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Methylation by DNMT1 is more Efficient in Chronic Lymphocytic Leukemia Cells than in Normal Cells

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Abstract

Binding of activators and repressors plays a significant role in transcriptional regulation of gene expression. The systems biology approach has been applied to predict and determine the efficiency of this process; however, most of the work is done in prokaryotic systems. Epigenetic regulation provides another dimension of transcriptional regulation, which does not involve changes in the coding region of genes. Methylation of specific CpG residues in the CpG islands of DNA by the enzyme DNA methyl transferase 1 (DNMT1) causes gene silencing. In cancer cells, the enzyme DNMT1 is highly expressed, and the methylation level at specific sites of genes, including tumor suppressor genes, is elevated. This results in the silencing of tumor suppressor genes for carcinogenesis. However, it is not known whether the increase in DNMT1 expression is proportional to the increase in the level of methylation. This paper developed a systems biology approach to determine whether DNMT1 acts more efficiently in cancer cells than in normal cells. The cooperativity of DNMT1 binding to eight selected genes was determined in chronic lymphocytic leukemia (CLL) cells using a modified Hill equation and compared these values with those obtained from normal lymphocytes and granulocytes. The cooperativity of DNMT1 was found to be positive in four cancer-associated genes, whereas their values were negative in both normal lymphocytes and granulocytes. These results show for the first time how to apply systems biology in the analysis of transcriptional gene regulation. This approach will be beneficial in determining the efficiency of genome-wide methylation in development and in diverse types of diseases.

Keywords: Genome-wide methylation; Lymphoma cells; Estrogen receptor; Granulocytes; Lymphocytes; Retinoblastoma

Abbreviations: DNMT1: DNA Methyl Transferase 1; CLL: Chronic Lymphocytic Leukemia; ER: Estrogen Receptor; E-cad: E-Cadherin; HIC1: Hypermethylated in Cancer 1; Rb: Retinoblastoma; GST-P1: Glutathione S-Transferase P1; AML: Acute Myeloid Leukemia

Introduction

Transcriptional regulation by an epigenetic mechanism is a major process that affects how gene expression is controlled in cells without altering the coding region of the gene. One of the epigenetic modifications that alters transcription is methylation at CpG residues in DNA. In addition, histone modifications, such as methylation and acetylation, also regulate transcription by changing open or closed chromatin structures. These modifications play a huge role in embryogenesis, stem cell biology, cellular development, and differentiation [1-3]. Aberration of this regulation generates many types of disorders and diseases [4]. Under normal circumstances, somatic cell gene methylation levels remain nearly constant. During DNA replication, the newly synthesized strand of DNA initially lacks methylation. The enzyme DNA methyl transferase 1 (DNMT1) then incorporates methyl groups onto the cytosine of CpG residues such that the methylation on the daughter strand matches the methylation on the template strand.

DNMT1 expression is often higher in cancer cells than that in normal cells [5]. Interestingly, tumor suppressor genes are selectively methylated in certain cancer cells as compared to normal cells, and this is one mechanism by which cancer cells can silence tumor suppressor genes. It is assumed that this increased expression of DNMT1 causes the observed increase in cancer cell methylation. Recently, it was proposed that epigenetic changes, including methylation at CpG residues, may play a significant role in cancer progenitor cell formation, cancer progression, and metastasis [1,2,4,6-9]. Thus, understanding the regulation of DNMT1 in cancer cells is necessary in order to fully understand carcinogenesis. This study examines such a process by testing the efficiency of methylation processes in response to altered DNMT1 expression levels in cancer cells as compared to healthy cells by applying a system biology approach.

