Aberrant Methylation of p16, DLEC1, BLU and E-Cadherin Gene Promoters in Nasopharyngeal Carcinoma Biopsies from Tunisian Patients

WAJDI AYADI[1], HÉLA KARRAY-HAKIM[1], ABDELMAJID KHABIR[2], LAMIA FEKI[1], SLIM CHARFI[2], TAHIA BOUDAWARA[2], ABDELMONEM GHORBEL[3], JAMEL DAOUD[4], MOUNIR FRIKHA[5], PIERRE BUSSON[6] and ADNANE HAMMAMI[1]

[1]*Laboratoire de Microbiologie,* [2]*Laboratoire d'Anatomopathologie,* [3]*Service d'Otorhino-laryngologie,* [4]*Service de Radiothérapie and* [5]*Service d'Oncologie, Hôpital Habib Bour-guiba, 3029 Sfax, Tunisie;* [6]*UMR 8126 Université Paris-sud et Institut de Cancérologie Gustave Roussy 94805 Villejuif, France*

Abstract. *Background: Aberrant methylation of tumor suppressor gene (TSG) promoters has been extensively investigated in nasopharyngeal carcinomas (NPC) from South East Asia but not from North Africa. Patients and Methods: The methylation status of p16, deleted in lung and esophageal cancer (DLEC1), zinc finger, MYND-type containing 10 (BLU) and E-cadherin gene promoters was investigated in 44 Tunisian NPC biopsies and three NPC xenografts, by methylation-specific PCR (MSP) combined with a quantitative assessment of some of the samples. Results: The frequencies of aberrant promoter methylation were similar to previous figures reported for Asian series: p16 27/44 (65%), DLEC1 38/44 (86.3%), BLU 15/44 (34.1%) and E-cadherin 35/44 (79.5%). Although in other malignancies, aberrant promoter hypermethylation increases with patient age, it was at the same high frequency in the juvenile and adult forms of Tunisian NPCs. However, there was a strong association between aberrant methylation of E-cadherin promoter and lymph node invasion (p<0.01). In addition, aberrant methylation of the BLU promoter was significantly correlated with an undifferentiated histological type (p=0.03). Conclusion: Aberrant methylation of tumor suppressor genes occurs with the same high frequency in NPCs from North Africa as in South East Asia, regardless of patient age.*

Nasopharyngeal carcinoma (NPC) is a malignancy with a remarkable racial and geographic distribution (1). In Tunisia, this cancer is a major public health issue as it represents the most frequent head and neck cancer, with an annual incidence varying from 3 to 4 cases /100,000 persons. The juvenile form, which is characteristic of the North African endemic area, accounts for 20% of Tunisian patients (1, 2).

The development of this Epstein-Barr virus (EBV)-associated cancer involves cumulative genetic changes in a background of multiple genetic and environmental risk factors (1). For the past few years, the definition of the cytosine guanine dinucleotide (CpG) methylation islands in promoters of tumor suppressor genes (TSGs) has led to a better understanding of the oncogenic processes (3). Aberrant methylation of CpG island promoters is recognized as a common mechanism for the transcriptional inactivation of TSGs in various human carcinomas (4, 5). Several studies performed on NPC specimens from South East Asia have shown that aberrant promoter methylation is highly prevalent in this malignancy. Silencing by promoter methylation has been reported for a variety of TSGs including Ras association domain family 1A (RASSF1), retinoic acid receptor β2 (RAR β2), death-associated protein kinase (DAP kinase), deleted in lung and esophageal cancer 1(DLEC1), p16$^{\text{inhibitor of cyclin dependent kinase 4a (INK4a)}}$ (p16), E-cadherin, and zinc finger, MYND-type containing 10 (BLU). These alterations have been detected in fresh biopsies as well as in NPC cell lines or xenografts (6-10). However, promoter methylation has not been investigated in NPC specimens from North Africa, although NPCs from this area are known to have specific clinical and biological characteristics. One major difference between North Africa and South East Asia is the age distribution. The juvenile form of NPC is not observed in Asia, where nearly all patients are above 40 years of age. We and others have found distinct characteristics of North African NPCs regarding the distribution of EBV polymorphisms (variants D, F, *Xho*+ *versus* C, f, *Xho*–) and associated-human leukocyte antigen (HLA) allele types

Correspondence to: Wajdi Ayadi, Laboratoire de Microbiologie, Faculté de Médecine de Sfax, Avenue Majida Boulila, 3029 Sfax, Tunisie. Tel: +21622821299, Fax: +21674246217, e-mail: zaidounemail@yahoo.fr

Key Words: NPC, methylation, p16, DLEC1, BLU, E-cadherin.

