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-epigenetic factors are believed to play a role as well. 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).

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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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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.


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-

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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 lesser 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 MgCl₂, 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 χ² 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).

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
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 ≤ 0.05) as shown in Table 1.

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

<table>
<thead>
<tr>
<th>Control</th>
<th>GC</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylated</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>H. pylori positive-IM</td>
<td></td>
<td>GC</td>
</tr>
<tr>
<td>Methylated</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>7</td>
<td>12</td>
</tr>
<tr>
<td>H. pylori negative-IM</td>
<td>GC</td>
<td></td>
</tr>
<tr>
<td>Methylated</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>10</td>
<td>12</td>
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<tr>
<td>H. pylori negative-IM</td>
<td>H. pylori positive-IM</td>
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<tr>
<td>Methylated</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>10</td>
<td>7</td>
</tr>
</tbody>
</table>

*p ≤ 0.05 was considered significant.

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 methylation 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, HRAALS, 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 ≤ 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.

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