



CpG Islands Methylation Analysis of CDH11, EphA5, and HS3ST2 Genes in Gastric Adenocarcinoma Patients

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Abstract

Purpose Gastric cancer is an aggressive disease which is the fourth prevalent malignancy in the world. Beside the genetic factors, epigenetic alterations such as promoter CpG island hyper methylation are involved in the emergence of gastric cancer. Herein, we investigated the methylation status of CDH11, EphA5, and HS3ST2 genes in patients with and without gastric adenocarcinoma for the first time.

Methods In the study 40 paraffin-embedded tissue sections from gastric adenocarcinoma patients and 40 specimens from patients with functional dyspepsia were taken. DNA extraction was performed using a modified salting out method. Epizyme DNA methylation kit was used to the bisulfite DNA conversion. The methylation status of CDH11, EphA5, and HS3ST2 genes were analyzed by methylation-specific PCR (MSP) technique.

Results Among the 80 specimens, 71 DNA samples were achieved (34 gastric adenocarcinoma patients and 37 control patients). The results showed that CDH11, EphA5, and HS3ST2 genes are methylated in 28 (82.45%), 19 (55.88%), and 26 (76.47%) of 34 DNA samples from gastric adenocarcinoma patients, respectively, whereas, these genes are methylated in 7 (18.91%), 9 (24.32%) and 7 (18.91%) of 37 samples from noncancerous patients, respectively. Statistical analyses using a chi-squared test showed that there is a statistically significant difference in methylation level of CDH11, EphA5, and HS3ST2 genes between gastric cancer and uncancerous patients ($p < 0.05$).

Conclusion To the best of our knowledge, this is the first report on methylation of CDH11, EphA5, and HS3ST2 promoters' in gastric adenocarcinoma patients using MSP. Identification of novel cancer-related molecular mechanisms can be useful in detection of new treatment strategies.

Keywords Gastric adenocarcinoma · Hyper methylation · MSP · CDH11 · EphA5 · HS3ST2

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Introduction

Gastric cancer is an aggressive disease which is the fourth prevalent malignancy in the world [1]. Despite the advent of many improvements in the diagnosis and treatment of the disease, gastric cancer is still the third leading cause of cancer-related death in terms of mortality rates [2]. Gastric cancer has a dark prognosis and becomes symptomatic in an advanced stage. Histologically, gastric cancer is divided into adenocarcinomas of the diffuse and the intestinal types which are different in clinical and epidemiologic characteristics [1]. Gastric adenocarcinoma is a multifactorial disease and different risk factors including *Helicobacter pylori* (*H. pylori*) infection, environmental and genetic factors have been identified for the aggressive disease [3]. Beside the genetic factors, epigenetic

alterations can be reasons for the emergence of gastric cancer [4]. Broadly, epigenetics alterations are heritable changes which can regulate gene expression without occurring changes in DNA sequence [5, 6]. DNA methylation, histone modifications and noncoding RNAs are the main mechanisms in epigenetic regulations [4]. DNA methylation is a common method for regulation of genes expression in eukaryotic cells. DNA methylation is known as important procedure in cell biology. It plays a key role in different stages of evolution such as chromosome X silencing and embryonic development [7]. In the mechanism, a methyl group (CH₃ moiety) covalently links to 5' position in the pyrimidine ring of cytosine in CpG sites. CpG sites abundantly are found in the promoters of protein-coding genes [8]. DNA methylation is regulated by the family of DNA methyltransferases (DNMTs) [8, 9]. Disruption of promoter DNA methylation is observed in different cancer cells [10]. Hyper-methylation in promoters of tumor suppressor gene leads to silencing of them and hypo-methylation in promoters of oncogenes leads to overexpression of these genes.

