Received: 2 January 2018 | Revised: 3 May 2018 | Accepted: 4 May 2018

DOI: 10.1002/cnr2.1111

ORIGINAL ARTICLE

WILEY Cancer Reports

Aberrant DNA methylation at *HOXA4* and *HOXA5* genes are associated with resistance to imatinib mesylate among chronic myeloid leukemia patients

Marjanu Hikmah Elias[1] | **Husin Azlan**[2] | **Sarina Sulong**[4] | **Abdul Aziz Baba**[3] | **Ravindran Ankathil**[4]

[1] Faculty of Medicine and Health Sciences, Universiti Sains Islam Malaysia, Nilai, Malaysia

[2] Haemato-Oncology Unit, Department of Internal Medicine, Universiti Sains Malaysia Hospital, Kota Bharu, Malaysia

[3] International Medical University, Kuala Lumpur, Malaysia

[4] Human Genome Centre, School of Medical Sciences, Universiti Sains Malaysia, Kelantan, Malaysia

Correspondence
Prof. Ravindran Ankathil, Human Genome Centre, School of Medical Sciences, Health Campus, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia.
Email: rankathil@hotmail.com

Funding information
Universiti Sains Malaysia, Malaysia, Research University (RU), Grant/Award Number: 1001/PPSP/812070

Abstract

Background: Imatinib mesylate is a molecularly targeted tyrosine kinase inhibitor drug. It is effectively used in the treatment of chronic myeloid leukemia (CML) patients. However, development of resistance to imatinib mesylate as a result of *BCR-ABL* dependent and *BCR-ABL* independent mechanisms has emerged as a daunting problem in the management of CML patients. Between these mechanisms, BCR-ABL independent mechanisms are still not robustly understood.

Aim: To investigate the correlation of *HOXA4* and *HOXA5* promoter DNA hypermethylation with imatinib resistance among CML patients.

Methods and results: Samples from 175 Philadelphia positive CML patients (83 good response and 92 *BCR-ABL* non-mutated imatinib resistant patients) were subjected to Methylation Specific High Resolution Melt Analysis for methylation levels quantification of the *HOXA4* and *HOXA5* promoter regions. Receiver operating characteristic curve analysis was done to elucidate the optimal methylation cut-off point followed by multiple logistic regression analysis. Log-Rank analysis was done to measure the overall survival difference between CML groups. The optimal methylation cut-off point was found to be at 62.5% for both *HOXA4* and *HOXA5*. Chronic myeloid leukemia patients with ≥63% *HOXA4* and *HOXA5* methylation level were shown to have 3.78 and 3.95 times the odds, respectively, to acquire resistance to imatinib. However, overall survival of CML patients that have ≤62% and ≥ 63% methylation levels of *HOXA4* and *HOXA5* genes were found to be not significant (*P*-value = 0.126 for *HOXA4*; *P*-value = 0.217 for *HOXA5*).

Conclusion: Hypermethylation of the *HOXA4* and *HOXA5* promoter is correlated with imatinib resistance and with further investigation, it could be a potential epigenetic biomarker in supplement to the *BCR-ABL* gene mutation in predicting imatinib treatment response among CML patients but could not be considered as a prognostic marker.

KEYWORDS

chronic myeloid leukemia, high-resolution melt analysis, hypermethylation, imatinib mesylate, resistance

1 | INTRODUCTION

Chronic myeloid leukemia (CML) accounts for 14% of all leukemias and 20% of all adult leukemias.[1] CML results from the aberrant tyrosine kinase activity of the *BCR-ABL* oncoprotein, which is encoded by the *BCR-ABL* oncogene, formed as a result of Philadelphia chromosome translocation t(9;22)(q34;q11). Imatinib mesylate (IM), the molecularly targeted drug, has shown remarkable efficacy against BCR-ABL oncogene protein and has become the current first line treatment for CML. Despite its remarkable efficacy, a significant proportion of CML patients treated with IM have been noted to have an unsatisfactory effect because of failure to respond, or to relapse because of primary or acquired resistance.[2,3] CML patients are judged to be IM resistant when response is lost or is not seen with a daily dose of >400 mg IM based on European LeukemiaNet.[4,5] Development of resistance to IM has emerged as a daunting problem in the management of CML patients.

Several pathways have been proposed and studied worldwide to understand the development of IM resistance in CML patients. Causative mechanisms of resistance to IM have been classified under two broad categories[1]: resistance involving *BCR-ABL* dependent pathways and[2] resistance involving *BCR-ABL* independent pathways. *BCR-ABL* dependent pathways, involving various *BCR-ABL* mutations,[6,7] were reported to be the most common cause of IM resistance.[8] However, several reports including ours, indicate that *BCR-ABL* mutations account for only 19% to 63% of resistance to IM in CML patients.[9-12] The cause of IM resistance among *BCR-ABL* mutation-free CML patients can be postulated to be due to aberrant *BCR-ABL* independent mechanisms involving other alternative signaling or epigenetic pathways. This is because, apart from BCR-ABL1 protein, several other proteins like transcription factors, transporter protein, and many other proteins are also important in regulating the high rate of proliferation and the suppression of apoptosis.[13,14]

Abnormal epigenetic regulation of the expression of CML-associated genes has been postulated[15] to play a critical role in its pathogenesis and in the mechanisms modulating therapeutic responsiveness.[16] Epigenetic silencing is a phenomenon whereby gene transcription may be suppressed through DNA methylation (a process that may regulate gene use) resulting in decreased protein expression. The methylation of the promoter is an alternative to coding region mutations in silencing tumor suppressor gene function and is observed in a non-random tumor-type specific manner. Aberrant hypermethylation has been observed in leukemia including CML.[16,17] Few studies[18-20] have suggested that hypermethylation might play a role in disease progression in CML as hypermethylation of several genes were noted in progression of CML. The observation that increased epigenetic silencing of potential tumor suppressor genes correlates with disease progression in a small proportion of patients treated with IM by Issa et al (2007) suggested a relationship between epigenetic silencing and development of IM resistance.[21]

