Research Article

Hypermethylation of tumor suppressor genes in gastric cancer: associations with demographic and clinicopathological characteristics

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Received: 04 April 2016
Accepted: 09 May 2016

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ABSTRACT

Background: Gastric cancer (GC) is one of the most common cancers worldwide. Despite the declining prevalence in Western countries, it is still a major health problem in Turkey and Asian countries. In the current study, we investigated the hypermethylation status of 25 TSGs in GC. Furthermore, the association between hypermethylation status of these TSGs and some demographic and clinicopathological characteristics were investigated.

Methods: Formalin-fixed paraffin-embedded tissue samples obtained from 27 patients with GC and genomic DNA isolated from these tissues. To compare the methylation status of 25 TSGs, methylation-specific multiplex ligation-dependent probe amplification (MS–MLPA) technique was used. Results were evaluated in terms of age, gender, positive lymph node status, lymphovascular invasion, perineural invasion, mortality and five-years of survival, retrospectively.

Results: Tumor suppressor gene hypermethylation was detected 16 (59.3%) of 27 GC tissues. Patients with hypermethylation-detected and patients with no hypermethylation-detected in their TSGs were classified as group 1 and group 2, respectively. The mean age of group 1 was 66.38±7.43 and the mean age of group 2 was found as 58.18±11.12 (p= 0.03). Hypermethylation was detected in 12 of 25 TSGs. Hypermethylation was detected as 51.8% for WT1, 40.7% for ESR1, 18.5% for CDH13, 14.8% for MSH6 and CD44, 7.4% for TP73 and PAX5 genes in the tumor tissues of patients with GC. Mean positive lymph node number was 8.81±5.38 in group 1 and 4.81±3.21 in group 2 (p= 0.037). Lymphovascular invasion, perineural invasion, mortality and five-years of mean survival were not statistically different between group 1 and group 2 (p>0.05 for all comparisons).

Conclusions: Hypermethylation frequency of certain tumor suppressor genes may increase with advancing age and with positive lymph nodes in gastric cancer patients.

Key words: Gastric cancer, Tumor suppressor gene, Hypermethylation, WT1, ESR1

INTRODUCTION

Gastric cancer (GC) is one of the most common cancers worldwide. Despite the declining prevalence in Western countries, it is still a major health problem in Turkey and Asian countries.1,2 Gastric tumors are resulting from environmental and genetic factors. Several environmental factors such as Helicobacter pylori infection, cigarette
smoking, and nutrition with salted and nitrated foods have been known to be related to GC development risk. GC develops only in a small percentage of individuals exposed to the known environmental risk factors. Genetic factors have also an important share within the etiologic reasons of GC. GC arises both from the effects of environmental factors from the effects of genetic and epigenetic alterations, which play essential roles in the carcinogenesis process.

Numerous studies reported that the silencing of tumor suppressor genes (TSGs) and other cancer-related genes have a pivotal role for the development of various human cancers including GC. In the last decade, epigenetic changes have considered as a key mechanism in carcinogenesis. During the tumor development, epigenetic changes are acceptable as an early event. Promoter CpG island hypermethylation has been known as an alternative mechanism which inactive TSGs, DNA repair genes, transcription factors and cell cycle regulators. Neoplastic transformation of normal cells occurs because of genetic changes on oncogenes, DNA repair genes and TSGs. Promoter CpG hypermethylation of certain TSGs are especially found in the transformed or malignant cells. In normal tissues, the vast majority of CpG islands are found as completely unmethylated, and it is not fully understood how and which alterations in DNA methylation in tissue-specific genes occur in cancer development.

Hypermethylation of TSGs plays a crucial role in the increasing of malignancy of cells. It has been suggested that methylation plays an important role in many stages of tumor transformation. In gastric carcinoma, by virtue of CpG island methylation, epigenetic silencing of tumor-related genes has been reported several times. Epigenetic silencing of a TSG could consider as the rate-limiting step for the development of normal cells to an invasive malignant tumor. Epigenetic changes have been considered as a crucial mechanism contributing to early gastric carcinogenesis. In the previous studies, the existence of epigenetic alterations in intestinal metaplasia and adenoma has been characterized.

