUNE2*, *PTPRO*)^{19,20,21,22} and B-cell development genes (*IL7* and *MCOLN2*)^{17,18}, and hypomethylated DMR that were associated with the upregulation of genes associated with aberrant hematopoiesis (*SOCS2*, *BID*).^{28,29} It is easy to envision that the aberrant epigenetic modifications identified in this study may be responsible for abnormal gene expression, thereby resulting in impaired B-cell development, a hallmark of ALL.

Methylation-mediated misregulation of these genes is hypothesized to play a role in the pathogenesis of pediatric ALL. Connections linking several of the potential driver genes to cancer and faulty hematopoiesis have previously been established. For example, *PTPRO*, a well-established tumor suppressor gene in the Wnt/ β -catenin pathway, has been shown to be hypermethylated and silenced in multiple cancers, including ALL.^{5,39,40,41} Further, *IL7* has been shown to be a vital cytokine for normal B-cell differentiation and it has been shown that the expression of *MCOLN2* is regulated by the B-cell related transcription factor, *PAX5*^{17,18} Finally, the upregulation of *SOCS2* has been shown to be linked to ineffective hematopoiesis resulting in increased expansion of immature precursor

populations in ALL.²⁸ Taken together, functional studies related to these genes in ALL is warranted.

Reliable relapse associated biomarkers are currently lacking in pediatric ALL. In this study, potential biomarkers were identified that were present in individuals that relapse and were absent in individuals that did not relapse. The 130-hypermethylated regions exclusive to patients that relapsed may be helpful in appropriately stratifying patients with favorable karyotypes at diagnosis into their correct risk group. The potential biomarkers were not associated with alterations in gene expression; therefore, the utility lies solely in appropriately stratifying patient risk of relapse. Interestingly, only 21 of the regions are located within CpG islands, whereas the majority (n=94) lay within intergenic sequences and outside of CpG islands. Milani and colleagues found that methylation levels outside of CpG islands had the highest variance among ALL patients and therefore served as the best classification markers in identifying recurrent subtypes of ALL.⁴² Hence, it is feasible that the CpGs identified outside of CpG islands have the potential to serve as predictive classifiers in the future for patients who relapse.

In conclusion, this study is the first to observe predominate hypomethylation in relapsed ALL. Genes involved in DNA replication and repair correlated with regulatory hypomethylation in this study at relapse and gene upregulation at relapse found in previous diagnosis and relapse paired studies. In addition, the

identification of potential epigenetic driver genes in ALL may provide insight into the mechanism(s) of how leukemic blasts in ALL resist chemotherapy and proliferate uncontrollably. Therefore, functional studies for those genes are an essential step in understanding their role in the tumorigenesis of ALL. Lastly, quantitating percent methylation differences of individual CpGs between the relapse and non-relapse cohorts of loci exhibiting the highest odds ratio would be a good starting point in the process of identifying bona fide relapse associated biomarkers at diagnosis.

Patient ID	Age (months)	WBC, 10 ³ /μl	Sex	Cytogenetics	Relapse Status	Months to Relapse
A4	4	7.8	M	hyperdiploidy	Yes	86
A15	36	7.8	M	hyperdiploidy	Yes	2
A18	17	4.3	F	46, XX-15der(1) t(1;?),del(6)(q21),t mar	Yes	39
A19	36	3.7	M	hyperdiploidy	Yes	56
A20	120	3.6	M	46, XY	No	-
A21	36	6.6	M	46, XY t(3;19)(p25;p13)	No	-
A22	60	2.5	F	47, XX +21; 48, XX	No	-
A23	180	2.3	M	46, XY del(6)(q21;q27)	No	-
A24	108	3.7	M	45, -7 -9 +der(9) t(8;9)(q112;p11)	No	-
A25	48	13.7	M	46, XY	No	-
A26	48	4.3	M	47, XY	No	-
A27	60	3.1	M	46, XY	Yes	30
A28	36	1.5	M	none available	No	-
A29	24	10.2	F	46, XX	No	-
A30	24	3.7	F	46, XX	No	-
A31	132	18.8	M	45, XY -7	No	-
A33	180	4.5	M	46, XY	Yes	31
A36	72	2.7	F	46, XX	No	-
A37	20	2.5	M	hyperdiploidy	No	-
A59	18	6.3	M	46,XY	Yes	8
A70	36	2.6	M	hyperdiploidy	Yes	8
A101	113	5.8	F	46, XX	Yes	7
A121	10	1.3	M	46, XY	Yes	28

Table 2.1 Patient characteristics

Figure 2.1. Genomic distribution of DMR that were hypomethylated in pediatric ALL patients at relapse.

Figure 2.2 BedGraphs illustrating average read count differences between diagnosis (orange) and relapse (green) within the 5' regulatory region of four genes (purple) with the largest $\log_2$ fold change: [*LRI03*, $\log_2\text{fc}=2.64$]^A, [*LYPD1*, $\log_2\text{fc}=2.63$]^B, [*MSX2*, $\log_2\text{fc}=2.61$]^C, [*PRDM5*, $\log_2\text{fc}=2.58$]^D. Note: CpG islands in dark green

Figure 2.3 BedGraphs illustrating average read count differences between diagnosis (orange) and relapse (green) within a 40kb stretch overlapping 5S ribosomal RNAs (red), *RHOA* and *DUSP5P1* (purple). **B.** Average read count differences within a 692kb region overlapping the $\alpha\beta\gamma$ protocadherin cluster (purple). Note: CpG islands in dark green

Hypomethylated genes with oncogenic potential at relapse		
	Gene name	Biotype
Histones	<i>H3F3C</i>	Protein-coding
	<i>HIST3H3</i>	Protein-coding
	<i>HIST1H2AA</i>	Protein-coding
	<i>HIST1H2BA</i>	Protein-coding
p53-signaling	<i>MDM4</i>	Protein-coding
	<i>CUL5</i>	Protein-coding
	<i>5S rRNA</i>	ncRNA
DNA replication/Cell-division	<i>TOP2A</i>	Protein-coding
	<i>PCNA</i>	Protein-coding
	<i>MCM6</i>	Protein-coding
	<i>PRIM1</i>	Protein-coding
	<i>CDC14C</i>	Pseudogene
	<i>LINC00857</i>	ncRNA
	<i>LINC00668</i>	ncRNA
MAP-kinase signaling	<i>MAP2K6</i>	Protein-coding
	<i>MAPK15</i>	Protein-coding
	<i>p38</i>	Protein-coding
	<i>DUSP5P1</i>	Pseudogene
	<i>LOC407835</i>	Pseudogene
Ras-signaling	<i>MRAS</i>	Protein-coding
Fibroblast growth factor receptor signaling	<i>FGFR2</i>	Protein-coding
	<i>LOC100335030</i>	Pseudogene
Inhibiting apoptosis	<i>AKT1</i>	Protein-coding
Wnt/β-catenin signaling	<i>FZD2</i>	Protein-coding
	<i>FZD7</i>	Protein-coding
	<i>FZD9</i>	Protein-coding
	<i>FZD10</i>	Protein-coding
	<i>WNT2</i>	Protein-coding
	<i>SOX10</i>	Protein-coding
	<i>SOX14</i>	Protein-coding
B-cell proliferation/differentiation	<i>CARD14</i>	Protein-coding
	<i>SOX11</i>	Protein-coding
miRNAs	<i>miR-543</i>	ncRNA
	<i>miR-632</i>	ncRNA
	<i>miR-922</i>	ncRNA
Stem cell proliferation/differentiation	<i>SOX2</i>	Protein-coding
	<i>SOX9</i>	Protein-coding

Table 2.2 Genes with oncogenic potential that were hypomethylated in pediatric ALL patients at relapse.