Melki et al. [10] published a comprehensive study of chronic lymphocytic leukemia (CLL), a malignant disease of lymphocyte maturation and proliferation, showing that expression levels of DNMT1, DNMT3a, and DNMT3b are much higher in leukemia cells than in normal lymphocytes and normal granulocytes. They also found that several oncogenes were hypomethylated and that several tumor suppressor genes were hypermethylated. Interestingly, hypermethylation was concentrated in CpG islands even though genome-wide methylation decreased. This paper expands on these findings and attempts to determine whether the increase in DNMT1 expression in CLL corresponds to the amount of increased methylation observed. The expression levels of DNMT1 along with the methylation level in adult blood cancer cells with CLL were obtained from [10]. For comparison, the expression levels of DNMT1 and methylation levels

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in normal lymphocytes and normal granulocytes were also obtained.

To determine the relationship between DNMT1 expression levels and methylation levels, the Hill equation, which is often used to calculate cooperativity in an allosteric enzyme system, was applied. The results of this study indicate that methylation levels in cancer cells are much higher than normal cells but that these methylation levels are not directly proportional to the increase in DNMT1 expression. This disparity may be due to DNMT1 cooperativity being much higher in CLL cells than in normal cells. These findings have important clinical implications and also show how systems biology can be applied to evaluate regulation of epigenetically modulated gene transcription.

Methods

Cell types

To correlate the methylation status of silenced genes with the level of DNMT1 expression in cancer cells and normal cells, published data from CLL patients was utilized [10]. These data were compared with lymphocytes from healthy volunteers. To introduce an internal control, the results of CLL were compared with normal granulocytes and normal lymphocytes collected from normal volunteers [10].

DNMT1 expression levels and cellular concentration

Relative DNMT1 expression levels normalized to beta-actin were obtained for both CLL and normal lymphocyte cells from a published paper [10]. To obtain the absolute concentration of DNMT1 in each sample, each relative value was multiplied by the average amount of beta-actin present in CLL and in normal lymphocytes [11].

Calculation of beta-actin

For each cell type (CLL, normal lymphocytes, normal granulocytes), the average beta-actin concentrations were calculated from total actin concentrations. The total actin concentration is the summation of the concentration of beta and gamma actin. The average concentration of total actin in these types of cells is 2.2 mg/10^9 cells. The cited beta:gamma-actin ratio is 2.2:1 for CLL and 2:1 for normal lymphocytes. The absolute amount of beta-actin present in CLL and normal lymphocytes was determined by using these ratios. To determine the concentration, amounts were divided by cellular volumes of $177*10^{-15}$ L in normal cells from healthy volunteers and $159*10^{-15}$ L in CLL cells from patients. For normal lymphocytes, the calculated beta- actin level was found to be 203.5 uM, and for CLL cells, beta-actin levels were calculated to be 139.8 μM. The beta-actin level in granulocytes was determined to be 100 uM based on published data [12].

Calculation of the methylation level of each tumor suppressor gene normalized to DNMT1 concentration

For each gene (calc, ER, E-cad, p15, p16, H1C1, Rb, GST-P1), in all samples from Melki et al. [10], the percent methylation of each gene was divided by the calculated concentration of DNMT1 in CLL, normal lymphocytes, and normal granulocytes.

Percent methylation (theta)

To calculate the cooperativity (n) for eight cancer related genes (calc, ER, E-cad, p15, p16, H1C1, Rb, GST-P1), Melki et al. [10] reported the percentage of acute myeloid leukemia (AML), CLL, and normal samples with hypermethylation. They determined methylation status for 18 AML samples, 6 CLL samples, and 9 normal samples using bisulfite sequencing and defined hypermethylation as greater than 25% [10].

Dissociation constant

Three different Kd values determined by Lee et al. [13] were used for unmethylated, hemimethylated, and methylated DNA template strands (Table 1). CLL cells which showed methylation levels (theta) near 35% were assigned the "hemimethylated" status for calculations. In contrast, all normal cells, which had methylation levels of less than 10% for genes of interest, were assigned an "unmethylated" status for calculations.

