ORIGINAL RESEARCH

CDX2 Protein Expression in Colorectal Cancer and Its Correlation with Clinical and Pathological Characteristics, Prognosis, and Survival Rate of Patients

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Abstract

Purpose Caudal-type homeobox transcription factor 2 (*CDX2*) is expressed in the nucleus of the intestinal epithelial cells and is essential for embryonic formation and differentiation of the intestine, whose reduced expression can result in metastatic tumors. This study was to investigate the association of *CDX2* expression level in colorectal cancer (CRC) with age, gender, microscopic histopathology, tumor staging, tumor grading, 3-year survival rate, and prognosis.

Methods After preparing paraffin tissue blocks, CDX2 protein expression was assayed by immunohistochemistry in 82 CRC patients. Hematoxylin and eosin staining was used to detect tumor histology, tumor grading, tumor staging, and blood-lymphatic, and neural invasion. The collected data includes age, gender, tumor site, and 3-year survival rate of patients after diagnosis.

Results The *CDX2* expression was significantly higher in men than in women, and it was significantly lower in right-sided tumors as in transverse colon and left-sided tumors. Also, the *CDX2* expression was significantly higher in adenocarcinoma than in mucinous. In addition, a significant correlation was found between downregulated *CDX2* and lymph node involvement. In tumor grading, there was a significant correlation between *CDX2* downregulation and high-grade tumor. Moreover, there was a significant correlation between downregulated *CDX2* expression and overall pathological staging.

Conclusion The downregulated *CDX2* expression is associated with female gender, right-sided tumors, mucinous tumors, lymph node involvement, high-grade tumor, and advanced overall pathological staging and can be considered as a possible prognostic factor for patients follow-up. However, our study is a preliminary study and further studies with larger sample sizes in different ethnic groups are required.

Keywords Colorectal cancer · *CDX2* · Immunohistochemistry · Histopathology · Prognosis

Introduction

Colorectal cancer (CRC; OMIM: 612592) is one of the most common malignancies in the world and the second cause of cancer death [1]. According to epidemiologic data, this cancer has been reported to be the fifth most common cancer in Iranian men and the third most common cancer in Iranian women [2]. Various genetic and environmental factors are related to the pathogenesis of CRC, the most important of which are inappropriate diet, wrong lifestyle, and family history. An increase in the daily diet of high-fat and low-fiber foods worldwide has resulted in a dramatic increase in the incidence of CRC [3]. Colorectal cancers are classified according to the histological examination of glandular differentiation into well, moderately, and poorly differentiated types [4]. The onset and the spread of this cancer are transformation processes from normal cells into malignant cells due to mutation in tumor suppressor genes and oncogenes. These alterations enable the cells to be proliferated without restriction, escape from apoptosis, invasion, and metastasis [5]. Many specific molecular markers have

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been identified for intestinal epithelium that are used to determine the nature of primary-stage and metastatic tumors, to find the origin of latent metastases to the lymph nodes, and to detect circulating tumor cells [6, 7]. Caudal-type homeobox transcription factor 2 (*CDX2*; OMIM: 600297) is a protein encoded by the *CDX2* gene, expressed in the nucleus of the intestinal epithelial cells, and is a specific intestinal transcription factor that is essential for embryonic development and differentiation of the intestine [8]. The *CDX2* transcription is limited to colon and small intestine cells of humans under normal conditions. The *CDX2* is a transcription regulator for a number of genes responsible for cell proliferation, differentiation, and migration [9]. It is clinically useful to detect the *CDX2* expression level in the tumoral tissue, since it is known to be an almost specific marker for gastrointestinal neoplasms, especially CRC, with a 70 to 100% expression incidence in tumor cells compared with normal cells [10, 11]. However, the *CDX2* can also be expressed in other primary-stage mucin-producing carcinomas such as bladder, ovarian, lung, pancreas, and biliary carcinomas [12]. On the other hand, the downregulated *CDX2* expression may lead to loss of differentiation and spread of cells and interfere with the occurrence of CRC, possibly through interactions with other genes [13]. There are studies in *CDX2* expression and its correlation with clinicopathologic findings, molecular characteristics, and prognosis of CRC patients, some of which showed *CDX2* expression is associated with invasive clinical behavior, invasive tumoral growth, higher staging and grading of tumors, less histological differentiation, less survival after chemotherapy, and less overall survival, while few inconsistently showed no correlation [14–16]. According to these studies, the *CDX2* is likely to be a prognostic factor in overall survival and a strong predictor for a negative response to treatment.

