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Genetic alterations of *CDX1*, *CYLD* and *CDKN2B* genes in CRC

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 *CDX1*, *CYLD*, and *CDKN2B*, are linked to tumorigenesis in CRC. As a result, this research was conducted in order to determine changes in the expression of these genes.

Materials and Methods: Specimens of CRC from 72 individuals with confirmation of pathology report,were provided and bought from the Bio banks. Real-time PCR was used to examine the expression of *CDX1*, *CYLD*, and *CDKN2B* genes in tumoral and non-tumoral tissues. These genes' histological associations with grading and staging for upregulation and downregulation were examined.

Result: The expression of *CYLD* (P = 0.01) and *CDKN2B* (P = 0.02) were upregulated significantly, but the *CDX1* (P = 0.03) gene expression was decreased. Correspondingly, there was no significant association between *CDX1* downregulation and *CDKN2B* upregulation with grade, stage, lymph-node metastasis (P= 0.02) and distant metastasis. Moreover, the *CYLD* 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).

Conclusion: The upregulation of *CYLD* and *CDKN2B* genes and downregulation of *CDX1* gene in tumoral tissues were impressive. Conclusively, the alteration of these genes expression can be considered as a colorectal cancer biomarker.

Keywords: Colorectal cancer, *CDX1*, *CYLD*, and *CDKN2B* genes, Alterations

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

✉ Email: zeynab_zhazaee_kohparc@yahoo.com

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Introduction

Colorectal cancer (CRC) is one of the most important causes of cancer mortality in the world (1). The major factor of CRC is the presence of polyps in the colon and also the changes of adenoma to carcinoma process. CRC is the growth of cancer cells in the colon part caused by uncontrolled growth of cells that can proliferate in other tissues irregularly (2). In this way, the term survival of patients with CRC has not been improved in a therapeutic manner. Strongly, there is a vital and emergency requirement for a better understanding in the molecular pathogenesis of CRC in order to recognize the novel biomarkers for prognosis and diagnosis of CRC (3). Correspondingly, molecular genetic methods especially based on DNA and RNA investigating are really practical and useful in diagnostic medicine (4).

CDX1 (caudal-type homeobox 1) is a transcriptional factor and controls enterocyte differentiation in the colon, where its expression is different from the crypt-base stem cell structure. Remarkably, *CDX1* is also a keyword to the capacity of a CRC cell line in differentiation, and it is classified as a negative marker of CRC stem cells. *CDX1* is required for the actual development of the homeostasis of the intestinal epithelium and also intestinal tract (5). Interestingly, *CDX1* is involved in the modulation of a variety of processes comprising cell adhesion, columnar morphology, proliferation, and apoptosis. *CDX1* is a primary controller of enterocyte differentiation and its expression is vital for the transcriptional regulation of a large number of intestine-specific genes essential for the maintenance of the intestinal phenotype, differentiation, and intestine development. Many markers in the differentiation process, containing villin and cytokeratin 20, have been indicated to be directly transcriptionally regulated by this gene. Many evidence indicates the loss or down-regulation of *CDX1* expression in colon cancer tumors and cell lines (6, 7).

Another important gene in gastrointestinal cancers particularly CRC, is the cylindromatosis (*CYLD*) gene, which was initially explored as a tumor suppressor mutated for familial cylindromatosis (8). In addition to skin tumors caused by *CYLD* loss, decreased *CYLD* expression has been described in several types of human cancers comprising breast cancer, hepatocellular carcinoma, cervical cancer, renal cell carcinoma, lung cancer, gastric cancer and also colon cancer. Remarkably, the expression profile and clinical significance of *CYLD* in patients with a series of co-colorectal lesions are so important (9-11).

