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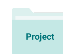

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https://doi.org/10.1007/s12253-019-00594-4

ORIGINAL ARTICLE

CDX1/2 and *KLF5* Expression and Epigenetic Modulation of Sonic Hedgehog Signaling in Gastric Adenocarcinoma

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Received: 30 June 2018 / Accepted: 15 January 2019
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Abstract

Gastric cancer is among the commonplace causes of cancer death worldwide. Sonic hedgehog (Shh) signaling is an important pathway which may be dysregulated in many cancers.*CDX1*/*2*, and *KLF5*are key transcription factors involved in Shh pathway and cancer stem cells. The aim of this study was to investigate the expression and epigenetic alterations of these genes in gastric cancer patients. DNA methylation's modifications of *CDX1*, *KLF5* and *CDX2* genes alongside with the expressions of these genes in gastric cancer tissues and their non-tumoral counterparts (margin tissues) were analyzed using methylation specific sequencing, and Real time PCR Taq man assays, respectively. The expression of *CDX1* ($P = 0.002$) and *KLF5* ($P = 0.010$) were decreased significantly, but it was considerably increased for *CDX2* ($P = 0.001$). Relatively, the results for the regulatory region methylation status of each CpG site had shown a notable fluctuation in these genes with no significant difference in most places. The creation of metastatic lymph nodes in patients was significantly associated with increased expression of *CDX2* gene. The modifications of these genes expression can be considered as a cancer biomarker in future studies. Methylation of the investigated genes is not the main mechanism of gastric cancer development.

Keywords Gastric cancer · DNA methylation · Gene expression · Epigenetics

Ali Akbar Samadani and Novin Nikbakhsh contributed equally to this work.

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Published online: 26 January 2019

Background

Gastric cancer is one of the most major causes of cancer related deaths worldwide. Discriminately, it is divided into two major types: intestinal metapalasia, and diffuse [1, 2].

Molecular researches on this hazardous disease in order to distinguish important genes involved in this disorder can be influencive and impressive, especially in prediagnosis or identification of its molecular mechanisms.

In this way, one of the effective factors that have been considered during the last decade is the function of cancer stem cells. Cancer stem cells are believed to have a close interaction with normal niche, which play a crucial role in gastric cancer development [3]. Cancer stem cells potentially have the ability of tumor initiation and are implicated in tumorogenesis [3]. Correspondingly, cancer stem cells may be derived and originated from gastric stem cells in gastric tissues or other tissues like bone marrow and mesenchymal stem cells [4, 5].

The most important and noteworthy genes that are detected in some main cancer stem cells include*: EGF, BMP, CDXI/2, SALL4, OCT3/4, WNTs, NOTCH, NANOG, SOX,KLF4/5, KLF1* and *FEM5* [6]. Considerably, one of the most important molecular pathways which may be implicated in cancer is sonic hedgehog signaling. In the adult digestive system, parietal cells in the stomach body express the sonic hedgehog protein and mRNA [6]. So, many human transcription factors like caudal type homeobox 1/2(*CDX1/2*), and kruppel like factor 5 (*KLF5*) are involved in this pathway. *CDX1* can have a significant role in final differentiation of intestine and also its expression in gastric tissue can lead to gastric cancer and stimulation of specialized genes such as Furin and Mucin [7, 8]. *CDX2* gene is a member of homeobox transcriptional factor. This gene has an impressive role in primary embryonic stage [9]. The expression of *CDX2* is reported in more than 85% of acute leukemia patients [9]. This gene also has a notable role in damaging to esophagus [10]. *KLF5* is a subfamily of zinc finger proteins. Hence, it is located in nucleus and connected to epidermal growth factor [11]. This transcriptional factor binds to GC boxes in the promoter region and activates the transcription of the corresponding gene [11]. *KLF5* has a noticeable relationship and correlation in making the embryonic stem cells and it controls and leads the regulation of cell proliferation in epithelium of intestine [12]. During the multi process of gastric cancer development which consists of atrophic gastritis, metaplasia, dysplasia, and cancer occurrence, respectively, *CDX2* gene plays a key role in the reprogramming of intestinal dysplasia [13]. Both *Cdx1* and *Cdx2* were able to induce intestinal metaplasia in transgenic mice [14]. *CDX1* can reprogram gastric epithelial cells into intestinal epithelial cells through induction of *KLF5* gene. This marker of stem cells can cause the expression of many downstream genes that are specific of stem cells as a gene regulator and prevent from the differentiation of these cells [15].

