RESEARCH ARTICLE

Epigenetic changes of *CDX2* in gastric adenocarcinoma

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Abstract Gastric cancer is one of the most commonplace and lethal cancers in the world. Molecular investigation of this disease, in order to obtain diagnostic and treatments achievements is important and vital. Relatively, in this research study, one of the most important epigenetic factors, the methylation of *CDX2* gene was investigated in tumoral and non-tumoral tissues of gastric cancer patients by bisulfite treatment followed by sequencing of the 5'UTR region of *CDX2* in both tissues. The results indicated a hypomethylation in tumoral tissues of adenocarcinoma. Consequently, the methylation amount of *CDX2* in tumoral tissues was significantly reduced compared with non-tumoral tissues.

Keywords Methylation · Gastric cancer · UTR · *CDX2*

Introduction

Gastric cancer is the forth common kind of cancer globally and it is classified into intestinal and diffuse type (Akhavan-

Highlights

- Fluctuation of epigenetic modifications of *CDX2* regulatory region was observed in gastric adenocarcinoma.
- *CDX2* becomes hypomethylated in gastric cancer.
- Specific CpG positions of 5'UTR of *CDX2* are prone to hypomethylation.

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Niaki and Samadani 2014; Nikbakhsh et al. 2016). It is confirmed that gastric cancer is the result of epigenetic and genetic fluctuations in oncogenes, tumor suppressor genes, DNA mismatch repair genes and regulators of cell cycle. In this account, DNA methylation represents one of the most important epigenetic factors. It can alter the activity of the DNA, specially leading to suppression or reduction of gene transcription. Correspondingly, DNA methylation is vital and indispensable for normal development and is related to many required functions including carcinogenesis, cell division and differentiation processes, X-chromosome inactivation and genomic imprinting (Akhavan-Niaki and Samadani 2013; Calcagno et al. 2013). Considerably, DNA methylation is divided into hyper and hypo methylation. In this way, hypermethylation usually happens at CpG islands in the promoter region and is directly related with gene function (Zhao and Bu 2012). Aberrant DNA methylation models including hypermethylation and hypomethylation are related in the progression and development of all kinds of cancers including gastric cancer, by different mechanisms (Hur et al. 2011; Niwa et al. 2010; Shin et al. 2010; Wani et al. 2012). In this account, aberrant methylation in CpG islands of tumor suppressor genes is a very impressive mechanism discovered in gastric cancer (Shi et al. 2012; Schneider et al. 2010). Considerably, there are many genes that are involved in gastric cancer and have a conspicuous relationship with DNA methylation (Esteller et al. 2000; Samadani and Akhavan-Niaki 2015; Tycko 2010). One of these genes is *CDX2*. It is a member of the caudal associated homeobox transcription factor gene family. This gene has many roles particularly in differentiation and cell growth (Barros et al. 2012; Camilo et al. 2012). It also plays a key role in early embryonic development of the intestinal tract. Aberrant expression of this gene is directly related with tumorigenesis and intestinal inflammation and disorders in digestive system

(Qin et al. 2012; Rugge et al. 2004). Conclusively, researches on molecular mechanisms of these epigenetic factors can lead to novel achievements especially in diagnosis and treatment. The aim of this study was the discovery of molecular fluctuations of *CDX2* methylation in gastric tumors.

Material and methods

Samples collection

The samples were gathered from patients who had gastric cancer with adenocarcinoma tissues (stages 1 to 4 and ages between 36 to 89 years, male and female) after gaining patients relative's agreement from hospitals of Babol city in Iran. Relatively, the tumoral and non-tumoral (normal) tissues were provided from surgery room for each patient. For confirmation of the kind of the tissues, all specimens were controlled with pathological reports. Eventually, tissue samples were stored at -80°C for further analyzes.

