



Expression and Promoter Methylation Status of *CDX1* and *CDX2* Genes in Chinese Patients with Gastric Cancer

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ABSTRACT

Gastric cancer is a multifactorial disease, the fourth most common cancer in the world and the second cause of cancer death. Changes in gene expression levels are one of the most important factors in the occurrence of cancer. Gene promoter methylation changes are considered a suitable target for therapeutic strategies. In the present study, the frequency of this epigenetic phenomenon and the expression of *CDX1* and *CDX2* genes and their relationship with pathological and clinical characteristics of Chinese patients with gastric cancer were investigated. In this study, in order to investigate the promoter methylation of *CDX1* and *CDX2* genes, one hundred thirty-three tissue samples were analyzed using the Methylation Specific PCR method and in order to investigate gene expression, sixty-one tumor tissue samples and eleven normal tissue samples were analyzed using the Real-Time RT-PCR method. According to the data obtained, there was no significant difference in the promoter methylation results of *CDX1* and *CDX2* genes in tumor tissue compared to normal tissues adjacent to the tumor and normal controls. Furthermore, changes in the expression of *CDX1* and *CDX2* genes show a significant relationship with increasing disease stages and lymph vascular and perineural invasions. It was concluded that increased frequency in the promoter methylation of *CDX1* genes in patients with gastric cancer compared to control tissues and its relationship with factors that confirm the poor prognosis of the disease cannot be introduced alone as a possible candidate for further studies to confirm the role of a poor prognostic biomarker in gastric cancer.

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Authors' Contribution

ZH and XZ participated in conceiving the design of the study and collecting and reviewing the data and coordination of project. LL and MH participated in doing literature review, collecting the data and analysis and in preparing the manuscript. MC and QM helped in critical revision and finalizing the manuscript. All authors read, revised, and approved the final manuscript.

Key words

CDX1, *CDX2*, Gastric cancer, Methylation, Gene expression, Epigenetics

INTRODUCTION

Cancer is the first and second cause of death in developed and developing countries respectively, gastric cancer is known as the fourth most common cancer and the second cause of death due to cancer in the world (Jemal *et al.*, 2011). Gastric cancer is one of the most common malignancies in the world; the prevalence of this cancer is caused by the process of creating cancer tissue in stomach in several stages and is part of multifactorial diseases and the reason for that is the creation of cancer due to the presence of infectious, environmental and genetic factors in people (Zabaleta, 2012). In general, stomach cancer is

a disease for old people, and the ratio of men to women is about 2 to 1 (Anderson *et al.*, 2011). This cancer is twice as common in black people as in white people with the same sex ratio (male to female 2 to 1) (Rondolph, 2010). According to 2005 statistics, the most cases of this cancer are observed in Japan, China and Russia, and the least cases are related to developed western countries (Inoue and Tsugane, 2005). In 1930 in the United States, gastric cancer was the second leading cause of death from cancer among men and the third leading cause of death from cancer among women, but today it is not even among the 10 most common causes of death (Anderson *et al.*, 2011). Gastric cancer is one of the most common cancers in China, which is increasing dramatically compared to Western countries (Yan *et al.*, 2023).

There is much evidence that gastric cancer is the result of multiple genetic and epigenetic changes on tumor suppressor genes, repair genes and cell adhesion molecules (Canale *et al.*, 2020). Recently, epigenetic changes have been investigated as important and valuable biomarkers in a variety of diseases, including cancer. Epigenetic events lead to heritable changes in gene expression and chromatin structure, without changes in DNA sequence.

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DNA methylation is the only epigenetic modification that affects DNA directly. This occurs mainly in CpG regions. Methylation regulates gene expression by affecting the gene promoter. Changes in the methylation pattern may lead to tumorigenesis and development of tumors (Dumitrescu, 2018).