Chromosome:Start-End	Regulatory Regions	Genomic Region	Gene Name
chr17:76130002-76130400	D, TF	Promoter	<i>TMC8</i>
chr3:194407802-194408200	TF	First exon	<i>FAM43A</i>
chr21:46117602-46117800		First exon	<i>KRTAP10-12</i>
chr1:154988802-154989000	TF	Exon	<i>ZBTB7B</i>
chr8:1900802-1901000		Exon	<i>ARHGEF10</i>
chr1:5006602-5006800		Intergenic	-
chr2:239403202-239403400	E,TF	Intergenic	-
chr5:133269802-133270000	E,D	Intergenic	-
chr7:403602-403800		Intergenic	-
chr12:122444402-122444600		Intergenic	-
chr12:132641402-132641800	TF	Intergenic	-
chr13:31416602-31416800		Intergenic	-
chr13:39899002-39899200		Intergenic	-
chr14:105122602-105122800	E,D,TF	Intergenic	-
chr16:3002802-3003000		Intergenic	-
chr17:216802-217000		Intergenic	-
chr21:40361002-40361200		Intergenic	-
chr2:131734602-131734800		Intron	<i>ARHGEF4</i>
chr3:13113802-13114200	E,D,TF	Intron	<i>IQSEC1</i>
chr3:195534802-195535000		Intron	<i>MUC4</i>
chr4:1835802-1836000	E,D,TF	Intron	<i>LETM1</i>
chr6:42007602-42007800	E,TF	Intron	<i>CCND3</i>
chr7:157462802-157463000		Intron	<i>PTPRN2</i>
chr7:2106002-2106200	D	Intron	<i>MAD1L1</i>
chr7:2177802-2178200	E,D,TF	Intron	<i>MAD1L1</i>
chr8:144599002-144599200		Intron	<i>ZC3H3</i>
chr10:126239002-126239200	TF	Intron	<i>LHPP</i>
chr12:529202-529400		Intron	<i>CCDC77</i>
chr16:1509202-1509400	E,D,TF	Intron	<i>CLCN7</i>
chr16:21552402-21552800	E,D,TF	Intron	<i>LOC23117</i>
chr17:3807802-3808000	TF,D	Intron	<i>P2RX1</i>
chr19:1967002-1967200		Intron	<i>CSNK1G2</i>
chr22:36724802-36725000	E,D,TF	Intron	<i>MYH9</i>
chr1:153744402-153744600	TF	Exon-intron junction	<i>INTS3</i>
chr16:1588002-1588200		Exon-intron junction	<i>TMEM204</i>
chr16:87734602-87735000	E,TF	Exon-Intron junction	<i>FLJ00104</i>
chr17:80866002-80866200		Exon-intron junction	<i>TBCD</i>
chr17:74674602-74674800		TTS	<i>MXRA7</i>

E = Enhancer region (H3K4me1 and H3K27ac overlap)

D = DNase I Hypersensitivity site

TF = Transcription factor bound site in the GM12878 lymphoblastoid cell line

TTS = Transcription termination site

Figure 2.4 Hypermethylated DMRs present in pediatric patients with ALL at diagnosis and at relapse. **B.** Hypomethylated DMRs present in pediatric patients with ALL at diagnosis and at relapse. **C.** Genomic distribution of DMRs present in pediatric patients with ALL at diagnosis and at relapse. **D.** DMRs present in pediatric patients with ALL at diagnosis and at relapse in the context of CpG islands.

	<u>Gene name</u>	<u>Methylation status</u>	<u>Function in cancer</u>	<u>Biotype</u>
<u>Inhibition of Src signaling</u>	<i>FRMD5</i> ¹⁹	Hypermethylated	Tumor suppressor	Protein-coding
<u>Inhibition of p53 signaling</u>	<i>MLF1</i> ²⁰	Hypermethylated	Tumor suppressor	Protein-coding
<u>Inhibition of Ras-signaling</u>	<i>PRUNE2</i> ²¹	Hypermethylated	Tumor suppressor	Protein-coding
<u>Inhibition of cell proliferation</u>	<i>LOC389332</i> ²³	Hypermethylated	Tumor suppressor	Protein-coding
	<i>LINC01018</i> ²⁷	Hypermethylated	Tumor suppressor	ncRNA
<u>Wnt/β-catenin signaling</u>	<i>PTPRO</i> ²²	Hypermethylated	Tumor suppressor	Protein-coding
<u>B-cell proliferation/differentiation</u>	<i>IL7</i> ¹⁷	Hypermethylated	Arrested B-cell lineage development	Protein-coding
	<i>MCOLN2</i> ¹⁸	Hypermethylated	Arrested B-cell lineage development	Protein-coding
<u>Cell-cycle regulator</u>	<i>SPDYE8P</i>	Hypomethylated	Unknown	Pseudogene
<u>miRNAs</u>	<i>miR-124-3</i> ²⁴	Hypermethylated	Tumor suppressor	ncRNA
	<i>miR-129-2</i> ²⁵	Hypermethylated	Tumor suppressor	ncRNA
	<i>miR-375</i> ²⁶	Hypermethylated	Tumor suppressor	ncRNA
<u>Leukemic hematopoiesis</u>	<i>SOCS2</i> ²⁸	Hypomethylated	Oncogene	Protein-coding
<u>Hematopoietic stem cell survival</u>	<i>BID</i> ²⁹	Hypomethylated	Oncogene	Protein-coding

Table 2.4 Classification of genes associated with aberrant methylation at diagnosis and at relapse

Figure 2.5 Differentially methylated loci between patients at diagnosis that did not relapse (left) and patients at diagnosis that did later relapse (right). Each row represents a DMR and each column represents a patient at original diagnosis. The regions were ranked based on odds ratio scores from largest (top) to smallest (bottom). Red cells represent read counts >50 and yellow cells

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CHAPTER 3: COMPARATIVE METHYLOME ANALYSIS IN CANINE AND HUMAN ACUTE LEUKEMIA

3.1 ABSTRACT

Background: Aberrant DNA methylation with resulting changes in expression that likely contribute to the pathogenesis of acute lymphoblastic leukemia have been identified in humans. Currently, the contribution of DNA methylation to disease phenotype remains unknown in canine acute leukemia (cAL). Therefore, identifying aberrant DNA methylation within regulatory regions of the same gene or intergenic loci in human ALL and cAL may aid in discovering novel therapeutic targets with the potential of also serving as functional or diagnostic biomarkers in human ALL. In order to identify aberrant DNA methylation, genome-wide methylation profiles were constructed for cAL and healthy dog using the methylated CpG island recovery assay (MIRA), followed by next-generation Illumina sequencing as previously performed in human ALL samples in our laboratory. A comparative methylome analysis between human ALL and canine AL was conducted. **Results:** First, the DNA methylation landscape in cAL was determined by comparing peaks against normal dog B lymphocytes, where 66,262 more peaks were present in cAL. Next, a differential methylation analysis was conducted between cAL and normal dog B lymphocytes, yielding 15,142 hypermethlated regions and 988 hypomethylated regions in cAL. Comparative analysis classified 409 genes exhibiting 5' regulatory hypermethylation in both human ALL and cAL. To identify potential functional biomarkers, we

incorporated gene expression data from our previous human ALL analysis, which filtered the gene list down to 10 that were concomitant with lower expression at diagnosis compared to healthy patients, including 3 tumor suppressor genes (*PRUNE2*, *PTPRO*, *RHOBTB1*). Lastly, to identify potential biomarkers outside of genic regions, DMRs within intergenic regions were queried for sequence conservation between human and dog, where one intergenic region was identified. **Conclusion:** The genes *PTPRO*, *PRUNE2*, and *RHOBTB1* are epigenetically misregulated in human ALL and cAL and therefore may serve critical biological function in human ALL. In addition, aberrant DNA methylation was identified in one conserved intergenic sequence between human and dog, where the regulatory potential in acute leukemia is unknown. Each of these may be valuable biomarkers.

