Epigenetic changes and their clinical relevance in Saudi diffuse large B-cell lymphoma

A molecular and tissue microarray analysis of 100 cases

Khawla S. Al-Kuraya, *MD, FCAP,* ***Abdul K. Siraj,*** *BSc,* ***Fouad A. Al-Dayel,*** *MD, FRCPA,* ***Adnan A. Ezzat,*** *MD,* ***Naif A. Al-Jommah,*** *BSc,* ***Valerie L. Atizado,*** *BS,* ***Rajeswari N. Narayanappa,*** *PhD.*

ABSTRACT

Objectives: The gene encoding the DNA repair enzyme O6-methylguanine-DNA methyltransferase (MGMT) is transcriptionally silenced by promoter hypermethylation in several human cancers including diffuse large B-cell lymphoma (DLBCL). We explored the aberrant promoter methylation of MGMT in Saudi diffuse large B-cell lymphoma and to investigate MGMT hypermethylation has an effect on patient's overall survival.

Methods: In a retrospective cohort study, 100 cases of DLBCL were collected from the Department of Pathology at King Faisal Specialist Hospital and Research Centre, Riyadh, Kingdom of Saudi Arabia. We used methylation specific polymerase chain reaction to analyze the MGMT promoter methylation status in 100 tumor DNA of Saudi DLBCL patients receiving multi drug regimens. Tissue microarray (TMA) of these cases was also constructed. The MGMT protein expression was analyzed immunohistochemically. Molecular data were correlated with clinical outcome.

Results: Seventy one percent (71%) of 100 DLBCL patients showed MGMT promoter hypermethylation in their lymphoma. The presence of MGMT methylation was associated with statistically significant increase in the overall survival (p=0.02). The MGMT promoter hypermethylation was independent and a strong prognostic factor.

Conclusion: The MGMT promoter hypermethylation appears to be useful marker for predicting survival in patient with DLBCL treated with multi drug regimens including cyclophosphamide, at the same time the study shows that TMA technology is useful for immunohistochemical analysis of large lymphoma populations.

Saudi Med J 2005; Vol. 26 (7): 1099-1103

Growing evidence has implicated aberrant promoter methylation of the gene encoding the DNA repair enzyme O6-methylguanine-DNA methyltransferase (MGMT) in the molecular pathogenesis of several human cancers including a fraction of diffuse large B-cell lymphoma (DLBCL).[1-4]

The MGMT protein protects cells from toxicity of alkylating agents which frequently target the O6 position of guanine.[5,6] Promoter methylation is an

From the Research Centre (Al-Kuraya, Siraj, Al-Jommah, Atizado, Narayanappa), Department of Pathology and Laboratory Medicine (Al-Dayel) and from the Medical and Clinical Operations (Ezzat), King Faisal Specialist Hospital and Research Centre, Riyadh, *Kingdom of Saudi Arabia.*

Received 10th January 2005. Accepted for publication in final form 12th April 2005.

Address correspondence and reprint request to: Dr. Khawla S. Al-Kuraya, Director, Biological Repository Centre, King Faisal Specialist Hospital and Research Centre, MBC 98-16, PO Box 3354, Riyadh 11211, *Kingdom of Saudi Arabia.* Tel. +966 (1) 2294444 Ext. 51813. Fax. +966 (1) 2293629. E-mail: kkuraya@kfshrc.edu.sa

epigenetic DNA modification targeting CpG islands located within the regulatory regions of human genes. As a consequence, methylation of CpG islands causes repression of gene transcription and is one of the common mechanisms for gene inactivation by tumor suppressors.[1-4]

In human cancer, the MGMT gene is not commonly mutated or deleted; however, loss of MGMT function is commonly due to epigenetic lesions, specifically promoter region methylation. Aberrant MGMT methylation has been associated with loss of messenger RNA (mRNA) transcription,[7] lack of MGMT protein[8,9] and loss of enzymatic activity.[9] The level of MGMT activity and expression varies widely in tumors and in different B-cell hematological malignancies. For example, lack of MGMT activity has been detected in approximately 36% of DLBCL and 23.8% of precursor B-cell neoplasia.[10,11]