About 20% of CpGs in the human genome are located in CpG islands. These islands have a length of 200 bp and a minimum content of 50% CpG. About 60% of human genes have CpG islands in their promoter region and in most tissues these regions are unmethylated (Ramalho *et al.*, 2018; Abbasi *et al.*, 2016). Methylation of gene promoter under the influence of external factors can affect gene expression and genome function. Methylated cytosines create specific patterns for different tissue types and disease states, and variable methylation positions, which are abbreviated as MPVs, are considered as epigenetic markers. Specific methylation changes may affect the process of response to different treatments in cancer, and the use of biomarkers to predict response to treatment is envisioned for them (Brancaccio *et al.*, 2020).

Changes and fluctuations between *CDX1* and *CDX2* genes are very significant and important in the carcinogenesis of different types of cancer, especially gastric cancer. In this regard, these genes with the origin of cancer stem cells in the Sonic Hitchcock (SHH) molecular pathway showed their effect in all types of gastrointestinal cancers, especially gastric cancer, and changes in the expression of these genes cause mutations and fluctuations in the cell signaling pathway and ultimately carcinogenesis (Fujii *et al.*, 2012; Soon *et al.*, 2011). Also, the association of *Helicobacter pylori* infection and the activity of these genes is important in the process of cancer progression.

Better understanding of the mechanisms involved in gastric cancer tumorigenesis can promote and advance treatment choices in groups with poor prognosis or prognosis of gastric cancer as well as its aggressive types. In this study, the expression of *CDX1* and *CDX2* genes and the prevalence of epigenetic silencing of these genes with promoter methylation in Chinese patients with gastric cancer were investigated in comparison with a control group in gastric tissue.

MATERIALS AND METHODS

Sampling

In this study, sampling was done from tumor tissue and normal tissue adjacent to the tumor in patients with gastric cancer and healthy gastric tissue samples from people who did not have any cancer and gastric diseases in themselves and their first-degree relatives. All subjects with gastric cancer after pathology confirmation were used

in this study. Data were collected from patients admitted to Wuhan First Hospital from January 2021 to January 2023. The samples of patients who were undergoing radiotherapy, chemotherapy or immunotherapy were not used in this study. Required paraclinical information such as age, tumor size, hormone receptor status, tumor grade status, and disease stage were obtained, which were used in secondary analyses. The disease stages were divided into four stages 1, 2, 3 and 4. In this study, 133 samples, including 61 tumor tissue samples, 61 normal tissue samples adjacent to the tumor, and 11 normal gastric tissue samples were examined. In terms of ethics, all sampling procedures were carried out in accordance with the ethics committee rules of the National Institute of Genetic Engineering and in accordance with the Helsinki rules. Consent was obtained from all the patients and people in the control group to use their biological samples in the research project without mentioning their names and no fees were imposed on the patients for the tests.

RNA extraction and cDNA synthesis

Total RNA was extracted from 100 mg of tissue using Trizol solution (Trizol Invitrogen Carlsbad USA) according to the relevant company's protocol. The purity and concentration of RNA were measured using a Nano drop device (Nano drop Spectrophotometr Bio-Tek-USA), and integrity was checked with 1% agarose gel electrophoresis. RNA was stored at -80°C until cDNA synthesis. cDNA synthesis from total mRNA was performed using Fermentazo's kit according to the company's protocol and at temperatures of 25°C for 5 min, 42°C for 60 min, and 70°C for 5 min. Concentration and purity were measured by Nano drop device based on the mentioned description. All cDNAs were stored at 20°C.

Quantitative real time PCR

The expression levels of *CDX1* and *CDX2* genes were measured by qRT-PCR using a thermocycler device (BIO-RAD USA). Reactions for these genes were performed using the TAKARA kit. The reactions were performed according to the company's protocol in a volume of 20 µL including 10µL sybrgreen0, 7µL nuclease free water, 1µL cDNA, 1µL primer F, and 1µL primer R. In order to determine efficiency, Real-Time_PCR reaction was performed with dilutions of 1, 0.1, 0.01, and 0.001 primers, and the slope of the standard curve that expressed the efficiency of the primers was obtained. Primer design was done by alleleID-7 software.

