RESEARCH ARTICLE

p16 Tumor Suppressor Gene Methylation in Diffuse Large B Cell Lymphoma: A Study of 88 Cases at Two Hospitals in the East Coast of Malaysia

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Abstract

   Introduction: *p16* gene plays an important role in the normal cell cycle regulation. Methylation of *p16* has been reported to be one of the epigenetic events contributing to the pathogenesis of diffuse large B-cell lymphoma (DLBCL) which occurring at varying frequency. DLBCL is an aggressive and high-grade malignancy which accounts for approximately 30% of all non-Hodgkin lymphoma cases. However, little is known regarding the epigenetic alterations of *p16* gene in DLBCL cases in Malaysia. Therefore, the objective of this study was to examine the status of *p16* methylation in DLBCL. **Methods:** A total of 88 formalin-fixed paraffin-embedded DLBCL tissues retrieved from two hospitals located in the east coast of Malaysia, namely Hospital Tengku Ampuan Afzan (HTAA) Pahang and Hospital Universiti Sains Malaysia (HUSM) Kelantan, were chosen for this study. DNA specimens were isolated and subsequently subjected to bisulfite treatment prior to methylation specific-PCR. Two pairs of primers were used to amplify methylated and unmethylated regions of *p16* gene. The PCR products were then separated using agarose gel electrophoresis and visualised under UV illumination. SPSS version 12.0 was utilised to perform all statistical analysis. **Result:** *p16* methylation was detected in 65 of 88 (74%) samples. There was a significant association between *p16* methylation status and patients aged >50 years old (p=0.04). **Conclusion:** Our study demonstrated that methylation of *p16* tumor suppressor gene in our DLBCL cases is common and significantly increased among patients aged 50 years and above. Aging is known to be an important risk factor in the development of cancers and we speculate that this might be due to the increased transformation of malignant cells in aging cell population. However, this has yet to be confirmed with further research and correlate the findings with clinicopathological parameters.

Keywords: DNA methylation- methylation specific PCR- *p16*- diffuse large B cell lymphoma

Introduction

   According to the Malaysian National Cancer Registry Report for the year 2007, there was a total of 776 lymphoma cases diagnosed in the government hospitals nationwide. Lymphoma was ranked as the sixth most common cancer among males and eighth among females (NCR Report, 2007). Diffuse large B-cell lymphoma (DLBCL) is one of major subtypes of non-Hodgkin lymphoma (Hagiwara et al., 2007). It is the most common type of lymphoma among adults worldwide and it accounts for approximately 30% of all lymphoid malignancies (Lossos et al., 2004; Zainuddin et al., 2011). DLBL cases are heterogeneous in morphologic appearance, immunophenotype and biologic pattern, as well as response to chemotherapy and overall survival (Lossos et al., 2004; Hiraga et al., 2006; Yang et al., 2007; Hunt and Reichard, 2008).

   DLBCL presents a high degree of genomic complexity as both somatic mutations and gross genomic alterations, such as chromosomal translocations and copy number changes of specific chromosomal regions, contribute to its pathogenesis (Testoni et al., 2015). Cell cycle deregulation, disturbances in repair of DNA damage and perturbations of transcriptional control of apoptosis are among the genetic alterations documented in DLBCL (Zainuddin et al., 2011). In addition to genetic aberrations, epigenetics changes have been implicated in the molecular pathogenesis of DLBL. As opposed to genetic mutations, epigenetic abnormalities are reversible (CHOO, 2011). Among the widely studied mechanisms it also include DNA methylation, chromatin remodelling and microRNA (CHOO, 2011). To date, DNA methylation has been

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shown to be the main epigenetic event taking place in lymphomagenesis leading to silencing of the tumour suppressor genes (Baylin, 2005).

p16 (also known as CDKN2A) is a tumour suppressor gene and a member of the INK family of cyclin-dependent kinase (CDK) inhibitors located on human chromosome 9p21.3 (Lee et al., 2010; Bihl et al., 2012). *p16* protein (*p16*) is a negative cell cycle regulator involved in the blockade of G1 phase progression of the cell cycle (Khor et al., 2013). Promoter methylation of *p16* has been reported to be one of the epigenetic events contributing to the pathogenesis of DLBCL and occurring at varying frequency (Zainuddin et al., 2011). This event causes the chromatin to be inactive and leads to gene silencing during the transcriptional process (Sulewska et al., 2007; Khor et al., 2013). It is possible that inappropriate gene inactivation by DNA methylation may result in malignant transformation due to the instability of genetic environment of the transformed cells (Deep et al., 2012).

