Immunohistochemical expression of cyclin D1 in colorectal adenomas: a clinicopathological study

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Background and objectives
The colorectum is the segment of the gastrointestinal tract most frequently affected by tumors. Most colonic tumors are benign epithelial polyps. There are many histologic types of polyps. The best characterized and most common cancer precursor is the adenomatous polyp. The size, number of adenomas, grade of dysplasia, and villous features predict the future risk for advanced neoplasia, including malignancy in patients who harbor adenomas. A number of studies have been published studies evaluating the clinical use of cyclin D1 immunohistochemical (IHC) expression as a predictor of malignant transformation in colorectal adenomas. The aim of the study was to evaluate the significance of IHC expression of cyclin D1 in colorectal adenomas as a marker for the prediction of malignant transformation.
Patients and methods
This study is a retrospective one in which a total of 39 formalin-fixed paraffin-embedded polypectomy specimens from patients with colorectal adenomas without concurrent or previous colorectal adenocarcinoma were retrieved from the archival materials during the period from March 2013 to March 2014. The histopathological diagnosis had been revised and all specimens were stained using IHC technique with cyclin D1.
Results
IHC expression of cyclin D1 had a significant correlation with villous type (P=0.003) and high-grade dysplasia in adenomas (P=0.021). However, there was no significant difference in the IHC expression of cyclin D1 according to the age and sex of the patients, and the size and site of colorectal adenomas (P>0.05).
Conclusion
Cyclin D1 potentially contributes to the multistep process of colorectal oncogenesis. It plays an important role in the malignant conversion of colorectal adenomas, as it is more likely to be expressed in advanced adenoma with high-grade dysplasia and villous histology and can be used as an ancillary marker for the risk for malignant transformation and as a target for chemoprevention with anti-inflammatory drugs.

Keywords:
colorectal adenomas, cyclin D1, dysplasia, immunohistochemistry, polyps

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Introduction

Colorectal cancer is a familiar cause of cancer-related death worldwide. Known genetic and epigenetic aberrations drive the formation of a benign adenoma and its progression to full-blown colorectal carcinoma [1]. The large majority of colorectal malignancies develop from an adenomatous polyp (adenoma). These can be defined as well-demarcated masses of epithelial dysplasia, with uncontrolled crypt cell proliferation [2].

The absorptive epithelium of the large intestine contains large numbers of cryptal cells. Differentiated cells (enterocytes, enteroendocrine cells, and goblet cells) occupy the crypt. The remaining part of the crypts is made up of stem cells and the proliferating progenitor compartment [3]. Stem cells reside near the bottom of the crypt and give rise to progenitor cells that are capable of differentiating toward all epithelial lineages. Stem cells self-renew to regenerate the epithelium after injury, whereas progenitor cells arrest their cell cycle and differentiate when they reach the tip of the crypt [4]. The presence of these cells renders the colonic epithelium the most rapidly self-renewing tissue. Epithelial renewal occurs in the crypts through a coordinated series of events such as proliferation, differentiation, and migration toward the large intestinal lumen [5]. All cells within the crypt are derived from the stem cells. One of the mitotic stem cells remains as a stem cell at the bottom of crypt and another cell is gradually pushed up to the luminal surface

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of the crypt as an epithelial cell. The cells that reached the uppermost part execute apoptosis and peel off without replicating or differentiating. Therefore, any mutations in these cells have essentially no impact on the normal turnover of mucosa. The cells with damaged DNA (mutated genes) do not cause apoptosis and reach the uppermost part in the crypt and continue proliferating. This is a precancerous change [6].

Biomarkers provide opportunities to understand cancer development and also to evaluate the efficacy of intervention. On the basis of epidemiologic, therapeutic, pathophysiological, clinical, and cost–benefit data, adenomas are considered to be endpoints in colorectal cancer (CRC) because removal of adenomatous polyps (adenomas) has been shown to reduce the risk of development of CRC. Much interest is currently directed toward research in the use of endpoint biomarkers that are altered early in colonic carcinogenesis, before polyp (adenoma) formation, to predict the clinical effectiveness of chemopreventive agents or drugs, because it takes 10–20 years for a normal cryptal cell to undergo molecular changes and to be clinically detected as a neoplasm [7]. A number of studies have been published evaluating the clinical use of cyclin D1 (CCND1) immunohistochemical (IHC) expression as a predictor of malignant transformation in colorectal adenomas. A significant correlation between the grade of dysplasia and CCND1 immunoreactivity was observed in some studies. Hence, the IHC analysis of CCND1 expression may be included as a part of routine pathological evaluation, useful in follow-up of patients with high-grade dysplastic colorectal adenomas [8].

