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Correlation between DNA methyltransferases expression and Epstein-Barr virus, JC polyomavirus and *Helicobacter pylori* infections in gastric carcinomas

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It' is accepted that aberrant expression of DNA methyltransferases (DNMTs) is responsible for hypermethylation in genes. However, there are limited data related to factors inducing aberrant expression of DNMTs. A total of 43 surgically resected gastrc carcinomas (GC) samples were analysed. Using immunohistochemistry assay we have determined expression level of DNMT1 and 3b. The presence of *H.pylori* was evaluated by histology, whereas JC polyomavirus (JCV) and Epstein-Barr virus (EBV) detection were carried out by PCR and *in situ* hybridization techniques, respectively. High expression of DNMT1 and 3b were detected in 46.5% and 53.5% of GC cases, respectively. Co-expression of DNMT1 and 3b were found in 37.2% of cases. Using different techniques, *H. pylori*, JCV and EBV were detected in 55.8%, 32.6% and 9%, respectively. Moreover, in 37% of cases, we noted the presence of JCV and/or EBV infections. *H.pylori* co-infection was found in 64.3% (9/14) of JCV positive cases and in 50% of EBV positive GC, without a reliable significant relationship. Correlation analyses have showed a marked increase in DNMT1 expression in EBV associated GC (P= 0.02). Also, co-expression of DNMT1 and 3b was significantly associated with EBV infection in GC (P=0.05). Similarly, JCV associated GC mostly displayed DNMT1 positive status, but the difference did not reach the significant threshold. Nevertheless, infection with JCV and/or EBV was significantly correlated with increased expression of DNMT1 in GC (P= 0.05). Our study suggests that EBV and JCV infections in GC correlated with deregulation of DNA methyltransferases.

Key words: DNA methyltransferases, Epstein-Barr virus, JC polyomavirus, Helicobacter pylori, Gastric neoplasms

Gastric carcinoma (GC) is the fourth most frequent malignancy and stands as the second common cause of cancer death in the world [1,2,3]. When diagnosed at an advanced stage, the disease is usually incurable with an adverse prognosis.

Molecular events of GC remain still not clearly understand. Nevertheless, it is widely recognized as an intricate multistep process that involves multiple etiologic factors and deregulation in proto-oncogenes, tumor suppressor genes, and other key cellular genes implicated in cell proliferation, differentiation, and genome integrity [4,5,6].

In GC, it is commonly agreed that *Helicobacter pylori* (*H.pylori*) infection is the major aetiological factor [7]. Indeed, infection with this bacterium has been associated with a significant increased risk of chronic gastritis and GC. *H pylori* acts by inducing chronic inflammation, which has the potential to damage DNA and proteins, can modulate enzymes activities and genes expression and so favour carcinogenesis [8,9]. Epstein-Barr virus (EBV) infection has also been reported to be associated in 5 to 10% of GC [10-14]. Likewise, several studies have suggested that JC polyomavirus (JCV) could be implicated in gastric carcinogenesis [15-17]. Conceptually, based on theirs oncogenic ability *in vitro*, EBV and JCV may have, in part, a role in the carcinogenesis of the stomach. These viruses, by itself, are not sufficient for cancer causation, but act as cofactor in conjunction with other alterations to ultimately contribute to gastric cancer.

A key feature in the pathogenesis of GC is methylation in promoter region, resulting in losses of parts or even whole gene expression. Hypermethylation of several gene promoters has been described in sporadic GC [5]. Furthermore, concurrent promoter hypermethylation of multiple genes, which is termed descriptively as the CpG island methylator phenotype (CIMP), was also described in gastric cancers development [4,5,18]. So far, three enzymes have been identified as responsible for methylation in mammals: DNA methyltransferase 1, putatively responsible for the maintenance activity, DNMT3a

and 3b, probably responsible for the *de novo* activity, in the malignant cell [19].

Although, aberrant expression of DNA methyltransferases (DNMTs) have been accepted as responsible for aberrant hypermethylation in genes, there are, however, limited data related to factors inducing aberrant expression of DNMTs. Interestingly, it has been reported in GC, that high expression of DNMT1 was strongly associated with EBV infection [20-22]. To the best of our knowledge, the relationship between DNMTs expression and JCV infection in GC has not been documented so far.

