

## Research Article

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# Impact of chromosomal rearrangement upon DNA methylation patterns in leukemia

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**Abstract:** Genomic instability, including genetic mutations and chromosomal rearrangements, can lead to cancer development. Aberrant DNA methylation occurs commonly in cancer cells. The aim of this study is to determine the effects of a specific chromosomal lesion, the *BCR-ABL* translocation t(9:22), in establishing DNA methylation profiles in cancer. *Materials and methods* We compared DNA methylation of 1,505 selected promoter CpGs in chronic myelogenous leukemia (CML), acute lymphoblastic leukemia (ALL) with and without the Philadelphia chromosome t(9:22), CD34+ hematopoietic stem cells transfected with *BCR-ABL*, and other tumors without *BCR-ABL* (acute promyelocytic leukemia (APL) and gastrointestinal stromal tumors (GIST)). In this study, the DNA methylation profile of CML was more closely related to APL, another myeloid leukemia, than Ph+ ALL. Although DNA methylation profiles were consistent within a specific tumor type, overall DNA methylation profiles were not influenced by *BCR-ABL* gene translocation in the cancers and tissues studied. We conclude that DNA methylation profiles may reflect the cell of origin in cancers rather than the chromosomal lesions involved in leukemogenesis.

**Keywords:** DNA methylation; Chronic Myelogenous Leukemia; Philadelphia Chromosome; Acute Promyelocytic Leukemia; Acute Lymphoblastic Leukemia; Gastrointestinal Stromal Tumor; 5-azacytidine

## 1 Introduction

The genesis of cancer in any patient can trace its origin to a single cell that has acquired multiple genetic and epigenetic alterations. It may lead to the abnormal gene expression and protein function responsible for the varied malignant biological phenotypes. Genetic mutations such as point mutations, chromosomal deletions, gene duplication and chromosomal translocations can lead to activation of oncogene or inactivation tumor suppressor gene [1]. Epigenetic alterations in DNA methylation, histone tail modifications and miRNA are also known to be associated with changes in gene expression level [2,3]. Aberrant DNA methylation at CpG islands located in promoter regions is a well-known phenomenon in cancer, and this DNA hypermethylation is associated with aberrant silencing of tumor suppressor genes [2]. The current dogma of cancer epigenetics states that aberrant DNA methylation leads to gene silencing, and this is supported by evidence of DNA methyltransferase inhibitors being reactivated in cancer through hypomethylation [4,5].

Here, we examined DNA methylation in chronic myelogenous leukemia (CML) and other cancers in order to better understand the etiology of aberrant DNA methylation in cancer. CML is a homogenous cancer in that the *BCR-ABL* oncogene is the sole chromosomal lesion that leads to the development of the disease phenotype in all cases. Previous studies have transfected the *BCR-ABL* fusion gene in cells or transgenic mice to confirm the dependence of this leukemia on this genetic event [6-8]. It is a clonal disease that originates from a single transformed hematopoietic stem cell or multipotent progenitor cell, where the Philadelphia translocation t(9:22) first occurred and is shared by all cancer cells [9,10]. Because

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of this shared chromosomal lesion, this cancer has a very homogenous clinical course and response to imatinib mesylate (Gleevec®). Imatinib mesylate is also used to treat gastrointestinal stromal tumors (GISTs) by inhibiting tyrosine kinase activity. Approximately 95% of GISTs stain positively for the CD117 antigen by immunohistochemistry and 80–85% of GISTs exhibit activating *KIT* mutations that are another target for imatinib mesylate therapy [11,12]. Epigenetic modifications, particularly increased gene promoter DNA methylation, have been shown to be associated with imatinib mesylate resistance [13–15]. To the best of our knowledge, the mechanism behind the association between gene promoter DNA methylation and response to imatinib mesylate treatment is not known. However, one possible mechanism might be an aberrant expression of genes related to the transport of imatinib mesylate to leukemic cells.

Acute promyelocytic leukemia (APL) is similar to CML in that it is a leukemia characterized by a specific genetic event. The translocation  $t(15:17)$  generates the oncogenic fusion protein *PML-RAR $\alpha$*  that is responsible for 99% of APL cases, which has distinct clinical features compared to CML [16]. Chromosomal abnormalities have been frequently described in acute lymphoblastic leukemia (ALL), and the most common is the Philadelphia chromosome (Ph). The *BCR-ABL* translocation is found in approximately 20–30% of adults and in 5% of pediatric cases of ALL, respectively [17,18]. It should be noted that two common translocation sites have been described for the *BCR-ABL* fusion gene in CML and Ph<sup>+</sup> ALL. One translocation creates a p210 fusion protein that is found in 95% of CML and one-third of ALL, whereas a slightly smaller p190 is found in less than 5% of CML patients, and two-thirds of adults and more than 90% of children with Ph<sup>+</sup> ALL [19]. However, the two forms of *BCR-ABL* are not exclusive for one disease or the other, and in fact, are interchangeable without significant changes in disease phenotype.

