# Pyrosequencing-based Quantitative Identification of *p16* Methylation in Diffuse Large B-cell Lymphoma at Two Centres in the East Coast of Malaysia

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*Abstract*— Introduction: Methylation of promoter region of p16 leading to gene silencing has been implicated in a wide range of malignancies including lymphomas. In diffuse large B cell lymphoma (DLBCL) particularly, a varying percentage of epigenetic inactivation of p16 promoter region was observed ranging from 16 - 54%. However, quantitative analysis of p16 promoter methylation in DLBCL has not been extensively studied in Malaysia. Objective: This study aims to quantitatively analyse p16 methylation in DLBCL samples using pyrosequencing technique. Methods: Genomic DNA was extracted from 16 formalin-fixed paraffin-embedded lymphoma tissue blocks from patients diagnosed with DLBCL. Samples were retrieved from Hospital Tengku Ampuan Afzan, Pahang and Hospital Universiti Sains Malaysia, Kelantan. Primers were designed to amplify bisulfite-treated DNA targeting p16 promoter region. Methylation status of 7 CpG sites was determined by pyrosequencing. Results: All the 16 samples studied showed promoter methylation of p16. The range of mean methylation percentage was between 18 to 81%. Conclusion: The present study has successfully measured the level of methylation of p16 in all 7 CpG sites despite the limitation in sample size. Since p16 methylation is a common event in our series of DLBCL cases, it is worth including a larger sample size in future studies to increase the chance of finding a significant correlation with clinical parameters.

***Keywords*— DLBCL, methylation, p16, pyrosequencing.**

## 1 INTRODUCTION

Diffuse large B cell lymphoma (DLBCL) is the most common type of lymphoma worldwide, accounting for nearly 30% of all lymphoid malignancies, and occurs in a broad age range with a median age of 70 years [1]. DLBCL is highly heterogeneous in immunophenotype, morphologic features, biologic performance and the response towards chemotherapy regimens [1]. To date, the aetiology of DLBCL is still largely unknown. Many research studies have been conducted on acquired modifications of genomic aberrations that may contribute to the pathogenesis of DLBCL. Amongst others these studies looked at the activation of the NF-kB pathway, translocation of *BCL6* gene, *TP53* mutation and *BCL2* gene alterations that have all been identified as common molecular dysregulations in DLBCL [2,3].

Lymphomagenesis does not only encompasses genomic alterations (such as mutations and single nucleotide polymorphism), but has also been shown to involve aberrant epigenetic-regulatory system. DNA methylation is a mechanism of epigenetic regulation of gene expression and its abnormality is believed to contribute significantly to the pathogenesis of lymphoma. Uniquely, DNA methylation involves no changes in the nucleotides and may be reversible [4]. Basically it is the addition of a methyl ($CH_3$) group to the cytosine base of CpG islands typically in the promoter region which may then lead to silencing of the particular gene [5, 6]. Cytosine methylation mediated gene silencing is linked to the poor expression of tumour suppressor gene and certain growth-regulatory proteins in various lymphoid malignancies [5]. The numerous findings made related to

epigenetic gene silencing in cancers have led to the development of epigenetic biomarkers and targeted therapy [4].

p16 gene on chromosome 9p21 is an important tumour suppressor gene involved in the p16/cyclin-dependent kinase (CDK) / retinoblastoma gene pathway of cell cycle control [7]. In addition to point mutations and deletion of *p16*, hypermethylation of promoter region of *p16* has been implicated in a wide range of malignancies including lymphomas [8,9]. In DLBCL particularly, a varying percentage of epigenetic inactivation of *p16* promoter region was observed ranging from 16-54% [10]. Information in this area for Malaysia is still lacking.

The most common type of assay used in DNA methylation study is methylation-specific PCR (MSP) following bisulfite treatment. It is sensitive and specific in detecting cytosine methylation in a CpG island [11]. Although it is a widely utilized and cost-effective method for determination of gene methylation status, the method does not quantify the degree of methylation at specific CpG sites [12,13,10]. Therefore, in order to allow for quantitative methylation analysis, several methods have been established. Methods that are capable of measuring the methylation level and pattern of either, a single CpG or few CpGs include High-Resolution Melting (HRM) analysis, Combined Bisulfite Restriction Analysis (COBRA), Bisulfite Genomic Sequencing, Methylation Sensitive-Single Nucleotide Primer Extension (MS-SNuPE), MethyLight and the recently established pyrosequencing technique [13].

