Expression of Cyclin D1 in Colorectal Cancer and Its Association With Clinicopathologic Parameters in Patients Underwent Colectomy

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Abstract- Colorectal cancer (CRC) is common cancer with a high mortality rate worldwide. Cyclin D1 is a gene that regulates cell cycle passage from stage G1 to S (G1/S checkpoint) and has recently been linked to the prognosis of a variety of cancers. Therefore, the aim of this study was to investigate the expression of cyclin D1 in colorectal cancers and its relationship with clinicopathologic factors. In this retrospective study, paraffin blocks of tumors of consecutive CRC patients registered in the histopathology laboratory of hospitals affiliated with Ahvaz Jundishapur University of Medical Sciences were used. Patients' clinicopathologic findings were extracted from their files, and using paraffin blocks, specific staining for cyclin D1 was performed using the immunohistochemistry method. Data were analyzed by SPSS software. In terms of staining, 11 samples (28.9%) scored 4, 11 samples (28.9%) scored 3, 8 samples (1/21%) scored 2, 3 samples (7.9%) scored 1, and 5 samples (2/13٪) scored zero. Staining intensity was severe in 10 cases (26.3%), moderate in 14 cases (36.8%), mild in 8 cases (21.1%), and negative in 6 cases (15.8%). The severity and extent of staining had no significant relationship with sex, age, tumor location, degree of differentiation (grade), depth of invasion, tumor size, lymph node involvement, and vascular and perineural invasion (P>0.05). Despite the high expression of cyclin D1 in colorectal carcinoma, no significant relationship was observed between its expression and prognostic factors, which is probably due to the small sample size.

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Keywords: Colorectal cancer; Cyclin D1; Immunohistochemistry; Clinical and pathological parameters

Introduction

Colorectal cancer (CRC) is the third most common cancer in the world after lung cancer and breast cancer. It is also the fourth most common cause of cancer death worldwide (1). CRC is the third most common cancer in men and the second most common cancer in women. In 2018, more than 10% of all new cancer cases were attributed to CRC, with approximately 1.8 million reported (2,3). The mortality rate from this cancer in both sexes and all ages was 9.2% of all cancer deaths and were equivalent to 880 thousand people. Colorectal cancer is the third most common cause of cancer death in men and women in the United States and the second leading cause of death due to cancer, regardless of gender (4). In the United States, in 2020, approximately 147,750 people were diagnosed with colorectal cancer, and 53,200 died of the disease, including 17,930 cases and 3,640 deaths in people under the age of 50 (5). In 2018, colorectal cancer accounted for 9864 new cases (in both men and women) out of 110115 new cases of all cancers (9%) in Iran, which was the third most common cancer after breast cancer (12.5%) and gastric cancer (10.6%) (6). The role of genetic modification in the formation of cancers, including CRC, has been well established. Problems in regulating cell proliferation are the main event in the formation and progression of cancerous masses (7). The role of genetic modification in the formation of cancers, including CRC, has been well established. Defects in the regulation of cell proliferation are the main event in the formation and progression of cancerous masses (8). This defect usually manifests itself by altering the cell cycle.

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Therefore, a better understanding of the cell cycle and its regulation is important for interpreting these changes (9). The goal of each cell cycle is to replicate the DNA of genetic material within the cell nucleus, and this cycle begins with the first interrupt phase (G1). The checkpoint in stage G1 is known as the restriction point. After this stage, the cell becomes independent, and no growth factor is required to enter the synthesis stage (S stage). After passing the checkpoint in G1, the cell is forced to complete the cycle (10,11). The controllers at these checkpoints are cyclins, especially cyclin D1, which precisely control the checkpoint. Cyclin D1 is encoded by the CCND1 gene on the 13th segment of chromosome 11 (12). Excessive expression of this protein disrupts the normal cell cycle, which in turn leads to the development and progression of cancer. Overexpression of cyclin D1 has been observed in many tumors, including the endometrium, thyroid, urinary tract, breast, brain glioma, and esophageal cancer (13). High regulation of cyclin D1 plays an important role in the pathogenesis and metastases of CRC and appears to be a useful prognostic marker for CRC (14). The aim of the present study was to investigate the expression pattern of cyclin D1 in colorectal cancer and its relationship with clinical and pathological parameters in patients referred to hospitals affiliated with Ahvaz Jundishapur University of Medical Sciences.