(B13, DRB*03, DRB*15 *versus* A2, B14, B46) (11-13). In addition, within the group of North African NPCs, we have reported striking differences between the juvenile and adult forms. Each form has specific biological characteristics, suggesting that it follows a distinct oncogenic pathway. Studies based on immunohistochemistry have shown intense EBV latent membrane protein 1 (LMP1) expression associated with low levels of p53 and Bcl2 in the juvenile form, contrasting with the adult form where, on average, LMP1 is less abundant whereas p53 and Bcl2 are strongly expressed (14-16). Regarding serological changes, peripheral blood IgA directed to EBV antigens is generally not found in the juvenile form in contrast with the adult form (17, 18). In order to determine whether promoter hypermethylations are as frequent in North African as in Asian NPCs and whether they have the same frequency in the juvenile and adult forms, aberrant promoter methylation of p16, DLEC1, BLU and E-cadherin was investigated in a series of 44 Tunisian NPC specimens. Thereafter, the data on TSG methylation status were related to clinicopathological parameters.

Patients and Methods

Patients and sample collection. Primary NPC biopsy samples were collected with informed consent from 44 patients, prior to any treatment, at Sfax University Hospital (Tunisia), between January 1998 and December 2004. Seventeen cases were of the juvenile form (patients aged less than 30 years). The clinical stage was determined according to the tumor, node and metastasis (TNM) classification of the American Joint Committee on Cancer/International Union Against Cancer (AJCC/UICC, 1997) (19, 20). The primary tumor was classified as T1-T2 in 12 cases and T3-T4 in 32 cases. Regional lymph node involvement was observed in 40 cases. The presence of distant metastasis was noted in 8 patients. The histological type of NPC was determined on tissue sections according to the World Health Organization (WHO, 2005) (21). Twenty-one cases were undifferentiated carcinoma of nasopharyngeal type (UCNT) and 23 cases were non-keratinizing carcinoma. Following 32 months of combined treatment of radio/chemotherapy, 31 patients were in complete remission, 6 patients had relapsed (bone, lung or liver) and the 7 remaining patients who had presented a distant metastasis at primary diagnosis had died.

All NPC biopsy samples were immediately frozen in liquid nitrogen, embedded in optimal cutting temperature (OCT) compound (Tissue Tek; Miles-Bayer Diagnostics, Puteaux, France) and subsequently stored at –80˚C until use. Hematoxylin and eosin-stained sections were examined by pathologists and shown to contain at least 70% of tumor cells. In addition, three biopsy samples totally devoid of malignant cells from nasopharyngeal tissues were used as negative controls. Three NPC xenografts C15, C17 and X666 were used as references. C15 and C17, routinely propagated in our laboratory (P. Busson, Villejuif, France), were derived from a North African primary NPC and from a cutaneous metastasis of a European NPC, respectively. X666 was derived from a Chinese NPC (kindly provided by Professor K.W. Lo, Chinese University of Hong-Kong).

DNA extraction and chemical modification. Five serial sections (30 µm-thick) of each tumor or non-tumor sample were digested in proteinase K lysis solution (100 µg/ml Proteinase K (Invitrogen, Cergy-Pontoise, France), 5 mM NaCl,, 50 mM Tris-HCl pH 7.5, 0.5% SDS and 10 mM EDTA) at 56˚C for 2-4 h under constant agitation. The DNA was then extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with sodium acetate and absolute ethanol. The DNA was collected, washed in 70% ethanol and rehydrated in sterile water.

One µg of purified DNA was subjected to bisulfite treatment for 9 h using the Methyl Detector Modification Kit (Active Motif Europe, Rixensart, Belgium) according to the manufacturer's instructions. This treatment deaminates unmethylated cytosines into uracil but does not affect 5-methylcytosines.

