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Research Article

Promoter Methylation Status of P53 and E-Cadherin Genes in Gastric Cancer and its Association with Epstein-Barr Virus and Helicobacter Pylori Infections

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Abstract

Objectives: Gastric cancer is one of the leading causes of morbidity and mortality worldwide. As in other cancers, the development and progression of gastric cancer has been attributed to many factors, including infection with *Helicobacter pylori (H. pylori)* and Epstein-Barr virus (EBV), genetic alterations, and epigenetic silencing due to aberrant methylation of tumor suppressor genes.

The purpose of this work is to analyze the promoter methylation status of P53 (*TP53*) and E-cadherin (*CDH1*) genes and its association with EBV and *H. pylori* infections in the gastric cancer patients of Grand Casablanca.

Methods: In this study, a total of 50 gastric cancer patients were recruited. Methylation in tumor tissues was detected by methylation-specific PCR (MSP), and the clinical relevance was statistically analyzed.

Results: Results revealed a methylation of *TP53* promoter observed in 36% of gastric carcinoma cases, which was significantly higher than adjacent normal tissue [p≤0.0001]. While no methylation of *CDH1* promoter was observed. Furthermore, the frequency of *TP53* promoter methylation was significantly different with intestinal and diffuse types of gastric cancer [22.3% vs 77.7%; <0.05]. Moreover cases with EBV infection had higher frequencies of *TP53* methylation [p<0.05], while no significant correlation between *H. pylori* and *TP53* methylation was observed.

Conclusion: Thus; the present data suggest a vital role of epigenetic alteration of *TP53* in the causation and development of gastric cancer, especially Epstein-Barr virus-associated gastric cancer in our population.

Keywords: Epstein-Barr virus, *CDH1*, gastric cancer, methylation, *TP53*, helicobacter pylori

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Gastric cancer (GC), or stomach cancer, is one of the most prevalent malignancy in the world.[1] Despite reduction in frequency and mortality rates in recent decades, it is still the fifth most common cancer and the third leading cause of cancer death worldwide, with An estimate of 1,033,701 new cases and 782,685 deaths related to GC recorded in 2018.[2]

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GC is a cancer with a recognized infectious etiology, involving bacteria and viruses, including *H. pylori* and EBV, and remains the well-established leading causes of GC.[3, 4] However, genetic alterations have been a major cause of increased risk of GC.[5] As in other malignancies, gastric carcinogenesis involves a steady accretion of various genetic and epigenetic alterations, leading to a gain of function in oncogenes and a loss of function in tumor suppressor genes.[6] Many studies have shown that silencing of tumor suppressor genes, is mainly caused by hypermethylation of CpG islands in promoters, which is essential for gastric carcinogenesis.[7, 8] This hypermethylation involves many genes, including *TP53*, *CDH1*, and several other genes involved in cancer.[9, 10]

The tumor suppressor gene *TP53* encodes a transcription factor that modulates the cellular stress response through transcriptional regulation of genes involved in DNA repair, cell cycle arrest and apoptosis.[11, 12] Mutations and methylation in the 5' promoter region of *TP53* have been shown to be associated with cancer development.[13] In addition, it has been reported that The expression of the *CDH1* gene that encodes E-dadherin, which is essential protein for maintaining cell polarity, cell adhesion and normal epithelial tissue architecture, was frequently lost or reduced in a range of epithelial tumors, resulting in increased tumor invasion and metastasis that has been shown to be associated with several solid malignancies, including GC.[14-16] Although methylation of *TP53* and *CDH1* genes has been well studied, data from the population of Grand Casablanca are lacking. In addition, some studies have linked DNA methylation to *H. pylori*-cagA+ and EBV infection, geographic location, and environmental exposure.[17] Thus, the present study aimed to investigate the methylation status of the *TP53* and *CDH1* genes and assess their association *with H. pylori* and EBV infections and also with clinicopathological characteristics of GC patients in the region of Grand Casablanca.

Methods

Patients and Samples

A total of 100 gastric tissue samples, including 50 tumor tissues and 50 corresponding adjacent normal tissues from patients who underwent gastric resection in the department of surgery of Ibn Rochd University Hospital Center, Casablanca, were included in this study.

The Research Ethics Boards of the respective institution approved this study and each subject signed an informed consent.

DNA Extraction

Tissue samples (both tumor and adjacent normal) were immediately frozen and stored at -80° C until use. DNA was extracted from the tissues using the Pure link Invitrogen® Genomic DNA mini kit, Thermo Fisher USA, according to the manufacturer's instructions. The quality and quantity of the DNA obtained were evaluated using NanoDrop 2000 (Thermo Scientific, USA).