Materials and Methods

Case selection and tissue preparation

In this descriptive-analytical retrospective, epidemiological study, paraffin-embedded blocks of patients who underwent colectomy with colorectal carcinoma were retrieved from the archive of the pathology department, Imam Khomeini Hospital, Ahvaz, Iran, between the years 2018-2020. The hematoxylin-eosin-stained slides were reviewed. Inclusion criteria were adequate tumoral mass, absence of necrosis/hemorrhage, presence of lymph node pathologic slides, and complete medical records. The total number of colorectal adenocarcinoma paraffin samples from the years 2018 to 2020 was considered as the sample size. Based on the inclusion criteria, 38 formalin-fixed, paraffin-embedded samples were included. Clinical information, including sex, age, tumor location, tumor size, tumor differentiation degree, depth of tumor invasion, lymph node status, vascular invasion (lymphovascular), and perineural were extracted from patients' pathology reports and recorded in a checklist.

Immunohistochemical test

The 5-μm paraffinized sections were soaked in the water-alcohol solution for 5 minutes. Slides were placed in a microwave oven for 30 minutes at 60° C. Deparaffinization was performed by soaking the slides in xylene and then alcohol (from 100% to 75% concentration) for 5 to 10 minutes. Sections were rinsed with 10% phosphate-buffered saline (PBS) followed by H2O2/methanol (1:9) and 10% PBS for 10 minutes. Then, the slides were heated in a microwave oven for 10 minutes in ethylenediaminetetraacetic acid (EDTA). The samples were left to reach room temperature; then were rinsed with PBS. Sections were incubated with 1 μg/mL diluted primary antibody against cyclin D1 for 1 hour at room temperature [rabbit monoclonal (SP4) to cyclin D1, 1:100 dilution; Abcam] and then were reincubated with biotinylated antibody for 30 minutes and soaked in 10%PBS for 10 minutes. Sections were incubated with conjugated enzyme for 30 minutes and developed in 3, 3`- diaminobenzidine hydrochloride (DAB). Hematoxylin stain was used to develop the ground contrast. In all stages, a Mantle cell lymphoma sample was used as a positive control to ensure the accuracy of staining. Finally, the samples were examined under a light microscope. PBS was used instead of specific antibodies as the negative control. After preparing the immunohistochemical slide, a microscopic examination of antibody-labeled sections was performed. For this purpose, antibody-labeled sections were counted for microscopic examination, at least 1000 cells per slide were counted, and the percentage of stained nucleus of cells in tumor epithelial cells was determined. Then, the percentage of cells-stained brown compared to the cells that were blue in each tissue was determined and, based on cancer samples, were divided into three groups.

Every tumor was given a score according to the intensity of nuclear staining.Staining intensity was scored negative (0), weak (+1), moderate (+2), and strong (+3). Also, the extent of staining of cyclin D1 in terms of the percentage of stained nucleus of tumor epithelial cells was scored as follows:

- Immune staining in <5% of cells (score 0)
- Immune staining in 5-25% of cells (score 1)
- Immune staining in 26%-50% of cells (score 2)
- Immune staining in 51-75% of cells (score 3)
- Immune staining in 76-100% of cells (score 4)

Statistical analysis

Descriptive statistics, including mean index and standard deviation for quantitative variables and frequency and percentage for qualitative variables, were

used. The *Chi*-square test and Fisher's exact test were used to examine the relationship between qualitative variables. Significance level $P<0.05$, and all analyzes were performed using SPSS software version 22.

Ethics

The study was accepted by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences. (IR.AJUMS.HGOLESTAN.REC.1399.121). Written, informed consent was obtained from each patient.

Results

In the present study, biopsy specimens of 38 patients with colorectal cancer with a mean age of 55.34±13.70 were examined. The clinical and pathological characteristics of patients are shown in Table 1.