Genomic mutations are associated with a high frequency of cancer-specific hypermethylation of particular loci, and these may be associated with distinct DNA methylation subgroups [20–23]. Aberrant DNA methylation has been investigated in several types of leukemia for further insight into cytogenetic subtypes [24–28]. However, there have been no studies into whether genomic translocations might be responsible for aberrant DNA methylation across the genome. Here, in order to examine the etiology and role of DNA methylation in cancer, DNA methylation profiling of CML was compared to APL and ALL, which have a unique genomic translocation. We also compared DNA methylation patterns between CML and GIST which are treated with the same drug, imatinib mesylate. The

role of the *BCR-ABL* translocation in DNA methylation was studied in more detail by examining DNA methylation profiles of Ph<sup>+</sup> ALL and hematopoietic stem cells transfected with *BCR-ABL* (hereafter called Ph<sup>+</sup> CD34<sup>+</sup>) [6]. We also examined DNA methylation changes that are involved in the biology of the ordered progression of CML from chronic phase to accelerated phase and finally blast phase. The effect of DNA methyltransferase inhibitors on DNA methylation in cancer was examined through the serial profiling of DNA methylation of a CML patient treated with the DNA methyltransferase inhibitor azacitidine.

## 2 Materials and methods

### 2.1 Patient information

Samples of patients with chronic myelogenous leukemia (CML), acute promyelocytic leukemia (APL), gastrointestinal stromal tumor (GIST) and acute lymphoblastic leukemia (ALL) were obtained from established tissue banks at University of Southern California and M. D. Anderson Cancer Center. All samples were collected according to the institutional review board of the University of Southern California and M. D. Anderson Cancer Center in accordance with institutional guidelines. A brief description of the study samples, such as gender, age, and specimen type, is provided in Supplementary Data 1. Treatment with the DNA methyltransferase inhibitor, 5-azacytidine, has been described previously [29]. CD34<sup>+</sup> hematopoietic stem cells isolated from umbilical cords and transfected with the *BCR-ABL* gene (Ph<sup>+</sup> CD34<sup>+</sup>) were generously provided by Dr. Ravi Bhatia [6]. Briefly, the controls expressed green fluorescent protein (GFP) alone, and the cell fractions were collected according to low or high BCR-ABL expression. The cells were cultured for 7 or 14 days before harvest to examine DNA methylation.

### 2.2 Genomic DNA extraction and DNA methylation analysis

DNA was extracted using phenol-chloroform and stored at -80°C until required. DNA extracted from the samples was modified with sodium bisulfite using the 96 Zymo EZ DNA Methylation kit (Zymo Research, Orange, CA, USA). DNA methylation status was assessed using the GoldenGate Assay for Methylation Cancer Panel I from Illumina (La Jolla, CA, USA) and performed at the University of South-

ern California Epigenome Center, as described elsewhere [30]. The commercially available DNA Methylation Cancer Panel I interrogates 1,505 independent CpG sites. Data were reported as beta values, with the methylation status of the interrogated CpG site calculated as the ratio of the fluorescent signal from methylated allele relative to the sum of both methylated and unmethylated alleles.  $\beta$  values with detection p-values  $> 0.01$  were removed from further analysis. The raw DNA methylation data will be available upon request. We used genomic DNA prepared following whole-genome amplification (WGA) as an ‘unmethylated’ control, and M.SssI-treated DNA as a fully ‘methylated’ control. WGA kits (REPLI-g, Qiagen, Doncaster, Australia) were used to amplify DNA from normal blood as recommended, and these were bisulfite-treated as described above. The methylation reactions were carried out in 1x M.SssI buffer with 160  $\mu$ M S-adenosyl methionine (SAM) (supplied with M.SssI by New England Biolabs, Ipswich, MA, USA), and DNA was treated three times with M.SssI prior to bisulfite treatment.

### 2.3 Bisulfite-PCR pyrosequencing measurements

Bisulfite-PCR pyrosequencing was performed for LINE-1 assay [26]. We used the PyroGold Reagent kit (Biotage, Uppsala, Sweden) on a Pyrosequencing 96HS as previously described [31]. In brief, 10  $\mu$ L PCR products for each sequencing reaction were immobilized onto streptavidin-coated beads (Streptavidin Sepharose HP, GE Healthcare Biosciences, Pittsburgh, PA, USA) in binding buffer (10 mM Tris-HCl, pH 7.6, 2 M NaCl, 1 mM EDTA, 0.1% Tween 20) for 10 min. The biotinylated template was purified with the Pyrosequencing vacuum prep tool (Biotage, Uppsala, Sweden) and incubated with 10 pmol/reaction individually with each sequencing primer in annealing buffer (20 mM Tris-acetate, pH 7.6 and 2 mM MgAc<sub>2</sub>). The DNA strands were denatured at 80°C for 2 min and reannealed at room temperature for 10 min. Sequencing was performed according to manufacturer’s instructions. The allele frequency (% cytosine or % thymidine) was calculated from the peak height, analyzed with the allele quantification module in the PSQ 96 HS software (Biotage, Uppsala, Sweden). Percentage of methylation was determined by the percentage of cytosine-to-thymidine conversion (methylation = % cytosine / (% cytosine + % thymidine)).

