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Regulating and changeable performance of *CDX2*, *CTNNBIP1*, and *FAT4* genes in colorectal cancer

Seyed Mohammad Taghi Hamidian [1], Rezvan Azadi [2], Pooya Rostami [3], Farnaz Azar Shabe [4], Zeynab Khazaee Kohparc [4*]

[1] Babol University of Medical Sciences, Department of Gastroenterology, Babol, Iran

[2] Shahid Beheshti University of Medical Sciences, Department of Medicine, Tehran, Iran

[3] New York University, Londgone Medical Center, Brooklyn, NY, USA

[4] Islamic Azad University of Tonekabon Branch, Department of Biology, Tonekabon, Iran

Abstract

Introduction: Colorectal cancer (CRC) is the third most frequent type of cancer in the world. In this explanation, genetic variation is associated in all cancers, particularly CRC, and modifications of numerous genes, such as *CDX2*, *CTNNBIP1*, and *FAT4*, are linked to tumorgenesis in CRC. As a result, this research was conducted in order to determine changes in the expression of these genes.

Materials and Methods: After obtaining patient consent and pathology department approval, from72 individuals with confirmation of pathology report,were provided and bought from the Bio banks. Real-time PCR was used to examine the expression of *CDX2*, *CTNNBIP1*, and *FAT4* genes in tumoral and non-tumoral tissues. These genes' histological associations with grading and staging for upregulation and downregulation were examined.

Result: *CDX2* (P = 0.01) and *CTNNBIP1* (P = 0.03) expression were highly increased, whereas *FAT4* (P= 0.05) expression was downregulated. Similarly, there was no evidence of a link between *CDX2* and *CTNNBIP1* overexpression and grade, stage, lymphnode metastasis, or distant metastasis. Furthermore, *FAT4* expression was linked to highe stage, high grade, distant metastasis and lymphnode metastasis (P 0.05).

Conclusion: *CTNNBIP1* and *CDX2* genes were upregulated in tumoral tissues, while *FAT4* genes were downregulated. Finally, changes in the expression of these genes can be used as a CRC biomarker.

Keywords: Colorectal cancer, Genes fluctuation, Regulation

[*]**Corresponding Author:** Zeynab Khazaee Kohparc

✉ Email: zeynab_zhazaee_kohparc@yahoo.com

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Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed cancer in adults. The third prevalent cancer in the world is CRC (1). CRC is a prevalent human cancer that requires a thorough knowledge of its molecular underpinnings. Initial therapy only cures a small percentage of people and is most effective when the disease is in its initial stages (2). CRC was among the first large epithelial malignancies in which molecular changes were observed systematically as the disease progressed. The discovery of new oncogenes and tumor suppressors would help us identify the biology of CRC and could lead to new effective treatments (3).

Since *CDX2* mutations are extremely rare events in CRCs, we hypothesized that epigenetic changes, such as promoter hypermethylation or histone deacetylation could be responsible for significant downregulation or absence of *CDX2*, particularly in the group of tumors displaying "serrated" molecular features. Human serrated adenomas with high-grade dysplasia have been shown to have significantly greater frequencies of *CDX2* hypermethylation than other polyp types (4). *CTNNBIP1* (β-catenin interacting protein 1) gene is an antagonist of Wnt signaling which binds to the β-catenin molecules. The *CTNNBIP1* function as a tumor suppressor gene or oncogene in different types of cancer is controversial. Several nuclear antagonists are known to regulate β-catenin-TCF mediated transcription. One such direct nuclear antagonist is *CTNNBIP1* (catenin, beta interacting protein 1; also known as ICAT) (5). *CTNNBIP1* binds to two different armadillo regions of β-catenin through its N-terminal and C-terminal domains leading to disruption of β-catenin-TCF interaction. The importance of *CTNNBIP1* in embryonic development and tissue differentiation process has been reported. Variable frequencies of expression of *CTNNBIP1* have been shown in metastatic and nonmetastatic human melanoma (6). The Fat gene family was originally identified in Drosophila as a member of the cadherin super-family with tumor suppressor functions. It regulates cell proliferation and planar cell polarity during Drosophila development by the Hippo signaling pathway. They encode a type 1 transmembrane protein with 34 cadherin repeats, 4 epidermal growth factor (EGF)-like repeats, a transmembrane domain and a cytoplasmic domain that is distinct from the classical cadherin proteins. In humans, four members of the Fat family have been identified, namely, FAT1, FAT2, FAT3 and *FAT4*, which are structurally similar to the Drosophila Fat protein. In mammals, *FAT4* is the true structural ortholog of the Drosophila FAT. *FAT4* functions as a tumor suppressor and previous findings have demonstrated that *FAT4* can inhibit the epithelial-to-mesenchymal transition (EMT) and the proliferation of gastric cancer cells. However, few studies have investigated the role of *FAT4* in the development of colorectal cancer (7).