Intrenal Control

Samples were checked by PCR with β-globin gene (internal control), Using the primers PCO4 (5'TCACCACAACTTCATCCACGTTCACC3') and GH20 (5'GAAGAGCCAAGGACAGGTAC3') (18). Only the samples that were positive for human ß-globin gene are the subjected To DNA methylation research.

Bisulfite Modification and Methylation-Specific PCR (MSP)

DNA conversion with sodium bisulfite was performed as described previously.[19] Bisulfite treatment was performed using an EZ DNA Methylation Gold Kit (ZYMO Research) according to the manufacturer's instructions. Briefly, 1–2 µg of genomic DNA was denatured with 2 M NaOH (final concentration of 0.2 M) at 50 °C for 20 min, followed by incubation with freshly prepared 2.5 M sodium bisulfite/1 M hydroquinone (pH 5.0) in a total volume of 520 ml, at 70 °C for 18 hours. DNA modification was completed by the addition of 5 ml of 3 M NaOH at room temperature for 10 min. The precipitation of the modified DNA was carried out through the addition of 75 ml of ammonium acetate 5 M (pH 7.0) and 350 ml of ethanol. The bisulfite-modified DNA was resuspended in 100 ml of sterile water and stored at 20 °C or immediately used for MS-PCR analysis.

PCR reaction was carried out with treated DNA in a 25 µl reaction mixture containing 0.25 mM of each primer pair, 200 mM of each dNTP, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl2, 1 unit of Taq DNA polymerase (Bioline). Using the primers as previously described (20, 21) Table 1.

Table1. Primer Sequences and PCR conditions for methylation-specific PCR (MSP) analysis

Genes	Primer name	Sequence	Amplicon Length	T (°C)	Ref.
TP53	P-F	GAGGTTTTTGGTATAAAGTTGGATAGT	172 bp	49	(20)
	P-R	CTCTAACTTACAAAATTTTCCACCC			
CDH1	P-F	TTAGGTTAGAGGGTTATCGCGT	115bp	61	(21)
	P-R	TAACTAAAAATTCACCTACC GAC			

Cycling conditions were as follows: denaturation at 95 °C for 5 min, followed by 35 cycles of 30 s at 95 °C, for 30 s at the specific temperature and 30 s at 72 °C, The reaction was finished with a 5 min extension at 72 °C. PCR products were size-fractionated in 2% agarose gel and visualized by ethidium bromide staining.

Statistical Analysis

Statistical analysis was performed using SPSS 23.0 statistical software (SPSS, Inc., Chicago, IL, USA). The correlation between the different disease parameters was analyzed by the Student's t-test, the Chi-square test and the fisher exact test. The difference was considered significant when the p-value was less than 0.05.

Results

Clinicopathological Characteristics

The clinical characteristics of the enrolled patients are illustrated in Table 2. The median age of the patients was 58 years (range, 36–72 years). Well and poor tumors differentiations were observed in 24% and 76% of patients, respectively. As shown in Table2, according to TNM classification, most samples were in high stage with a metastasis to lymph nodes.

Methylation status

Hypermethylation of the promoter region of *TP53* was observed in 36% of GC cases, which was significantly higher than adjacent normal tissue (p≤0.0001) Table 3. While no

Table 2. Patients' clinicopathological characteristics

Characteristics	%
Age	
<58 yrs	50
≥58 yrs	50
Gender	
Male	56
Female	44
Lauren's classification	
intestinal type	42
diffuse type	58
Histopathological differenciation	
Well	24
Moderate/Poor	76
Lymph node metastasis	
Negative	28
Positive	72
Stage	
Low (I and II)	30
High (III and IV)	70

Table 3. TP53 Promoter methylation in Normal and Tumor Tissue

	Normal	Tumor	p
Methylated	6	18	<0.0001
Unmethylated	44	32	

CDH1 promoter methylation was observed. Furthermore, the frequency of *TP53* promoter methylation was significantly different with intestinal and diffuse types of GC (22.3% vs. 77.7%; p=0.034), as well as with histopathological differentiation (p=0.036) Table 4. Moreover cases with EBV infection had higher frequencies of *TP53* methylation (p=0.004), while no significant correlation between *H. pylori* and *TP53* methylation was observed Table 5.

