RESEARCH ARTICLE

Editorial Process: Submission:05/27/2019 Acceptance:08/22/2019

Prognostic Significance of *Cyclin D1* Over-expression in Colorectal Cancer: An Experience from Madinah, Saudi Arabia

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Abstract

Background and study aim: *Cyclin D1* is a key regulatory protein in the cell cycle and is over-expressed in many tumors, including endometrial, thyroid, urothelial, breast, brain gliomas, and esophageal cancers. The main aim of the present study is to examine the expression pattern of *cyclin D1* and its correlation with the different clinicopathological features in patients with colorectal camcer (CRC) from the Madinah region of Saudi Arabia. **Patients and methods:** The archival tumor blocks were analyzed using immunohistochemistry for *Cyclin D1* over-expression in 324 CRC patients diagnosed from January 2006 to December 2017, at the Department of Pathology, King Fahad Hospital, Madinah, Saudi Arabia. **Results:** *Cyclin D1* over-expression was absent in normal mucosa, while 15% cases of adenoma showed its over-expression. In CRC, *Cyclin D1* was expressed at high levels in 24.1% of case. No significant correlation was observed between *Cyclin D1* over-expression and age, gender, tumor size, type and location. However, *Cyclin D1* over-expression exhibited a significant correlation with tumor differentiation (p=0.04), lymph node involvement (p=0.001), lymphovascular invasion (p=0.001), distant metastasis (p=0.006) and AJCC staging (p=0.001). The Kaplan-Meir analysis revealed a shorter period of survival with *Cyclin D1* over-expression (p=0.000). The Cox-regression model analysis showed that *Cyclin D1* over-expression was an independent prognostic marker in CRC (p=0.000). **Conclusion:** *Cyclin D1* over-expression increases during normal-adenoma-carcinoma sequence. The significant association observed between *Cyclin D1* over-expression, advanced tumor stage and short survival period clearly suggest the role of *Cyclin D1* in the carcinogenesis and progression of CRC.

Keywords: *Cyclin D1*- colorectal- cancer- prognosis

Asian Pac J Cancer Prev, **20 (8)**, 2471-2476

Introduction

Colorectal cancer (CRC) is one of the most common cancers, being the second most common cancer in females and the third in males. Colorectal cancer statistics in 2013 showed an incidence of nearly 14.1 million cases and 694,000 deaths around the globe (Alao, 2007). The majority of cases of CRC occur in the developed nations, the incidence being lower in Asian and African countries. The Kingdom of Saudi Arabia (KSA) is an area of low incidence; however, according to the latest Saudi cancer registry, CRC is the second most common cancer, and the trend, disturbingly, is upward (Albasri et al., 2014; Al-Maghrabi et al., 2015). In spite of a better diagnostic approach and multimodal therapeutic regimens, CRC is nevertheless associated with a poor outcome, and approximately 50% to 60% of patients die within five years of diagnosis (Arber et al., 1996). Moreover, in KSA, a recent histopathology-based study from the Madinah region by Albasri et al., (2014) reported that CRC was diagnosed at an advanced stage in their cohort.

The multi-step genetic alteration in CRC is well studied: a series of events leads to an alteration of the normal epithelium, then to precancerous lesionsand further to adenocarcinoma, and subsequently to the development of metastasis (Bahnassy et al., 2004). Abnormal regulation of cell proliferation is the central event in tumor progression, generally reflected by alteration in the cell cycle. Therefore, a better understanding of cell cycle regulation is important for interpreting the significance of alterations. During the cell cycle process, a cell is committed to DNA replication, and the cycle starts with the Gap 1 (G1) phase (Bahnassy et al., 2004; Balcerczak et al., 2005). The checkpoint in the G1phase is known as the restriction point, beyond which the cell becomes independent and no growth factors are required for entering the synthesis phase (S phase); the cell is then committed to complete the cycle. These checkpoints are carefully controlled by

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cyclins, especially *cyclin D1* (Bali et al., 2004; Chen et al., 2007). *Cyclin D1*, a key regulatory protein in the cell cycle, is encoded by the CCND1 gene, located on chromosome 11q13, and over-expression of this protein interrupts normal cell cycle control, thus encouraging the development and progression of cancer (Cunningham et al., 2010). *Cyclin D1* over-expression has been observed in many tumors, including endometrial, thyroid, urothelial, breast, brain gliomas, and esophageal cancers (Elkablawy and Albasri, 2015). The up-regulation of *cyclin D1* plays an important role in the pathogenesis and metastases of CRC, and seems to be a useful prognostic marker for CRC (Bali et al., 2004; Chen et al., 2007). The main objective of the present study is to examine the expression pattern of *cyclin D1* and its correlation with the different clinicopathological features in patients from the Madinah region of Saudi Arabia.

