ORIGINAL ARTICLE

Hypermethylation Analysis of $p16^{INK4a}$ and $p15^{INK4b}$ Promoters in Chronic Lymphocytic Leukaemia Patients and Normal Individuals

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ABSTRACT

Introduction: Chronic Lymphocytic Leukaemia (CLL) is a common type of leukaemia in persons of predominantly European descent but is rare in the Asian population. Disparities in CLL incidence among people of Asian and European descent may be related to the genetic make-up of the two different populations. Hypermethylation event might be one of the silencing mechanisms that inactivate the tumour suppressor genes in CLL. The aim of this study was to determine the hypermethylation status of $p16^{INK4a}$ and $p15^{INK4b}$ among CLL patients and normal individuals. **Materials & Methods:** A total of 25 CLL patients and 25 normal individuals were recruited for this study and their genomic DNA were extracted from the peripheral blood. The hypermethylation status of $p16^{INK4a}$ and $p15^{INK4b}$ were determined using Methylation Specific-PCR (MS-PCR) whereas DNA sequencing method was applied to selected samples for validation of the MS-PCR results. We also evaluated the association between hypermethylation of these genes with the clinical and demographic characteristics of each group of subjects. **Results:** Among the CLL patients, $p15^{INK4b}$ partial-methylation occurred in 6 (24%) subjects while methylation occurred in 1 (4%) subject. All the remaining patients were unmethylated at $p15^{INK4b}$. All the samples showed unmethylation at $p16^{INK4a}$. Statistically significant associations were found between $p15^{INK4b}$ hypermethylation with the presence of CLL (p=0.01) and with race (p=0.02). **Conclusion:** Further study using a larger sample size is warranted to explore the significance of DNA methylation incidence among the CLL patients of the Malaysian population. Hence, we suggest that hypermethylation at $p15^{INK4b}$ has a huge influence that kick-starts CLL disease among Malaysians and MS-PCR technique is applicable to be used in methylation study.

KEYWORDS: Chronic Lymphocytic Leukaemia, Methylation-specific PCR, DNA sequencing, $p16^{INK4a}$, $p15^{INK4b}$

INTRODUCTION

Leukaemia is one of the most frequent cancers and is ranked 6th among the ten most common cancers in Malaysia.[1] It is characterized by diffuse replacement of the bone marrow by neoplastic cells which leads to suppression of normal haematopoiesis. Leukaemia can be classified as acute or chronic, lymphoid or myeloid to produce four main types of diseases: acute lymphoblastic (ALL), acute myeloid (AML), chronic lymphocytic (CLL) and chronic myeloid leukaemia (CML). According to Parker *et al* (1997), CLL has been reported to be the most common leukaemia in the Western population, but not in Asians.[2] The lower incidence of CLL in Asians may be associated with the genetic mechanisms that highlight the differences in the genetic component between these two populations. DNA methylation is catalysed by a DNA methyltransferase enzyme that results in addition of a methyl group to the 5-carbon of the

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cytosine ring in CpG nucleotides.[3] The CpG islands are regions of DNA which are GC rich, typically 0.5-4.0 kb in length and remain unmethylated in normal tissues when found in the 5' region of genes.[4,5] These CpG islands are frequently located within the promoter regions of human genes, and methylation within the islands has been shown to be associated with transcriptional inactivation of the corresponding gene.[6] Both hyper- and hypomethylation might play important roles in the tumorigenic process but the increased methylation at CpG islands is by far the most studied mechanism and has a much clearer role in carcinogenesis.[6]

Hypermethylation of CpG islands in a human tumour was first reported in 1986.[7] Multiple genes have been shown to be inactivated by hypermethylation of CpG islands in many types of cancer including haematological malignancies. However, the role of gene hypermethylation events in CLL has not been adequately studied, particularly in Asia. It has been proposed that methylation of the promoter region should be included in the Knudson's two hit hypothesis for inactivation of tumour suppressor genes.[8] The first hit may be a mutation in the DNA sequence or promoter methylation and the second inactivating hit may be either loss of heterozygosity or a further mutational or methylating event in the second allele. Hypermethylation which is one type of epigenetic events is accomplished by the enzymatic addition of methyl groups to CpG dinucleotides in an organized reaction which consists of DNA methyltransferases, methyl-binding domain proteins and histone deacetylase.[9,10]