The majority of investigations consider that the expression level of DNMT3a is not a critical determinant in gastric cancer. Thus, in this study, we examined proteins expression level of DNMT1 and DNMT3b, along with infections with JCV and other known etiologic agents as *H.pylori* and EBV in gastric carcinomas from Tunisian patients. We attempt to give insight whether DNMTs expression is a target for the oncogenic agents in GC.

Material and methods

Clinical samples. A total of 43 GC samples, from Tunisian patients who had undergone tumor resection, were enrolled in this study. The demographic and clinical data of patients were reviewed and recorded. None of the patients have received preoperative therapy. Histological assessment was performed according to the Lauren classification criteria [23]. Penetration of the gastric wall and the presence of lymph node metastases were recorded for all patients using the standard criteria for TNM staging with the unified international gastric cancer staging classification [24].

Immunohistochemical analysis of DNMT1 and 3b. Immunohistochemistry analyses of DNMT1 and 3b proteins expression were conducted using Envision kit (DakoCytomation, Glostrup, Denmark). Briefly, formalin-fixed, paraffin-embedded tissues were cut into 4μm thick sections and mounted on poly-L-lysine-coated glass slides. Slides were deparaffinized in xylene, rehydrated in graded alcohols and washed in Tris-buffered saline (TBS) (0.05 mM Tris-HCl; 1.15 mM NaCl, pH 7.6). Endogenous peroxidase activity was blocked by incubating sections in 3% H_2O_2 for 7 min. For antigen retrieval, slides were heated in a water bath for 45 min with 10 mM sodium citrate buffer (pH 6.0). Then, the slides were thoroughly washed with TBS. After that, the slides were incubated with specific primary antibodies that recognized DNMT1 (rabbit polyclonal, dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), DNMT3b (mouse monoclonal, clone 52A1018, dilution 1:200, Imgenex, Santa Diego, CA, USA), respectively. All primary antibody incubations were conducted at 4°C overnight. Following washes with TBS, the slides were incubated for 15 min with the secondary biotinylated antibody at room temperature for 30 min. Antigen-antibody complexes were visualized using a diaminobenzidine as a chromogen. Slides were counter-stained with hematoxylin, dehydrated, and analyzed under a light microscope.

Two pathologists evaluate the immunostaining results independently. Cases with nuclear staining in more than 30% of tumor cells were considered to have a high expression of DNMT1 and 3b, respectively.

Two kinds of control were used in this assay, infiltrating lymphocytes that show a positive immunoreactivity for DNMTs were used as a positive internal control for all sections. Tris-buffered saline (TBS), instead of the primary antibody, was used for negative control sections. The results were reported without knowledge of JCV, EBV and *H.pylori* status.

H. pylori, JCV and EBV detection. For *H.pylori* review, 5μm thick sections were routinely processed for hematoxylin-eosin and modified Giemsa staining for histological evaluation and assessment of the presence of *H.pylori*. Patient was considered as *H.pylori* positive when visible curved, rod-shaped bacteria were identified.

PCR assay was used as a routine method for the detection of JCV DNA in GC. We used specific PCR primers which allowed us to detect different JCV regions. SV40 and BKV polyomavirus are also searched in this study. The primer sequences, annealing temperature, and product sizes used for analysis were listed in our previous investigation [17].

Detection of EBV DNA in GC was carried out by *in situ* hybridization analysis as described in our previous study [14]. A case was regarded to be EBV associated GC, if it showed, under microscopy, a positive signal as a dark blue/black color in the nuclei of tumor cells. As positive control, we used a specimen of EBV positive nasopharyngeal carcinoma.

Statistical analysis. Data were analyzed with SPSS 17.0 statistics software (SPSS, Chicago, IL). JCV, EBV and *H.pylori* status was analyzed with regard to clinicopathological parameters and immunohistochemical findings using Chi 2 or Fisher's exact tests. All *P*-values presented are two-tailed and the statistical significant threshold was set at 0.05.