DNA sequencing

In order to confirm the identity of the PCR fragments, the region containing each band was cut from the gel, and

each band was sequenced by Bioneer, South Korea, and compared with the sequence in the Gene Bank, which 100% similarity for *CDX1* and 99% similarity for *CDX2* confirm the result.

DNA extraction and treatment by sodium bisulfite

In order to study the promoter methylation of *CDX1* and *CDX2* genes, genomic DNA was extracted from the tissue of the patients and healthy people. After extracting the DNA samples, the quantity and quality of the extracted DNAs were analyzed using the optical spectrum absorption assay using the Nanodrop spectrophotometer, and then the DNAs with desired quality were subjected to bisulfite treatment using the EpiTect Bisulfite kit produced by Qiagen, Germany. Treatment of the target DNA with sodium bisulfite leads to the conversion of unmethylated cytosines to uracil. Meanwhile, methylated cytosines remain unchanged. This change obtained after the treatment makes it possible to examine the different pattern of two methylated and non-methylated forms.

Designing suitable primers for conducting PCR specific to methylation study

In the design of MS-PCR primers, the sequence of genomic DNA treated with sodium bisulfite is considered as a target sequence. After DNA treatment with sodium bisulfite, all cytosines, except for the cytosines that are in the form of CpG and methylated, are converted to uracil and finally to thymine. Therefore, to design a specific primer for this type of PCR, first, the sequence of the desired promoter region was determined and the primer was designed by considering the type of bases after bisulfite treatment. After receiving the desired sequence to create game changes resulting from sodium bisulfite treatment, Meth Primer Software was used to determine the best primers in the regions rich in cytosine and guanine (CpG island) bases related to the promoters.

Methylation specific PCR (MS-PCR)

In the MS-PCR method, bisulfite-treated DNA was used as a template and two specific methylated and unmethylated primers for *CDX1* and *CDX2* genes. The MSP reaction was performed in a final volume of 10 microliters using the Qiagen EpiTect MSP methylation master mix. For the positive control of methylated and non-methylated samples, PCR Kit Methylation Specific controls of Qiagen company were used. The PCR reaction was carried out in 40 cycles with an initial 5 min incubation at 95°C, repeating a temperature cycle of 30 sec at 95°C, 30 sec at 55°C, and then extension at 72°C for 30 sec and finally the final extension for 10 min 72 °C. After the MS-PCR reaction, the product was electrophoresed on a 1.5%

agarose gel. In this method, a separate PCR was performed for each methylated and non-methylated primer pair.

Statistical analysis

Statistical evaluation and measurement of data was done using SPSS-16 software. P-value less than 0.05 was accepted as an acceptable level of significance. The Kolmogorov-Smirnov test was used to measure whether the data has a normal or non-normal distribution. Then, due to the non-normality of the data, non-parametric Mann-Whitney tests were used for comparison between two groups and Kruskal-Wallis method for significant comparison between several groups. Chi-square method was used to statistically analyze the data related to the promoter methylation status of *CDX1* and *CDX2* genes in the experimental and control groups as a frequency percentage.

RESULTS

133 gastric cancer patients took part in the study. The gender distribution was 30 female and 42 male patients aged between 25 and 90 years, with a M(SD) age of 57.89(12.4) years (Table I). Tables I and II show demographic and pathologic-related variables of the patients who included in the study.

Table I. Description of demographic characteristics.

	Controls (n=11)		Cases (n=61)	
Mean age ± SD	57.66±12.3		58.32±12.5	
Age group	Men (%)	Women (%)	Men (%)	Women (%)
25-45	0(00.0)	1(9.1)	7(11.5)	3(4.8)
46-60	2(18.2)	1(9.1)	10(16.4)	14(23.0)
61-75	3(27.2)	2(18.2)	13(21.3)	7(11.5)
76-90	2(18.2)	0(00.0)	5(8.2)	2(3.3)
Total	7(63.6)	4(36.4)	35(57.4)	26(42.6)

Table II. Pathobiological features of study patients.