The aim of the present research was to evaluate the significance of IHC expression of cyclin D1 as a marker for prediction of malignant transformation in colorectal adenomas in relation with different clinicopathologic parameters.

Patients and methods

In this retrospective study, 39 formalin-fixed paraffin-embedded polypectomy specimens from patients having single or two colorectal neoplastic mass, without concurrent or previous colorectal adenocarcinoma, were retrieved from the archival materials during the period from March 2013 to March 2014. Clinicopathological parameters including the age and sex of the patients, site, size, histological type of the polyp, and grade of dysplasia were obtained from the available histopathological reports. For each case, one representative section was stained with hematoxylin and eosin and the histopathological diagnosis was revised, whereas the second section was put on positively charged slides and stained IHC for CCND1.

The monoclonal antibody used in the current study was monoclonal mouse antihuman CCND1 manufactured by Dako (Denmark) (code number K0673). It is intended for laboratory use to identify qualitatively with light microscopy CCND1-positive cells in normal and neoplastic tissues using IHC test methods. The detection kit was immunophosphatase secondary detection system manufactured by Dako (DakoCytomation LSAB+System-HRP, code number K0673).

In each immunohistochemistry run, technical negative control slides were obtained by omitting the primary antibody of CCND1, and this was undertaken under identical test condition. The sections from a normal tonsil that were known to be immunoreactive for CCND1 were used as positive control according to the manufacturer's instructions.

Procedure of immunohistochemistry

Immunohistochemistry or immunoperoxidase staining technique is the localization of antigens or proteins in tissue sections with the use of labeled antibodies as specific reagents through antigen–antibody interactions that are visualized with the labeling method using three steps – the indirect streptavidin method. The procedure was carried out in accordance with the manufacturer's instructions.

Four-micrometer-thick sections were obtained from formalin-fixed paraffin-embedded tissue blocks and mounted on Fisherbrand positively charged slides. The slides were baked in a hot air oven at 65°C overnight, and then deparaffinization and rehyd ration were performed by immersing the slides sequentially in xylene and then in descending concentrations of ethanol at room temperature. The tissue sections were then placed in the recommended antigen retrieval solution and heated in hot air oven for 30 min at 95°C. Sufficient drops of peroxidase block reagent were applied onto the tissue, covering the whole section and incubated at room temperature for 5 min in a humid chamber. Sufficient drops of primary antibody were applied onto each section and incubated at 37°C overnight in a humid chamber; drops of secondary antibody (biotinylated link) were applied onto the sections and incubated at 37°C for 30 min in a humid chamber. Streptavidin reagent was

applied to tissue sections and incubated at 37°C for 30 min in a humid chamber. Substrate-chromogen solution was applied on each section and incubated in darkness at room temperature for 10 min. Counter staining was carried out with hematoxylin. The slides were mounted with an aqueous-base mounting medium.

Assessment of immunohistochemical staining [8]

In this study, we considered the cell stained for cyclin D1 when the nucleus was being stained; the pattern of stain is diffuse and color of stain was brown.

Expression was graded as follows:

5% or lower expression=negative;
6–25% expression=1+;
26–50% expression=2+;
51–75% expression=3+;
Over 76% expression=4+.

Statistical analysis

Statistical analysis was performed with statistical package for social sciences (SPSS, 10.01, SPSS Inc., Delaware, US) and also Excel 2010 programs (Excel 10: Microsoft, Las Vegas, US). Data analysis was performed using *t*-test and the χ^2-test for tables with frequencies, percentages, range mean, and SD. Values were considered statistically significant when *P*-value less than 0.05.