Results

DNA Methyltrasferases expression. Expression of DNMT1 and 3b was evaluated by immunohistochemistry assay. Among 43 tumor samples analyzed, high expression of DNMT1 and 3b were detected in 20 (46.5%) and 23 (53.5%) of GC cases, respectively. Immunoreactivity for DNMT1 and 3b were specifically detected in the nuclei of tumor cells. In normal gastric mucosa, the expression levels of DNMT1 and T3b are very low. Representative examples for DNMT1 and 3b high expression in GC were shown in Figure 1 panels (i-l) and panels (m-p). Co-expression of DNMT1 and 3b were found in 37.2% (16/43) of cases.

JCV, EBV and H.pylori detection. *H.pylori* infection was evaluated by histology using Giemsa stain and was identified in 55.8% (24/43) of patients. Results from PCR experiments, have identified JCV DNA sequence in 32.6% (14/43) of GC samples. None of the samples were positive for SV40 or BKV.

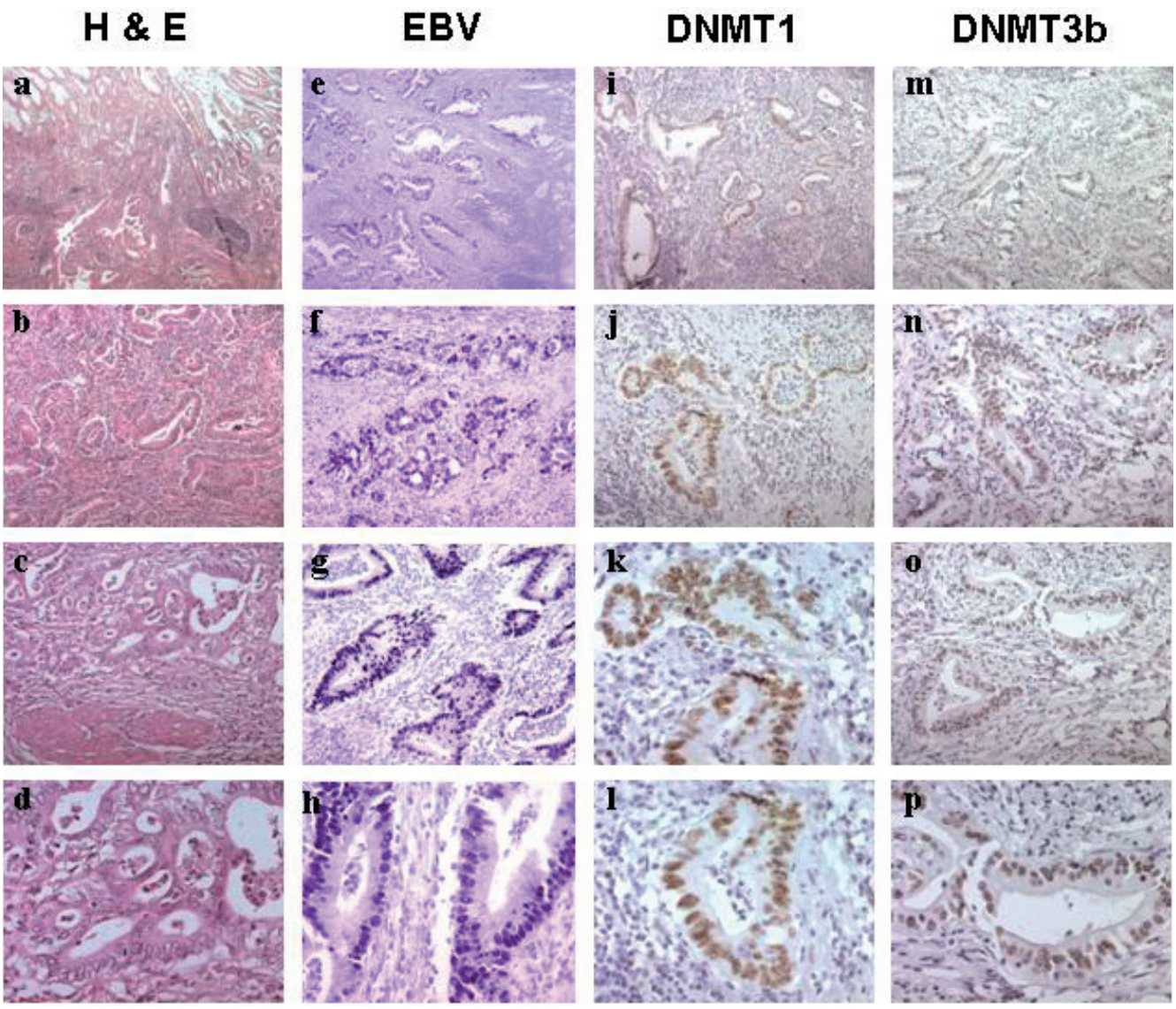

Figure 1. Representative illustrations of an EBV-positive gastric carcinoma case showing strong expression of DNMT1 and DNMT3b. Panels a - d: Haematoxylin and eosin (H&E) staining showing a well-differentiated gastric adenocarcinoma of intestinal type; panels e-h: Detection of Epstein-Barr virus (EBV) in the nuclei of the tumor cells using EBER *in situ* hybridization (dark blue/black stain); immunohistochemical staining showing strong expression (brown stain) of DNMT1 (panels i-l) and DNMT3b (panels m-p) in the nuclei of the tumor cells (Original magnifications, ×40, ×100, ×200, ×400).

Using *in situ* hybridization, EBV was demonstrated in 4 cases of GC samples. In the EBV positive cases, signals were specifically localized in the nuclei of tumor cells Figure 1 panels (e-h).

Moreover, in 37% (15/43) of GC cases, we noted the presence of JCV and/or EBV infections. *H.pylori* co-infection was found in 64.3% (9/14) of JCV positive cases and in 2 cases of EBV positive GC, without a reliable significant relationship (Figure 2).