Table 1. Clinical features and pathology of patients

variable		Frequency	Percent
Gender	Female	20	52/6
	Male	18	47/4
Grade tumor	G1	13	34/2
	G2	20	52/6
	G3	5	13/2
Depth of tumor	PT1	1	2/6
	PT2	4	10/5
	PT3	23	60/5
	PT4a	6	15/8
	PT4b	3	7/9
	PTis	1	2/6
Tumor site	Rectum	13	34/2
	Cecum	2	5/3
	Sigmoid	14	36/8
	Colon	9	23/7
Lymph node involvement	PN0	21	55/3
	PN1a	1	2/6
	PN1b	6	18/8
	PN1c	3	7/9
	PN2a	3	7/9
	PN2b	4	10/5
Vascular invasion	No	21	55/3
	Yes	17	44/7
Perineural invasion	No	25	65/8
	Yes	13	34/2
Age (Mean±SD)		55/34±13/70	

In terms of the degree of staining cyclin D1 marker, out of 38 samples, 11 samples (28.9%) have a score of 4, 11 samples (28.9%) have a score of 3, 8 samples (21.1%) have a score of 2, 3 samples (9/7). 2) had a score of 1, and 5 cases (13.2%) had a score of zero. In terms of staining intensity cyclin D1 marker, out of 38 samples, staining intensity was intense in 10 cases (26.3%), medium in 14 cases (36.8%), and mild in 8 cases (21.1%). In 6 cases (15.8%), it was negative (Figures 1-3).

The results of the *Chi*-square test showed that there was no significant relationship between gender with both degree and staining intensity of cyclin D1 marker ($P>0.05$) (Table 2).

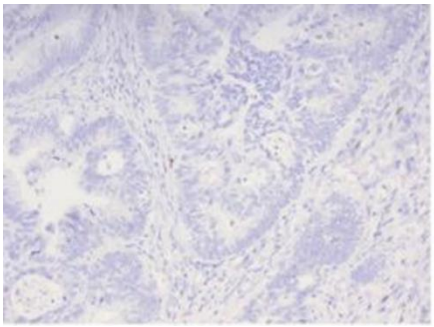

Figure 1. Negative staining for cyclin D1

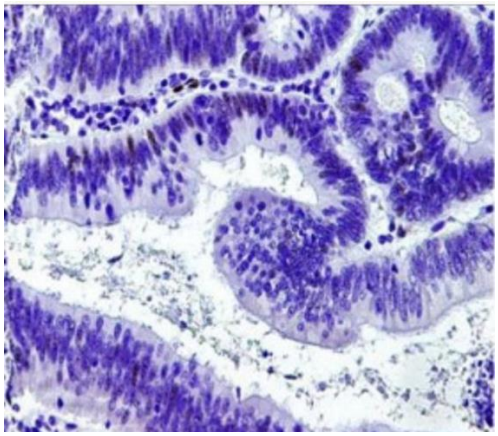

Figure 2. Poor staining for cyclin D1

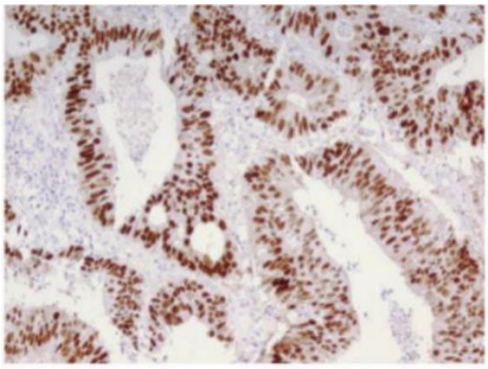

Figure 3. Strong staining for cyclin D1

Table 2. Relationship between gender with staining degree and staining intensity

Variable		Gender		*P*
		Female	Male	
Staining degree	0	(٪ 15/0) 3	(٪ 11/1) 2	0/892
	1	(٪ 5/0) 1	(٪ 11/1) 2	
	2	(٪ 25/۰)5	(٪ 16/7) 3	
	3	(٪ 30/0) 6	(٪ 27/8) 5	
	4	(٪ 25/0) 5	(٪ 33/3) 6	
Staining intensity	Intense	(٪ 30/0) 6	(٪ 22/2) 4	0/741
	Medium	(٪ 30/0) 6	(٪ 44/4) 8	
	Mild	(٪ 20/0) 4	(٪ 22/2) 4	
	Negative	(٪ 20/0) 4	(٪ 11/1) 2	

The one-way analysis of variance (ANOVA) demonstrated that there was no significant relationship between age, both degree and staining intensity of cyclin D1 marker (P>0.05) (Table 3).

The results of the *Chi*-square test showed that there was no significant relationship between variables gender, tumor location, tumor grade (well, moderate, and poorly differentiated), depth of tumor invasion, lymph node involvement, vascular invasion, and perineural invasion with both degree and staining intensity of cyclin D1 marker (P>0.05) (Table 4,5,6,8,9 and 10).