Materials and Methods

The present study was a retrospective study involving archival paraffin blocks of tumor from 324 consecutive patients with CRC diagnosed between January 2006 and December 2017 in the histopathology laboratory of King Fahad Hospital (a tertiary care hospital) in the Madinah region of Saudi Arabia. The clinicopathological data included sex, age, tumor site, size, type, grade, lymph node status, lymphovascular invasion,distant metastasis status, and the American Joint Committee on Cancer (AJCC) stage, all of which were obtained from patients' medical records. The present study did not involve patients' personal information or have any implicationsfor the management plan. Hence, no ethical approval was sought, according to the principles of the Helsinki Declaration. Twenty cases of normal colonic mucosa and 40 cases of colorectal adenomas were used as controls in the present study. Only histopathologically confirmed invasive CRC cases who underwent total colectomies, hemi-colectomies,and local resection were included. In-situ lesions, recurrences, biopsies, benign lesions, and metastatic lesions were excluded from the analysis.

Immunohistochemical procedures

Tissue cores were extracted from archival blocks of the primary CRC cases and used in the construction of a tissue mini-array (TmA), asdescribed previously by Elkablawy and Albasri (Ferlay et al., 2013). Four micro-meter-thick tissue sections were cut from the TmA blocks, mounted on positively charged poly-l-lysil slides, and immunohistochemistry was performed on them using the avidin-biotin detection system, following the instructions of the manufacturer.The human anti-cyclin D1 monoclonal antibody was used (diluted 1:70 in blocking solution; Ventana, Inc., Tucson, AZ, USA). The immunohistochemistry procedure was done on an automated immune-stainer (Ventana Bench Mark Xt; Ventana, Inc.). The positive control was achieved by using a case of breast cancer, and the negative control was achieved by substitution of the primary antibody with serum.

Interpretation of immunohistochemical staining

The percentage of nuclear staining of *cyclin D1* was reported as: 0, less than 5%; 1, 5–25%; 2, 26–50%; 3, 51–75%; and 4, more than 75%. The staining pattern was scaled from 0 to 3, where 0 was negative, 1 was weak, 2 was moderate, and 3 was strong. The final expression score was calculated as follows: '-' for score 0, '+' for scores 1–3, '++' for scores 4–6, and '+++' for scores >6. For the purpose of statistical analysis, the cases that scored '-'and '+'as a low score were comparedwith the cases that scored '++' and '+++' as a high score. No cytoplasmic staining of *cyclin D1* was found in any of the studied cases.

Statistical analysis

Data analysis was performed using the Statistical Package for Social Sciences (SPSS) Version 22.0 for Windows XP (BMCorp., Armonk, NY, USA). To determine inter-observer reproducibility,correlation analysis of the two data sets was performed. The chi-square test and Fisher's exact tests were performed to extract the significant level of association between *cyclin D1* over-expression and the clinicopathological parameters. The cumulative survival analysis was assessed by the univariate Kaplan–Meir method, and the long-rank test was performed for comparison of the survival curves. Risk factor analysis of factors independently associated with survival was carried out by the Cox proportional hazards linear regression model. A p value of <0.05 was considered as significant for all the statistical analyses. The present study was a retrospective study involving the archival histopathology material and record; and did not involve patients' personal information or any implication upon the management; thus, was according to the principles of Helsinki Declaration. Hence no ethical approval was mandatory in the present study.

Results

Clinicopathological characteristics of the cases

In the present study, a total of 324 cases of CRC was included. Therewere 196 male patients (60.5%) and 128 female patients (39.5%), making a male to female ratio of 1.5:1.The ages of the studied cases ranged from 22 to 96 years, with a mean age of 56.9 years. Left- and right-sided tumors were seen in 63.5% and 36.5% of cases, respectively, and the size of the tumor was >4 cm in 84% of cases. The commonest histological type was adenocarcinoma, seen in 279 cases (86.1%), and the majority of the tumors were moderately differentiated. AJCC stages I, II, III, and IV were reported in 10.5%, 44.7%, 31.5%, and 13.3% of cases, respectively. Approximately 55.2% of cases had positive lymph node metastasis and lymphovascular invasion. Distant metastases were seen in 30.3% of cases. Table 1 summarizes the clinicopathological characteristics of the 324 CRC cases.