In this study, the PCR-based technique, pyrosequencing assay was used to quantitatively analyse *p16* methylation in DLBCL samples, a sequencing-by-synthesis method that quantitatively evaluates the real-time incorporation of nucleotides through the enzymatic conversion of released pyrophosphate into a proportional light signal [14]. After bisulfite treatment and PCR, the degree of each methylation at each CpG position in a sequence is determined from the ratio of T and C [15,16, 17]. Therefore, the aim of this study then was to examine *p16* gene methylation in DLBCL tumour tissues using pyrosequencing assay.

## 2 METHODOLOGY

A total of 16 formalin-fixed paraffin-embedded (FFPE) lymphoma tissue blocks from patients diagnosed with DLBCL according to WHO classification were retrieved from Hospital Tengku Ampuan Afzan, Pahang and Hospital Universiti Sains Malaysia, Kelantan, of these six were females and 10 were males. The age range was 27 to 84 years. The study was approved by the Ethics Committees from the International Islamic University Malaysia (IREC 170) and Universiti Sains Malaysia (USM/JEPeM/15100447).

Genomic DNA was extracted from the tissue block sections using E.Z.N.A® FFPE DNA Omega Biotek kit as specified by the manufacturer. The purity and quantity of DNA was determined using NanoDrop 1000 (Thermo Scientific, US). DNA purity of the samples was measured as 260/280 ratio, and a ratio of ~1.8 was accepted as purified dsDNA. The DNA samples were subsequently subjected to bisulfite treatment using EZ DNA Methylation-Lightning™ Kit (Zymo Research, Orange, CA) according to protocol stipulated by the manufacturer. In brief, 200-500 ng of each DNA sample was incubated with Lightning Conversion reagent at 98°C for 8 minutes and 54°C for 60 minutes. In a Zymo-Spin™ IC Column, the solution was further incubated at room temperature for 20 minutes following the addition of L-Desulphonation Buffer. The bisulfite-treated DNA was subsequently eluted in a total volume of 20 µL. Sodium bisulfite converts unmethylated cytosine to uracil while 5-methylcytosine remains unchanged and it therefore identifies all methylated and unmethylated CpG positions present in the genomic DNA [12].

The primers were designed using PyroMark Assay Design 2.0 software. The following primers were used for amplification of bisulfite-treated DNA targeting *p16* promoter region: Forward 5'-AGAGGATTTGAGGGATAGGG-3', reverse 5'-biotin-TACCTACTCTCCCCCTCT-3'. The amplicon length was 135 base pairs covering 7 CpG positions (Figure 1a). The PCR reaction was performed in a final volume of 25µl, containing 12.5 µl of PyroMark PCR Master Mix (1X), 2.5 µl of coral load, 5 µl of Q-solution, 0.75 µl of each forward and reverse primers (0.3 µM), 2 µl of bisulfite-treated DNA and 1.5 µl distilled water. The PCR cycling conditions were as follows: initial denaturation (95°C, 15 minutes); followed by 50 cycles of denaturation, annealing, and elongation at 94°C for 30 seconds, 57°C for 40 seconds, and 72°C for 40 seconds respectively. The final extension step was done at 72°C for 10 minutes.

Approximately 20 µl of the PCR product were immobilized on streptavidin-coated Sepharose beads (Streptavidin Sepharose™ High Performance, GE Healthcare, Sweden). The PCR products bound to the beads were purified and denatured into single-stranded DNA using PyroMark Q96 Vacuum Workstation according to the manufacturer's instructions. The single-stranded DNA templates were subsequently added to the annealing buffer containing 0.3µM of sequencing primer
5'-GGTTGGTTGGTTATTAGA-3'.

