ORIGINAL PAPERS

Adv Clin Exp Med 2016, **25**, 3, 465–470
DOI: 10.17219/acem/38842

ISSN 1899–5276

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Alteration in Methylation Pattern of Retinoblastoma 1 Gene Promotor Region in Intestinal Metaplasia with or without *Helicobacter pylori* and Gastric Cancer Patients*

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article

Abstract

Background. *Helicobacter pylori*, intestinal metaplasia (IM), and gene methylation play important roles in gastric carcinogenesis. However, the association among *H. pylori* infection, IM, gastric cancer (GC), and gene methylation is not fully understood. Cell cycle control involving retinoblastoma 1 (RB1) gene is one of the main regulatory pathways reported to be altered in gastric carcinogenesis.
Objectives. The purpose of this research is to assess the methylation status of *RB1* gene in GC and IM with or without *H. pylori* infection, and to discuss the possible role of *H. pylori*-induced *RB1* gene methylation in the mechanism of gastric carcinogenesis.
Material and Methods. The methylation profile of *RB1* gene was analyzed by sodium bisulfite modification and methylation-specific PCR in GC (n = 24), IM patients with *H. pylori* positive (n = 20) and negative (n = 20), and control subjects (n = 20).
Results. According to methylation levels in *RB1* gene; the high correlation values were detected between *H. pylori* positive-IM group and GC group, and between *H. pylori* positive-IM and *H. pylori* negative-IM groups ($p < 0.05$). No correlations between *H. pylori* negative-IM and GC groups and between GC and control groups were detected in methylation status of *RB1* gene.
Conclusions. High methylation levels in *RB1* gene in *H. pylori* positive individuals may suggest an elevated risk of gastric cancer occurrence (**Adv Clin Exp Med 2016, 25, 3, 465–470**).

Key words: gastric cancer, *Helicobacter pylori*, intestinal metaplasia, methylation, retinoblastoma 1.

Although the incidence and the mortality rates of the GC have been decreasing for more than 50 years, today it is still the fourth most common cancer type with one million new cases per year [1, 2]. The GC incidence in Turkey is 9.6 and 5.7 cases per 100,000 people in men and women, respectively [3]. Histologically, human GC is classified into 2 groups; diffuse-type and more commonly intestinal-type, which is mostly affiliated with gastric atrophy and intestinal metaplasia (IM). Chronic stomach inflammation in older patients causes GC, which is mostly associated with *Helicobacter pylori* infection. A hypothesis explaining the mechanism of GC progression due to *H. pylori* was suggested by Correa [4]. *H. pylori* are shown to play role in GC progression from healthy gastric mucosa to superficial gastritis, chronic active gastritis, atrophic gastritis, and to IM [2, 4]. Although its etiology is not clearly known, diet, alcohol, *Epstein-Barr* virus (EBV) infection, and genetic-epigene-

* The study was supported by Celal Bayar University Scientific Research Projects.

tic factors can be held responsible for gastric carcinogenesis in addition to *H. pylori* infection [4, 5].

RB1 gene at chromosome 13q14 was originally identified as the gene responsible for the development of retinoblastomas and is the first tumor suppressor gene cloned. It represses the transcription of S phase genes by binding the transcription factor E2F. Inactivation of *RB1* gene may cause cell proliferation, which leads to tumorigenesis. It resides on a 200 kb area encoding *pRb* nucleoprotein with a major role in G1-S transition during mitosis [6]. This specific gene region harboring *RB1* gene is found to be altered in retinoblastoma, bone and soft tissue sarcomas, small cell lung cancer, hematologic malignancies, breast cancer, and gastric tumors [6, 7].

Epigenetic alterations of genomic DNA are known to have an effect in the progression of cancer. The accumulation of genetic and epigenetic changes is thought to activate oncogenes and inactivate tumor suppressor genes resulting in GC. One of the epigenetic changes is methylation of the gene regulatory elements resulting in gene inactivation. Replication and transcription initiation may be hindered if the CpG islands in promoter regions are methylated. The transcriptional inactivation of tumor suppressor genes such as *RB1* gene by promoter methylation is a major epigenetic event in the origin of many cancers, including GC [8, 9]. Therefore, identification of novel tumor suppressor genes inactivated by promoter methylation will be of great importance in the understanding of the GC progression, and could be utilized as biomarkers for the early detection of cancer [9, 10].

There are many studies related to the promoter methylation of tumor suppressor genes, which plays a role in GC. However, there are very rare studies about *RB1* gene methylation in GC. Thus, the role of *RB1* in GC has not been fully understood and needs further research. To our knowledge, there is no report about the promoter methylation status of *RB1* gene in GC and IM with or without *H. pylori* infection. Therefore, we examined *RB1* gene methylation status in GC, *H. pylori* positive-IM, and matched *H. pylori* negative-IM groups. The aim of the study is to understand the relationship among *H. pylori*, *RB1* gene, and their roles in gastric carcinogenesis.

