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The Use of Polycomb Gene Targets Methylation as Biomarkers for Hematological Malignancies

Adel Mohammed Abuzenadah

Faculty of Applied Medical Sciences,
King Abdulaziz University, Jeddah, Saudi Arabia

Abstract: The methylation frequencies of polycomb gene targets (PCGTs) [Slit Homolog 2 protein (*SLIT2*), Secreted Frizzled-Related protein 1 (*SFRP1*), Hypermethylated in Cancer 1 (*HIC1*) and Myogenic Differentiation 1 (*MOYD1*)] have been recently acknowledged as a potential biomarker in solid tumors. However, there is no data to support the use of PCGTs (*SLIT2, SFRP1, HIC1 and MOYD1*) in acute myeloid leukemia (AML) and myeloproliferative disorders (MPD). This study utilized DNA samples from AML and MPD patients from King Abdulaziz University Hospital (KAUH) collected during the period from January 2009-July 2013 and determined the methylation frequency of these four genes using the MethyLight technique. The methylation of PCGT genes represented by SLIT2, SFRP1 and MYOD1 are significantly associated with AML (*P= 0.002, 0.003, 0.003 and 0.049*) respectively. Overall, AML shows a worse prognosis than MPD (*P<0.0001*). The methylation of the PCGT genes is associated with poor survival in AML (*p<0.0001*). Individually, the methylation of SLIT2, HIC1 and SFRP1 demonstrate an association with AML overall survival (*P=0.011, 0.031* and *0.047*) respectively. The results point to the potential of PCGT methylation to serve as a prognostic marker for poor prognosis in AML.

Key words: Polycomb Group Proteins · Acute Myeloid Leukemia · DNA Methylation · Myeloproliferative Disorders

INTRODUCTION

AML and MPD are hematological malignancies that affect the myeloid cell lineages. AML is a clonal hematopoietic disorder that is derivative from a hematopoietic stem cell or a progenitor cell from specific lineage [1]. It has two peaks; the first one occurs in infants (<1year). The peak then decline in older children and adolescents while second peak is usually occurs among adults of 40 years old [2]. In contrast to solid tumors, chromosomal abnormalities (translocation, inversion, deletion, or insertion) are a common cause of AML and this leads to different aspects of diagnosis, treatment and prognosis [3]. The clinical presentation of AML is nonspecific and it reflects the low production of normal bone marrow elements. The diagnosis of AML is characterized by elevated white blood cell (WBC) count with the presence of myeloblasts [2, 4]. As AML affects blood cells and bone marrow, which are the main components of the immune system, AML patients are more vulnerable to infections [6]. On the other hand, MPD can be defined as a group of slowing blood cancers that result in enormous production and excess accumulation of myeloid cells (red cells, granulocytes and platelets) in the bone marrow peripheral blood and body tissues [2, 6]. MPD reflects the chronic pattern of myeloid leukemia. It includes the distinctive clinical disease of chronic myelogenous leukemia (CML), polycythemia vera (PV), essential thrombocytopenia (ET) and myelofibrosis (MF). AML and MPD have different prognostic factors such as cytogenetic, molecular genetics and flowcytometry [2].

Recently, the methylation of PCGTs (SLIT2, SFRP1, HIC1 and MYOD1) has been shown to have a prognostic potential in sporadic breast cancer and colorectal cancer [7, 8]. Polycomb group (PCG) proteins are transcriptional repressors that inhibit developmental regulators in

Corrsponding Author: Adel Mohammed Abuzenadah, Faculty of Applied Medical Sciences, King Abdulaziz University, Jeddah, Saudi Arabia, P. O. Box 80324 Zip Code 21589. Tel: +966 12 69551673

embryonic stem cells and silence tumor suppressor genes in cancer [9, 10]. Chromatin modifications are the form of the repression mechanism of PcG and it is reversible. This mechanism allows stem cells and multipotent progenitors to differentiate into committed cell-lineages by expressing specific PCGTs [11]. However, several studies have shown that the PCGTs in the human embryonic stem cell (HESC) are more likely to undergo cancer-specific promoter DNA hypermethylation than non-target genes, which implying a new model called a stem-cell origin model of cancer. In this new model, PCGTs are methylated and they would permanently lock cells in an undifferentiated status of self-renewal and as a result, predispose them to subsequent cancer transformation [12- 14]. In this study, the methylation of these genes will be evaluated in Acute Myeloid Leukemia (AML) and performed correlations with known clinic-pathological parameters.

