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Experimental Study

Immunoexpression of cyclin D1 in colorectal carcinomas is not correlated with survival outcome

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ABSTRACT

Background: Colon and colorectal cancer (CRC) research has entered a new era with recent updates of molecular events and prognostic markers. Among other prognostic markers, exaggerated expression of nuclear CCND1 has key role in tumour pathogenesis and metastases of CRC and has also been claimed to predict response to treatment.
Objectives: This study was designed to evaluate the prognostic and predictive value of CCND1 in CRC and the correlation of CCND1 expression with the different clinicopathological parameters.
Methods: Paraffin blocks from 117 primary CRC were retrieved from the archives of the Department of Pathology at King Abdulaziz University. Tissue microarrays were designed and constructed. The immunostaining of CCND1 was performed and analysed.
Results: There were more cases with low nuclear immunoexpression of CCND1in both primary tumours and nodal metastasis ($p < 0.001$). Cyclin D1 did not show association with clinicopathological features except with lymphovascular invasion. Low nuclear immunoexpression of CCND1 was associated with negative lymphovascular invasion ($p = 0.046$). There was no statistically significant correlation between CCND1 immunoexpression and survival probability (Log Rank = 2.474, $p = 0.116$).
Conclusion: Our study indicates that CCND1 immunoexpression cannot be used as a predictor of survival in CRC. It also shows no significant correlation with clinicopathological features except with lymphovascular invasion.

1. Introduction

New advances in the molecular pathology of cancer over the past two decades described key signalling pathways involved in malignant progression of colorectal carcinoma (CRC). Upregulation of nuclear cyclin D1 (CCND1) plays an important role in pathogenesis and metastases of CRC [1,2]. High nuclear CCND1 expression occurs in one-third of CRC [3]. Sensitive biological markers are needed to maximise the benefit of therapeutic approaches in CRC [4]. Study of molecular events and prognostic factors is therefore important in CRC research. In CRC cell lines, CCND1 downregulation has anti-APC mutation effect in transgenic mice [5].

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CCND1 interacts with other proteins such as DNA repair proteins [6,7]. Between CCND1a and CCND1b, only CCND1a transfer to nuclear chromatin sufficiently provokes DNA damage response (DDR) [6]. Reduction of endogenous CCND1 in CRC cells also reduces the DDR in response to 5-FU treatment [6].

Arrays of core biopsies obtained from paraffin-embedded tissues are called as tissue microarrays (TMAs). These arrays serve as an excellent means for high-throughput gene or protein expression analysis among population based large study groups of cancer patients on a single slide. This technology is increasingly being used for the high throughput analysis of the diagnostic, predictive, or prognostic value of biomarkers in tissue specimens. Immunohistochemical (IHC) methods seem to be most ideal for validation since the tissue based studies are present in the form of formalin fixed paraffin-embedded tissue blocks [8]. The principle of TMA is miniaturisation and a high throughput gene or protein expression analysis. TMA technology has been implicated widespread in cancer studies including CRC [9,10].

The aim of this study was to evaluate the prognostic and predictive value of cyclin D1 in colorectal carcinomas and the correlation of cyclin D1 expression with the different clinicopathological features in patients attending King Abdulaziz University Hospital from 1995 to 2012.

2. Materials and methods

2.1. Patients

The study included paraffin wax blocks of tumour from 117 patients with CRC and 29 corresponding to nodal metastases. Blocks were collected from the Department of Pathology at King Abdulaziz University, Jeddah, Saudi Arabia from 1995 to 2012. Patients' demographic data is listed in Table 1. The study was approved by the Research Committee of the Biomedical Ethics Unit, Faculty of Medicine, King Abdulaziz University. Disease-free survival (DFS) was calculated as the time from diagnosis to the appearance of recurrent disease (or date last seen disease-free).

2.2. Tissue microarray (TMA) construction

TMAs were constructed as described by Kallioniemi [11]. New sections were prepared from the donor blocks and stained with haematoxylin–eosin (H&E). These slides were used to guide the samplings from morphologically representative regions of the tissues. TMA block was constructed using one punch from each colorectal carcinoma depending on the most cellular region of the tumour marked by a pathologist. A tissue arrayer (Tissue Micro Array Master 3D Histech, EU) was used to create TMAs.