Correspondingly, the direct correlation of epigenetic factors like DNA methylation is very remarkable in carcinogenesis. DNA methylation is a commonplace trait in vertebrates and is an important epigenetic mechanism in controlling gene expression which is divided into hyper and hypo methylation [16]. Many studies have been performed on fluctuations of DNA methylation in stem cells and also the role of this alteration in cancer development [17]. So, the investigation of gene expression in conjunction with DNA methylation in order to find their dependency can be important and significant. The aim of this study was to investigate the expression pattern of *CDX1, KLF5* and *CDX2* genes and their methylation modifications in adenocarcinoma and non-tumoral (margin) tissues in human's stomach.

Materials and Methods

Samples Collection

The research was performed on 30 people (23 male and 7 female) diagnosed with stomach cancer with different stages and grades and who have had a surgical operation. Details about histopathological status of patients were reported previously. After obtaining patients' relatives consent, 30 tumoral and 30 non-tumoral (margin tissue surrounding the tumor) tissues were prepared directly in surgery room for each patient by deep freezing in liquid nitrogen. Ultimately, tissue samples were stored at −80 °C for further analyzes.

Nucleic Acids Extraction

The RNA extraction was accomplished using Trizol (R Ambion, USA) after crushing and grinding the tissues with liquid nitrogen as described previously. Relatively, the DNA extraction was performed with DNA extraction kit (Bioneer, South Korea) according to the manufacturer's instructions. The quality and quantity assessment of the extracted nucleic acids was accomplished by agarose gel electrophoresis and spectrophotemetric measurement with NanoDrop® ND-1000 (Thermo Fisher Scientific, USA).

Gene Expression Analysis

cDNA preparation was accomplished with cDNA Kit (Quanti Test Reverse transcription kit, Qiagen),using approximately 2 pg RNA for each reaction. The primers were designed by Allel ID version 6 software. The first cDNA strand was synthesized using a stem- loop sequence specific primer according to Fattahi et al. [18, 19]. The sequences of forward and

reverse primers as well as the universal probe used are given in Table 1.

The real time PCR tests were performed in a Step one instrument (Applied Biosystem, USA) using cDNA by Taqman method in the presence of a universal probe. An amount of 1 μl cDNA from each sample was subjected to amplification. *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) was used as a housekeeping gene. Amplification took place in a 20 μl final volume by initial incubation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 1 min. The range of up regulation or down regulation in each sample was investigated carefully using the $-2^{\blacktriangle\blacktriangle ct}$ method. All reactions were performed as triplicate.

DNA Methylation Analysis

For investigating DNA methylationof regions such as promoter, 5'UTR and CpG islands, Meth Primer software was used to find GC rich regions of *CDX1, KLF5 and CDX2* genes. Specific primers (Table 2) were designed for amplification. The Gold TM methylation kit (Zymo research, USA) was employed alongside with sequencing of amplicons corresponding to select the CpG rich regions, as described previously. Table 2 shows the sequences of specific primers used to amplify DNA after bisulfite treatment. Sequencing was performed in an ABI sequencer (Bioneer, South Korea). The obtained sequencing data corresponding to tumoral and non-tumoral tissues were analyzed by QUMA (Quantification tool for Methylation Analysis web-based bisulfite sequencing analysis tool available at http://quma.cdb.riken.jp/ and used for CpG methylation analysis.

Statistical Analyzes

Data were analyzed using the ANOVA test and SPSS software, 2010. *P* values <0.05 were considered as statistically significant.

Table 2 The sequences of primers used for amplification of methylated DNAs

Reverse CDX1	5′ AAGCCTCCGRRCCGCGAATCA3′
Forward CDX1	5′ GGAAGACTCGTGTATGTATGTGYATATGTG3′
Forward KLF5	5′ GAGTTGGGTGAAATAGAGG3′
Reverse KLF5	5'TCGAATAAACTCCTCARACA3′
Forward CDX2	5′ TAGTTTGYGGGGYTGYTGTA3′
Reverse CDX2	5′ GCCATATACRTAARCTACCTCCT3′

Results

Gene Expression Evaluation in Tumoral Tissues

The analysis of expression levels of tumoral and corresponding non-tumoral tissues for *CDX1, KLF5* and *CDX2* genes indicated that *KLF5* and *CDX1* were down regulated in tumoral tissues in comparison with their non-tumoral counterparts, with more than 70% of samples showing decreased expression (P value for *CDX1* = 0.005 and for *KLF5* = 0.049). On the contrary, the *CDX2* expression level had increased significantly ($P = 0.004$) in more than 70% of samples (Fig. 1).