Since the prediction of clinical outcome and prognosis of patients with CRC are primarily done based on the clinical and pathological characteristics, such as tumor grade and stage [17], it is useful to find and use novel biomarkers with prognostic value in follow-up and recommendation for diet regimens to develop patients' treatment. The findings of various studies on the onset and development of CRCs due to downregulated *CDX2* expression suggest the tumor suppressor activity of this gene [18], but its correlation with prognosis and patient survival and clinical and pathologic characteristics are still under discussion. For this purpose, this study measured the expression level of CDX2 protein in tumor tissue and examined its correlation with age and gender, tumor site, histopathological features, tumor grading, tumor staging, blood-lymphatic vessel invasion, and neural invasion as effective parameters in prognosis, as well as correlation with death from CRC 3 years after diagnosis.

Materials and Methods

Study Population and Tissue Samples

The tumor samples were collected from patients with CRC, without history of other cancers, who undergo colectomy and confirmed by a pathologist at the pathology laboratory of Shahid Beheshti Hospital of Kashan during 2010–2013. The tumors were classified according to the fifth edition of the UICC TNM Classification [19]. The patients did not undergo chemotherapy and radiography prior to surgery. Postoperative cares were performed based on Danish Colorectal Cancer Groups recommendations [17]. The patients were followed up for recurrence and death because of CRC for maximum of 3 years after diagnosis. After screening of samples and for experimental studies, 82 from CRC patients were enrolled. The tumor specimens were obtained from the colectomy specimen of CRC patients. Tumor tissue specimens were fixed in formalin buffer 10% (> 48 h).

The Immunohistochemistry

As usual, paraffin blocks and tissue cross-sections with a thickness of 5 μm were prepared, a series of slides were stained with the usual hematoxylin-eosin method, and similar series of slides were stained with immunohistochemistry (IHC) technique according to the following procedure:

1. Rehydration of cross-sections with passage of slides from xylene and alcohol with different percentages (100%, 80%, 70%, and 50% respectively) and distilled water
2. Antigen retrieval using heating method by placing the slides in citrate (pH = 6) at a temperature of 95 °C and then reaching the slides to room temperature inside the buffer and washing three times with distilled water and 2 min each time
3. Removal of endogenous peroxidase activity by placing the cross-sections for 5–10 min in a solution of 0.1–1% hydrogen peroxide in distilled water and then washing 2 times with PBS and 5 min each time
4. Addition of anti-CDX2 primary antibody (DAKO, Diagnostic Bio System, Mouse/Rabbit Monoclonal Antibody to CDX2 plus HRP/DAB detection system, LOT: 1459) with a dilution of 1: 100 to the cross-sections and incubation at 4 °C overnight
5. Washing with PBS, 3 times each time for 5 min
6. Addition of horseradish peroxidase–conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) to the cross-section and the incubation for 30 min at room temperature
7. Washing with PBS, 3 times each time for 5 min

8. Placing the cross-sections in DAB solution (diamino-benzidine tetrahydrochloride dihydrate) for 1 to 5 min for peroxidase reaction
9. Field staining using hematoxylin
10. Dehydration of cross-sections by alcohol solutions (60–80% and 100%) and 100% xylene
11. Mounting with mounting medium

At this stage, the slides are ready for examination with optical microscope. The histopathologic detection was performed based on the slides stained by hematoxylin and eosin method. Based on the microscopic findings, the following were determined: blood-lymphatic vessel invasion, neural invasion, tumor grade, and tumor pathologic staging including the depth of tumor, microscopic invasion, and regional lymph node involvement [19]. The slides stained by IHC method were explored for the CDX2 protein expression through observing the appearance of yellow-to-brown color in the nucleus of the gland epithelial cells and classified into positive and negative categories and positive samples were graded based on the staining intensity from + 1 to + 3 as follows [20]: negative or poorly stained specimens in less than 10% of cells as negative; weak-to-moderate staining in 10 to 29% of cells as + 1 or weak; moderate-to-severe staining in 30 to 49% of cells as + 2 or moderate; strong staining in more than 50% of the cells as + 3 or strong (Fig. 1).

Statistical Analysis

Data from each sample including tumor site, histopathologic characteristics, tumor grading, tumor staging, blood-lymphatic vessel invasion, and neural invasion, as well as
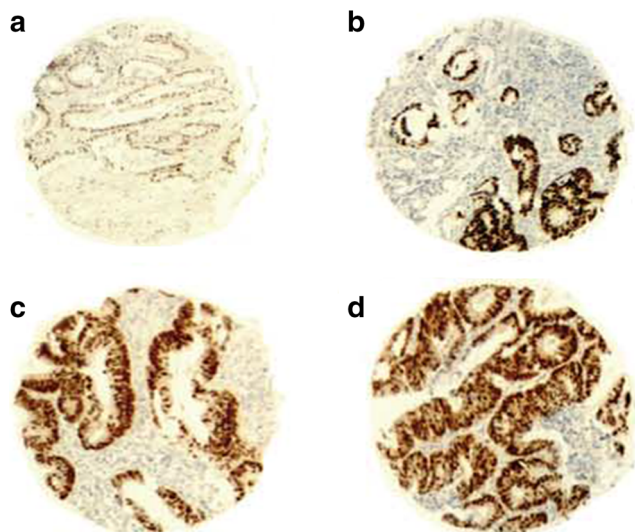
Fig. 1 Immunohistochemical staining of CDX2 protein. **a** Non-stained samples as negative control. **b** Poorly to moderately stained samples as + 1 or weak. **c** Moderately to severely stained samples as + 2 or moderate. **d** Strongly stained samples as + 3 or strong (magnification × 40)