3.2 INTRODUCTION

Canine acute leukemia (cAL) is a bone marrow malignancy of neoplastic hematopoietic cells in the bone marrow associated with poor responsiveness to treatment and is ultimately fatal in dogs. cAL can be of immature lymphoid or myeloid origin with white blood cell counts (WBC) greater than $5 \times 10^9/\text{L}$ (50,000/ μL), but in some cases a limited number of circulating cells at diagnosis is observed.¹ Immunophenotyping is also a very important diagnostic tool used to identify lineage specific cell surface markers and is routinely practiced in both dog and human acute leukemia.² For example, immunophenotyping can help distinguish between chronic and acute canine leukemia by evaluating the

presence of stem cell marker CD34+ blast populations, which are associated with acute leukemia. In canine acute lymphoblastic leukemia (ALL), B-lymphocytes display CD20, CD21 and/or CD79a, whereas T lymphocyte lineage shows CD3, CD5, and either CD4 or CD8 antigens.^{1,3} In human pre-B cell ALL pertinent surface markers for diagnosis and subclassification are CD19, CD20, CD22, CD24, and CD79a, while T-ALL surface markers present CD1a, CD2, CD3, CD4, CD5, CD7 and CD8 antigens.⁴ AML-M5a (monocytic) is the most common type in dog that lack MHCII- expression but display differential cell surface markers that contain stem cell antigens CD34/CD90 and monocyte antigen CD14.⁵ AML in human can be defined based on expression of at least two of the following myeloid cell surface markers: CD13, CD15, CD33, CD65, and CD117.⁶

In human ALL, epigenetic alterations, such as DNA methylation have been extensively shown to play an instrumental role in regulating gene expression. This would entail mechanistic promoter specific hypermethylation of tumor suppressor and lineage dependent genes.⁷ Currently, there has been only one genome-wide DNA methylation study in canine AML that observed heterogeneity of either preferential hypomethylation, hypermethylation, or no difference in 5-mC compared to healthy dogs.⁸ However, individual genes and functional groups affected by aberrant DNA methylation in cAL have not been investigated. Therefore a more complete DNA methylation analysis is warranted for canine AL. Utilizing the methylated CpG island recovery assay, followed by next-generation sequencing (MIRA-seq), methylome profiles were constructed for dogs

diagnosed with AL and CD21+ healthy control dogs. Our first aim was to characterize the DNA methylation pattern observed in cAL by comparing the methylation peak signature in both the groups. The second aim of this study was to identify potential tumor suppressors, hematopoietic related genes, and functional groups displaying aberrant 5' regulatory methylation using MEDIPS R package to calculate differentially methylated regions (DMRs) between AL and healthy dog lymphocytes. Lastly, we conducted an epigenetic comparative analysis between human and dog to identify genes that exhibited aberrant regulatory DNA methylation in canine AL that was also present in our previous DNA methylation analysis in human acute lymphoblastic leukemia.⁹ In addition, aberrant methylation occurring within conserved intergenic sequences between canine AL and human ALL were investigated. This may provide insight into genes that are of functional relevance to acute lymphoblastic leukemia that may also serve as biomarkers in human ALL.

3.3 MATERIALS AND METHODS

3.3.1 Patient samples

Peripheral blood was extracted from 4 cAL and 6 healthy dogs and was used in this study with Animal Care and Use Committee approval of the University of Missouri. Flow cytometry was conducted on 3 out of the 4 cAL dogs at the University of Missouri Veterinary Health Center, in which all were positive for hematopoietic stem cell marker CD34+ (**Table 3.1**). Myeloid and leukoid lineages were undetermined from the flow cytometry. However, one dog had a

subset of CD14+/MHCII+ cells, which may indicate a blast population of AML origin. All 4 dogs were euthanized within a two-week span after their diagnosis. CD21+ cells were isolated by flow cytometry from peripheral blood from 6 healthy dogs and were used as control samples. Previously generated DNA methylation (MIRA-seq) and gene expression (RNA-seq) data derived from 20 bone marrow aspirates from mostly cytogenetically normal and other subtypes of human ALL patients compared against ten healthy human umbilical blood (HCB) donor patients, where pre-BI/pre-BII (CD19+,CD34-,CD45^{low/med}) cells were used.⁹

3.3.2 Genomic DNA isolation and MIRA-seq profile construction from patient samples

Using the QIAamp DNA Micro Kit (Qiagen cat. no. 56304), genomic DNA was extracted from bone marrow aspirates in dog. Approximately 1µg of DNA in was used as the starting concentration in construction of each MIRA-seq library. The DNA was sonicated with the Diagenode Bioruptor (Diagenode; cat. no. B01010002) at 4°C, creating a range of 200 to 700bp fragments. Sonicated DNA (~150ng) was run on 1% agarose gel at ~96V for 45 minutes to verify the 200 to 700bp fragmented range. The sonicated DNA was purified using the MinElute PCR purification kit (Qiagen; cat. no. 28004) and eluted with 12µL nuclease-free water. End repair and dA tailing of the fragments was performed using the NEBNext DNA Library Prep Master Mix Set for Illumina kit (New England BioLabs; cat. no. E6040S) with a DNA purification step using Agencourt AMPure XP Beads (Beckman Coulter; cat. no. A63881). After End repair and dA tailing,

NEB-Next Adaptors were added following another DNA purification step with 1x the concentration AMPure XP Beads and 10 μ L nuclease-free water elution. For methylated DNA fragment enrichment, methylated CpG island recovery assay MIRA was performed with the Methyl Collector UltraTM kit (Active Motif; cat. no. 55005). This pull-down method involves a methyl binding protein complex (MBD2b/MBD3L1) conjugated with nickel beads that compartmentalize the methylated fragments towards the magnetic stand, where the supernatant is exposed and removed with subsequent wash steps in between using AMPure XP Beads. The MIRA product was run on 1% agarose gel for 45 minutes at ~96V and bands between 200 to 700bps were excised and purified using the MinElute Gel Extraction kit (Qiagen; cat no. 28604). These methylated fragments were PCR enriched with the NEBNext DNA Library Prep Master Mix Set for Illumina kit with multiplex oligos for 12 cycles. The DNA product was purified again with AMPure XP Beads and eluted with 15 μ L nuclease-free water. Fragment analysis and Qubit quantitation was performed for each library prior to sequencing. MIRA-seq libraries were multiplexed in 10nM concentrations with 100bp single-end sequencing by the DNA Core facility at the University of Missouri on the HiSeq 2000.

3.3.3 Sequence processing and peak identification of dog MIRA-seq libraries

Quality control check of FASTQ file reads were done using FastQC v0.11.4¹⁰, then adaptor sequences were removed, including reads < 50bps, using cutadapt v1.7.1.¹¹ Read alignment was performed using Bowtie 2 v2.2¹², mapping to the canine reference genome (canFam3) with default settings and allowing no more than two mismatches. The output SAM files were then converted to BAM files and sorted accordingly using “view” and “sort” options with samtools version 1.2.1.¹³ Picard-tools version 1.92 under “MarkDuplicates” option, removed duplicate reads. The final BAM output files were then indexed with samtools “index” option. Methylation peaks for each sample were created using MACS2 v2.0¹⁴ “callpeak” function. A common methylation peak signature was identified in both the cAL and healthy dog cohorts using bedtools (version 2.17.0)¹⁵ “intersectBed” to find overlapping coordinates that were merged as one unified peak using “mergeBed.” The peaks from the two cohorts were then compared against each other.

3.3.4 Identifying DMRs between cAL and healthy dog

Differentially methylated regions between cAL and healthy dogs were identified in the same manner as our previous human ALL study and are based off Chavez and colleagues’ computational method.^{9,16} Sufficient coverage depth was observed in each sample file (BAM file) using a saturation analysis (saturation correlation > 0.90). When performing differential coverage between the different

dog cohorts, local CpG density biases would affect read coverage in the same way. We acknowledge that there may be impacts due to certain genetic components like copy number alterations. However, we reason that these genetic alterations would need to occur within the same locus among many dogs to have sizable impact. Therefore, input control samples were not needed when identifying DMRs. The reads were accumulated in 200bp windows along with a 200bp extension (average fragment size of ~400bp). These windows are termed regions of interest (ROIs). ROIs with a mean read count across all samples < 20 counts were excluded to avoid bias and enhancing power, while reducing false discoveries.¹⁷ In determining if a ROI is a DMR, the statistical parameters of a false discovery rate (Benjamini-Hochberg) < 5% and a $\log_2$ fold change ≥ 1 had to be met. Normalized RPKM values (Reads Per Kilobase Per Million) were computed for each 200bp window and used to identify DMRs. DMRs were merged as separate entities in respect to hypomethylation and hypermethylation, if the adjacent 200bp window met the previously described criteria.

