Analysis of Promoter Methylation, Polymorphism and Expression Profile of Cytotoxic T-Lymphocyte-Associated Antigen-4 in Patients with Gastric Cancer

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Received: 19.05.2014
Accepted: 21.07.2014

ABSTRACT

Background & Aim: Cytotoxic T lymphocyte-associated antigen-4 (CTLA4) is a crucial immune-checkpoint receptor regulating T-cell activation. The current study was carried out to evaluate the function of CTLA4 gene in patients with gastric cancer.

Methods: The methylation of CTLA4 gene promoter was evaluated by methylation-specific polymerase chain reaction (MSP) technique using 85 paraffin-embedded gastric cancer tissue samples and normal tissue on the tumor margins as control tissue samples. Expression analysis was performed on paraffin-embedded tissue samples (25 each of cancerous and normal tissues) using Real-time PCR.

Results: Statistically significant differences were observed between the tumor and margin-cell areas with respect to promoter methylation status (OR = 4.829, 95% CI: 2.46–9.48, p < 0.001) and CTLA4 expression profile (mean ± SD = 7.56 ± 17.35, p = 0.04).

Conclusion: To the best of our knowledge, the current study is the first one highlighting the association between promoter hypermethylation of CTLA4 gene, decreased CTLA4 expression, and increased risk of gastric cancer.

Key words: methylation – gastric cancer – CTLA4 – gene.

INTRODUCTION

Gastric cancer is the fourth most common cancer and second leading cause of cancer-related death worldwide [1], even though the incidence of gastric cancer has shown a dramatic decrease over the past half-century. The highest prevalence of gastric cancer has been reported in Japan, China, South America, Eastern Europe, and the Middle East. In Iran, gastric cancer is the leading and second major cause of cancer-related mortality among men and women, respectively. It is a multi-fatorial disease, with a wide variety of genetic and environmental factors involved in its etiology, including chronic inflammation, Helicobacter pylori infection, exposure to diverse carcinogens, genetic susceptibility, tobacco smoking, high-salt intake, diets with insufficient antioxidant levels, and lifestyle factors [2, 3]. Gastric carcinoma can be divided into two broad categories based on clinicopathologic factors: i) early gastric cancers (EGCs) limited to mucosa and submucosa, and including the protruded, superficial, and excavated types, and ii) adenocarcinomas, which extend at least into muscularis propria [3]. Epigenetic mechanisms contribute to all the steps of cancer development and progression, and deregulation of DNA methylation, a reversible process, is known to exert profound influence on cancer. In normal cells, CpG islands in the promoter regions of genes are unmethylated, whereas repetitive elements and transposons are mostly methylated [4]. Abrupt methylation of promoter regions is an epigenetic abnormality of the human genome, which is highly characteristic of cancer.

Cytotoxic T-lymphocyte-associated antigen-4 (CTLA4), encoded by the human CTLA4 gene, is a receptor belonging to the immunoglobulin superfamily, and is expressed on the surface of activated T cells. CD28 and CTLA4 share the ligands B7.1 and B7.2, which share identical functions [5, 6]. CTLA4 consists of 149 amino acids, and is expressed exclusively in activated CD4+ and CD8+ T-cells.
T lymphocytes [7]; it binds to B7 molecules on antigen-presenting cells, and could be considered a two-blade knife, as its optimal expression ensures an effective, but at the same time, safe immune response [8].

CTLA4 has much higher overall affinities for both CD80 and CD86 ligands compared to CD28; it has been proposed that CTLA4 expression on the surface of T cells regulates T-cell activation by outcompeting CD80 for ligand binding, as well as by actively delivering inhibitory signals to the T cell [9]. However, CTLA4 also confers "signaling-independent" T-cell inhibition through sequestration of CD80 and CD86 ligands from CD28 engagement. Furthermore, CTLA4 plays a pivotal role in cancer development and progression [10]. Increased levels of regulatory T cells (T-reg) and CTLA4 have been observed in patients with different types of cancer compared to control individuals, and higher CTLA4 expression has also been shown to prevent tumor growth [11-13]. Moreover, antibodies that block the activity of this receptor have been reported to improve survival in patients with dissimilar types of cancer [9]. The human CTLA4 gene is located on the long arm of chromosome 2q33 within 25–150 kb of the gene encoding CD28. There are at least three polymorphic markers associated with CTLA4, a C to T polymorphism at position 49 of exon 1, resulting in a substitution of Thr17 to Ala, and an (AT)n dinucleotide repeat polymorphism located in the 3’ UTR of exon 4 [14]. The aim of this study was to analyze promoter methylation, polymorphism, and expression level of CTLA4 in patients with gastric cancer.