Bisulfite sequencing. To investigate the methylation pattern of the four TSG promoters in NPC, DNA samples from the three NPC xenografts (C15, C17 and X666) were subjected to bisulfite sequencing analysis. After bisulfite treatment, PCR amplification was performed on 100 ng of modified DNA in a final reaction mixture of 100 µl containing 1x PCR buffer, 1 µM of each appropriate primer, 200 µM of each dNTP, 2 mM $MgCl_2$ and 2.5 units Taq DNA polymerase (Promega, Lyon, France). The primer sequences, product size and annealing temperature of each TSG promoter are shown in Table I. The amplified fragments were purified using the Wizard SV gel and PCR clean-UP System (Promega) and subjected to cloning into a pGEM-T Easy vector (Promega) in accordance with the manufacturer's instructions. Three to four clones of each sample were sequenced with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) with an ABI 377 DNA auto-sequencer (Applied Biosystems).

Methylation-specific polymerase chain reaction (MSP). The methylation status of each TSG promoter in the NPC xenografts and biopsies was examined by two-stage nested MSP. The primers of the stage-1 PCR were also used for the preceding amplifications which recognize the bisulfite-modified template, but do not discriminate between methylated and unmethylated sequences (Table I). Stage-1 PCR was performed on 10 ng of modified DNA in a final reaction mixture of 30 µl containing 1x PCR buffer, 1 µM of each appropriate primer, 200 µM of each dNTP, 2 mM $MgCl_2$ and 1.25 units Taq DNA polymerase (Promega). After this, the PCR products were diluted 100-fold and 2 µl was subjected to stage-2 PCR with two different mix reactions containing independent primers specific to the methylated and unmethylated templates respectively. The primer sequences, product sizes and annealing temperatures are shown in Table I. Finally, the PCR products of the methylated and unmethylated DNA sequences were separated on a 2% agarose gel and visualized by ethidium bromide staining.

Statistical analysis. Fisher's test was used to evaluate the relationship between the promoter methylation status and the clinicopathological parameters. A p-value ≤0.05 was considered statistically significant.

Results

Methylation patterns in NPC xenografts. The number of CpG sites shown by sequencing in the *p16, DLEC1, BLU* and *E-cadherin* promoters was 28, 18, 29 and 32 respectively (Figure 1A). The status of each potentially methylated

Table I. *Summary of primers used in the present study.*

Forward primers: (5' → 3')	Reverse primers: (5' → 3')	Position[a]	Product size (bp)	Annealing temperature
p16				
Bisulfite sequencing or MSP (Stage-1 PCR)				
GGAGGAAGAAAGAGGAG	CTACAAACCCTCTACCCA	1892-2168	277	50˚C
MSP (Stage-2 PCR)				
M-TTATTAGAGGGTGGGGCGGATCGC	M-GACCCCGAACCGCGACCGTAA	1919-2069	150[b]	65˚C
U-TTATTAGAGGGTGGGGTGGATTGT	U-CAACCCCAAACCACAACCATA	1919-2070	151[b]	60˚C
DLEC1				
Bisulfite sequencing or MSP (Stage-1 PCR)				
TAAAAGGATAATGTTGAAGATATA	AACTTCCAAATAAACTAACTAAAAC	19572-19831	260	55˚C
MSP (Stage-2 PCR)				
M-GATTATAGCGATGACGGGATTC	M-ACCCGACTAATAACGAAATTAACG	19608-19800	193[c]	60˚C
U-TGATTATAGTGATGATGGGATTTG	U-CCCAACTAATAACAAAATTAACACC	19607-19799	193[c]	60˚C
BLU				
Bisulfite sequencing or MSP (Stage-1 PCR)				
TTGGGAATTTAAATATTATG	AACAACAATTCCAAATCTC	13036-13334	299	50˚C
MSP (Stage-2 PCR)				
M-GCGGGTTAGAGATTCGTTC	M-TCGAAACCGAAATCCGACG	13080-13310	231[d]	55˚C
U-GGTGGGTTAGAGATTTGTTT	U-ATATCAAAACCAAAATCCAACA	13079-13313	235[d]	51˚C
E-Cadherin				
Bisulfite sequencing or MSP (Stage-1 PCR)				
TTTTAGGTTAGAGGGTTAT	CTACAACAACAACAACAAC	844-1193	350	50˚C
MSP (Stage-2 PCR)				
M-GGTGAATTTTTAGTTAATTAGCGGTAC	M-CATAACTAACCGAAAACGCCG	945-1148	204[e]	59˚C
U-GGTAGGTGAATTTTTAGTTAATTAGTGGTA	U-ACCCATAACTAACCAAAAACACCA	941-1151	211[e]	59˚C