3.3.5 Custom annotation of genomic coordinates in canine (canFam3)

Annotating genomic coordinates in dog is partially challenging because many genes in the dog genome remain unclassified in sequence databases like RefSeq and Ensembl. In order to annotate genomic coordinate BED files containing either methylation peaks or DMRs in dog patients, HOMERv4.3¹⁸ (Hypergeometric Optimization of Motif EnRichment) custom annotation option was implemented. To create a custom annotation for dog, a gene transfer format

(GTF) file containing the Ensembl Genes track was created using the canFam3 assembly from the Table Browser tool in the UCSC Genome Browser platform.¹⁹ Ensembl ID's for each coordinate were then converted into gene names utilizing the database-to-database conversion tool from the biological DataBase network²⁰, in which many ensembl IDs lack a gene prediction in dog. The X and Y-chromosomes were excluded from this analysis.

3.3.6 Comparative DNA methylation analysis in human and dog and bedGraph illustration

First, genes were filtered based on possession of DMR(s) within the 5' regulatory region (promoter/5'UTR/first exon) in canine AL and human ALL data. Next, in order to find a common gene signature with aberrant DNA methylation in canine AL and human ALL, the compiled list of genes that contained hypermethylated or hypomethylated DMR(s) in each species was compared against each other, respectively. Lastly, the resulted list of shared genes between human and dog were filtered based on their expression in human ALL. Because shared hypomethylation was not observed, only hypermethylated genes were investigated for downregulated expression in human ALL. Using the "genomeCoverageBed" function from bedtools¹⁵, bedGraph illustrations were created for each candidate gene in the context of human ALL vs. HCB (control) and canine AL vs. healthy dog. Each bedGraph contains peaks representing average read counts per base coordinate across all patient samples in a given cohort within the 5' regulatory region of a gene.

3.3.7 Identifying conserved intergenic sequences with aberrant methylation between human ALL and canine AL

The entire list of DMR coordinates in canine AL was uploaded to UCSC genome browser's LiftOver tool interface.²¹ Next, dog coordinates from the canFam3 assembly were converted to the human hg19 assembly under the strict parameter, where 0.95 minimum ratio of bases from the dog sequence must remap to the human sequence. A total of 421 regions were successfully converted into hg19 coordinates and then filtered upon their overlap of hyper or hypomethylated regions found in our human ALL study. One hypermethylated intergenic region was identified in both canine AL and human ALL. Using CLC Sequence Viewer 8²² software, the respective dog and human sequence was aligned against each other to examine the degree of conservation. Transcription factor binding motif analysis was done using Tomtom Motif Comparison Tool under The MEME Suite v5.0.2²³ with default parameters.

3.4 RESULTS

3.4.1 DNA methylation peak analysis in canine AL

Methylome profiles were created for 10 dog samples, resulting in a total of 250 million reads for cAL samples and 272 million reads for healthy dog with an average alignment rate of 93%. In order to characterize the methylation landscape of cAL and healthy canine, methylation peaks were identified in each sample derived from high quality reads. An overall genome-wide increase of

DNA methylation was observed in AL with 136,758 methylation peaks present in cAL and 70,496 methylation peaks among healthy dog patients. The genomic distribution of peaks revealed methylation within intergenic and intronic loci for both cohorts (**Figure 3.1**). Further, 2,221 more peaks were identified within the 5' regulatory regions (promoter and first exon) in cAL compared to healthy dog. In the context of CpG islands (CGI), the majority of peaks were distributed outside CGI sequences (**Figure 3.1**). The distribution of peaks within CGIs displayed 8,988 more peaks in cAL than healthy dog.

3.4.2 Predominant hypermethylation in canine AL

The methylation pattern specific to cAL was explored by identifying DMRs between cAL and healthy dog. Predominant hypermethylation was observed in cAL with 15,142 regions gaining methylation and only 988 regions losing methylation at diagnosis. While the majority of hypermethylated DMRs overlapped intergenic and intronic sequences, many DMRs coincided with 5' regulatory regions (promoter n=1247, first exon n=655) (**Figure 3.2**). Due to the significant role DNA methylation plays in the 5' regulatory region, we analyzed functional groups likely affected by aberrant methylation. Using DAVID's functional annotation clustering tool with a benjamini cutoff value < 0.05, 1,106 genes with 5' hypermethylation were analyzed. Among the functional groups identified, included genes with Homeodomains (Enrichment Score: 18.00, Gene count: 56), genes involved in the WNT pathway (Enrichment Score: 4.76, Gene count: 9), and histones (Enrichment Score: 2.70, Gene count: 8). Functional

annotation clustering of the 20 genes in cAL coinciding with hypomethylated DMR within the 5' regulatory region did not reach statistical significance.

Next, individual genes were sought that exhibited regulatory hypermethylation in conjunction with functional involvement in hematopoiesis, epigenetics, apoptosis, acute leukemia or inhibition of pathway signaling. A total of 52 genes were identified, in which genes involved in hematopoiesis were the most represented (n=14) (**Table 3.2**). Hematopoietic related genes include essential transcription factors such as *IKZF1*, *PAX5*, *SOX2*, *ETV2* and cell surface markers *CD19*, *CD40*, and *CD79B*. Genetic and epigenetic lesions within these genes are frequently observed in human acute leukemia. Among epigenetic associated genes were histones, where the majority of the variants belonged to the H2A and H2B family. Lastly, the Wnt/ β -Catenin pathway contributed to ~44% of overall identified negative regulators of pathway signaling, which included genes *SOX9*, *PTPRO*, and *DKK2*. The remaining negative regulators included genes such as *CDKN2B* of cell cycle signaling and tumor suppressors *PRUNE2* and *RHOBTB1*.

3.4.3 Comparative DNA methylation analysis in canine AL and human ALL

Comparative epigenetic analyses across species can aid in identify genes of pathological relevance to cancer and have the potential to serve as biomarkers. Therefore, we incorporated DNA methylation and gene expression data from our previous study in human acute lymphoblastic leukemia (ALL) patients and examined genes with aberrant methylation in the 5' regulatory that existed in both

human ALL and canine AL. A total of 409 genes were hypermethylated in both human ALL and canine AL. Shared regulatory hypomethylation was not found between human and dog leukemia. The list of 409 hypermethylated genes was then filtered based on inverse correlation with gene expression found in human ALL, resulting in 10 genes concomitant with downregulation at diagnosis (**Table 3.3**). Among the 10 genes listed included 3 reported tumor suppressors: *PTPRO*, *PRUNE2*, and *RHOBTB1*. BedGraph illustrations highlight the distinct read count differences observed in the 5' regulatory region for each of the 3 candidate biomarker genes in both human ALL and canine AL (**Figure 3.3**). The read count peaks are much higher and are distributed across the 5' regulatory region in the leukemia groups for both species compared to their healthy counterparts, who exhibit very low read count peaks in all 3 genes.

In order to identify potential biomarkers outside of intragenic regions, DMR coordinates belonging to dog were converted into human coordinates with the objective of identifying conserved intergenic sequences that displayed aberrant DNA methylation in both species. Utilizing the LiftOver tool from the UCSC genome browser, 421 hypermethylated and 39 hypomethylated coordinates in canine (canFam3) were successfully converted to the human (hg19) assembly. These coordinates were intersected with hyper- and hypomethylated DMR found in human ALL, where 77 hypermethylated loci were shared and no overlapping regions of hypomethylation were found. The 5' regulatory region (n=47) made up most of the 77 hypermethylated DMR and only 1 intergenic region on

chromosome 12 in human and chromosome 26 in dog was identified. The conservation of the intergenic region was investigated in human and dog by aligning the sequence from both assemblies against one another. Approximately 72% of the bases aligned between human and dog and 8 base pair gaps in intervals of no more than 3 were observed in the dog sequence (**Figure 3.4**). The intergenic sequences in both species lay in between transmembrane gene *TMEM132B* and a long intergenic noncoding RNA. Interestingly, the human intergenic sequence lies within a CpG shore, whereas the dog sequence is greater than 4kb from the nearest CpG island.

Finally, the human DNA sequence was explored for any known transcription factor (TF) binding motifs using The MEME Suite. DNA motif analysis identified 4 transcription factors belonging to the ETS family (ETV2, ETV4, ETS1, ERG) and two within with zinc-finger family (GATA2, ERG) that were statistically significant (p-value < 0.05). Hematopoietic transcription factor ETV2 was the most statistically significant motif finding (p-value= 5.31e-04) with an optimal alignment sequence of 5'-TTCCTG-3'.