The sequence to be analysed was
5'-GGGTGGGGYGGATYGYGTG YGTTYGGYG GTTGYGGAGAGGGGGAGAGTAGGTAG-3'.
The sequencing reaction was accomplished by PyroMark Q96 ID (Qiagen) using the PyroMark® Gold Q96 Reagents (Qiagen). Triplicates were performed in samples with sufficient DNA. Protocol was performed until the pyrosequencer determined the results as PASS. The percentage of methylation was assigned to each of the CpG sites of each sample by evaluating the C/T ratio. The mean percentage of methylation was obtained for each of the analysed samples. SPSS version 12.0 was used to perform the statistical analysis. Spearman's correlation analysis was used to find any correlation between mean methylation level of *p16* with age and gender of patients. Significance level was set at 0.05.

Biotin-forward PCR pyro primer →
5'- ***AGAGGATTTGAGGGATAGGG***TCGGAGGG
GGTTTTTTCGTTAGTATCGGAGGAAGAAAGG
AGGGGTTGGTGGTTATTAGAGGGTGGGGCG
ATCGCGTGCGTTCGGCGGTTGCG
***GAGAGGGGAGAGTAGGTA***GCGGGCGGCGG
← Reverse PCR pyro primer

**Figure 1a.** Schematic diagram of *p16* promoter region (bisulfite-treated) analysed showing the starting points for PCR reactions of pyrosequencing. The shaded regions 'C' were the CpG sites analysed.

## 3 RESULTS

DLBCL tissues from 16 patients were evaluated for p16 methylation. Seven different CpG sites were assessed. The methylation level for DNA control set; unmethylated and methylated was 4% and 94%, respectively as shown in Table 1. All the 16 samples studied showed promoter methylation of p16. The range of mean methylation percentage was 18-81%. All samples except for 'Sample ID 13' (in which CpG 1 and CpG 3 sites showed absence of methylation) exhibited methylation of all the 7 CpG sites analysed. A representative pyrogram illustrating the methylation level detected in seven analysed CpGs within the *p16* gene sequences is shown in Figure 1b. Using Spearman non-parametric correlation, there was no association between p16 mean methylation percentage and the age (p=0.83) and gender (p=0.29) of the patients (Table 2).

**Table 1.** The methylation level of the seven CpG sites analysed in 16 samples by pyrosequencing assay.

| Sample ID | Methylation level (%) CpG 1 | CpG 2 | CpG 3 | CpG 4 | CpG 5 | CpG 6 | CpG 7 | Mean (±SD) |
|---|---|---|---|---|---|---|---|---|
| **1** | 12 | 15 | 22 | 25 | 21 | 16 | 18 | 18(5) |
| **2** | 4 | 21 | 21 | 37 | 19 | 16 | 22 | 20(10) |
| **3** | 8 | 28 | 20 | 41 | 25 | 26 | 26 | 25(10) |
| **4** | 18 | 28 | 37 | 42 | 44 | 26 | 25 | 31(10) |
| **5** | 20 | 42 | 33 | 41 | 41 | 34 | 28 | 34(8) |
| **6** | 15 | 42 | 15 | 38 | 41 | 38 | 48 | 34(13) |
| **7** | 25 | 38 | 34 | 49 | 37 | 35 | 32 | 36(7) |
| **8** | 47 | 50 | 45 | 44 | 45 | 45 | 44 | 46(2) |
| **9** | 39 | 55 | 43 | 58 | 56 | 51 | 54 | 51(7) |
| **10** | 29 | 54 | 48 | 70 | 66 | 53 | 50 | 53(13) |
| **11** | 46 | 69 | 48 | 63 | 70 | 58 | 61 | 59(9) |
| **12** | 49 | 75 | 63 | 87 | 91 | 59 | 55 | 68(16) |
| **13** | 0 | 100 | 0 | 94 | 100 | 92 | 100 | 69(48) |
| **14** | 51 | 81 | 63 | 84 | 81 | 65 | 65 | 70(12) |
| **15** | 71 | 86 | 68 | 81 | 82 | 79 | 82 | 78(7) |
| **16** | 26 | 96 | 53 | 96 | 100 | 93 | 100 | 81(29) |
| **Mean for each CpG** | 28.7 | 55 | 38.3 | 59.3 | 57.4 | 49.1 | 50.6 | |
| **M** | 93 | 100 | 94 | 94 | 100 | 86 | 93 | 94(5) |
| **U** | 3 | 5 | 3 | 3 | 5 | 3 | 4 | 4(1) |