The homeobox (*HOX*) genes are a family of homedomain containing transcription factors present in four separate clusters (*HOXA*, *HOXB*, *HOXC*, and *HOXD*) which are key regulators of embryonic development,[22] haematopoitic differentiation, and leukemogenesis.[23-26] The *HOX* gene is a 183 BP DNA sequence and codes a 61 amino acid domain known as homeodomain. It is typically located at a terminal or sub-terminal position of the corresponding homeoprotein and is responsible for recognizing and binding to the sequence specific targeted DNA.[25] The *HOX* gene networks are believed to encode master regulators in haematopoiesis and are associated with the development of haematologic malignancies, prognosis, and treatment response.[27] Interestingly, DNA methylation was reported to have an important role in aberrant control of *HOX* gene expression during the development of leukemia. DNA hypermethylation of several *HOX* genes was believed to be associated with progression of CML into blast phase.[28,29] We hypothesized that DNA hypermethylation of several *HOXA* genes is correlated with resistance to IM in CML patients. Thus, 2 *HOXA* genes, the *HOXA4* gene (OMIM#142953) and *HOXA5* gene (OMIM#142952), that are located at 7p14.2 to 7p15 were selected as candidate genes to be investigated for their involvement in mediating IM resistance. In our previous study,[30] we only reported on the correlation between *HOXA4* promoter hypermethylation and resistance to IM in CML patients. Further to that, the present study incorporates (1) data of *HOXA4* with more number of patients, (2) original data on methylation levels of *HOXA5*, (3) the correlation of *HOXA4*/*HOXA5* promoter hypermethylation (alone or combination) with IM resistance, and (4) the correlation of overall survival with *HOXA4*/*HOXA5* promoter hypermethylation.

2 | METHODOLOGY

The current study was done at Human Genome Centre, Universiti Sains Malaysia. This study has been approved by the Ministry of Health, Malaysia (NMRR-10-1206-7127) as well as Research and Ethics Committee of Universiti Sains Malaysia. A total of 175 Philadelphia chromosome-positive CML patients were recruited. Those CML patients were in chronic, accelerated, or blast phase that has been treated for at least 12 months with IM as a frontline treatment. The diagnosis of each patient was confirmed hematologically, cytogenetically as well as via molecular analysis.

The CML patient's response to IM treatment was assessed based on the hematologic, cytogenetic, and molecular responses results according to European LeukemiaNet 2010.[31] In European LeukemiaNet 2010, an IM resistant CML patient was defined as showing lack of complete cytogenetic response (CCyR) by 12 months and/or major molecular response by 18 months after started the treatment.[31] Based on these assessment criteria, the CML patients were categorized according to their response to IM (good response and resistance).

2.1 | Sample collection and DNA extraction

Peripheral blood (3 mL) from CML patient was collected in EDTA tube. Genomic DNA was extracted and was kept in −40°C before subjected to bisulfite treatment. Genomic DNA of patients and controls was extracted and purified using the GENTRA PUREGENE Blood Kit (Qiagen, Germany) according to the recommendation. DNA

concentration was quantitated by using NanoQuant Infinite M200 (Tecan, Switzerland), and the quality of the DNA was confirmed using agarose gel electrophoresis.

2.2 | Bisulfite treatment

Five hundred nanograms of the DNA was treated with bisulfite using EZ DNA Methylation-Gold Kit (ZYMO Research, USA) following manufacturer's recommendation. The concentration of the bisulfite treated DNA was measured as described previously.[30]

The 100% and 0% methylation DNA control used were universal methylated DNA and unmethylated DNA (ZYMO research, USA). Both DNA methylation control were treated with bisulfite, and a series of methylation dilution were subsequently prepared according to the ratio of 10%, 25%, 50%, and 75%. This set of serial DNA methylation percentages was included in each experimental run.

2.3 | MS-HRM analysis

MS-HRM primers (Table 1) were designed using Methyl Primer Express v1.0 Software (Applied Biosystem, USA) based on criteria suggested by Wojdacz et al (2008) with some modifications.

MS-HRM analysis was done using CFX Real Time PCR Detection System (Bio-Rad Laboratories, USA). MS-PCR amplification was monitored using CFX Manager Software, and the HRM data was analysed using Bio-Rad Precision Melt Analysis Software. Total volume of PCR reaction used was 10 µL, comprising of 1X Precision Melt Supermix (Bio-Rad Laboratories, USA), 200 nM of each primer, and 20 ng of bisulfite-treated DNA. Technical replication was performed in triplicate. PCR condition used was as follows: (1) initial denaturation at 95°C for 2 minutes and 50 cycles of (2) denaturation at 95°C for 10 seconds, (3) annealing at 50°C to 54.5°C (as shown in Table 1) for 30 seconds, and (4) extension at 72°C for 30 seconds. Heteroduplex formation was done after PCR amplification at 95°C for 30 seconds and subsequently at 60°C for a minute. HRM analysis was performed immediately after heteroduplex formation by 0.2°C increments, and the temperature was held for 10 seconds of each increment. The increments were done from 65°C to 95°C. A no template control and set of serial percentage control (0%, 10%, 25%, 50%, 75%, and 100%) in triplicate were included in each experimental run.

MS-HRM results were then used to construct difference curve. Normalized melt curve was then developed by choosing the 0% melt curve as the reference relative fluorescent unit (RFU), using Bio-Rad Precision Melt Analysis Software. The peak reading of difference-RFU from the difference curve for all the 0%, 25%, 50%, 75%, and 100% methylation control levels was plotted, and a "best-fit" straight line through the data was drawn, and the linear regression analysis was applied to the data using excel (Figure 1). Methylation level percentages of all IM treated CML patients were subsequently calculated based on the linear regression equation of the difference-RFU peaks.