Association of Genes Expression with Clinicopathological Variables

Clinicopathological consequences of *CDX1*, *CDX2* and *KLF5* genes expression were evaluated in gastric cancer patients. The analysis of different clinicopathological variables and genes expression correlation is presented in Table 3. *CDX2* expression was significantly associated with tumor cell metastasis to lymph nodes ($P = 0.013$). Of 20 patients with lymph node metastasis, 14 (70%) over expressed *CDX2* (mean ± SD: 248.7026 ± 382.8491). Other clinicopathological features did not show an association with examined genes expression.

Table 1 The sequences of primers and probe used for cDNA syntheses and Real time PCR

Reverse *CDX1*	5'GTCGTATCAGTGCTGCGACCGTATGGATGTGTCTGCGGCGTTTTATCATG CACTGGATACGACGACGAC3'.
Froward *CDX1*	5'GGAAGACTCGTGTATGTATGTGYATATGTG3'
Forward *KLF5*	5'CGATTTGGAGAAACGACGCATC3'
Reverse *KLF5*	5'GTCGTATCCAGTGCTGCGACCGTATGGATGTGTCTGCGGGGTTT TATCATGCACTGGATACGACTGTGCAACCAGG'3'
Forward *CDX2*	5'ACCTGTGCGAGTGGATGCG3'
Reverse *CDX2*	5'GTCGTATCCAGTGCTGCGACCGTATGGATGTGTCTGCGGCGT TTTATCATGCACTGGATACGACCTGGTTTTCACTTG3'
Probe	FAM 5′ TGGATGTGTCTGCGGCGTTTTATCAT 3′ BHQ-1

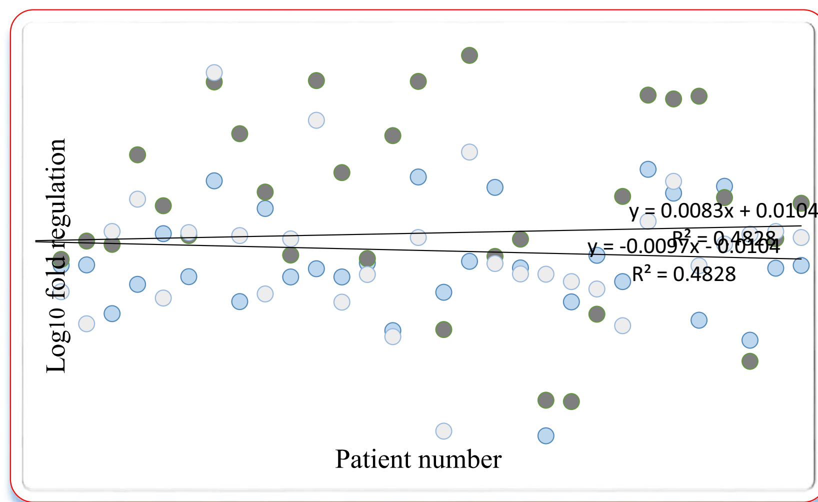

Fig. 1 Scatter plot analyzes of relative expression of *CDX1*, *KLF5* and *CDX2* in gastric cancer patients. The Y axis indicates the logarithm of relative gene expression. Horizontal black lines represent cut-off values logarithms for 2 fold changes in expression. The upper part of the graphs indicates up-regulation in the tumoral compared to the non-tumoral tissue; the lower part of the graph indicates down-regulation in the tumoral compared to the non-tumoral tissue (differences in expression ≥2; $P < 0.05$). ● *KLF5*; ● *CDX2*; ● *CDX1*

DNA Methylation Evaluation

The alteration of methylation of *CDX 1,CDX2* and *KLF5* were notable in tumoral and non-tumoral tissues. CpG positions for each gene are shown in Fig. 2. Remarkably, 29 CpGs located upstream of *CDX1* and in its promoter region, 37 CpGs located in the first exon of *CDX2* and 29 CpGs present in the 5'UTR of *KLF5* were studied. In this way, in *CDX1* gene the tumoral tissues became methylated in comparison with non tumoral tissues. Considerably, the tumoral tissues became unmethylated in comparison with non tumoral tissues in both *CDX2* and *KLF5* genes.