DNA Methyltrasferases expression with regard to infectious agents in gastric cancer. DNA methyltrasferase 1 and 3b protein expressions according to the status of JCV, EBV and *H. pylori* infections in GC from Tunisian patients are summarized in Tables 1 and 2.

In the present study, we found that the expression profile of DNMT1 and 3b remain the same in GC with and without association of *H.pylori* (Table 1). However, EBV positive cases displayed high expression of DNMT1 and 3b (Table 1). Indeed, the presence of EBV in GC was significantly associated with DNMT1 protein overexpression (P=0.02). Also, EBV infection was significantly associated with co-expression of DNMT1 and 3b (P=0.05) in GC (Table 1).

As for the relationship with the presence of JCV infection and proteins expression levels of DNMT1 and 3b, there was no significant correlation between JCV infection and DNMT1 protein high expression (P= 0.1) nor DNMT3b protein high

expression (P =0.73) in GC (Table 1). However, EBV and/or JCV infection was significantly correlated with increased expression of DNMT1 in GC (P= 0.05; Table 2).

Clinicopathologic findings with regard to infectious agents in gastric cancer. The clinical and pathological features of JCV, EBV and *H.pylori* positive gastric carcinomas in Tunisian patients are summarized in Tables 3 and 4.

Patients with *H.pylori* positive tumor showed less aggressive clinical feature than negative cases. Indeed statistical analysis showed an inverse correlation between the presence of *H.pylori* and lymph node invasion (P=0.004; Table 3). With regard to viral infection, four GC cases were EBV positives and were all male patients, older than 50 years and of high tumor extension (T3 or T4 stages) (Table 3). On the other hand, JCV infections were more frequently detected in older patients (P=0.02; Table 3) and in gastric cancer of intestinal histological type than in diffuse one (P=0.01; Table 3). In the same tumor, coinfection with two or more infectious agents was also correlated with some clinicopathologic characteristic of GC. The main results are presented in Table 4.