MATERIALS AND METHODS

Patients: The material of this retrospective study consisted of a series of 109 AML and MPD peripheral blood samples collected from King Abdulaziz University Hospital, Jeddah, Kingdom of Saudi Arabia, covering the period from January 2009 to July 2013. Sample collection procedures followed were in accordance with the local ethical guidelines. Additionally, samples clinicopathological features and survival data were collected from the participant's records. DNA was extracted from peripheral blood using the QIAGEN® blood DNA extraction kit, following the manufacturer's guidelines. DNA obtained from non-leukemic individuals was used as a control group in this study (n= 72).

Bisulfite `DNA Modification and Methy Light Assay: Up to 0.5 µg of DNA was used for bisulfite conversion using the Qiagen Epitect Bisulfite Conversion kit. DNA methylation analysis was performed using MethyLight (a modification of the Taqman® approach) as described elsewhere [15]. The methylation levels of *SLIT2*, *SFRP1*, *MYOD1* and *HIC1* were analyzed using primer-probe combinations (Table 1) according to previously published reports [16-19]. A probe targeting bisulfite-modified *COL2A1* promoter sequences was used to normalize for input DNA. The specificity of the reaction was ascertained using *sssl*-treated and bisulfite-modified positive control DNA (Qiagen) and the negative control DNA (Qiagen). The percentage of fully methylated reference (PMR) was calculated by dividing the gene:COL2A1 ratio of a sample by the gene: COL2A1 ratio of the positive control DNA and multiplying by 100. Samples with PMR=10 is considered positive for methylation, whereas samples with PMR<10 will be considered negative (*i.e.* unmethylated). The PMR=10 is considered positive as it indicates a very likely hypermethylation-mediated loss of expression for the genes analyzed.

Statistical Analysis: All statistical tests were performed using IBM SPSS Statistics version 19. Fisher's exact test was used to identify the statistical significance of the correlation between methylation events and clinicopathological factors. The primary endpoints of the study included overall diseased specific survival calculated from the date of diagnosis to the last recorded date of being alive or death caused by leukemia. In calculating overall survival, participants who died of other or unknown causes were censured. All survival times were calculated by univariate Kaplan-Meier analysis and equality of the survival functions between the strata was tested by log-rank (Mantel-Cox) test. All tests are two-sided and p-values <0.05 were considered statistically significant.

RESULTS

This study was performed in order to evaluate the methylation profile and the prognostic value for PCGT in AML and other hematological malignancies (MPDs and MDS). The cohort studied consisted of DNA samples from patients diagnosed with AML or

Table 1: Forward, reverse primers and probe sequences used for the MethyLight assay

GENE	FORWARD PRIMER SEQUENCE	REVERSE PRIMER SEQUENCE	PROBE OLIGO SEQUENCE
SFRP1	CAACTCCCGACGAAACGAA	CGCGAGGGAGGCGATT	6FAM-CACTCGTTACCACGTCCGTCACCG-BHQ1
MYOD1	GAGCGCGCGTAGTTAGCG	TCCGACACGCCCTTTCC	6FAM-CTCCAACACCCGACTACTATATCCGCGAAA-BHQ1
HIC1	GTTAGGCGGTTAGGGCGTC	CCGAACGCCTCCATCGTAT	6FAM-CAACATCGTCTACCCAACACACTCTCCTACG-BHQ1
SLIT2	CAATTCTAAAAACGCACGACTCTAAA	CGGGAGATCGCGAGGAT	6FAM-CGACCTCTCCCTCGCCCTCGACT-BHQ1
COL2A1	TCTAACAATTATAAACTCCAACCACCAA	GGGAAGATGGGATAGAAGGGAATAT	VIC- CCTTCATTCTAACCCAATACCTATCCCACCTCTAAA -BHQ1

Table 2: Clinicopathological features of the cohort (No. of patients) in this study

CHARACTERISTICS	NUMBER OF PATIENTS	PERCENTAGES
Diagnosis		
AML= 1	51	46.8%
Other= 0	58	53.2%
Age		
≥40 years= 0	46	42.2%
<40 years= 1	55	50.5%
Unknown	8	7.3%
Gender		
Male= 0	34	31.2%
Female= 1	72	66.1%
Unknown	3	2.7%
Status		
Alive= 1	49	45.0%
Dead= 0	14	12.8%

other hematological malignancies (n=109). As a control, DNA from non-leukemia cases (n=72) were also analyzed. The control group showed no significant methylation for any of the genes analyzed (PMR<10).