Recently, Esteller et al[11] have reported that the presence of MGMT promoter region methylation in DLBCL is indeed a strong predictor of response and overall survival in patients treated with alkylating agent such as cyclophosphamide. However, this genetic information on DLBCL is derived from studies performed on European and United States (US) patients. To date, the role of aberrant promoter hypermethylation in Saudi DLBCL has not been investigated in detail; therefore, we aimed in this study to investigate whether MGMT inactivation by hypermethylation could provide prognostic information for DLBCL patients in Saudi Arabia.

Methods.

Patient population and tumor samples. This study was based on 100 tumor samples of the clinicopathological spectrum of DLBCL recognized by World Health Organization classification. The samples were collected between 1990-2000 from the Department of Pathology, King Faisal Specialist Hospital and Research Centre, Riyadh, Kingdom Saudi Arabia. Tumor samples were derived from lymph nodes. Diagnosis was based on morphology and immunophenotypic analysis of cell surface markers and was complemented in most cases by immunogenotypic analysis of an antigen receptor gene rearrangement and chromosomal translocation.

Treatment of patients varied depending on the stage, date of diagnosis and prognostic factors. All patients however, were treated with cyclophosphamide anthracycline containing regimen. The study was approved by institutional Research Advisory Council (RAC # 2030 019).

Clinical samples and cell lines. The SW48 cell line was used as a control for methylation, and was obtained from ATCC. The SW48 cell lines were grown in RPMI 1640, supplemented with 10% fetal bovine serum in the presence of glutamine, 100 U/ml penicillin and 10 µg/ml streptomycin in an atmosphere of 5% CO_2 at 37ºC. In addition, in vitro methylated (IVM) DNA was used as a positive control for methylation in MS-PCR. Normal PBL was used as a negative control.

DNA extraction. Genomic DNAs were extracted from the patient samples and cell lines using Gentra Kit (Minneapolis, MN, USA) according to the manufacturer recommendations.

Bisulfite modification. The procedure for bisulfite modification was based on the method published previously by Warnecke et al.[12]

Briefly, 2 µg of DNA in a volume of 50 µl was denatured by incubation with 0.4 M NaOH for 30 minutes at 42ºC. Sodium bisulfite was added at the final concentration of 3 M (Sigma, St. Louis, MO) while hydroquinone (Sigma, St. Louis, MO) at 10 mM. The reaction was performed at 55 ºC for 16 hours. Modified DNA was purified using Wizard DNA Purification System (Promega Corp., Madison, WI) and modification was completed by a final treatment with 0.4M NaOH for 15 minutes at 37 ºC, followed by ethanol precipitation. The DNA was re-suspended in water and was used immediately or stored at –80 ºC.

Methylation-specific (MSP) PCR amplification. The 200 ng of sodium bisulfite-modified genomic DNA was PCR amplified. The PCR mixture contained 2.5 µl of 10-x PCR buffer, 20 pmol of each primer, 2 µl of dNTP's, 3.5 mM $MgCl_2$ for methylated and 4.5 mM $MgCl_2$ for unmethylated reaction and 1 U of Hotstar Taq DNA polymerase in a final volume of 25 µl reaction. Primer sequences for unmethylated reaction were 5'-TTT GTG TTT TGA TGT TTG TAG GTT TTT GT-3' (sense) and 5'-AAC TCC ACA CTC TTC CAA AAA CAA AAC A -3' (antisense). Primer sequences for methylated reaction were 5'-TTT CGA CGT TCG TAG GTT TTC GC-3' (sense) and 5'-GCA CTC TTC CGA AAA CGA AAC G -3' (antisense). Unmethylated reaction was amplified for 35 cycles at 56ºC annealing while methylated reaction was amplified at 57 ºC for 35 cycles. Negative control samples without DNA were included for each set of PCR. PCR products were analyzed on 4% agarose gels containing ethidium bromide.