Cadherin 11 (CDH11) is a member of the superfamily of cadherin which plays a vital role in calcium-dependent cell–cell adhesion, proliferation, and invasive cells [11]. CDH11 acts as tumor suppressor gene which can inhibit cell proliferation and invasiveness [11]. Recent studies have shown that CDH11 is downregulated and often methylated in several types of tumors [12, 13]. In addition, the studies showed that the amount of CDH11 promoter methylation is different in various types of tumors [14]. This event confirmed that CDH11 promoter methylation is tumor specific. Ephrin type-A receptor 5 (EphA5) belongs to the ephrin receptor subfamily of the protein-tyrosine kinase family [15]. EphA5 plays a vital role in regulation of carcinogenesis and cancer progression as other Eph subtypes [16]. Hyper methylation and downregulation of EphA5 is reported in several tumor types as breast and prostate cancers [17, 18]. HS3ST2 gene encodes heparan sulfate (glucosamine) 3-O-sulfotransferase 2, a member of the heparan sulfate biosynthetic enzyme family. The protein has heparin glucosamineamine 3-sulfotransferase activity and modifies glycosaminoglycan chains [19]. These changes are very important in the specific binding of heparan sulfate proteoglycans to proteins and, therefore, to their regulatory role. Hyper methylation of HS3ST2 gene is reported in various cancers including breast, lung, pancreatic, and cervical cancer [19–22].

Up to now, CDH11, EphA5, and HS3ST2 promoters' methylation in gastric cancer patients has not been investigated. Therefore, the aim of the present study is the assessment of promoter methylation of EphA5, HS3ST2, and CDH11 genes in patients with and without gastric cancer. Identification of novel cancer-related molecular mechanisms can be useful in detection of new treatment strategies.

Material and Methods

Collection of Tissue Samples from Patients

In the study, from October 2016 to November 2018, 40 paraffin-embedded tissue sections were taken from patients with gastric adenocarcinoma who had been admitted to the Imam Reza Hospital, Tabriz, Iran. The patients consisted of 40 men whose ages were 57–104 years old. Also, in order to reduce the parameters of intervention, samples from 40 men patients (age range; 55–94 years old) with functional dyspepsia and without gastric cancer and *H. pylori* infection were chosen as control group. The control patients were classified according to endoscopic diagnosis into individuals with gastritis (32/40) and ulcers (8/40). Patients in the two groups were match the age, sex, and smoking. Present study was approved by the Ethics and Research Committees of the Tabriz University of Medical Sciences.

DNA Extraction

The total DNA was extracted from samples using an improved salting out method. Briefly, at first, the collected sections of paraffin-embedded gastric biopsies were deparaffinized by xylene (Merck, Germany) twice for 10 min, and then they were re-hydrated by alcohols 100 and 75%, respectively for 10 min. After centrifugation, the pellet was dissolved in P buffer containing; EDTA 0.5 M, Tris-HCL 1 M and NaCl 5 M. In the following, SDS 10% (Merk, Germany) and proteinase K (Sigma-Aldrich) were added, respectively. For the salting out, NaCl 5 M for 20 min at –20 °C and then NaCl 0.5 M for 20 min at –20 °C were used respectively. Then, alcohols 100 and 75% were used for sedimentation of the DNA. DNA was dissolved in TE buffer-containing Tris 1 M and EDTA 0.5 M.

DNA Treatment with Sodium Bisulfite

DNA treatment using sodium bisulfite resulted in deamination and conversion of cytosine residues to uracil, while 5-mC residues remain the same. Therefore, cytosine residues are recognized as thymine in subsequent PCR amplification by using specific methylation primers [23]. In the study, we used Epizem DNA Methylation Kit (Gendepot, USA) for the bisulfite DNA conversion and also clean-up the converted DNA as the manufactory instructions.

Methylation-Specific PCR (MSP)

The modified DNA was used as a template for methylation-specific PCR analysis using the specific methylated and unmethylated primer sets. The primer sequences are summarized in Table 1. The PCR reaction was performed according

Table 1 Primer sequences which used for the determination of EphA5, HS3ST2, and CDH11 genes methylation profile in MSP

Primer	Sequence
Methylation EphA5	Forward: 5'-ATTGAGTCGTTTCGGGATAGC-3' Reverse: 5'-GTCGAAATACAAAATAACAACCGA-3'
Unmethylation EphA5	Forward: 5'-GATTGAGTTGTTTGGGATAGTGG-3' Reverse: 5'-CCATCAAAATACAAAATAACAACCA-3'
Methylation HS3ST2	Forward: 5'-TGTTTTTTCGGAAATTATGATTTTC-3' Reverse: 5'-GTAAAAACGAAAAACAACCTACG-3'
Unmethylation HS3ST2	Forward: 5'-TTTTTTTGGAAATTATGATTTTGG-3' Reverse: 5'-AACATAAAAAACAAAAACAACCTACAC-3'
Methylation CDH11	Forward: 5'-TTATTTTTGTTATTAGCGCGTTC-3' Reverse: 5'-CCATTCACAAATCAACGACG-3'
Unmethylation CDH11	Forward: 5'-TTTTTATTTTTGTTATTAGTGTGTTT-3' Reverse: 5'-TCCCATTTCACAAATCAACAACA-3'

to the following program: initial denaturation at 95 °C for 3 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C (for all genes), extension at 72 °C for 1 min, and final extension at 72 °C for 8 min. Amplicons were separated on 3% agarose gels and visualized under ultraviolet illumination.

