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Artikel Asli/*Original Articles*

MGMT and *SPOCK2* Promoter Methylation in Diffuse Large B-Cell Lymphoma: A Study in Two Tertiary Health Centres in the East Coast of Malaysia

(Metilasi Promoter *MGMT* dan *SPOCK2* dalam *Diffuse Large B-cell Lymphoma*: Satu Kajian yang Dijalankan di Dua Pusat Kesihatan di Pantai Timur Malaysia)

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ABSTRACT

MGMT (O^6-Methylguanine-DNA Methyltransferase) suppresses tumor development by removing alkyl adduct, while SPOCK2 (SPARC/Osteonectin CWCV and Kazal-like domains proteoglycan) abolishes the inhibition of membrane-type matrix metalloproteinases (MT-MMP) which leads to angiogenesis. Hence, MGMT methylation may initiate malignant cells transformation. In contrast, SPOCK2 methylation is hypothesized not to be a common event in diffuse large B-cell lymphoma (DLBCL). In this study, we examined the methylation status of MGMT and SPOCK2 in DLBCL as in Malaysia the information is extremely lacking. A total of 88 formalin-fixed paraffin-embedded tissue of patients diagnosed with DLBCL from the year 2006 to 2013 were retrieved from Hospital Universiti Sains Malaysia, Kelantan and Hospital Tengku Ampuan Afzan, Pahang. Methylation-specific polymerase chain reaction (MSP) was used to examine the methylation status of both genes. Interestingly, methylation of MGMT was detected in all the 88 DLBCL samples, whereas SPOCK2 was found to be methylated in 83 of 88 (94.3%) DLBCL cases. Our study showed a remarkably high percentage of promoter methylation of both MGMT and SPOCK2 genes. Our finding also negates initial expectation that SPOCK2 methylation would be an uncommon event in the majority of DLBCL cases. This study has shown a very high percentage of promoter methylation of MGMT and SPOCK2 in the DLBCL cases studied by MSP, using archival lymphoma tissues. Nonetheless, additional research is needed to quantitatively evaluate MGMT and SPOCK2 methylation, and to analyse gene expression and/or protein expression in order to further understand the role of MGMT and SPOCK2 methylation in the pathogenesis of DLBCL.

Keywords: MGMT; SPOCK2; MSP; DLBCL; methylation

ABSTRAK

MGMT (O^6-Methylguanine-DNA Methyltransferase) merencat perkembangan tumor dengan menyingkirkan alkyl adduct, manakala SPOCK2 (SPARC/Osteonectin CWCV and Kazal-like domains proteoglycan) menghalang perencatan matriks metaloproteinase jenis membran (MT-MMP) yang menyebabkan angiogenesis. Oleh itu, metilasi MGMT mampu menyebabkan inisiasi transformasi sel malignan. Sebaliknya, secara hipotesis, metilasi SPOCK2 bukanlah penyumbang yang lazim dalam pembentukan diffuse large B-cell lymphoma (DLBCL). Dalam kajian ini, status metilasi MGMT dan SPOCK2 pada DLBCL telah ditentukan memandangkan kurangnya kajian tentang ini di Malaysia. Sebanyak 88 tisu parafin pesakit yang didiagnosis dengan DLBCL dari tahun 2006 hingga 2013 telah dikumpulkan daripada Hospital Universiti Sains Malaysia, Kelantan dan Hospital Tengku Ampuan Afzan, Pahang. Reaksi rantaian polimerase yang spesifik-metilasi (MSP) telah diaplikasikan bagi memeriksa status metilasi kedua-dua gen. Menariknya, metilasi MGMT dikesan dalam kesemua 88 sampel DLBCL, manakala metilasi SPOCK2 dikesan dalam 83 daripada 88 (94.3%) kes DLBCL. Kajian ini menunjukkan peratusan metilasi promoter gen MGMT dan SPOCK2 yang amat tinggi. Penemuan ini juga menafikan jangkaan awal yang metilasi SPOCK2 adalah jarang berlaku dalam kebanyakan kes DLBCL. Kajian ini telah menunjukkan peratusan metilasi promoter MGMT dan SPOCK2 yang sangat tinggi dalam kes DLBCL yang dikaji melalui kaedah MSP menggunakan arkib tisu limfoma. Walau bagaimanapun, penyelidikan lanjut diperlukan untuk menilai metilasi MGMT dan SPOCK2 secara kuantitatif, dan untuk menganalisa ekspresi gen dan/atau ekspresi protein untuk lebih memahami peranan metilasi MGMT dan SPOCK2 dalam patogenesis DLBCL.