Results

Description of the sample

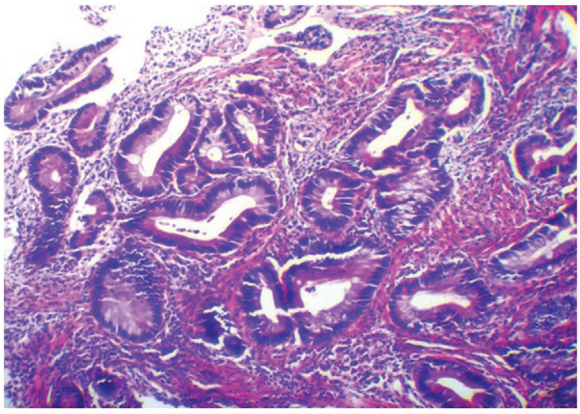
The mean age of patients having adenomatous polyp was 51.05±2.81 years. The median age was 52 years. Their ages ranged from 8 to 80 years. As regards sex distribution, there was a slight male predominance, with a male : female ratio of 1.17 : 1.

As regards the site and type and grade of dysplasia of colorectal adenomas, the distal colon was predominant site and represented 24 (62%) cases, compared with two (5%) cases with adenomas in the proximal site and 13 (33%) cases with adenomas in the rectum. The tubular adenomas (Fig. 1) have the greatest frequency, affecting 23 (59%) cases, compared with 11 (28%) cases for tubulovillous (Fig. 2) and five (13%) cases for villous type (Fig. 3). There were 12 (31%) cases with high-grade dysplasia and 27 (69%) cases with low-grade dysplasia (Figs 1–3) in different types of adenomas.

Cyclin D1 expressions in different clinicopathological parameters

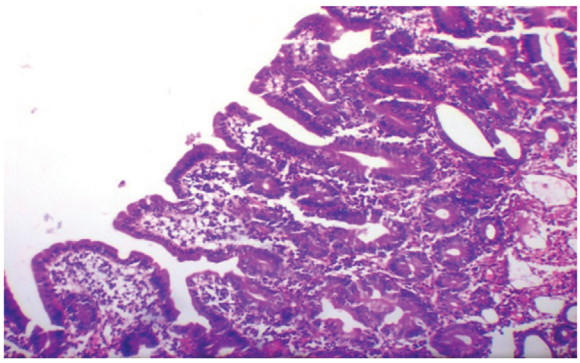
The mean Cyclin D1 expression was 35.9%, with a range of 0–80%. The median was 30% (Table 1).

Figure 1



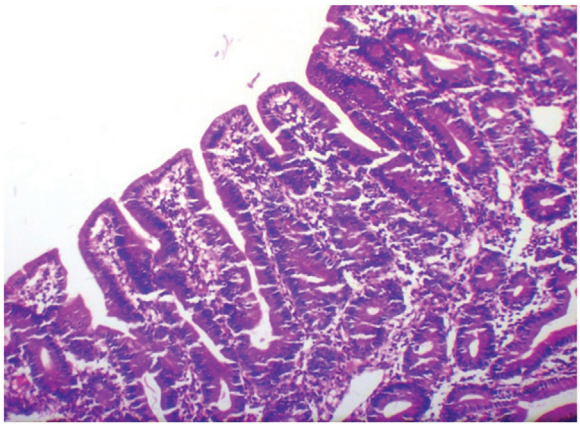
Tubular adenoma showing low-grade dysplasia. Hematoxylin and eosin stain, ×10.

Figure 2



Tubulovillous adenoma showing low-grade dysplasia. Hematoxylin and eosin stain, ×4).

Figure 3



Villous adenoma showing low-grade dysplasia Hematoxylin and eosin stain, ×10).

There was no significant correlation between the age and the IHC expression of cyclin D1 in different types of colorectal adenomas (*P*=0.729; *r*=0.057).

There was no significant difference in the IHC expression of cyclin D1 with respect to sex of patients. The mean cyclin D1% in male patients was 34.95±6.14%, whereas in female patients it was 37.0 ±6.65%; *P*-value was 0.822. Moreover, there was no significant association between the site of colorectal adenomas and the IHC expression of cyclin D1 (*P*=0.243) (Table 2).