CYLD was recognized identified as a gene mutated in familial cylindromatosis (FC), a genetic case that predisposes patients for the progression of skin tumors, termed cylindroma. Cylindromas are benign tumors that emerge on the scalp and interestingly is to be derived from hair follicles of stem cells (12). The cylindromatosis patients possess heterozygous germ-line mutations in the *CYLD* gene, but the wild-type *CYLD* allele undergoes loss of heterozygosity (LOH) and rarely somatic mutations in different tumors as tumor suppressor gene. The human *CYLD* gene is situated on chromosome 16q12.1 and encodes a protein of 956 amino acids. The C-terminal region of *CYLD* includes a catalytic domain with sequence homology to USP family members (9, 13). The second important gene is *CDKN2B* which is referred to the *CDKN2A* tumor suppressor gene in a region at 9p21 and this gene is regularly mutated and omitted in many different tumors. Considerably, this gene encodes a cyclin-dependent kinase inhibitor, and it is considered as *CDKN2B* protein, which is a cell cycle regulator (14). The *CDKN2B* gene encodes for *CDKN2B*, which is a member of the *INK4* class of cell cycle inhibitors. Noticeably, *CDKN2B* has ankyrin repeats that permit it to bind and interact of cyclin-dependent kinase *(CDK) 4/6* with cyclin D, through inhibiting the function of *CDK4/6*. Given the critical role of *CDK4*/6 and cyclin D in improving development through the G1 checkpoint, *CDKN2B* performs as a significant inhibitor of cell cycle and cell proliferation (15, 16).

Materials and Methods

Samples collection

The research was performed on 72 patients (53 female and 19 male) which was confirmed by the pathology department and also an agreement by patients. The histopathological status of patients is shown in Table 2. 72 tumoral and 72 non-tumoral (margins tissues) were provided and bought from the Bio banks. In this way, DEPC (diethylpyrocarbonate) was employed to clean and treat all sampling instruments during providing the biopsies (tumoral and nontumoral tissues) in order to avoid RNAs enzyme. Correspondingly, after sampling operation, all specimens were transferred to liquid nitrogen for deep freezing. Vitimately, tissue samples

were stored at − 80 °C for long preservation and study. RNA isolation from human tumoral and nontumoral tissues was performed using a commercial reagent, Trizol (Invitrogen cat no 15596-025, USA.) Less than 1cm of each tissue was crushed in order to powder them by a mortar and pestle in the presence of liquid nitrogen, and 40– 80 mg of powdered tissue was used for RNA isolation according to the manufacture's protocol. RNA quantity was measured by A260/A280 ratio using NanoDrop spectrophotometer (TC100, USA) and also controlled by electrophoresis on agarose gel 2% in order to observe all RNA bands (5S, 18S and 28S).

Relatively, cDNA synthesis was done in the presence of 1 pg total RNA, 4 µL 5X reaction buffer, 10 mM each of dNTPs, and 1 µL (200 U/ µL) by QuantiTect Reverse Transcription Kit (cat no 20S313, USA) in a final volume of 20 µL, by 60 min incubation at 44°C. Meanwhile, Real-time PCR was done on Exicycler q6, Bioneer, USA by using a universal reverse primer and Universal Taqman-specific probe and also the expression levels of all these genes were normalized against GAPDH, RNA as control. The 20 µL PCR comprised 1µµL RT yeild, 0.25 mM universal-specific probe, 0.5 mM each forward and reverse primers. The PCR reagents were all from Qiagen HotStarTaq reagent set (Qiagen, cat no 203205). The mixtures were incubated at 96 °C for 5 min, followed by 43 cycles of 90 °C for 45 s, and 63 °C for 1 min. All reactions were done in triplicate. The CTs were described as the fractional cycle number.

The primers were designed by Allel ID version 7 software. The first cDNA strand was synthesized. The sequences of forward and reverse primers used are given in Table 1. The Real-time PCR tests were accomplished in a Step one instrument (Applied Biosystem, USA) using cDNA. An amount of 1 µl cDNA from each sample was determined for amplification. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was employed as a housekeeping gene. Amplification occurred in a 20 µl final volume by initial incubation at 96 °C for 5 min, followed by 43 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 measured using the $2^{-\blacktriangle\blacktriangle ct}$ method.

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

	Primer sequence (5′–3′)
Forward *CDX1*	5´-AAGCCTCCGRRCCGCGAATCA-3´
Reverse *CDX1*	5'-GGAAGACTCGTGTATGTATGTGY ATATGTG-3'
Forward *CYLD*	5'-ATGGATAACCCTATTGGCAACTG-3'
Reverse *CYLD*	5'-GTATCCAGTGCTGCGACCGT-3'
Forward *CDKN2B*	5'- TGGCCGGAGGTCATGATG -3'
Reverse *CDKN2B*	5'- GGGCAGCATCATGCACCG -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 *CDX1*, *CYLD* and *CDKN2B* genes indicated that the *CYLD* and *CDKN2B* were down regulated in tumoral tissues in comparison with their non-tumoral counterparts ($P = 0.02$). On the contrary, *CDX1* expression level had decreased significantly in 70% of samples (Figure 1,2,3).