An abundance of genes has been investigated for hypermethylation in different types of malignancies by utilizing various laboratory techniques. For instance, oestrogen receptor gene located on chromosome 6q was found to be methylated in lymphomas, acute myeloid leukaemia (AML), acute lymphoblastic leukaemia (ALL) and chronic myeloid leukaemia (CML).[11-13] *HIC-1* was frequently methylated and silenced in haematological neoplasms such as newly diagnosed ALL (53%), non-Hodgkin's lymphoma (NHL) (25%), chronic myeloid leukaemia in chronic phase (CML-CP) (50%) and in almost all cases of recurrent ALL and blastic transformation of CML (CML-BT). However, it was rarely methylated in newly diagnosed AML (10%). Thus it is inferred that methylated *HIC-1* may contribute to disease progression.[14]

It has been suggested that the human chromosome 9p21 region is a frequent site of deletion and rearrangement in many tumour types including leukaemia; implicating the existence of a tumour suppressor gene within 9p21 which is involved in tumour formation.[14,15] The 9p21 locus harbours the tumour suppressor genes $p16^{INK4a}$, its alternative splice product $p14^{ARF}$, and $p15^{INK4b}$. Alterations of these genes in human cancers can occur through a variety of mechanisms including point mutation, homozygous deletion and hypermethylation of promoter region.

According to Chim *et al* (2006), $p16^{INK4a}$ and $p15^{INK4b}$ promoter hypermethylation was each detected in 14.3% and 36% of Chinese patients with newly diagnosed CLL.[16] Their study involving 56 CLL patients also suggested that both genes might be important in pathogenesis of CLL. Another study by Tsirigotis *et al* (2006) carried out in 34 CLL patients found a similar frequency as reported by Chim *et al* (2006) with 6 cases (17.6%) detected to have $p16^{INK4a}$ hypermethylation.[16,17] Papageorgiou *et al* (2007) also detected promoter hypermethylation of $p15^{INK4b}$ in 11.8% of CLL patients in Greece and suggested that this abnormality represents an early event in tumour progression.[18] Both genes are the neighbourhood genes located on the same region of chromosome 9p21 and are commonly reported to be hypermethylated in leukemic patients. Among Chinese children with CLL, 42% (39/93) had methylation at $p15^{INK4b}$.[19] This finding is in line with studies by Gardiner *et al* (2012) and Kim *et al* (2009), where they found methylation of $p15^{INK4b}$ in 67% (32/48) of Canadian ALL paediatric patients and 34.4% (21/61) of Korean ALL paediatric patients.[20,21] Taken together, it was confirmed that methylation of $p15^{INK4b}$ is frequent in childhood ALL.[19] However, the difference in hypermethylation status of these genes between CLL patients and normal controls remain unclear.

There are many laboratory methods to determine hypermethylation depending on the objective of a study. These methods may be enzymatic based, antibody based, bisulphite-treatment based and restriction enzyme with bisulphite-treatment based. A novel method, methylation-specific PCR (MSP) is useful and rapid in determining the methylation status of CpG islands.[22] This PCR assay uses bisulphite treatment to convert all unmethylated, but not methylated, cytosine to uracil, followed by

amplification with primers specific for methylated and unmethylated DNA.[22] As reported by Herman *et al* (1996) and Ku *et al* (2011), MS-PCR assay is a rapid, specific and sensitive technique for detecting hypermethylation of virtually any block of CpG sites in the CpG islands.[22,23] It can also assist in recognition of small quantity of methylated alleles, permit DNA study from limited samples and is capable of examining all the CpG sites beyond sequences recognized by hypermethylation-sensitive restriction enzymes.[22] In this current study, hypermethylation status of leukaemic samples are detected using gold standard techniques: bisulphite modification followed by PCR amplification of the bisulphite-modified genomic DNA (MS-PCR), gel electrophoresis and DNA sequencing techniques.

