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Correlation of 2,4-DDT with CDH1 Gene Promoter Methylation in Gastric Cancer

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Abstract

Background: Methylation alterations of tumor suppressor gene promoters are important epigenetic changes in gastric cancer. *CDH1* encodes a protein (cadherin1) with essential roles in cell-cell adhesion. In this study, the association of organochlorine pesticide (OCP) serum levels with the methylation profile of this gene was investigated in gastric cancer, intestinal metaplasia (IM), and functional dyspepsia (FD) patients.

Methods: Gastric cancer ($n=34$), IM ($n=8$), and FD ($n=48$) patient serums were analyzed for the determination of OCP levels by gas chromatography. The methylation status of *the CDH1* gene promoter was examined by the methylation-specific PCR (MSP) method. Immunohistochemistry (IHC) was used to confirm the reduced protein expression of this gene in methylated samples.

Results: Our findings revealed significant hypermethylation of the *CDH1* gene promoter and its reduced expression in gastric cancer patients compared with IM and FD patients. Furthermore, there was a significant association between *CDH1* promoter hypermethylation and 2,4-DDT (OR = 1.183; 95% CI = 1.001–1.398; $P=0.048$) serum levels in gastric cancer patients.

Conclusion: Our results suggest an association between 2,4-DDT OCP levels in gastric cancer patient serums with *CDH1* gene promoter hypermethylation. Additionally, this gene promoter's methylation may play a role in the progression of pre-cancerous IM towards gastric cancer.

Keywords: Organochlorine pesticides, *CDH1,* DNA methylation, Gastric cancer, Intestinal metaplasia

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Introduction

Gastric cancer is an extremely lethal malignancy, with an annual incidence of over 1 million individuals. It has been approximated that in the year 2018, approximately 800 000 individuals succumbed to this cancer on a global scale. Functional dyspepsia (FD) is a disorder that impacts the upper sections of the gastrointestinal tract in a non-ulcerous manner, with a prevalence of 11%–29.2% of the global population (1,2). It has been observed that gastritis leading to gastric mucosa atrophy may progress into a condition known as intestinal metaplasia (IM) (3). The development of IM has been associated with a sixfold risk of gastric cancer (4).

Conversely, FD can potentially advance into IM (5). Epigenetic alterations play a crucial role in the initiation and progression of gastric cancer (6). Among these alterations, hypermethylation of the promoter region of tumor suppressor genes stands out as a prominent mechanism that typically leads to decreased expression of these genes (6). In individuals with gastric cancer, the evaluation of the methylation status of the cadherin1 (CDH1) gene promoter holds great significance due to the involvement of the gene's product in cell-cell interactions and cell migration, which, if compromised as a result of

promoter hypermethylation, could potentially contribute to the metastatic behavior of gastric cancer cells (6). Organochlorine pesticides (OCPs) are commonly used in agriculture to eliminate pests (7). Human exposure to OCPs occurs extensively through various routes, including food consumption, liquid intake, inhalation of air, and even occupational contact (7). The association between the use of pesticides, including OCPs, and the incidence of gastric cancer has been established (8).

Recent findings have indicated that pesticide exposure can trigger epigenetic modifications, such as alterations in the methylation of gene promoters under laboratory conditions (9). The primary objective of this investigation was to evaluate whether there is a correlation between hypermethylation of the CDH1 gene promoter, a known tumor suppressor gene in gastric cancer, and elevated levels of OCPs in patients diagnosed with gastric cancer, IM, chronic gastritis, and FD. Furthermore, the potential impact of this gene promoter methylation on the progression from IM to gastric cancer was a critical aspect examined in this study. Additionally, immunohistochemistry (IHC) was employed to validate the decrease in cadherin protein expression due to promoter hypermethylation. To achieve this goal, we assessed the serum levels of seven OCPs, namely α-HCH, β-HCH, γ-HCH, 2,4-DDE, 4,4-DDE, 2,4-DDT, and 4,4-DDT, as well as the status of CDH1 gene promoter methylation in gastric tissues collected from individuals diagnosed with gastric cancer.