For early diagnosis of various cancer types, methylation analysis has a great potential. Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) has been performed in several studies, and this method was found to correlate with other methylation-detection methods. In the present study, we aimed to investigate the methylation status of 25 TSGs in GC, also to find the association between hypermethylation status of these TSGs and some demographic and clinicopathological features.

METHODS

In the present study, 27 patients with GC who admitted to Department of General Surgery, Faculty of Medicine, Cukurova University, Sivas, Turkey in 2006-2011 were selected randomly and retrospectively. Their paraffin blocks were obtained from Department of Pathology. The mean age of patients was 63.04±9.81 years (range 37-81). Of 18 (66.7%) these patients were males and 9 (33.3%) of these were females.

Histological classification of tumors, lymphovascular invasion rate, perineural invasion rate and positive lymph node number of patients were obtained from their pathology reports. Patients were analyzed retrospectively for the 5-year survival and mortality rate. Survival information of patients was obtained by medical records and phone calls. Patients with hypermethylation-detected in their TSGs and patients with no hypermethylation-detected in their TSGs were called as group 1 and group 2, respectively.

Preparation of FFPE tissue and DNA isolation

Formalin fixed-paraffin embedded (FFPE) tissues were selected from the archives of Department of Pathology. The tissues were stored at -20 °C for approximately 3 hours to cut by microtome. Meanwhile, microtome device, especially in contact with the tissue sections, were cleaned by wiping with 70% ethyl alcohol. Ten micron thick sections were taken from these samples. After each cutting, blade of microtome was changed and microtome was cleaned with 70% ethyl alcohol.

FFPE tissue sections were placed into labeled, sterile tubes, and stored at +4 °C until the DNA isolation. DNA isolation was performed using a commercial spin column kit with some minor modifications (PureLink® Genomic DNA Mini Kit, Invitrogen, California, USA). Concentration and purity of the isolated total genomic DNAs were measured with using spectrophotometric method.

Determination of the methylation patterns of target genes

Promoter methylation of TSGs was assessed using SALSA MLPA probemix ME002-B1 Tumour Suppressor 2, Version 14 (MRC-Holland, Amsterdam, The Netherlands). This commercial kit contains 27 MS-MLPA probes, detecting the promoter methylation status of 25 different TSGs. These TSGs were: Tumor protein P53 (TP53), Ataxia telangiectasia mutated (ATM), Phosphatase and tensin homolog (PTEN), Breast cancer 1 (BRCA1), Breast cancer 2 (BRCA2), Paired box 5 (PAX5), Cadherin 13 (CDH13), O-6-methylguanine-DNA methyltransferase (MGMT), Checkpoint with forkhead and ring finger domains (CHFR), Tumor protein P73 (TP73), Wilms tumor 1 (WT1), von Hippel-Lindau tumor suppressor (VHL), Glutathione S-transferase pi 1 (GSTM1), estrogen receptor 1 (ESR1), Retinoblastoma 1 (RB1), Cell adhesion molecule 1 (CADM1), Thrombospondin 1 (THBS1), mutS homolog 6 (MSH6), Serine/threonine kinase 11 (STK11), Retinoic acid receptor, beta (RARB), PYD and CARD domain.
containing (PYCARD), Cyclin-dependent kinase inhibitor 2A (CDK2NA), Paired box 6 (PAX6), GATA binding protein 5 (GATA5), CD44 molecule (CD44). Additionally, 14 reference genes are included which are not affected by HhaI digestion.18,19

Amplification products analyzed using a genetic analyzer device (ABI-3130, Applied Biosystems, California, USA). Experimental procedures of the MLPA, data analysis and cutoff selection were carried out by the manufacturer’s instructions.20 At every turn, reference samples isolated from a healthy stomach tissue and 3 cancer tissues were worked together. Methylated regions were determined by comparison of results with reference samples.

Statistical analysis

All statistical analyses were performed by Statistical Package for the Social Sciences (SPSS) version 22.0 (SPSS IBM, New York, USA). In the comparisons of the means of one variable for two groups, firstly, normality test performed to data, then analyzed using Independent samples t-test or Mann-Whitney U test. Chi-square test or Fisher’s exact test were applied to compare the frequency of methylation of each gene between groups. Descriptive values were expressed as mean ± standard deviation (SD) and range or frequency and percent. A p value of less than 0.05 was considered as significant, using two-sided comparisons. All results were expressed with a 95% confidence interval.