### 2.4 Data analysis

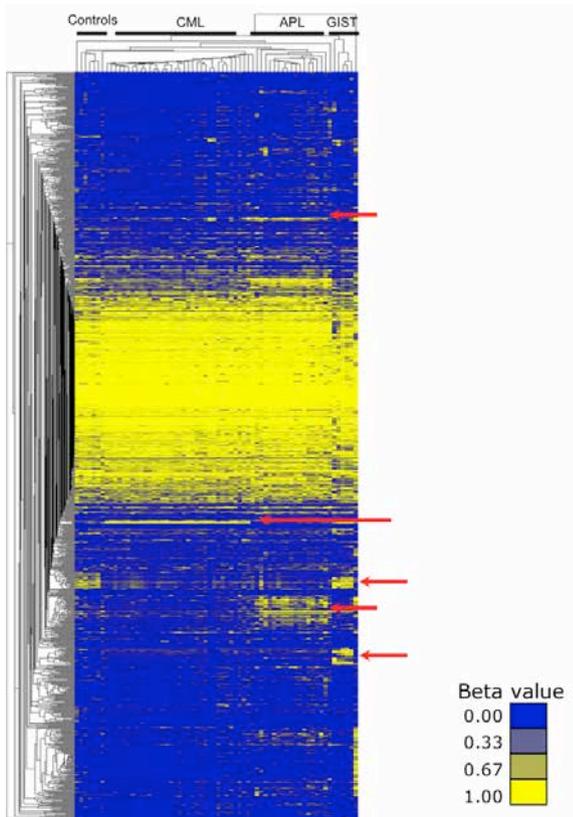
Data from the Illumina GoldenGate Methylation microarrays were analyzed using the open-source programs Cluster 3.0 and Java Treeview. Cluster 3.0 was used to perform an unsupervised hierarchical cluster analysis by applying complete linkage clustering with a Euclidean distance metric. The basic statistic and scatter plots were done using JMP, Version 7 (SAS Institute Inc., Cary, NC, USA). X-chromosome linked loci, loci that possibly contain single nucleotide polymorphisms (SNPs) and/or repetitive elements were eliminated to increase the strength of analysis [31].

## 3 Results

### 3.1 DNA methylation analysis of CML, APL and GIST

We examined DNA methylation using the Illumina GoldenGate Methylation Assay, which simultaneously analyzes methylation at 1,505 CpG sites in 807 genes, in 36 patients with CML, 18 patients with APL, five patients with GIST and six healthy controls. We analyzed 972 of these loci, as 533 loci are located on the X-chromosome or have previously been described to contain SNPs or repeat sequences that affect the DNA methylation analysis [31]. An unsupervised two-dimensional cluster analysis of these homogenous cancers demonstrated clear clustering by disease type, consistent with the theory that aberrant DNA methylation is not random but highly consistent within a specific cancer type, and that the DNA methylation profile is unique within a specific cancer (Figure 1). We found a high degree of correlation between DNA methylation profiles within each cancer (Pearson’s correlation CML = 0.927, APL = 0.915, and GIST = 0.892), and the correlation between cancers was lower (Pearson’s correlation = 0.852).

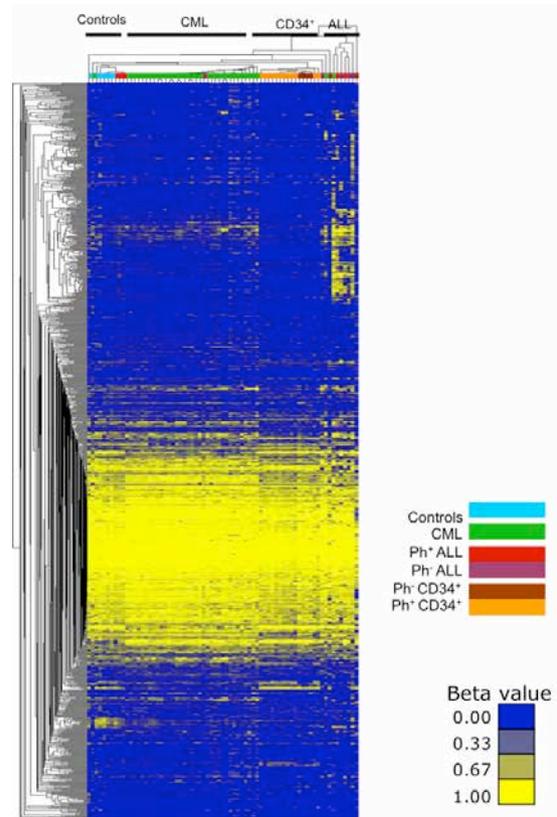
Unique DNA methylation profiles existed for CML, APL, and GIST by visual inspection (Figure 1, red arrows). The leukemia-specific methylated loci and the potential function of genes differentially methylated in leukemia were also analyzed by Ingenuity pathway analysis and listed in Supplementary Data 2. The DNA methylation profiles in these tumors enabled clustering by disease type, and the DNA methylation profiles were shared by individuals with the same disease. Therefore, DNA methylation profiles were similar between specific cancers, but different between CML, APL and GIST cancers.



**Figure 1:** DNA methylation analysis of homogenous cancers. Two-dimensional unsupervised cluster analysis of DNA methylation in 36 CML, 18 APL and five GIST patients, and six samples of peripheral blood from healthy controls. DNA methylation at 972 CpG sites are shown, with probes corresponding to sites on the X-chromosome and those known to contain SNPs were sequestered and not used for downstream analysis. Red arrows indicate clusters of DNA methylation that are specific for each disease. Blue represents low levels of DNA methylation (beta value close to 0) and yellow represents high level of DNA methylation (beta value close to 1).