Hypermethylation of tumour suppressor genes such as $p16^{INK4a}$, $p14^{ARF}$ and $p15^{INK4b}$ has been suggested to play a role in leukaemogenesis. This study may provide some information on the effect of these hypermethylated genes in CLL and may impact future therapy where the use of demethylation agents could be used to suppress hypermethylation events in leukaemic patients. Recently, it has also been suggested that hypermethylation of $p16^{INK4a}$ is not a disease specific event as methylated DNA of this gene is present not only in leukaemic patients, but also in the mononuclear cells from normal subjects.[24] Thus, it is important to reveal the frequency of hypermethylation in these genes among both leukaemic patients and normal individuals.

MATERIALS AND METHODS

Peripheral blood from 25 chronic lymphocytic leukaemia (CLL) patients and 25 normal individuals were collected into EDTA tubes after informed consent were obtained from the subjects. CLL samples were recruited from Hospital USM, Kelantan and Hospital Ampang, Selangor. This study was approved by the Research and Ethics Committee, Clinical Sciences, USM (Reference number: USMKK/PPP/JEPeM [234.3.(07)]) and was approved by the National Medical Research Register (NMRR) (NMRR-11-822-8307). The authors state that there is no conflict of interest.

Two types of cell pellets derived from HL60 and Raji cell lines were kindly provided by Prof. Dr. Rosline Hassan (Universiti Sains Malaysia, Kelantan) and Prof. Dr. Lim Yang Mooi (Universiti Tunku Abdul Rahman, Selangor), respectively. Genomic DNA was extracted from both peripheral blood and the cell pellets using QIAamp® DNA Blood Mini kit (QIAGEN, Hilden, Germany). The extracted genomic DNA was quantified using NanoDrop 2000 Spectrophotometer (Thermo Scientific, Massachusetts, USA) before proceeding to bisulphite treatment.

Bisulphite-modification

Bisulphite treatment, or bisulphite modification method, was developed by Frommer, McDonald [(20)], and it can completely deaminate unmethylated cytosine to uracil. Following PCR of the modified sequences, all original cytosines will be displayed as thymines while methylated cytosines will remain as cytosines in the final sequence pattern. In this study, this treatment was done using CpGenome Modification kit (Chemicon Europe Ltd., Middlesex, UK). DNA modification Reagents I, II, III and IV were included in the kit.

A total of 7 µl of 3M NaOH and 2 µl of DNA Modification Reagent IV were added to 0.25 µg of genomic DNA in 100 µl of distilled deionized water (2.5 ng/µl) in a 1.5 ml tube. The mixture was incubated for 10 minutes at 50°C to separate the double stranded DNA to its single strand using temperate heat at an alkaline pH. Next, 550 µl of freshly prepared DNA Modification Reagent I was added and the whole mixture was vortexed to ensure a homogenous mixture.

Then, the homogenous mixture was incubated at 50° C for 16 hours (overnight) in a water bath protected from light. After the long hour incubation, a total of 5 µl of well suspended DNA Modification Reagent III and 750 µl of DNA Modification Reagent II were added to the sample. The reagent was incubated for 5 to 10 minutes and then spun down for 30 seconds at 8000 rpm. Most of the times, a small white pellet appeared. If it did not appear, another 5 µl of DNA Modification Reagent III was added to the sample and it was again incubated and centrifuged. Then, the supernatant was discarded. After that, the sample underwent repeated centrifugation and re-suspension thrice in 70% EtOH for desalting process. After the supernatant from the third wash had been discarded, another centrifugation was carried out at high speed for 2 minutes and the remaining

supernatant was removed with a white pipette tip. Next, a total of 50 µl of 20 mM NaOH/90% EtOH solution was added. The mixture was vortexed and incubated at room temperature for 5 minutes, followed by centrifugation for 30 seconds at 8000 rpm to concentrate all contents to the tip of the tube. Desalting was carried out by adding 1 ml of 90% EtOH to the sample and vortexed to wash the pellet before it was spun down and the supernatant discarded. This step was done twice. After the supernatant from the second spin was discarded, the sample was centrifuged at the highest speed for three minutes. All the remaining supernatant was again discarded with a small pipette tip. The sample was set aside for about 10 minutes at room temperature until the alcohol odour diminished. Then, a total of 25 µl of TE Buffer was added to the sample, followed by incubation for 15 minutes at 50 -60˚C to elute the DNA. Lastly, the sample was centrifuged at the highest speed for 3 minutes and the remaining supernatant was transferred to 0.2 ml tubes using a plastic pipette tip. Then, the sample was immediately used for MSP or stored at -80˚C for up to 2 weeks.