DNA extraction

DNA preparation was accomplished with DNA extraction Kit (Bioneer – Accu plep, South Korea) according to the manufacturer's instructions. The evaluation of quantity and quality of extracted DNA was performed by agarose gel electrophoresis and spectrophotometric measurement (NanoDrop® ND-1000, Thermo Fisher Scientific, USA) at 260 nm.

DNA methylation evaluation

For investigation of DNA methylation, initially, bioinformatics software (Meth primer) was recruited to find GC rich regions of *CDX2* gene. Specific primers (Forward: 5'GCCATYTTTTAAAATGYAAATTATGTTT3', Reverse: 5'AAACCTCACCATRCTRCT3') were designed for amplification of 5'UTR region of *CDX2* gene by PCR method. The methylation kit (Zymoresearch, EZ DNA methylation – Gold TM kit, USA) was used according to the manufacturer's

instruction for bisulfite conversion of 20 μl genomic DNA. (15–30 ng/ μl). 3–4 μl bisulfite treated genomic DNA was amplified in a 25 μl final volume using forward and reverse primers by initial incubation at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s and 57°C for 50 s and 72°C for 50 s and a final extension at 72°C for 14 min. Relatively, for investigation of methylation pattern of amplicons, confirmation and identifying of different methylated nucleotides, all PCR products were sequenced in a ABI set (Bioneer, South Korea). The acquired sequencing data were analyzed in tumoral and non-tumoral tissues by QUMA (QUantification tool for Methylation Analysis) a web-based bisulfite sequencing analysis tool for CpG methylation analysis available at <http://quma.cdb.riken.jp/>.

Statistical analysis

Data were analyzed using the ANOVAs test and SPSS software. *P* value <0.05 was considered as statistically significant.

Results

The sequence of *CDX2* gene spanning from nucleotide -39 to $+291$ (NCBI code: accession, EU544225) show all investigated CpG sites (Fig. 1). The methylation situation of the 27 CpG sites of 5'UTR of *CDX2* gene in tumoral (Fig. 2) and non-tumoral (Fig. 3) tissue samples of 4 gastric cancer patients were investigated and analyzed. Relatively, some sections like CpG 1 and 3 (nucleotides 1 and 22) became methylated in tumoral in comparison to non-tumoral tissues. Remarkably, CpG sites number 3 (nucleotide 22) and 20 (nucleotide 266) showed significant increase of methylation status with *P* values of 0.04 and 0.00047, respectively. Other CpG positions showed lower or no methylation pattern in tumoral tissues in comparison to their normal counterparts. Figure 4 represents sequencing data corresponding to a tumoral and a non-tumoral tissues.

Fig. 1 The 5'UTR sequence of *CDX2* gene. Sequences of 336 nucleotides spanning from position -39 to $+291$ of *CDX2*. 27 investigated CpG positions are shown in yellow highlight

CGAGGGGTTGTGCGTAGAGTGCGCGCTGCGCCTCGACGTCTCAACCATTGGTGTCTGTG
TCATTACTAATAGAGTCTTGTAACACTCGTTAATCAGGAAGGC CGCGGCCTGGGGCT
CGGCA CGCCAGCCTGTGGCGGGTCTTCCC CGCCTCTGCAGCCTAGTGGGAAGGAGGTGG
GAGGAAAGAAGGAAGAAAGGGAGGGAGGGAGGAGGCAGGCCAGAGGGAGGGACCGCC
TCGGAGGCAGAAGAGCGCGAGGAGCCAGCGGAGCACCGCGGGCTGGGGCGCAGCCAC
CGGCGCTCCTCGAGTCCCTCGCCCTTTCCCTTC

Fig. 2 The methylation's fluctuations in tumoral tissues. Panel **a** represents the 27 CpG sites analyzed from left (position 1, nucleotide 1) to right (position 27, nucleotide 316). Each row represents the methylation status of one patient with ● representing a methylated site and ○ representing an unmethylated site. Panel **b** represents the

percentage of methylated (*upper black columns*) and unmethylated (*lower white columns*) cases for each CpG position, and panel **c** represents the relative amount of methylation for each CpG position. These panels (**a**, **b** and **c**) show the reduced amounts of methylation in tumoral tissues