Tissue microarray. The DLBCL TMA was constructed as described[13] using semi automated tissue arrayer (Beecher Instruments, Silverspring, MD). Briefly, an hematoxylin and eosin (H&E) stained section was made from each block to define representative tumor regions. Tissue cylinders with diameters of 0.6 mm were then punched from tumor areas of each donor tissue block and brought into 4 different recipient paraffin blocks each containing between 100 individual samples using the tissue

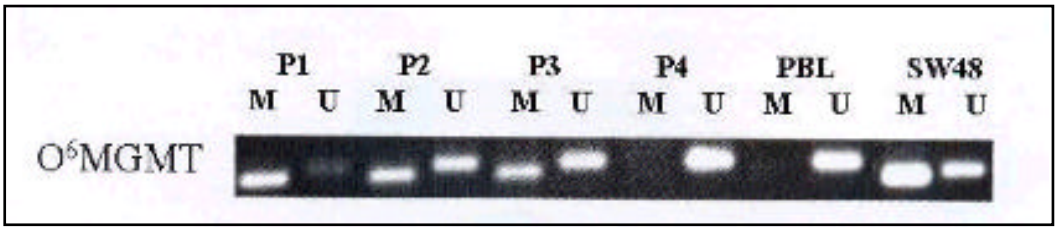

Figure 1 - Depicts MSP analyses of O6-methylguanine-DNA methyltransferase (MGMT). Methylation-specific polymerase chain reaction (PCR) analyses of MGMT promoter methylation status in tumor DNA of patients with diffuse large B-cell lymphoma. Four representatives diffuse large B-cell lymphoma samples from complete data. M=methylated reaction and U=unmethylated reaction. The cancer cell line SW-48 was used as positive control for methylation. Normal peripheral blood lymphocytes (PBL) were used as negative control.