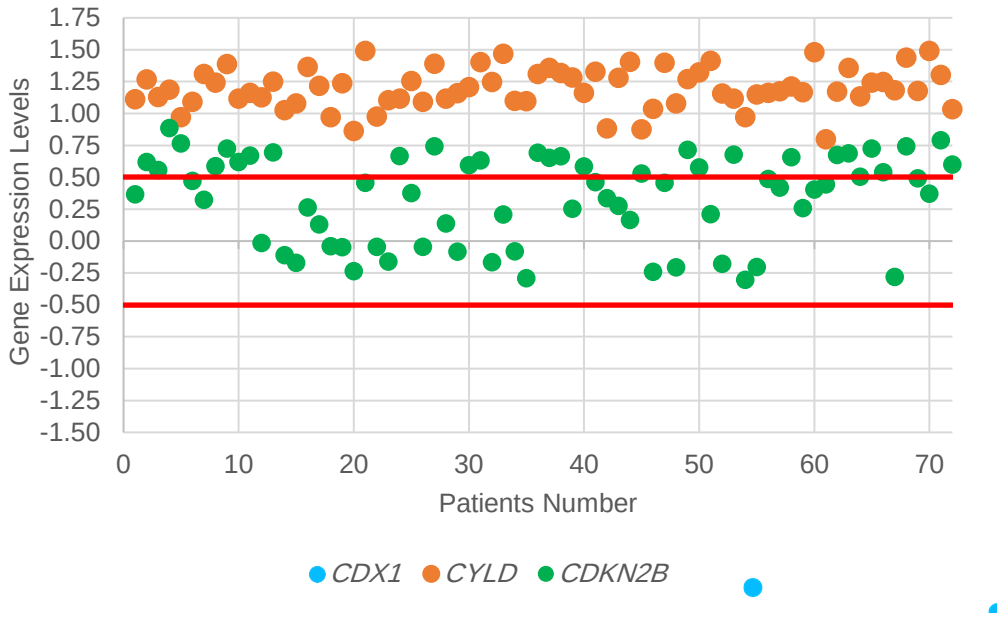


Figure1. Scatter plot analysis of relative expression of *CDX1*, *CYLD* and *CDKN2B* in colorectal cancer patients. The Y-axis indicates the logarithm of relative gene expression. Horizontal red lines represent cut-off values logarithms for two-fold changes in expression (FC≥2.0, p<0.05). The upper part 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 *CYLD* (P = 0.01) and *CDKN2B* (P = 0.02) expression level had increased and *CDX1* (P = 0.03) expression level had decreased significantly in tumoral compared to the non-tumoral samples.