Material and methods

Subjects

Our investigation was conducted on individuals who were diagnosed with FD, IM with chronic gastritis, and gastric cancer. Specimens were collected from Afzalipour Hospital in Kerman between July 2018 and April 2020. The study groups consisted of recently diagnosed patients with FD (n = 48), IM with chronic gastritis (n = 8), and gastric cancer (n = 34). These patients' diagnoses were confirmed based on clinical observations made during endoscopy performed by gastrointestinal subspecialists and documented in pathological reports.

All participants in this study were new cases and had no prior medical history. They had not received any medications, supplements, or narcotics. The consent form was thoroughly read and signed by all patients. Patients were specifically diagnosed with primary gastric cancer. Those with a previous history of gastric cancer or other cancers that had metastasized to the stomach from elsewhere were excluded from this study. This study strictly adhered to the ethical guidelines outlined in the Declaration of Helsinki. The Kerman University of Medical Sciences Ethics Committee approved this study under the ethical code IR. KMU. REC.1398.335.

Sample collection

Fasting blood samples were gathered from each participant and subjected to centrifugation to separate the serum from the cells. The centrifugation process was carried out at 3000 rpm for 10 minutes. Subsequently, the obtained serum samples were carefully transferred to sterile containers designed for specimen storage. These containers were maintained at a temperature of -70 °C for subsequent analysis. Additionally, approximately 25 mg of gastric tissue was obtained from each patient's antrum using an endoscopy needle under the supervision of a gastroenterology subspecialist. After collection, the gastric tissue was also preserved at a temperature of -70 °C for further analysis.

Measurement of OCPs in patient's serum

The standards for OCP components, including α-hexachlorocyclohexane (α-HCH), β- hexachlorocyclohexane (β-HCH), γ- hexachlorocyclohexane (γ-HCH), 2,4-dichlorodiphenyldichloroethylene (2,4-DDE), 4,4-dichlorodiphenyldichloroethylene (4,4-DDE), 2,4-dichlorodiphenyltrichloroethane (2,4-DDT) and 4,4- dichlorodiphenyltrichloroethane (4,4-DDT) were provided from Ehrenstorfer company (Germany). The internal standard, 4,4-dichlorobenzophenone (DBP), was obtained from Supelco (Sigma-Aldrich, PA, USA). OCPs were extracted using hexane and sulfuric acid using the method applied by Zumbado et al (10). Briefly, 20 µLof the internal standard was added to 500 µL of the serum samples. Samples were extracted twice with 2 mL of hexane. The organic part of the extract was separated by adding 200 µL of concentrated sulfuric acid to the extracts. Then, the sample was dehydrated using 200 mg of anhydrous sodium sulfate. After centrifuging, the organic layer was concentrated at room temperature. Following solvent evaporation, 100 µL of ethyl acetate was added to the samples. Then, the samples were injected into the gas chromatography instrument with a flame ionization detector (11), which used capillary columns (HP-5) for the identification of OCPs (Agilent 7890A, USA).

Modification with sodium bisulfite and methylation-specific PCR (MSP)

MSP is used to investigate the methylation profile of CpG islands based on the PCR method. First, DNA samples were treated with sodium bisulfite to convert unmethylated cytosine to uracil, while methylated cytosines remained unchanged. Moreover, this is the basis for distinguishing methylated and unmethylated DNA samples in MSP. The method used for DNA sodium bisulfite treatment was based on the work done by Tiwari et al (12). Briefly, NaOH solution (Merck KGaA, Darmstadt, Germany) was added to about one microgram of DNA to the final concentration of 0.3 M of NaOH. For efficient denaturation of DNA strands,