MATERIAL AND METHODS

Patients and tissue samples

Eighty five samples of paraffin-embedded gastric adenocarcinoma tissues were obtained from the Pathology service of Alzahra Hospital in Isfahan, Iran. The samples were derived from patients who had undergone a curative gastrectomy during the period 2009–2012. Control samples, also 85 in number, were obtained from the unaffected normal tissue at the tumor margins; they were confirmed as non-cancerous by pathologists.

DNA extraction, modification, and methylation-specific PCR (MSP)

Genomic DNA was extracted from paraffin-embedded tissues as previously described. Bisulfite modification for the conversion of unmethylated cytosine to uracil, while leaving methylated cytosine unaltered, was carried out using 2 µg of DNA. Wizard® DNA Clean-Up System (Promega, Madison, WI) was employed for sodium bisulfite conversion according to the supplier's protocol. Methylation-specific PCR (MSP) was performed using primers specific for the methylated (M) or unmethylated (U) alleles of CTLA4 gene; the primer sequences and annealing temperatures are summarized in Table I. Each PCR reaction contained 1 µL of bisulfite-modified DNA, 0.5 µL of HS-Taq DNA polymerase (2.5 U/µL, Cat. No. 01011, Pars Tous Biotechnology), 2.5 µL of 10X buffer, 0.8 mM dNTP mix, 0.4 mM of each primer, and 1.5 mM Mg2+; the reaction was made up to a final volume of 25 µL using RNase-free double-distilled water. The MSP amplification conditions included initial denaturation step at 95°C for 10 min, 40 cycles of 95°C for 30 s, 63°C (M) or 59°C (U) for 40 s, and 72°C for 40 s, followed by 1 cycle of final extension at 72°C for 10 min. The PCR products were analyzed by electrophoresis using 3% agarose gel followed by ethidium bromide staining and visualization using UV transilluminator.

RNA extraction and RT-PCR

Isolation of total RNA from paraffin-embedded tissues was performed using CinnaPure RNA kit (Cat. No. PR891620, Tehran, Iran) according to the manufacturer’s protocol. Subsequently, first-strand cDNA was synthesized from 1–10 µg of total RNA using 2-steps RT-PCR kit (Cat. No. RTP112, Vivantis Technologies) according to the supplier’s protocol. Real-time PCR was performed in reactions of 16 µl volume containing Power SYBR Green PCR Master Mix (Applied Biosystems); Applied Biosystems Real-Time PCR instrument

<table>
<thead>
<tr>
<th>Genes</th>
<th>Sequences(5’-3’)</th>
<th>Product size</th>
<th>Annealing temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTLA4-M</td>
<td>F: GATTGTTATTGGTGTCGTCGTC&lt;br&gt;RR: GCCAACGCTATCTTACTCCGAA</td>
<td>131</td>
<td>47</td>
</tr>
<tr>
<td>CTLA4-U</td>
<td>F: GATTGTTATTGGTGTCGTCGTC&lt;br&gt;RR: GCCAACGCTATCTTACTCCGAA</td>
<td>140</td>
<td>48</td>
</tr>
<tr>
<td>CTLA4</td>
<td>F: GGGGCAGAGCAGTGGAAAAAT&lt;br&gt;RR: CACATCATCATCTGGCTCCAGATGG</td>
<td>162</td>
<td>60</td>
</tr>
<tr>
<td>RNA 185</td>
<td>F: GTAAACCCGTGGACACCCATT&lt;br&gt;RR: CCATCCCAATCGGTAGTACCG</td>
<td>120</td>
<td>60</td>
</tr>
<tr>
<td>+49CTLA4 (A/G)</td>
<td>F0: GATTGTTATTGGTGTCGTCGTC&lt;br&gt;R0: TCCATCCTGATCTGCTCAAAAAAGTCGATC&lt;br&gt;F1(G): GACAAGGCTAGTGGAACCTGGAGT&lt;br&gt;R1(A): ACAGGAGAGTGCAGGGCCAGGTCGACAGT&lt;br&gt;F2(G): ACAGCAGAGGTGACAGGGCCAGGTCGACAGT&lt;br&gt;R2(A): ACAGCAGAGGTGACAGGGCCAGGTCGACAGT</td>
<td>229</td>
<td>62</td>
</tr>
<tr>
<td>-318CTLA4 (C/T)</td>
<td>F0: CAATGAAATGAATTTGAGCTGATG&lt;br&gt;R0: TGCAACCAGCAAGAAGCTTGGAATA&lt;br&gt;F1(C): CTCCACCTAGTGTACGATCATC&lt;br&gt;R1(T): ACTGAAGCTTATGCTCATCTA</td>
<td>296</td>
<td>58</td>
</tr>
</tbody>
</table>