### 3.2 The Philadelphia Chromosome and DNA methylation in leukemia

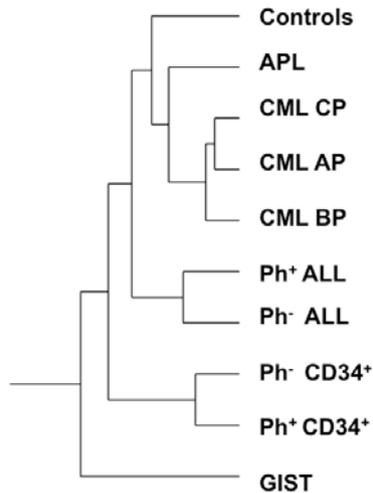
In order to understand the DNA methylation changes driven by t(9,22) in CML, we examined the DNA methylation profiles of CML and Ph<sup>+</sup> ALL, a lymphoid malignancy which is also characterized by t(9:22). In addition, we examined CD34<sup>+</sup> hematopoietic stem cells isolated from umbilical cords and transfected with the *BCR-ABL* gene (Ph<sup>+</sup> CD34<sup>+</sup>)[6]. Two-dimensional unsupervised cluster analysis was performed using DNA methylation data from CML, ALL (Ph<sup>+</sup> and Ph<sup>-</sup>), and CD34<sup>+</sup> (Ph<sup>-</sup> and Ph<sup>+</sup>) (Figure 2) [6]. Here again, we found clear clustering by disease and cell type, but not by the presence or absence of *BCR-ABL*. Partial discrimination between CML and Ph<sup>+</sup> ALL was possible despite the shared *BCR-ABL* genomic translocation, and CML did not cluster with Ph<sup>+</sup> CD34<sup>+</sup>. Ph<sup>+</sup> ALL and Ph<sup>-</sup> ALL showed a distinct pattern of DNA methylation



**Figure 2:** DNA methylation profiles of Ph<sup>+</sup> leukemias. Two-dimensional unsupervised cluster analysis of DNA methylation profiles associated with the Philadelphia chromosome (CML, Ph<sup>+</sup> CD34<sup>+</sup>, and Ph<sup>+</sup> ALL) and counterparts (Ph<sup>-</sup> CD34<sup>+</sup> and Ph<sup>-</sup> ALL). Blue represents low DNA methylation (beta value close to 0), and yellow represents high DNA methylation levels (beta value close to 1). Different leukemia types are represented by different color bars on the top of heatmap.

independently of the presence of the Philadelphia chromosome.

Next, a consensus DNA methylation profile for each disease was determined by calculating the mean DNA methylation beta value at each locus. The hierarchical clustering tree of mean methylation of all samples also supported the notion that the myeloid malignancy, CML (three phases based on clinical characteristics; chronic phase, accelerated phase, and blast crisis phase), is more closely related to the other myeloid malignancy, APL, than Ph<sup>+</sup> CD34<sup>+</sup> cells or Ph<sup>+</sup> ALL (Figure 3). The difference in correlation was subtle, nonetheless consistent with the cluster analysis, and the highest correlation shown with CML was APL ( $r=0.9475$ ), and not Ph<sup>+</sup> CD34<sup>+</sup> hematopoietic stem cells ( $r=0.9343$ ) or Ph<sup>+</sup> ALL ( $r=0.9416$ ). Interestingly, there was a highly consistent DNA methylation profile between Ph<sup>+</sup> and Ph<sup>-</sup> ALL, and between Ph<sup>+</sup> CD34<sup>+</sup> and Ph<sup>-</sup> CD34<sup>+</sup> cells, despite differences in Philadelphia chromosome status ( $r=0.9621$ ,  $p=2.2 \times 10^{-16}$  and  $r=0.9633$ ,  $p=1 \times 10^{-10}$ , respectively) (Figure 4).



**Figure 3:** The hierarchical tree of leukemias with mean DNA methylation. Relationship of DNA methylation profile by clustering based analysis with different leukemias, GIST,  $\text{Ph}^{+/-}$   $\text{CD34}^{+}$  hematopoietic stem cells, and healthy controls. A close relationship is demonstrated for the different phases of CML and myeloid leukemias (CML and APL), lymphoid leukemias ( $\text{Ph}^{+}$  ALL and  $\text{Ph}^{-}$  ALL) and hematopoietic stem cells ( $\text{Ph}^{+/-}$   $\text{CD34}^{+}$ ). Relationships identified by clustering are independent of *BCR-ABL* status.