2.3. Immunohistochemical staining

Immunostaining staining for the CCND1 to sections of the formalin-fixed colonic biopsies microarray was carried out. Four micrometre thick sections were prepared from paraffin blocks and mounted on positive charged slides. Sections were deparaffinised and rehydrated. Slides were immersed in H_2O_2 (0.3%) for 12 min to block the endogenous peroxidase activity. Slides were then pre-treated in microwave oven in 10 mM citrate buffer (pH 6) for three cycles of 5 min each. Immunostaining with CCND1 using Ventana "ready to use" kit was performed in an automated immunostainer (BenchMark XT, Ventana Medical Systems Inc., Tucson, AZ, USA) according to the instruction manual attached. Subsequently, sections were washed, counterstained with haematoxylin and mounted. Negative control (by substitution of primary antibody with Tris-buffered saline) was used. Positive control was used as those from breast cancer.

Table 1
Clinicopathological parameters of CRC ($n = 117$) patients attending King Abdulaziz University Hospital, Jeddah.

Parameter		Number (%)
Sex	Male	59 (50.4%)
	Female	58 (49.6%)
Grade	Well-differentiated	32 (27.4%)
	Moderately differentiated	70 (59.8%)
	Poorly differentiated	15 (12.8%)
Age	<60 years	58 (49.6%)
	≥60 years	59 (50.4%)
Tumour location	Right colon	37 (31.6%)
	Left colon	71 (60.7%)
	Rectum	9 (7.7%)
Tumour size	<5 cm	51 (43.6%)
	≥5 cm	66 (56.4%)
Primary tumour	T1	2 (1.7%)
	T2	21 (17.9%)
	T3	87 (74.4%)
	T4	7 (6%)
Nodal metastasis	Negative	68 (58.1%)
	Positive	49 (41.9%)
Distant metastasis	Negative	86 (73.5%)
	Positive	31 (26.5%)
Lymphovascular invasion	Negative	104 (88.9%)
	Positive	13 (11.1%)
Margin status	Free	112 (95.7%)
	Involved	5 (4.3%)
Relapse	No relapse	79 (67.5%)
	Relapse	38 (32.5%)

T1: Tumour involves submucosa.
T2: Tumour involves muscularis propria.
T3: Tumour crosses through the muscularis propria into the subserosa or into non-peritonealised pericolic or perirectal tissues.
T4: Tumour directly involves other organs/structures, and/or perforates visceral peritoneum.

2.4. Scoring of immunohistochemistry

The intensity of the staining was graded as: 0, no staining; 1, mild staining; 2, moderate staining; and 3, marked staining. The percentage of staining was reported as: 0, less than 5%; 1, 5–25%; 1, 26–50%; 3, 51–75%; and 4, more than 75%. The final score was calculated by the sum of intensity and percentage as follow: 0, 0–1; 1, 2; 2, 3–5; 3, 6–7 [12]. For statistical purpose, CCND1 immunoscores were dichotomised as low expression (0 and 1), and high expression (2 and 3).

Table 2
Categories of immunoexpression of CCND1 in primary CRC and nodal metastases among patients attending King Abdulaziz University Hospital, Jeddah.

	Primary tumour ($n=117$)	Nodal metastasis ($n=29$)	p value
Low expression	90 (76.9%)	20 (69%)	0.34**
High expression	27 (23.1%)	9 (31%)	
p value	<0.001*	<0.001*	

* One sample non-parametric chi-square test.
** Mann–Whitney test.

2.5. Statistical analysis

Differences between two groups of patients on one variable were tested by using Mann–Whitney test. To test association procedure in three groups of patients on one independent variable the Kruskal–Wallis test was used. Chi-square was used to test association between CCND1 expression and clinicopathological features. Multivariate analysis was used to predict nodal metastasis, distant metastasis, surgical resection margins, lymphovascular invasion, and recurrence in relation immunoexpression of cyclin D1. The Kaplan–Meier procedure was used to calculate the survival probabilities and the Log Rank test was used to compare the difference between survivals. The endpoint for patients was death from tumour (disease-free). Disease-free survival (DFS) was calculated as the time from diagnosis to the appearance of recurrent disease (or date last seen disease-free). Statistical procedures were performed using SPSS® Release 16.0. Statistical significance was determined at p value of ≤ 0.05 and was 2-sided.