The results of the Kruskal-Wallis test demonstrated that there was no significant relationship between tumor size and degree staining of cyclin D1 marker (P>0.05). The results of the Kruskal-Wallis test indicated that there was a significant relationship between tumor size and staining intensity, so that in cases with larger tumor size, staining intensity was lower (P<0.05) (Table 7).

Table 3. Relationship between age with staining degree and staining intensity

		Age (mean±SD)	*P*
Staining degree	0	6312±	0.518
	1	10.69±57.66	
	2	15.79±56.75	
	3	12.50±54.45	
	4	14.67±50.72	
Staining intensity	Intense	11.12±53.70	0.410
	Medium	15.99±60.00	
	Mild	14.35±50.12	
	Negative	10.10±54.16	

Table 4. Relationship between tumor site with staining degree and staining intensity

		Tumor site				*P*
		Rectum	Cecum	Sigmoid	Colon	
Staining degree	0	(%15.4) 2	(%0.0) 0	(%21.4) 3	(%0.0) 0	0.646
	1	(%0.0) 0	(%0.0) 0	(%14.3) 2	(%11.1) 1	
	2	(%30.8) 4	(%50.0) 1	(%7.1) 1	(%22.2) 2	
	3	(%38.5) 5	(%0.0) 0	(%21.4) 3	(%33.3) 3	
	4	(%15.4) 2	(%50.0) 1	(%35.7) 5	(%33.3) 3	
Staining intensity	Intense	(%30.8) 4	(%0.0) 0	(%28.6) 4	(%22.2) 2	0.884
	Medium	(%38.5) 5	(%50.0) 1	(%28.6) 4	(%44.4) 4	
	Mild	(%7.7) 1	(%50.0) 1	(%28.6) 4	(%22.2) 2	
	Negative	(%23.1) 3	(%0.0) 0	(%14.3) 2	(%11.1) 1	

Table 5. Relationship between tumor differentiation with staining degree and staining intensity

Variable		Differentiation			*P*
		G1	G2	G3	
Staining degree	0	(%23.1) 3	(%5.0) 1	(%20.0) 1	0.582
	1	(%15.4) 2	(%5.0) 1	(%0.0) 0	
	2	(%23.1) 3	(%25.0) 5	(%0.0) 0	
	3	(%23.1) 3	(%30.0) 6	(%40.0) 2	
	4	(%15.4) 2	(%35.0) 7	(%40.0) 2	
Staining intensity	Intense	(%38.5) 5	(%25.0) 5	(%0.0) 0	0.279
	Medium	(%38.5) 5	(%40.0) 8	(%20.0) 1	
	Mild	(%15.4) 2	(%15.0) 3	(%60.0) 3	
	Negative	(%7.6) 1	(%20.0) 4	(%20.0) 1	

Table 6. Relationship between depth tumor with staining degree and staining intensity

		Depth of tumor						*P*
		PT1	PT2	PT3	PT4a	PT4b	PTis	
Staining degree	0	(%0.0) 0	(%0.0) 0	(%0.0) 0	(%16.7) 1	(%0.0) 0	(%100.0) 1	0.160
	1	(%0.0) 0	(%0.0) 0	(%13.0) 3	(%0.0) 0	(%0.0) 0	(%0.0) 0	
	2	(%100) 1	(%50.0) 2	(%17.4) 4	(%0.0) 0	(%33.3) 1	(%0.0) 0	
	3	(%0.0) 0	(%25.0) 1	(%39.1) 9	(%0.0) 0	(%33.3) 1	(%0.0) 0	
	4	(%0.0) 0	(%25.0) 1	(%17.4) 4	(%83.3) 5	(%33.3) 1	(%0.0) 0	
Staining intensity	Intense	(%100) 1	(%25.0) 1	(%26.1) 6	(%16.7) 1	(%0.0) 0	(%100.0) 1	0.315
	Medium	(%0.0) 0	(%75.0) 3	(%39.1) 9	(%0.0) 0	(%66.7) 2	(%0.0) 0	
	Mild	(%0.0) 0	(%0.0) 0	(%17.4) 4	(%50.0) 3	(%33.3) 1	(%0.0) 0	
	Negative	(%0.0) 0	(%0.0) 0	(%17.4) 4	(%33.3) 2	(%0.0) 0	(%0.0) 0	