samples were incubated for 15 minutes at 50 °C. Then, 50 µL of low melting point (LMP) agarose 2% (V2111, Promega, Madison, WI, USA) was added to the mixture, which was then incubated for 15 minutes at 50 °C. After that, 15 µL of the mixture was added to a microtube containing 300 µL of mineral oil (Sigma, Saint Louis, MO, USA) and incubated at -4 °C for 30 minutes. It causes the solidification of the reaction mixture drops in the mineral oil and the formation of agarose beads. Next, 700 µL of bisulfite solution (5 M sodium bisulfite and 125 mM hydroquinone; both Merck, KGaA, Darmstadt, Germany; pH = 5.0) was added to each microtube containing a single agarose bead. The microtubes were shaken gently to transform the beads into the aqueous phase and were then incubated in a dark place for 4 hours at 55 °C. In order to stop the reaction, 1 mL of 1 × TE (Tris-HCl and EDTA, Merck, Darmstadt, Germany) (2 × 15 min) was added and then was desulfonated by adding 500 mL of NaOH (0.2 M) (2 × 10 minutes) to the mixtures. The agarose beads were used in PCR after washing with 1 ml of 1 × TE and then 1 ml ddH2O (1 × 15 min).

MSP was performed as follows. For CDH1 gene promoter methylated primary amplicon, denaturation at 95 °C for 5 minutes, 34 cycles of amplification comprising denaturation at 95 °C for 30 seconds, annealing at 52 °C for 30 seconds, extension at 72 °C for 30 seconds, and final extension at 72 °C for 5 minutes. For CDH1 gene unmethylated amplicon, denaturation at 95 °C for 5 minutes, 34 cycles of amplification comprising denaturation at 95 °C for 42 seconds, annealing at 56.1 °C for 42 seconds, extension at 72 °C for 42 seconds, and final extension at 72 °C for 5 minutes. Primer sequences are indexed in Table 1. Then, the amplicons were separated by electrophoresis on 1.5% (W/V) agarose gel in the 0.5% TAE buffer and finally examined by ultraviolet light.

Measuring of Helicobacter pylori-specific antibodies in serum

Anti-*Helicobacter pylori* immunoglobulin G was assayed by a commercially available enzyme-linked immunosorbent assay (Trinity Biotec, Ireland). Following manufacturer guidelines, the results were obtained as immune status ratio (ISR), and the values equal to or bigger than 1.1 were considered positive.

Immunohistochemical (IHC) staining of gastric tissues for E-cadherin

In order to understand whether hypermethylation of the CDH1 gene could contribute to the reduced expression of coding protein (E-cadherin), staining of gastric tissues in FD samples (whose CDH1 gene was not methylated) and gastric cancer samples with methylated CDH1 gene was performed using ready-to-use ZYTOMED rabbit anti-E-cadherin (Berlin, Germany, BRB047) based on manufacturer protocols. Briefly, pre-treatment for heat-induced epitope retrieval was performed using EDTA buffer (pH 9.0). Then, tissues were incubated for 45 minutes.

Statistical analyses

Continuous variables were reported as the mean and standard deviation, and categorical variables were presented as percentages. The normality of the variables was verified by conducting the Kolmogorov-Smirnov test. A paired t-test was employed to compare the quantitative variables among patients. The relationship between OCPs and the methylation status of the CDH1 gene in gastric cancer patients was assessed through continuous logistic regression. The potential association between clinical and demographic information and the methylation status of CDH1 was assessed using Pearson's chi-square test. All statistical analyses were conducted using SPSS 21.0 for Windows (IBM/SPSS Inc., New York, USA). Statistical significance was established with *P* values lower than 0.05.

Results

Methylation status of CDH1 gene promoter

The results of our study demonstrated that hypermethylation of the CDH1 gene promoter was present in 10 out of 34 individuals diagnosed with gastric cancer. The gene promoter exhibited hypermethylation in two patients with IM, and only one patient with familial dysplasia (FD) displayed hypermethylation of the gene promoter (Figure 1). Upon examination of Table 2, it can be inferred that there were no significant associations between the age, gender, and differentiation status of gastric cancer patients and the methylation of the promoter region of the CDH1 gene.

IHC staining of gastric tissues for E-cadherin

It was revealed that gastric cancer samples with hypermethylated CDH1 gene promoter had reduced expression of E-cadherin protein compared to FD samples, whose CDH1 gene was not methylated (Figure 2).