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was employed, and the reaction conditions included initial
denaturation at 95°C for 10 min, 40 cycles of 95°C for 15 s,
60°C for 30 s, and 72°C for 40 s, followed by 1 cycle of final
extension at 72°C for 10 min; finally, the melting curve was
obtained over the range 60–95°C. The sequences of the primers
used for expression analysis are listed in Table I. The expression
of CTLA4 was normalized to 18s rRNA internal control, and
analyzed using the 2−ΔΔCT method.

Statistical analysis
SPSS Version 20 was used for statistical analyses. Categorical
data was analyzed using chi-squared test (χ2), and the association between
CTLA4 gene methylation and the association between
categorical data was analyzed using chi-squared test (χ2),
and the association between
CTLA4 gene promoter in tumor samples and their
methylation of the
CTLA4 gene in patients with gastric cancer 251
margins was 78.82% and 43.53%, respectively. Table IV details
the frequency of methylation patterns corresponding to
each clinical characteristic of the tumor samples. Significant
differences were observed between CTLA4 expression in
cancerous tissue (n = 25, mean ± SD: 7.56 ± 17.35) and their
normal margins (n = 25, mean ± SD: 15.45 ± 7.96, p < 0.001),
as shown in Table V.

The frequencies of -318 C/T and +49 A/G polymorphisms
of CTLA4 gene in gastric cancer patients are shown in Table VI.

Table IV. Analysis of methylation status link with various clinical
characteristics

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>UU</th>
<th>MM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histologic grade, n (%)</td>
<td></td>
<td></td>
<td>0.63</td>
</tr>
<tr>
<td>Poorly</td>
<td>10</td>
<td>32</td>
<td>(76.19)</td>
</tr>
<tr>
<td>Moderate</td>
<td>6</td>
<td>21</td>
<td>(77.78)</td>
</tr>
<tr>
<td>Well</td>
<td>2</td>
<td>14</td>
<td>(87.5)</td>
</tr>
<tr>
<td>Histologic type, n (%)</td>
<td></td>
<td></td>
<td>0.42</td>
</tr>
<tr>
<td>Intestinal</td>
<td>12</td>
<td>51</td>
<td>(80.95)</td>
</tr>
<tr>
<td>Diffuse</td>
<td>6</td>
<td>16</td>
<td>(72.72)</td>
</tr>
<tr>
<td>Mixed</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Stage of cancer, n (%)</td>
<td></td>
<td></td>
<td>0.75</td>
</tr>
<tr>
<td>Stage I</td>
<td>1</td>
<td>9</td>
<td>(90)</td>
</tr>
<tr>
<td>Stage II</td>
<td>4</td>
<td>16</td>
<td>(80)</td>
</tr>
<tr>
<td>Stage III</td>
<td>12</td>
<td>37</td>
<td>(75.51)</td>
</tr>
<tr>
<td>Stage IV</td>
<td>1</td>
<td>5</td>
<td>(83.33)</td>
</tr>
</tbody>
</table>

Demographic characteristics

| Mean age (years) | Male | 69.46 | 62.92 | 0.37 |
| Gender | Female | 51 | 60.53 | |

Table V. Comparison of relative gene expression for CTLA4 gene in patients
with gastric cancer between tumors and their margins