immunohistochemistry results from CDX2 expression level, were analyzed statistically along with data including age, gender, and 3-year survival rate of patients as prognostic factors. After collecting, describing, and displaying data, the statistical analysis was performed by SPSS software (Version 22; SPSS Inc., IBM Corp Armonk, NY, USA) using independent sample t test, ANOVA, Fisher's exact test, chi-square, and Pearson correlation coefficient at the significance level of less than 0.05.

Results

This study was performed on 82 CRCs including 39 (47.6%) males and 43 (52.4%) females. The mean age of the patients was 64.5 ± 14.9 years, the mean age of males was 64.2 ± 12.8 years, and the mean age of females was 65.4 ± 16.8 years. Of the 82 CRC patients at 3 years after diagnosis, 23 (28%) died from cancer and 59 (72%) survived. Of the 82 CRC samples, 29 cases (35.37%) were negative for CDX2 and 53 (64.63%) were positive for CDX2. Of the 53 positive CDX2, 14 cases (26.41%) were with weak expression (1+), 26 cases (49.06%) were with moderate expression (2+), and 13 cases (24.53%) were with strong expression (3+). Based on the data, the CDX2 expression showed a significant difference in gender ($p = 0.011$), meaning that the strong positive CDX2 expression in males is towards moderate and weak in females. It should be noted that the CDX2 expression level was not significantly different in terms of 3-year survival (death from cancer) ($p = 0.58$). Concerning the tumor site, 25 cases (30.5%) were located on the right side of the colon and 56 cases (68.3%) on the left side of the colon and one case (1.2%) in the transverse colon. Data analysis showed that the CRC samples with any grade of CDX2 expression were different in terms of tumor site and statistically significant ($p = 0.019$). The CDX2 expression also tends to be negative in the right-sided tumor relative to the left-sided and the transverse colon tumors. The histological examinations of the CRC samples showed 62 (75.6%) cases of conventional adenocarcinoma and 20 (24.4%) cases of mucinous carcinoma, with CDX2 expression level different from tumor histology and negative significant statistically ($p = 0.003$), meaning the CDX2 expression tends to be negative from the adenocarcinoma to the mucinous. In terms of tumor grading, 77 (93.9%) were low grade and 5 (6.1%) were high grade, with a significantly negative correlation between degree of tumor differentiation (grade) and CDX2 (positive and negative) ($p = 0.031$); CDX2 tends to be negative from the low to high grade. In the microscopic examination of samples in terms of blood-lymphatic vessel invasion, 31 cases (37.8%) were negative and 51 (62.2%) were positive, with no significant difference in blood-lymphatic vessel invasion ($p = 0.69$). In the microscopic examination of the samples in terms of neural invasion, 52

cases (63.4%) were negative and 30 cases (36.6%) were negative, which showed no significant difference in the *CDX2* expression in terms of neural invasion ($p = 0.99$).

In the pathologic staging classification, the following results were observed by examining the tumor invasion depth (*T*) and lymph node involvement (*N*). The depth of invasion (expansion to the intestinal wall) in 82 CRC cases revealed 4 (4.9%) with mucosal and submucosal invasion (T1), 18 (22%) with muscular propria invasion (T2), 46 (56.1%) with subserosal invasion (T3), and 14 cases (17.1%) with invasion to visceral peritoneum or involvement of surrounding organs (T4), with no significant difference in the *CDX2* expression in terms of invasion depth ($p = 0.28$).

Concerning regional lymph node involvement, 60 cases (73.2%) were not involved (n0) and 22 cases (26.8%) were involved (n1, n2), with a significant difference in the *CDX2* expression in terms of lymph node involvement ($p = 0.008$), which means that the downregulated *CDX2* expression was observed from n0 to n1 and n2. The overall staging classification showed 19 (23.2%) with stage 1 and 43 (52.4%) with stage 2 and 20 (24.4%) with stage 3, with negatively difference in the *CDX2* expression level in terms of overall staging ($p = 0.036$), meaning the *CDX2* expression tend to be negative from stage 1 to stages 2 and 3.