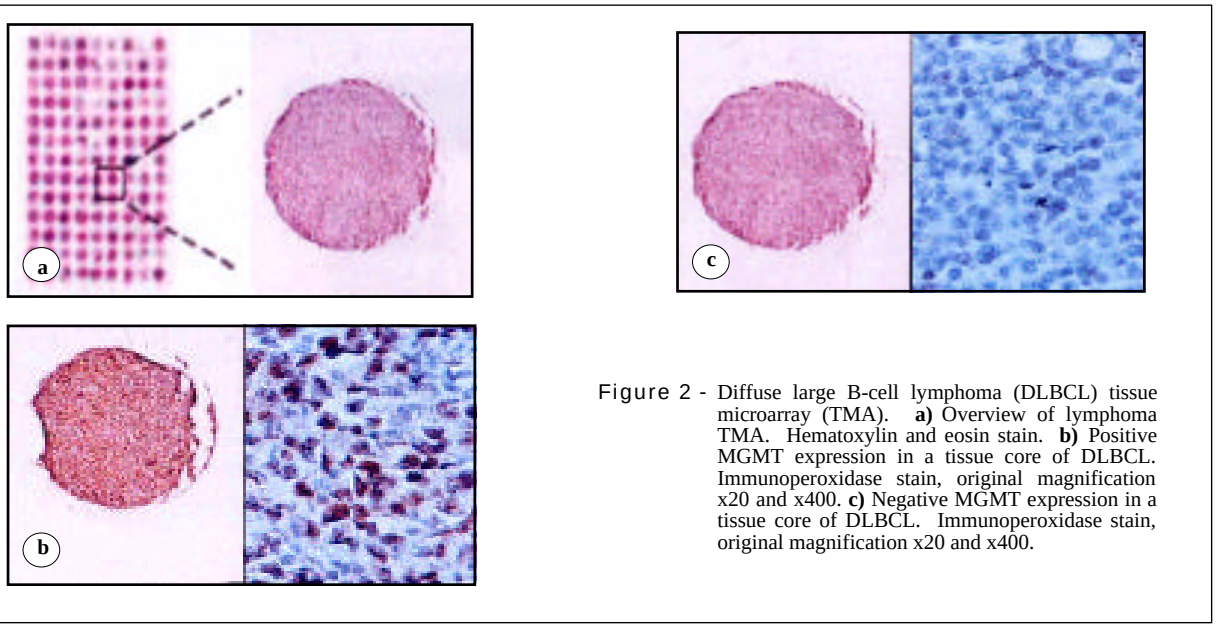

Figure 2 - Diffuse large B-cell lymphoma (DLBCL) tissue microarray (TMA). **a)** Overview of lymphoma TMA. Hematoxylin and eosin stain. **b)** Positive MGMT expression in a tissue core of DLBCL. Immunoperoxidase stain, original magnification x20 and x400. **c)** Negative MGMT expression in a tissue core of DLBCL. Immunoperoxidase stain, original magnification x20 and x400.

arrayer. Four µm sections of the resulting TMAs were transferred to an adhesive coated slide system (Instrumedics Inc., Hackensack, New Jersey). An overview of an H&E stained DLBCL TMA section is shown in **Figure 2**.

Immunohistochemistry. The TMA sections were used for immunohistochemical staining of MGMT protein. The correlation between MGMT methylation status and MGMT protein expression was assessed in TMA section of 100 samples of DLBCL. Paraffin embedded tissue sections were deparaffinized with xylene, dehydrated by using a graded series of ethanol and treated for 30 minutes in TEC (Tris-EDTA-Citrate) solution (pH 7.8) in a microwave oven at 250 W. Immunohistochemistry was performed using the Avidin-biotin-peroxidase complex (ABC) method (ABC-Elite kit, Vector, Burlingame, CA, USA) with diaminobenzidine as the chromogen. Commercially available mouse anti-MGMT monoclonal antibody (clone MT3.1; Chemicon Intl., Temecula, CA) at a 1:100 dilution was used.[14] This antibody was previously demonstrated to be useful for immunohistochemistry and to correlate with MGMT activity.[8,15] Scoring of the immunohistochemical staining followed a published guidelines.[14] All MGMT analyses by immunohistochemistry (IHC) were interpreted without a prior knowledge of the MGMT methylation status and vice versa.

Statistics. Contingency table analysis and Chi-square tests were used to study the relationship between MGMT methylation and MGMT immunostaining. Survival curves were plotted according to Kaplan-Meier. A log rank test was applied to examine the relation between MGMT methylation and raw survival. Analyses were performed with the use of JMP 3.1 (SAS Institute, Inc., Cary, NC).

Results. We examined MGMT promoter hypermethylation in tumors of 100 patients with DLBCL. The MGMT hypermethylation was found in 71% of the samples (representative DLBCL cases are shown in **Figure 1**). Lymphoma samples

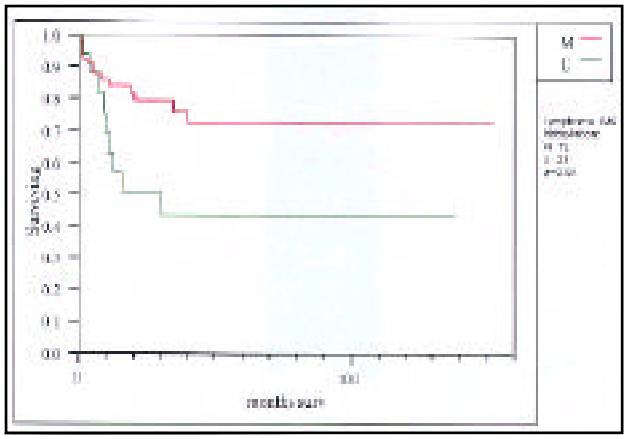

Figure 3 - Kaplan-Meier analyses of O6-methylguanine-DNA methyltransferase (MGMT) promoter hypermethylation in patients with diffuse large B-cell lymphoma and its impact on survival.

carrying unmethylated MGMT alleles accounted for 23% of the samples.