2.4 | Statistical analysis

Statistical Package for the Social Sciences (SPSS) Software version 20.0 was used for statistical analysis. ROC curve was generated from the numerical data to determine the optimal cutoff point of *HOXA4*

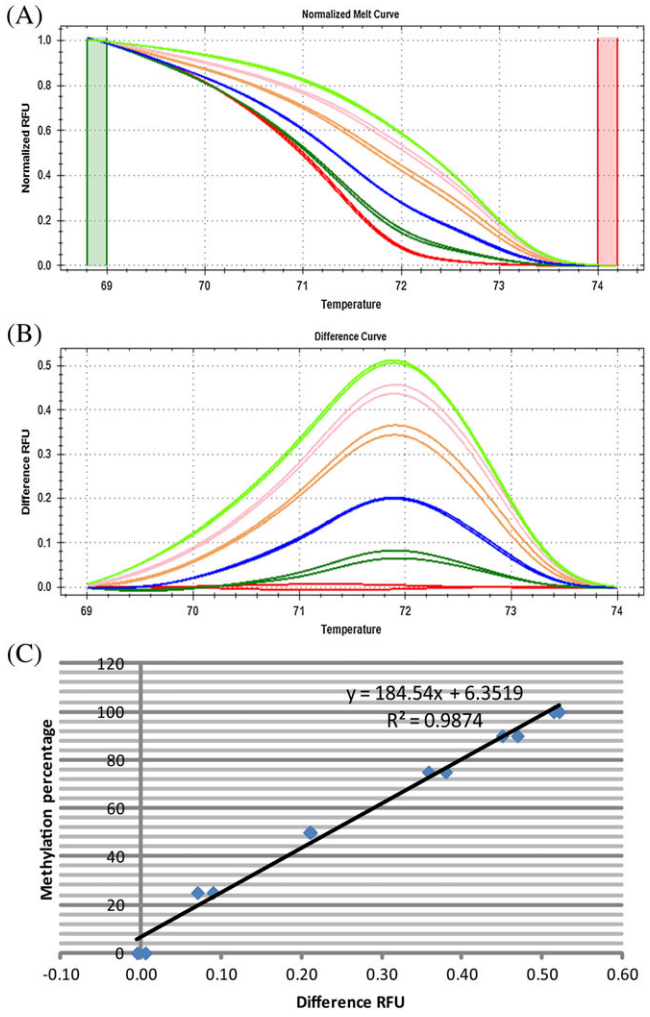

FIGURE 1 A, Normalized melt curve, B, difference curve of the 0%, 25%, 50%, 75%, and 100% methylation control, and C, standard curve from difference curve peak of all methylation control that was obtained from MS-HRM data, plotted, and the equation generated was used for the samples methylation percentage calculation of the samples

TABLE 1 Primer sequences used for MS-HRM of *HOXA4* and *HOXA5* promoter amplification

Gene	Name	Sequence	Size of Amplicon	Number of CpG	Annealing Temperature
HOXA4	*HOXA4*_fw *HOXA4*_rv	5′ TTTTGAAGGATACGAAGTTTGA 3′ 5′ TCCTCTCGAAAACCCTCTAC 3′	115 BP (−519 to −399)	9 CpG	50.0 °C
HOXA5	*HOXA5*_1fw *HOXA5*_1rv	5′ AGAGCGGTAGGGATATTGTTT 3′ 5′ ACTAACCGTCCTACCAACAACT 3′	169 BP (−754 to −586)	7 CpG	51.2 °C
	*HOXA5*_2fw *HOXA5*_2rv	5′ AGGTAGGATTTATGATTGGATAAT 3′ 5′ ACATCTATAACACCCTTACACAAT 3′	140 BP (−186 to −47)	6 CpG	54.5 °C

and *HOXA5* methylation levels in discriminating the IM good response and resistance CML patients. From the ROC curve, an optimal cutoff point was identified by employing the "points on curve closest to the (0, 1)" method.[32] Later, the numerical data of *HOXA4* and *HOXA5* methylation levels were divided into 2 groups according to the cut-off point calculated. Simple logistic regression was then used to identify the risk of developing IM resistance by determining the crude odd ratios (ORs) and 95% confidence interval (CI). Multiple logistic regression analysis was used to evaluate the relationship between *HOXA4* as well as *HOXA5* promoter methylation levels and IM response of CML patients by calculating the ORs and 95% CI. The analysis was performed by SPSS software (version 20) with all *P*-values as 2-sided. A 5-year survival analysis was performed by using Cox proportional hazard model involving simple Cox regression deriving hazard ratio for univariate analysis and Kaplan Meier curves were plotted. The survival distributions for univariate were compared using log-rank test. A *P*-value of less than 0.05 was considered as statistically significant.

3 | RESULTS

3.1 | Demographic profile

In this study, 175 Philadelphia chromosome positive CML patients comprising both IM good response (n = 83) as well as IM resistant (n = 92) groups were recruited. Among the 83 IM good response CML patients, 42 are male and 41 are female with mean age of 42 (SD = 14.33) while among 92 IM resistant CML patients, 43 are male and 49 are female with mean age of 43 (SD = 14.54). Among the *BCR-ABL* non-mutated IM resistant CML patients, 32 (34.8%) were in warning group, 40 (43.5%) in primary resistance group, and 20 (21.7%) in secondary resistance group.

3.2 | Correlation between methylation level and IM response

In the MS-HRM analysis, the well-separated and nearly equal deviation between all percentages of the methylation control in the normalized melt curve (Figure 1A) and the difference curve (Figure 1B) showed an acceptable sensitivity of the analysis. In each run, the difference curve peak of all methylation controls was obtained, plotted, and the coefficient of determination (r^2) was found to be higher than 0.9 (Figure 1C).

From the MS-HRM analysis of IM good response and resistant CML patients, the methylation percentage of the *HOXA4* (519 to −399 from transcription start site [TSS] of *HOXA4*) was found to be scattered from 10% to 100%. However, for *HOXA5*, all patients regardless of their response to IM showed methylation level of more than 75% when analysed using *HOXA5_1* primers set (Table 1). This indicated that promoter region at −754 to −586 of *HOXA5* TSS is highly methylated regardless of their response to IM. Thus, this region was regarded as not appropriate to be an epigenetic marker for IM resistance identification. Interestingly, *HOXA5*_2 primer set (Table 1) used to amplify −186 to −47 from *HOXA5* TSS showed the methylations percentage were scattered from 0% to 100%.