Table 4
Multivariate analysis for CCND1 immunoexpression among CRC patients attending King Abdulaziz University Hospital, Jeddah.

Variable	Adjusted R square	p value
Nodal metastasis	0.006	0.552
Distant metastasis	0.002	0.365
Surgical resection margins	0.000	0.322
Lymphovascular invasion	0.036	0.025
Recurrence	0.000	0.311

3. Results

In Table 2 we present the categories of immunoexpression in primary CRC and nodal metastases. Twenty-seven patients showed high CCND1 immunoexpression and its correlation to clinicopathological parameters is presented in Table 3. In Table 4 we present the multivariate analysis for CCND1 immunoexpression. Fig. 1 presents the CCND1 nuclear expression in CRC.

Table 3
Distribution of high CCND1 immunoexpression ($n=27$) in relation to clinicopathological parameters among CRC patients attending King Abdulaziz University Hospital, Jeddah.

		Number (%)	p value
Grade	Well-differentiated	10 (37%)	0.257*
	Moderately differentiated	14 (51.9%)	
	Poorly differentiated	3 (11.1%)	
Sex	Male	13 (48.1%)	0.829**
	Female	14 (51.9%)	
Age	>60 years	15 (55.6%)	0.517**
	≥60 years	12 (44.4%)	
Tumour location	Right colon	5 (18.5%)	0.087*
	Left colon	19 (70.4%)	
	Rectum	3 (1.1%)	
Tumour size	<5 cm	12 (40%)	0.545**
	≥5 cm	15 (60%)	
Depth of invasion (pT)	T1	2 (7.4%)	0.441*
	T2	5 (18.5%)	
	T3	18 (66.7%)	
	T4	2 (7.4%)	
Nodal metastasis ($n=29$)	Negative	16 (59.3%)	0.537**
	Positive	11 (40.7%)	
Distant metastasis	Negative	22 (81.5%)	0.269**
	Positive	5 (18.5%)	
Lymphovascular invasion	Negative	21 (77.8%)	0.046**
	Positive	6 (22.2%)	
Margin status	Free	25 (92.6%)	0.326**
	Involved	2 (7.4%)	
Relapse	Relapse	20 (74.1%)	0.268**
	No relapse	7 (25.9%)	

* Kruskal–Wallis test.
** Chi square test.