Calculation of cooperativity (n) of binding efficiency of DNMT1 to individual genes

Data from multiple sources were used to construct a dataset of theta and DNMT1 values associated with a single (sample, gene) combination. For theta, on average, the percent methylation for a given gene was assumed to equal the percentage of samples with hypermethylation. To create CLL data points, the CLL cell DNMT1 concentrations and the CLL cell methylation levels were crossed for eight cancer-related genes. To generate normal lymphocyte and granulocyte data points, the normal lymphocyte DNMT1 concentrations and the normal lymphocyte and granulocyte methylation levels were crossed for the same eight genes.

In other words, the set of CLL data points=$[DNMT1]_i$ X $theta_j$, i denotes sample {A,B,C,D,E}, j denotes sample {calc, ER, E-cad, p15, p16, H1C1, Rb, GST-P1}, and the set of normal lymphocyte data points=$[DNMT1]_i$ X thetaj, i denotes sample {1,2,3,4}, j denotes sample {calc, ER, E-cad, p15, p16, H1C1, Rb, GST-P1}.

The Hill equation

The classical Hill equation can be written as: theta=L^n/(Kd+L^n), where the variables are defined as follows: theta represents the fraction of possible ligand binding sites (the sequence of DNA of each gene) on the enzymes that actually have ligand (DNMT1) bound, L represents the concentration of ligand, Kd is the dissociation constant for the enzyme-ligand interaction, and n is the cooperativity. An increasing n suggests increasing cooperativity. In the classical model, n values less than 1 indicate cooperativity is negative. In other words, as more ligand binds, subsequent ligands are less likely to bind. When n=1, no cooperativity is demonstrated, and the reaction is just as likely to proceed regardless of the amount of bound ligand. When n>1, positive cooperativity is suggested, meaning that as more ligands bind the enzyme, subsequent ligands become more likely to bind. The actual methylation of the newly synthesized DNA strand is assumed to proceed very rapidly, while the binding of DNMT1 to the template strand is assumed to be the rate-limiting step in the reaction.

The generated cooperativity values (n) are endpoints, one from untransformed normal cells and another from completely transformed cancer cells. No data points are from the presumed transition states during which the DNA becomes methylated initially and continues to be increasingly methylated with time. The log methylated/unmethylated was plotted against log DNMT1 concentration in CLL cells, as well.

Methylation Status of DNA Template Strand	Kd (nM)	Cell types
Unmethylated	29.9	Normal
Hemimethylated	22.9	CLL
Methylated	46.3	--

Table 1: Dissociation constants and associated cell types.

Results

DNMT1 concentration in leukemia and normal cells and ratio of methylation versus DNA concentration of different genes

To determine the efficacy of methylation in cancer cells as compared to the normal cells, data were compiled from a published paper on CLL that analyzed the extent of methylation in the upstream region of different cancer related genes, including tumor suppressor genes [10]. The paper also measured DNMT1 expression levels in CLL and compared both methylation levels and DNMT1 expression levels with normal lymphocytes and normal granulocytes. As described in the methods, all results were converted to quantitative levels of DNMT1 expression, and then the ratio of methylation levels to the DNMT1 expression levels was calculated in CLL, normal lymphocytes, and normal granulocytes. The efficacy of methylation (n) was determined by using a modified Hill equation for 8 candidate genes (calc, ER, E-cad, p15, p16, H1C1, Rb, GST-P1) in CLL cells, normal lymphocytes, and granulocytes.

Building on this previous work, this paper calculated the concentration of DNMT1 enzyme in CLL lymphocytes and compared them to normal lymphocytes and normal granulocytes. This calculation is described in the methods section. Figure 1 clearly shows that DNMT1 levels are higher in all CLL lymphocytes as compared to that in normal lymphocytes and granulocyte cells. These differences are significant as reflected from the p values.

As shown in Figure 1, DNMT1 concentrations are elevated in CLL cells, so it is expected that methylation levels, at least in select genes and CpG islands [1], will also be elevated. However, it is not clear whether the increase in DNMT1 concentration is proportional to the increase in methylation level. To determine this fact, the amount of methylation in eight selected cancer-related candidate genes, normalized to DNMT1 concentrations in CLL, normal lymphocytes, and normal granulocytes were plotted. Figure 2 shows that the methylation levels of calc, E-cad, HIC-1, and p15 in CLL cells are disproportionately higher than would be predicted based on the increased DNMT1 concentrations.