In *HOXA4*, the mean methylation levels were found to be higher in IM-resistant CML patients (mean = 64.52; SD = 19.51) compared with IM good response CML patients (mean = 52.25; SD = 17.04). Similarly in *HOXA5*, the mean methylation levels were found to be higher in IM resistant CML patients (mean = 71.42; SD = 20.98) compared with IM good response CML patients (mean = 52.34; SD = 27.28). Subsequently, from the ROC curve analysis, the methylation levels of *HOXA4* and *HOXA5* promoter between IM good response and IM resistance CML patients were discriminated, considering the optimal methylation level cutoff point as 62.5 for both *HOXA4* (0.644 sensitivity, 0.723 specificity) and *HOXA5* (0.741 sensitivity, 0.402 specificity). Figure 2A,B shows the ROC curve for *HOXA4* and *HOXA5*, respectively. Based on the methylation cutoff point, 60.0% of the CML patients showed a parallel methylation level in HOXA4 and HOXA5. A total of 45.8% of the IM good response CML patients had methylation lower than 62.5% in both HOXA4 and HOXA5, while 50.0% of the IM resistant CML patients had methylation higher than 62.5% in both HOXA4 and HOXA5. Others showed different methylation status between HOXA4 and HOXA5.

Based on the final model of multiple logistic regression, a CML patient with ≥63% *HOXA4* and *HOXA5* methylation level had 3.78 and 3.95 times, respectively, the odds to acquire resistance to IM when adjusted for each other (Table 2). For a better clinical point of view, European LeukemiaNet 2013 classification was employed and the risk association of *HOXA4* and *HOXA5* methylation levels with the IM warning, primary resistant, and secondary resistant groups of

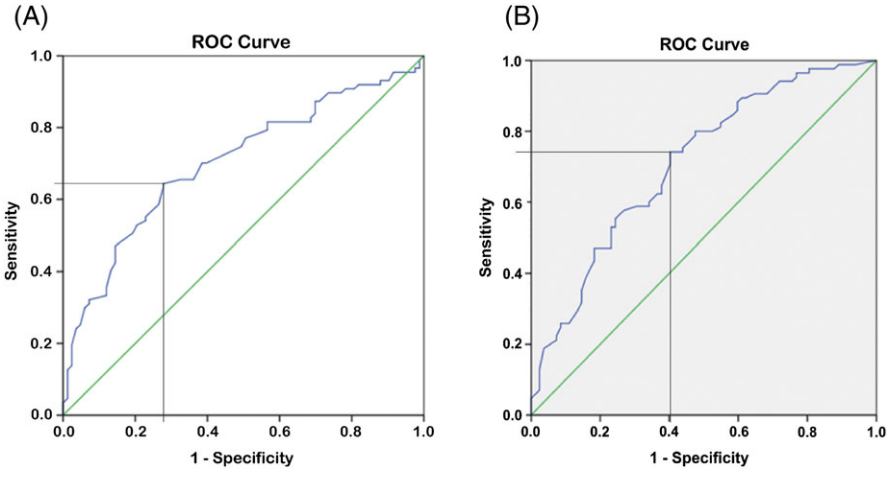

FIGURE 2 ROC curve of A, HOXA4 with optimal cut-off point at 62.5% (0.644 sensitivity, 0.723 specificity, 95% CI 0.624, 0.782) and B, HOXA5 with optimal cut-off point at 62.5% (0.741 sensitivity, 0.598 specificity, 95% CI 0.63, 0.79)

TABLE 2 Risk association of *HOXA4* and *HOXA5* methylation levels with the development of IM resistance in CML patients

Genes	Methylation Percentage	Simple Logistic Regression			Multiple Logistic Regression[a]		
		Regression coefficient (b)	Crude OR (95% CI)	*P* value	Regression coefficient (b)	Adjusted OR (95% CI)	*P* value
HOXA4	0–62% 63–100%	0 1.55	1.00 4.71 (2.46,9.03)	<0.001*	0 1.15	1.00 3.78 (1.89,7.55)	<0.001*
HOXA5	0–62% 63–100%	0 1.45	1.00 4.26 (2.22,8.17)	<0.001*	0 1.38	1.00 3.95 (2.00,7.82)	<0.001*

*P value <0.05 is significant.

[a]Forward LR multiple logistic regression model was applied.

Multicollinearity and interaction term checked and not found.

Hosmer-Lemeshow test, (P-value = 0.940), classification table (overall correctly classified percentage = 68%) and area under the ROC curve (74.9%) were applied to check the model fitness.

CML patients was evaluated. The mean promoter methylation level of *HOXA4* among the IM optimal response group was significantly different from IM primary (P < 0.001) and secondary resistant (P < 0.001) CML patients, but not from the IM warning response group (P = 0.093). Interestingly, unlike in *HOXA4*, the mean promoter methylation level of *HOXA5* among the IM optimal response CML patients was significantly different from all IM warning group (P = 0.005), IM primary (P = 0.001), and secondary resistant (P = 0.006) CML patients (Table 3).

3.3 | Correlation between methylation level and overall survival

Overall survival of IM treated CML patients was also evaluated based on their *HOXA4* and *HOXA5* promoter hypermethylation levels. Figure 3A,B shows the Kaplan Meier curves for *HOXA4* and *HOXA5*, respectively. However, from the Log-Rank analysis, there was no significant difference in the overall survival between CML patients in group that have ≤62% and ≥63% methylation levels (P-value = 0.126 for *HOXA4*; P-value = 0.217 for *HOXA5*). Table 4 shows the Cox regression analyses for the *HOXA4* and *HOXA5* methylation levels.