Statistics

Data analyzing was used by statistical package for the Social Sciences (SPSS) software v.19. Chi-squared test was used to check if there is a significant difference between the two groups in terms of methylation. $P < 0.05$ was considered to be statistically significant.

Results

Among the 80 paraffin-embedded gastric biopsy specimens, 71 DNA samples were achieved using modified salting out method of DNA extraction (34 gastric adenocarcinoma patients and 37 control patients). The adequate amount of DNA from each samples were treatment with sodium bisulfite and then the treatment and untreated DNAs were used in PCR reaction using the pair primers specific for methylated and unmethylated cytosine in the promoters of CDH11, EphA5, and HS3ST2 genes (Fig. 1).

The results showed that of 34 DNA samples from gastric adenocarcinoma patients 28 samples are methylated in CDH11 gene. However, seven of 37 samples from control patients were methylated. In the case of EphA5 gene, 19 of 34 and nine of 37 samples from gastric adenocarcinoma patients and noncancerous patients were methylated, respectively. Also, 26 of 34 DNA samples were methylated in HS3ST2 gene in cancerous patients, while only seven samples were methylated in this gene in control patients. In addition, in the case of HS3ST2 gene, one of the 34 tumor samples had both

methylated and unmethylated bands. More details are summarized in Table 2.

Statistical analyses using a chi-squared test showed that there is a statistically significant difference in methylation level of CDH11 ($p < 0.001$), EphA5 ($p < 0.001$), and HS3ST2 ($p < 0.001$) genes between gastric cancer and uncancerous patients.

Discussion

Currently, gastric cancer alone accounts for 10% of all cancers in the world. This type of cancer is the fourth most important cancer in the world, and more than 870,000 new cases are reported every year worldwide [24]. Today, it has been identified that epigenetic alterations such as promoter CpG island hyper methylation are involved in emergence of different tumors such as gastric adenocarcinoma [25].

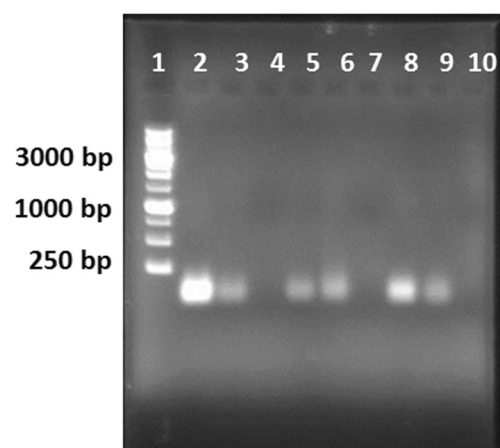


Fig. 1 MSP analysis. 2, 5, and 8 are methylated and 3, 6, and 9 are unmethylated samples for HS3ST2, CDH11, and EphA5 genes, respectively

Table 2 Comparison of methylation status in the promoters of EphA5, HS3ST2, and CDH11 genes in adenocarcinoma and control group patients

	Gastric adenocarcinoma patients <i>n</i> = 34			Control group <i>n</i> = 37		
	Methylated	Unmethylated	Methylated/ Unmethylated	Methylated	Unmethylated	Methylated/ Unmethylated
EphA5	19	15	0	9	25	3
HS3ST2	26	7	1	7	30	0
CDH11	28	6	0	7	29	1

In the present study, we verified the promoter methylation of EphA5, HS3ST2, and CDH11 genes in patients with and without gastric cancer using MSP technique, for the first time. These genes act as tumor suppressor genes, therefore the inactivation of them can be lead to cancer promotion. CDH11 as a member of the superfamily of cadherin has an important role in cell–cell adhesion, proliferation, and invasive cells [11]. Yuan et al. investigated the promoter methylation of CDH11 in colorectal cancer (CRC) tissues using MSP [13]. They reported that CDH11 is methylated in CRC cells. CDH11 acts as an antagonist of Wnt/ b-catenin and AKT/Rho A signaling in the cells. Also, Lin et al. verified the methylation of CDH11 in bladder cancer tissue specimens [14]. The results showed that CDH11 promoter is frequently methylated in the specimens, and it is correlated with malignant behavior in bladder cancer. Therefore, they introduced CDH11 as an independent prognostic biomarker [14]. Our results showed that CDH11 promoter is also methylated in gastric adenocarcinoma specimens. Therefore, it seems that the mechanism of CDH11 inactivation in cancer cells is due to its promoter methylation.