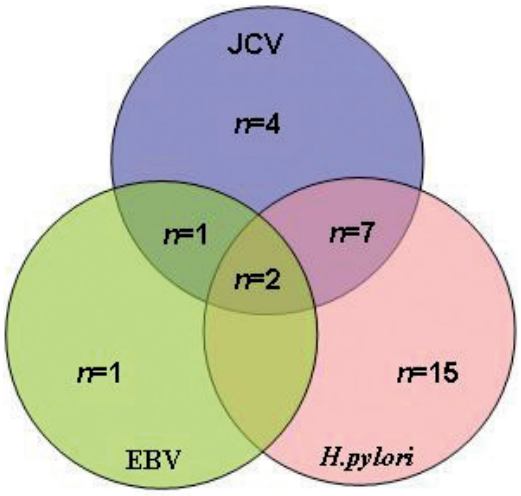

Figure 2. Interrelationships between *H. pylori*, JCV and EBV in gastric cancer. A Venn diagram summarizing the distribution of numbers of positive cases for *H. pylori*, JCV and EBV among a series of 43 gastric carcinoma cases from Tunisia.

Table 1. DNA methylransferases expression and infectious agents in gastric cancer

DNMT expression	Total	*H. pylori*		*P* value	EBV		*P value*	JCV		*P value*
		(+)	(-)		(+)	(-)		(+)	(-)	
Number of cases	43	24	19		4	39		14	29	
DNMT1										
High expression	20	9	11	0.18	4	16	**0.02**	9	11	0.1
Low expression	23	15	8		0	23		5	18	
DNMT3b										
High expression	23	12	11	0.6	3	20	0.19	8	15	0.73
Low expression	20	12	8		1	19		6	14	
DNMT1+DNMT3b										
Positive	16	8	8	0.55	3	1	**0.05**	7	9	0.22
Negative	27	16	11		1	28		7	20	

All statistical tests were two-sided; p-values were calculated by the χ^2 or Fisher exact test; significant p-values are shown in bold. (+) positive case; (-) negative case.

Table 2. DNA methyltransferases expression and multiple infections pattern in gastric cancer

DNMT expression	Total	*H.pylori* and/or EBV		*P value*	*H.pylori* and/or JCV		*P value*	EBV and/or JCV		*P value*	*H.pylori* and/or EBV and/or JCV		*P value*
		(+)	(-)		(+)	(-)		(+)	(-)		(+)	(-)	
Number of cases	43	26	17		29	14		15	28		30	13	
DNMT1													
High expression	20	11	9	0.54	13	7	0.75	10	10	**0.05**	14	6	1
Low expression	23	15	8		16	7		5	18		16	7	
DNMT3b													
High expression	23	14	9	0.94	15	8	0.73	9	14	0.53	16	7	0.97
Low expression	20	12	8		14	6		6	14		14	6	
DNMT1+DNMT3b													
Positive	16	10	6	0.83	11	5	1	8	8	0.1	12	4	0.73
Negative	27	16	11		18	9		7	20		18	9	

All statistical tests were two-sided; p-values were calculated by the χ^2 or Fisher exact test; significant p-values are shown in bold. (+) positive case; (-) negative case.

Table 3. Clinicopathologic parameters and infectious agents in gastric cancer

Clinicopathologic parameters	Total	*H. pylori*		*P* value	EBV		*P* value	JCV		*P* value
		(+)	(-)		(+)	(-)		(+)	(-)	
Number of cases	43	24	19		4	39		14	29	
Gender										
Male	27	13	14	0.18	4	23	0.27	9	18	0.88
Female	16	11	5		0	16		5	11	
Age (years)										
≤ 50	9	4	5	0.46	0	9	0.5	0	9	**0.02**
> 50	34	20	14		4	30		14	20	
Tumor location										
Cardia	14	7	7	0.48	3	11	0.09	5	9	1
Non cardia	29	17	12		1	28		9	20	
Histological type[1]										
Intestinal	19	9	10	0.32	3	16	0.3	10	9	**0.01**
Diffuse	24	15	9		1	23		4	20	
Depth of invasion [2]										
Early	8	7	1	**0.05**	0	8	1	4	4	0.4
Advanced	35	17	18		4	31		10	25	
Lymph node metastasis										
Positive	19	6	13	**0.004**	2	17	1	5	14	0.43
Negative	24	18	6		2	22		9	15	

[1]Histological classification according to Lauren's criteria (1965): intestinal; diffuse
[2]Depth of invasion: Early (pT1-pT2), Advanced (pT3-pT4)
All statistical tests were two-sided; *p*-values were calculated by the χ^2 or Fisher exact test; significant *p*-values are shown in bold. (+) positive case; (-) negative case.