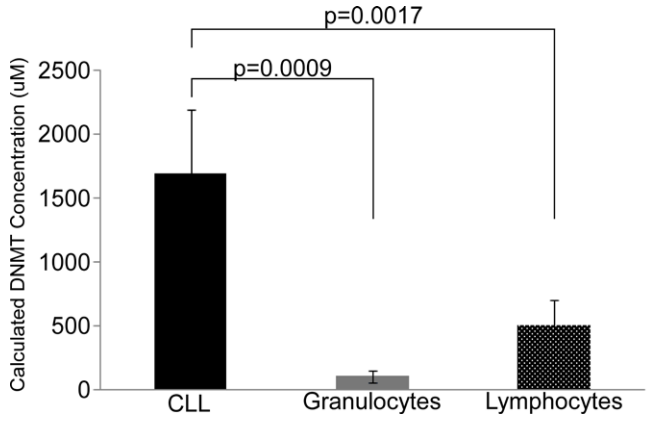

Figure 1: DNMT1 Concentrations. The left bar represents the average DNMT1 concentration in five CLL samples. The middle bar represents the average DNMT1 concentration in granulocytes from normal donors. The right bar represents the average DNMT1 concentration in lymphocytes from normal donors. DNMT1 concentrations were calculated as described in the methods section.

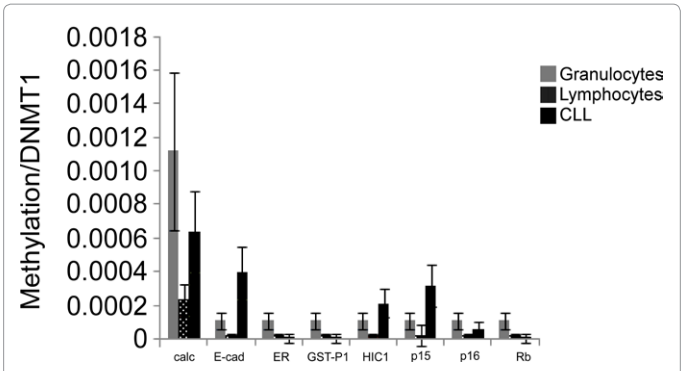

Figure 2: Level of methylation normalized to DNMT1 concentration. The X-axis shows eight tumor suppressor genes. The Y-axis describes the ratio of methylation levels normalized to the DNMT1 concentration in CLL, granulocytes from normal donors, and lymphocytes from normal donors.

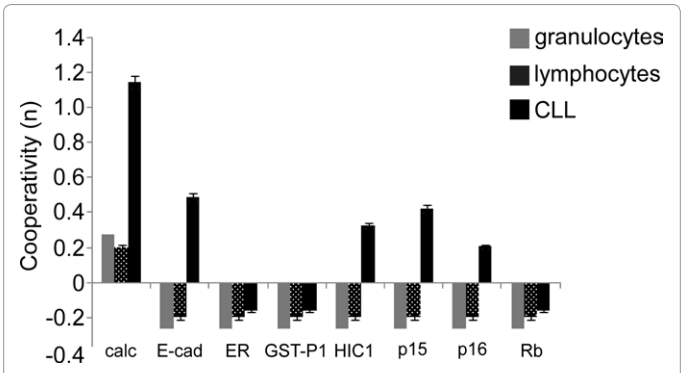

Figure 3: Cooperativity (n) of DNMT1 binding to Individual genes. The X-axis shows eight tumor suppressor genes. The Y-axis describes the cooperativity (n) value, which denotes the efficiency of binding of DNMT1 to the respective genes.