4 | DISCUSSION

It is well documented that hypermethylation of gene promoter associated CpG islands leads to inactivation of gene expression. The *HOX* genes are known to play crucial roles in the control of differentiation of adult haematopoietic cells.[25] According to Shah and Sukumar (2010), normal *HOX* gene expression disruption in cancer affects various pathways that promote tumorigenesis, and metastasis,

TABLE 3 Risk association of the *HOXA4* and *HOXA5* methylation groups, divided based on the 62.5% cutoff point towards the 4 response groups to IM treatment

IM response Groups	HOXA4			HOXA5		
	Regression coefficient (B)	Crude odds ratio (95% CI)	*P*-value	Regression coefficient (B)	Crude odds ratio (95% CI)	*P*-value
Optimal Warning	0 0.75	1.00 2.12 (0.88,5.09)	- 0.093	0 1.33	1.00 3.78 (1.50,9.54)	- 0.005*
Primary Secondary	1.95 2.12	7.04 (2.95,16.82) 8.35 (2.74,25.41)	<0.001* <0.001*	1.50 1.53	4.48 (1.88,10.69) 4.61 (1.54,13.79)	0.001* 0.006*

*P < 0.05, significant at 95% CI.

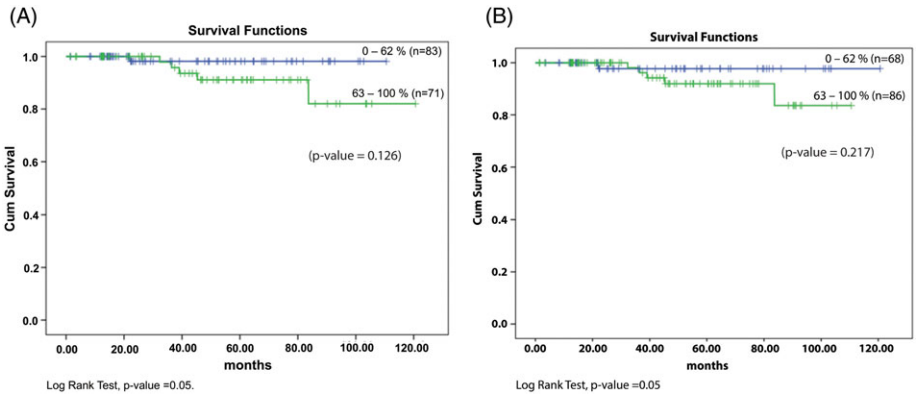

FIGURE 3 Kaplan Meier curves of A, HOXA4 and B, HOXA5

TABLE 4 Cox regression analyses showing the value of *HOXA4* and *HOXA5* methylation level in predicting the risk of mortality in IM treated CML patients

Variables		Regression Coefficient (b)	Crude Hazards Ratio (95% CI)	Wald Statistic	*P*-Value
HOXA4	0%–62% 63%–100%	0 1.53	1 4.60 (0.54, 39.38)	- 1.94	- 0.164
HOXA5	0%–62% 63%–100%	0 1.27	1 3.55 (0.42, 30.39)	- 1.34	- 0.248

*P value <0.05 was significant.

including the activation of anti-apoptotic pathways, suppression of differentiation,[33,34] and necroptosis.[35] From what is known so far, homeobox proteins interact with numerous regulatory pathways, including fibroblast growth factor, bone morphogenic protein, retinoic acid, sex steroid signaling, and also proteins involved in cell matrix interaction, such as integrins and intracellular adhesion molecule.[36] From all clusters of *HOX* genes, aberrant methylation was reported to be most frequently occurring in the promoter regions of six *HOXA* cluster genes, with some of them showing leukemia type-specific hypermethylation in all leukemia cell lines.[37] Among the *HOXA* cluster genes, *HOXA4* and *HOXA5* genes are transcription factors that are known to regulate haematopoiesis[38] and are associated with acute myeloid leukemia development.[39] *HOXA4* was proven to produce mature myeloid and lymphoid progeny in hematopoietic stem cells and was shown to be important in the regulatory mechanisms of controlling hematopoiesis within its natural context.[40] *HOXA5*, on the other hand, works by regulating normal proliferation and apoptosis, and hypermethylation of *HOXA5* is also known to regulate myeloid differentiation[29] and necroptosis.[35] Studies focusing on the potential oncogenic and tumour suppressive role of *HOX* gene family members in leukemia development have shown that inactivation of *HOXA4* is associated with poor prognosis. In both adult myeloid and lymphoid leukemias, *HOXA4* and *HOXA5* were found to be frequently inactivated by promoter hypermethylation.

Increased level of DNA methylation was observed in *HOXA4* gene and was reported to be associated with transcriptional repression in chronic lymphocytic leukemia[41] and acute myeloid leukemia.[29] In CML, inactivation of *HOXA4* and *HOXA5* genes by hypermethylation is strongly correlated with progression into blast crisis.[29] Thus, the changes in level of HOXA4 and HOXA5 protein production will perturb the normal regulation of haematopoiesis and subsequently promote leukemogenesis.[29] Even though *HOXA4* and *HOXA5* were reported to be associated with the poor prognosis in CML patients, their involvement in mediating IM resistance among IM-treated CML patients has not been explored earlier. To assess the possible epigenetic component of IM resistance, we compared the methylation levels of *HOXA4* and *HOXA5* in Malaysian CML patients responsive and resistant to IM.[41,29]

The present finding involving 175 CML patients also suggested that the methylation level of *HOXA4* has a strong correlation with IM resistance among CML patients, which is in agreement with our earlier report on 95 CML patients.[30] Additionally, in the present study, the involvement of *HOXA5* methylation level towards IM resistance was also evaluated. Interestingly, instead of 50% cutoff point, a new optimal methylation level cutoff point of 62.5% was considered for both *HOXA4* and *HOXA5*. This new cutoff point was determined via ROC curve which is more reliable than the methylation cutoff point used in our previous study. Based on the 62.5% methylation cutoff point, the response towards IM treatment among CML patients was more significantly differentiated ($P < 0.001$) for both *HOXA4* and *HOXA5* promoter methylation levels. Interestingly, from the association study, *HOXA4* promoter methylation level was able to differentiate IM primary and secondary resistant CML patients from the IM optimal response but not from the IM warning response. However, apart from IM primary and secondary resistant CML patients, *HOXA5* promoter methylation level was able to differentiate IM warning group also, from the IM optimal response group of CML patients. This present study demonstrated that DNA hypermethylation of *HOXA4* and *HOXA5* promoters could be a biomarker for predicting resistance towards IM treatment in CML patients. To the best of available knowledge, there are no other studies till date that had reported the contribution of *HOXA4* and *HOXA5* in mediating IM resistance in CML patients.