3.5 DISCUSSION

Methylation peak and DMR analysis from our previous human ALL study revealed global losses in DNA methylation in conjunction with regulatory hypermethylation. Using the same methodical approach (MIRA-seq) in human ALL, our canine AL samples displayed gains of global DNA methylation with

limited losses of methylation⁹. However, this observation is not representative of all dogs with acute leukemia as global hypomethylation has also been documented in AML.⁸ In most human ALL cases, the global methylation pattern can be attributed to specific cytogenetic aberrations.^{24,25} As for canine AL, cytogenetic analysis is not readily available for clinical application due to the difficulty of karyotyping 76 acrocentric autosomes of similar shape and size. Yet, cytogenetic alterations observed in human leukemia, such as BCR-ABL translocation, has been reported in individual AML cases.²⁶ Thus, cytogenetic lesions may have an impact on the observed gains of DNA methylation in our canine AL samples, but are currently unknown.

The genomic distribution of CpG islands between dog and human differ considerably and may influence the number of genes affected by regulatory methylation. The DMR analysis in canine AL resulted in identifying 1,798 genes exhibiting 5' regulatory hypermethylation with 692 of the genes currently not annotated in the canFam3 assembly. In human ALL, hypermethylation was observed in 2,063 genes. Intriguingly, the dog genome has over 20,000 more CpG islands in their genome than the human genome. However, there are approximately 3,000 more promoter-associated CpG islands in human, which may contribute to the higher number of genes with 5' regulatory hypermethylation detected in human ALL.²⁷

We hypothesized that many genes involved in hematopoiesis and tumor suppression would possess regulatory hypermethylation in both canine AL and human ALL. Moreover, a comparative analysis of the identified genes in dog and their functional groups may also be affected by aberrant methylation in human ALL. Functional annotation clustering of genes with regulatory hypermethylation in canine AL and human ALL show similar affected groups and individual genes. Both species have enrichment in homeodomain (gene count: 82 human, 56 dog) and Wnt-signaling (gene count: 9 in human and dog) related genes. Aberrant hypermethylation of homeodomain genes is a well-known phenomenon in many cancers and has been linked to poorer outcomes in human ALL and breast cancer patients.^{28,29} In addition, methylation induced silencing of Wnt-signaling antagonist is a hallmark of human leukemia and has also been implicated in both relapsed ALL and AML.^{30,31,32}

Further, the comparative analysis identified 3 candidate biomarker genes: *PTPRO*, *PRUNE2*, *RHOBTB1*, which may play a distinct functional role in human ALL pathogenesis that may be regulated by DNA methylation. Each gene has been reported as a functional tumor suppressor in cancer. For example, *PTPRO* has been identified as a novel receptor in the Wnt/ β -catenin signaling pathway and is epigenetically silenced by DNA methylation in esophageal, lung, and ALL cancers.^{9,33,34,35} Tumor suppressor activity of *RHOBTB1* has also been described in breast and colon cancer, where a loss of function mutation leads to Golgi fragmentation.^{36,37} Although the function of *PRUNE2* is unknown, induced

expression resulted in decreased proliferation of prostate cancer cells.³⁸

Therefore, the candidate genes represent potential biomarkers in terms of diagnostic and therapeutic intervention in human ALL.

Conserved intergenic sequences have previously been observed in mammals, specifically between human and murine.³⁹ In the context of other potential biomarkers, the UCSC genome browser's LiftOver tool allowed for exploration of aberrant methylation occurring within conserved intergenic DNA sequences between human and dog. As one might expect, all but one of the dog to human converted genomic coordinates lay within a conserved genic loci, primarily the 5' regulatory region. Since genic regions had been investigated in terms of aberrant DNA methylation and gene expression between human and dog, intergenic regions were queried for sequence conservation. We found aberrant methylation within a homologous intergenic sequence between human and dog containing predominately ETS binding motifs in the human DNA sequence. In relation to hematopoiesis, ETV2 has been reported as a key player in hematopoietic progenitor development in murine through enhancer binding and subsequent activation of downstream target *LMO2*.^{40,41} Therefore, further methylation in conjunction with TF binding studies may be warranted within this intergenic region.

In conclusion, comparative methylome analysis between dog and human is a powerful tool in finding common functional groups and individual genes that likely contribute to acute leukemia. This is especially important in unraveling epigenetic lesions that are currently unknown and may lead to therapeutic targets, which are desperately needed in canine AL. In addition, conserved intergenic sequences in human and dog with aberrant methylation could unveil novel enhancer or repressor like regulatory regions. Moreover, this study has narrowed down potential epdrivers of human ALL in *PTPRO*, *PRUNE2*, and *RHOBTB1* genes with tumor suppressing activity. Thus, validating the need for additional studies on their role in human ALL.

ID	Breed	Age	Sex	Diagnosis	Treatment	Outcome
8151731	Labrador Retriever	9 yrs	Female	Acute leukemia: CD34+MHCII- Flow cytometry did not reveal markers for myeloid or leukoid lineage.	L-asparaginase, steroids, vincristine, cytosine arabinoside	Euthanized 1 week later due to disease
7164929	Golden Retriever	9 mths	Male	Acute leukemia: Flow cytometry not done.	None	Euthanized at diagnosis
7136328	Mixed breed dog	8 yrs 7 mths	Female	Acute leukemia: CD34+ with population of CD14+MHCII	Prednisone	Euthanized 2 weeks later due to disease
7147625	German Shepherd	7 yrs	Female	Acute leukemia Poorly differentiated	None	Euthanized at diagnosis

Table 3.1 Clinical characteristics of 4 dogs diagnosed with acute leukemia.

Figure 3.1 Genomic distribution of methylation peaks in canine that are healthy and diagnosed with acute leukemia. **B.** Peak distribution in the context of CpG islands, shores, and shelves of healthy and acute leukemic canine.

Figure 3.2 Genomic distribution of differentially methylated regions in canine acute leukemia patients.

<u>Gene</u>	<u>Log2 fold change</u>	<u>Gene</u>	<u>Log2 fold change</u>
<u>Hematopoiesis</u>		<u>Pro-apoptotic</u>	
<i>SOX2</i>	4.53	<i>PERP</i>	5.58
<i>PAX5</i>	4.06	<i>NTN1</i>	2.55
<i>TNFRSF13B</i>	3.11	<u>Negative regulators</u>	
<i>CD40</i>	2.83	<u>Wnt/β-Catenin</u>	
<i>CD79B</i>	2.50	<i>SOX9</i>	3.41
<i>FCMR</i>	2.40	<i>WNT5A</i>	3.33
<i>NODAL</i>	2.34	<i>SFRP4</i>	3.03
<i>SYK</i>	1.93	<i>PTPRO</i>	2.84
<i>IKZF1</i>	1.83	<i>APCDD1</i>	2.10
<i>CD19</i>	1.63	<i>DKK2</i>	2.05
<i>CTF1</i>	1.36	<i>PCDH10</i>	2.03
<i>SEMA4D</i>	1.15	<i>WNT3</i>	1.65
<i>AKNA</i>	1.12	<i>SOX10</i>	1.41
<i>ETV2</i>	1.06	<i>WNT11</i>	1.27
<u>Epigenetic regulators</u>		<u>Cell-cycle signaling</u>	
<u>Histones</u>		<i>CCNA1</i>	3.06
<i>HIST1H2BJ</i>	3.44	<i>CDKN2B</i>	2.78
<i>HIST1H1A</i>	3.29	<i>PKMYT1</i>	1.03
<i>HIST1H3A</i>	1.99	<u>Tumor-suppressor</u>	
<i>HIST1H2AC</i>	1.78	<i>PRUNE2</i>	3.74
<i>HIST1H2BB</i>	1.42	<i>RHOBTB1</i>	3.22
<i>HIST1H2AB</i>	1.78	<i>CDX1</i>	2.94
<i>HIST3H2A</i>	1.37	<i>FRMD3</i>	2.69
<i>HIST1HC</i>	1.23	<i>TWIST2</i>	2.50
<u>Methyltransferase</u>		<i>CCDC8</i>	2.08
<i>BHMT2</i>	1.05	<i>CEACAM1</i>	1.88
<u>Histone methyltransferase</u>		<i>KLK10</i>	1.80
<i>PRDM14</i>	4.62	<i>DAB2IP</i>	1.72
<i>SUV39H2</i>	1.23	<i>CAV1</i>	1.71
<u>RNA methyltransferase</u>			
<i>NSUN7</i>	4.55		
<u>Kinetochores assembly</u>			
<i>CENPW</i>	2.08		

Table 3.2 Classification of genes associated with 5' regulatory hypermethylation in canines with acute leukemia.