Here, also we investigated the methylation status of EphA5 in gastric adenocarcinomas. Our result indicated that the gene is hyper methylated in the tissues. Hyper methylation of EphA5 has been also reported in several tumor types. Li et al. verified the expression and an epigenetic change of EphA5 in prostate cancer cell lines [17]. The results indicated that EphA5 expression is decreased in these cells due to hyper methylation of its promoter CpG sites. Also, the immunohistochemical analysis of EphA5 in clear cell renal cell carcinoma (ccRCC) tissues by Wang et al. indicated that EphA5 expression is downregulated in the tissues [26]. Paradoxically, two study in hepatocellular carcinoma reported that the expression of EphA5 gene was upregulated [27, 28]. It seems that the expression of EphA5 in tumor cells is related to tissue type, ligand, or ligand-dependent signaling.

The verification of methylation status of HS3ST2 gene is the last goal of this study. The results showed that the methylation HS3ST2 gene is statistically significant in gastric adenocarcinoma patients. The same results have been reported from other study which investigated the methylation profile of this gene in different tumors including the breast, lung, pancreas, and cervix [19–22].

In summary, here, for the first time, we showed that the promoter CpG islands in CDH11, EphA5, and HS3ST2 genes are hyper methylated in gastric adenocarcinoma patients in comparison with control dyspepsia patients without gastric cancer. Therefore, it seems that the mechanism of CDH11, EphA5, and HS3ST2 genes downregulation in gastric cancer cells is promoter CpG island hyper methylation.

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Compliance with Ethical Standards

The present study was approved by the Ethics and Research Committees of the Tabriz University of Medical Sciences.

Conflict of Interest The authors report no conflicts of interest in this work.

References

- Sitarz R, Skierucha M, Mielko J, Offerhaus GJA, Maciejewski R, Polkowski WP. Gastric cancer: epidemiology, prevention, classification, and treatment. *Cancer Manag Res*. 2018;10:239–48.
- Rugge M, Fassan M, Graham DY. Epidemiology of gastric cancer. In: *Gastric cancer*: Springer; 2015. p. 23–34.
- Ajani JA, Lee J, Sano T, Janjigian YY, Fan D, Song S. Gastric adenocarcinoma. *Nat Rev Dis Primers*. 2017;3:17036.
- Patel TN, Roy S, Ravi R. Gastric cancer and related epigenetic alterations. *Ecancermedicalsecience*. 2017;11.
- Takeshima H, Yamada H, Ushijima T. Cancer epigenetics: aberrant DNA methylation in cancer diagnosis and treatment. In: *Oncogenomics*: Elsevier; 2019. p. 65–76.
- Kanwal R, Gupta K, Gupta S. Cancer epigenetics: an introduction. In: *Cancer epigenetics*: Springer; 2015. p. 3–25.
- Li S, Zhu Y, Zhi L, Han X, Shen J, Liu Y, et al. DNA methylation variation trends during the embryonic development of chicken. *PLoS One*. 2016;11(7):e0159230.
- Lim DH, Maher ER. DNA methylation: a form of epigenetic control of gene expression. *Obstet Gynaecol*. 2010;12(1):37–42.
- Du J, Johnson LM, Jacobsen SE, Patel DJ. DNA methylation pathways and their crosstalk with histone methylation. *Nat Rev Mol Cell Biol*. 2015;16(9):519–32.