Pathobiological criteria	Cases (n=61)	
Cancer stage	Stage 1	3(4.9)
	Stage 2	15(24.6)
	Stage 3	22(36.1)
	Stage 4	18(29.5)
Perineural invasion	Present	42(68.9)
	Absent	19(31.1)
Lymph vascular invasion	Present	53(86.9)
	Absent	8(13.1)

Table III. Comparison of gene expression between groups.

Gene	Normal tissue control	Normal tissue adjacent to tumor	Tumor tissue	P value
<i>CDX1</i>	1	1±0.25	0.5±1.41	0.035
<i>CDX2</i>	1	1±0.34	2.1±1.07	0.029

The average expression of *CDX1* and *CDX2* genes show a significant difference in tumor tissue samples from patients with gastric cancer compared to normal tissue samples adjacent to the tumor and the normal control group that include gastric tissue samples from people not suffering from gastric cancer and any gastric disorder and malignancy in themselves and their first degree relatives (Table III). The comparison results of *CDX1* and *CDX2* genes expression in different stages of gastric cancer shows that with the disease stage increase, the average expression of *CDX1* and *CDX2* genes shows a significant decrease and increase, respectively (Table IV). Furthermore, changes in the expression of *CDX1* and *CDX2* genes show a significant relationship with lymph vascular and perineural invasions.

Table IV. Comparison of gene expression in different stages of gastric cancer.

Gene	Stage 1	Stage 2	Stage 3	Stage 4	P-value
<i>CDX1</i>	2±0.47	1.7±0.64	1.2±0.83	0.4±0.94	0.043
<i>CDX2</i>	2±0.39	2.4±0.67	2.7±0.92	3.7±0.1.33	0.002

The comparison results of the promoter methylation of *CDX1* and *CDX2* genes in the study groups, tumor, normal adjacent to tumor and normal control, are shown in Table V. The data obtained from this research show that about 74% of tumor samples have methylation in the promoter

region of *CDX1* gene, while only 8.2% of normal samples adjacent to the tumor have methylation in the promoter region of *CDX1* gene, and none of the tissue samples of the normal control group showed a promoter methylation of this gene ($P>0.05$). The results also show for *CDX2* that about 77% of tumor samples have methylation in the promoter region of *CDX2* gene, while only 3.3% of normal samples adjacent to the tumor have methylation in the promoter region of *CDX2* gene, and none of the tissue samples of the normal control group showed a promoter methylation of this gene ($P>0.05$).

DISCUSSION

Considering that gastric cancer is a multifactorial disease, investigating the factors involved in it can be complex and different and show significant results. On the other hand, gastric cancer occurs through many genetic disorders that include oncogenes, tumor suppressor genes and DNA repairs genes (mismatch repairs). Molecular studies show the existence of certain carcinogenic pathways for gastric cancer, in this regard, DNA methylation is one of the most important epigenetic changes in the development of gastric cancer, and identifying the signaling mechanism and methylation of genes that are present in the occurrence of gastric cancer (epigenetic alteration) are very important. So that it can be effective even in formulating treatment strategies. It is noteworthy that these events are observed in the early stages of carcinogenesis and have a direct relationship with the severity and increase of the disease. Hypermethylation of tumor suppressor genes also causes cancer, and these events are mostly observed in CPG parts and changes the chromatin structure and causes the transcription of these genes to be turned off. Therefore, simultaneous examination of the expression of genes involved in carcinogenesis and comparing their function with DNA methylation can achieve significant results.

Table V. Classification of the promoter methylation status of gene based on types of tumor and normal samples.