In Malaysia, Lee et al., (2010) revealed that more than 70% of the DLBCL cases in their study exhibited loss of *p16* gene as evident by monosomy 9, hemizygous and homozygous deletions. However much less is known with regard to the epigenetic alterations of *p16* gene in DLBCL cases in Malaysia (Lee et al., 2010). Therefore, this has prompted us to examine the status of *p16* methylation in DLBCL locally.

Materials and Methods

Samples

Twenty-five and 63 formalin-fixed, paraffin-embedded (FFPE) tissue blocks of DLBCL cases from which diagnosis was made from year 2006 to 2013 have been retrieved from HTAA, Pahang and HUSM, Kelantan, respectively. The selection of specimens were based on the availability of completed sociodemographic data (age and gender) and the quality of extracted DNA. Overall, the archived samples were from 35 male and 53 female patients with a median age of 55 years old (range, 1-84 years). All samples were diagnosed and reconfirmed by pathologists as DLBCL according to the WHO classification. Five normal lymph node tissue blocks were also obtained from HTAA. The protocol of this study was approved by the International Islamic University Malaysia Research Ethics Committee (IREC 170) and Human Research Ethics Committee USM (USM/JEPeM/15100447).

DNA Extraction and Bisulfite Treatment

Genomic DNA was extracted from the tissue block sections using E.Z.N.A ® FFPE DNA (Omega Biotek, US) kit as specified by the manufacturers. The purity and quantity of DNA was determined using NanoDrop 1000 (Thermo Scientific, US) with purity of the samples measured as 260/280 ratio in which ~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 the manufacturers. Briefly 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 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 then eluted in a total volume of 20 µL.

Sodium bisulfite identifies all methylated and unmethylated CpG islands present in the genome (Sulewska et al., 2007). It converts all unmethylated cytosines to uracil which is located on single-stranded DNA and leaves methylated cytosines intact (Chim et al., 2001; Nygren et al., 2005; Wang et al., 2008).

Methylation-Specific Polymerase Chain Reaction Analysis (MSP)

The methylation status at promoter region of *p16* was assessed by MSP using methylation specific primers. The primer sequences were as previously reported (Table 1) (Wang et al., 2008). The PCR mixture contained 10 ng of bisulfite-treated DNA, ZymoTaqTM PreMix (1X), and 0.2 µM of each primer in a final volume of 25 µL. The PCR cycling conditions were as follows: 95°C for 10 minutes; then 38 cycles at 95°C for 30 seconds, 60°C (*p16* unmethylated) or 64°C (*p16* methylated) for 40 seconds and 72°C for 40 seconds; a final extension of 7 minutes at 72°C. Human methylated and non-methylated sets (Zymo Research, Orange, CA) were used as positive and negative controls, respectively. Finally, 5 µL of the PCR products were visualised on 2% ethidium-bromide stained agarose gels under UV illumination. For each case, MSP results were recorded as methylated when a clear visible band on the agarose gel at methylated lane is observed, with or without unmethylated band.

Statistical Analysis

The relationship between *p16* methylation status and patients' demographic parameters was examined using Pearson's Chi-square test. The results were reported as statistically significant when p<0.05. Confidence intervals of 95% were used to determine the effect of each variable on outcome.

Results

Sixty five (65) out of 88 (74%) DLBCL samples were found to exhibit *p16* gene methylation. There was no *p16* methylation identified in the five normal lymph nodes samples. The representative results of PCR products are illustrated in Figure 1. Using Pearson's Chi-square analysis, we found a significant relationship between *p16* methylation status and patients' age of > 50 years old (p=0.04) (Table 2). The categorization of age is based on

Table 1. PCR Primers of *p16* for MSP Analysis

Genes	Primer sequences (5´ -> 3´)	Product size (bp)
p16 M F	TTATTAGAGGGTGGGGCGGATCGC	150
p16 M R	GACCCCGAACCGCGACCGTAA	
p16 U F	TATTAGAGGGTGGGGTGGATTGT	151
p16 M R	CAACCCCAAACCACAACCATAA	

M, methylated; U, unmethylated; F, forward; R, reverse; bp, base pair

Table 2. Correlation between *p16* Gene Methylation Status and Patients' Demographic Data in DLBCL