RESULTS

In the present study, tumor tissues of 27 patients GC were determined and their formalin fixed paraffin embedded tissues were obtained for the study. Genomic DNAs were isolated from FFPE tissue sections. Demographic and clinicopathological characteristics of patients with GC have shown in Table 1.

Table 1: Demographic and clinicopathological features of gastric cancer patients.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients (n=27)</th>
<th>Group 1 (n=16)</th>
<th>Group 2 (n=11)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>63.04 ±9.81</td>
<td>66.38±7.43</td>
<td>58.18±11.12</td>
<td>0.03*</td>
</tr>
<tr>
<td>Male gender</td>
<td>18 (66.66%)</td>
<td>13 (81.25%)</td>
<td>5 (45.45%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Histological type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDA</td>
<td>7 (25.93%)</td>
<td>4 (25%)</td>
<td>3 (27.27%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>MDA</td>
<td>14 (51.85%)</td>
<td>10 (62.5%)</td>
<td>4 (36.36%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>WDA</td>
<td>4 (14.28%)</td>
<td>1 (6.25%)</td>
<td>3 (27.27%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Lyposarcoma</td>
<td>1 (3.57%)</td>
<td>-</td>
<td>1 (9.1%)</td>
<td>-</td>
</tr>
<tr>
<td>PDSRCC</td>
<td>1 (3.57%)</td>
<td>1 (6.25%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tumor state</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>3 (11.11%)</td>
<td>1 (6.25%)</td>
<td>2 (18.18%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>T3</td>
<td>13 (48.15%)</td>
<td>7 (43.75%)</td>
<td>6 (54.55%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>T4</td>
<td>11 (40.74%)</td>
<td>8 (50%)</td>
<td>3 (27.27%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Lymphovascular invasion rate</td>
<td>18 (66.66%)</td>
<td>10 (62.5%)</td>
<td>8 (72.73%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Perineural invasion rate</td>
<td>15 (55.55%)</td>
<td>8 (50%)</td>
<td>7 (63.64%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Metastasis</td>
<td>4 (14.28%)</td>
<td>2 (12.5%)</td>
<td>2 (18.18%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Survival (months)</td>
<td>27.52±17.76 (1-59)</td>
<td>28.81±19.84 (1-59)</td>
<td>25.82±14.59 (1-51)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>5-years survival rate (%)</td>
<td>18 (66.67)</td>
<td>10 (62.75)</td>
<td>8 (72.73)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Mortality</td>
<td>9 (33.33%)</td>
<td>6 (37.5%)</td>
<td>3 (27.27%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Positive lymph nodes number</td>
<td>7.18±4.96</td>
<td>8.81±5.38</td>
<td>4.81±3.21</td>
<td>0.037*</td>
</tr>
</tbody>
</table>

*The difference between group 1 and group 2 was statistically significant. Data were presented as number and percentage or mean ± standard deviation and range as appropriate. Group 1: Hypermethylation-detected group, Group 2: No hypermethylation-detected group, PDA: Poorly-differentiated adenocarcinoma, MDA: Moderately-differentiated adenocarcinoma, WDA: Well-differentiated adenocarcinoma, PDSRCC: Poorly-differentiated signet-ring cell carcinoma

TSG hypermethylation-detected and no hypermethylation-detected groups were called as group 1 and group 2, respectively. The mean age of group 1 was 66.38±7.43 and the mean age of group 2 was found as 58.18±11.12. The difference between groups was statistically significant (p=0.03, Table 1, Figure 1). In this study, hypermethylation was detected in 12 of 25 TSGs in patients with GC. The most frequent hypermethylation was observed in WT1 and ESR1 genes. Hypermethylation was detected as 51.8% for WT1, 40.7% for ESR1, 18.5% for CDH13, 14.8% for MSH6 and CD44, 7.4% for TP73 and PAX5 genes in the tumor tissues of patients with GC (Table 2, Figure 2).
In the present study, while targeted tumor suppressor gene hypermethylation was detected 16 of 27 (59.3%) stomach cancer tissues (group 1), no hypermethylation observed remaining 11 cases (group 2). Six (37.5%) patients within group 1 have died. Mortality rate was 3/11 (27.2%) within group 2. Mortality rate was found higher in hypermethylated group, but the difference detected between groups was not found statistically significant (p>0.05). Five-years of survival rate were 62.75% and 72.73% in group 1 and group 2, respectively. The difference between groups was not statistically significant (Table 1).