Helicobacter pylori-specific antibody measurement in serum

It was revealed that 24 out of 34 gastric cancer patients

Table 1. Primer sequences, annealing temperatures, and amplicon sizes applied in MSP of CDH1 gene promoter

Amplicon name	Primer sequences (5′→ 3′)	Annealing temperature	Product size (bp)
Methylated	TTAGTTAATTAGCGGTACGGGG TAAAATCTAAACTAACTTCCGCA	52	125
Unmethylated	TAGTTAATTAGTGGTATGGGG AACTAAAATCTAAACTAACTTCCACAA	56.1	127

Table 2. Association of CDH1 gene promoter hypermethylation with demographic and clinical characteristics of gastric cancer patients based on Pearson's chi-square test

Variables		Gastric cancer (n = 34)	*P* value
Age by year (mean ± SD)		63.82 ± 16.29	0.217
Gender	Number of males with hypermethylated promoter	7 out of 26	0.566
	Number of females with hypermethylated promoter	3 out of 8	
Differentiation status	Number of poorly differentiated subjects with hypermethylated promoters	7 out of 18	0.198
	Number of moderately differentiated subjects with hypermethylated promoters	3 out of 16	

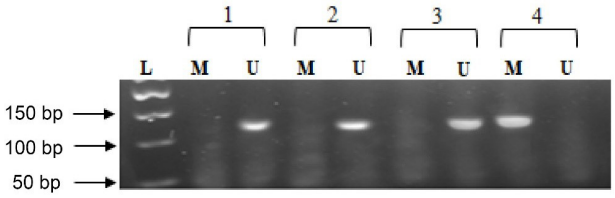

Figure 1. An electrophoresis picture of the methylation status of the CDH1 gene performed by MSP. The methylated and unmethylated amplicon of each patient (4 patients) have been shown here. As presented here, patient 4 has methylated, and other patients have the unmethylated status of this gene. The methylated amplicon is 125 bp, and the unmethylated amplicon is 127 bp long. M: methylated, U: unmethylated, L: DNA ladder, bp: base pair

were positive for *H. pylori* (including two patients who had hypermethylated CDH1 genes), and 3 of 8 IM patient serums had reactivity for *H. pylori*.

Association of OCP serum levels with methylation status of CDH1 gene promoter

The mean pesticide levels in the hypermethylated and unmethylated pattern of the CDH1 gene promoter in gastric cancer patients are shown in Table 3. The mean levels of 2,4-DDT ($P = 0.020$) were significantly different in unmethylated CDH1 genes compared to hypermethylated genes. Considering the logistic regression analysis, the odds ratios revealed that there was a significant association between CDH1 gene promoter hypermethylation and 2,4-DDT (OR = 1.183; 95% CI = 1.001–1.398; $P = 0.048$) in gastric cancer and IM patients (Table 4).

Discussion

In the current investigation, we conducted a survey to assess the methylation status of the promoter region of the CDH1 gene in patients diagnosed with gastric cancer, IM, and FD. Additionally, we explored the potential association between the methylation profile of these patients and the levels of certain OCPs in their serum. Our findings indicate a significant association between CDH1 gene promoter hypermethylation in gastric cancer patients and the 2,4-DDT pesticide, as determined by logistic regression analysis of the odds ratio (OR). Our results align with those presented by Zhang et al., who demonstrated that various pesticides, including OCPs, can induce hypermethylation of tumor suppressor gene promoters (9). However, in contrast to our observations,

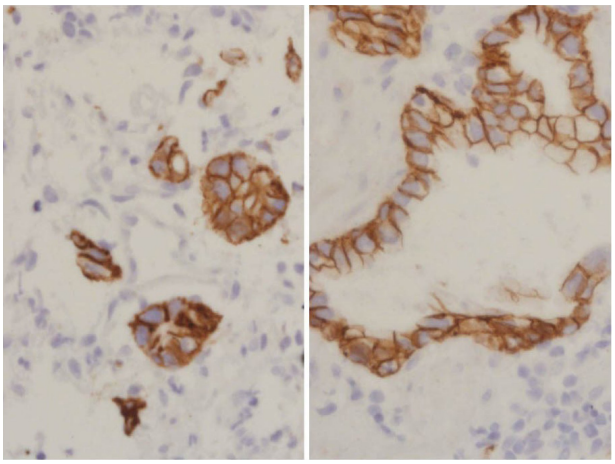

Figure 2. IHC staining of gastric tissues for E-cadherin. On the right, immunostaining of an FD sample whose CDH1 gene was not methylated. On the left, a gastric cancer sample immunoreaction for E-cadherin with methylated CDH1 gene promoter. As shown, the expression of E-cadherin protein is reduced in gastric cancer samples compared to FD samples