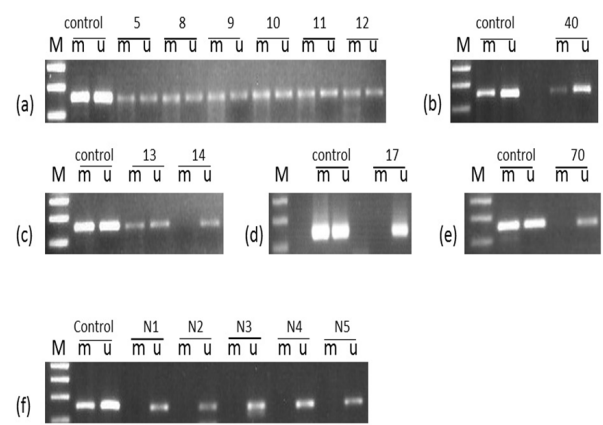| Characteristics	All	*p16*	
		U (n=23)	M (n=65)
Age			
≤50 years	31	12	19
>50 years	57	11	46
			0.04*
Gender			
Female	53	13	40
Male	35	10	25
			0.67

*Chi-square p<0.05; M, methylated; U, unmethylated

Figure 1. Representative Results of Methylation-Specific PCR analysis. (a) 5, 8, 9, 10, 11 and 12, methylated; (b) 40, methylated; (c) 13, methylated; 14, unmethylated; (d) 17, unmethylated; (e) 70, unmethylated; (f) Normal lymph nodes (N1 – N5), unmethylated. m , marker; m, methylated; u, unmethylated

Discussion

In the present study, we have investigated a cancer-related *p16* gene methylation qualitatively using methylation-specific PCR on DLBCL specimens. Interestingly, *p16* methylation was detected at a high frequency, which was 65 (74%) out of 88 samples. To the best of our knowledge, this is the first study identifying *p16* gene methylation in DLBCL in Malaysia.

In a normal cell cycle, during the early G1 phase, retinoblastoma protein (pRb) is hypophosphorylated, while in the later phase of G1, in order for a cell to enter the S phase, pRb is progressively hyperphosphorylated by CDK4 and CDK6 (Satyanarayana and Rudolph, 2004; Li et al., 2011). The role of *p16* is observed at the point of G1-to-S phase transition in which it inactivates the kinase (CDK4 and CDK6) catalytic activities needed for phosphorylation of pRb and thereby inducing blockage of the cell cycle progression (Li et al., 2011). Methylation mediated silencing of *p16* gene might therefore trigger unregulated cell proliferation due to aggressive process of cell cycle leading to the malignant transformation and has been proposed as one of the early events in the development of lymphoid malignancies (Takino et al., 2005). There also appears to be evidence supporting the role for *p16* inactivation in the transformation of low-growth fraction lymphomas into their aggressive variants (Baur et al., 1999; Huang et al., 2004). Takino et al., (2005) documented *p16* loss due to methylation in MALT lymphoma (60%) with transformation to DLBCL. In essence, the role of aberrant DNA methylation in the pathogenesis of DLBCL would require further comprehension of the biochemical and biological effects of epigenetic modifications on lymphomagenesis (Shaknovich and Curr, 2011). Also, as epigenetic abnormalities are reversible, further understanding of *p16* methylation role in the pathogenesis of DLBCL could pave the way for a targeted therapeutic intervention.

p16 silencing event through methylation in non-Hodgkin lymphoma occurs in a varied frequency ranging from 9% to 72% (Zainuddin et al., 2011). Guney et al., (2012) showed that *p16* inactivation by deletion or methylation was observed in 42.7% of DLBCL cases studied while Zainuddin et al., (2011) revealed *p16* methylation in 37% of their cases. In this present study, a higher percentage of DLBCL cases (74%) with *p16* methylation was obtained. As we only performed qualitative assessment of the *p16* promoter region methylation, it is possible that the number of positive DLBCL cases could be higher if a quantitative technique such as pyrosequencing is employed in determining the methylation status of this gene as shown by Zainuddin et al., (2011).