### 3.3 DNA methylation changes with CML progression

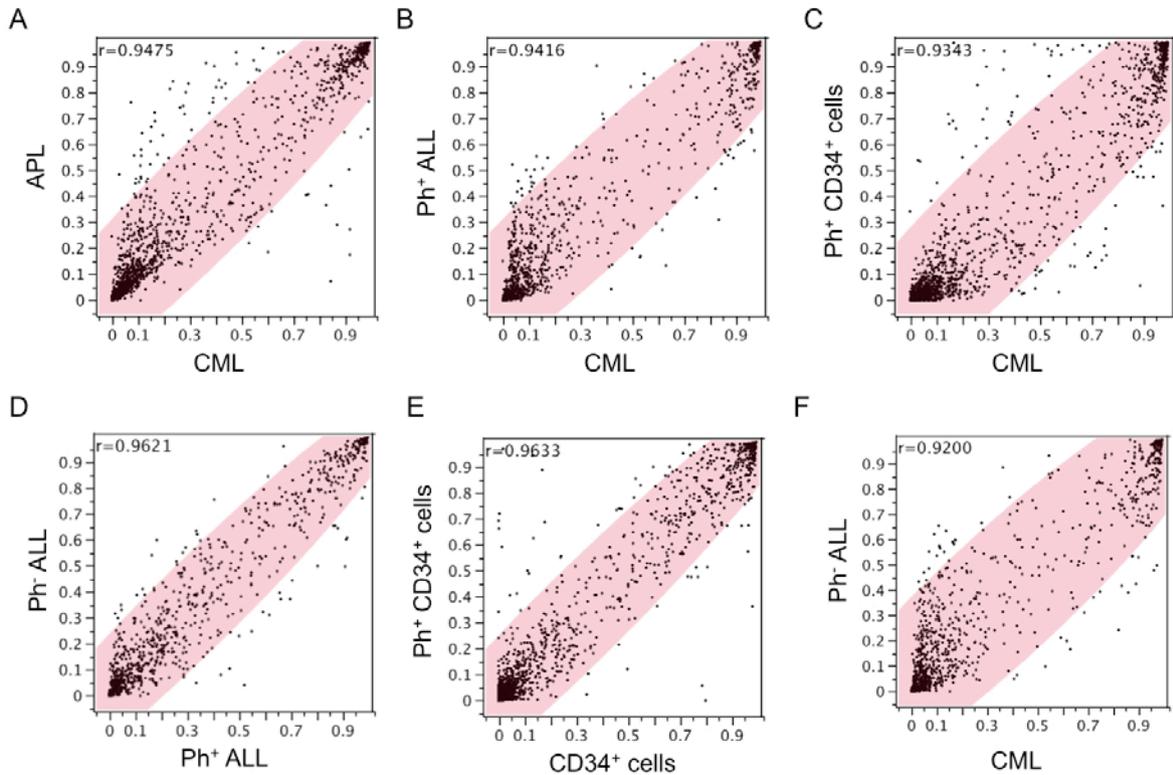
The clinical course of untreated CML typically follows an ordered progression for almost all patients from an early chronic phase (CP) through an accelerated phase (AP) to terminal blast crisis phase (BP). The consistency of this progression among patients likely reflects the homogeneity of the disease, and also enabled us to examine DNA methylation changes involved in the progression of CML. Thus, we determined the DNA methylation changes found in different phases of CML. Subset analysis of our CML samples by chronic phase ( $n=23$ ), accelerated phase ( $n=5$ ), and blast phase ( $n=8$ ) revealed 84 loci that clearly showed increased DNA methylation (mean  $\beta > 0.01$  and  $p$  value  $< 0.05$ ) and 66 loci that showed a decrease in DNA methylation ( $\beta < 0.01$  and  $p$  value  $< 0.05$ ) with CML progression (Figure 5A). The list of genes and Ingenuity pathway analysis are listed in Supplementary Data 3. Interestingly, we had a single patient for whom we had a series of blood samples taken at the time of diagnosis (chronic phase) and from the accelerated and blast phases. As a further validation of these identified loci, we plotted DNA methylation levels at 20 hyper- and hypomethylated loci in an independent set of serial samples from this single patient at the chronic, accelerated and blast phases (Figure 5B). DNA methylation changes of hypermethylation or hypo-

methylation with CML progression from 36 individual CML patients were well validated in the serial samples from a single patient.

### 3.4 The effect of azacitidine (5-azacytidine, Vidaza®) treatment upon DNA methylation changes in CML patient

We next wanted to determine the plasticity of the disease-specific DNA methylation changes in CML and changes involved in CML progression. We studied serial DNA methylation changes in a CML patient who became resistant to imatinib mesylate and developed blast crisis. The patient was then treated in a clinical trial with the DNA methyltransferase inhibitor, azacitidine (5-azacytidine, Vidaza®) for three courses and responded with hematological improvement of white blood cell count (Clinical trial reported previously by Bernstein *et al.* [29] Patient #2, Table 1). Eventually, a more potent tyrosine kinase inhibitor, dasatinib (Sprycel®), became available and the patient's therapy was switched. Serial peripheral blood samples were collected and the genome-wide changes in DNA methylation profile were studied by Illumina GoldenGate Methylation Assay and by bisulfite PCR-pyrosequencing to assess the LINE-1 DNA repetitive element in samples from the chronic phase, blast phase, azacitidine treatment and dasatinib treatment.