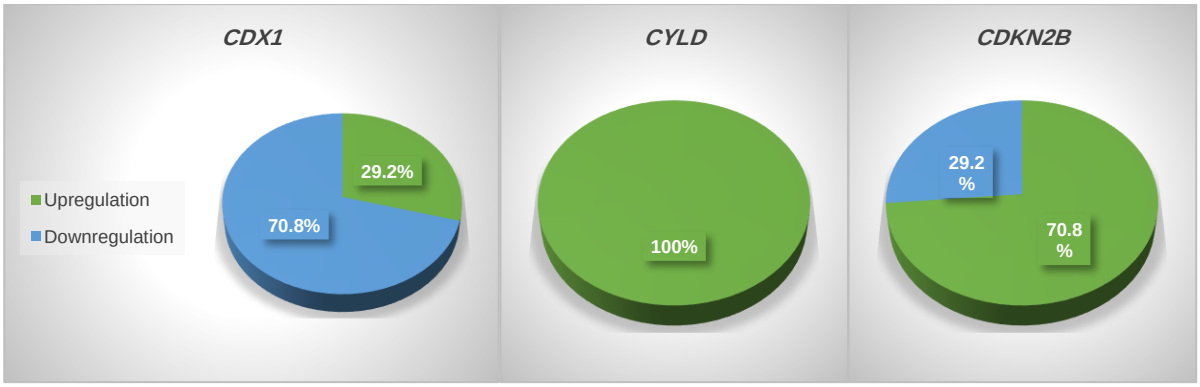
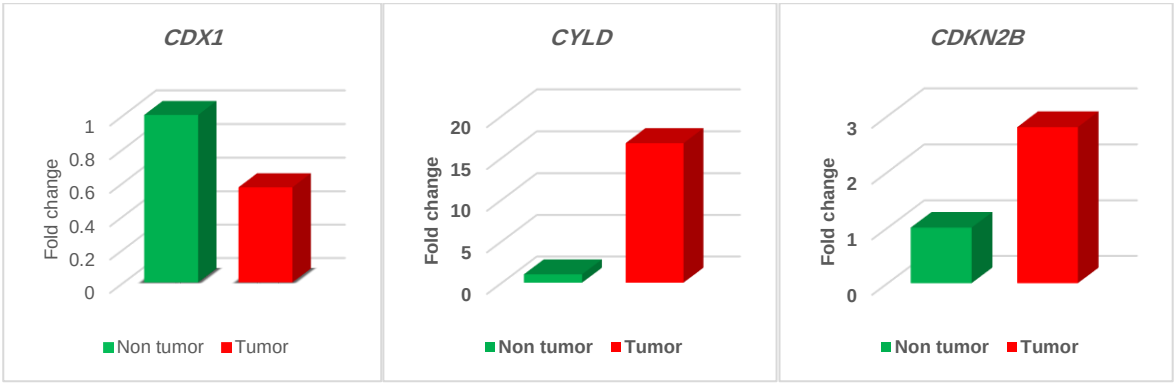


Figure 2. The *CYLD* and *CDKN2B* were down-regulated in tumoral tissues in comparison with their non-tumoral counterparts (P < 0.05).



(a) **(b)** **(c)**

Figure 3. Fold change of **(a)** *CDX1* (P= 0.05), **(b)** *CYLD* (P= 0.02) and **(c)** *CDKN2B* (P= 0.04) expression in tumoral tissues in comparison with non-tumoral (tumor margin) tissues.

Clinicopathological analysis

Clinicopathological consequences of *CDX1*, *CYLD* and *CDKN2B* 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 (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 stage (Stages III–IV), and high-grade histology. Lymph node metastasis and distant metastasis were observed in more than 60% of the patients.

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)

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 using SPSS 25 and also Chi-Square test and T-test.

The expression of *CDX1*, *CYLD* and *CDKN2B* was matched with different clinicopathological data of the colorectal cancer patients (summarized in Table 2). There was no significant association between *CDX1* downregulation and *CDKN2B* upregulation with the grade, stage, lymph-node metastasis (P= 0.02) and distant metastasis. Moreover, the *CYLD* 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 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

	CDX1		P value	*CYLD*		P value	*CDKN2B*		P value
Tumor Stage	↓/−	↑		↓/−	↑		↓/−	↑	
I-II	18	12	0.7	0	30	0.03	12	18	0.5
III-IV	33	9		0	42		7	35	
Tumor Grade									
I-II	19	11	0.1	0	30	0.03	13	17	0.6
III-IV	30	10		0	42		6	36	
LM									
Yes	30	14	0.4	0	44	0.05	24	22	0.3
No	21	7		0	28		11	15	
DM									
Yes	32	12	0.5	0	44	0.05	21	23	0.2
No	19	9		0	28		15	13	

LM: Lymph node Metastasis, DM: Distance Metastasis

The Association of *CDX1*, *CYLD* and *CDKN2B* expression with clinicopathological qualifications