Materials and Methods

Samples collection

The study sample consisted of 72 tumoral and 72 non-tumoral (margins tissues) from 53 females and 19 males were provided and bought from the Bio banks. Information on histological status is shown in Table 1. Then, all tissues were delivered to liquid nitrogen for deep freezing. Tissue samples were kept at a temperature of 80 °C for long-term conservation and investigation. Trizol (Invitrogen cat no 15596-025, USA.) was used to isolate RNA from tissues. The spectrophotometer (TC100, USA) was used for quantitative RNA analysis and electrophoresis (2% agarose gel) was used for qualitative analysis.

cDNA was prepared using the cDNA Kit (Quanti Test Reverse transcription kit, Qiagen) with around2 pg RNA per reaction. The first cDNA strand was generated utilizing a stem-loop sequence-specific primer. Table 2 lists the forward and reverse primer sequences. The real-time PCR assays were carried out on cDNA by using the SYBR Green technique in Step one equipment (Applied Biosystem, USA). A total of 1 liter of cDNA from each tissue was used for amplification. As a housekeeping gene, GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was employed. Early incubation at 95 °C for 5 minutes was proceeded by 40 cycles of 95 °C for 30 s and 60 °C for 1 min in a 20 l final volume. Using the 2-ct approach, the range of up-regulation or down-regulation in each sample was extensively studied. All of the reactions were carried out in triplicate.

Table 1. Sequences of primers employed for Real-time PCR action.

Primer sequence (5′–3′)	
Forward *CDX2*	5′-TAGTTTGYGGGGYTGYTGTA-3′
Reverse *CDX2*	5′-GCCATATACRTAARCTACCTCCT-3'
Forward *CTNNBIP1*	5′-GGAAGATGGGATCAAACCTGA CAG-3′
Reverse *CTNNBIP1*	5′-TCGTATCCAGTGCTGCGACCGTAT GGATGTGTCTGCGGCGTTTTATCATG CACTGGATACGAC AAC GCCATCA CC-3′
Forward *FAT4*	5'-ACACTGTGATTGCCAGGAGAG-3'
Reverse *FAT4*	5'-GGATGTGTCTGCGGCGTTTTAT CATGCACTGGATACGACCAAGAGTC CAGTC-3'

Statistical Analyses

All the acquired data from Real-time PCR were analyzed by exercycle set. Correspondingly, the significant difference was statistically interpreted by paired Student's t-test. $P < 0.05$ was considered statistically significant. Analyses were accomplished using commercially available statistical software (SPSS Statistics software, version 25, Chicago).

Results

Gene expression evaluation in tumoral tissues

The analysis of expression levels of tumoral and corresponding non-tumoral tissues for *CDX2*, *CTNNBIP1* and *FAT4* genes indicated that the *CDX2* and *CTNNBIP1* were upregulated in tumoral tissues in comparison with their non-tumoral counterparts. On the contrary, *FAT4* expression level had decreased significantly in 50% of samples (Figure 1, 2,3).