Gene/sample type	N	Methylated promoter	Unmethylated promoter	Both promoters	P value
<i>CDX1</i>					
Tumor tissue	61	45(73.8%)	12(19.7%)	4(6.5%)	0.089
Normal tissue adjacent to tumor	61	5(8.2%)	14(22.9%)	42(68.9%)	0.144
Normal tissue control	11	0(00.0%)	11(100.0%)	0(00.0%)	0.213
<i>CDX2</i>					
Tumor tissue	61	47(77.0%)	11(18.0%)	3(5.0%)	0.331
Normal tissue adjacent to tumor	61	2(3.3%)	12(19.7%)	47(77.0%)	0.412
Normal tissue control	11	0(00.0%)	11(100.0%)	0(00.0%)	0.172

Examining the expression of different genes and finding the relationship between their expression and different histopathological characteristics such as different stages of disease and the clinical conditions of patient is very importance because it can lead to candidate biomarkers with different values for diagnosis, prediction and prognosis of the disease. For this purpose and due to the importance of *CDX1* and *CDX2* genes in various cancers including gastric cancer, the expression of these genes in different histopathological groups of Chinese patients with gastric cancer was investigated in the present study. Also, the methylation of the promoter region of these genes was investigated as an effective epigenetic factor in changing gene expression.

Review studies show that expression disorders in *CDX1* and *CDX2* genes have been reported in many types of cancer, including colorectal cancers (Kim *et al.*, 2005; Guo *et al.*, 2004; Lu *et al.*, 2008), gastric cancer (Gharakhyli *et al.*, 2023; Nakayama *et al.*, 2018; Bornschein *et al.*, 2013), and breast cancer (Adli *et al.*, 2019). In the present study, the average expression of *CDX1* gene in tumor tissue compared to the normal tissue adjacent to the tumor and the normal control tissue showed a significant decrease in expression, while the average expression of the *CDX2* gene in the tumor tissue compared to the normal tissue adjacent to the tumor and the control normal tissue showed a significant increase in expression. This difference in expression level is interpreted based on the clinicopathological status of the patients in such a way that the highest level of expression in *CDX1* and *CDX2* genes is observed in stages one and four of the disease, respectively, and the lowest level of expression in these genes is observed in stages four and one of the diseases, respectively. A possible interpretation of this is that *CDX1* and *CDX2* genes play a role as tumor suppressor genes (Hryniuk *et al.*, 2014; Bonhomme *et al.*, 2003).

The studies conducted confirm that many and various factors are involved in the occurrence of gastric cancer, among which we can mention family history, nutrition, age, and epigenetic changes such as DNA methylation. The study of the promoter methylation pattern of *CDX1* and *CDX2* genes in the present research reported the frequency of the methylation pattern in the tissue samples of patients with gastric cancer to be more than 73.8%, which indicates the prevalence of the methylation pattern in the promoter of this gene during gastric cancer. But since there is no significant difference in the promoter methylation results of *CDX1* and *CDX2* genes in tumor tissue compared to normal tissues adjacent to the tumor and normal controls, the role of epigenetics in controlling these genes in gastric cancer cannot be mentioned alone. Examining the methylation

pattern of these genes in the normal tissue adjacent to the tumor compared to the non-diseased gastric tissue showed that the normal tissue adjacent to the tumor showed non-significantly a double methylated and unmethylated pattern in more than 68.9% for *CDX1* and 77% for *CDX2* ($P>0.05$). Meanwhile, the normal tissue of the gastric has a dominant pattern of unmethylated. With this result, we cannot say for sure that the normal tissues adjacent to the tumor area are somehow affected by the signals received from the tumor cells and epigenetic changes are created in them.

CONCLUSION

The results showed that methylation of *CDX1* and *CDX2* genes as an epigenetic phenomenon may be effective along with other factors in Chinese gastric cancer patients, which can be further investigated. The role of methylation of *CDX1* and *CDX2* genes in the etiology of patients may be considered as a possible prognostic factor, whose identification will be effective in the diagnosis and monitoring of gastric cancer treatment in the pre-invasive period to metastasis. Therefore, future studies in more samples and follow-up of patients condition are necessary to evaluate the usefulness of these prognostic biomarker candidates.

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IRB approval

This study was approved by the Advanced Studies Research Board of Wuhan First Hospital, Wuhan, Hubei430030, China.

Ethical approval

The study was carried out in compliance with guidelines issued by ethical review board committee of Wuhan First Hospital, China. The official letter would be available on fair request to corresponding author.

Statement of conflict of interest

The authors have declared no conflict of interest.

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