[a]Positions of primers for *p16*, *DLEC1*, *BLU* and *E-cadherin* are according to GenBank accession numbers DQ406745, AP006309, AC002481 and L34545 respectively. [b,c,d and e]See references 23, 8, 9 and 7, respectively. M, methylated-specific primers; MSP, methylation-specific PCR; U, unmethylated-specific primers.

cytosine (CpG site) was analyzed in three or four clones and, based on these data, each CpG site was classified as methylated, partially methylated or unmethylated. As shown in Figure 1A, the *p16* promoter had a very high density of methylated cytosines in all three NPC xenografts (C17, C15 and X666). This was also the case for the *DLEC1* promoter in the C15 and X666 xenografts. In contrast, several CpG sites were unmethylated in the *DLEC1* promoter for the European NPC xenograft C17. Regarding the *E-cadherin* and *BLU* promoters, a noticeable number of their CpG sites remained unmethylated, especially in the Chinese NPC xenograft X666 and to a lesser extent in the C17 xenograft.

Results consistent with the sequencing data were obtained when the MSP technique was applied to the xenograft DNA (Figure 1B). Therefore, the same experimental conditions were used to assess promoter methylation in the NPC biopsies.

Frequency of methylation in NPC biopsies. As shown in Figure 1C, all four promoters were unmethylated in the three non-tumoral nasopharyngeal specimens. In contrast among the NPC cases, only one had retained the four promoters unmethylated. The frequency of methylation involving 1, 2, 3 or 4 promoters was 11.3% (5/44), 22.7% (10/44), 50% (22/44) and 13.6% (6/44) respectively. The overall frequency of aberrant methylation for each promoter was as follows: 65% (27/44) for p16, 86.3% (38/44) for *DLEC1*, 34.1% (15/44) for *BLU* and 79.5% (35/44) for *E-cadherin*. Amplicons corresponding to unmethylated sequences were detected in all the NPC cases and can be explained by the presence of nonmalignant cells contained in the tumor fragments.

Clinicopathological significance of promoter methylation. Table II shows the results of statistical analysis of the correlations between promoter methylation status and the clinicopathological parameters age, histology type, tumor size, lymph node involvement, distant metastasis and clinical evolution.

The frequencies of aberrant methylation in these four TSG promoters were not significantly different in the juvenile and adult forms. However, other types of clinicopathological correlations were recorded. There was a strong association