another study reported the absence of CDH1 gene promoter hypermethylation in the blood cells of individuals exposed to organophosphate pesticides (13).

This paradox may be interpreted by considering pesticide varieties, dietary factors, ethnicity, and the specific tissue type on which the MSP procedure was performed. It is worth noting that each of these parameters has the potential to impact the methylation profile of tumor suppressor genes (11). Research studies have demonstrated that the CDH1 gene promoter is hypermethylated in gastric tissue (14) and blood cells (15) of individuals who have gastric cancer. These findings are in accordance with our results. Our study revealed that 10 out of 34 gastric cancer patients exhibited hypermethylation of the CDH1 gene promoter, which is in line with a prior investigation indicating that 54.8% of gastric cancer patients had hypermethylation in the promoter CpG islands of this gene (6).

Moreover, our study found a significantly higher CDH1 gene promoter hypermethylation occurrence in gastric cancer patients (29.4%) and patients with IM (25%). Furthermore, the noticeably elevated serum levels of OCPs in these patients may indicate increased exposure to these pesticides (13). Our results have demonstrated that, among the 48 patients diagnosed with FD, only one individual exhibited hypermethylation of

Table 3. The mean levels of OCPs in gastric cancer patients in both hypermethylated and unmethylated CDH1 gene promoters.

	Gastric cancer (n = 34)		
OCPs (ng/mL)	**Unmethylated (n = 24) Mean ± SE**	**Hypermethylated (n = 10) Mean ± SE**	***P* value**
α-HCH	0.475 ± 0.021	0.49 ± 0.40	0.096
β-HCH	0.264 ± 0.087	3.356 ± 2.95	0.108
γ-HCH	3.946 ± 1.70	26.157 ± 19.14	0.082
2,4-DDE	1.486 ± 0.69	4.276 ± 2.59	0.166
4,4-DDE	0.499 ± 0.27	0.314 ± 0.114	0.679
2,4-DDT	0.804 ± 0.200	5.210 ± 2.80	0.020
4,4-DDT	1.721 ± 1.00	3.071 ± 2.04	0.511

OCP: organochlorine pesticides; α-HCH: α-hexachlorocyclohexane; β-HCH: β-hexachlorocyclohexane; γ-HCH: γ-hexachlorocyclohexane; 2,4- DDE: 2,4- dichlorodiphenyldichloroethylene; 4,4- DDE: 4,4- dichlorodiphenyldichloroethylene; 2,4- DDT: 2,4- dichlorodiphenyltrichloroethane; 4,4- DDT: 4,4- dichlorodiphenyltrichloroethane; SE: standard error of the mean. Serum OCPs were measured by GC.
All data were expressed as mean ± SE.
P values less than 0.05 were considered significant.

Table 4. Association of OCP serum levels with CDH1 gene promoter hypermethylation in gastric tissues of gastric cancer patients

OCPs (ng/mL)	**OR (95% CI)**	***P* value**
α-HCH	1.837 (0.834–4.048)	0.131
β-HCH	0.997 (0.971–1.024)	0.841
γ-HCH	1.049 (0.993–1.109)	0.088
2,4-DDE	1.152 (0.999–1.329)	0.052
4,4-DDE	0.876 (0.411–1.869)	0.732
2,4-DDT	1.183 (1.001–1.398)	0.048
4,4-DDT	1.013 (0.917–1.119)	0.801