Table 2: Distribution of methylation-detected genes according to gender, age and lymph node number in patients with gastric cancer.

<table>
<thead>
<tr>
<th>TSGs</th>
<th>Patients (n=27)</th>
<th>Gender</th>
<th>Age</th>
<th>Lymph node number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT1</td>
<td>14 (51.8%)</td>
<td>11 (78.6%)</td>
<td>3 (21.4%)</td>
<td>0.17</td>
</tr>
<tr>
<td>ESR1</td>
<td>11 (40.7%)</td>
<td>9 (81.8%)</td>
<td>2 (18.2%)</td>
<td>0.16</td>
</tr>
<tr>
<td>CDH13</td>
<td>5 (18.5%)</td>
<td>5 (83.3%)</td>
<td>1 (16.7%)</td>
<td>0.16</td>
</tr>
<tr>
<td>CD44</td>
<td>4 (14.8%)</td>
<td>3 (75%)</td>
<td>1 (25%)</td>
<td>0.7</td>
</tr>
<tr>
<td>MSH6</td>
<td>4 (14.8%)</td>
<td>4 (100%)</td>
<td>-</td>
<td>0.12</td>
</tr>
<tr>
<td>TP73</td>
<td>2 (7.4%)</td>
<td>1 (50%)</td>
<td>1 (50%)</td>
<td>0.6</td>
</tr>
<tr>
<td>PAX5</td>
<td>2 (7.4%)</td>
<td>2 (100%)</td>
<td>-</td>
<td>0.3</td>
</tr>
<tr>
<td>VLH</td>
<td>1 (3.7%)</td>
<td>1 (100%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MGMT</td>
<td>1 (3.7%)</td>
<td>1 (100%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CADM1</td>
<td>1 (3.7%)</td>
<td>1 (100%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PAX6</td>
<td>1 (3.7%)</td>
<td>1 (100%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>1 (3.7%)</td>
<td>1 (100%)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*p=0.025; **p=0.047 , WT1: Wilms tumor 1; ESR1: Estrogen receptor 1; CDH13: Cadherin 13; CD44: CD44 molecule; MSH6: mutS homolog 6; TP73: Tumor protein P73; PAX5: Paired box 5; VLH: von Hippel-Lindau tumor suppressor; MGMT: O-6-methylguanine-DNA methyltransferase; CADM1: Cell adhesion molecule 1; PAX6: Paired box 6; CDK2NA: Cyclin-dependent kinase inhibitor 2A.

Figure 1: Comparison of mean age between hypermethylation-detected and no hypermethylation-detected patients.

Five-years of mean survival of 27 GC patients in our study were 27.52±17.76 months. While 5-years survival in group 1 was 28.81±19.84 months, five-year survival in group 2 was 25.82±14.59 months. When 5-years survival is compared between two groups, the difference was not statistically significant (p>0.05, Table 1).

Figure 2: Number of hypermethylation of tumor suppressor genes in patients with gastric cancer.

Lymphovascular invasion rate was 62.5% and 72.73% in group 1 and group 2, respectively. Perineural invasion rate was 50% in group 1 and 63.64% in group 2. Statistically significant difference was not detected between two groups in terms of lymphovascular invasion rate and perineural invasion rate (p> 0.05, Table 1). Mean positive lymph node number was 8.81±5.38 in group 1 and 4.81±3.21 in group 2. Statistically significant difference was detected between the two groups (p=0.037, Table 1). In the GC tissues, hypermethylation...
frequency of TSGs was increased with T stages of tumor (T2=33.3%, T3=53.8% and T4=72.7%, Figure 3). However, this difference was not statistically significant (p>0.05).