As regards the type of adenoma, there was a significant correlation of the IHC expression of cyclin D1 with adenomas of villous histology (villous and tubulovillous) (*P*=0.003; *r*=0.464) (Figs 4–7), whereas no significant correlation with the size of colorectal adenomas was established (*P*=0.674; *r*=0.076) (Fig. 8).

There was a significant correlation between IHC expression of cyclin D1 and high-grade dysplasia (*P*=0.021) (Figs 9–11).

Discussion

Although the present study is not a large epidemiological one that expresses the prevalence and incidence of different clinicopathological features of colorectal adenomas, its uniqueness in being the first study conducted on Iraqi patients having colorectal adenomatous polyps for examining the role of CCND1 as a predictive marker for carcinogenesis renders it important. However, the limitations of the current study include the homogeneity of the population with regard to race/ethnicity and other characteristics.

Table 1 Classification of patients according to cyclin D1 score

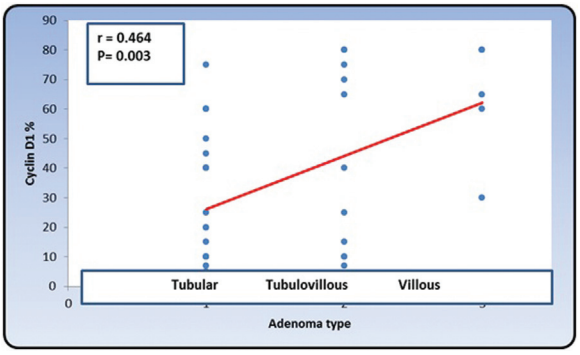
Cyclin D1 scores	*N* (%)
Negative	5 (12.82)
+1	13 (33.33)
+2	8 (20.51)
+3	9 (23.08)
+4	4 (10.26)
Total	39 (100.00)

Table 2 Association between site and cyclin D1% expression in colorectal adenomas

Cyclin D1 scores	Proximal [*N* (%)]	Distal [*N* (%)]	Rectum [*N* (%)]	Total [*N* (%)]
Negative	1 (50.00)	2 (8.33)	2 (15.38)	5 (12.82)
1	1 (50.00)	7 (29.17)	5 (38.46)	13 (33.33)
2	0 (00.00)	6 (25.00)	2 (15.38)	8 (20.51)
3	0 (00.00)	8 (33.33)	1 (7.69)	9 (23.08)
4	0 (00.00)	1 (4.17)	3 (23.08)	4 (10.26)
Total	2 (10.00)	24 (10.00)	13 (10.00)	39 (100.00)
P	0.243			

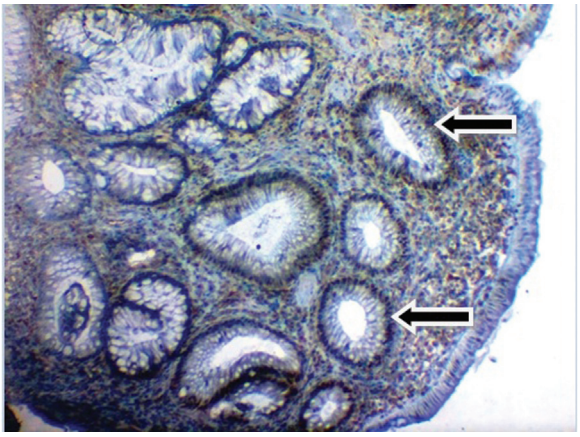
Accurate, long-term risk predictors for CRC development in patients with sporadic adenomas are lacking [9]. In this study, we validate biomarker

Figure 4



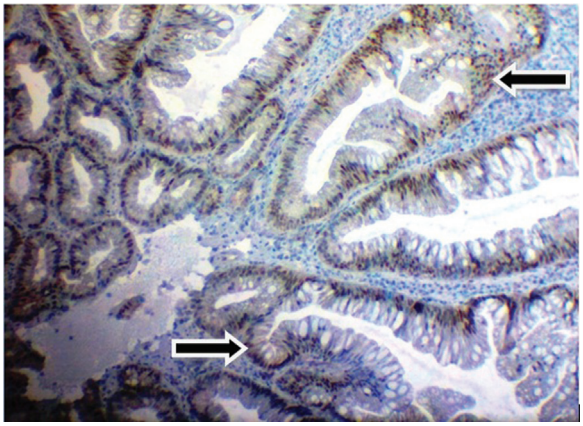
Correlation between type of adenoma and cyclin D1% expression in the studied group.