Methylation specific-PCR (MS-PCR)

MS-PCR analysis was mainly performed in a total volume of 10 µl reaction mixture, which comprised 1X PCR Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 u/µl AmpliTaq Gold DNA polymerase, 0.2 µM forward and reverse primers, approximately 50 ng of bisulphite treated DNA template and ddH_2O. The reagents were prepared in one master mix for each gene; 8 µl of the mixture was aliquoted into 0.2 ml microcentrifuge tube with 2 µl of DNA template to obtain a final volume of 10 µl. PCR buffer, $MgCl_2$, dNTP and AmpliTaq Gold DNA polymerase were purchased from Applied Biosystems (California, USA) while primers were purchased from First BASE Laboratories Sdn. Bhd. (Selangor, Malaysia). The primer sequences and other details are listed in Table I.

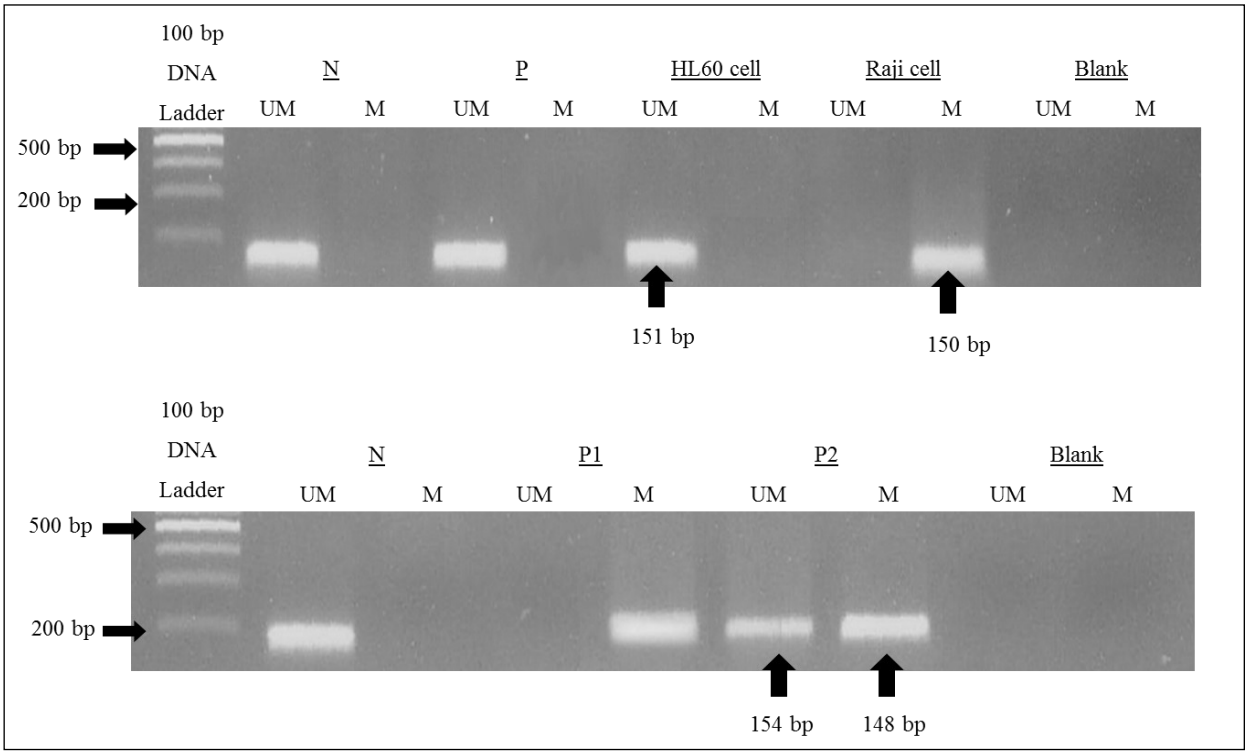

Figure 1

a) Methylation - Specific PCR assay of *p16*^{INKa}. Electrophoresis with 3% agarose gel using PCR amplicons of target gene and positive controls, HL60 and Raji cell lines. Primer sets used for amplification is designated as unmethylated (UM) and methylated (M). The HL60 and Raji cell lines serve as unmethylated and methylated controls respectively. N indicates normal sample; P, CLL sample; and H_2O, blank. At *p16*^{INKa}, normal sample and HL60 cell line show unmethylated status, whereas CLL sample and Raji cell line show methylated status.

b)Methylation-Specific PCR assay of *p15*^{INK4b}. Primer sets used for amplification is designated as unmethylated (U) and methylated (M). N indicates normal sample; P1, first representative of CLL sample; P2, second representative of CLL sample; and H_2O, blank. Normal sample shows unmethylated status at *p15*^{INK4b} whereas P1 sample shows methylated and P2 sample shows partial-methylated.

Amplification of all four genes was done and optimised using the Touch down-PCR protocol and GeneAmp® PCR System 9700 (Applied Biosystems, California, US) thermal cycler machine. The standard protocol of TD-PCR using annealing temperature of 57ºC was used for all genes. Below are the protocols of TD-PCR for $p16^{INK4a}$ and $p15^{INK4b}$: initial denaturation at 95˚C for 10 minutes, followed by 14 cycles (20 seconds at 94˚C, 1 minute at 64-57˚C, 1 minute at 72˚C) and further 32 cycles (20 seconds at 94˚C, 1 minute at 57˚C, 1 minute at 72˚C), final extension at 72˚C for 5 minutes and storage at 10˚C.