Several studies have proven that hypermethylation of *HOXA4* is able to promote inactivation of its gene expression.[39,41,42] HOXA4 and HOXA5 proteins are known as DNA-binding transcription factors which may regulate gene expression, morphogenesis, and differentiation. Thus, it is reasonable to suggest that the suppression of HOXA4 and HOXA5 protein production by hypermethylation-induced gene silencing might be promoting resistance to IM. Hypermethylation of *HOXA4* was reported to be involved with the development of leukemia in several studies.[39] In 2009, Zangenberg et al reported that 77% of their acute myeloid leukemia (AML) patients exhibited hypermethylation of *HOXA4* promoter region.[42]

It is known that p53 gene plays an important role in regulating apoptosis. This programmed cell death associated gene was reported to be transcriptionally regulated by the *HOXA5*.[43] *HOXA5* has been shown to function as a transactivator of the P53 gene[43] and to induce apoptosis by P53 dependent and P53 independent mechanisms.[44] DNA methylation of the CpG island located in the 5′ end of the *HOXA5* gene has been identified in breast[43] and lung cancer[45] and has been associated with loss of *HOXA5* expression.[43] As in breast cancer, where overexpression of *HOXA5* has been found to induce apoptosis by upregulating the expression of p53,[44] *HOXA5* may also behave as a tumor suppressor gene in CML. Likewise hypermethylation of *HOXA5* results in downregulation of its expression. Thus, downregulation of *HOXA5* might be consequently reducing p53 protein and blocking the guardian effects of p53 on the genome which may lead to uncontrolled cell growth and disease progression.

Furthermore, Chen et al (2005) had demonstrated that *HOXA5* may trigger the receptor-mediated apoptotic pathways via the activation of NF-κB signaling pathway. During leukemogenesis, NF-κB

signaling is blocked by the activation of PI3K-Akt signaling pathway, a downstream pathway of *BCR-ABL*, subsequently leading to reduced apoptosis. Therefore, it is reasonable to suggest that even though IM blocks *BCR-ABL* tyrosine kinase activity, the disease continues to progress via the inactivation of NF-κB by the down regulation of *HOXA5*. Thus, apart from *BCR-ABL* gene, the failure in the regulation of normal *HOXA5* expression, most probably due to hypermethylation, will also lead to the uncontrolled leukemic cell accumulation in leukemogenesis of CML.[44]

From the present study, it is reasonable to speculate that DNA hypermethylation induced gene silencing can provide an alternative to *BCR-ABL* mutations in mediating resistance to IM in CML patients. Thus, further studies are needed for hypermethylation status of *HOXA4* and *HOXA5* to be applied as an additional marker of resistance to IM in supplement to *BCR-ABL* mutation analysis. This is because, no known mechanism of IM activity towards *HOXA4* and *HOXA5* hypermethylation has been reported so far. Hence, mechanistic studies are still needed to prove how hypermethylation of *HOXA4* and *HOXA5* causes poor response to IM. Furthermore, the cause of hypermethylation particularly in both HOXA4 and HOXA5 is still not fully revealed. Arising-mutation of DMNT3a could be eliminated as one of the causes of DNA hypermethylation as DMNT3a has been associated with lower level of DNA methylation in many genes, including HOXA4 and HOXA5.[46] Another gene associated with epigenetic regulation is TET2. This TET2 gene is commonly mutated in CML patients; thus, it is related to the pathogenesis of CML as well as resistance towards TKI treatment.[47] However, no studies have shown the association of TET2 mutation and HOXA4 and HOXA5 methylation level.

The association of *HOXA4* and *HOXA5* gene expression levels with overall survival of these CML patients was also investigated in the present study. From the overall survival analysis, no significant difference in the overall survival between patients with ≤62% and ≥63% of *HOXA4* and *HOXA5* methylation levels was observed. Thus, the levels of *HOXA4* and *HOXA5* promoter methylation could be considered as epigenetic markers for predicting the response to IM but could not be considered as a prognostic marker as the methylation level was not significantly associated with the poor prognosis among imatinib-treated CML patients. There might be differences between particular genes being hypermethylated and their prognostic impact. We can hypothesize that hypermethylation of genes involved in specific cellular pathways may make these cells more prone and sensitive to chemotherapy treatment.

However, recent studies on myeloproliferative neoplasm have revealed that 5-mC is converted to an intermediate state of 5-hmC before further demethylated into C.[48,49] Unfortunately, MS-HRM which was used in this study utilized the principle of bisulfite analysis that recognizes both 5-mC and 5-hmC as C. Thus, the level of expression could be affected in all methylation-positive samples as those samples also may contain 5-hmC. Oxidative MS-HRM together with MS-HRM will help to distinguish 5-mC and 5-hmC for more accurate determination of methylation level that may reflect the expression level of HOXA4 and HOXA5.

In conclusion, the present study result suggests that promoter hypermethylation of *HOXA4* and *HOXA5* genes is correlated with IM resistance and could be a potential epigenetic biomarker in supplement to the *BCR-ABL* gene mutation in predicting IM treatment response among CML patients.