Table 7. Relationship between tumor size with staining degree and staining intensity

		Tumor size (Mean±SD)	*P*
Staining degree	0	1.28±4.22	0.216
	1	2.08±6.33	
	2	2.12±3.77	
	3	2.78±5.40	
	4	2.54±6.09	
Staining intensity	Intense	2.63±4.18	0.007
	Medium	1.25±4.21	
	Mild	2.22±7.56	
	Negative	2.59±5.91	

Table 8. Relationship between lymph node involvement with staining degree and staining intensity

		lymph node involvement						*P*
		PN0	PN1a	PN1b	PN1c	PN2a	PN2b	
Staining degree	0	(%19.0) 4	(%0.0) 0	(%0.0) 0	(%0.0) 0	(%0.0) 0	(%25.0) 1	0.800
	1	(%9.5) 2	(%0.0) 0	(%0.0) 0	(%33.3) 1	(%0.0) 0	(%0.0) 0	
	2	(%23.8) 5	(%0.0) 0	(%33.3) 2	(%0.0) 0	(%33.3) 1	(%0.0) 0	
	3	(%28.6) 6	(%100) 1	(%33.3) 2	(%0.0) 0	(%33.3) 1	(%25.0) 1	
	4	(%19.0) 4	(%0.0) 0	(%33.3) 2	(%66.7) 2	(%33.3) 1	(%50.0) 2	
Staining intensity	Intense	(%19.0) 4	(%100) 1	(%33.3) 2	(%33.3) 1	(%66.7) 2	(%0.0) 0	0.144
	Medium	(%52.5) 11	(%0.0) 0	(%33.3) 2	(%33.3) 1	(%0.0) 0	(%0.0) 0	
	Mild	(%9.5) 2	(%0.0) 0	(%33.3) 2	(%33.3) 1	(%0.0) 0	(%75.0) 3	
	Negative	(%19.0) 4	(%0.0) 0	(%0.0) 0	(%0.0) 0	(%33.3) 1	(%25.0) 1	

Table 9. Relationship between vascular invasion with staining degree and staining intensity

		Vascular invasion		*P*
		NO	Yes	
Staining degree	0	(%19.0) 4	(%5.9) 1	0.532
	1	(%9.5) 2	(%5.9) 1	
	2	(%23.8) 5	(%17.6) 3	
	3	(%28.6) 6	(%29.4) 5	
	4	(%19.0) 4	(%41.2) 7	
Staining intensity	Intense	(%23.8) 5	(%29.4) 5	0.101
	Medium	(%52.4) 11	(%17.6) 3	
	Mild	(%9.5) 2	(%35.5) 6	
	Negative	(%14.3) 3	(%17.6) 3	

Table 10. Relationship between perineural invasion with staining degree and staining intensity

		Perineural invasion		*P*
		NO	Yes	
Staining degree	0	(%18.2) 4	(%6.3) 1	0.388
	1	(%13.6) 3	(%0.0) 0	
	2	(%18.2) 4	(%25.0) 4	
	3	(%27.3) 6	(%31.2) 5	
	4	(%22.7) 5	(%37.5) 6	
Staining intensity	Intense	(%27.3) 6	(%25.0) 4	0.613
	Medium	(%40.9) 9	(%31.3) 5	
	Mild	(%13.6) 3	(%31.3) 5	
	Negative	(%18.2) 4	(%12.5) 2	

Discussion

In the present study, the expression of cyclin D1 marker in 38 colorectal cancer specimens and its relationship with clinical and pathological parameters were examined. 11 samples (28.9%) had a score of 3, 8 samples (1/21%) had a score of 2, 3 samples (7.9%) had a score of 1, and 5 samples (2.13%) had a score of 0. Staining intensity was reported to be severe in 10 cases (26.3%), moderate in 14 cases (36.8%), mild in 8 cases (21.1%), and negative in 6 cases (15. 8%).

In the study of Albasri *et al*., (2019) (14), overexpression of cyclin D1 was not observed in normal mucosa, while 15% of adenoma cases and 24.1% of CRC cases had high expression levels (4). In the present study, the rate of staining for grade 4 and the intensity of staining for cyclin D1 was 28.9% and 26.3%, respectively, which is consistent with our study.