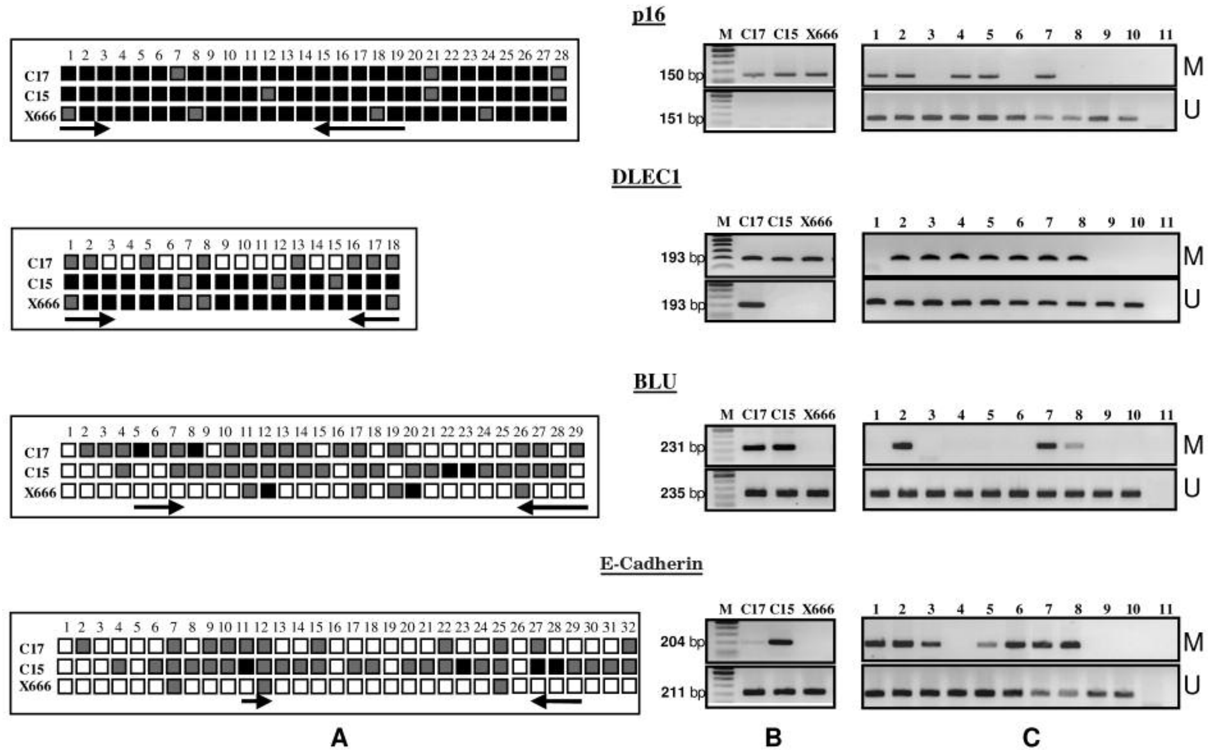

Figure 1. *A) Sequencing results of p16, DLEC1, E-cadherin and BLU promoter genes from three NPC xenografts (C17, C15 and X666). The methylation status of CpG residues is indicated by shading: black (methylated), white (unmethylated) and grey (partially methylated). The arrows indicate the different positions of primers used by MSP (stage-2 PCR). B) MSP results of each promoter analyzed from the three NPC xenografts. C: Representative results of MSP analysis in tumor biopsies (lines 1-8) and non-tumors (lines 9 and 10). Line 11, negative control (sterile distilled water). M and U represent the amplification of methylated and unmethylated sequences respectively.*

between aberrant methylation of the *E-cadherin* promoter and lymph node involvement (N+) in the NPC patients (p<0.01). This methylated promoter was also significantly associated with distant metastastatic events detected at primary diagnosis or when the disease relapsed (p=0.02). *E-cadherin* promoter methylation was detected in all the NPC patients with a distant metastasis at the time of diagnosis (8/8) and the patients who relapsed after complete remission (6/6). On the other hand, aberrant methylation of the *BLU* promoter was significantly correlated with an undifferentiated histological type (p=0.03). For the *p16* and *DLEC1* genes, no significant correlation with the clinico-pathological parameters was found.

Discussion

In all three of the NPC xenografts, many CpG sites were partially methylated instead of being either methylated or unmethylated. This pointed to intra-tumoral heterogeneity of gene promoter status, which may have resulted from the presence of heterogeneous malignant cell subclones and/or intracellular allelic variability.

The *INK4/p16* locus on chromosome 9p21 is one of the most frequently altered in NPCs. Silencing of the *p16* gene often results from the loss of one allele combined with hypermethylation of the remaining allele (22). The dense methylation of this promoter in 27 out of the 44 Tunisian NPC biopsies (65%) was consistent with previous findings in Southern Chinese cohorts in which the frequency of aberrant methylation in tumor and brushing specimens was 52% and 46.4% respectively (23, 24). *p16* inactivation can be suspected in a greater number of cases by other genetic mechanisms such as homozygous deletion, which has been reported in 35% of primary tumor cases from South China (25). Finally, the p16 protein can be functionally inactivated by the viral oncoprotein LMP1 (26).

Alterations of the 3p21 and 22 loci are also highly prevalent in NPCs often with a combination of loss of heterozygosity (LOH) and aberrant promoter methylations (10). The *DLEC1* and *BLU* genes map to this region. A high frequency of aberrant methylation in the *DLEC1* promoter (86.3%) was found in the present study not dissimilar to the frequency of 71.4 % reported for Chinese primary NPCs (8). In contrast, the *BLU* gene showed a lower frequency of

Table II. *Association between TSG promoter methylation and clinicopathological parameters*.