Calculation of cooperativity (n) of DNMT1 binding to individual genes in CLL and normal lymphocytes and granulocytes

To understand the efficiency of methylation in cancer cells compared to the normal cells, the cooperativity of DNMT1 for binding efficiency to the CpG residues in the DNA as described in the methods section was calculated. A level of "n" greater than zero describes increased efficiency of DNMT1 binding to the CpG residues in the DNA. Calc, E-cad, p15, and p16 showed a positive cooperativity of DNMT1 for DNA in CLL. The other four genes, ER, GST-P1, HIC-1, and Rb, did not show positive cooperativity in CLL cells. Compared to CLL, the binding cooperativity of DNMT1 to all eight genes in normal lymphocytes and granulocytes was negative (Figure 3).

Next, the average binding cooperativity (n) of DNMT1 was calculated for all eight genes in order to get a general pattern in CLL as compared to normal lymphocytes and granulocytes. As shown in Figure 4, it is clear that CLL has a positive cooperativity towards the binding of DNMT1 to DNA, while normal lymphocytes and granulocytes have negative cooperativity. The large standard deviation values are expected, as the level of individual gene cooperativity is widely variable.

Figure 5 shows a plot comparing average methylation levels with differing DNMT1 concentrations in different CLL cells (A-E) [10]. In an ideal enzymatic reaction, there should be an increasing trend when plotting the log [substrate] vs log [enzyme]. However, this figure does

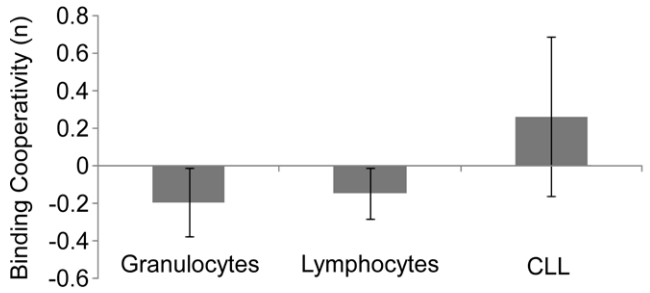

Figure 4: Average binding cooperativiy of DNMT1 in CLL, normal Lymphocytes and in normal Granulocytes. The Y-axis represents the average binding cooperativity (n) of DNMT1 in CLL, normal granulocytes, and normal lymphocytes. The average value was calculated from the binding cooperativity of the eight tumor suppressor genes shown in Figure 3. Percent methylation of select genes in normal and CLL cells.

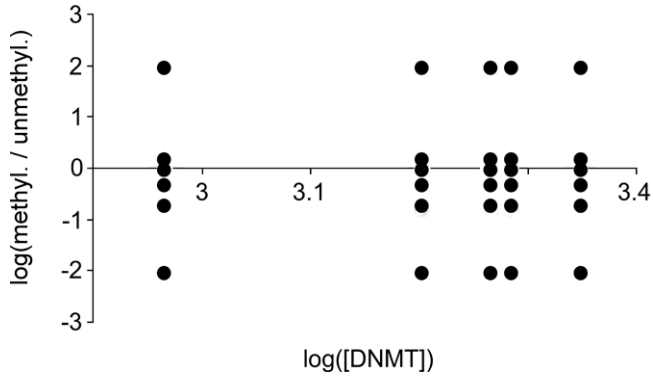

Figure 5: Scatter plot of gene methylation pattern in CLL. The X-axis shows the log DNMT1 concentrations in CLL cells. The Y-axis represents the log of fraction methylated divided fraction unmethylated for each gene shown in Figure 3.

not reflect that increase. One explanation of this apparent discrepancy is that the data obtained from the published papers are the end point data. The cancer cells have achieved the highest level of methylation that can possibly occur with the amount of DNMT1 present in these cells, and calculations are based on these end point data. To get a clear picture of the increasing level of cooperativity, the kinetics of methylation in cancer cells must be determined and compared to DNMT1 levels, and this is technically very difficult. However, *in vitro* studies have previously shown that the efficacy of DNMT1 activity increases as these enzymes bind hemimethylated DNA [14].