The experimental group (n=109) consisted of DNA from 72 male patients, 34 female patients and 3 cases with unknown details. The mean age for the experimental group was 42.2 years. There were 55 patients above the age of 40, 46 patients were 40 or below and 8 patients were of unknown age. AML was the confirmed diagnosis in 51 out of 109 patients while 58 were diagnosed with other hematological malignancies (mainly myeloproliferative disorders). 49 out of 109 were reported alive, 14 were reported dead and 46 were of unknown status (Table 2).

For the experimental group, SLIT2 had the highest frequency of methylation with 15.6% of the samples showing positive methylation and using the PMR of =10 as a cut off. SFRP1 methylation frequency was 12.8%, HIC1 methylation frequency was 11.9% and lastly MYOD1 with the least methylation frequency with 6.4% (Figure, 1). The methylation frequency SLIT2 was higher in AML cases (27.4%) compared to the MPD group (5.1%). SFRP1 showed the second largest methylation in AML with frequency of 23.5% while in MPD it only showed 3.4%. HIC1 showed the largest methylation in MPD with a frequency of 6.8%. However, there were only 17.6% of AML cases were HIC1 methylated. MYOD1 has the least frequency of methylation among all the other genes in AML and MPD with frequencies of 11.7% and 1.7% respectively. The methylation of PCGT were only reported in AML cases and it had a frequency of 9.8% (Figure, 2).

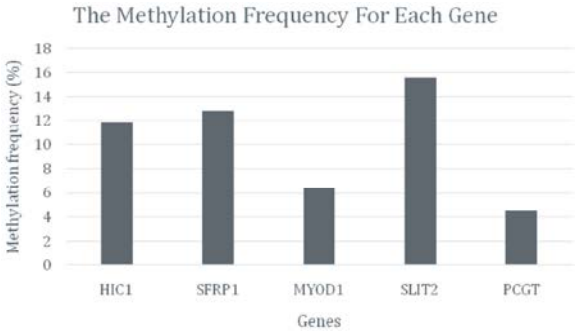

Fig 1: Bar chart demonstrating the methylation frequency of each gene in the experimental group.

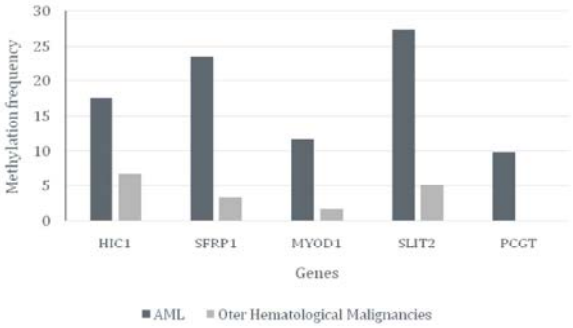

Fig. 2: Comparison of methylation frequency in AML and other diagnosis in the experimental group.

Next, a Fisher's exact test was conducted to determine if there were any statistically significant associations between the methylation of the four genes. Inter-gene correlations were analyzed using Pearson's correlation test. MYOD1, SFRP1, HIC1 and SLIT2 methylation were associated together (P= 0.005). SFRP1 methylation and SLIT2 methylation were also associated together (P=0.008). There were no statistically significant difference between the methylation of HIC1 and SFRP1, HIC1 methylation and MYOD1 and MYOD1 methylation and SLIT2 (P= 0.062, 0.196 and 0.075 respectively).

Fisher's exact test was also conducted to determine the association between each gene methylation and the study variables (diagnosis, age, gender and status). SFRP1, SLIT2, MYOD1 and PCGT methylations were significantly associated with AML (P= 0.003, 0.003, 0.049 and 0.002 respectively). SFRP1 was also more predominant in male patients (P= 0.034). Moreover, SFRP1 and PCGT methylations were significantly associated with worse disease-specific survival (P= 0.001 and 0.047 respectively). There was no statistically significant relationship between HIC1 methylation with the study variables (P= 0.137, 0.245, 0.725 and 0.646 respectively for diagnosis, age, gender and status).