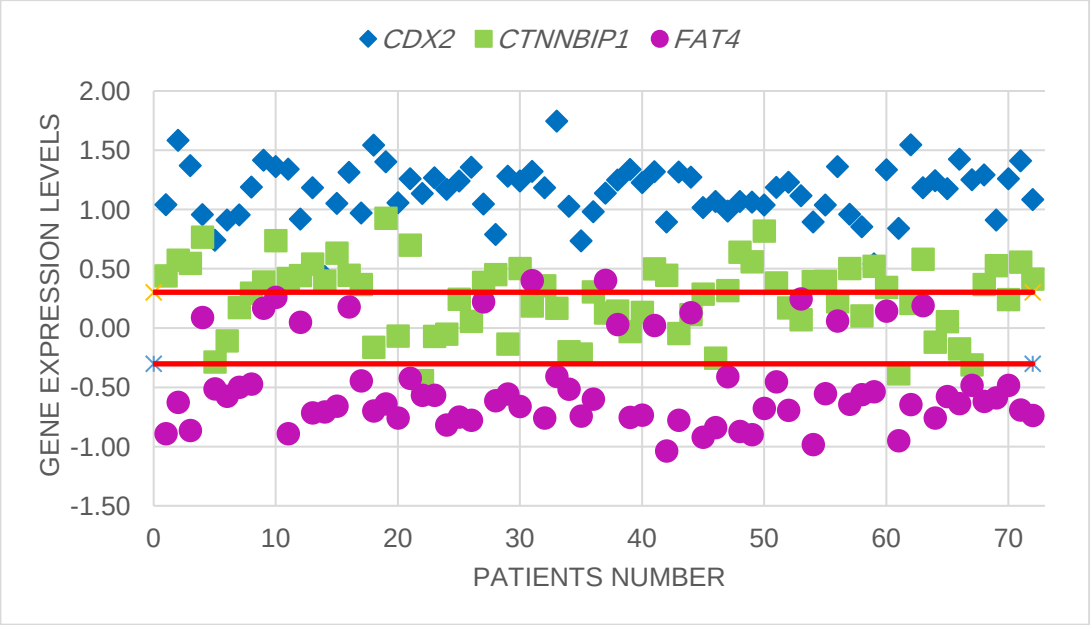


Figure1. Scatter plot analysis of relative expression of *CDX2*, *CTNNBIP1* and *FAT4* in colorectal cancer patients. The Y-axis indicates the logarithm of relative gene expression. Horizontal red lines represent cut-off values logarithms for two-fold changes in expression (FC≥2.0, $p<0.05$). The upper 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; $P < 0.05$). The *CDX2* ($P = 0.01$) and *CTNNBIP1* ($P = 0.03$) expression level had increased and *FAT4* ($P = 0.05$) expression level had decreased significantly in tumoral compared to the non-tumoral samples.

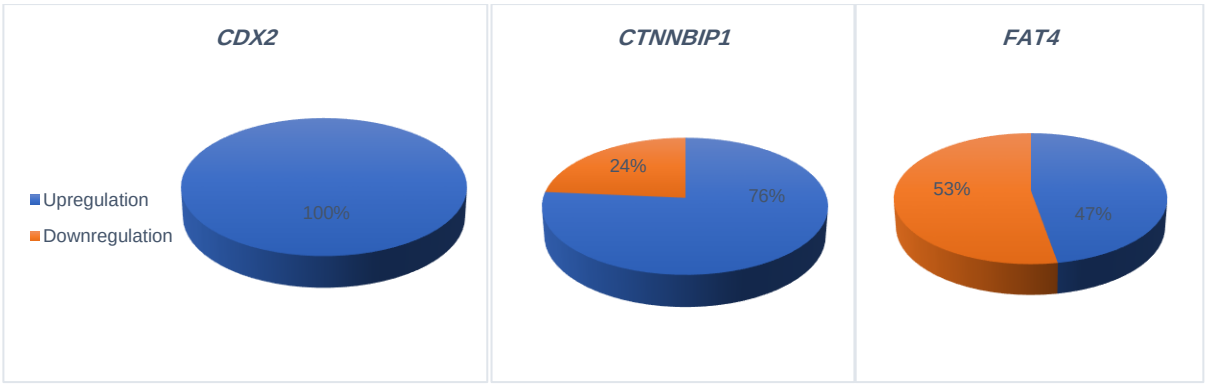


Figure 2. The data revealed a significant upregulation of *CDX2* and *CTNNBIP1* expression and downregulation of *FAT4* in colorectal cancer (P < 0.05.