Discussion

During replication, when the template strand of DNA contains no methylation, DNMT1 does not effectively promote methylation. Conversely, when the template strand is hemimethylated, DNMT1 is very effective at catalyzing the methylation of newly synthesized DNA strands [15]. In cancer cells, DNMT1 expression is usually elevated so that CpG island methylation levels, primarily in tumor suppressor genes, are maintained and these genes silenced. It is still not clear how higher levels of methylation specifically target tumor suppressor genes in cancer cells as opposed to oncogenes, which remain hypomethylated, and whether the increase in DNMT1 expression is proportional to the increase in methylation level in silenced genes.

This study determines that the efficacy of DNMT1 is much higher in CLL cells than in normal lymphocytes and normal granulocytes (Figures 3 and 4). Interestingly, the efficacy was increased for the 4 genes Calc, E-cad, p15, and p16, which are usually epigenetically silenced by methylation in cancers, including CLL. Conversely, for the other 4 genes (ER, GST-P1, HIC-1, Rb), which may not be epigenetically regulated in CLL, the efficacy of DNMT1 binding is not increased and is similar to the normal lymphocytes and granulocytes (Figure 3). Methylation levels were also low for these genes with the exception of HIC-1 (Figure 2). Estrogen receptor (ER) is often silenced in breast cancer; however, it is not always silenced in leukemias [16]. Similarly, GSTP1 is epigenetically silenced in prostate cancers, but its expression correlation with leukemia is not well defined [17]. Lastly, Rb protein is silenced by histone deacetylase 2 [18].

The evidence presented here of enhanced DNMT efficacy related to increased methylation of certain genes in cancer cells opens the field to expanded genome-wide studies of this phenomenon in a wider range of cell types. Although it is not presently known how DNMT1 selectively increases the methylation of specific genes, the current study provides novel evidence that the cooperativity of DNMT1 is correlated with higher methylation in cancer cells. Figure 5 does not show a gradual increase in methylation levels of all genes studied, but this scatter plot can be improved by the inclusion of data from no methylation to gradual increased methylation levels. Unfortunately, such data is extremely difficult to assess *in vivo* when cancer is in progress. However, *in vitro* studies with synthetic hemimethylated DNA demonstrated that indeed, DNMT1 shows positive cooperativity [15], which supports the results presented here.

Computational biology studies use enhancer analysis to predict genome-wide gene regulation and expression, which are often cell type specific. An interesting observation of these studies is that the enhancer pattern alterations are at par with epigenetic changes rather than mutational changes in gene sequences [19]. Thus, alterations in gene expression during different diseases including cancer involve epigenetic regulation. In addition, epigenetic alterations significantly regulate gene expression by repressing enhancer promoter interactions at transcription looping CTCF sites. These sites primarily silence lineage specific developmental regulatory genes to maintain stem cell pluripotency. This region is characterized by the binding of polycomb proteins around nucleosomes containing histone H3 trimethylated at lysine 27. The formation of these insulated regions suppresses many genes, while the disruption of this bound complex re- express many genes [20]. Interestingly, in cancer cells, genes which are methylated and silenced are located in such insulated region [21]. However, it is not known how methylation changes in cancer cells impact the maintenance and creation of insulated sites and thus, the downstream silencing or expression of genes. Regardless, the primary signal for gene expression will relate to the methylation level of the region of interest. Therefore, the enhanced efficacy of the DNMT1 enzyme in cancers possibly regulates this process by methylating regions of DNA in cancer cells normally demethylated. It was previously postulated that the trigger of cancer initiation by cancer progenitor cell formation occurs through epigenetic events [1,2,6]. Increased methylation by more efficient DNMT1 activity which can alter gene expression patterns specifically at locations tightly regulated in cells may contribute to this process. However, this phenomenon needs further investigation in order to fully understand how this enhanced efficacy in particular diseases selectively regulates some genes but not others.

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