For *KLF5*, in non tumoral tissues, the nucleotides numbers 1, 5 and 22 became methylated. Their methylated amounts were 100%, 37.5%, and 3.4%, respectively; while in tumoral tissues nucleotides numbers 1, 5 showed 94.1% and 14.3% methylation, respectively. Other studied positions were unmethylated (Fig. 3). The *P* value of methylation analysis between tumoral and non tumoral tissues in CpG site corresponding to nucleotide number 1 was 0.01288.

For *CDX1,* intumoral tissues, all examined CpG sites were fully (100%) methylated except CpG sites corresponding to nucleotides number: 298, 405, and 408 which became methylated at 66.7, 36.7, and 46.7%, respectively. For non tumoral tissues, all CpG sites became 100% methylated except CpG sites corresponding to nucleotides number 298, 314, 405, and 408 with 96.7, 96.7, 46.7, and 63.3%, respectively (Fig. 3). The *P* value of CpG site number 28 (nucleotide 405) was 0.115618, and for CpG site number 29 (nucleotide 408) was 0.032821.

In *CDX2* of tumoral tissues, only CpG sites corresponding to nucleotides number 12 and 78 became methylated with 100 and 10.7% amount respectively; while in normal tissues, the nucleotides number 1, 16, 27, and 36 became methylated with different amounts of 100, 24, 22.7, and 17.9%, respectively (Fig. 3). The P value of CpG site number 14 (nucleotide 78) was 0.000645.

Table 3 The Association of genes expression with clinicopathological qualifications. The amount of gene expressions of all samples were compared and investigated with the stage and grade of all patients. There was a significant association in *CDX2* gene expression with tumor cell metastasis to lymph nodes. Statistical analyzes were performed with using SPSS (2014, version 7, New York) and also Chi Square test and T test. L.M: lymph node metastasis; D.M: distant metastasis; ↓/−: decrease or no change of gene expression; ↑: increase of gene expression

	CDX1		P value	CDX2		P value	KLF 5		P value
Tumor stage	↓/−	↑	0.66	↓/−	↑	0.12	↓/−	↑	0.46
1–2	13	4		10	7		13	4	
3–4	10	3		4	9		11	2	
Tumor Grade									
1–2	9	2	0.48	6	5	0.38	8	3	0.37
3–4	14	5		8	11		16	3	
L.M									
Yes	15	5	0.57	6	14	0.013	15	5	0.32
No	8	2		8	2		9	1	
D.M									
Yes	17	4	0.34	11	10	0.12	18	3	0.23
No	6	3		2	7		6	3	

Discussion

DNA methylation is one of the most important epigenetic alterations in creation of gastric cancer. Correspondingly,