Figure 3: Distribution of hypermethylation-detected and no hypermethylation-detected patients by the T stages.

In the group 1, 13 (81.25%) of 16 patients were male, on the other hand, 5 (45.45%) of 11 patients were male in group 2. Considerably high number of male patients has been detected in group 1. However, this difference detected between two groups was not statistically significant (p=0.097). In addition, the distribution of hypermethylation-detected genes by gender were evaluated, and methylation of WT1, ESR1, CDH13, MSH6, CD44 and PAX5 genes were detected in men at a higher frequency compared to women. Due to other methylation-detected genes is restricted with only one patient, statistical analysis not performed (Table 2).

Whereas, mean positive lymph node number of patients who have hypermethylated ESR1 gene was 9.45±5.95, mean positive lymph node number of patients with no hypermethylation detected ESR1 gene was 5.62±3.57. This difference was statistically significant (p=0.047). The difference of other methylation-detected genes in terms of mean positive lymph node number was not statistically significant. The mean age of patients who have methylated CD44 gene was 73.0±6.2 and statistically significant difference was found between methylated and unmethylated patients in terms of age (p=0.025). The mean age of patients who have methylation in their other TSGs was not statistically significant (Table 2).

DISCUSSION

In this study, hypermethylation frequencies of 25 tumor suppressor genes were studied in the tumor tissues of 27 patients with GC. In addition, the association between hypermethylation status of these TSGs and demographic and clinicopathological characteristics of patients were investigated.

In the current study, a total of 25 TSGs including DNA repair-related genes (MGMT, WT1, MSH6, BRCA1, BRCA2, PYCARD and ATM), cell cycle regulation-related genes (TP53, PAX5, VHL, GSTP1, CHFR, THBS1, RB1, STK11, CD44 and PAX6), signal transduction-related genes (PTEN, CDKN2A and RARB), tissue invasion and metastasis-related genes (CADM1 and CDH13), transcription factors (ESR1, GATA5), and an angiogenesis-related gene (TP73) were investigated. These TSGs are found frequently methylated in tumor cells, but they are found as unmethylated in the DNA of healthy individuals.18

In recent years, DNA methylation of tumor cells has become an important field of investigation. In gastric carcinogenesis, promoter methylation acts as an important alternative to genetic changes for the gene inactivation.16 The TSG hypermethylation status in GC is very important whether it was successful treatment against tumor growth and disease development.5,14 Because of the potential ability to reverse DNA methylation, DNA methylation markers have been suggested as markers response to treatment and have important prognostic value. Epigenetic changes occur early and at high frequency in different human malignancies including GC. Hypermethylation of tumor suppressor genes is significantly associated with clinicopathological characteristics in GC.5,11

In the current study, the highest hypermethylation frequency was determined in WT1 gene (51.8%). WT1 gene plays an important role in cell proliferation, organ development, differentiation, apoptosis, and the maintenance of several adult tissues.21 Zheng Y et alreported hypermethylation frequency of WT1 gene in GC tissues as 50%. Moreover, they reported that this level of hypermethylation could be improved with 5-aza-2'-deoxycytidine treatment in cell culture studies.

Estrogen receptor alpha (ER-α), is a nuclear receptor that activated by estrogen and synthesized by ESR1 gene and composed of several domains for hormone binding, DNA binding, and activation of transcription.23 ESR1 gene locus has been known as a frequent loss of heterozygosity (LOH) site in GC. In GC cell lines, it has been demonstrated that inactivation of ER-α gene expression associated with CpG island methylation of ESR1 gene.24 In this study, hypermethylation of ESR1 gene was detected as 40.7% in the GC tissues.

CDH13 or T-cadherin, is a member of cadherin family. T-cadherin has important function including regulation of cell growth, survival and proliferation of cells. T-cadherin loss in tumor cells is associated with metastasis, tumor malignancy and invasiveness.25 T-cadherin is expressed in both normal and malignant gastric mucosa, and its expression is decreased in gastric carcinoma tissues and associated with larger tumor size, invasion into surrounding tissues, poor-differentiation, lymph node metastasis, and higher TNM stage.26,27 Hibi K et al.28
reported CDH13 promoter hypermethylation in 23 (35%) out of 66 patients with GC. They also reported that abnormal methylation was frequently found at all clinical stages of patients with GC. For the early detection of GC, CDH13 methylation could be used as a tumor marker in serum and stool of patients with GC. In the current study, hypermethylation of CDH13 was detected in 18.5% of the GC tissues.