Interestingly, we also found statistical significant association between *p16* methylation status and patients aged of more than 50 years old. There was a number of researchers associating *p16* methylation in cancer and the age of patients. However, we could not find similar studies which highlight on the evidence of *p16* methylation and older age in human DLBCL, except for a study by Alhejaily et al., (2014) in follicular lymphoma patients, where CDKN2A deletion or methylation was found to be associated with more advanced age. Aging is known to be an important risk factor in the development of cancers (Ahuja et al., 1998) and we speculate that this might be due to the increased transformation of malignant cells in aging cell population. The process of cellular senescence which causes irreversible growth arrest can be triggered by multiple mechanisms; one of which is epigenetic depression of *p16* which in turn serves as an excellent molecular marker of aging (Collado et al., 2007). It is also noteworthy that *p16* gene methylation is not the sole determinant of *p16* expression (Keyes et al., 2007). DNA-damages, oxygen radicals, ionizing radiation, chemotherapeutic agents and telomere dysfunction including molecular positive regulator of ERK-mediated activation of Ets1/2 are known to cause *p16* elevation (Kim and Sharpless, 2006).

Although *p16* promoter methylation occurs in DLBCL and may be one of the significant events in human lymphomagenesis, its role as a biomarker for diagnostic

of accuracy will need to be further studied (Guney et al., 2012). Amara et al., (2008) found that hypermethylated *p16* of DLBCL was associated with a more aggressive disease and poorer outcome, whereas Zainuddin et al., (2011) questioned its role as a negative factor. We, however in this study, were not able to investigate on the impact of *p16* methylation on the clinical outcomes of our patients due to lack of data. In addition, as there appears to be a good agreement of *p16* methylation with lack of protein expression in B-cell lymphomas (Huang et al., 2004), further research to examine on *p16* expression along with *p16* methylation is highly recommended.

As far, the current study has demonstrated the highest occurrence of *p16* methylation in DLBCL cases obtained from two medical institutions located in the East Coast of Malaysia, and this was achieved using the MSP technique. It indicates that the contribution of *p16* tumour suppressor methylation in DLBCL development might be of high importance. There might be an increased transformation of malignant cells in the aging cell population, as we have found a significant increase in *p16* methylation among patients aged 50 years and above. Moreover, this valuable discovery is supported by the absence of *p16* methylation observed in five normal lymph node tissue samples. Nevertheless, due to the lack of complete clinical data of all patients, we were unable to confirm on the diagnostic or prognostic values of *p16* methylation in our DLBCL samples. Therefore, more research related to quantitative evaluation of *p16* methylation, the mRNA and protein expression, as well as the correlation to clinicopathological parameters are needed as *p16* might be a valuable prognostic biomarker for DLBCL in Malaysia. Other pathways involved in the *p16* DNA methylation-related metastasis phenotype are also worth to be explored.

Statement conflict of Interest

There is no conflict of interest between authors.

References

Ahuja N, Li Q, Mohan AL, Baylin SB, Issa JPJ (1998). Aging and DNA methylation in colorectal mucosa and cancer. *Cancer Res*, **58**, 5489–94.

Alhejaily A, Day AG, Feilotter HE, Baetz T, Lebrun DP (2014). Inactivation of the CDKN2A tumor-suppressor gene by deletion or methylation is common at diagnosis in follicular lymphoma and associated with poor clinical outcome. *Clin Cancer Res*, **20**, 1676-86.

Amara K, Trimeche M, Ziadi S, et al (2008). Prognostic significance of aberrant promoter hypermethylation of CpG islands in patients with diffuse large B-cell lymphomas. *Ann Oncol*, **19**, 1774-86.

Baur A, Shaw P, Burri N, et al (1999). Frequent methylation silencing of p15INK4b (MTS2) and *p16*INK4a (MTS1) in B-Cell and T-Cell Lymphomas. *Blood*, **94**, 1773-81.

Baylin S (2005). DNA methylation and gene silencing in cancer. *Nat Clin Prac Oncol*, **2**, 4-11.

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

Chim C, Liang R, Tam C, Kwong Y (2001). Methylation of p15 and *p16* genes in acute promyelocytic leukemia: potential diagnostic and prognostic significance. *J Clin Oncol*, **19**, 2033-40.

Choo KB (2011). Epigenetics in disease and cancer. *Malaysian J Pathol*, **33**, 61-70.

Collado M, Blasco MA, Serrano M (2007). Cellular senescence in cancer and aging. *Cell*, **130**, 223–33.

Deep J, Sidhu S, Chandel A, Thapliyal S, Garg C (2012). Aberrant methylation in promoters of GSTP1, *p16*, p14, and RASSF1A genes in smorkers of North India. *ISRN*, 1-6.

Guney S, Jardin F, Bertrand P, et al (2012). Several mechanisms lead to the inactivation of the CDKN2A (*P16*), P14ARF, or CDKN2B (P15) genes in the GCB and ABC molecular DLBCL subtypes. *Genes Chromosomes Cancer*, **51**, 858-67.