Figure 5



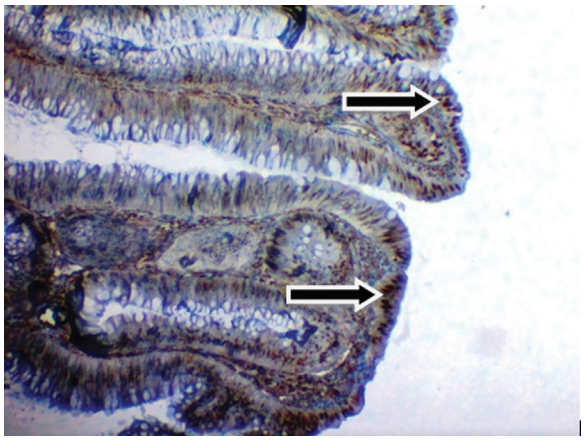
Immunohistochemical expression of cyclin D1 in low-grade tubular adenoma showing positive brown nuclear staining (arrows). ×10.

Figure 6



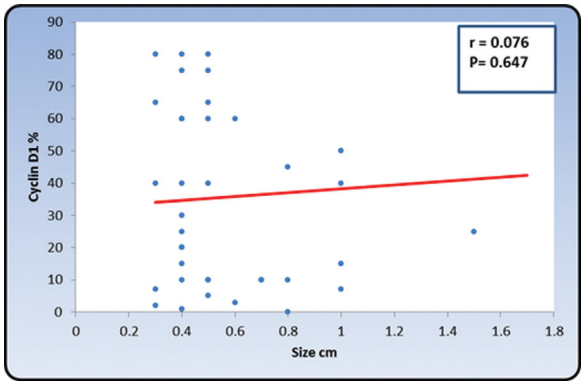
Immunohistochemical expression of cyclin D1 in low-grade tubulovillous adenoma showing positive brown nuclear staining (arrows). ×10.

Figure 7



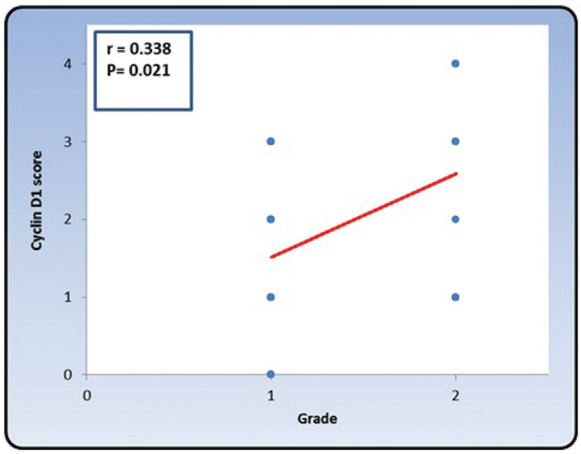
Immunohistochemical expression of cyclin D1 in high-grade villous adenoma showing positive brown nuclear staining (arrows). ×10.

Figure 8



Correlation between size of tumor and cyclin D1%.

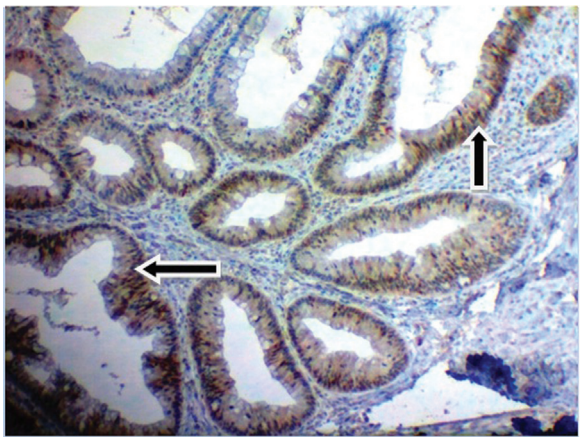
Figure 9



Correlation between grade of dysplasia of adenomas and cyclin D1% in studied group.