<table>
<thead>
<tr>
<th>Gene</th>
<th>Case</th>
<th>Control</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTLA4</td>
<td>25</td>
<td>25</td>
<td>0.04</td>
</tr>
<tr>
<td>n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DISCUSSION

Our results indicate that the methylation of CTLA4 gene
is associated with an increased risk of gastric cancer, and
significantly lower expression of this gene could be observed
in cancer tissues relative to their normal margins. Along
these lines, Zhou et al (2013) reported that CTLA4 expression
in ALL patients was higher just prior to their death than
at the time of recruitment [15]. Polymorphisms in CTLA4
are associated with various types of cancer, and increased
expression of CTLA4 mRNA or protein has been found in
different tumor types; for instance, increased CTLA4 expression
was observed in a majority of non-small cell lung cancer
(NSCLC) patients [16]. A few studies have demonstrated that
blocking of CTLA4 receptor results in antitumor activity,
reduced metastatic behavior, and durable tumor regressions
in certain patients (Kwon et al 1999, Erfani et al 2012) [10].
Lung tumors have been shown to contain large numbers of
T-reg cells with high CTLA4 expression, which selectively
inhibit host immune response and thereby contribute to the


CONCLUSION

While previous studies have shown high levels of CTLA4 mRNA or protein in the blood of patients with multiple types of cancer, the present study indicates that gene silencing and reduction in CTLA4 expression are possibly related to promoter hypermethylation of its gene, and that it can be considered a risk factor for gastric cancer. These results raise the possibility that CTLA4 could be considered as a potential therapeutic target for gastric cancer. As the current study is the first one dealing with this subject, there is a limitation with regard to comparing the current data with that of other studies. Further investigation of all the components involved in the CTLA4 pathway, in a study involving larger sample sizes and various populations, is therefore required for a better understanding of the role of CTLA4 gene in the development of gastric cancer and its prognosis.

Conflicts of interest. None to declare.

Authors’ contribution: DMKT made substantial contribution to concept, design, and analysis and interpretation of data. SD, TB and SH participated in collection of laboratory data, drafting the article and providing samples.

Acknowledgements. The authors wish to thank the University of Sistan and Baluchestan for financially supporting this project. In addition, the authors would like to thank the patients and healthy subjects who willingly participated in the study.

REFERENCES


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Table VI. Frequencies of cytotoxic T lymphocyte antigen-4 (CTLA-4) -318 C/T and +49 A/G polymorphisms in patients with gastric cancer

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Patients (%)</th>
<th>Alleles</th>
<th>Patients (%)</th>
<th>Gene combination</th>
<th>Patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-318 C/T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/C</td>
<td>5 (5.88)</td>
<td>C</td>
<td>55 (32.35)</td>
<td>CC/AA</td>
<td>1 (1.17)</td>
</tr>
<tr>
<td>C/T</td>
<td>45 (52.94)</td>
<td>T</td>
<td>115 (67.64)</td>
<td>CC/AG</td>
<td>2 (2.35)</td>
</tr>
<tr>
<td>T/T</td>
<td>35 (41.17)</td>
<td></td>
<td></td>
<td>CC/GG</td>
<td>2 (2.35)</td>
</tr>
<tr>
<td>+49 A/G</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/A</td>
<td>5 (5.88)</td>
<td>A</td>
<td>80 (47.05)</td>
<td>CT/AG</td>
<td>40 (47.05)</td>
</tr>
<tr>
<td>A/G</td>
<td>70 (82.35)</td>
<td>G</td>
<td>90 (52.94)</td>
<td>TT/AA</td>
<td>2 (2.35)</td>
</tr>
<tr>
<td>G/G</td>
<td>10 (11.76)</td>
<td></td>
<td></td>
<td>TT/AG</td>
<td>28 (32.94)</td>
</tr>
</tbody>
</table>

Information obtained until now suggests that various factors, including genetic, epigenetic, and environmental factors, could be involved in gastric cancer development. Gastric cancer is among the most common malignancies worldwide, and is often diagnosed only in advanced stages. Therefore, identification of genetic and epigenetic alterations and factors affecting the incidence of this cancer can help in early detection and treatment of this dangerous disease.

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