Table 4. Clinicopathologic parameters and multiple infections pattern in gastric cancer

Clinicopathologic Parameters	Total	*H.pylori* and/or EBV		*P value*	*H.pylori* and/or JCV		*P value*	EBV and/or JCV		*P value*	*H.pylori* and/or EBV and/ or JCV		*P value*
		(+)	(-)		(+)	(-)		(+)	(-)		(+)	(-)	
Number of cases	43	26	17		29	14		15	28		30	13	
Gender													
Male	27	15	12	0.52	17	10	0.41	10	17	0.7	18	9	0.56
Female	16	11	5		12	4		5	11		12	4	
Age (years)													
≤ 50	9	4	5	0.44	4	5	0.13	0	9	**0.01**	4	5	0.1
> 50	34	22	12		25	9		15	19		26	8	
Tumor location													
Cardia	14	8	6	0.6	9	5	0.72	5	9	0.86	9	5	0.72
Non cardia	29	16	11		20	9		10	19		21	8	
Histological type[1]													
Intestinal	19	10	9	0.35	13	6	0.9	10	9	**0.03**	13	6	0.86
Diffuse	24	16	8		16	8		5	19		17	7	
Depth of invasion [2]													
Early	8	7	1	0.11	8	0	**0.03**	4	4	0.41	8	0	0.08
Advanced	35	19	16		21	14		11	24		22	13	
Lymph node metastasis													
Positive	19	7	12	**0.01**	8	11	**0.002**	6	13	0.68	9	10	**0.004**
Negative	24	19	5		21	3		9	15		21	3	

[1]Histological classification according to Lauren's criteria (1965): intestinal; diffuse
[2]Depth of invasion: Early (pT1-pT2), Advanced (pT3-pT4)
All statistical tests were two-sided; *p*-values were calculated by the χ^2 or Fisher exact test; significant *p*-values are shown in bold. (+) positive case; (-) negative case.

Discussion

Despite the high incidence of GC, knowledge of the molecular events leading to this tumor is still limited. Aberrant DNA cytosine methylation is one of the most consistent epigenetic changes in GC. DNA hypermethylation occurs frequently in CpG islands near regulatory regions of genes and affects the transcription of specific genes notably tumor suppressor genes [5,6,25]. In addition, several lines of evidence have suggested that elevated DNMTs expression might play a causal role in DNA hypermethylation and thus in gastric carcinogenesis [19,20,26,27].

To understand the background behind of DNMTs, we examined its expression along with etiological factors considered to be involved in GC. Indeed, cancers of the stomach belong to the group of infection-related neoplasms. The main mechanisms by which infections agents may induce GC are still to be elucidated.

JC polyomavirus (JCV) is a ubiquitous virus, well adapted to humans. JCV infection is subclinical, but in immunocompromised individuals may be associated with neural disease as progressive multifocal leukoencephalopathy (PML). Recently, JCV genomic DNA sequences and T-antigen expression have been detected in a broad range of human tumors of the gastrointestinal tract notably in esophagus [28], gastric [15-17], colon [29], colorectal [30,31] and bladder [32] cancers. JCV infection was even reported as a potential risk factor for colorectal cancer [33].

Although JCV's etiologic involvement in cancer is strongly suspected, its precise role in carcinogenesis is still unclear. Like other polyomaviruses, JCV encodes a version of a large T-antigen that can bind to and inactivate tumor suppressor proteins p53 and pRB and interfere with several cell-signaling pathways [34].