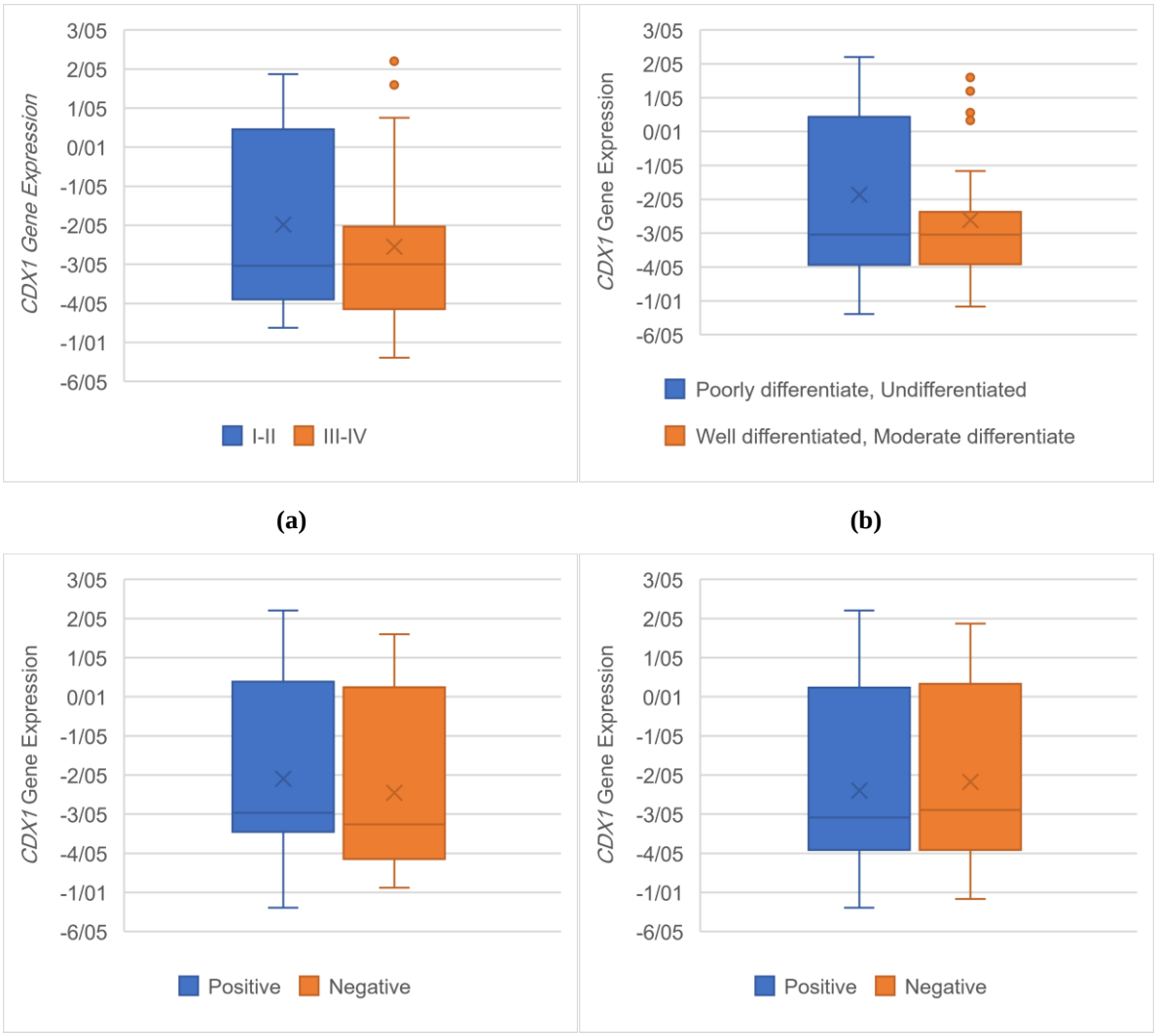
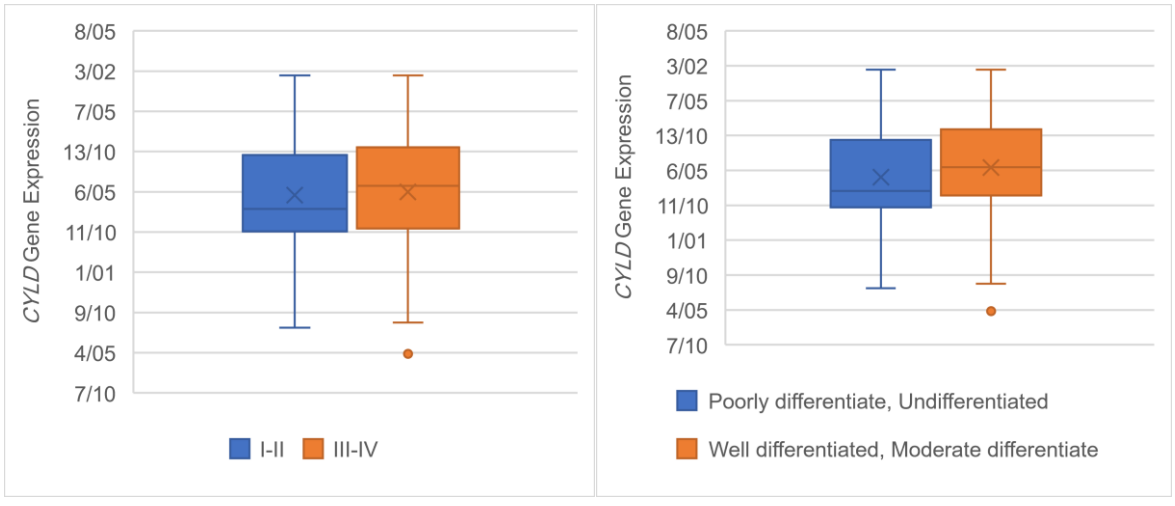
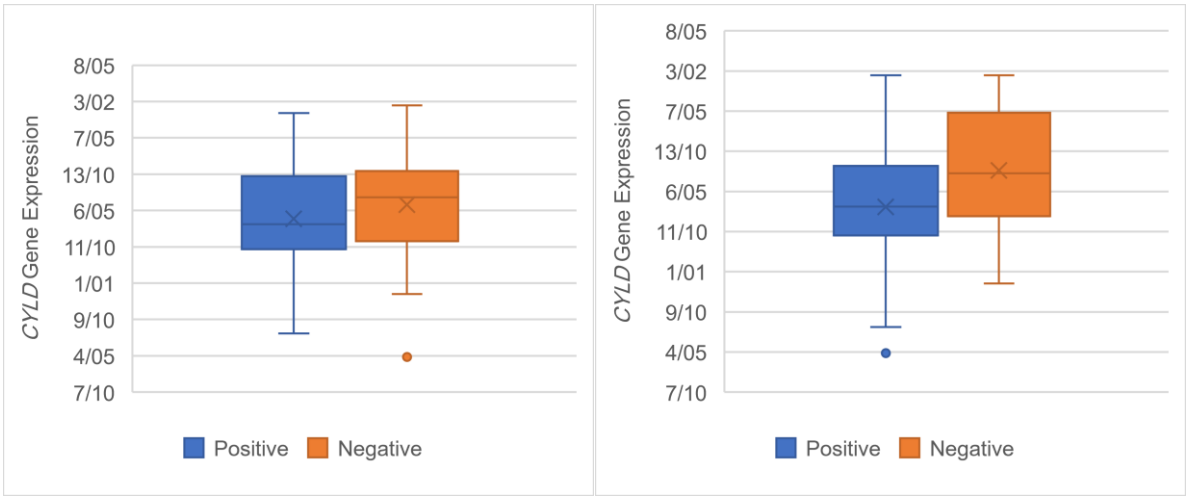