The patient was a 33-year-old female who was originally diagnosed with CML in 1999. The patient was treated with hydroxyurea and then interferon, and finally imatinib mesylate in 2001, when it became available. The patient's best response to therapy was only a partial cytogenetic response with 31% of cells being  $\text{Ph}^{+}$  by fluorescence in situ hybridization (FISH) after 18 months of therapy (March of 2003). The patient presented with a markedly elevated white blood cell (WBC) count of 306,000 cells/ $\text{mm}^3$  and was found to be in myeloid blast crisis. The patient was started on the DNA methyltransferase inhibitor, azacitidine as part of a clinical trial at a dose of 25  $\text{mg}/\text{m}^2$  intravenously for five consecutive days in Course 1 (28 day courses), 75  $\text{mg}/\text{m}^2$  intravenously for Course 2, and 75  $\text{mg}/\text{m}^2$  subcutaneously for Course 3 [29]. There was a dramatic decrease in the patient's WBC count to normal by Course 2 of therapy (342,800 cells/ $\text{mm}^3$  to 5,200 cells/ $\text{mm}^3$ ) (Figure 6A). This corresponded with a subtle but significant decrease in global DNA methylation, as assessed by measuring DNA methylation of the LINE-1 repetitive element by pyrosequencing (Pearson's correlation 0.67,  $p$  value=0.049), and a decrease in the average DNA methylation of 1,006 loci as measured by Illumina GoldenGate

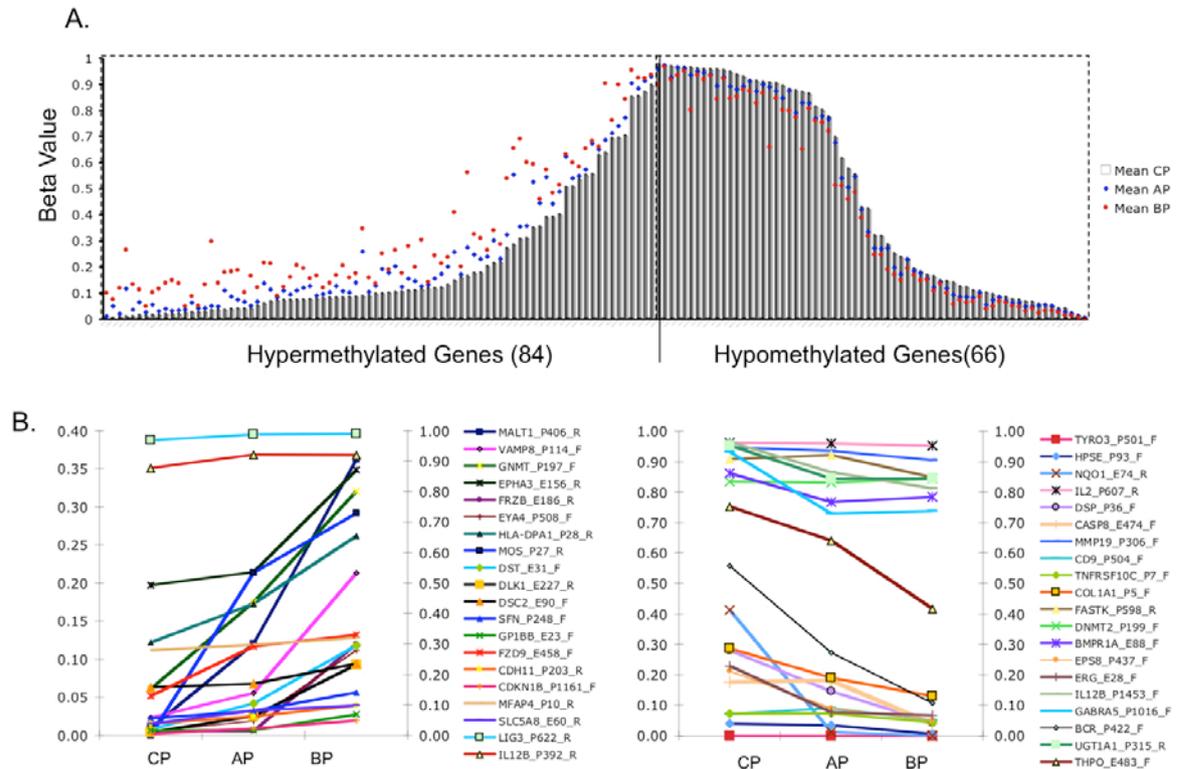


**Figure 4:** Direct comparison of DNA methylation profiles for different leukemias. DNA methylation profiles were compared for various leukemias (CML, APL, Ph<sup>+</sup> ALL, Ph<sup>-</sup> ALL, Ph<sup>-</sup> CD34<sup>+</sup>, and Ph<sup>+</sup> CD34<sup>+</sup> cells). Scatter plots were generated for direct comparisons of mean methylation beta values. (A) CML with APL ( $r=0.9475$ ), (B) CML with Ph<sup>+</sup> ALL ( $r=0.9416$ ), (C) CML with Ph<sup>+</sup> CD34<sup>+</sup> cells ( $r=0.9343$ ) (D) Ph<sup>-</sup> ALL with Ph<sup>-</sup> ALL ( $r=0.9621$ ), and (E) Ph<sup>+</sup> CD34<sup>+</sup> cells with Ph<sup>-</sup> CD34<sup>+</sup> cells ( $r=0.9633$ ), and (F) CML with Ph<sup>-</sup> ALL ( $r=0.9200$ ).