**M = methylated control; U = unmethylated control.**

**Table 2.** Correlation between *p16* methylation percentage and age and gender

| Characteristics | Methylation percentage |
|---|---|
| Age (27-84 years) | 0.83 |
| Gender Male (n=10) Female (n=6) | 0.29 |

Significant correlation p<0.05

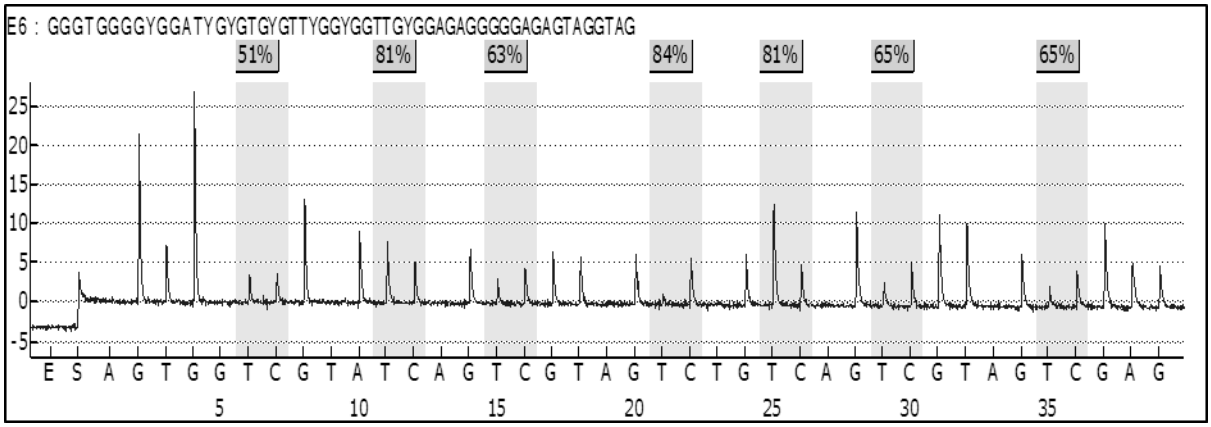


**Figure 1b**. Representative pyrogram for sample 4 showing quantification of methylation for 7 CpGs (shaded).

## 4 DISCUSSION

In this study, we examined the promoter methylation of *p16* gene in sixteen archived specimens diagnosed with DLBCL using the pyrosequencing analysis, and to our knowledge, this is the first study done in Malaysia.

Pyrosequencing is a PCR-based method. It quantifies the presence of gene methylation using small sized PCR products (100-150bp). This is considered an advantage particularly when the starting material is isolated genomic DNA obtained from archival samples such as FFPE tissues, as in the present work [14]. In the analysis of DNA methylation patterns, pyrosequencing reproducibly and accurately measure of degree of methylation at several CpGs in close proximity with high quantitative resolution [16].

Our experience indicates that pyrosequencing is a simple method, cost effective and non-time consuming as the method can analyse 96 samples within a few hours (in a single assay). It reproducibly analyses both CpG rich and CpG poor regions [18]. Although we did not analyse any normal tissue, this method can be utilized to determine the threshold that discriminate DNA methylation in normal versus tumour tissues [18].

We found that all our 16 DLBCL samples (100%) exhibited promoter methylation of *p16*. Though we were not able to analyse normal (non-tumorous) samples for pyrosequencing and perform dilution series to validate our results, we have included 100% methylated and non-methylated controls in the analysis, of which the quality has been validated by the manufacturing company. Previously, Zainuddin *et.al* [10] reported a lower percentage (37.2%) using the pyrosequencing assay. They however examined a larger number of cases (113) on four CpG sites. It has to be noted that the difference in methylation percentages was also due to the different CpG sites analyzed. Other methylation studies in DLBCL did not apply the pyrosequencing technology, hence a direct comparison on the similarities or differences of methylation percentages could not be made. On the other hand, using the qualitative method MSP, Yoon *et.al* [19] showed that 52% of their DLBCL cases harbour *p16* methylation. Hypermethylation of *p16* promoter was also demonstrated by Amara *et.al* [20] in 21 (46%) of 46 DLBCL cases studied. Although MSP is a cost effective method to study DNA methylation, it is associated with high rate of false positives and several limitation in the analysis of CpG-poor regions with lack of ability to quantify the results, hence providing only information related to presence or absence of methylation [18].