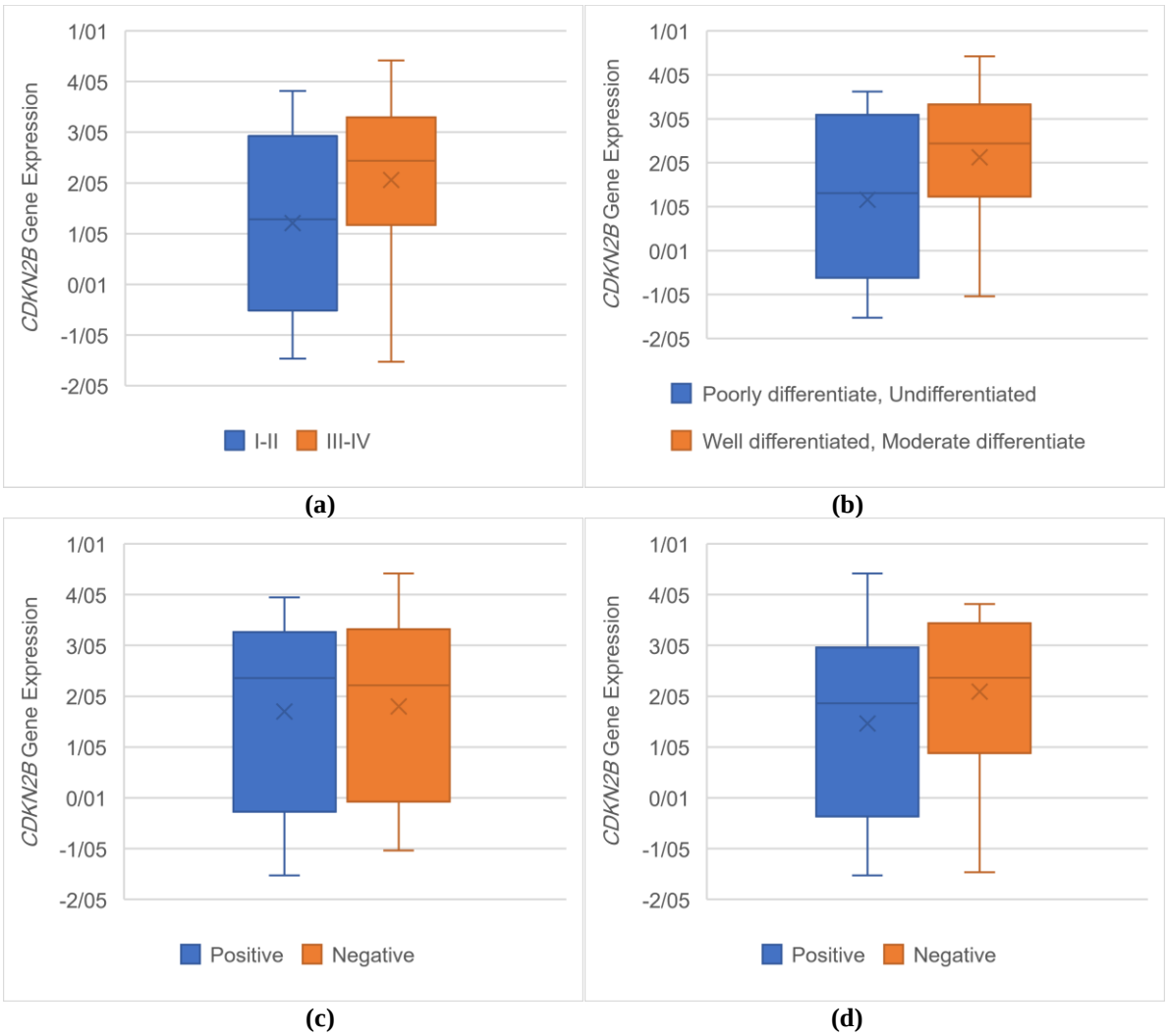
Figure 4. The Association of *CDX1* expression with clinicopathological qualifications. There was no significant association between *CDX1* downregulation with **(a)** tumor stage (P =0.7), **(b)** tumor grade (P =0.1), **(c)** lymph-node metastasis (P= 0.4) and **(d)** distance metastasis (P= 0.5).