Kata kunci: MGMT; SPOCK2; MSP; DLBCL; metilasi

INTRODUCTION

Diffuse large B cell lymphoma (DLBCL) is an aggressive non Hodgkin lymphoma accounting for 30% of lymphoid cancers, with median age of 70 years (Hunt & Reichard 2008). Overall, in Malaysia, lymphoma is ranked the sixth most common cancer among males and eighth in females (National Cancer Registry Report 2007). DLBCL is

heterogeneous in morphologic features, immunophenotypes, genetic changes and clinical behaviour (Scott et al. 2014; Wang et al. 2010). Research in recent years has provided valuable insight into the molecular mechanisms involved in the pathogenesis of DLBCL. Amongst the findings include pathways leading to immune escape, genetic lesions leading to constitutive NF-κB activity and chronic active BCR signaling, deregulation of BCL6 activity, disruption of the terminal differentiation pathway and epigenetic modification (Pasqualucci 2013).

Epigenetic modification in particular DNA methylation has been intensively studied and is implicated in lymphomagenesis. DNA methylation is a covalent addition of a methyl group (CH3) to the 5' carbon position of cytosine by DNA methyltransferases (DNMTs), thus triggering histone deacetylases (HDACs) to deacetylate and literally change the chromatin structure (Baylin 2005). Hence, the genes would not be in a readable state and would fail to be transcribed by transcriptional factors. In cells of the normal state, during tissue differentiation and development, transcription-relevant control regions in the genome become selectively de-or upmethylated to allow for transcription of a restricted set of genes within a given tissue (Li et al. 2013). In the pathogenesis of DLBCL, DNA methylation has been shown to be disrupted. The aberrant methylation involving hypermethylation of gene regulatory regions can lead to transcriptional silencing and hence resulting in poor expression of tumour suppressor gene and certain growth-regulatory proteins. Recent studies have further suggested that regulation of gene expression by DNA methylation patterning is complex and it is not a simple "on" or "off" switch for gene expression (Jiang et al. 2013).

The *MGMT* (*O^6-Methylguanine-DNA Methyltransferase*) is a DNA repair gene located on chromosome 10q26. It encodes DNA repair protein that removes alkyl groups from the O^6 positions of guanine an important site for DNA alkylation to the active cysteine site within its own sequence thereby preventing the formation of lethal cross-links and other mutagenic effects (Esteller et al. 1999, Mehrzad et al. 2014; Smith-Sørensen et al. 2002). This single-enzymatic pathway is termed as suicidal reaction as for each lesion repaired, one *MGMT* molecule would be inactivated (Esteller et al. 1999). When *MGMT* expression is repressed in cancers, this is often due to methylation of its promoter region (Hegi et al. 2005). The presence of *MGMT* methylation was reported in many types of human malignancies such as gliomas, colorectal carcinoma, non-small cell lung carcinoma and lymphoma (Mulero-navarro & Esteller 2008). A methylation profile study conducted by Yoon et al. demonstrated that epigenetic inactivation of *MGMT* ranging from 12.5% to 37.5% among the different types of non-Hodgkin lymphoma (Yoon et al. 2008). Specifically, they found *MGMT* gene to be methylated in 30.4% of DLBCL cases. Additionally, the immunohistochemistry (IHC) detection of *MGMT* gene hypermethylation in DLBCL was validated by Ucella et al. (2009) using the quantitative real-time methylation-specific PCR assay. 27% of the DLBCL cases in their study were *MGMT*-negative at IHC; however, there was a good correlation between the presence of MGMT expression and the unmethylated status of the DLBCL cases.

SPOCK2 (SPARC/Osteonectin CWCV and Kazal-like domains proteoglycan) gene located on chromosome 10q22 encodes a protein testican 2, a member of the testican group of extracellular chondroitin and heparin sulfate proteoglycans. Testicans are involved in the regulation of extracellular protease cascade (Chung et al. 2008; Hartmann et al. 2012). Testican 2 may contribute to ECM remodeling by regulating function(s) of other testican family members and the role has mainly been explored in the central nervous system. Nakada et al. (2003) found that testican 2 uniquely abrogates the inhibition of membrane-type matrix metalloproteinases (MT-MMP) enzyme by other testican proteins and for that, testican 2 was suggested to be associated with malignant behaviour of astrocytic tumours. Matrix metalloproteinases (MMPs) are a family of ECM degrading enzymes, which play important role in the angiogenesis related to tumour cells growth (Sounni et al. 2011).