	Number of samples	*p16*	p^a	*DLEC1*	p^a	*BLU*	p^a	*E-Cadherin*	p^a
Age (years)									
<30	17	10		15		4		14	
≥30	27	17	0.96	23	0.57	11	0.39	21	0.51
Histological type[b]									
UC	21	14		18		11		18	
NKC	23	13	0.70	20	0.62	4	0.03	17	0.27
T-stage[c]									
T1-T2	12	7		10		2		8	
T3-T4	32	20	0.53	28	0.52	13	0.25	27	0.18
N-stage[c]									
N0	4	2		3		1		0	
N+	40	25	0.50	35	0.45	14	0.57	35	<0.01
Metastasis[c]									
M0	36	23		30		12		27	
M+	8	4	0.36	8	0.27	3	0.56	8	0.13
Metastatic relapse									
No	38	22		32		14		29	
Yes	6	5	0.23	6	0.39	1	0.31	6	0.22
Metastatic events[d]									
No	30	18		24		11		21	
yes	14	9	0.95	14	0.08	4	0.43	14	0.02

[a]Based on the Fisher's test. A *p*-value ≤0.05 was considered statistically significant. [b]Histological type; UC, undifferentiated carcinoma; NKC, non-keratinizing carcinoma. [c]Clinical stage at time of diagnosis: primary tumor according to AJCC/UICC (1997); regional lymph node classified N0 in the absence of clinical or radiological evidence of lymph node invasion, N+ in the other cases; metastatic status defined as M0 in the absence of clinical or radiological evidence of distant metastasis, M+ in the other cases. [d]The sum of metastatic events at primary diagnosis and when the disease relapsed.

promoter hypermethylation (34.1%) in the Tunisian series compared to 66% in Chinese NPCs (9). Since aberrant methylation of *BLU* was strongly associated with the undifferentiated histological type (*p*=0.03), the difference with the Chinese series might be related to a difference in the relative percentage of undifferentiated *versus* non-keratinizing forms, a point that would require further investigation.

Aberrant methylation of the *E-cadherin* promoter, in the 16q chromosome region, was also frequently found in the Tunisian NPC specimens (79.5%). This frequency was higher than that reported for South East Asian NPC patients (23.8 to 52.4% of cases) (7, 24, 27, 28). Since there was a strong relationship between methylation of the *E-cadherin* promoter and metastatic involvement, this difference might be related in part to the earlier detection of NPCs in some parts of South East Asia compared to Tunisia. The relationship between E-cadherin promoter methylation and metastatic involvement was consistent with many previous clinicobiological investigations showing correlations between the loss of E-cadherin expression and advanced disease stage or shorter survival (29). It was also consistent with data obtained *in vitro,* as well as in animal model systems (30, 31). Overall, the present data confirmed that TSG promoter hypermethylation was at least as frequent in North African as in Asian NPCs.

It has been suggested that aberrant promoter hypermethylation is more common in malignancies occurring in older individuals (32-34). This general assumption is not confirmed by the present study, since the frequency of TSG promoter methylation was not different in the juvenile and adult forms of Tunisian NPCs. However, the methylation process in NPC could be driven by unusual mechanisms, such as mechanisms related to latent EBV-infection. Interestingly, it has been shown in transfected cells that the EBV oncoprotein LMP1 can induce hypermethylation of the *E-cadherin* promoter *via* the overexepression of cellular DNA methyltransferases (35). As previously mentioned, we have shown that LMP1 is abundantly expressed in the juvenile form of NPC (16).

As a next stage of our study, we intend to analyze, in the two age-forms of NPC, the methylation status of the EBV gene promoters, especially those of the major transactivator Rta and the main oncoprotein LMP1.

Acknowledgements

We thank N. Ben Hamed, proficient in the English language, for checking the manuscript.

References

1 Busson P, Keryer C, Ooka T and Corbex M: EBV-associated nasopharyngeal carcinomas: from epidemiology to virus-targeting strategies. Trends Microbiol *12*: 356-360, 2004.

2 Boussen H, Bouaouina N, Mokni-Baizig N, Gamoudi A, Chouchane L, Benna F and Ladgham A: Nasopharyngeal carcinoma. Recent data. Pathol Biol *53*: 45-51, 2005.

3 Santini V, Kantarjian HM and Issa JP: Changes in DNA methylation in neoplasia: pathophysiology and therapeutic implications. Ann Intern Med *134*: 573-586, 2001.

4 Herman JG, Graff JR, Myohanen S, Nelkin BD and Baylin SB: Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci *93*: 9821-9826, 1996.

5 Herman JG, Merlo A, Mao L, Lapidus RG, Issa JP, Davidson NE, Sidransky D and Baylin SB: Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. Cancer Res *55*: 4525-4530,1995.