Discussion

The development and progression of gastric cancer involves a number of genetic and epigenetic alterations of tumor suppressor genes.[22, 23] Promoter hypermethylation of tumor suppressor genes has emerged as a potential mechanism for gene silencing and is still being explored in basic molecular biology, clinical oncology, and medical research.[24] Aberrant hypermethylation of CpG islands in promoter regions can permanently inactivate tumor suppressor genes, as do mutations and chromosomal abnormalities.[25] In GC, genes are more frequently inactivated by aberrant methylation than by gene mutations.[26] A large number of genes inactivated by hypermethylation have been reported in GC.[8, 27, 28] This study therefore focused on elucidating the role of epigenetic alteration of the *CDH1* and P53 genes in the pathogenesis of gastric cancer, and its association *with H. pylori* and EBV infections in the GC population of Grand Casablanca. Hypermethylation of the *CDH1* promoter leading to gene silencing in sporadic gastric cancers ranges from 11% to 75%.[29, 30] In our study, no *CDH1* promoter methylation was detected.

TP53 As a crucial tumor suppressor gene, its silencing or deficiency could alter cell growth arrest and apoptosis, leading to the initiation of carcinogenesis.[31] *TP53* silencing or deficiency has been detected in almost all types of cancers.[32] In the present study, our result revealed a methylation of *TP53* promoter observed in 36% of gastric carcinoma cases, which was significantly higher than adjacent normal tissues. Furthermore, clinicopathological features are essential elements in the detection of gastric cancer and routinely measured in clinical practice, and the identification of molecular subtypes of GC is essential because of their significant value for diagnosis and prediction of response to treatment. In the present study, the frequency of *TP53* promoter methylation was significantly different with intesti-

Table 4. Association of TP53 Promoter methylation with Various Clinicopathological features of Gastric Cancer Patients

Clinicopathological features	Cases	Methylation state of the tumor		p
		Methylated	Unmethylated	
Age groupe				
<58 yrs	25	11	14	0.24
≥58 yrs	25	7	18	
Gender				
Male	28	9	19	0.52
Female	22	9	13	
Tumor size				
<5 cm	10	2	8	0.3
≥5 cm	40	16	24	
Histopathological differentiation				
well	12	1	11	0.036
Moderate/Poor	38	17	21	
Lauren's classification				
intestinal type	21	4	17	0.034
diffuse type	29	14	15	
Lymph node metastasis				
Negative	14	3	11	0.18
Positive	36	15	21	
Lymphatic duct vessels invasion				
Negative	15	5	10	0.8
Positive	35	13	22	
Stage				
Low (I and II)	15	5	10	0.8
High (III and IV)	35	13	22	

Table 5. Association of TP53 Promoter methylation with H. pylori and EBV infections in Gastric cancer

Clinicopathological features	Cases	Methylation state of the tumor		p
		Methylated	Unmethylated	
H. pylori statuts				
Negative	33	13	20	0.5
Positive	17	5	12	
EBV statuts				
Negative	30	6	24	0.004
Positive	20	12	8	

nal and diffuse types of gastric cancer (22.3% vs. 77.7%), as well as with histopathological differentiation, whereas none of the other clinicopathological parameters showed a significant association with *TP53* methylation status.

More importantly, *H. pylori* and EBV infections have been shown to be closely associated with varying degrees of CpG island methylation, which may contribute to gastric carcinogenesis.[33] Some studies have shown that *CDH1* methylation is present in *H. pylori*-infected non-neoplastic gastric mucosa and in gastric carcinoma, especially in poorly differentiated adenocarcinoma, and that this methylation can be reversed after *H. pylori* eradication.[34] In our study, no methylation of the *CDH1* promoter was detected. Furthermore *H. pylori* strains expressing high levels of CagA, which is a virulence factor produced by *H. pylori* and known to be involved in the methylation of some tumor suppressor genes, more strongly suppress p53 expression compared to low-risk strains.[35] Other functional studies show that viral genes are involved in oncogenic modulation of host gene expression, including CpG methylation of cellular machinery components.[36] Our results showed that there was no significant correlation between *H. pylori* and

TP53 methylation, whereas EBV-infected cases had higher frequencies of *TP53* methylation, suggesting that EBV may contribute to carcinogenesis through the induction of aberrant methylation in gastric epithelial cells.

Conclusion

In conclusion, our results suggest that promoter methylation plays a major role in inactivating tumor suppressor genes, where methylation in P53 assumes an important role. EBV may contribute to carcinogenesis through the induction of aberrant methylation in gastric epithelial cells, although further study is needed to elucidate the detailed molecular mechanisms underlying the induction of promoter methylation in response to EBV infection. Understanding these mechanisms could clarify the process of gastric carcinogenesis, and application of this knowledge to clinical use could aid in diagnosis, risk management, and prevention.

Disclosures

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Ethics Committee Approval: The study was approved by the Biomedical Research Ethics Committee of the School of Medicine and Pharmacy of Casablanca. All patients consented to participate in the study according to the ethical standards.

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