**MSH6** is a subunit of the mutS homolog 2 (MSH2)-MSH6 mispaired base recognition factor required for mismatch repair. MSH6 also contributes to the ADP and ATP binding process and lead to be activated of ATPase. Products of this gene play an important role in binding to double-stranded DNA. 29 CD44 gene produces the CD44 antigen. CD44 antigen is a cell surface glycoprotein, which has important roles in cell-cell interactions, cell adhesion and migration. CD44 gene transcription is associated with beta-catenin and Wnt signaling which responsible for tumor progression. 30 31 In this study, hypermethylation of MSH6 and CD44 genes was detected in 14% of patients.

PAX5 gene belonging to paired box (PAX) transcription factors family and this protein has an important regulatory role in the neoplastic transformation. 32 33 Patients with GC who have PAX5 gene methylation had a significant poor survival compared with the unmethylated patients. 34 TP73 is a TSG and similar to p53, structurally and functionally. TP73 plays an important role in cell cycle regulation and apoptosis induction. 35 Bernal C et al detected TP73 hypermethylation status in the level of 27% in stomach cancer. 36 We found hypermethylation frequencies of PAX5 and TP73 gene in GC tissues as 7.4%.

In the present study, frequency of hypermethylation in the other genes (RB1, MGMT, GSTP1, BRCA1, TP53, PTEN, VHL, CHFR, ATM, BRCA2, RARB, GATA5, CDK2NA, PAX6, THBS1, CADM1, STK11, PYCARD) have been identified as less than 5% in the GC tissues.

In the previous studies, researchers reported that hypermethylation in TSGs and tumor-associated genes have increased with advancing age. 37 In several cancers, higher age-related hypermethylation seems to be gene-specific. 38 In the current study, frequency of hypermethylation in TSGs increased with advancing age in patients with GC. In spite of the mean age of hypermethylation observed patients was 66.38, mean age of no hypermethylation observed patients was 58.18.

In previous studies, it has been suggested that the occurrence of hypermethylation on TSGs have increased depending on the gender. 15 In a study conducted with gastric carcinoma patients, researchers showed that mutL homolog 1 (MLH1) methylation was found to be more common in males (82.1%) compared to females (35.75%). 39 In the current study, male patients (81.25%) are composed of the majority of individuals in hypermethylation observed group. In addition, methylation of WT1, ESR1, CDH13, MSH6, CD44 and PAX5 genes were detected at a higher frequency in men compared to women. In the light of previous findings and our findings, we suggest that gender may be considered as an important factor affecting the hypermethylation of tumor suppressor genes in GC development.

Lymph node metastasis is considered as an important prognostic factor predicting recurrence in patients with GC. Some TSGs were found as correlated with lymph node metastasis of GC. 40 In the current study, mean positive lymph node number was found statistically significantly higher in hypermethylation-detected cases. Furthermore, mean positive lymph node number of patients who have hypermethylated ESR1 gene was found statistically significantly higher than patients with unmethylated ESR1 gene.

Major important limitation of our study is relatively small sample size. Furthermore, in the current study, number of male gender is higher than number of female gender. For these reasons, we believe that further studies in homogenous and larger groups are required to clarify the role of hypermethylation of these tumor suppressor genes in GC development.

**CONCLUSION**

In the current study, the higher frequency of hypermethylation was detected in WT1, ESR1, CDH13, MSH6, CD44, TP73 and PAX5 TSGs. Furthermore, mean positive lymph node number and mean age were higher in hypermethylation-detected patients. Coexistence with hypermethylation of tumor suppressor genes and advancing age or positive lymph nodes could be considered as an important risk factor for the development and growth of gastric cancer.

*Funding: This study was supported by the Scientific Research Project Fund of Cumhuriyet University (CUBAP) under the project number T-449.*

*Conflict of interest: None declared*

*Ethical approval: The study was approved by the Institutional Ethics Committee*

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