6 Chow LS, Lo KW, Kwong J, To KF, Tsang KS, Lam CW, Dammann R and Huang DP: *RASSF1A* is a target tumor suppressor from 3p21.3 in nasopharyngeal carcinoma. Int J Cancer *109*: 839-847, 2004.

7 Tsao SW, Liu Y, Wang X, Yuen PW, Leung SY, Yuen ST, Pan J, Nicholls JM, Cheung AL and Wong YC: The association of E-cadherin expression and the methylation status of the E-cadherin gene in nasopharyngeal carcinoma cells. Eur J Cancer *39*: 524-531, 2003.

8 Kwong J, Chow LS, Wong AY, Hung WK, Chung GT, To KF, Chan FL, Daigo Y, Nakamura Y, Huang DP and Lo KW: Epigenetic inactivation of the deleted in lung and esophageal cancer 1 gene in nasopharyngeal carcinoma. Genes Chromosomes Cancer *46*: 171-180, 2007.

9 Qiu GH, Tan LK, Loh KS, Lim CY, Srivastava G, Tsai ST, Tsao SW and Tao Q: The candidate tumor suppressor gene BLU, located at the commonly deleted region 3p21.3, is an E2F-regulated, stress-responsive gene and inactivated by both epigenetic and genetic mechanisms in nasopharyngeal carcinoma. Oncogene *23*: 4793-4806, 2004.

10 Lo KW and Huang DP: Genetic and epigenetic changes in nasopharyngeal carcinoma. Semin Cancer Biol *12*: 451-462, 2002.

11 Ayadi W, Feki L, Khabir A, Boudawara T, Ghorbel A, Charfeddine I, Daoud J, Frikha M, Hammami A and Karray-Hakim H: Polymorphism analysis of Epstein-Barr virus isolates of nasopharyngeal carcinoma biopsies from Tunisian patients. Virus Genes *34*: 137-145, 2007.

12 Mokni-Baizig N, Ayed K, Ayed FB, Ayed S, Sassi F, Ladgham A, Bel Hadj O and El May A: Association between HLA-A/-B antigens and -DRB1 alleles and nasopharyngeal carcinoma in Tunisia. Oncology *61*: 55-58, 2001.

13 Goldsmith DB, West TM and Morton R: HLA associations with nasopharyngeal carcinoma in Southern Chinese: a meta-analysis. Clin Otolaryngol Allied Sci *27*: 61-67, 2002.

14 Khabir A, Sellami A, Sakka M, Ghorbel AM, Daoud J, Frikha M, Drira MM, Busson P and Jlidi R: Contrasted frequencies of p53 accumulation in the two age groups of North African nasopharyngeal carcinomas. Clin Cancer Res *6*: 3932-3936, 2000.

15 Khabir A, Ghorbel A, Daoud J, Frikha M, Drira MM, Laplanche A, Busson P and Jlidi R: Similar BCL-X but different BCL-2 levels in the two age groups of North African nasopharyngeal carcinomas. Cancer Detect Prev *27*: 250-255, 2003.

16 Khabir A, Karray H, Rodriguez S, Rosé M, Daoud J, Frikha M, Boudawara T, Middeldorp J, Jlidi R and Busson P: EBV latent membrane protein 1 abundance correlates with patient age but not with metastatic behavior in north African nasopharyngeal carcinomas. Virol J *2*: 1-7, 2005.

17 Karray H, Ayadi W, Fki L, Hammami A, Daoud J, Drira MM, Frikha M, Jlidi R and Middeldorp JM: Comparison of three different serological techniques for primary diagnosis and monitoring of nasopharyngeal carcinoma in two age groups from Tunisia. J Med Virol *75*: 593-602, 2005.

18 Dardari R, Khyatti M, Benider A, Jouhadi H, Kahlain A, Cochet C, Mansouri A, El Gueddari B, Benslimane A and Joab I: Antibodies to the Epstein-Barr virus transactivator protein (ZEBRA) as a valuable biomarker in young patients with nasopharyngeal carcinoma. Int J Cancer *86*: 71-75, 2000.

19 American Joint Committee on Cancer (AJCC): Cancer Staging Manual, Fifth Edition. Fleming ID, Cooper JS, Henson DE, Hutter RV, Kennedy BJ, Murphy GP, O'Sullivan B, Sobin LH and Yarbro JW (eds.). Philadelphia, Lippincott-Raven, pp. 31-39, 1997.