	<u>Canine AL</u>	<u>Human ALL</u>	
	Hypermethylated log ₂ fc	Hypermethylated log ₂ fc	Downregulated log ₂ fc
<i>PKHD1L1</i>	1.49	2.66	-5.38
<i>KHDRBS2</i>	2.64	2.84	-4.83
<i>PTPRO</i>	2.83	3.65	-4.65
<i>PDE4C</i>	1.45	3.43	-3.82
<i>RHOBTB1</i>	2.11	3.84	-2.75
<i>OSMR</i>	6.01	3.96	-2.38
<i>CDC42BPB</i>	2.64	4.12	-1.98
<i>SLC13A5</i>	1.96	3.34	-1.71
<i>PRUNE2</i>	3.74	4.56	-1.47
<i>LMNA</i>	2.66	2.27	-0.91

Table 3.3 Downregulated genes in human ALL accompanied with 5' regulatory hypermethylation in canine and human leukemia. (p-value < 0.05)

Figure 3.4 Sequence alignment of a hypermethylated intergenic region found in human ALL and canine AL. The 208 bases (chr12:12618099-12618306) on the (+) strand from the human sequence were derived from the canFam3 to hg19 assembly conversion of 200 bases (chr26:4428401-4428600) on the (-) strand in canine, shown by 8 gaps created in the canine sequence.

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CHAPTER 4: RELAPSE ASSOCIATED BIOMARKERS IN ACUTE LYMPHOBLASTIC LEUKEMIA

4.1 Abstract

Although the 5 year survival rate for ALL has broken the 90% mark, patients who relapse have a dismal outcome. Therefore patients at diagnosis would greatly benefit from relapse-associated biomarkers like DNA methylation so that an alternative therapeutic approach may be put in place and monitored closely through the course of treatment. Previously, we identified 130 loci (200bp) that were hypermethylated in a cohort of patients (n=10) at diagnosis that went on to relapse compared to healthy controls. Hypermethylation in these regions was absent in another cohort of patients (n=13) at diagnosis who did not relapse. Odds ratio calculations for 3 read count cutoffs (50, 75, 100) identified 38 statistically significant regions (p-value < 0.05) to quantitate individual CpG methylation differences between the two patient cohorts using pyrosequencing. Among the 4 regions tested in both cohorts by pyrosequencing, region #1 contained 3 CpGs that were hypermethylated in the relapse cohort (n=10) compared to the non-relapse cohort (n=8). In addition, the area under the curve (AUC) was greater than or equal to .925 for all 3 CpGs (p-value < 0.05).

4.2 Introduction

Risk stratification remains a significant problem for ALL patients. It is crucial that patients be categorized into their respective low-standard or high-risk group of relapsing prior to induction treatment. Failure in doing so vastly increases the likely-hood of failed response to treatment and subsequent relapse.

Consequences of inaccurate stratification include high risk patients treated under a less intensive treatment regimen or increased chemotoxicity exposure for patients misclassified as low-standard risk. Specifically, patients exhibiting favorable cytogenetic characteristics, such as diploidy (46 chromosomes) or high-hyperdiploidy ($50 < \text{chromosomes}$) are placed into the low-standard risk treatment protocol. However, many of these patients have unidentified genetic and/or epigenetic characteristics that would qualify them as high-risk. Therefore, additional strategies implemented alongside primary stratification methods of cytogenetic testing, minimal residual disease (MRD) monitoring and white blood cell count (WBC) based stratification are needed at diagnosis and throughout treatment.

DNA methylation-based methods would be an attractive addition to patient stratification because of the robust nature and stability of DNA, which may also offer a non-invasive approach to monitoring patient treatment response via peripheral blood as opposed to bone marrow aspiration. For example, past studies have demonstrated the ability to assign patients into their correct cytogenetic subtype with high precision using a CpG panel developed from 450K

microarray data.¹⁻⁴ Furthermore, CpG island methylator phenotype (CIMP) classification is a method that has stratified ALL patients at diagnosis and relapse into low-standard and high-risk groups based on the bimodal distribution of methylation derived from a panel consisting of 1,293 individual CpG sites.^{5,6}

This study's objective was to identify CpG sites that could differentiate a small sample size of patients at diagnosis who either did or did not relapse. Using next-generation Illumina sequencing, our previous study identified 130 loci that were hypermethylated in normal and diploidy ALL patients at diagnosis that later went on to relapse (n=10) compared to healthy patient controls (n=10). Importantly, these 130 loci were not differentially methylated in a subset of normal or diploidy patients that had not gone on to relapse (n=13) when compared to healthy patients. Based on odds ratio calculations applied to 3 different read count cutoffs (50, 75, 100) for each region for a given patient, 38 regions reached statistical significance ($p < 0.05$) among the 130. Given that read count data lack the quantitative means to measure individual CpG sites, pyrosequencing can overcome this shortcoming by producing percent methylation ratios (C/T) of individual CpG sites. In addition, pyrosequencing remains one of the most accurate technologies used in a clinical setting with minimal false-positive and false-negative results.⁷ Therefore, incorporating pyrosequencing technology provides a gold standard and optimal resolution for measuring differential methylation of individual CpG sites within our 38 loci of interest.

4.3 Methods

4.3.1 Patient samples

Bone marrow aspirates containing >87% leukemic blasts from ALL patient samples were used under full ethical approval of the Institutional Review Board at the University of Missouri (**Table 4.1**). MIRA-seq libraries were created for A27, A59, A70, A101, and A121 patient samples at diagnosis who relapsed and for all ten matched relapsed samples in this study. These MIRA-seq profiles (hg19 build) are accessible through the NCBI Sequence Read Archive (Accession PRJNA470682). Previously generated MIRA-seq libraries for A4, A15, A18-A26, A28-A31, A33, A36, A37 diagnosis patient samples and ten normal human umbilical blood (HCB1-HCB10) were used in this study. The previously generated MIRA-seq profiles can be found in the NCBI Sequence Read Archive (Accession SRP058314).

4.3.2 Genomic DNA isolation and pyrosequencing experimentation

Using the QIAamp DNA Micro Kit (Qiagen cat. no. 56304), genomic DNA was extracted from 18 ALL patients (n= 8, non-relapse cohort) (n= 10, relapse cohort). Bisulfite conversion of genomic DNA (200 ng) was performed using the EZ DNA methylation kit (D5006, Zymo Research, Irvine, CA, USA) per the manufacturer's instructions. Bisulfite PCR was performed using PyroMark PCR Kit (Qiagen) using 20ng of bisulfite converted DNA under Qiagen's

recommended HotStarTaq cycling conditions (45 cycles). All primer sequences designed were chosen based on a quality score of at least “Medium quality” using the PyroMark Assay design software 2.0 (Qiagen, Valencia, CA, USA) and can be found in (**Table 4.2**). Streptavidin-coated Sepharose beads (GE Healthcare) were used to purify biotin labeled PCR products. Subsequent purification, washing, and denaturing steps (NaOH solution) were applied to the PCR products before sequencing. A pyrosequencing primer annealed to the single stranded PCR product and pyrosequencing was then performed on the PyroMark Q24 per manufacturer’s instructions. Internal controls for bisulfite conversion were implemented by including non-CG sequence(s) into the target sequence. In addition, a water control was included as a negative control. The percent methylation within CpG sites of interest were calculated using instrumental software (Qiagen, PSQ24MA 2.1), resulting in pyrogram illustrations that provide a visual of peak heights representing nucleotide incorporation to the single stranded DNA and therefore a C/T ratio at the CG site of interest could be calculated.

4.3.3 Statistical analysis of pyrosequencing results

Nonparametric statistical analysis of relative percent methylation differences between the relapse and non-relapse cohorts was performed with SPSS Statistics software (IBM Corp.). Wilcoxon rank-sum test and receiver operator characteristic (ROC) curve was applied to the percent of methylation values of

each CpG site measured across 18 ALL patient samples. P-values < 0.05 were considered statistically significant.