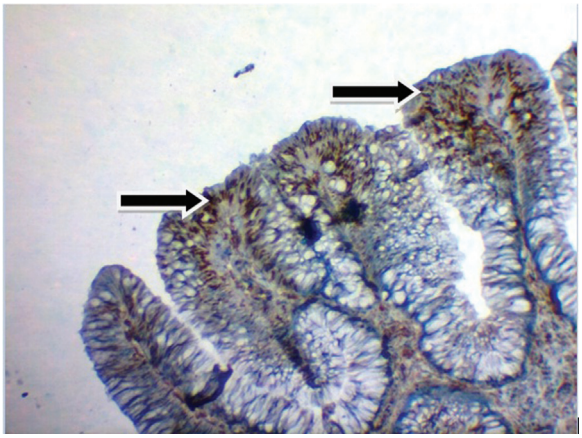
predictive of metachronous CRC development in patients with sporadic colorectal adenomas, using 39 consecutive patients.

Figure 10



Immunohistochemical expression of cyclin D1 in high-grade tubulo-villous adenoma showing positive brown nuclear staining (arrows). ×10.

Figure 11



Immunohistochemical expression of cyclin D1 in high-grade villous adenoma showing positive brown nuclear staining (arrows). ×10.

In the current research, there were 39 adenomatous polyp cases, of which 11 were of tubulovillous type, five were of villous type, and 23 cases of tubular type. Thus, the current study revealed that tubular type is the predominant type. This is in agreement with several previous studies conducted in Iran, Iraq, and Europe [10–12] that revealed tubular adenoma to be the most common type.

Studies conducted in Iraq, the USA, and Russia reported that about 68.9% of cases detected were mainly in the age group 50–69 years [11,13–15]. This is nearly similar to the result of the current study that revealed that 30.77% of patients were in the age group 61–70 years. The age of the patients varied from 8 to 80 years (mean: 51.05 years).

As regards sex, in the present study, there was a slight male predominance [21 (54%) male patients and 18

(46%) female patients]. This result is in accordance with the studies conducted in Iran, Iraq, and the USA, which also reported a slight male predominance [10,11,13,15].

This study revealed that more than half of the cases, regardless of the histopathological type of adenomas, were found in the distal colon (62%), which is the region of the colon that extends from the distal one-third of the transverse colon, including the descending and the sigmoid colon, and only 5% of cases were found in the proximal colon, which is the region of the colon that extends from the cecum, including the ascending colon and two-thirds of the transverse colon; 33% of cases were found in the rectum. Our findings are in concordance with other studies conducted in Egypt, Japan, and three studies in the USA, which found that adenoma in 40% of cases affect the distal side of the colon, 40% affect the proximal side, and 20% affect the rectum [15–20].

As regards dysplasia of colorectal adenomas in the current study, regardless of the association with any parameter, there were 27 (69%) cases of low-grade dysplasia and 12 (31%) cases of high-grade dysplasia. This expected finding is in agreement with other studies conducted in Iraq and Portugal [11,15,21].

Assessment of tumor cell proliferation may predict tumor behavior. Quantification of cell proliferative activity in neoplasia is currently the subject of considerable investigation. Thus, it provides simple method for assessment of growth fraction of tumors in paraffin-embedded sections [22].

The current work demonstrated that mean cyclin D1 expression in colorectal adenomas was 35.9%. Ayhan *et al.* [23] showed that only 9.1% of adenomas were overexpressing cyclin D1. However, Hur *et al.* [24] observed that IHC expressions of cyclin D1 were increased in both adenomas and adenocarcinomas, but that it is undetectable in normal colonic mucosa, indicating that the degree of induction of these proteins during carcinogenesis may be related to oncogenic transformation.

As regards the age, sex, site, and size of adenoma in the present study, there were no significant correlations between CCND1 expression and these parameters ($P>0.05$); this result is supported by a study completed in the USA in 1996 [25].