In this study we examined the methylation status of the 2 genes, *MGMT* (*O^6-Methylguanine-DNA Methyltransferase*) and *SPOCK2* (SPARC/Osteonectin CWCV and Kazal-like domains proteoglycan) in DLBCL as in Malaysia information is still lacking. In fact, published studies related to *SPOCK2* and lymphoma is scarce. We hypothesise that promoter methylation of *SPOCK2* would not be a common event in DLBCL.

MATERIALS AND METHODS

This study was approved by the Research Ethics Committee of International Islamic University Malaysia (IREC) and Universiti Sains Malaysia (JEPeM). In all, 25 and 63 formalin fixed paraffin embedded (FFPE) DLBCL tissue blocks were retrieved from the Department of Pathology of Hospital Tengku Ampuan Afzan (HTAA) Kuantan, Pahang and Hospital Universiti Sains Malaysia (HUSM) Kubang Kerian, Kelantan, respectively. The inclusion criteria are cases which have been diagnosed as DLBCL according to WHO classification 2008. The exclusion criteria are cases of DLBCL with inadequate tissue samples, external referral cases and/or incomplete demographic data. The samples included consisting of more than 70% tumour cells which were collected from year 2006 to 2013. However, due to incomplete data on stages and grades, as well as cancer progression, we were not able to classify all cases according to the aforementioned criteria. Overall, the samples were from 35 males and 53 females with a median age of 55 years old (range, 1-84 years). Five reactive lymph node tissues were obtained from HTAA.

Genomic DNA was extracted using the E.Z.N.A® FFPE DNA kit (Omega Biotek, US) following protocol

specifications by the manufacturer. Extracted DNA was subjected to bisulfite treatment using the EZ DNA Methylation-Lightning™ kit (Zymo Research, Orange, CA). For each of the samples, 200-500 ng DNA 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 reincubated at room temperature for 20 minutes after the addition of L-Desulphonation Buffer. Finally, the bisulfite-treated DNA was eluted in a total volume of 20 µL in Elution Buffer. Sodium bisulfite converts all unmethylated cytosines to uracil leaving the methylated cytosines intact.

The *MGMT* and *SPOCK2* fragments were amplified using the methylation specific primers as shown in Table 1. In this study, we designed the primers for *SPOCK2* by using MethPrimer, a software that is available online (Li & Dahiya 2002). The PCR mixture contained 10 ng of bisulfite-treated DNA, Zymo*Taq*™ PreMix (1X), and 0.4 µM (*MGMT*) or 0.2 µM (*SPOCK2*) of each primer in a final volume of 20 µL. The PCR cycling conditions were as follows: 95°C for 10 minutes; then 40 cycles of 95°C for 30 seconds, 40 seconds at 60°C (*MGMT* unmethylated), 40 seconds at 59°C (*MGMT* methylated) and 40 seconds at 55°C (*SPOCK2* unmethylated and methylated), and 72°C for 40 seconds; and a final extension of 7 minutes at 72°C. A mixture of 5 µL of each PCR product and 2 µl of DNA loading dye was electrophoresed on a 2% ethidium-bromide stained agarose gel, and visualised under UV illumination.

Pearson's Chi-square test of SPSS version 12.0 was used to identify the association between *MGMT* and *SPOCK2* methylation status with the demographic parameters of the patients. $p < 0.05$ was considered to indicate a statistically significant difference with 95% confidence intervals in order to determine the effect of each variable on outcome.

TABLE 1. Primer Sequences

Gene			Primer sequences in 5'-3' orientation	Product sizes (bp)
MGMT	M	F	TTTCGACGTTCGTAGGTTTTCGC	81
		R	GCACTCTTCCGAAAACGAAACG	
	U	F	TTTGTGTTTTGATGTTTGTAGGTTTTTGT	93
		R	AACTCCACACTCTTCCAAAAACAAAACA	
				(Yoon et al. 2008)
SPOCK2	M	F	GATTTTCGGTAATTTTATGGAGGAC	113
		R	CTTAACAACCCTACGAACTCACGT	
	U	F	TTTTTGGTAATTTTATGGAGGATGA	111
		R	CTTAACAACCCTACAAACTCACATC	
				(Li & Dahiya 2002)

Abbreviations: M = methylated; U = unmethylated; F = forward; R = reverse; bp = basepair.