A) *KLF5*

TGGGTGAAATAGAGGCGGGCGTCAAGTGTCAGTAGTCGCGGGGCAGGTACGTGCGC

TCGCGGTTCTCTCGCGGAGGTCGGCGGTGGCGGGAGCGGGCTCCGGAGAGCCTGAG

AGCACGGTGGGGCGGGGCGGGAGAAAGTGGCCGCCCGGAGGACGTTGGCGTTTACG

TGTGGAAGAGCGGAAGAGTTTTGCTTTTCGTGCGCGCCTTCGAAAACTGCCTGCCGC

B) *CDX2*

GCCCATGGCTGCGGCCGGGGAGCCACCGTTGAGGCCGTGAGCCACG

GCGTTGGCGGCGGCCGCGGCGCCTCCGGGCGCGTAGCCATTCCAGTCCTC

CCGGAGTGGGGCGCCATACGCTGCCGGCCAGGATGGCCCCGGGGACTGCG

CGCTGTCCAAGTTCGCTGCCGCTGCAGCTGCGGCCGCCACGTGGTAACCG

CCGTAGTCCGGGTACTGCGGGGGGCTGACGAAGTTCTGCGGCGCCAGGTT

GAGGCCGCCAGAGTGGCGCACGGAGCTAGGGTACATGCTCACGTCCTTGT

CC

C) *CDX1*

CATTTCCCCAGGGAAAAACATCCACAGCTTCCATGACGAGAGGGGTCGTGACCCCT

CCCCGCCAAAAGATTAAGGACCTGCGATCCTACAGACCGGAGCCCTGTTTGAAGTCT

GCGTTGCCCCTCACCTCAAGCTGGTCACTGTGTGAAGTTGGCCTAGAATCCCCCGGC

CCCTGGGAGCTTGTTCCTCCGCCTGTAAAATGGGGCTGCAGGGCCGTCCACGCGGCC

ACCGGAAGGACAAGGTGTTCAGGCCGCTAGGCCGCTCCCTGGCAAGCGATTCCCAC

TCGCAGCGCGGCCTCGACCCTCGCCCAAGACGCGCCCTCCGCGCCCCCACCCCCTCC

AGGCCCTGGCCAGTCCACCTCCCGCTTGGGGCGGCAATTTGTCTCCTTTTGAACCCC

CCGCCCCCGACGGGTTTCCCC CTT

Fig. 2 The sequences of CpG islands studied for *KLF5*, *CDX2*, and *CDX1*. The upstream sequence of *CDX1* comprising 421 nucleotides spanning from position −530 to −110 (NCBI code: accession, NG_046970.1) The 5'UTR sequence of *KLF5* comprising 225 nucleotides spanning from position +181 to +405 (NCBI code: accession, NM_001730.4). Sequences of 298 nucleotides of the first exon of CDX2 spanning from c.21 to c.318. (NCBI code: accession, NM_001265.4). Investigated CpG positions are shown in yellow highlight. 37 CpG position were studied for *CDX2*, 29 for each

simultaneous study of expression of genes involved in carcinogenesis pathways and comparing their fluctuation with DNA methylation can obtain considerable results. According to the study of human tumor samples, there is a direct relationship between the down regulation of *KLF5* gene and disease severity or risk of tumor formation [20]. But, the reason for the down regulation of this gene has not been studied yet. *CDX1* gene that belongs to homeobox transcription factor gene family [21] have exhibited its effective role together with *CDX2* in growth of intestinal epithelial tissue cells, and their expression in stomach tissue provokes gastric cancer and also stimulation of intestine function with special genes like Furin and Mucin. It was proved that the DNA methylation and histone modifications had direct relationship with *CDX2* promoters function in colon cancers [22]. DNA sequencing performed upon bisulfite treatment and methylation specific PCR, expressed that *CDX1,2* promoters revealed no methylation in *HT29* cell line, but neither genes were expressed [23]. On the contrary, *CDX1* was methylated in Caco2/Tc7 cell line. Also, regarding *CDX1*, *2* and *KLF5* genes, it became distinct that in gastric cancer cell lines, after inactivating the *KLF5* gene, *CDX1* gene expression has decreased, too. Therefore, it seems that *CDX1* gene expression decreases in stem cells, and also causes transformation of gastric epithelilal cells into stem cells-like as a result of cell reprogramming [24]. Our data revealed a significant upregulation of *CDX2* expression in gastric adenocarcinomas. This finding was in concordance with another study performed on transgenic mice [25].

We found a decrease of *KLF5* expression in the present study. Relatively, in a recent research conducted on mice, *Klf5* knock down was shown to destabilize the maintenance of intestinal barrier function both in vitro and in vivo [26].

Regarding *CDX1*, we found a remarkable down regulation in most tumoral tissues. A research conducted on murine colorectal tumor model in which the expression, activation, and methylation of *Cdx1* gene was studied, indicated that this gene acts as a tumor suppressor [27]. Conspicuously, in another research in transgenic mice animal model, *Cdx1* gene expression in the gastric mucosa induced gastric intestinal metaplasia [14]. In this account, it was shown that *CDX1* causes the