4.3.4 Determining 38 loci of interest for pyrosequencing analysis

The 130 regions of interest were identified as hypermethylated in the relapse cohort compared to normal healthy controls, but were not hypermethylated in the non-relapse cohort. The regions were then ranked based on odds ratios, where larger scores represented a greater presence of individuals with read counts of either > 50, 75, 100 (cutoffs) in the relapsed cohort in correlation with the absence of read counts > 50,75,100 within the non-relapsed cohort. Odds ratio scores, including 95% confidence intervals, and their respective p-values were calculated using MEDCALC software v 12.4 (MedCalc Software, Ostend, Belgium). Regions where read count values were > 50,75,100 were labeled in red and regions < 50, 75, 100 read counts were labeled in yellow for a given patient. Haldane-Anscombe correction was applied to the data for each region, where a constant equal to 0.5 was added to each cell. P-values < 0.05 were considered statistically significant.

4.4 Results

4.4.1 38 loci are associated with relapse outcome in ALL patients

In order to determine loci with statistical association to relapse outcome from the aforementioned list of 130 regions, odds ratio was applied to the sequencing data under three arbitrary read count cutoffs (50, 75, 100). This resulted in identifying

38 loci that reached statistical significance (p -value < 0.05), of which 16 had an overlap in at least two of the three read count cutoffs (**Figure 4.1**). The vast majority of the 38 loci were located in repetitive satellite intergenic elements of the genome ($n=34$), where the remaining 4 loci overlapped intronic sequences within 4 separate genes. The most observed genes in proximity of the 38 loci included many pseudogenes: Actin-related pseudogenes *ACTR3BP5* ($n=4$) and *ACTR3BP3* ($n=3$), zinc finger pseudogene *ZNF733P* ($n=4$), and ankyrin repeat pseudogene *ANKRD26P1* ($n=3$).

4.4.2 3 CpG sites are hypermethylated in ALL patients who relapsed compared to ALL patients who did not relapse

In order to quantitate methylation differences between relapse and non-relapse patients at diagnosis, individual CpGs within the 38 loci of interest were investigated for assaying compatibility (primer design and PCR product visibility) with pyrosequencing. A total of 4 loci: Region #1(chr2: 92272401-92272600(CpGs=3)). Region #2(chr6: 58774601-58774800 (CpGs=1)), Region #3 (chr2: 92272401-92272600 (CpGs=2)), Region #4 (chr2: 92274801-92275000 (CpGs=1)) were tested. The 3 CpGs tested in region #1 were hypermethylated in the relapse cohort ($n=10$) compared to the non-relapse cohort ($n=8$) (p -value <0.05). In addition, ROC curve analysis of all 3 CpGs demonstrated the ability to distinguish between the two cohorts based on methylation percentage with the area under the curve (AUC) being greater than at least 0.925 for all 3 CpGs tested (p -value <0.05) (**Figure 4.2**). The distribution of percent methylation

plotted across all 18 patients for the 3 CpGs, illustrates a clear separation between patients at diagnosis who did or did not relapse (**Figure 4.3**). Furthermore, the line graph highlights patient outliers for 1 or more CpGs: A19, A15 (relapse cohort in red) and A23, A28 (non-relapse cohort in blue).

4.5 Discussion

Considering only 1 of the 4 loci tested were statistically significant between relapse and non-relapse ALL patients, the remaining loci should also be assessed for methylation differences. The regions were prioritized based on primer constraints from Qiagen software where at least a medium quality score had to be met before proceeding. However, this does not exclude the possibility of lower quality or other custom designed primers from producing robust results. Moreover, alternative methods to measure CpG methylation within these regions may be used to circumvent the primer limitations of bisulfite specific PCR and subsequent pyrosequencing, such as quantitative methylation specific PCR (qMSP).

Many of our listed 130 loci (n=103), including the 4 loci tested, contain novel CpG sites that have yet to be explored on an individual or genome-wide scale in ALL/relapsed ALL due to the absence of probes in 450K methylation array experiments. This observation may also be attributed to the fact that primer development is difficult in these regions as many lay within repeat sequence motifs.

The potential regulatory significance of region #1 has yet to be determined. Although, according to ENCODE ChIP-seq data from the UCSC genome browser consortium, both enhancer histone markers (H3K4me1, H3K27ac) from lymphoblastoid cell line GM12878 overlap the region.⁸ Interestingly, the intergenic region lays between genes denoted as abparts (antibody various regions). In addition, methylation-mediated enhancer regulation of immunoglobulins and other elements such as eRNA has been shown to be an essential component to normal B cell function.^{9,10}

A glaring limitation of this study includes a small sample size tested (n=18) relative to most pyrosequencing studies used to find clinical significance between various treatment and diseased groups.¹⁰⁻¹³ In addition, the few patient outliers observed in region #1 may increase as the sample size goes up. Nonetheless, region #1 clearly distinguishes between relapse and non-relapse ALL at diagnosis in our sample set and therefore warrants further patient testing. Because the methylation pattern is specific to cytogenetic aberrations in ALL, a follow up study would include patients consistent with this study that present with either a normal karyotype or hyperdiploidy, then potentially branching out to the other predominate patient subtypes.¹⁴

Patient ID	Age (months)	WBC, 10 ³ /μl	Sex	Cytogenetics	Relapse Status	Months to Relapse	Performed Pyrosequencing
A4	4	7.8	M	hyperdiploidy	Yes	86	Yes
A15	36	7.8	M	hyperdiploidy	Yes	2	Yes
A18	17	4.3	F	46, XX-15der(1) t(1;?),del(6)(q21),t mar	Yes	39	Yes
A19	36	3.7	M	hyperdiploidy	Yes	56	Yes
A20	120	3.6	M	46, XY	No	-	No
A21	36	6.6	M	46, XY t(3;19)(p25;p13)	No	-	No
A22	60	2.5	F	47, XX +21; 48, XX	No	-	Yes
A23	180	2.3	M	46, XY del(6)(q21;q27)	No	-	Yes
A24	108	3.7	M	45, -7 -9 +der(9) t(8;9)(q112;p11)	No	-	Yes
A25	48	13.7	M	46, XY	No	-	No
A26	48	4.3	M	47, XY	No	-	No
A27	60	3.1	M	46, XY	Yes	30	Yes
A28	36	1.5	M	none available	No	-	Yes
A29	24	10.2	F	46, XX	No	-	
A30	24	3.7	F	46, XX	No	-	Yes
A31	132	18.8	M	45, XY -7	No	-	Yes
A33	180	4.5	M	46, XY	Yes	31	Yes
A36	72	2.7	F	46, XX	No	-	Yes
A37	20	2.5	M	hyperdiploidy	No	-	Yes
A59	18	6.3	M	46,XY	Yes	8	Yes
A70	36	2.6	M	hyperdiploidy	Yes	8	Yes
A101	113	5.8	F	46, XX	Yes	7	Yes
A121	10	1.3	M	46, XY	Yes	28	Yes

Table 4.1 Patient characteristics

Region #	Genomic coordinate (hg19)	Forward Primer (5'→3')	Reverse Primer (5'→3')	Pyrosequencing Primer (5'→3')	CpGs analyzed	Quality score
1	Chr2: 89844201-889844400	AGGGAATTAAATGGAATAAATGTTAAATTG	CTATTCATACCCATTAAATTATTTACAC	GAATGTAATGGAATGGAAA	3	73
2	Chr6: 58774601-858774800	AGAAATATTTTTGTAGAAATTTGGAAGTGT	CAAAAAATCCAAATATCCACTAACAT	TTTCTACCATAAATCTCAAAAC	1	63
3	Chr2: 92272401-92272600	TGATGATGATTTGGTTTGGTTTAA	ATTCCCCTTTCTTAAAAATCCTCCAACTA	ATGATTTGGTTTGGTTTAAAT	2	68
4	Chr2: 92274801-92275000	TGGATTATATGGAGGTTTAATTGTGA	CTTTAACTAAAAATAACATCCCAAAAT	ATTTGGATAGATTGAGGAT	1	76

Table 4.2 Bisulfite specific primer sequences.