Agarose gel electrophoresis

3% agarose gel was prepared, and the subsequent wells were loaded with samples of either gDNA or PCR product by mixing together 1 µl loading dye (Promega Co., Madison, USA), 1 µl 200X Ultra Power DNA Stain (BioTeke Corporation, Beijing, China) and 3 µL of samples. Samples were run at 90V for 40 minutes, using a power pack (Wealtec Corp., Taiwan). The result of agarose gel electrophoresis was viewed under UV lights and the images produced were captured using a UV transilluminator (Wealtec Corp., Taiwan).

DNA sequencing

Authentication of MS-PCR and the identity of the methylated and unmethylated sequences were verified using DNA sequencing approach. All sequenced PCR product samples were analysed by forward primers except for PCR products of $p15^{INK4b}$ which were analysed by reverse primers. Up to 30 µL PCR products together with 5 µL of 10 µM forward or reverse primer for each sample were sent to First BASE Laboratories Sdn. Bhd. (Selangor, Malaysia) for the full services of purification and DNA sequencing. The results, which was in the form of electropherograms, was delivered via email and subsequently retrieved using BioEdit Sequence Alignment Editor.[21] Every CpG site was monitored carefully to see if they were compatible to the gel electrophoresis result obtained before the samples were sent for DNA sequencing service.

Statistical analysis

Statistical Package for the Social Sciences (SPSS) software version 21 (IBM Corporation, New York, USA) was used to perform all the analyses. Pearson Chi-square test or Fisher's Exact test was used to test the categorical differences and determine the association between hypermethylation status (unmethylated, methylated and partial-methylated) with both cases (CLL patients) and control (normal individuals) groups as well as demographic and clinical data. If more than 20% of the cells were having an expected count of less than 5, Fisher's exact test was used. If less than 20% of the cells were having an expected count of less than 5, Pearson Chi-square test was used. A *p*-value of less than 0.05 ($p<0.05$) was considered as statistically significant.