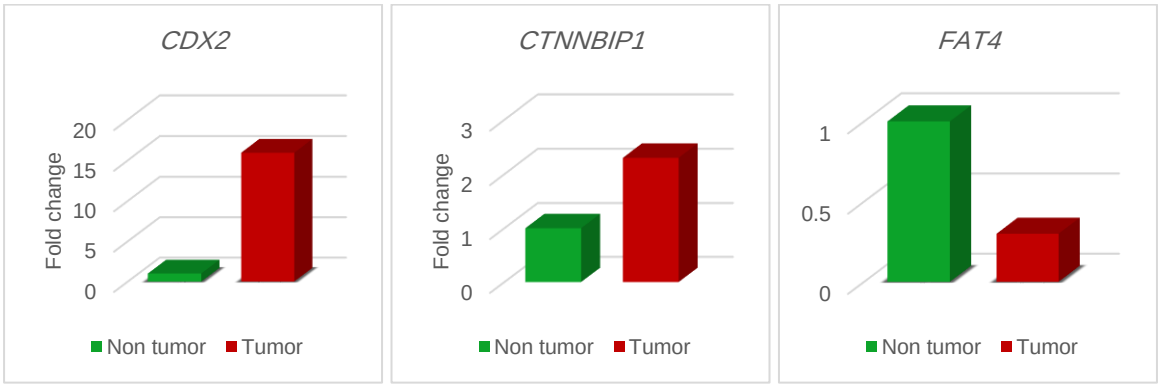


Figure 3. Fold change of **(a)** *CDX*2 (P= 0.02), **(b)** *CTNNBIP1* (P= 0.02) and **(c)** *FAT4* (P= 0.04) expression in tumoral tissues in comparison with non-tumoral (tumor margin) tissues.

Clinicopathological analysis

Clinicopathological consequences of *CDX2*, *CTNNBIP1* and *FAT4* genes expression were evaluated in 72 patients diagnosed with adenocarcinoma of the colorectal. Patients' clinicopathological characteristics are summarized in Table 2. The analysis of different clinicopathological variables and genes expression correlation is presented in Table 3 (up/down). The mean age of patients was 58.9±12.5 years at the time of diagnosis (female to male ratio, 4:1; age range, 37–88 years). In general, more than half of the patients had advanced T-stage (Stages III-IV), and high-grade histology. Lymph-node metastasis and distant metastasis were observed in more than 60% of the patients.

The number of gene expressions of all samples was compared and investigated with the stage, grade, lymph node metastasis and distance metastasis of all patients.

The analysis of different clinicopathological variables and genes expression correlation is presented in Table 3. Statistical analyzes were performed with using SPSS 25 and also Chi Square test and T test.

The expression of *CDX2*, *CTNNBIP1* and *FAT4* was matched with different clinicopathological data of the colorectal cancer patients (summarized in Table 2). There was no significant association between *CDX2* and *CTNNBIP1* expression with grade, stage, lymph-node metastasis (P= 0.02) and distant metastasis. Moreover, the *FAT4* expression was also significantly associated with high grade (P = 0.03), high stage (P = 0.03), lymph-node metastasis (P= 0.05) and distant metastasis (P= 0.05) (Figure 4, 5, 6).

Table 2. Clinicopathological characteristics of colorectal cancer cases.

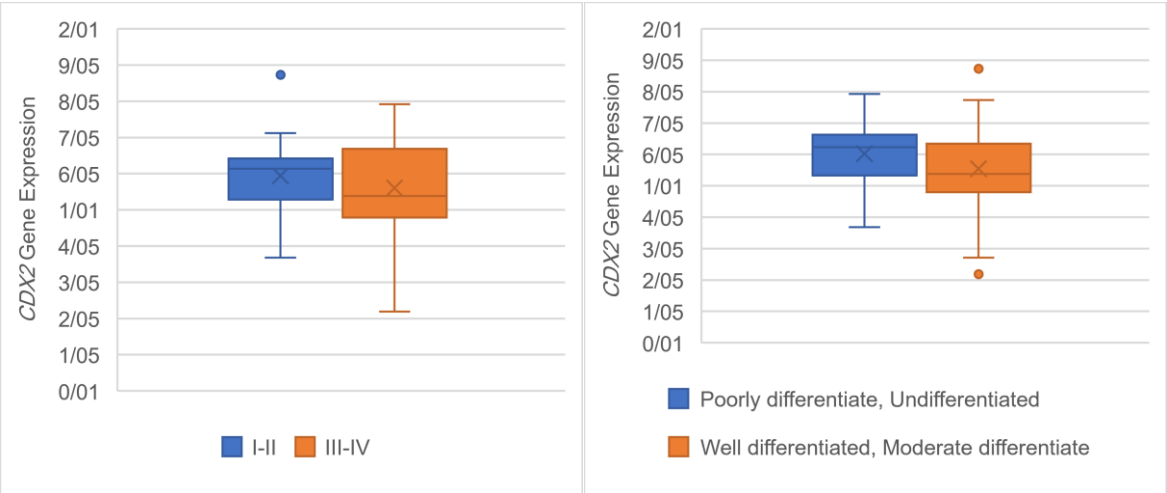
Characteristics	Total (N=72) Patients (%)
Gender	
Female	53 (73.6)
Male	19 (26.4)
Age	
< 60 years	38 (52.8)
≥ 60 years	34 (47.2)
Stage	
I	6 (8.3)
II	24 (33.3)
III	38 (52.8)
IV	4 (5.6)
Grade	
Well-differentiated	4 (5.6)
Moderate differentiate	26 (36.1)
Poorly differentiate	39 (54.1)
Undifferentiated	3 (4.2)
LM	
Yes	45 (62.5)
No	27 (37.5)
DM	
Yes	44 (61.1)
No	28 (38.9)