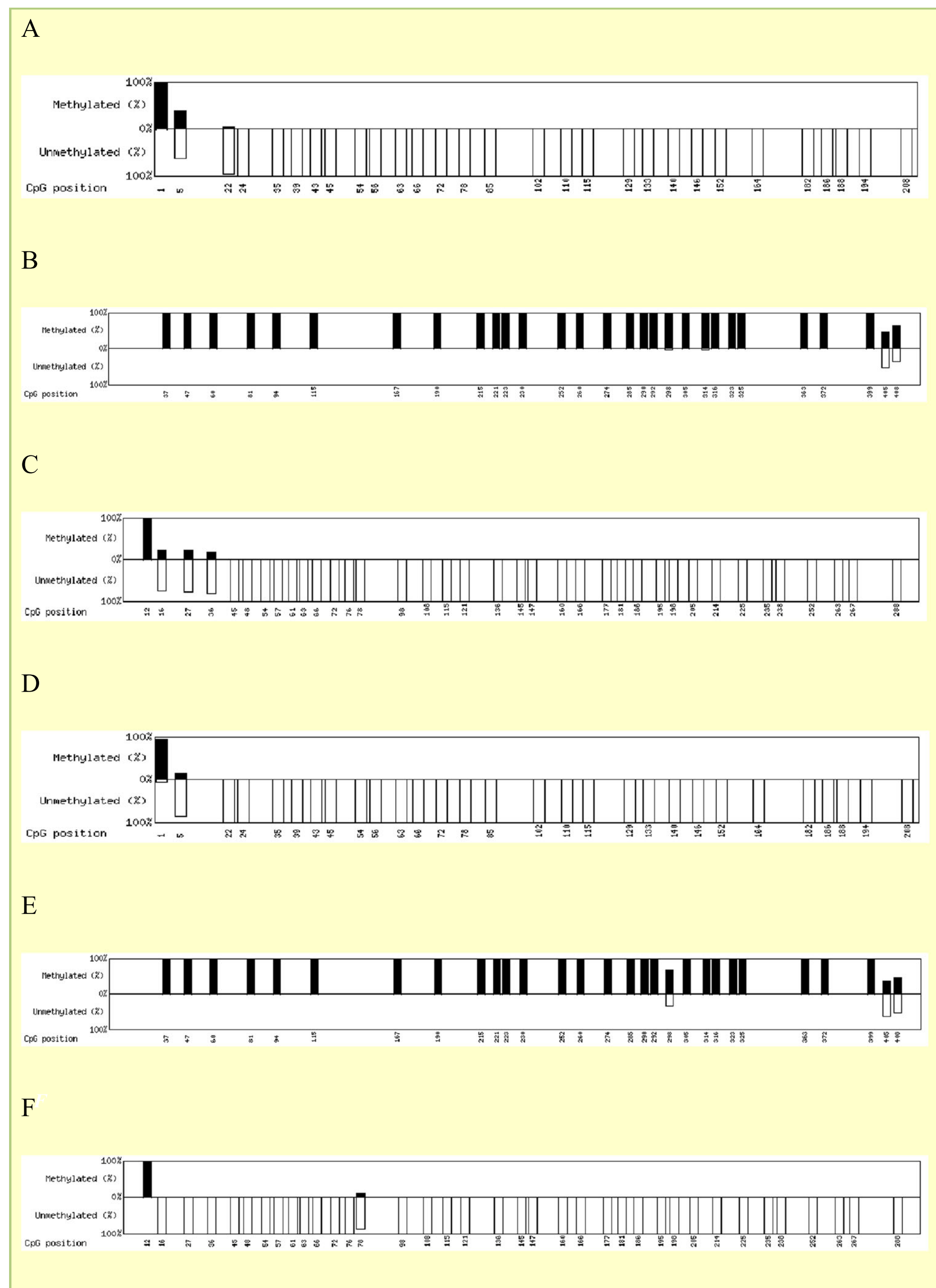

Fig. 3 Methylation status in different investigated positions of *CDX1*, *CDX2* and *KLF5* analyzed by QUMA software. These diagrams show the CpG positions of each gene and their methylation status in both tumoral and non-tumoral tissues. **a**, **b**, **c** methylation of non tumoral tissues at *KLF5*, *CDX1* and *CDX2*, respectively; **d, e, f**: methylation of non tumoral tissues at *KLF5*, *CDX1* and *CDX2*, respectively. The percent of methylated and unmethylated regions are shown as scale bars

increasing of programming factors alongside with stem cells factors like *KLF5* and *SALL4*, and these alterations lead to the development of gastric cancer [7]. These up and down regulation alterations of *CDX1* are perhaps due to the dual nature of this gene.