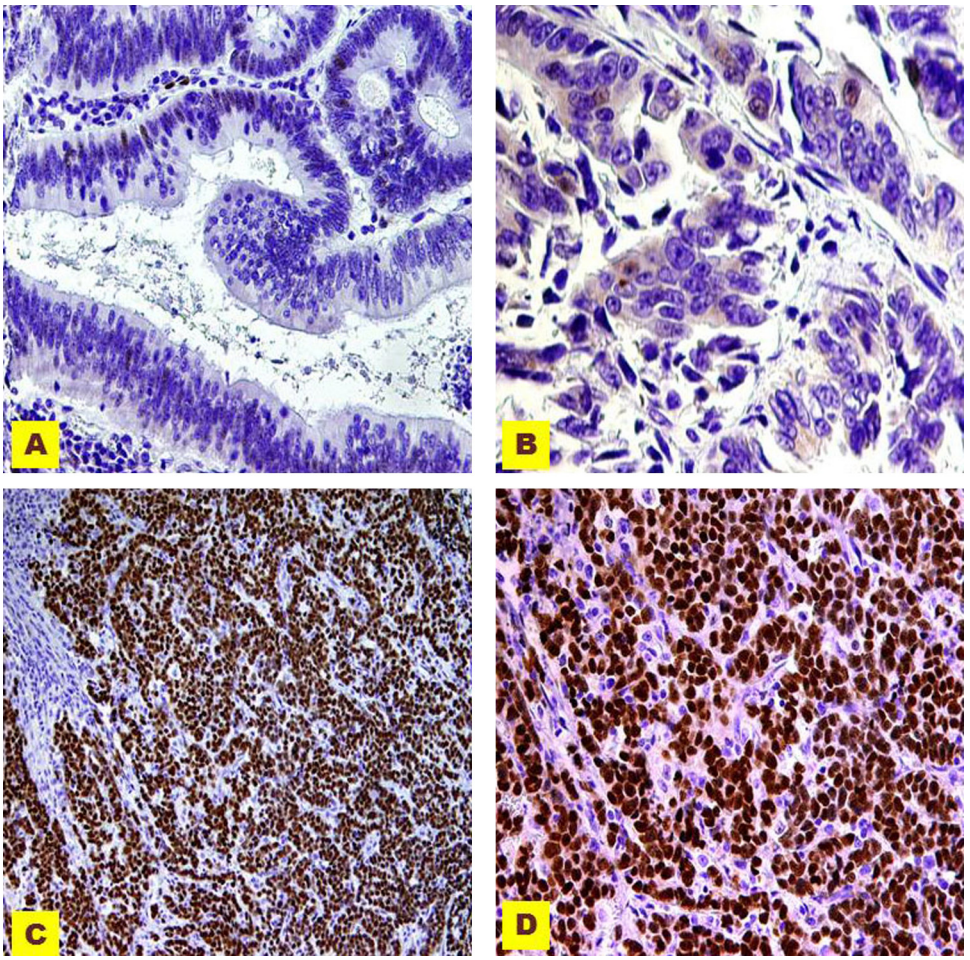

Fig. 1. (A and B) Low nuclear CCDN1 expression (IHC, 200×). (C and D) High nuclear CCDN1 expression (IHC, 200×).

3.1. Immunohistochemistry of CCND1

There were more cases with low CCND1 immunostaining in both primary tumours and nodal metastasis ($p < 0.001$). There was no difference between CCND1 immunoexpression in primary tumours and nodal metastasis.

3.2. Relation of CCND1 immunostaining to clinicopathological features and survival probability

CCND1 immunoexpression did not show any association with clinicopathological features except with lymphovascular invasion. Low nuclear expression of CCND1 was associated with negative lymphovascular invasion ($p = 0.046$). There was also no relation between CCND1 immunostaining and survival probability (Log Rank = 2.474, $p = 0.116$) (Fig. 2).

4. Discussion

Colorectal carcinoma (CRC) is one of the most frequent cancers in the Western world and with the changes of life behaviours; CRC has reportedly become more and more frequent in China [12,13]. CRC is the third most common cancer in men and the second in women worldwide. Almost 55% of the cases occur in more developed regions [14]. According to the Saudi Arabian National Cancer Registry, CRC is accounting for 11.3% of all newly diagnosed cases in year 2009. This cancer ranked first among male population and third among female in Saudi population.

The pathogenesis and progression of CRC are results of multiple genetic alterations occurring in a systematic fashion. In the past decade research has identified multiple molecules regulating CRC in an effort to highlight biologically aggressive tumours and appropriately select patients for adjuvant systemic or targeted therapies [12].

The role of CCND1 is known as a key player molecule in control of the shift of cell cycle from phase G1 to S phase by pRb mediation. CCND1/Cyclin-Dependent Kinase (CDK) 4–6 complexes initiate the phosphorylation of pRb and cyclin E/CDK2 complex completes the procedure in late G1 phase. Alterations in cyclin and CDK expression result in increased cell proliferation and contribute to malignancy [15]. CCND1 protein stimulated cellular proliferation and contributes to oncogenesis [16]. CCND1 gene is disrupted in the cancer cell genome usually by the process of gene amplification or chromosome translocation which may be involved in malignancy [17]. In humans over-expression