Hagiwara K, Nagai H, Li Y, et al (2007). Frequent DNA methylation but not mutation of the ID4 gene in malignant lymphoma. *J Clin Exp Hematopathol*, **47**, 15-17.

Hiraga J, Kinoshita T, Ohno T, et al (2006). Promoter hypermethylation of the DNA-repair gene O6-Methylguanine-DNA Methyltransferase and p53 mutation in diffuse large B-cell lymphoma. *Int J Hematol*, **84**, 248-55.

Huang Q, Ai L, Zhang ZY, Chun-Yang F, Weiss LM (2004). Promoter hypermethylation and protein expression of the *p16* gene: analysis of 43 cases of B-cell primary gastric lymphomas from China. *Mod Pathol*, **17**, 416-22.

Hunt K, Reichard K (2008). Diffuse Large B-Cell Lymphoma. *Arch Pathol Lab Med*, **132**, 118-24.

Keyes MK, Jang H, Mason JB, et al (2007). Older age and dietary Folate are determinants of genomic and *p16*-Specific DNA methylation in Mouse colon. *J Nutr*, **137**, 1713–17.

Khor GH, Froemming GA, Zain R, et al (2013). DNA methylation profiling revealed promoter hypermethylation-induced silencing of *p16*, DDAH2 and DUSP1 in primary oral squamous cell carcinoma. *Int J Med Sci*, **10**, 1727-39.

Kim WY, Sharpless NE (2006). Review the regulation of INK4/ARF in cancer and aging. *Cell*, **127**, 265–75.

Lee ES, Kim LH, Abdullah W, Peh SC (2010). Expression and alteration of *p16* in diffuse large b cell lymphoma. *Pathobiology*, **77**, 96-105.

Li J, Poi MJ, Tsai MD (2011). The regulatory mechanisms of tumor suppressor *p16*INK4a and relevence to cancer. *Biochemistry*, **50,** 5566-82.

Lossos I, Czerwinski D, Alizadeh A, et al (2004). Prediction of survival in diffuse large-b-cell lymphoma based on the expression of six genes. *N Eng J Med*, **350**, 1828-37.

Mehrzad J, Hashemi M, Jamshidi M, et al (2014). Aberrant methylation in promoter region induced silencing of *P16*/NK4a in colorectal cancer in Iranian patients. *J Bio Environ Sci*, **4**, 94-103.

Nygren AOH, Ameziane N, Duarte HMB, et al (2005). Methylation-Specific MLPA (MS-MLPA): simultaneous detection of CpG methylation and copy number changes of up to 40 sequences. *Nucleic Acids Res*, **33**, 1-9.

Satyanarayana A, Rudolph K (2004). *p16* and Arf: activation of teenage proteins in old age. *J Clin Invest*, **114**, 1237-40.

Shaknovich R, Curr AR (2011). Epigenetics and B-cell Lymphoma. *Opin Hematol*, **18**, 293–99.

Sulewska A, Niklinska W, Kozlowski M, et al (2007). Detection of DNA methylation in eucaryotic cells. *Folia Histochem Cytobiol*, **45**, 315-24.

Takino H, Okabe M, Li C, et al (2005). *p16*/INK4a gene methylation is a frequent finding in pulmonary MALT lymphomas at diagnosis. *Mod Pathol*, **18**, 1187-92.

Testoni M, Zucca E, Young KH, Bertoni F (2015). Genetic

lesions in diffuse large B-cell lymphomas. *Ann Oncol*, **26**, 1069–80.

Wang JM, Sasco AJ, Fu CW, et al (2008). Aberrant DNA methylation of *p16*, MGMT, and hMLH1 genes in combination with MTHFR C677T genetic polymorphism in esophageal asquamous cell carcinoma. *Cancer Epidemiol Biomarkers Prev*, **17**, 118-25.

Yang E, Kang H, Koh K, et al (2007). Frequent inactivation of SPARC by promoter hypermethylation in colon cancers. *Int J Cancer*, **121**, 567–75.

Zainal AO, Nor Saleha IT (2011). National cancer report 2007, Ministry of Health, Malaysia.

Zainuddin N, Kanduri M, Berglund M, et al (2011). Quantitative evaluation of *p16*ink4a promoter methylation using pyrosequencing in de novo diffuse large b-cell lymphoma. *Leukemia Res*, **35**, 438-43.