ACKNOWLEDGEMENT

This study was supported by Universiti Sains Malaysia, Malaysia, Research University (USM-RU) Grant/Award Number: 1001/PPSP/812070. Exceptional appreciation to Dr Hoh Boon Peng and staff of Institute of Medical Molecular Biotechnology, Faculty of Medicine, Universiti Teknologi MARA for permitting to use the facilities used in this study. The co-operation and support of staff, Human Genome Center, Universiti Sains Malaysia, and all the patients who have participated in this study are also gratefully acknowledged.

AUTHORS' CONTRIBUTIONS

All authors had full access to the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. *Conceptualization*, R.A.; *Methodology*, M.H.E.; *Investigation*, M.H.E.; *Formal Analysis*, M.H.E.; *Resources*, M.H.E.; *Writing*, M.H.E.; *Original Draft*, M.H.E.; *Writing-Review & Editing*, R.A., H.A., S.S., A.A.B.; *Visualization*, R.A.; *Supervision*, R.A.; *Funding Acquisition*, R.A.

ORCID

Marjanu Hikmah Elias http://orcid.org/0000-0001-9343-7954

REFERENCES

1. Fehniger TA, Welch JS, Pluard TJ. The Washington Manual of Oncology: Lippincott Williams & Wilkins; 2007.
2. Quintas-Cardama A, Kantarjian HM, Cortes JE. Mechanisms of primary and secondary resistance to imatinib in chronic myeloid leukemia. *Cancer Control*. 2009;16(2):122-131.
3. Zhang WW, Cortes JE, Yao H, et al. Predictors of primary imatinib resistance in chronic myelogenous leukemia are distinct from those in secondary imatinib resistance. *J Clin Oncol*. 2009;27(22):3642-3649.
4. Baccarani M, Saglio G, Goldman J, et al. Evolving concepts in the management of chronic myeloid leukemia: recommendations from an expert panel on behalf of the European LeukemiaNet. *Blood*. 2006;108(6):1809-1820.
5. Kantarjian HM, Talpaz M, O'Brien S, et al. Dose escalation of imatinib mesylate can overcome resistance to standard-dose therapy in patients with chronic myelogenous leukemia. *Blood*. 2003;101(2):473-475.
6. Azevedo AP, Reichert A, Afonso C, Alberca MD, Tavares P, Lima F. BCR-ABL V280G mutation, potential role in Imatinib resistance: first case report. *Clin Med Insights Oncol*. 2017;11:1179554917702870.
7. Kaplan JB, Platanias LC. Another tyrosine kinase inhibitor-resistance mutation within the BCR-ABL kinase domain: chasing our tails? *Leuk Lymphoma*. 2017;58(7):1526-1527.
8. Kantarjian HM, Talpaz M, Giles F, O'Brien S, Cortes J. New insights into the pathophysiology of chronic myeloid leukemia and imatinib resistance. *Ann Intern Med*. 2006;145(12):913-923.
9. Elias MH, Baba AA, Husin A, et al. Contribution of BCR-ABL kinase domain mutations to imatinib mesylate resistance in Philadelphia chromosome positive Malaysian chronic myeloid leukemia patients. *Hematol Rep*. 2012;4(4):e23.
10. Kim SH, Kim D, Kim DW, et al. Analysis of Bcr-Abl kinase domain mutations in Korean chronic myeloid leukaemia patients: poor clinical outcome of P-loop and T315I mutation is disease phase dependent. *Hematol Oncol*. 2009;27(4):190-197.