Discussion

The CDX2 protein is expressed in the nucleus of the intestinal epithelial cells and is a specific intestinal transcription factor and essential for embryonic development and differentiation of the intestine. Downregulation of the *CDX2* expression can result in the loss of differentiation and metastatic tumors [9]. Although tumor suppressor activity has been suggested for this gene, the possible correlations of the *CDX2* expression level and pattern in CRC with the prognosis and the survival of patients are controversial and unclear. This study examined the expression of CDX2 protein in the CRC tissue using the IHC method and its correlation with age, gender, tumor site, microscopic histopathology (histology type, grading, pathologic staging, and blood-lymphatic vessel invasion) and 3-year survival rate as factors affecting prognosis. In this study, out of 82 CRCs, 64.6% were positive for *CDX2* and 35.4% were negative for *CDX2*. Different studies showed the expression of *CDX2* protein between 70 and 100% [10, 11, 21]. The *CDX2* positive tumors included 26.4% weak, 49.1% moderate, and 24.5% with strong grading. In this study, the negative or positive *CDX2* showed no correlation with age and these results differed from those of Kim et al., who showed a negative correlation of *CDX2* with higher age [14]. In this study, the different degrees of positive *CDX2* expression showed a significant correlation with gender, so that the positive *CDX2*

expression in men was stronger than that of women, which was similar to the results of studies by Bae et al. and Baba et al., which showed a downregulated *CDX2* expression in female gender [15, 16]. In this study, the tendency of the right-sided colon tumors to negative *CDX2* and left-sided tumors to positive *CDX2* was similar to those of Olsen et al. and Bae et al., which showed the downregulated *CDX2* expression in relation to the right-sided tumors [9, 15]. However, in the study of Baba et al., there was no correlation between the *CDX2* expression and the tumor site [16]. In this study, the correlation of negative *CDX2* with poorly differentiation (high-grade tumor) was observed and the correlation of positive *CDX2* with well to moderately differentiated (low grade) tumors, which were similar to studies by Olsen et al., Kim et al., Brody, and Baba et al. [9, 14, 16, 22]. In this study, the histologic type showed the correlation of mucinous tumors with negative *CDX2* and the correlation of adenocarcinoma types with positive *CDX2*, coinciding with Bae et al. [15]. In this study, the pathologic staging did not show a significant correlation between tumor invasion depth and *CDX2* expression, different from Kim et al. and Dawson et al., who reported the correlation between downregulated *CDX2* expression and deeper invasion [14, 23]. In this study, the downregulated *CDX2* expression was also associated with a greater involvement of regional lymph nodes, similar to those of Kim et al., Platet et al., and Bae et al., who stated that the *CDX2* expression knockdown facilitated the ability of the invasion [14, 15, 24]. In this study, in terms of overall staging, more advanced tumors were associated with negative *CDX2* and early-stage tumors were associated with positive *CDX2*, consistent with studies conducted by Kim et al., Bae et al., and Baba et al. [14–16]. Contrarily, Olsen found no correlation between *CDX2* expression and tumor stage [9]. In this study, there was no significant correlation between *CDX2* expression and both blood-lymphatic vessel invasion and neural invasion, which is different from that of the study conducted by Dawson et al. [23]. In this study, there was no significant correlation between the *CDX2* expression and cancer death (survival), but studies by Dalerba et al., Kim et al., and Bae et al. suggested that the downregulated *CDX2* expression was correlated with less overall survival [14, 15, 25] and Baba et al. and Lugli et al. did not find such correlation similar to this study [16, 26]. The contradictory results from different results can be due to the influence of racial factors, environmental parameters, life style, and type of diet. Many factors could interfere with the alteration of gene expression and among them, genetic and epigenetic variations have major role. For example, genetic mutations and variations in the promoter region of the gene and also promoter methylation could alter the gene expression profile [27, 28]. Therefore, these alterations in the genetic and epigenetic profile of *CDX2* gene could explain the changes in the gene expression in patients with CRC.

Conclusion

According to the results obtained from the present study, the expression of CDX2 protein is probably correlated with prognostic factors but not with the overall survival of patients. This study was conducted for the first time in Iran. Due to the research limitations, it is hoped that future studies will be conducted at a wider level with a larger sample size and prolonged follow-up of patients for correlation with a 5-year survival rate. It is also suggested that the prognosis of patients should be performed with further markers to improve patients follow-up and use of beneficial therapies. Besides, gene-gene interaction interactions would be useful to obtain more accurate conclusion. However, our study could be considered as a preliminary study and further studies with larger sample sizes in different ethnic groups are required.

Compliance with Ethical Standards

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