Figure 4.1 Venn diagram of 38 statistically significant loci based on odds ratio (p-value < 0.05) applied to 130 regions of different read count cutoffs (50, 75, 100).

Figure 4.2 Statistical analysis of 3 CpGs tested in region #1. Median value differences of DNA methylation were observed between relapse and non-relapse cohorts using Wilcoxon rank-sum test (p-value < 0.05). The ROC curve analysis found the ability of the experimental test of 3 CpGs to distinguish between ALL patients at diagnosis who did or did not go on to relapse (AUC > .925) (p-value < 0.05).

Figure 4.3 Line graph representing the percent methylation of 3 CpGs observed across ALL patients at diagnosis who relapsed (n=10) or did not relapse (n=8). Outliers observed in 2 patient from the relapse cohort (A15,A19) and 2 patients from the non-relapse cohort (A23, A28).

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CHAPTER 5: SUMMARY AND FUTURE DIRECTIONS

The main objectives of this study were to 1) characterize the DNA methylation landscape of relapsed ALL and 2) identify potential epigenetic driver genes and 3) biomarkers associated with relapse ALL within a mostly cytogenetically homogenous cohort (diploidy or hyperdiploidy). Our first experiment characterized the genomic landscape as predominantly hypomethylated at relapse. It is imperative to point out the differences between the technique that was used in this study (MIRA-seq) and past microarray studies that also evaluated matched diagnosis/relapse patients methylation differences and found predominate hypermethylation.^{1,2} The 450K and 27K arrays were designed to contain probes mostly within a CpG island context. Theoretically, the affinity-based approach (MIRA-seq) pulls down double stranded DNA fragments (~250-500bp) that contain at least 2 methylated CpGs.³ This notion is consistent with our results that observed 70,702 hypomethylated DMRs at relapse, where 30,208 DMRs were found in regions that did not overlap an existing probe within the 450K cassette. Furthermore, the majority of the 30,208 DMRs are located within non-CpG island intergenic sequences where the CpG density is lower and therefore the CpG proximity to one another is farther. Our results add an element to relapsed ALL that suggests a global demethylation event that may have implications for retrotransposable element activation and genomic instability in ALL patients and should be further examined. It is also important to stress the fact that the cytogenetic subtype strongly influences the methylation landscape of

ALL and therefore this phenomenon may be observed to a greater or lesser extent in other patient subtypes not tested in this study. Further, predominant global hypomethylation was the trend observed in our patients at diagnosis compared to pre-B cells from healthy individuals with 61% of 25,282 identified DMRs labeled as hypomethylated.⁴

Our second objective was to identify potential epigenetic driver genes related to relapsed ALL. This was accomplished by comparing diagnosis and relapse samples to healthy patients (HCB), respectively and identifying common DMRs located in 5' regulatory sequences between [diagnosis vs. HCB] and [relapse vs. HCB]. A large portion of the common hypermethylated regions (n=818/2170) was indeed in either a promoter, 5' UTR, or first exon region. The decision to include those three regions was made based on studies that correlated first exon methylation with transcriptional silencing and our own observation which found many genes exhibiting promoter hypermethylation that extended up to the first exon.⁵ The epigenetic driver genes identified highlight tumor suppressor genes *FRMD5*, *MLF1*, *PRUNE2*, *PTPRO* and B-cell development related gene *MCOLN2*. These genes are putative epigenetic driver genes in that they exhibit regulatory hypermethylation and were downregulated at diagnosis when compared to healthy patients. Importantly, only *PTPRO* has been documented as a potential epigenetic driver of ALL. Further, its leukemogenic properties have been revealed in decitabine treated ALL cell line studies that linked activation of *PTPRO* to increased apoptosis.⁶

Hence, the remaining four putative epidriver genes should also be explored as potential targets of relapsed ALL.

In addition, analyzing DNA methylation changes in canine acute leukemia (cAL) reinforced the notion that leukemia is an epigenetically related disease as much as it is a genetic one. Around 13% of the hypermethylated regions found in cAL lay within the 5' regulatory region. We have identified numerous genes pertaining to hematopoiesis, epigenetics, and negative regulation of pathways that are hallmarks of ALL. Filtering of hypermethylated genes found in cAL that were concordant between hypermethylation and downregulation in our human ALL study, resulted in identifying a total of 10 genes in common between the two species: *PKHD1L1*, *KHDRBS2*, *PTPRO*, *PDE4C*, *RHOBTB1*, *OSMR*, *CDC42BPB*, *SLC13A5*, *PRUNE2*, *LMNA*. Besides *PTPRO*, the other nine genes have not been identified or tested for tumor suppressor or B cell function in ALL. Moreover, these results support the existence of a common epigenetic signature that may be essential in establishing ALL pathogenesis. In addition, this strengthens the potential functional significance of *PTPRO* and *PRUNE2* as it pertains to relapsed ALL.

Our third objective utilized MIRA-seq profiles from human ALL patients at diagnosis who either did or did not relapse later on. 130 regions were hypermethylated in the relapse cohort compared to healthy patients. However no statistical differences were identified in the non-relapse cohort. The question

remains, whether or not these regions were differentially methylated between the two patient cohorts themselves. Odds ratio calculations were applied to read counts, which narrowed down 38 regions that were most likely hypermethylated in the relapse cohort compared to the non-relapse cohort. One 200bp region containing 3 CpGs was hypermethylated in the relapse group (p-value < 0.05). Given that the sample size was low for this experiment, the results should be interpreted as preliminary. Moreover, methylation data for ALL patients is absent within this region and likely variable between different subtypes. However, all 38 regions should be investigated considering only 4 regions were tested due to theoretical primer constraints on regions composed predominately of intergenic loci and also in part due to limited resources. Pyrosequencing remains an attractive option in forthcoming methylation studies in ALL and has been an instrumental method as shown in clinical trial studies used to identify chemo-resistant patients by quantitating CpG sites within the promoter of *MGMT*. Further, one study compared pyrosequencing among 4 other methods in analyzing the promoter methylation status of *MGMT* in glioma patients: Methylation-specific polymerase chain reaction (MSP), MethyLight (quantitative-MSP), methylation-sensitive high-resolution melting (MS-HRM), and immunohistochemistry.⁷ The study concluded that pyrosequencing was superior in that it displayed the highest reproducibility and sensitivity among the other methods and was further validated in additional frozen or FFPE glioma patient samples (n=139) from 8 independent clinical centers.

Future work should also include designing functional assays to investigate the role of potential relapse associated epidrivers identified in this study. Because of the scarcity of treatment options for relapsed ALL patients, new targets are desperately needed. These experiments may include transfection assays delivering a vector carrying the transcriptionally active form of the gene of interest to ALL cell lines or primary patient cells. Successful lentiviral-mediated transfections has been demonstrated in lymphoblastoid cell lines and may offer an effective way to express a gene of interest that may otherwise be epigenetically silenced.⁸ One would then evaluate gene expression changes and determine if there is any correlation with tumor suppressor function by measuring apoptotic activity, cell morphology, and cell viability. In addition, genes involved in proper B cell development such as methylation-induced downregulation of *MCOLN2* should be further explored. Other than the fact that B cell transcription factor *PAX5* binds to the promoter and regulates *MCOLN2* in some fashion, very little is known about the regulation and function of *MCOLN2* in the context of B cell development.⁹ With the improvement of cellular delivery and stability, next generation hypomethylating agents such as guadecitabine may allow researchers to better understand epidrivers in a more ideal sense.¹⁰ Further, low dosage of next-generation hypomethylated drugs could be an essential component to combined therapy options in the future.

In conclusion, DNA methylation has the future potential to serve as a biomarker to identify new gene targets and aid in ALL patient risk stratification at diagnosis

and relapse stages. The implications of bonafide methylation-based biomarkers can be envisaged as a noninvasive approach to risk stratification that is compatible within a clinical setting. At initial ALL diagnosis, DNA methylation has been utilized to subtype patients with higher accuracy when coupled with standard cytogenetic testing. In addition, CpG panels may help identify patients at greater risk for relapse prior to and throughout the course of treatment as demonstrated by the several individual CpGs correlating with relapse and poorer overall survival. Future studies should scrutinize previously identified loci in ALL and novel loci that were observed in this study as well.

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VITA

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