RESULTS

Review of demographic and clinical data in CLL patients

Age of CLL patients ranged between 52 to 78 years (median age 65.8 ± 7.8 years) while the normal subjects' age ranged between 23 to 42 years (mean age 27.9 ± 4.3 years). There were 17 (81%) male CLL patients and 8 (19%) females. Subjects were categorized into Malays and non-Malays. Fifteen of CLL patients were Malays (60%) and the other 10 CLL patients were non-Malays (40%). For normal individuals, 22 of them were Malays (88%) and the remaining 3 normal individuals were non-Malays (12%). The distribution of CLL patients and normal individuals based on age, gender and race are summarized in **Table II.**

Determination of hypermethylation status at $p16^{INK4a}$ and $p15^{INK4b}$

Analysis of methylation status of $p16^{INK4a}$ and $p15^{INK4b}$ were performed on bisulphite treated DNA samples using MS-PCR analysis. All 25 DNA samples from each group of CLL patients and normal individual were screened for the methylation status. For $p16^{INK4a}$, HL60 and Raji cell lines were used as the positive control for unmethylated and methylated $p16^{INK4a}$ respectively. The existence of 151 bp product in the unmethylated (UM) lane indicated the sample was unmethylated and a 150 bp product in the methylated (M) lane indicated the sample was methylated (Fig 1 (a)). For $p15^{INK4b}$, the size of the amplified amplicons was 154 and 148 bp for unmethylated (UM) and methylated (M) $p15^{INK4b}$ respectively (Fig 1 (b)).

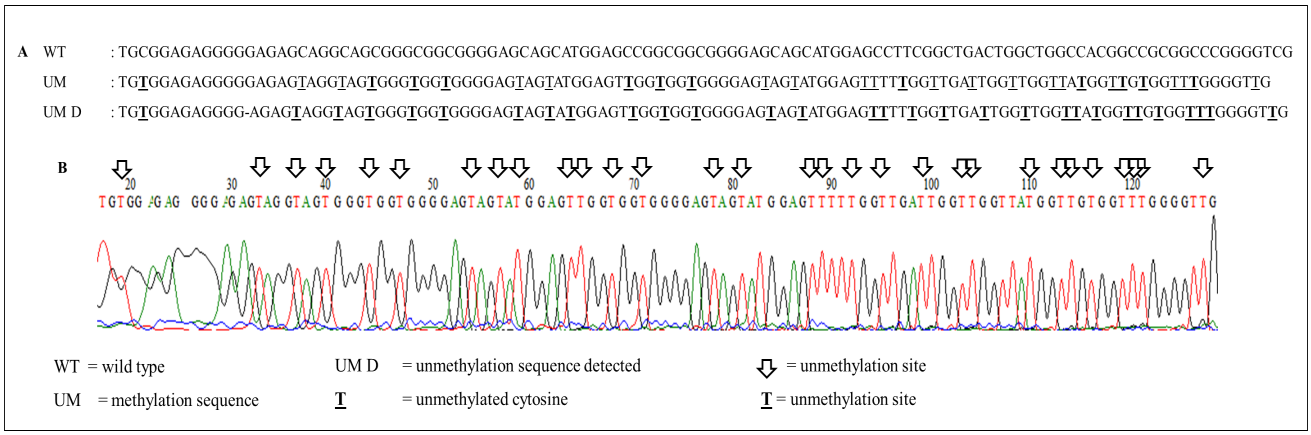

Fig 2 Sequencing result and electropherogram picture of sample unmethylated at *p16*^{INK4a}

a) Sequencing alignment of unmethylated normal sample at *p16*^{INK4a} with unmethylated bisulphite-treated *p16*^{INK4a} sequence which was taken from Methyl Primer Express v1.0 software as reference. Note the presence of many thymidines (T) in the sample sequence after bisulphite conversion and MSP. The Ts represent unmethylated cytosines. Therefore, this sample is confirmed to be unmethylated.

b) Electropherogram picture shows successful Ts' formation which represent the unmethylated cytosine.

