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 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.
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.

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 MgCl₂ 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).
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
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 metastatic 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
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 overexpression 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


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