Shaw *et al.* [21] and Bihl *et al.* [7] have also utilized the pyrosequencing technology to determine the percentage of *p16* methylation in different types of cancers, namely oral cancer and colorectal cancer. To be highlighted, the study conducted by Shaw *et.al* [21] showed that promoter methylation analysis using this method generated valuable quantitative data from several CpG sites as compared to qualitative data in MSP. Havik *et al.* [17] used both pyrosequencing and quantitative MSP methods to study *MGMT* methylation in gliomas and found that pyrosequencing is a better analytical method as well. It has been established through many studies that epigenetic silencing of the tumor suppressor genes namely *p16* gene is an important and common event in the pathogenesis of malignancies [22, 9]. It acts as an alternative mechanism to homozygous deletion and point

mutations in which it equally contributes to the loss of gene expression [8,23]. As *p16* is involved in regulating the cell cycle through inhibition of kinase enzymes (CDK4 and CDK6) in order to control the progressive entry from G1 to S phase, transcriptionally silenced or lack of *p16* has the potential to initiate inappropriate cell proliferation [9]. In aggressive lymphoma, lack of p16 protein in the absence of *p16* gene deletions or mutations, and only cytosine methylation has been reported [24].

Other studies have also demonstrated that inactivation of *p16* epigenetically and loss of *p16* protein expression was common events in high-grade B cell lymphoma and progression of low-grade lymphoma into DLBCL [23,25]. DLBCL with hypermethylated *p16* has been reported to commonly show a biologically aggressive phenotype and is associated with a worse prognosis [20]. There were also significant associations with B symptoms, high International Prognostic Index (IPI) scores, and advanced clinical stage [10, 20]. Though it is well-noted that the information on clinical stages of DLBCL is crucial particularly for survival analysis, we regrettably do not have complete information on stages for most of the samples. Moreover, this study has limited number of samples with high quality DNA for analysis. Nevertheless, the samples used in this study have been confirmed histologically to show >70% of cancer cells.

The study has successfully utilized the pyrosequencing technology to identify promoter methylation of *p16* in all 16 cases of DLBCL. However, the sample size utilized was limited, hence no correlation could be made with the clinical parameters. Since *p16* methylation is a common event in our series of DLBCL cases, future studies should include a bigger sample size, clinicopathological information of the cases and analysis of the non-tumorous (normal) tissue.

## CONFLICTS OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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## REFERENCES

[1] Hunt K.E. and Reichard K.K. *Diffuse large B-Cell Lymphoma*. Arch. Pathol. Lab. Med. 2008; 132:pp.118-124.

[2] Chan W.C. *Pathogenesis of diffuse large B cell lymphoma*. Int. J. Hematol. 2010; 92:pp.219-230.

[3] Perry A.M., Mitrovic Z. and Chan W.C. *Biological Prognostic Markers in Diffuse Large B-Cell Lymphoma*. Cancer Control. 2012; 19:pp.214-226.

[4] Choo, K.B. *Epigenetics in disease and cancer*. Malaysian Journal of Pathology. 2011; 33:pp.61-70.

[5] Baylin S.B. *DNA methylation and gene silencing in cancer*. Nat. Clin.Prac. Oncol. 2005; 2:S4-S11.

[6] Mulero-Navarro S. and Esteller M. *Epigenetic biomarkers for human cancer*: *The time is now*. Oncol. Hematol. 2008; 68:pp.1-11.

[7] Bihl M.P., Foerster A., Lugli A. and Zlobec I. *Characterization of CDKN2A(p16) methylation and impact in colorectal cancer: systemic analysis using pyrosequencing*. J. Transl. Med. 2012; 10:pp.1-10.

[8] Drexler H.G. *Review of alterations of the cyclin-dependent kinase inhibitor INK4 family genes p15, p16, p18 and p19 in human leukemia-lymphoma cells.* Leuk. 1998; 12:pp.845-859.

[9] Li J., Poi M.J. and Tsai M.D. *The Regulatory Mechanisms of Tumor Suppressor P16INK4A and Relevance to Cancer.* Biochem. 2011; 50:pp.5566-5582.