The present study revealed a significant correlation between markers expressed by villous adenomas, as opposed to tubular and tubulovillous adenomas; thus, it would be another candidate for a marker of malignant progression, given the different malignant potential of these two precancerous lesions. IHC approaches were applied to detect these markers in tissues; this is in concordance with the result obtained in a study conducted in the USA [26].

Some studies concluded that severity of dysplasia correlates significantly with expression of CCND1; thus, expression for CCND1 was strong in adenomas with high-grade dysplasia compared with low-grade dysplasia [8]; this supports the result of the present study ($P=0.021$). In other words, kinase with higher expression levels in colon polyps would be a potential biomarker for the development of colon cancer. However, a study conducted in the USA in 1996 by Arber *et al.* [25] is contradictory to this study, and this may be due to several factors including the difference in population, environment, in addition to different resources of CCND1 and different methods for CCND1 mutation analysis.

Multiple intestinal neoplasias (Min) mice have a heterozygous, dominant mutation in the adenomatous polyposis coli (*Apc*) gene, causing inappropriate regulation of cellular β-catenin pools, which is the motivating force in *Apc*-induced colonic neoplasia. In the early research studies with *APC*-mutated colon carcinoma cells, it was reported that β-catenin is present in the nucleus as a part of the T-cell factor/lymphoid enhancer factor (Tcf/Lef) transcription factor in the transcriptionally active type, and that reintroducing wild-type APC proficiently removes β-catenin from the Tcf/Lef complex [27]. The cellular levels of β-catenin are amplified at all stages of colon carcinogenesis, including dysplastic aberrant crypt foci, adenomas, and invasive carcinomas, in patients with colon cancer [28–32]. In the nucleus, Tcf/Lef-β-catenin complex activates a wide diversity of Wnt-responsive genes such as *cyclin D1* [33], which is often overexpressed in colon cancer [25,34,35]. Zhang *et al.*[36] also showed that cyclin D1 immunoreactivity in the intestinal epithelium of multiple intestinal neoplasias mouse intestine was restricted to the adenomatous areas, with a significantly higher percentage of positively staining nuclei in high-grade dysplasia versus low-grade dysplasia. Morphologically normal areas of intestinal epithelia were uniformly negative for cyclin D1 immunoreactivity. Immunoblot analysis of lysates from surgical specimens revealed increased levels of cyclin D1 in the majority of intestinal adenomas from human familial adenomatous polyposis patients in

comparison with the adjacent grossly normal colonic mucosa. They accomplished that augmented cyclin D1 immunoreactivity is associated with more severe dysplasia and that abnormal upregulation of these important G1 cell cycle proteins is a comparatively early event in intestinal carcinogenesis and that these changes may contribute to malignant progression within those lesions [36].

A large diversity of anti-inflammatory substances exert their chemopreventive properties in colorectal cancer by inhibiting nuclear accumulation of β-catenin and expression of cyclin D1 [37–40]. Studies in human colon cancer cells have found that inhibition of nuclear β-catenin by NSAIDs, such as sulindac and indomethacin, results in a dramatic downregulation of its transcriptional targets, including *cyclin D1* and *c-myc* [41,42]. In agreement with these *in-vitro* findings, Greenspan *et al.* [43] also found an association between nuclear β-catenin expression and positive cyclin D1 staining in the sporadic colon adenomas. They demonstrated that 75% of adenomas with nuclear β-catenin staining also exhibited nuclear cyclin D1 expression. In addition, the suppression of nuclear β-catenin by either ibuprofen or aspirin was associated with a reduction in nuclear cyclin D1 expression. They concluded that these data indicate that nuclear expression of β-catenin in adenomas can activate proliferative *Wnt* target genes, which can also be suppressed by NSAID intake.

Conclusion

Cyclin D1 potentially contributes to the multistep process of colorectal oncogenesis. It plays an important role in the malignant conversion of colorectal adenomas as it is more likely to be expressed in advanced adenoma with high-grade dysplasia and villous histology and can be used as an ancillary marker for the risk for malignant transformation and as a target for chemoprevention by anti-inflammatory drugs.

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Conflicts of interest

There are no conflicts of interest.

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