(c) **(d)**

Figure 5. The Association of *CYLD* expression with clinicopathological qualifications. The *CYLD* 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)**.**



(a) **(b)**

(c) **(d)**

Figure 6. The Association of *CDKN2B* expression with clinicopathological qualifications. There was no significant association between CDKN2B 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).

Discussion

Transgenic expression of *CDX1* in mouse gastric epithelium causes intestinal transdifferentiation, which protects this consideration that *CDX1* is up-regulated in Barrett's metaplasia of the esophagus. Considerably, many transcriptional targets and effective activities of *CDX1* have been recognized, there remains much to learn about the mechanisms by which it encourages differentiation and, also, those by which it inhibits stemness *CDX1* action as transcription factors regulate a wide range of cellular mechanisms (6).

Additionally, *CDX1*, an intestine-specific transcription factor, is a candidate tumor suppressor gene and it manages the intestine-specific gene transcription and regulates the intestinal epithelial cell phenotype. Past investigation illustrated that the murine *CDX1* overexpression in rat normal intestinal epithelial cells regulates proliferation as a conclusion of inducing cell cycle arrest. Meaningly, this antiproliferative role may be mediated through down-regulation of the D-type cyclins (17). The *CDX1* gene is expressed in a collaborative model during intestinal progression. *CDX1* expression will last in the intestinal epithelium throughout life, notably in the crypt. The same model of *CDX1* expression was discovered in the human small intestine. Many searches have described that the *CDX1* expression is markedly down-regulated in both adenomas and carcinomas of the colon. Little is known about the molecular mechanisms that regulate the developmental and spatial patterns of the *CDX1* expression in normal intestine or what induces the down-regulation in colonic adenomas and cancers (18). Wong et al. have shown that the loss or reduction of *CDX1* is often induced by promoter methylation. Together, these observations indicate a potential role of *CDX1* loss in tumor development (19).