[10] Zainuddin N., Kanduri M., Berglund M., Lindell M., Amini R.M., et al. *Quantitative evaluation of p16INK4a promoter methylation using pyrosequencing in de novo diffuse large B-cell lymphoma.* Leuk. Res. 2011; 35:pp.438-443.

[11] Herman J.G., Graff J.R., Myohanen S., Nelkin B.D. and Baylin S.B. *Methylation-specific PCR: A novel PCR assay for methylation status of CpG islands.* Med. Sci. 1996; 93: 9821-9826.

[12] Mikeska T., Bock C., El-Maari O., Hubner A., Ehrentraut D., et al. *Optimization of Quantitative MGMT Promoter Methylation Analysis Using Pyrosequencing and Combined Bisulfite Restriction Analysis*. J. Mol. Diagn. 2007; 9:pp.368-381.

[13] Mikeska T., Candiloro I.L.M. and Dobrovic A. *The implications of heterogeneous DNA methylation for the accurate quantification of methylation*. Epigenomics. 2010; 2:pp.561-573.

[14] Colella S., Shen L., Baggerly K.A., Issa J.P.J. and Krahe R. *Sensitive and quantitative universal PyrosequencingTM methylation analysis of CpG sites.* BioTechniques. 2003; 35:pp.146-150.

[15] England R. and Pettersson M. *Quantitative analysis of methylation at multiple CpG sites by Prosequencing™*. Nature Methods. 2005.

[16] Tost J. and Gut I.G. *DNA methylation analysis by pyrosequencing*. Nat Protoc. 2007; 2:pp.2265-2275.

[17] Havik A.B., Brandal P., Honne H., Dahlback H.S.S.,

Scheie D., et al. *MGMT promoter methylation in gliomas assessment by pyrosequencing and quantitative methylation-specific PCR.* J. Transl. Med. 2012; 10:pp.1-13.

[18] Roessler J. and Lehmann U. Quantitative DNA Methylation Analysis by Pyrosequencing. In Pyrosequencing Methods and Protocols, Methods in Molecular Biology. pp 175-188. Springer Science+Business Media New York. 2015.

[19] Yoon S.O., Kim Y.A., Jeon Y.K., Kim J.E., Kang G.H., et al. *Diffuse Large B Cell Lymphoma Shows Distinct Methylation Profiles of the Tumor Suppressor Genes among the Non-Hodgkin's Lymphomas*. Korean J. Pathol. 2008; 42:pp.16-20.

[20] Amara K., Trimeche M., Ziadi S., Laatiri A., Hachana M., et al. *Progonostic significance of aberrant promoter hypermethylation of CpG islands in patients with diffuse large B-cell lymphoma*. Ann. Oncol. 2008; 19:pp.1774-1786.

[21] Shaw R.J., Lilogluo T., Rogers S.N., Brown J.S., Vaughan E.D., et al. *Promoter methylation of P16, RARβ, E-cadherin, cyclin A1 and cytoglobin in oral cancer: quantitative evaluation using pyrosequencing.* Br. J. Cancer. 2006; 94:pp. 561-568.

[22] Delgado M., Piqueras J.F., Garcia M.J., Arranz E., Rivas C., et al. *Hypermethylation of a 5' CpG island of p16 is a frequent event in non-Hodgkin's lymphoma.* Leuk. 1997; 11:pp.425-428.

[23] Baur A.S., Shaw P., Burri N., Delacretazz F., Bosman F.T., et al. *Frequent Methylation Silencing of $p15^{INK4b}$ (MTS2) and $p16^{INK4a}$ (MTS1) in B-Cell and T-Cell Lymphoma*. Blood. 1999; 94:pp1773-1781.

[24] Pinyol M., Cobo F., Bea S., Jares P., Nayach I., et al. *p16INK4a Gene Inactivation by Deletions, Mutations, and Hypermethylation Is Associated With Transformed and Aggressive Variants of Non-Hodgkin's Lymphomas.* Blood. 1998; 91:pp.2977-2984.

[25] Villuendas R., Beato M.S., Martínez J.C., Saez A.I., Delgado B.M., et al. *Loss of p16/INK4A Protein Expression in Non-Hodgkin's Lymphomas Is a Frequent Finding Associated with Tumor Progression.* Am. J. Pathol. 1998; 153:pp.887-897.