In a study by Nosho *et al*., (2008) on 865 patients with colorectal cancer, 246 cases (28.4%) showed overexpression of cyclin D1 in immunohistochemistry (15). In a study by Al-Maghrabi *et al*., (2015) on 117 patients with primary colorectal cancer in Saudi Arabia, high expression of cyclin D1 was observed in 23.1% of primary tumors and 31% of tumors with nodular metastasis (16). In the Formentini study *et al*., (2012) on 140 patients with colorectal cancer, paleness and high immune response of cyclin D1 were present in 98 (70%) and 42 (30%) of cancers, respectively (17). The results of the above 3 studies were consistent with the present study. Differences in the detection rate of cyclin D1 expression can be due to several factors, including different tumor phenotypes, different scoring systems, antibodies used, and different ethnic groups of patients (18).

In the present study, there was no significant relationship between gender and age of patients with the intensity of staining for cyclin D1 in immunohistochemical staining. In a study by Albasri *et al*., (2019) on 324 CRC patients in Saudi Arabia, the

expression of cyclin D1 had no significant relationship with age and sex and was consistent with the present study results (14).

In a study by Al-Maghrabi *et al*., (2015) on 117 patients with colorectal cancer in Saudi Arabia, cyclin D1 expression was not significantly related to age and gender (19). In the study of Lam *et al*., (2000), the expression of cyclin D1 in oral squamous cell carcinoma was not related to age and sex (20). In the study of John *et al*., (2018) on 60 patients with oral cancer, there was no significant relationship with age and sex (18). The last 3 studies are also consistent with the present study. However, in a meta-analysis study of 22 observational articles and a total of 4150 patients with CRC, Li *et al*., (2014) reported that overexpression of cyclin D1 in CRC was significantly related with age in older patients (≥60 years) but has not been related to gender (21).

In the present study, the intensity and level of staining for cyclin D1 in immunohistochemistry technique had no significant relationship with tumor location, differentiation, depth of invasion, tumor size, lymph node involvement, vascular and perineural invasion. Tumor invasion, vascular invasion, perineural invasion, and lymph node involvement were related, but no relation was observed for staining intensity.

A study by Al-Maghrabi *et al*., (2015) on 117 patients with primary colorectal cancer in Saudi Arabia found that cyclin D1 was not significantly related to tumor size, tumor location, tumor grade, depth of tumor invasion, and nodal metastasis, and only with lymphovascular invasion was significantly related (16). In a study by Jang *et al*., (2012) on 220 CRC patients, expression of cyclin D1 was not related to tumor location, tumor size, tumor differentiation, nodal metastasis, and lymphovascular metastasis but was significantly related to recurrence and/or metastasis (22). The relationship between cyclin D1 and pathological findings was consistent with the above two studies.

Contrary to the above studies in the study of Albasri *et al*., (2019) on 324 CRC patients in the pathology department of King Fahd Hospital in Saudi Arabia, cyclin D1 had no significant relationship with tumor size, type, and location, but tumor differentiation, lymph node involvement, lymphovascular invasion, Distant metastasis, and AJCC staging were significantly related (14). In a meta-analysis study of 22 observational articles and a total of 4150 patients with CRC, Li *et al*., (2014) stated that overexpression of cyclin D1 in CRC was significantly related with tumor invasion, and lymph node involvement, and distant CRC invasion (21). In a study by Bahnassy *et al*., (2004) on 60 patients with colorectal cancer, the Ki-67 staining index, cyclin A and D1 had a significant relationship with tumor size, tumor invasion depth, and nodal metastasis (23) due to the discrepancies in studies on the relationship between cyclin D1 expression and pathological findings require further studies.

Out of 38 colorectal cancer samples studied, 11 samples (28.9%) scored 4, 11 samples (28.9%) scored 3, 8 samples (21.1%) scored 2 in terms of immunohistochemical staining for cyclin D1, 3 samples (7.9%) had a score of 1, and 5 samples (2.13%) had a score of 0. Staining intensity was severe in 10 cases (26.3%), moderate in 14 cases (36.8%), mild in 8 cases (21.1%), and negative in 6 cases (15.8%). The severity and extent of staining had no significant relationship with sex, age, tumor location, degree of differentiation, invasion depth, tumor size, lymph node involvement, and vascular and perineural invasion (P>0.05).

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