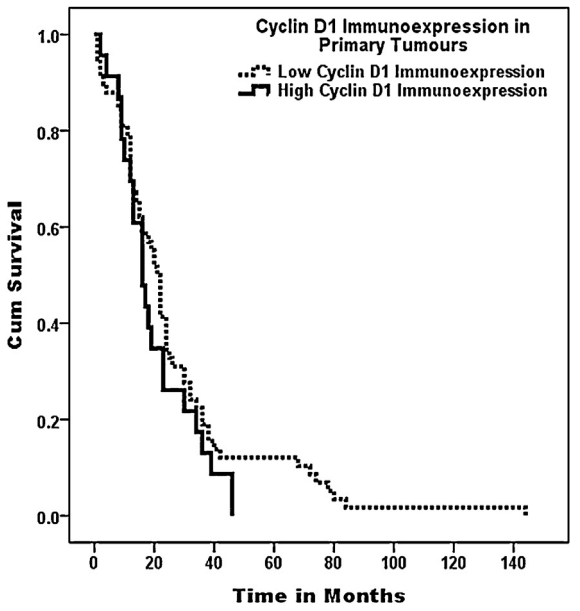

Fig. 2. Correlation between disease-free survival curve (Kaplan–Meier) and CCND1 immunoexpression. (1) Low CCND1 immunoexpression; (2) high CCND1 immunoexpression (Log Rank = 2.474, $p = 0.116$).

of CCND1 is seen in many tumours including colorectal cancers [12].

Although lots of studies have been performed on CCND1 expression in CRC, they seem to be ending up with conflicting conclusions. Some found that CCND1 is of prognostic importance in CRC. However, they did find association between CCND1 and survival. The findings were also conflicting regarding which is favourable low or high CCND1 [1,4,18–22]. Since all these studies on CCND1 have used immunostaining the variability in results could be due to the use different anti-CCND1 antibody clones and using different cut off points for immunostaining scoring. Other factors that differ among these studies are the number of cases, and techniques used. However, the results in the present study are in concordance with a previous study [20] supporting the observations that there seems to be no statistically significant correlation between CCND1 expression and overall survival probability or clinicopathological features.

Ogino et al. [22] examined the relation between CCND1 expression and survival of patients in stage I to IV CRC. They found that CCND1 overexpression was independent of clinicopathological features and other related molecular variables such as p53, p21, p27, KRAS, BRAF, LINE-1 methylation, MSI, and the CIMP. All of these characteristics are potential confounders in analysis of tumoral CCND1 status and patient survival. They concluded that CCND1 expression in colon cancer is associated with superior prognosis [22]. In addition, CCND1 expression in colon cancer is related with microsatellite instability (MSI), the CpG island methylator phenotype (CIMP), and BRAF mutation [23].

Studies have suggested strong association of CCND1 expression with prolonged survival among male with CRC [19] adding further momentum to the existing evidence that CRC is a hormone-dependent cancer, for which prognostic and treatment predictive molecular biomarkers should be evaluated based on patients gender [19].

Zhang et al. [24] reported that knockdown of paired box (PAX2), a transcription factor, inhibits the activity of AP-1, a transcription factor that induces CCND1 expression, implying that PAX2 induces CCND1 through AP-1 (a transcription factor) in CRC. PAX2 plays a critical role in embryogenesis. When aberrantly expressed in adult tissues, it generally exhibits oncogenic properties [24]. The mRNA cap-binding protein, eukaryotic initiation factor 4E (eIF4E) is critical in translation initiation due to its limiting amount in the cell. eIF4E levels were moderately correlated with VEGF and CCND1 in colon cancer supporting the role for eIF4E in translational regulation of proteins related to angiogenesis and growth [25]. Myklebust et al. [4] investigated the roles of CCND1a and CCND1b, as prognostic markers in CRC in a cohort. In CRC, combined stage 1 and 2 in addition to stage III, CCND1a nuclear overexpression was found to be a predictive marker for benefit from 5-fluorouracil and levamisole comparable to surgery. Contrary, low nuclear immunoexpression of CCND1a has no effect on treatment outcome [4]. CCND1b has neither prognostic nor predictive association in CRC [4,25].

Limitations of the current study include: TMA uses only a small part of a tissue specimen that may not be represent the actual gene or protein nature and distribution within a tumour which is also prone to exhibit heterogeneous territorial staining patterns [3,12,26]. Regarding using immunohistochemistry to highlight CCND1, although the method is sensitive, results are not quantitative and there are no standardised scoring systems or uniformly accepted threshold for positivity which are major limitations to interpretations.

5. Conclusion

Our study indicates that CCND1 immunoexpression cannot be used as a predictor of survival in CRC. It also shows no significant correlation with clinicopathological features except with lymphovascular invasion. However, further validation studies for the prognostic role of CCND1 in CRC are required clinically.

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