Material and Methods

Ethics Statement

This study was performed in accordance with the ethical standards laid down in the 2000 Declaration of Helsinki as well as the Declaration of Istanbul 2009. Celal Bayar University Institutional Review Board approved this clinical trial (No.: 343) on May 20, 2009. Each patient signed an informed consent form prior to any study-related procedures.

Cases and Tissue Samples

The study was conducted at the Departments of Gastroenterology and Medical Biology in Celal Bayar University Faculty of Medicine in Manisa, from September 2009 to August 2012. Endoscopic biopsy method was performed with sterile forceps to obtain the gastric mucosa samples from the upper corpus and antral regions in the lessor curvature. For detection of *H. pylori*, a set of biopsy specimens were fixed in formalin, embedded in paraffin, and stained with a modified toluidine blue. For methylation study, another set of biopsy specimens were immediately frozen in 0.1 mol/L phosphate-buffered saline and stored at –80°C until further processing.

Eighty-four tissue biopsies including distal gastric carcinomas (n = 24), *H. pylori* positive-IM (n = 20), *H. pylori* negative-IM (n = 20), and control group (n = 20) were obtained from endoscopic samples. The control group includes those who had applied to the gastroenterology outpatient clinic with the complaint of dyspepsia symptoms such as epigastric pain, bloating, early satiation, fullness, epigastric burning, nausea, and vomiting and were found normal endoscopically, radiologically, and pathologically.

Patients excluded from the study were those 1) who were on continuous treatment with acid suppression in the preceding two weeks before endoscopy, 2) who were on continuous treatment for *H. pylori* eradication (while cases receiving *H. pylori* eradication treatment are excluded from the study, this was not an exclusion criteria for patients with gastric carcinoma), 3) who had undergone previous upper gastrointestinal surgery such as gastrectomy, and 4) who had severe gastroparesis or esophageal varices.

DNA Isolation

Genomic DNA from stomach mucosa biopsy samples was isolated using a PureLink™ Genomic DNA Mini Kit (Invitrogen Technologies, Inc., CA, USA) according to the manufacturer's instructions. The concentration of DNA was quantified by Nanodrop 1000 (Nanodrop, Wilmington, USA). A260/A280 ratios in the range of 1.8–2.0 were considered satisfactory for purity standards. All DNA samples were stored –20°C until further processing.

Sodium Bisulfite Modification

Bisulphite modification of genomic DNA was performed as reported [11]. Using a CpGenome™ fast DNA modification kit (Chemicon International, Inc., USA), genomic DNA (1 μg) in a volume of 100 μL was denatured with 7 μL of 3 M NaOH freshly prepared at 37°C for 10 min. Freshly prepared 550 μL of DNA modification reagent was then added and the reaction was performed at 55°C for 16–20 h. The modified DNA was cooled on ice for 5 min before 750 μL of binding buffer was added. After centrifugation, the products were washed with 750 μL of 1x washing buffer and denatured with 50 μL of 20 mM NaOH/90% EtOH for 10 min followed by an additional 750 μL of 1x washing buffer. The eluted products were stored at –20°C for later use. Bisulfite treatment converts unmethylated cytosine residue to uracil while methylated ones remain unchanged.

Methylation-Specific PCR

Methylation-Specific PCR (MSP) is a technique that distinguishes unmethylated alleles from methylated ones based on sequence changes following bisulfite treatment of DNA, and subsequent PCR using primers designed for either methylated or unmethylated DNA. MSP was performed with CpG WIZ® *RB1* Amplification Kit (Chemicon International, Inc., USA). MSP assay was performed in 25 μL of reaction mixture containing 50 ng of bisulfite-treated DNA, 2 mM $MgCl_2$, 200 μM each of deoxynucleotide triphosphate mixture, 200 mM of forward and reverse primers (primers used for the methylated MSP (M-MSP) and unmethylated MSP (U-MSP)), 1x PCR buffer, and 0.5 U of Taq DNA polymerase (Invitrogen Technologies, Inc., USA).

DNA from normal gastric mucosa donors was used as negative control, while an enzymatically methylated control DNA, CpGenome Universal Methylated DNA, (Chemicon International, Inc., MA, USA) was used as positive control in all experiments.

MSP was performed using Eppendorf Mastercycler Gradient PCR (Hamburg, Germany) with the following cycling conditions: after a 4 min denaturation at 95°C the reaction was run for 35 cycles each comprising 45 s of denaturation at 95°C, 45 s of annealing at 56°C, and 45 s of extension at 72°C, with a final extension at 72°C for 5 min as the last step.