Recently, the expression monitoring of *CYLD* in many colorectal-related lesions and the clinical significance of *CYLD* expression in CRC have remained unclear, although, past investigation indicating that both the transcription function and the protein level of *CYLD* were downregulated in colon cancer in comparison with normal colon tissues. The difference of *CYLD* expression in the normal colorectal epithelium, benign adenoma, primary CRC and metastatic lesions was explored (20). Of particular interest, we wondered whether *CYLD* expression played a part in tumor development, progression, or metastasis and whether reduced *CYLD* expression was a good or poor prognostic factor for CRC patients. These findings strengthened the fact that *CYLD* functioned as a tumor-suppressor gene not only in the skin tumor but also in CRC. In addition, reduced *CYLD* expression was an independent factor for poor prognosis of CRC patients. Based on the evidence above, our results also recommended that the downregulation of *CYLD* might be involved in a series of important biological properties of colorectal cancer cells, such as carcinogenesis, tumor progression and metastasis (21). These findings also have implications on the tumor suppressor function of *CYLD*, as colonic inflammation in IBD patients is a risk factor for colorectal cancer. The potential association of *CYLD* gene suppression with colon cancer is more directly suggested by a study showing reduced expression of *CYLD* in colon cancer cell lines and tissue samples It is currently unknown how the *CYLD* gene is suppressed in IBD and colon cancer cells. Nevertheless, the mechanistic insight of *CYLD* gene repression has been provided by studies using other cancer models (22).

In another study, *CYLD* expression was analyzed in two of the most common human carcinomas worldwide. Colon carcinoma derives from intestinal epithelial cells and HCC derives from hepatocytes. We found reduced *CYLD* mRNA expression in all three HCC cell lines and eight colon carcinoma cell lines examined compared with normal primary cells. Additionally, reduction or loss of *CYLD* expression was found in situ in most hepatocellular and colon carcinoma compared with non-neoplastic tissue samples. Analysis on protein level confirmed these findings. Functional assays with *CYLD* transfected cell lines revealed that *CYLD* expression decreased NF-κB activity. Thus, functional relevant loss of *CYLD* expression may contribute to tumor development and progression, and may provide a new target for therapeutic strategies (11). *CDKN2B* is a cyclin-dependent kinase inhibitor and functions as a cell growth regulator that controls cell cycle G1 progression. Last investigations have acknowledged *CDKN2B* as a required tumor suppressor, and deletion of its enhancer element is related to many different malignancies. Silencing of *CDKN2B* gene expression by epigenetic modification characterize in multiple

myelomas gastric adenocarcinoma (23). Reexpression of *CDKN2B* in tumor-derived cells significantly attenuates the tumorigenic potential of the cells and delays tumor progression (24). Fluctuation of *CDKN2B*'s expression has been announced in association with many malignancies particularly, prostate, colorectal, breast, and liver cancer. Considerably, *CDKN2B* were ubiquitously expressed in colon cancer at different stages of tumorigenesis (25).

CDKN2B encoded by the INK4b-ARF-INK4a locus. It is an acknowledged tumor suppressor gene that can form a complex with *CDK4* or *CDK6* and inhibits the activation of the cyclin-dependent kinase and progression of the cell cycle. The INK4b-ARF-INK4a locus is organized by Polycomb repressive complexes. In this way, downregulation of *CDKN2B* was investigated in cancers (26). The epigenetic investigation of these genes alongside gene expression and also a mutation of other genes which are involved in GI cancers is recommended strongly.

Conclusion

It is concluded that the upregulation of *CYLD* and *CDKN2B* genes and downregulation of *CDX1* gene in tumoral tissues were impressive. Conspicuously, the modification of these genes expression can be accepted as the main biomarker in colorectal cancer.

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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