Table 3. The association of genes expression with clinicopathological qualification. LM: Lymph node Metastasis, DM: Distance Metastasis; ↓/−: decrease or no change of expression; ↑: increase of gene expression.

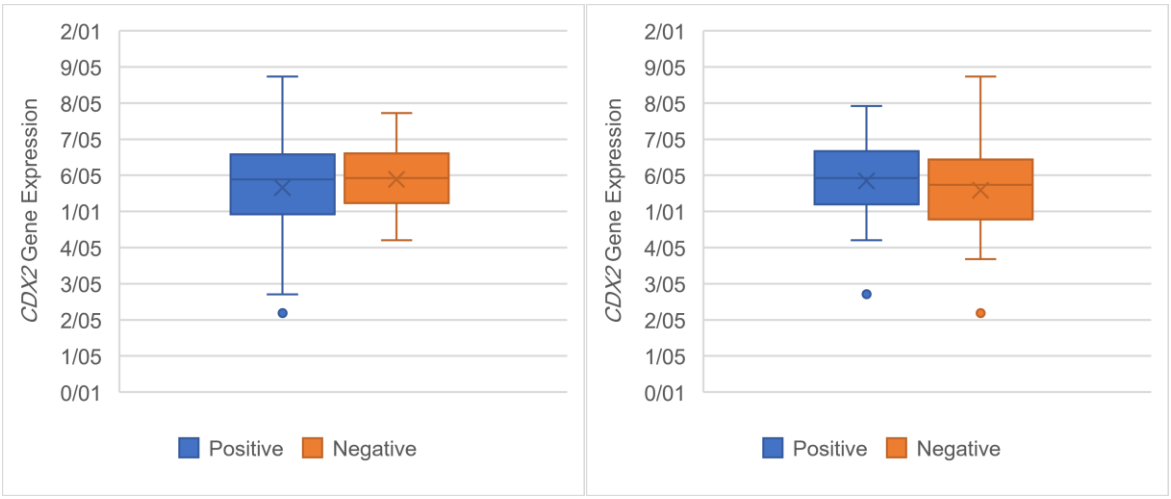
	CDX2		P-value	*CTNNBIP1*		P-value	*FAT4*		P-value
Tumor Stage	↓/−	↑		↓/−	↑		↓/−	↑	
I-II	0	30	0.5	12	18	0.7	25	5	0.03
III-IV	0	42		5	37		32	10	
Tumor Grade									
I-II	0	30	0.6	13	17	0.1	23	7	0.03
III-IV	0	42		6	36		35	7	
LM									
Yes	0	44	0.3	24	22	0.4	36	8	0.05
No	0	28		11	15		20	8	
DM									
Yes	0	44	0.2	21	23	0.5	36	8	0.05
No	0	28		15	13		21	7	

LM: Lymph node Metastasis, DM: Distance Metastasis

The Association of *CDX2*, *CTNNBIP1* and *FAT4* expression with clinicopathological qualifications