Methylation Assay (including the original 972 loci analyzed in this study and those loci located on the X-chromosome loci). The maximum decrease in LINE-1 DNA methylation was 2.5% after treatment with azacitidine. It took three courses of treatment to reach this point, and this was consistent with the average gene-specific change at the 1,006 loci, which showed a mean beta value decrease of 0.03 after three courses of treatment (Figure 6B). According to visual inspection, changes in DNA methylation (the average of promoter gene methylation and global DNA methylation) occurred prior to changes in WBC levels (azacitidine Course 1 Day1, Course 2 Day 3, and off trial). The mean DNA methylation value of the CML progression loci (hypo- or hyper-) also responded to the DNA methyltransferase inhibitor drug (Figure 6C). The loci that were shown to be increasingly methylated during CML progression became less methylated during azacitidine treatment (mean beta value 0.61 to 0.48), and those loci at which methylation decreases during CML progression became increasingly methylated with azacitidine treatment (mean beta value 0.14 to 0.42) (Figure 6C).

## 4 Discussion

A unique aspect of our study was the ability to compare the DNA methylation profiles 1) between Ph<sup>+</sup> leukemias and Ph<sup>-</sup> leukemias, 2) leukemias containing other chromosomal translocations (t(15,17), and 3) other solid tumors (GIST). Therefore, we were able to assess whether specific chromosomal lesions lead to the unique DNA methylation changes observed, or if the chromosomal lesions are independent of the observed DNA methylation profiles. We determined the aberrant DNA methylation profile involved in CML and its progression, and compared this profile to other cancers. We found that the DNA methylation profiles in CML were more closely related to those of another myeloid leukemia, APL, than other cancers that have the same chromosomal lesion, t(9:22). In addition, the DNA methylation profiles within ALL were similar and independent of the presence of the t(9:22). Finally, transfection of the *BCR-ABL* gene into hematopoietic stem cells inducing a CML-like phenotype did not show a CML-like DNA methylation profile. Thus, the chromosomal lesion responsible for CML, t(9:22), did not induce the overall DNA methylation profile observed in the leukemia.



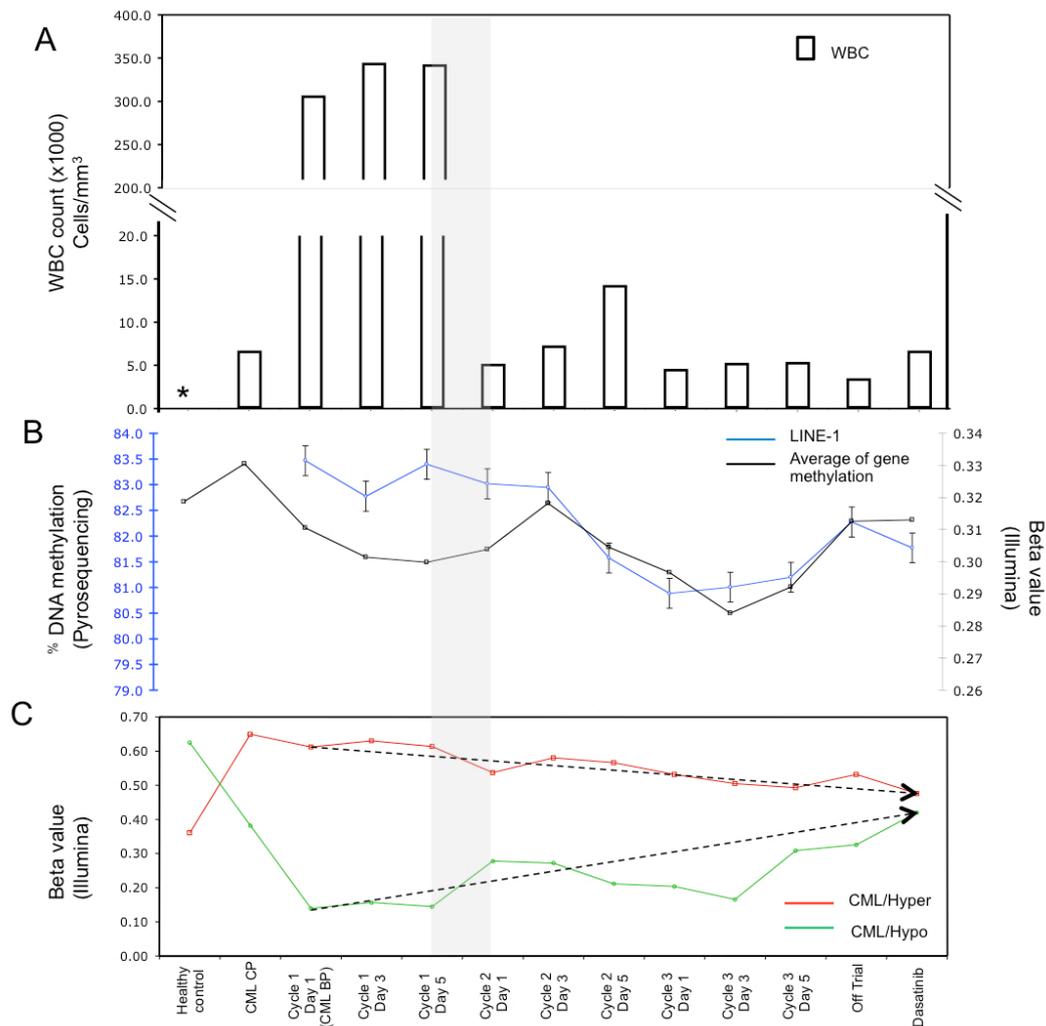
**Figure 5:** DNA methylation changes associated with CML progression. (A) DNA methylation levels at 84 loci showed a significant increase while 66 loci showed a significant decrease during progression of CML from chronic phase (bars) to accelerated phase (blue dots), and blast crisis phase (red dots). (B) Examination of 20 hypermethylated (left) and 20 hypomethylated (right) CML progression related loci with serial samples from a single patient.