Fig. 3 The methylation's fluctuations in non-tumoral tissues. Panel **a** represents the 27 CpG sites analyzed from left (position 1, nucleotide 1) to right (position 27, nucleotide 316). Each row represents the methylation status of one patient with ● representing a methylated site and ○ representing an unmethylated site. Panel **b** represents the

percentage of methylated (*black columns*) and unmethylated (*white columns*) cases for each CpG position, and panel **c** represents the relative amount of methylation for each CpG position. These diagrams (**a**, **b** and **c**) show the increased amounts of methylation in non-tumoral tissues

Fig. 4 Representative electrophoregrams of *CDX2* methylation pattern. These electrophoregrams represent the sequence of nucleotides +175 to +291 of *CDX2* corresponding to (a): a non-tumoral tissue and (b): a tumoral tissue examined after bisulfite conversion of genomic DNA and sequencing. All cytosines are converted to thymine while methylated cytosines remain unchanged and are read as cytosine. Red arrow indicates unmethylated CpG and blue arrow indicates methylated CpG

Discussion

Gastric cancer is a serious disease which is driven by progressive genetic and epigenetic

origination. The key role of DNA methylation in the pathogenesis of gastric cancer is proven and remarkable. This epigenetic modification collaborates also with gene mutations in the development of normal gastric mucosa. Remarkably, these modifications in DNA methylation help to the molecular heterogeneity changes of gastric cancer. Calculating and counting the amount of methylated and unmethylated parts of DNA is of particular importance on the diagnosis and prevention in all kinds of cancers especially in gastric one. Obviously, it is confirmed that the section of active DNA demethylation depends on the function of the cell programming (19). Relatively, previous studies and searches indicated that *CDX2* gene had transcriptional function in *CDX2*-expressing gastric cancer (Camilo et al. 2014). Correspondingly, it was shown that the expression of *CDX2* was silenced by unknown repressing pathway(s) other than DNA methylation. At least two CpG islands exist in the promoter and 5'UTR of the *CDX2* gene, with the methylation status of the lower site being related with expression of *CDX2* (Kang et al. 2011). In this way, the alterations of *CDX2* gene in tumoral and non tumoral tissues had different effect and the situation of promoter is involved in this process. Conspicuously, in the present study, the tumoral and non-tumoral tissues indicated to have both methylated and unmethylated modifications of *CDX2* in gastric cancer patients. It is confirmed that the methylation is not a dominant process in carcinogenesis (Ebrahimi-Askari et al. 2015). In a comparison of methylation fluctuation between *P15* gene in esophagus cancer and *CDX2* in our research, other mechanisms like loss of heterozygosity (LOH) can have a more important role in inactivation of these genes (Rotkrue et al. 2011). Transparently, the amount of methylation of *P15* gene in tumoral tissues was 16.6% and it is approved that the methylation has not a significant correlation in esophagus cancer (Rotkrue et al. 2011). Similarly, the methylation amount of *CDX2* was different and some sections became methylated in tumoral in comparison to non-tumoral tissues. The amount of *P* value of CpG sites number 3 and 20 of the 5'UTR was 0.04 and 0.00047, respectively. Thus the present data

strongly suggest methylation fluctuations of *CDX2* in tumoral tissues of adenocarcinoma. Therefore, the methylation amount of *CDX2* in tumoral tissues is significantly reduced compared to non-tumoral tissues.

Conclusively, investigating the molecular mechanisms of cancer development and identifying critical regions exposed to methylation fluctuations will permit a better understanding of the role of epigenetic modifications in all types of cancer particularly in gastric cancer and will lead to therapeutic and preventive approaches.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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