The results of the current study on *CDX1* gene alterations have suggested that the methylation is not the only direct factor in carcinogenesis. The promoter region of *CDX1* was investigated, and most CpG sites were methylated in both non-tumoral and tumoral tissues. Relatively, the tumoral tissue was methylated about 94.8% and the non-tumoral tissue became methylated about 96.7%. Relatively, a research conducted on colorectal tissue specimen showing a decrease of *CDX1* expression showed that *CDX1* promoter was hypermethylated in those tissues [28]. The position of investigated regions of *CDX2* and *KLF5* genes were the first exon. Similarly, we found no relation between methylation's fluctuations and expression of these genes. Interestingly, in KLF5 about only 2 to 3% of CpG sites were unmethylated, and more than 95% of CpG sites were methylated.

Some histopathologic features of patients had meaningful relationship only with *CDX2* gene expression in our research.

The lymph node metastasis had meaningful relationship with *CDX2* up regulation ($P = 0.013$). 14 out of 20 patients (70% having lymph node metastasis, have shown *CDX2* up regulation.

Zeraati et al. found that *CDX2* expression had a significant correlation with Lauren classification, TNM stage, and lymph node metastasis. Also no correlation of methylation and gene expression became distinct [29]. In concordance with our study, the up regulation and association of increasing expression with lymph node metastasis were observed.

Generally, it can be explained that, cancer has a very complicated mechanism and usually is created from the coordination and corporation of at least 6 mutations or oncogenes in cells functional modifications of some effective gene involved in different signaling pathways like WNT, SHH, Notch and etc. Consequently, it can be illustrated that maybe the main differences between DNA methylation and mutation, may be the most important epigenetic mechanism, cause of reversibility, and high flexibility [30, 31].

Regarding to these conditions, it is likely that lack of DNA methylation cause the genomic instability and activation of oncogenes in carcinogenesis. Against the genetics, epigenetic mechanisms like DNA methylation are expressed with high flexibility and heritability [32]. Moreover, it should be emphasized that the tumoral or non-tumoral tissues are heterogeneous and may represent a mixture of cells at different states of differentiation, adding more complexity to data interpretations. Correspondingly, performing such studies on different established cancerous or non-cancerous gastric cell lines may add precision to the current data.

Interestingly, the implication of components of hedgehog signaling pathways in gastric cancer development, may further facilitate targeted therapy. Correspondingly, several studies demonstrated that small molecules capable to inhibit selectively *KLF5* expression, show anti-proliferative activity in colon cancer or triple negative breast cancer cells [33, 34]. Ultimately, existence of large numbers of methylated sites in different types of cancers and also, identifying the methylation ̓s positions can lead to treatment and preventive approaches in cancer pathobiology. Noticeably, other epigenetic modifications such as microRNAs modulation should be considered in future studies [35].

Conclusion

It was concluded that *CDX1* and *KLF5* expression were decreased, but *CDX2* expression was increased significantly. Likewise, it became distinct that creation of lymph node metastasis had a meaningful relationship with *CDX2* up regulation in patients. There was no difference in most methylation sites studied. The expression modifications of these genes can be considered as a cancer biomarker, and must be taken into consideration in future studies. Also, results suggest that the methylation of studied genes is not the main mechanism of gastric cancer progression.

Acknowledgments This work was supported by the Research deputy of Babol University of Medical Sciences (Grant No 9237527), and a grant by Iranian National Science Foundation (INSF project code: 92017552). Authors appreciate the staffs of Cellular and Molecular Biology Research Center, Rouhani and Shahid Beheshti hospitals, Razi Medical Laboratory, and all patients who participated in this project.

Compliance with Ethical Standards

Institutional Review Board This study was approved by the ethics committee of Babol University of Medical Sciences, Iran.

Informed Consent Written informed consent was obtained for all subjects before their inclusion in the study. All experiments and data comparisons were carried out in compliance with relevant laws and guidelines and in accordance with the ethical standards of the Declaration of Helsinki.

Conflict of Interest The authors declare that they have no conflict of interest.

Abbreviations cDNA, complementary DNA; *CDX1/2*, caudal type homeobox 1/2; GC, gastric cancer; *KLF5*, kruppel like factor 5; PCR, polymerase chain reaction

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