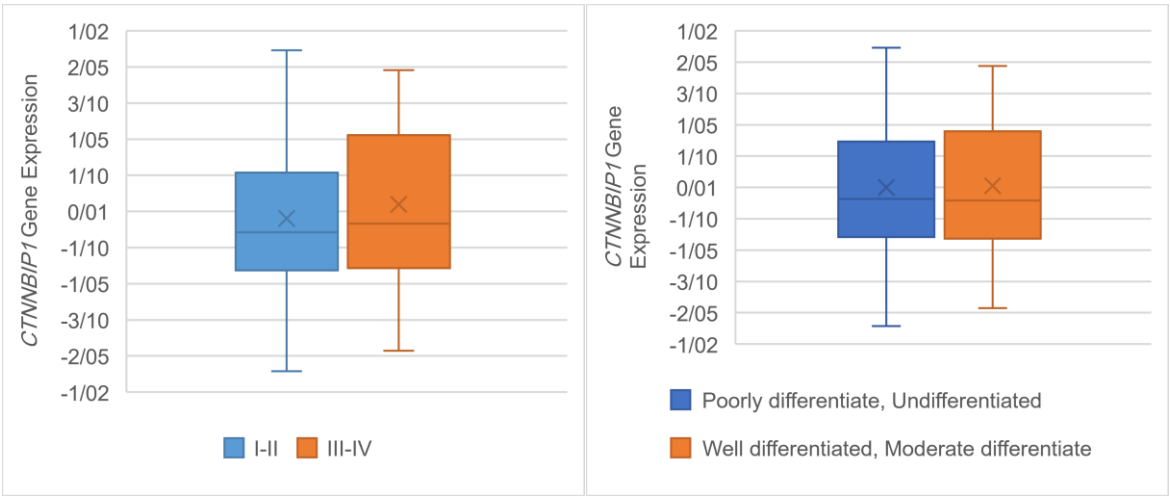


(a) **(b)**

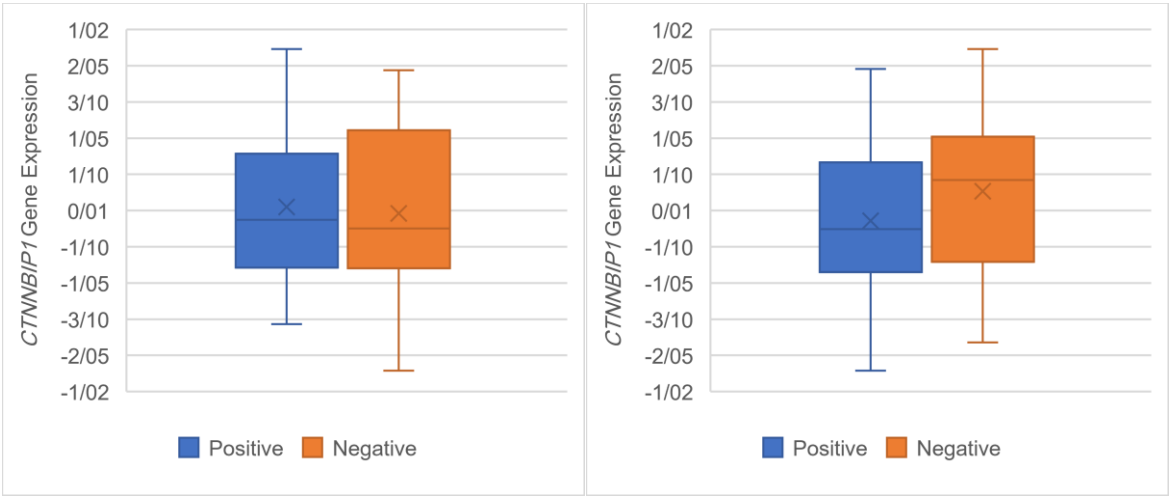


(c) **(d)**

Figure 4. The Association of *CDX2* expression with clinicopathological qualifications. There was no significant association between *CDX2* upregulation with **(a)** tumor stage (P =0.5), **(b)** tumor grade (P =0.6), **(c)** lymph-node metastasis (P= 0.3) and **(d)** distance metastasis (P= 0.2).



(a) **(b)**



(c) **(d)**

Figure 5. The Association of *CTNNBIP1* expression with clinicopathological qualifications. There was no significant association between *CTNNBIP1* downregulation with **(a)** tumor stage (P =0.7), **(b)** tumor grade (P =0.1), **(c)** lymph-node metastasis (P= 0.4) and **(d)** distance metastasis (P= 0.5).