Morphologic classification of leukemias into myeloid and lymphoid malignancies is the clinical standard, and the DNA methylation analysis we present supports this. The use of DNA methylation profiles to cluster leukemias is consistent with morphologic classification by stem cell of origin, such as myeloid versus lymphoid. DNA methylation profiles could distinguish between different types of leukemias, but CML was more closely related to APL than ALL or GIST. Thus, global DNA methylation profiles for CML did not resemble ALL even if they share  $Ph^+$ , nor did CML and GIST share DNA methylation profiles even if both cancers are responsive to imatinib mesylate. It has been observed that cell lineage and tissue of origin determines promoter hypermethylation in cancers, but our study, to the best of our knowledge, is the first to show that cell of origin determines the DNA methylation pattern in leukemia more than genetic defects [32,33].

Previous studies have shown that hematopoietic stem cells transfected with *BCR-ABL* phenotypically resemble CML cells [31]. However, we did not observe substantial changes in DNA methylation after transfection. Thus, it appears that DNA methylation profiles are largely based on the cell type of origin rather than the tumor development process or chromosomal lesions involved.

There are some cancer-specific DNA methylation changes, as we found a minority of loci for which hyper and hypomethylation occurred with progression of CML. Interestingly, when a CML patient was treated and responded to a DNA methyltransferase inhibitor, azacitidine, hypermethylated loci involved in CML progression displayed a decrease in methylation, while hypomethylated loci unexpectedly displayed an increase in DNA methylation levels. This suggests that changes in DNA methylation levels after treatment with DNA methyltransferase inhibitor may reflect a phenotypic change in response to therapy, rather than purely a direct effect on DNA methylation repression. However, the conclusion was based on one patient and these experiments need to be replicated in a greater number of individuals.

Our data demonstrates that global DNA methylation profiles observed in CML and other cancers do not solely reflect the genetic events such as the translocation of the *BCR-ABL* gene. Instead our data suggest that DNA methylation profiles in cancer reflect the cancer cell of origin, such as myeloid versus lymphoid. Furthermore, treatment of CML patients with the DNA methyltransferase inhibitor azacitidine led not only to decreases in DNA methylation at some loci, but also to an increase in methylation



**Figure 6:** Clinical response and DNA methylation changes induced by azacitidine (Vidaza®) treatment in a patient with CML. A) WBC count, B) DNA methylation of the LINE-1 repetitive element, and the mean DNA methylation of 1,007 CpG sites for Illumina GoldenGate analysis, and C) CML progression hyper- and hypomethylated loci following a course of azacitidine treatment.

at another subset of hypomethylated loci. This suggests that the DNA methylation changes observed after azacitidine treatment may not solely reflect direct inhibition of DNA methyltransferase, but also changes originating from other therapeutic effects.

Our study is limited by primarily using peripheral blood instead of bone marrow stem cell samples from some of the patients, and by the limited clinical data available to us. The use of bone marrow specimens may be preferable over peripheral blood specimens, however, the relative ease of collecting peripheral blood over bone marrow enabled the collection and assessment of serial samples. In addition, cancer type was the predominant factor in determining methylation pattern, and not specimen type, as we observed the clustering by cancer type and not by tissue (Figure 1 and 2). Detailed clinical information on each subject may have also been useful, but we

were limited due to the retrospective nature of the analysis we performed and the requirement of staying within the institutional guidelines. Additionally, advances in technology would have enabled a far greater number of loci to be interrogated at once, as with Illumina's Infinium 27k and 450k microarrays. At the time of the analysis, we used the latest technology available for assessing global DNA methylation, but technology has progressed sufficiently to enable an increase of two orders of magnitude in the number of loci that can be analyzed in such a manner. Although we concede that more detailed studies might reveal additional information, we expect the major insights from our study will not change. The Illumina GoldenGate platform provides sufficient genome wide coverage to demonstrate that the genetic events of leukemogenesis do not induce the DNA methylation profiles observed. In addition, the observed DNA methylation

profiles reflected the tissue of origin and not the genetic events, such as *BCR-ABL*.

In summary, the chromosomal lesion responsible for CML, t(9:22), did not induce the overall DNA methylation profile observed in leukemia. Our results may contribute to understanding the epigenetic patterns in CML in relationship to the genomic translocation, and the therapeutic effects of DNA methyltransferase in treating CML.

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#### List of Abbreviations

ABL: abelson  
 ALL: acute lymphoblastic leukemia  
 AP: accelerated phase  
 APL: acute promyelocytic leukemia  
 BCR: breakpoint cluster region  
 BP: blast crisis phase  
 CML: chronic myelogenous leukemia  
 CP: chronic phase  
 EDTA: Ethylenediaminetetraacetic acid  
 Ph: Philadelphia chromosome  
 Ph<sup>+</sup> CD34<sup>+</sup>: hematopoietic stem cells transfected with *BCR-ABL*  
 GIST: gastrointestinal stromal tumor  
 WBC: white blood cell  
 WGA: Whole Genome Amplification

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