Nine microliters of PCR products were loaded and run on 1.5% agarose gel, and visualized under ultraviolet light after staining with ethidium bromide. With complete chemical modification reaction, U-MSP primers amplified only unmethylated DNA (162 bp) and M-MSP primers amplified only methylated DNA (159 bp).

Statistical Analysis

Statistical analysis was performed using SPSS software v. 15.0. Demographic and clinical characteristics of the patients were determined by descriptive statistics, means, and frequencies. Correlations between methylation statuses and clinopathological parameter (categorical variables) were evaluated with Fisher's exact of χ^2 tests. The statistical significance threshold is 0.05 and all p-values indicated are two-tailed.

Results

Age and Sex

The mean ages ± standard deviations (SD) were 46.13 ± 10.4 years for the control group, 54.22 ± 13.6 years for the GC group, 52.76 ± 14.7 years for *H. pylori* negative-IM group, and 51.6 ± 12.2 years for *H. pylori* positive-IM group. There were 9/20 males (45%) and 11/20 females (55%) in the control group, 14/24 males (58.4%) and 10/24 females (41.6%) in the GC group, and 11/20 males (55%) and 9/20 females (45%) in both *H. pylori* negative-IM and *H. pylori* positive-IM groups (Fig. 1).

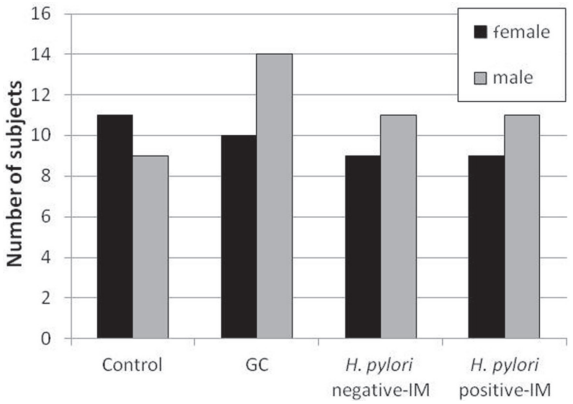

Fig. 1. Gender distribution in the study groups

Methylation Status

We examined promoter methylation of *RB1 gene* using MSP approach in our study group. Methylation levels of 4 groups were compared in Table 1. When we considered the *RB1* gene methylation profile of the entire study population; 43/84 subjects (51.2%) had methylated *RB1* gene

Table 1. Statistical comparison of the methylation status of *RB1* gene in study groups

	Control	**GC**	**p-value***
Methylated	8	12	0.16
Unmethylated	12	12	0.16
	H. pylori positive-IM	GC	
Methylated	13	12	0.03
Unmethylated	7	12	0.03
	H. pylori negative-IM	GC	
Methylated	10	12	1.00
Unmethylated	10	12	1.00
	H. pylori negative-IM	*H. pylori* positive-IM	
Methylated	10	13	0.03
Unmethylated	10	7	0.03

* $p \leq 0.05$ was considered significant.

and 41/84 subjects (48.8%) had unmethylated *RB1* gene. The percent of methylated *RB1* genes was significantly higher in *H. pylori* positive-IM group (65%) than in other groups (GC group 50%, *H. pylori* negative-IM group 50%, control group 40%).

According to methylation levels in *RB1* gene, there were no significant differences between the GC group and the control group, and between *H. pylori* negative-IM group and the GC group ($p > 0.05$). However, there was significant difference in patients with *H. pylori* positive-IM group and the GC group, and *H. pylori* positive-IM and *H. pylori* negative-IM groups ($p \leq 0.05$) as shown in Table 1.

Discussion

Although GC can be observed at all ages, it is seen most frequently between the 5th and 7th decades [12]. Sixty-two and a half percent of the participants who had GC and accepted to be enrolled into the study were between the 5th and 7th decades (n = 15), 25% of participants were under the 5th decade (n = 6), and 12.5% of them were above the 7th decade (n = 3). Although mean age of the participants in the control group is younger than the ones in other groups, there was no significant difference between GC, *H. pylori* positive-IM, and *H. pylori* negative-IM groups.

Male/female ratio in GC is usually declared as 1.5–2.5 [13, 14]. In the national studies carried out in Turkey, male/female ratio was also defined as similar to this ratio [15]. In this study, male/female ratio (n = 15/n = 9) was found to be 1.67, which correlates with the data from the world and Turkish studies [16].

Early diagnosis is of critical importance in cancer treatment. The use of molecular markers can improve cancer diagnosis by allowing further subclassification of the tumors [17]. Methylation profile can be used as a molecular marker to help categorize types or subtypes of tumors; moreover, it can help to evaluate the potential responses to chemotherapeutic agents and survival. Methylation often occurs before malignant features are detectable, which makes them suitable for early diagnosis of cancer. Abnormal promoter methylations can silence the tumor associated genes. In support of this, promoter methylation cases are reported more frequently than gene mutations in GC [17, 18]. Thus, we focused on methylation as a diagnostic marker for early diagnosis and prognostic evaluation of GC in our study.