11. Qin Y, Chen S, Jiang B, et al. Characteristics of BCR-ABL kinase domain point mutations in Chinese imatinib-resistant chronic myeloid leukemia patients. *Ann Hematol*. 2011;90(1):47-52.
12. Sherbenou DW, Hantschel O, Kaupe I, et al. BCR-ABL SH3-SH2 domain mutations in chronic myeloid leukemia patients on imatinib. *Blood*. 2010;116(17):3278-3285.
13. Kalle AM, Sachchidanand S, Pallu R. Bcr-Abl-independent mechanism of resistance to imatinib in K562 cells: induction of cyclooxygenase-2 (COX-2) by histone deacetylases (HDACs). *Leuk Res*. 2010;34(9):1132-1138.
14. Nambu T, Araki N, Nakagawa A, et al. Contribution of BCR-ABL-independent activation of ERK1/2 to acquired imatinib resistance in K562 chronic myeloid leukemia cells. *Cancer Sci*. 2010;101(1):137-142.
15. Machova Polakova K, Koblihova J, Stopka T. Role of epigenetics in chronic myeloid leukemia. *Curr Hematol Malig Rep*. 2013;8(1):28-36.
16. Heller G, Topakian T, Altenberger C, et al. Next-generation sequencing identifies major DNA methylation changes during progression of Ph+ chronic myeloid leukemia. *Leukemia*. 2016;30(9):1861-1868.
17. Toyota M, Kopecky KJ, Toyota MO, Jair KW, Willman CL, Issa JP. Methylation profiling in acute myeloid leukemia. *Blood*. 2001;97(9):2823-2829.
18. Jelinek J, Gharibyan V, Estecio MR, et al. Aberrant DNA methylation is associated with disease progression, resistance to imatinib and shortened survival in chronic myelogenous leukemia. *PLoS One*. 2011;6(7):e22110.
19. Wang XJ, Li J, Fu BJ, Guo LL, Zhang JH, Huang SA. Methylation status of JunB and CDH13 gene promoter in CD34(+)CD38(−) chronic myelogenous leukemia cells. *Zhongguo Shi Yan Xue Ye Xue Za Zhi*. 2009;17(6):1405-1408.
20. Wang YL, Qian J, Lin J, et al. Methylation status of DDIT3 gene in chronic myeloid leukemia. *J Exp Clin Cancer Res*. 2010;29(1):54.
21. Issa JP. DNA methylation as a therapeutic target in cancer. *Clin Cancer Res*. 2007;13(6):1634-1637.
22. Deschamps J, Duboule D. Embryonic timing, axial stem cells, chromatin dynamics, and the Hox clock. *Genes Dev*. 2017;31(14):1406-1416.
23. Abramovich C, Humphries RK. Hox regulation of normal and leukemic hematopoietic stem cells. *Curr Opin Hematol*. 2005;12(3):210-216.
24. Buske C, Humphries RK. Homeobox genes in leukemogenesis. *Int J Hematol*. 2000;71(4):301-308.
25. Cillo C, Cantile M, Faiella A, Boncinelli E. Homeobox genes in normal and malignant cells. *J Cell Physiol*. 2001;188(2):161-169.
26. Grier DG, Thompson A, Kwasniewska A, McGonigle GJ, Halliday HL, Lappin TR. The pathophysiology of HOX genes and their role in cancer. *J Pathol*. 2005;205(2):154-171.
27. Kontro M, Kumar A, Majumder MM, et al. HOX gene expression predicts response to BCL-2 inhibition in acute myeloid leukemia. *Leukemia*. 2017;31(2):301-309.
28. Esteller M. Cancer epigenetics: DNA methylation and chromatin alterations in human cancer. *Adv Exp Med Biol*. 2003;532:39-49.
29. Strathdee G, Holyoake TL, Sim A, et al. Inactivation of HOXA genes by hypermethylation in myeloid and lymphoid malignancy is frequent and associated with poor prognosis. *Clin Cancer Res*. 2007;13(17):5048-5055.
30. Elias MH, Baba AA, Husin A, et al. HOXA4 gene promoter hypermethylation as an epigenetic mechanism mediating resistance to imatinib mesylate in chronic myeloid leukemia patients. *Biomed Res Int*. 2013;2013:129715.
31. LeukemiaNet E. Recommendations from the European LeukemiaNet for the Management of chronic myeloid leukemia (CML). update 2010 ed: European LeukemiaNet; 2010.
32. Kumar R, Indrayan A. Receiver operating characteristic (ROC) curve for medical researchers. *Indian Pediatr*. 2011;48(4):277-287.
33. Shah N, Sukumar S. The Hox genes and their roles in oncogenesis. *Nat Rev Cancer*. 2010;10(5):361-371.
34. Domsch K, Papagiannouli F, Lohmann I. The HOX-apoptosis regulatory interplay in development and disease. *Curr Top Dev Biol*. 2015;114:121-158.
35. Alharbi RA, Pandha HS, Simpson GR, et al. Inhibition of HOX/PBX dimer formation leads to necroptosis in acute myeloid leukemia cells. *Oncotarget*. 2017;8(52):89566-89579.
36. Rodini CO, Xavier FC, Paiva KB, et al. Homeobox gene expression profile indicates HOXA5 as a candidate prognostic marker in oral squamous cell carcinoma. *Int J Oncol*. 2012;40(4):1180-1188.
37. Fang MH, Liu WL, Meng FK, Sun HY. Aberrant methylation at promoter region of HOX a gene cluster in leukemia cells. *Zhonghua Xue Ye Xue Za Zhi*. 2009;30(7):468-472.
38. Lawrence HJ, Largman C. Homeobox genes in normal hematopoiesis and leukemia. *Blood*. 1992;80(10):2445-2453.
39. Musialik E, Bujko M, Kober P, et al. Promoter DNA methylation and expression levels of HOXA4, HOXA5 and MEIS1 in acute myeloid leukemia. *Mol Med Rep*. 2015;11(5):3948-3954.
40. Fournier M, Lebert-Ghali CE, Krosl G, Bijl JJ. HOXA4 induces expansion of hematopoietic stem cells in vitro and confers enhancement of pro-B-cells in vivo. *Stem Cells Dev*. 2012;21(1):133-142.
41. Strathdee G, Sim A, Parker A, Oscier D, Brown R. Promoter hypermethylation silences expression of the HoxA4 gene and correlates with IgVh mutational status in CLL. *Leukemia*. 2006;20(7):1326-1329.
42. Zangenberg M, Grubach L, Aggerholm A, et al. The combined expression of HOXA4 and MEIS1 is an independent prognostic factor in patients with AML. *Eur J Haematol*. 2009;83(5):439-448.
43. Raman V, Martensen SA, Reisman D, et al. Compromised HOXA5 function can limit p53 expression in human breast tumours. *Nature*. 2000;405(6789):974-978.
44. Chen H, Rubin E, Zhang H, et al. Identification of transcriptional targets of HOXA5. *J Biol Chem*. 2005;280(19):19373-19380.
45. Shiraishi M, Sekiguchi A, Oates AJ, Terry MJ, Miyamoto Y. HOX gene clusters are hotspots of de novo methylation in CpG islands of human lung adenocarcinomas. *Oncogene*. 2002;21(22):3659-3662.
46. Hajkova H, Markova J, Haskovec C, et al. DNMT3A mutations are connected with lower DNA methylation levels and numbers of concurrently Hypermethylated genes in AML patients. *Blood*. 2011;118(21):4629.
47. Kim T, Tyndel MS, Kim HJ, et al. Spectrum of somatic mutation dynamics in chronic myeloid leukemia following tyrosine kinase inhibitor therapy. *Blood*. 2017;129(1):38-47.
48. Mori N, Ohwashi-Miyazaki M, Yoshinaga K, et al. Tumor suppressor gene methylation on the short arm of chromosome 1 in chronic myelogenous leukemia. *Eur J Haematol*. 2017;98(5):467-477.
49. Pronier E, Almire C, Mokrani H, et al. Inhibition of TET2-mediated conversion of 5-methylcytosine to 5-hydroxymethylcytosine disturbs erythroid and granulomonocytic differentiation of human hematopoietic progenitors. *Blood*. 2011;118(9):2551-2555.

How to cite this article: Elias MH, Azlan H, Sulong S, Baba AA, Ankathil R. Aberrant DNA methylation at *HOXA4* and *HOXA5* genes are associated with resistance to imatinib mesylate among chronic myeloid leukemia patients. *Cancer Reports*. 2018;1:e1111. https://doi.org/10.1002/cnr2.1111