All 100 cases tested were representative by H&E morphology. There was no tissue at the position 12 of the TMA; and at the position 9 tumor was not observed. However, each sample was punched 4 times, therefore at least one valuable core was present in almost all cases. All cases were examined for MGMT protein expression. However, due to the factors related to the TMA technology, 10 cases contained no tissue on the slides and therefore could not be evaluated. Therefore, IHC was interpretable in 90% of lymphoma cases, and showed strong nuclear staining in 12/90 cases (13%); absence of staining in tumor cells was seen in 60/90 cases (66.7%). Representative examples of MGMT positive and negative tumors are shown in **Figure 2**.

The MGMT hypermethylation was associated with absent MGMT protein expression p=0.001. Since all lymphoma samples carrying MGMT hypermethylation had failed to express the protein as tested by immunohistochemistry.

The MGMT methylation status in DLBCL patients was strongly associated with overall survival. Overall survival was significantly increased among patients with increased MGMT methylation p=0.02 shown in **Figure 3.**

Discussion. Diffuse large B-cell lymphoma is a common malignancy in Saudi Arabia, according to the recent study at KFSH & RC in Riyadh, KSA during the period 1999-2002. The DLBCL accounted for 62% of the lymphoma cases.[16-18] Molecular information is increasingly being used for optimized therapy selection. The vast majority of molecular information on DLBCL and other tumors is from studies performed on European and US patients. However, growing evidence suggests relevant molecular differences between cancers of patients from different ethnic groups. Our study indeed demonstrates that MGMT inactivation through promoter methylation occurs with different frequencies in DLBCL patients from different ethnic groups. While MGMT promoter hypermethylation is seen in 20-40% of European,[10, 11] our study shows that this epigenomic event reached up to 70% of the Saudi patients with DLBCL. However, despite the higher number of methylation in Saudi Arabia DLBCL cases, the expected positive prognostic effect of gene methylation on DLBCL patients receiving alkylating agents could be confirmed in our study (p=0.02).

Several hypotheses may explain the prognostic role of MGMT in predicting survival of patients with DLBCL treated with alkylating agents. One hypothesis is that MGMT inactivation may render DLBCL cells more prone to the genotoxic effects of alkylating agents, as it has been proposed recently in a case of glioma.[19] In fact, the DNA repair protein MGMT is one of the key factors mediating resistance to these agents, and several reports suggest that MGMT does play a role in modulating the activity of cyclophosphamide at least in-vitro, as demonstrated in lung cancer.[20]

Increased sensitivity to alkylating agents conferred by MGMT inactivation may result in complete elimination of all transformed cells, which would increase the patient survival.

Although, the reasons for increased frequency of MGMT hypermethylation in Saudi DLBCL patients are unclear, it could highlight the potential molecular differences between non-Western countries. The practical implication of these results is that the clinical and diagnostic criteria that are used to predict prognosis and response to cytotoxic therapy in Western tumor may be different in tumors from other ethnic groups.

Moreover, further studies from other institutions and patients with different ethnic background would be helpful to evaluate the prognostic significance of MGMT methylation.

Finally, our ability to correlate the MGMT inactivation by promoter hypermethylation as confirmed by MGMT protein expression by IHC utilizing the tissue microarray technology, could suggest future value of MGMT expression analyses by immunohistochemistry in assessing methylation status in DLBCL patients. Additionally, our findings also highlights the importance of this high-throughput technology in facilitating the analysis of large patient cohorts and expedite discoveries due to projects that have previously been considered unmanageable can be completed more rapidly and with expenditure of significantly less resources.