Loss of *RB1* function by loss of heterozygosity has been reported in GC, glioblastomas, breast cancer, renal carcinoma, and laryngeal cancer [19]. However methylation studies on the promoter region of *RB1* gene in relation to the GC and its pathogenesis are limited. In some of the studies, Zhao et al. [20] found that the percent of positive methylation bands for *RB1* gene was 44.6%, similar to Liu et al. [21] study of EBV-negative gastric carcinoma (*EBVnGC)* (50%), but less than that of EBV-associated gastric carcinoma (*EBVaGC)* cases (80%), which provides further support that *EBV* induces *RB1* gene methylation in *EBVaGC*. However, there are no studies about *RB1* gene methylation in GC and IM with or without *H. pylori* infection. Our study is the first that demonstrates *RB1* gene promoter methylation in relevance to GC, IM, and *H. pylori* infection. In this study, 43/84 (51.2%) patients were found to have methylated *RB1* gene and 41/84 (48.8%) patients were found to have unmethylated *RB1* gene.

H. pylori are known to be a major risk factor for GC progression. It causes chronic active inflammation in the gastric mucosa and has the capacity to colonize human stomach persistently [4, 5]. Abnormal methylations in promoter regions of several genes are induced by *H. pylori* in gastric mucosa including cell growth-related genes *p16(INK4a), p14(ARF),* and *APC;* DNA-repair genes, *hMLH1, BRCA1,* and *MGMT;* the cell adherence gene E-cadherin; as well as *LOX, FLNC, HRASLS, HAND1, THBD,* and *p41ARC,* which are known to be methylated in GC patients [22–24]. Individuals with *H. pylori* infection have increased level of gene methylations,

which decrease in the absence of the bacteria, consistent with the notion that methylations are induced by the bacterial infection [4, 22, 24, 25]. Non-cancerous gastric tissue shows lower levels of methylation compared to the GC tissue, suggesting a mechanistic approach for *H. pylori*-induced carcinogenesis [22]. In the current study, methylated *RB1* genes have shown significantly the highest in *H. pylori* positive-IM group (65%) (GC group 50%, *H. pylori* negative-IM group 50%, and control group 40%) and high correlation between *H. pylori* positive-IM and GC groups ($p \leq 0.05$). This high correlation suggests that the methylation in the promoter region of tumor suppressor *RB1* gene in combination with the *H. pylori* infection and IM might play a strategic role in the gastric carcinogenesis. It is known that the reason for induced chronic inflammation, cell proliferation, and IM could well be *H. pylori* infection. One of the factors promoting DNA methylation has been suggested to be cell proliferation. Moreover, inflammatory processes repress the expression of a number of genes and methylation is known to be promoted by the decrease in gene expression [25]. Similar results to those found in our study were also reported by Lui et al. In their study, Lui et al. [26] determined high methylation in *EBVaGC,* which suggests that *RB1* methylation is elevated in virus infection as well as in bacterial infection shown in our study. The lower methylation percentage detected in GC cases compared to *H. pylori* positive-IM cases may be explained with the absence of heterozygosity of *RB1* gene in several cases of GC group [19]. The fact that *RB1* methylation level is 40% in the control group suggests that clinically, radiologically, and pathologically normal individuals may not be normal at molecular level, and monitoring the methylation levels may increase the efficiency of tumor screening procedures.

Our results showed no correlation between the methylation status of GC and control groups and also between *H. pylori* negative-IM and GC groups ($p > 0.05$). The fact that there was no correlation between the control and the GC groups in our study suggested that carcinogenesis occurred in GC patients through some mechanisms excluding methylation or the inactivation of *RB1* gene occurred during carcinogenesis process in the GC patients. The undetectable correlation between *H. pylori* negative-IM and GC groups suggested that *H. pylori* are more effective in *RB1* methylation in GC than in IM.

In this study, we analyzed the methylation status in the promoter region of tumor suppressor *RB1* gene in GC and *H. pylori* positive and negative IM cases. Our results suggest that *RB1* promoter methylation may be associated with GC and *H. pylori* infection. Through this association, we aimed to demonstrate the role of *H. pylori* infection in the development of GC and the role of *RB1* gene promoter methylation on *H. pylori* infection and GC.

Acknowledgments. This research has been supported by Celal Bayar University Scientific Research Projects. Authors would like to thank Dr. Olcay Boyacioglu for his help in writing the manuscript.

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Conflict of interest: None declared

Received: 12.01.2015
Revised: 1.02.2015
Accepted: 24.03.2015