RESULTS AND DISCUSSION

In all, a total of 88 FFPE tissue blocks of patients diagnosed with DLBCL were examined for *MGMT* and *SPOCK2* methylation status. Methylation of *MGMT* was detected in all the 83 cases including the five reactive lymph nodes tissues analysed. *SPOCK2* was found to be methylated in 83 of the 88 (94.3%) DLBCL cases. We also detected *SPOCK2* methylation in three of the five reactive lymph nodes. Representative results of PCR products for *MGMT* and *SPOCK2* genes are as illustrated in Figure 1 and 2, respectively. No association between gene methylation status with age and gender of patients were seen.

This study reports promoter methylation of *MGMT* in all the DLBCL cases studied and the result is considered remarkably high. As no similar studies have been conducted in Malaysia or even in the South East Asia region, we are not able to compare our results regionally. However, a study from the Middle East revealed a high percentage of *MGMT* methylation (71%; 71 of 100 cases studied) in DLBCL (Al-Kuraya et al. 2005). Studies from Korea and Japan reported a much lower percentage, 30.4% and 38.8% respectively (Hiraga et al. 2006; Yoon et al. 2008). An Italian series also reported a lower percentage of *MGMT* methylation (36%, 30/84 cases) (Esteller et al. 2002). Recently, Kristensen et al. (2013) examined *MGMT* methylation in DLBCL using MSP and allelic MSP-pyrosequencing analyses and revealed that by the respective methods, 22% and 19% of 148 DLBCL cases exhibited *MGMT* methylation.

Inactivation of *MGMT* may initiate carcinogenesis as it codes for a protein that removes alkyl adducts from the O^6 position of guanine. Alkylated guanine is likely to mispair with thymine during DNA replication (Candiloro & Dobrovic 2009). Alkylation damage from a variety of environmental sources (such as alkylating environmental pollutants and tobacco-specific carcinogens) is a common occurrence and thus loss of *MGMT* function may give rise to a mutator phenotype (Candiloro & Dobrovic 2009). Although promoter methylation of *MGMT* has been shown in various studies to be a useful marker for predicting increased overall survival in patients with DLBCL and a predictive marker related to respond to chemotherapy with alkylating agents, for most cancers, studies that relate *MGMT* activity to therapeutic outcome following O^6-alkylating

drugs are still lacking (Candiloro & Dobrovic 2009; Christmann et al. 2011; Hiraga et al. 2006; Lee et al. 2009;). We however did not determine the prognostic significance of this methylation status in our patients.

Interestingly, we found the five reactive lymph nodes analysed also exhibited *MGMT* methylation. Sidhu and colleagues reported that the frequency of epigenetic *MGMT* inactivation was significantly higher among smokers, alcohol drinkers and meat eaters as compared to non-smokers, non-alcoholic drinkers and vegetarians (Sidhu et al. 2010). Unhealthy social habits appear to harm the normal regulation of epigenetic homeostasis. We are not able to verify these factors in our cases as this study was on archival tissues, nevertheless the findings by Sidhu et al. (2010) may partially explain the methylation observed in the reactive lymph nodes (Sidhu et al. 2010). Also thus far available data shows that the expression of *MGMT* varies greatly in normal tissues and in some cases this has been related to cancer predisposition (Christmann et al. 2011). Quantitative methylation studies such as by pyrosequencing would be beneficial as it could determine the threshold that discriminate DNA methylation in normal versus tumour tissues.

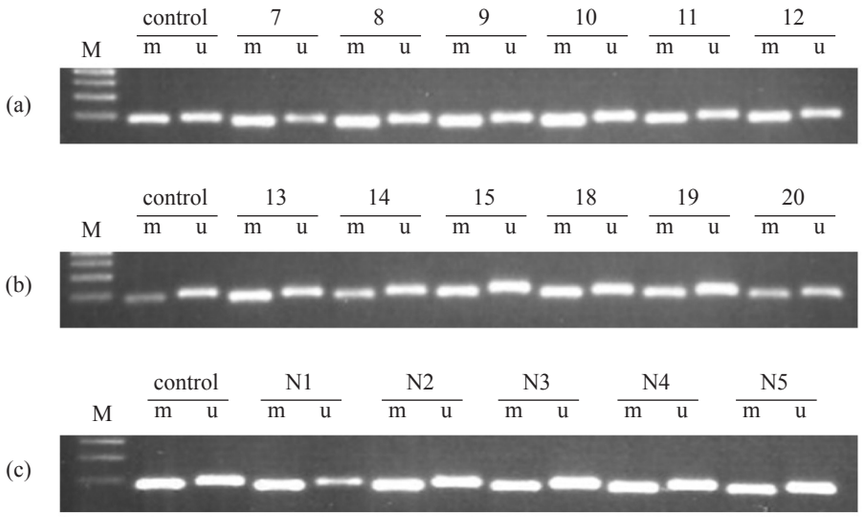

FIGURE 1. Representative MSP results for MGMT. (a) Sample 7, 8, 9, 10, 11 and 12 = methylated (b) Sample 13, 14, 15, 18, 19 and 20 = methylated (c) Reactive lymph nodes, N1, N2, N3, N4 and N5 = methylated. Abbreviations: M = marker; m = methylated, u = unmethylated; N = reactive lymph nodes