20 International Union Against Cancer (UICC): TNM Classification of Malignant Tumors, 5th Edition. Sobin LH and Wittekind CH (eds.). New York, Wiley-Liss, pp. 25-32, 1997.

21 Chan JKC, Pilch BZ, Kuo TT, Wenig BM and Lee AWM: Tumors of the nasopharynx: introduction. *In*: Pathology and Genetics of Head and Neck Tumours (World Health Organization Classification of Tumours). Barnes L, Eveson JW, Reichart P and Sidransky D (eds.). Lyon, France, IARC press, pp. 82-84, 2005.

22 Lo KW, Cheung ST, Leung SF, van Hasselt A, Tsang YS, Mak KF Chung YF, Woo JK, Lee JC and Huang DP: Hypermethylation of the p16 gene in nasopharyngeal carcinoma. Cancer Res *56*: 2721-2725, 1996.

23 Kwong J, Lo KW, To KF, Teo PM, Johnson PJ and Huang DP: Promoter hypermethylation of multiple genes in nasopharyngeal carcinoma. Clin Cancer Res *8*: 131-137, 2002.

24 Wong TS, Tang KC, Kwong DL, Sham JS, Wei WI, Kwong YL and Yuen AP: Differential gene methylation in undifferentiated nasopharyngeal carcinoma. Int J Oncol *22*: 869-874, 2003.

25 Lo KW, Huang DP and Lau KM: P16 gene alterations in nasopharyngeal carcinoma. Cancer Res *55*: 2039-2043, 1995.

26 Ohtani N, Brennan P, Gaubatz S, Sanij E, Hertzog P, Wolvetang E, Ghysdael J, Rowe M and Hara E: Epstein-Barr virus LMP1 blocks $p16^{INK4a}$-RB pathway by promoting nuclear export of E2F4/5. J Cell Biol *162*: 173-183, 2003.

27 Li Z, Lin SX and Liang YJ: Influence of *E-cadherin* promoter methylation and mutation of *beta-catenin* on invasion and metastasis of nasopharyngeal carcinoma cells. Zhonghua Zhong Liu Za Zhi *25*: 238-242, 2003.

28 Tan SH, Ida H, Goh BC, Hsieh W, Loh M and Ito Y: Analyses of promoter hypermethylation for RUNX3 and other tumor suppressor genes in nasopharyngeal carcinoma. Anticancer Res *26*: 4287-4292, 2006.

29 Zheng Z, Pan J, Chu B, Wong YC, Cheung AL and Tsao SW: Down-regulation and abnormal expression of E-cadherin and beta-catenin in nasopharyngeal carcinoma: close association with advanced disease stage and lymph node metastasis. Hum Pathol *30*: 458-466, 1999.

30 Vleminckx K, Vakaet LJ, Mareel M, Fiers W and van Roy F: Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. Cell *66*: 107-119, 1991.

31 Oka H, Shiozaki H, Kobayashi K, Inoue M, Tahara H, Kobayashi T, Takatsuka Y, Matsuyoshi N, Hirano S, Takeichi M and Mori T: Expression of E-cadherin cell adhesion molecules in human breast cancer tissues and its relationship to metastasis. Cancer Res *53*: 1696-1701, 1993.

32 Issa JP: Aging, DNA methylation and cancer. Crit Rev Oncol Hematol *32*: 31-43, 1999.

33 Ahuja N, Li Q, Mohan AL, Baylin SB and Issa JP: Aging and DNA methylation in colorectal mucosa and cancer. Cancer Res 58: 5489-5494, 1998.

34 Li Q, Jedlicka A, Ahuja N, Gibbons MC, Baylin SB, Burger PC and Issa JP: Concordant methylation of the ER and N33 genes in glioblastoma multiforme. Oncogene *16*: 3197-3202, 1998.

35 Tsai CL, Li HP, Lu YJ, Hsueh C, Liang Y, Chen CL, Tsao SW, Tse KP, Yu JS and Chang YS: Activation of DNA methyltransferase 1 by EBV LMP1 Involves c-Jun NH(2)-terminal kinase signaling. Cancer Res *66*: 11668-11676, 2006.

Received March 31, 2008
Revised May 22, 2008
Accepted May 27, 2008