10. Tahara T, Arisawa T. DNA methylation as a molecular biomarker in gastric cancer. *Epigenomics*. 2015;7(3):475–86.
11. Li L, Ying J, Li H, Zhang Y, Shu X, Fan Y, et al. The human cadherin 11 is a pro-apoptotic tumor suppressor modulating cell stemness through Wnt/ β -catenin signaling and silenced in common carcinomas. *Oncogene*. 2012;31(34):3901–12.
12. Carmona FJ, Villanueva A, Vidal A, Munoz C, Puertas S, Penin RM, et al. Epigenetic disruption of cadherin-11 in human cancer metastasis. *J Pathol*. 2012;228(2):230–40.
13. Yuan S, Li L, Xiang S, Jia H, Luo T. Cadherin-11 is inactivated due to promoter methylation and functions in colorectal cancer as a tumour suppressor. *Cancer Manag Res*. 2019;11:2517–29.
14. Lin YL, Gui SL, Ma JG. Aberrant methylation of CDH11 predicts a poor outcome for patients with bladder cancer. *Oncol Lett*. 2015;10(2):647–52.
15. Pasquale EB. Eph receptors and ephrins in cancer: bidirectional signalling and beyond. *Nat Rev Cancer*. 2010;10(3):165–80.
16. Chen X, Wang X, Wei X, Wang J. EphA5 protein, a potential marker for distinguishing histological grade and prognosis in ovarian serous carcinoma. *J Ovarian Res*. 2016;9(1):83.
17. Li S, Zhu Y, Ma C, Qiu Z, Zhang X, Kang Z, et al. Downregulation of EphA5 by promoter methylation in human prostate cancer. *BMC Cancer*. 2015;15(1):18.
18. Brantley-Sieders DM, Jiang A, Sama K, Badu-Nkansah A, Walter DL, Shyr Y, et al. Eph/ephrin profiling in human breast cancer reveals significant associations between expression level and clinical outcome. *PLoS One*. 2011;6(9):e24426.
19. Hwang J-A, Kim Y, Hong S-H, Lee J, Cho YG, Han J-Y, et al. Epigenetic inactivation of heparan sulfate (glucosamine) 3-O-sulfotransferase 2 in lung cancer and its role in tumorigenesis. *PLoS One*. 2013;8(11):e79634.
20. Vijaya Kumar A, Salem Gassar E, Spillmann D, Stock C, Sen YP, Zhang T, et al. HS3ST2 modulates breast cancer cell invasiveness via MAP kinase-and Tcf4 (Tcf712)-dependent regulation of protease and cadherin expression. *Int J Cancer*. 2014;135(11):2579–92.
21. Miyamoto K, Asada K, Fukutomi T, Okochi E, Yagi Y, Hasegawa T, et al. Methylation-associated silencing of heparan sulfate D-glucosaminyl 3-O-sulfotransferase-2 (3-OST-2) in human breast, colon, lung and pancreatic cancers. *Oncogene*. 2003;22(2):274–80.
22. Lim EH, Ng SL, Li JL, Chang AR, Ng J, Ilancheran A, et al. Cervical dysplasia: assessing methylation status (Methylight) of CCNA1, DAPK1, HS3ST2, PAX1 and TFPI2 to improve diagnostic accuracy. *Gynecol Oncol*. 2010;119(2):225–31.
23. Leontiou CA, Hadjidaniel MD, Mina P, Antoniou P, Ioannides M, Patsalis PC. Bisulfite conversion of DNA: performance comparison of different kits and methylation quantitation of epigenetic biomarkers that have the potential to be used in non-invasive prenatal testing. *PLoS One*. 2015;10(8):e0135058.
24. Organization WH. Global cancer rates could increase by 50% to 15 million by 2020. In: *Global cancer rates could increase by 50% to 15 million by 2020*; 2003.
25. Ferreira HJ, Esteller M. CpG islands in cancer: heads, tails, and sides. In: *CpG Islands*: Springer; 2018. p. 49–80.
26. Wang X, Xu H, Wu Z, Chen X, Wang J. Decreased expression of EphA5 is associated with Fuhrman nuclear grade and pathological tumour stage in cc RCC. *Int J Exp Pathol*. 2017;98(1):34–9.
27. Sun B, Wu J, Zhang T, Wang C. High-resolution analysis of genomic profiles of hepatocellular carcinoma cells with differential osteopontin expression. *Cancer Biol Ther*. 2008;7(3):387–91.
28. Wu J-C, Sun B-S, Ren N, Ye Q-H, Qin L-X. Genomic aberrations in hepatocellular carcinoma related to osteopontin expression detected by array-CGH. *J Cancer Res Clin Oncol*. 2010;136(4):595–601.

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