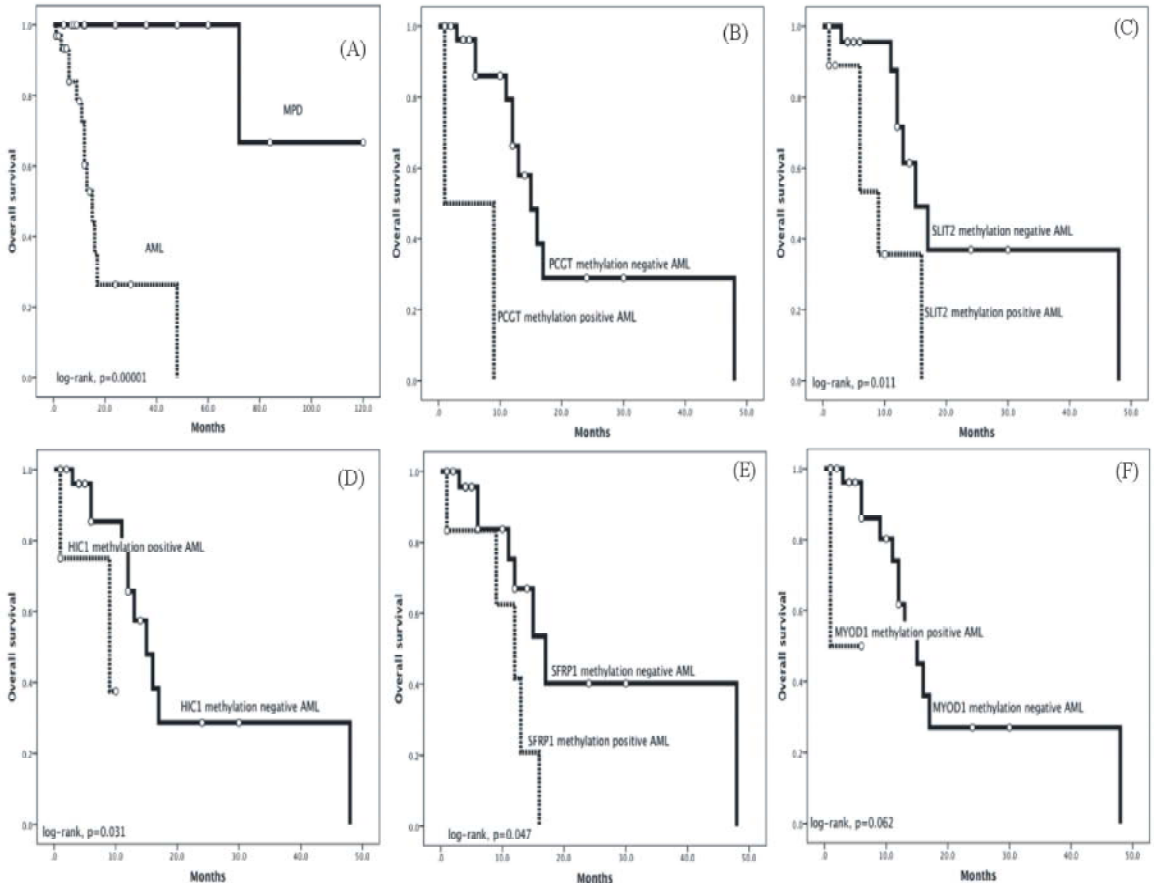

Fig. 3: Kaplan-Meier curve showing the comparison of the (A) overall survival of patients diagnosed with AML or other hematological abnormalities (myeloproliferative disorders), (B) the overall survival of AML patients with positive PCGT methylation versus negative PCGT methylation, (C) the overall survival of AML patients with positive SLIT2 methylation versus negative SLIT2 methylation, (D) the overall survival of AML patients with positive HIC1 methylation versus negative HIC1 methylation, (E) the overall survival of AML patients with positive SFRP1 methylation versus negative SFRP1 methylation and (F) the overall disease-free survival of AML patients with positive MYOD1 methylation versus negative MYOD1 methylation.

Univariant Kaplan-Meier analysis was conducted in order to evaluate the overall DFS within the two diagnosis; AML and MPDs. There was statistically significant difference between AML and MPDs in term of prognosis. AML had a significantly worse prognosis than MPDs (P<0.0001), Figure 3. Next, we evaluated the prognostic value for each gene in AML cases only. The results point to the potential of PCGT methylation to serve as a prognostic marker for poor prognosis in AML (*P<0.0001)*. Similar findings could be reported for SLIT2 methylation (*P= 0.011)*, HIC1 methylation (*P= 0.031)* and SFRP1 (P= 0.047), fig3b, 3c, 3d and 3e. For MYOD1, there was no statistically significant association between MYOD1 methylation and AML prognosis (P= 0.062), Figure 3.