OR: odds ratio; CI: confidence intervals for the OR; OCP: organochlorine pesticides; α-HCH: α-hexachlorocyclohexane; β-HCH: β-hexachlorocyclohexane; γ-HCH: γ-hexachlorocyclohexane; 2,4 -DDE: 2,4- dichlorodiphenyldichloroethylene; 4,4- DDE: 4,4- dichlorodiphenyldichloroethylene; 2,4- DDT: 2,4- dichlorodiphenyltrichloroethane; 4,4- DDT: 4,4- dichlorodiphenyltrichloroethane. *P*-values less than 0.05 are considered significant.

the CDH1 gene promoter. This observation suggests that hypermethylation of the CDH1 gene promoter is not a characteristic feature of FD patients. Furthermore, it is worth noting that the hypermethylation of the CDH1 gene promoter is an early epigenetic alteration observed in patients with IM and gastric cancer. This epigenetic modification may play a role in the progression of the pre-cancerous lesion IM towards gastric cancer.

Additionally, our findings have revealed that two patients diagnosed with gastric cancer and exhibiting methylated CDH1 gene were also infected with *H. pylori*. This finding indicates that *H. pylori* infection may contribute to epigenetic alterations, including methylation of the CDH1 gene, which is consistent with previous research studies (16). Our research findings demonstrated a statistically significant correlation between the levels of 2,4-DDT in the serum and the hypermethylation of the promoter of the CDH1 gene in patients with gastric cancer. Stated differently, our findings indicate that exposure to OCP may lead to epigenetic alterations, such as hypermethylation of the CDH1 gene promoter in patients with gastric cancer. Consequently, we can assert that exposure to OCPs is linked to the hypermethylation of the CDH1 gene promoter and may be associated with gastric cancer. Various studies support this result. Lind et al (17) reported that DDE can induce human DNA methylation.

Additionally, exposure to OCPs may contribute to the hypermethylation of the promoter region of tumor suppressor genes in malignancy-afflicted cells (18). Conversely, cancer cells tend to exhibit an upregulation of DNA methyltransferases (DNMTs), which are responsible for the hypermethylation of gene promoters due to exposure to OCPs (19). Furthermore, it has been found that exposure to 2,4-DDT has the potential to induce methylation alterations in certain genes within the blood cells of individuals; however, the CDH1 gene did not manifest significant methylation among these subjects (20). These findings may indicate that the impact of 2,4-DDT on various organs' methylation patterns is inconsistent. Moreover, gastric carcinogenesis is multifaceted and gradual, affected by *H. pylori* infection, inflammation, stem cells, and generalized and specific genetic and epigenetic alterations (21). Taking into consideration these facts, as well as the strong correlation between intestinal-type gastric cancer and *H. pylori* infection (22) and CDH1 gene methylation (23), it is plausible to suggest that the two parameters analyzed in our study may have associations with the process of gastric carcinogenesis.

Furthermore, following a previous study, CDH1 gene methylation in gastric cancer samples has been linked to reduced expression of the cadherin1 protein, which is encoded by this gene (24). To our knowledge, our study is one of the first investigations indicating a potential correlation between exposure to OCPs and hypermethylation of the CDH1 gene promoter in gastric cancer patients. However, it is important to acknowledge the limitations of our study, which include time constraints and a relatively small number of patients. Surveying methylation of other tumor suppressor gene promoters and histone modifications, particularly in larger populations, could greatly contribute to our understanding of the role of OCPs in the pathogenesis of IM and gastric cancer.

Conclusion

The results of our study unveiled a significant

correlation between the levels of serum 2,4-DDT and the hypermethylation of the CDH1 gene promoter in individuals diagnosed with gastric cancer. These discoveries indicate the association between OCPs and the hypermethylation of the CDH1 gene promoter. Furthermore, they may also suggest the involvement of the epigenetic silencing of this gene in the progression from pre-cancerous IM lesions to gastric cancer.

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Competing Interests
The authors declare that there is no conflict of interest.

Consent to Participate
All patients participating in this study were informed about the project and willingly signed the informed consent form**.**

Consent for Publication
All authors declare their consent for the publication of this paper.

Ethical Approval
The Research Ethics Committee of Kerman University of Medical Sciences has approved this study (ethics code: IR.KMU.REC.1398.335).

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