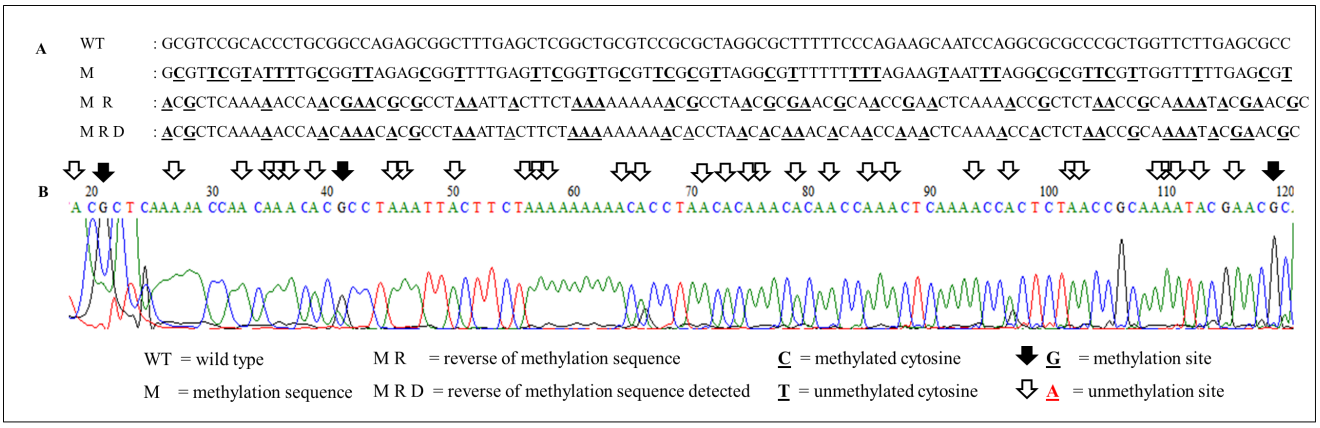

Fig 3 Sequencing result and electropherogram picture of sample partially methylated at *p15*^{INK4b}

a) Sequencing alignment of methylated normal sample at *p15*^{INK4b} with methylated bisulphite-treated *p15*^{INK4b} sez quence taken from Methyl Primer Express v1.0 software as reference. Note the presence of many adenine (A) and guanine (G) after bisulphite conversion and MSP. The As mark the presence of unmethylated cytosine whereas Gs mark the presence of methylated cytosine. Therefore, this sample is confirmed to be partially methylated.

b) Electropherogram picture shows successful formation of adenine and guanine resulting from complete conversion of unmethylated and methylated cytosines.

All the CLL patients and normal individuals showed unmethylated products of 151 bp suggesting that these samples had unmethylated region of *p16*^{INK4a}. The presence of both 154 and 148 bp products, suggesting the occurrence of partial methylation of *p15*^{INK4b} was detected in 6 CLL patients. Only 1 patient showed methylated regions of *p15*^{INK4b} while the remaining 18 patients showed unmethylated regions of *p15*^{INK4b}.

Association between hypermethylation status at *p16*^{INK4a} and *p15*^{INK4b} with a status of samples are shown in Table III. Only descriptive analysis is shown in Table IV as all samples show unmethylation at *p16*^{INK4a}. There is a statistically significant (p=0.01) association between the status of hypermethylation at *p15*^{INK4b} and the presence of CLL (Table IV).

DISCUSSION

Aberrant hypermethylation has emerged as a key player in the pathogenesis of CLL since two decades ago. Hypermethylation in gene promoter regions silences the gene inappropriately and leads to tumour progression, thus serving as the only mechanism for loss of function of many genes in tumours, including *p16*^{INK4a} and *p15*^{INK4b}.[25] In this study, hypermethylation at the promoter regions of the two different tumour suppressor genes were

examined in both CLL patients and normal individuals. None of the patients' and control samples showed hypermethylation at $p16^{INK4a}$ hence there was no significant association in this study between methylation at $p16^{INK4a}$ with status of samples (p-value=1.000). A previous study by Tsirigotis *et al* (2006) showed CLL patients had hypermethylation at $p16^{INK4}$ in 17.6% (6/34) of patients.[17] A study by Baur *et al* (1999) discovered that in the same sample of patients with B-cell lymphoma, only 32% (15/47) of the samples have $p16^{INK4a}$ hypermethylation, which was much lower than the 64% (30/47) samples with hypermethylation of $p15^{INK4b}$ at 64% (30/47).[26] According to Chim *et al* (2006), hypermethylation of $p16^{INK4a}$ (14%) was lower compared to $p15^{INK4b}$ (36%) in Chinese patients with newly diagnosed CLL and these irregularities did not correspond with age, stage of cancer and overall survival. However, in this study only a small number of patients had hypermethylation at $p16^{INK4a}$ which might imply that the aberrant hypermethylation observed is either a random event with very low frequency or very