References

1. Singal R, Ginder GD. DNA Methylation. ***Blood*** 1999; 93: 4059-4070.
2. Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile of human cancer. ***Cancer Res*** 2001; 61: 3225-3229.
3. Robertson KD. DNA methylation, methyltransferases, and cancer. ***Oncogene*** 2001; 20: 3139-3155.
4. Santini V, Kantarjan HM, Issa JP. Changes in DNA methylation in neoplasia: pathophysiology and therapeutic implications. ***Ann Intern Med*** 2001;134: 573-86.
5. Ludlum DB. DNA alkylation by the haloethylnitroureases: nature of modifications-produced and their enzymatic repair or removal. ***Mutat Res*** 1990; 233: 117-126.
6. Pegg AE, Dolan ME, Moschel RC. Structure, function and inhibition of O6-alkylguanine-DNA alkyltransferease. ***Prog Nucleic Acid Res Mol Biol*** 1995; 51: 167-223.
7. Esteller M, Toyota M, Sanchez-Cespedes M, Capella G, Peinado MA, Watkins DN, et al. Inactivation of the DNA repair gene O6-methyguanine-DNA methyltransferase by promoter hypermethylation is associated with G to A mutations in K-ras in colorectal tumorigenesis. ***Cancer Res*** 2000; 60: 2368-71.
8. Esteller M, Hamilton SR, Burger PC, Baylin SB, Herman JG. Inactivation of the DNA repair gene O6-methyguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. ***Cancer Res*** 1999; 59: 793-797.
9. Herfarth KK, Brent TP, Danam RP, Remack JS, Kodner IJ, Wells SA Jr., et al. A specific CpG methylation pattern of the MGMT promoter region associated with reduced MGMT expression in primary colorectal cancers. ***Mol Carcinog*** 1999; 24: 90-98.
10. Rossi D, Capello D, Gloghini A, Franceschetti S, Paulli M, Bhatia K, et al. Aberrant promoter methylation of multiple genes throughout the clinico-pathologic spectrum of B-cell neoplasia. ***Haematologica*** 2004; 89: 154-164.
11. Esteller M, Gaidano G, Goodman SN, Zagonel V, Capello D, Botto B, et al. Hypermethylation of the DNA repair gene O6-methylguanine-DNA methyltransferase and survival of patients with diffuse large B-cell lymphoma. ***J Natl Cancer Inst*** 2002; 94: 26-32.
12. Warnecke PM, Stirzaker C, Melki JR, Milar DS, Paul Clark SJ. Detection and measurement of PCR bias in quatitative methylation analysis of bisulfite DNA. ***Nucleic Acids Res*** 1997; 25: 4422-4426
13. Kononen J, Bubendorf L, Kallioniemi A, BarSchraml P, Leighton S, et al. Tissue microarrays for highthroughput molecular profiling of tumor specimens. ***Nat Med*** 1998; 4: 844-847.
14. Brent TP, Von Wronski M, Pegram CN, Bigner DD. Immunoaffinity purification of human O6-alkylguanine-DNA alkyltransferase using newly developed monoclonal antibodies. ***Cancer Res*** 1990; 50: 58-61.
15. Reese JS, Koc ON, Lee KM, Liu L, Allay JA, Philips WP Jr, et al. Retroviral transduction of a mutant methylguanine DNA methyltransferase gene into human CD34 cells confers resistance to O6 benzylguanine plus 1,3-bis (2-chloroethyl-1-nitrosouurea. ***Proc Natl Acad Sci USA*** 1996; 93: 14088-14093.
16. 1999 Cancer Incidence Report, GCCC Cancer Registry 2003.
17. Tumor Cancer Registry, KFSH&RC Annual Report 2001.
18. Ibrahim EM, Ezzat AA, Raja MA, Rahal MM, Ajarim DS, Mann B, et al. Primary gastric non-Hodgkin's lymphoma: clinical features, management, and prognosis of 185 patients with diffuse large B-cell lymphoma. ***Ann Oncol*** 1999; 10: 1441-1449.
19. Esteller M, Garcia-Foncillas J, Andion E, Goodman SN, Hidalgo OF, Vanaclocha V, et al. Inactivation of the DNA repair gene MGMT and the clinical response of gliomas to alkylating agents. ***N Engl J Med*** 2000; 343: 1350-1354.
20. Mattern J, Eichhorn U, Kaina B, Volm M. O6-methylguanine-DNA methyltransferase activity and sensitivity to cyclophosphamide and cisplatin in human lung tumor xenografts. ***Int J Cancer*** 1998; 77: 919-922.