Nowadays, little information is available regarding the molecular features of JCV positive GC. In our previous study, we have found that JCV positives GC display higher frequencies of methylation of *P16* and *P14* than that in JCV negative cases [17]. Similarly, Yamaoka et *al.* have presumed that hypermethylation of CpG islands can be a mechanism of silencing of some tumor suppressor genes in JCV positive GC [35]. No study was available indicating that JCV directly or indirectly causes the aberrant CpG island methylation of the host genome. To the best of our knowledge, our study is the first analysis of JCV DNA with regard to DNMTs expression in GC patients. Interestingly, we found that tissues from GC patients, with DNMT1 and 3b high expression, had higher incidence of JCV infection as compared with the patients without JCV infection, although the difference was not statistically significant.

This result can supports the transient effects of JCV in cellular transformation, as it can promote DNMTs expression in onset gastric carcinogenesis, in the same manner than that previously proposed for EBV infection in GC [20,21,22].

Apart from the JCV involvement, EBV has also proved to be potently implicated in gastric carcinogenesis. EBV is well known for its oncogenic potential, and has been associated with GC. In our previous investigation [14], we have demonstrated the presence of EBV in GC from Tunisian people. Our finding is in keeping with studies in Mexico, Peru, Asian countries and in African continent [10,11,12].

The role of EBV in carcinogenesis of the stomach is not completely understood. But, it is agreed that EBV positive carcinomas have distinct molecular characteristics.

The p16INK4a-Rb pathway plays a critical role in preventing inappropriate cell proliferation. This pathway is often targeted by viral oncoproteins. Kang *et al.* [36] have reported that the methylation frequency of p16INK4a in the EBV positive GCs was more than three times higher than that in the EBV negative GCs. In addition, several studies have showed that EBV positive GCs display frequently down regulation of many genes through aberrant methylation in malignant cells than in EBV negative GCs [36,37].

However, the mechanism by which EBV can promote aberrant CpG island methylation of host genome is yet to be elucidated. There is no convincing evidence indicating that EBV directly or indirectly causes the methylation of the host genome. Previous, *in vitro,* studies [21,22] have provided evidence that transfection of the EBV's oncoprotein LMP1 induces the expression and activity of DNMT1 in cultured cancer cells. Etoh et *al.* [22], in a retrospective study among gastric tissues samples, have found that all the four EBV positive cases showed DNMT1 protein overexpression. The authors have suggested that EBV infection and other aetiological factors may be associated with DNMT1 overexpression in GC. In the present study**,** we have found that the presence of EBV in GC was significantly associated with DNMT1 protein overexpression (P=0.02). In addition, EBV infection was significantly associated with co-expression of DNMT1 and DNMT3b in GC (P=0.05). All together, these observations suppose that DNMT1 may be a target of EBV and promote aberrant DNA methylation during gastric carcinogenesis.

Although JCV and EBV can have a role in the pathogenesis of gastric cancer, it cannot be considered the sole causative agent in this malignancy. There are other risk factors that clearly contribute and perhaps the strongest is *H.pylori* infection in gastric carcinogenesis.

In 1994, the World Health Organization and the International Agency for Research on Cancer consensus group stated that there was sufficient evidence to classify *H.pylori* as a class I human carcinogen. Infections with this bacterium have been associated with a significantly increased risk of chronic gastritis and gastric cancer [7,8,38]. Studies of conventional GC have revealed the presence of *H.pylori* in 50-80% of cases worldwide [38].

The role of *H.pylori* infections in the development of GC has been thoroughly characterized. Few studies have linked DNA hypermethylation with *H.pylori* of cagA+ genotype [9,39]. In this investigation, we have found that GC did not

showed different DNMT1 and 3b expression profile with regard to *H.pylori* infection. Similar result has been reported in GC [20]. Indeed, Etoh et *al.* [20] have found that DNMT1 protein overexpression was not significantly associated with the incidence of *H.pylori* infection in corresponding noncancerous mucosa of gastric samples.

Conclusions

Gastric cancer arising in Tunisian people has a multifactor aetiology where *H.pylori*, JCV and EBV are important risk factors in the development of this neoplasm. EBV and JCV infections in GC correlated with deregulation of DNA methyltransferases.

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