DISCUSSION

In this study, we have analyzed 181 samples distributed among two groups; control and experimental. The control group (n= 72) consisted of DNA samples collected from non-leukemic individuals. On the other hand, the experimental group (n= 109) consisted of DNA samples collected from AML and other hematological malignancies (mainly MPD). The reason we chose MPD in this cohort was its similarities with AML as they share the same origin (CFU-GEMM), characteristics and diagnosis. Moreover and in general, MPD patients may show aggressive cellular growth and transforms to AML [2]. The control group showed no significant methylation events (PMR<10). While for the experimental group, there

was significant methylation especially in the AML cases. We were the first to evaluate the methylation of these PCGTs combined in AML and MPD. AML had a larger overall methylation levels than MPD. SLIT2 had the largest methylation frequency overall (15.6%) and was more frequent in AML cases (27.4%). This could be added to the pattern of SLIT2 methylation in leukemia as Dunwell *et al*, [20] reported the methylation frequency of SLIT2 in acute lymphocytic leukemia (ALL), both B-ALL and T-ALL, as 58% and 83% respectively. AML and ALL have completely different cell lineages, they only share the same stem cell (pluripotent stem cell) [2] and this could explain the methylation of SLIT2 in both of them. They also determined the methylation of SLIT2 in CLL and interestingly, showed an 80% methylation [2]. This last finding contradict our findings of SLIT2 methylation in MPD, which is considered as the chronic phase of myeloid leukemia, as SLIT2 was methylated only in 3 out of 58 cases (5.1%).

SFRP1 was the second highest methylated gene in the entire experimental group with 12.8%. It also had the second highest methylation frequency in AML cases with 23.5%. In addition, we found that SFRP1 methylation was more predominant in male patients with a ratio of 13:1 (P= 0.034). This is in agreement with Cheng *et al*. (2011) [21] who reported that SFRP1 was methylated in 34% of AML cases and it was more associated with male patients (P= 0.034) [21].

HIC1 methylation was present in both diagnosis AML and MPD (17.6 and 6.9% respectively). However, there was no association between HIC1 methylation and the study variables. HIC1 methylation found to be associated with several solid tumors such as pancreatic cancer [22], renal cell carcinoma [23], prostate cancer [24] and colorectal cancer [8]. This could be due to the distinct carcinogenesis mechanism of the solid tumors that totally differ from carcinogenesis mechanism of the hematological malignancies. MYOD1 methylation frequency was weakly associated with AML, ($P= 0.049$). However, the number of samples that are methylated with MYOD1 were low in our cohort (6.8%) and thus, an association between MYOD1 methylation and AML cannot be reliable.

PCGT methylation is also examined as defined by the co-methylation of 3 or more genes. Although the number of PCGT methylation was very low ($n= 5$), we were the first to find that PCGT methylation was highly associated with AML as all the five samples were only methylated in AML ($P=0.002$).

Furthermore, we have conducted Univariant Kaplan-Meier survival analysis for 63 samples of our cohort with known survival data. The other 46 samples were of unknown status (alive or dead). This could be explained by the source of these samples. King Abdulaziz University Hospital is a general diagnostic hospital in the city of Jeddah, Kingdom of Saudi Arabia. Once diagnosed, most of the cancer patients are referred to a specialized cancer center to complete their treatment and prognosis. Among these 63 samples, AML had significantly worse prognosis over MPD ($P<0.0001$) with a ratio of (13:1). AML patients in this cohort had an average of approximately 4 years of overall survival which is lower than worldwide average of 5 years [24]. We also showed that the methylation of PCGT, SLIT2 and HIC1 were hinting to be potential biomarker for AML ($P<0.0001$, $P= 0.011$, and $P= 0.031$ respectively). A cautionary note to this finding would be the low number of AML samples utilized in this study ($n= 51$). Therefore, a clear and a final judgment on the prognostic value for these genes will require future studies that utilize high number of AML samples.

In conclusion, it was shown in this study that methylation of Polycomb genes can be potentially used as biomarkers of hematologic malignancies.

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