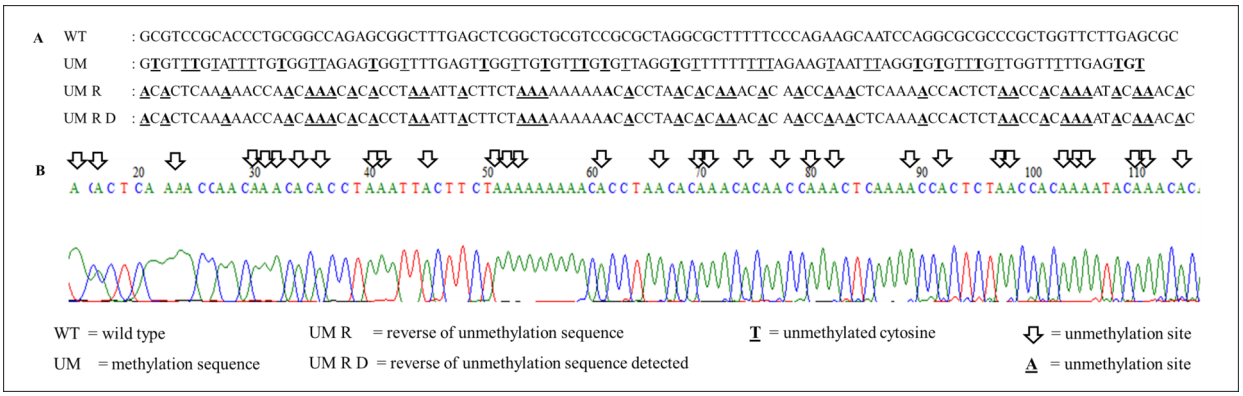

Fig 4 Sequencing result and electropherogram picture of unmethylated sample which was partially methylated at $p15^{INK4b}$

a) Sequencing alignment of unmethylated normal sample at $p15^{INK4b}$ with unmethylated bisulphite-treated $p15^{INK4b}$ sequence which was taken from Methyl Primer Express v1.0 software as reference. Note the presence of many thymidines (T) in the sample sequence after bisulphite conversion and MSP. The Ts represent unmethylated cytosines. Therefore, this sample is confirmed to be unmethylated.

b) Electropherogram picture shows successful Ts' formation which represent the unmethylated cytosine.

In the current study, it is found that for $p15^{INK4b}$, all the control samples as well as 18 samples from patients were unmethylated, while 6 of the patients showed partial methylation with 1 patient showing hypermethylation. It is demonstrated that partial methylation of $p15^{INK4b}$ has a significant association with a status of samples with p=0.01. The MSP technique showed bands corresponding to the amplification of unmethylated $p15^{INK4b}$ in many of the healthy samples.

This finding was expected by the researchers since mononuclear cells isolated from B-CLL patients usually represent a mixture of normal and malignant cells, resulting in the existence of both methylated and unmethylated $p15^{INK4b}$ bands. In a previous study by Baur *et al*, (1999) three out of eight patients with small lymphocytic non-Hodgkin's lymphoma were hypermethylated at $p15^{INK4b}$ gene.[26] A previous study also showed that $p15^{INK4b}$ promoter methylation was detected in 36% of Chinese patients with newly diagnosed CLL and was not associated with age, stage of disease, sex and overall survival.[16] In the current study, methylation at $p15^{INK4b}$ also showed high association significance with race (p=0.036) and clinical features (p=0.042) of CLL patients.

As a conclusion, MS-PCR assay followed by DNA sequencing are applicable for detecting gene methylation status. Our study suggested that $p15^{INK4b}$ could be an important target gene for hypermethylation in carcinogenesis of CLL. Further study using a larger sample size is warranted to explore the significance of DNA methylation incidence among the CLL patients of the Malaysian population.

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