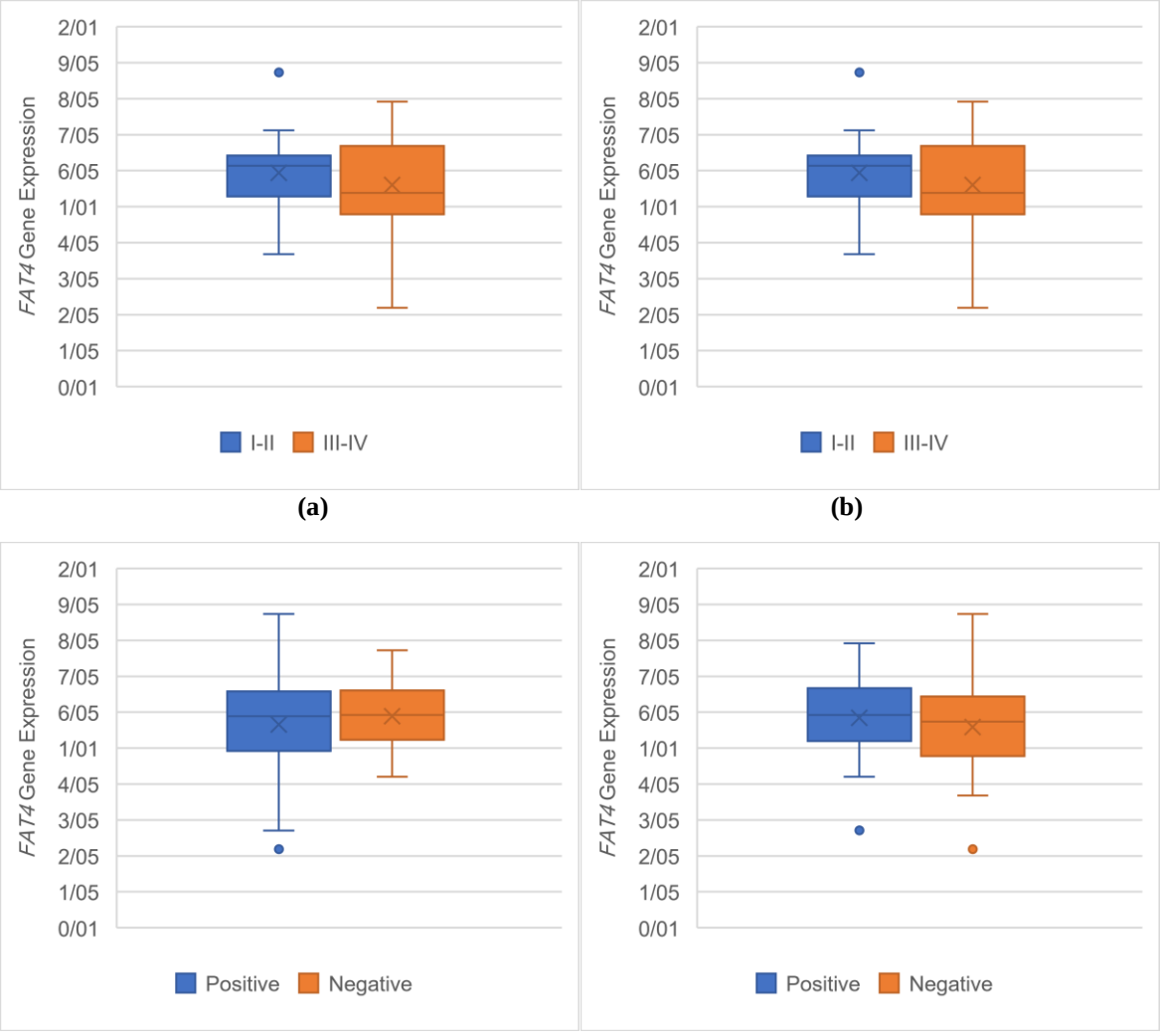


Figure 6. The Association of *FAT4* expression with clinicopathological qualifications. The *FAT4* expression was significantly associated with **(a)** tumor stage (P =0.03), **(b)** tumor grade (P =0.03), **(c)** lymph-node metastasis (P= 0.05) and **(d)** distance metastasis (P= 0.05)**.**

Discussion

Reduced *CDX2* protein expression is related to certain molecular alterations during colorectal tumorigenesis. Previous work shows that nearly all sporadic microsatellite unstable (MSI) cancers show some degree of loss of the protein in the tumor, whether in a small or substantial percentage of cells. This loss is not however limited to MSI-high cancers but is also found in microsatellite stable (MSS) tumors with BRAF mutation and high-level CpG island methylator phenotype (CIMP), in other words, in cancers deriving from the so-called serrated pathway (4). The previous research showed *CDX2* expression was increased significantly in gastric cancer. *CDX2* expression had a significant correlation with TNM stage and lymph node metastasis.