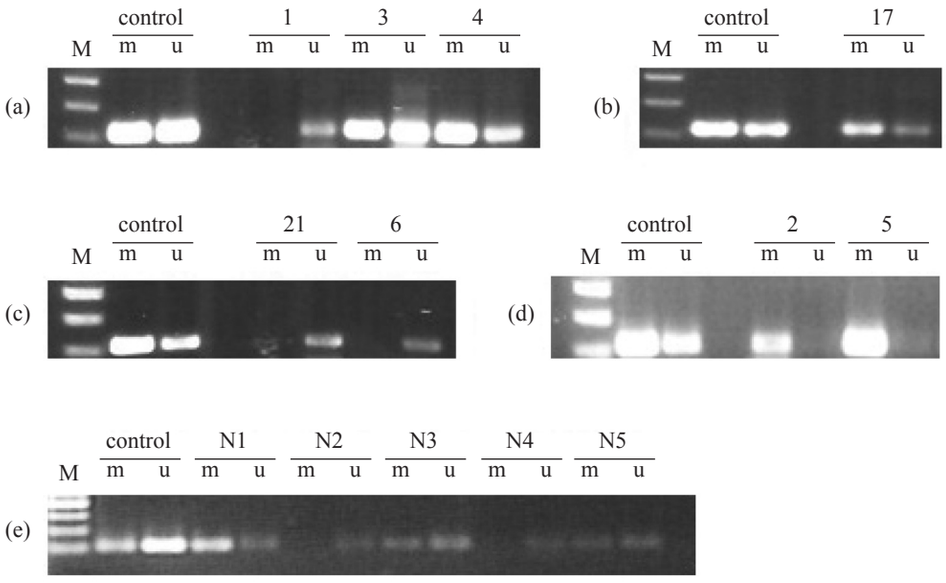

FIGURE 2. Representative MSP results for SPOCK2. (a) Sample 1 = unmethylated, sample 3 and 4 = methylated (b) Sample 17 = methylated (c) Sample 21 and 6 = unmethylated (d) Sample 2 and 5 = methylated (e) Reactive lymph nodes, N1, N3 and N5 = methylated, N2 and N5 = unmethylated Abbreviations: M = marker; m = methylated, u = unmethylated; N = reactive lymph nodes

This study also identifies promoter methylation of *SPOCK2* in 94.3% of DLBCL cases. Three of the five reactive lymph nodes analysed also exhibited methylation of *SPOCK2*. This finding negates our initial expectation that *SPOCK2* methylation would be an uncommon event in the majority of DLBCL cases. In fact only 5 cases (6%) studied were negative for the methylation. Initially we had postulated that promoter methylation of *SPOCK2* would not be a common event in DLBCL since this gene codes for testican 2 that functions to abolish the inhibition of membrane-type matrix metalloproteinases (MT-MMP) enzyme by other testican proteins. MMPs are a family of ECM degrading enzymes thereby may play crucial role in promoting angiogenesis related to tumour cells growth and metastasis (Georges et al. 2012; Nakada et al. 2003; Sounni et al. 2011). Aggressive tumour cells that express MT-MMP have been shown to proliferate rapidly due to high development rate of vascularisation within accumulated tumour cells (Seiki & Yana 2003). High expression of testican 2 was formerly reported in malignant astrocytic tissues (Nakada et al. 2003). Chung et al. (2008) reported that *SPOCK2* was found to be epigenetically silenced in the large proportion of prostate and colon cancers but did not discuss the possible contributions of methylated *SPOCK2* in the pathogenesis of those cancers. Thus, our finding of a very high percentage of *SPOCK2* need to be further explored.

CONCLUSION

This study has shown very high percentage of promoter methylation of *MGMT* and *SPOCK2* genes in the DLBCL cases studied by MSP, using archival lymphoma tissues. Nonetheless, more research is needed to quantitatively evaluate *MGMT* and *SPOCK2* methylation, and to analyse gene expression and/or protein expression in order to further understand the role of *MGMT* and *SPOCK2* methylation in the pathogenesis of DLBCL and to correlate the findings with the clinicopathological parameters.

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