Previous findings have shown that transfection of *CDX2* cDNA, and human HT29 CRC cell line to

express *CDX2* protein, indicated the oncogenic potential of the abovementioned cells, and metastasis of related cells markedly decreased while cell sensitivity for apoptosis significantly increased. The results have shown that in comparison to the normal population, the degree of methylation of the promoter region of *CDX2* in lesion tissue of patients with CRC was higher than that of the normal population. The protein expression in the control and lesion sections of CRC patients showed that the expression level of *CDX2* in the lesion section of patients with CRC was lower. This finding suggested that there was a certain correlation between *CDX2* and CRC or the decrease in the degree of *CDX2* gene promoter methylation to a certain extent, promotes the risk of CRC (8).

Previous research indicates the downregulation of *CTNNBIP1* gene which corresponds to a tumor suppressor role for *CTNNBIP1* in GC. Also, the expression level of *CTNNBIP1* was extremely lower in female patients than males. According to our findings, the tumor-suppressing function of *CTNNBIP1* in GC is mostly associated with initiation procedures, because well-differentiated tumors showed significant downregulation of *CTNNBIP1* compared with other malignant grades. *CTNNBIP1* expression associated with EBV and CMV infections suggests that the Wnt/β-catenin dysregulation is affected by these agents in GC.

CTNNBIP1 is a suppressor of lung cancer progression. The *CTNNBIP1* protein is important, in that it can control lung cancer cell migration via the coordinated regulation of the β-catenin pathway. A low expression of *CTNNBIP1* is correlated with a high level of expression of MMP7, and there is also an upward trend in terms of the pathological stage and poorer patient survival, which suggests that *CTNNBIP1* may be able to serve as a prognostic biomarker for lung cancer (9).

FAT4 is a tumor suppressor in CRC. Moreover, *FAT4* silencing inhibits CRC cell autophagy and stimulates the invasion and migration of these cells as well as the EMT, whereas the overexpression of *FAT4* yields the opposite results and increases autophagy. Furthermore, the stimulatory effects of *FAT4* on autophagy occur through the upregulation of LC3 and the downregulation of P62 and the effects of *FAT4* on the EMT, as evidenced by the detected changes in the expression levels of E-cadherin and Twist1. Moreover, an increase in *FAT4* leads to a reduction in xenograft tumor growth in vivo, whereas the opposite outcome was obtained with *FAT4* knockdown. Therefore, we conclude that *FAT4* regulates the activity of PI3K to promote autophagy and inhibit the EMT, and these effects are partly achieved through the PI3K/AKT/mTOR and PI3K/AKT/GSK-3β signaling pathways. We anticipate that this study will provide a basis for establishing new strategic approaches for the development of effective CRC therapies (10).

Cai et al, found that *FAT4* has a tumor suppressor role mediated by the modulation of Wnt/β-catenin signaling, providing potential novel targets for the treatment of gastric cancer (11).

Conclusion

The overexpression of CDX2 and *CTNNBIP1* expression in tumoral tissues, as well as the downregulation of *FAT4*, were found to be outstanding. Interestingly, changes in the expression of these genes can be used as a primary biomarker in CRC.

Author contributions

RZ, PR, and FAS collected data and accomplished some sections of the study and manuscript, SMTH collected all the biopsies directly in Omid clinic and hospital by himself and also confirmed the clinical qualifications of all the patients as a gastroenterologist. ZKK controlled and confirmed the data quality, evaluated and optimized the informatics database, wrote the paper and edited it, some other essential functions containing study design, controlling the project and protocol development and also data analysis. All authors revised the article carefully, read and acknowledged the final version of the paper.

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Conflict of interests

Authors declare no conflict of interest.

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