Cyclin D1 over-expression profiles

Evaluation of *cyclin D1* protein over-expression on TmA sections containing normal colonic mucosa showed a complete absence of its expression. By contrast, *cyclin*

Table 1. The Clinicopathological Features of CRC Cases

Variable	No of Patients	% of Total
Age (yr)		
<40	177	54.6
≥40	147	45.4
Sex		
Female	128	39.5
Male	196	60.5
Tumor site		
Colon	168	51.9
Rectum	156	48.1
Tumor size		
<4cm	52	16
≥4cm	272	84
Tumor type		
Adenocarcinoma	279	86.1
Mucinous adenocarcinoma	45	13.9
Tumor differentiation		
Well	49	15.1
Moderate	260	80.2
Poor	15	4.7
Lymph Node		
Negative	179	55.2
Positive	145	44.8
Lymphovascular invasion		
Negative	179	55.2
Positive	145	44.8
Distant metastasis		
Negative	281	86.7
Positive	43	13.3
AJCC		
I	34	10.5
II	145	44.7
III	102	31.5
IV	43	13.3

D1 over-expression was noted in six out of 40 cases (15%) ofcolorectal adenoma. In CRC cases, *cyclin D1* was expressed at a low level in 246 (75.9%) cases, while 78 (24.1%) cases showed high levels ofexpression (Figure 1).

Correlation of cyclin D1 over-expression with the clinicopathological parameters

Cyclin D1 over-expression did not reveal any significant correlation with age, gender, tumor size, tumor histologic type,ortumor location. However, *cyclin D1* over-expression exhibited a significant correlation with tumor differentiation (p=0.04), lymph node involvement (p=0.001), lymphovascular invasion (p=0.001), distant metastasis (p=0.006), and AJCC staging (p=0.001). A summary of our observations of correlation of *cyclin D1* over-expression with clinicopathological parameters ispresented in Table 2.

Univariate and multivariate long-term survival analysis

The significant findings (p<0.05) associated with survival for all the 324 CRC cases in the present study are shown in Table 3. The significant survival curves for *cyclin D1* over-expression are shown in Figure 2. The patients with a high score forcyclin D1 over-expression experienced a significantly lower survival period than the patients with a low score of *cyclin D1* over-expression (p=0.000). High *cyclin D1* expression (p=0.000), AJCC stage (p<0.001), lymph node metastasis (p<0.001), lymphovascular invasion (p<0.001), distant metastasis (p<0.001), and histological tumor grade (p<0.001) were the only significant independent prognostic indicators, as determined by multivariate analysis usingthe Cox regression model.

Discussion

The incidence of CRC has reportedly increased in nations where it was historicallyless prevalent (Asaad et al., 2000; Hilska et al., 2005). In the past decades, advances in molecular carcinogenesis have described

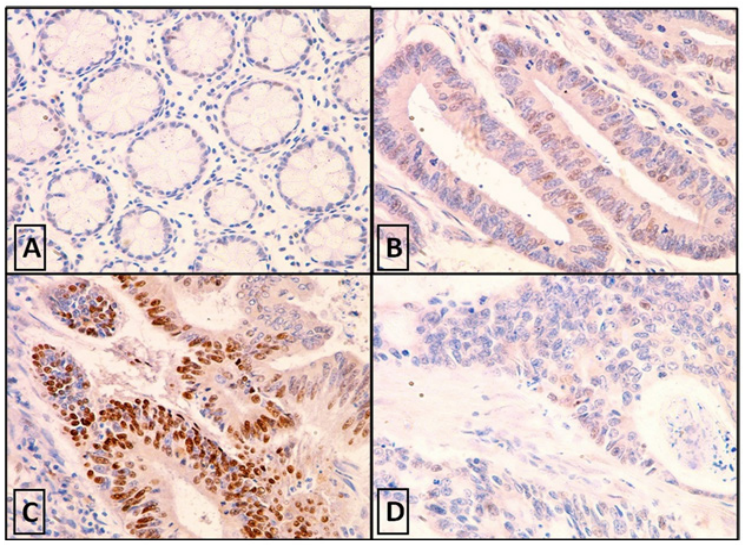

Figure 1. Different Patterns of Immunohistochemical Cylin D1 Expression in A. Normal colon (no staining), B. Colonic adenoma (mild to moderate nuclear positivity), C. Colorectal carcinoma (marked nuclear expression), D. Colorectal carcinoma (low nuclear expression).

Table 2. Correlation of Cylin D1 Over-expression with the Clinicopathological Variables

Variable	Low Cycline D1	High Cycline D1	P value
Age (yr)			= 0.091
<40	141	36	
≥40	105	42	
Sex			= 0.59
Male	151	45	
Female	95	33	
Tumor site			= 0.897
Colon	127	41	
Rectum	119	37	
Tumor size			= 0.72
<4cm	41	11	
≥4cm	205	67	
Tumor type			= 0.70
Adenocarcinoma	213	66	
Mucinous adenocarcinoma	33	12	
Tumor differentiation			= 0.04
Well	43	6	
Moderate	194	66	
Poor	9	6	
Lymph Node			= 0.001
Negative	149	30	
Positive	97	48	
Lymphovascular invasion			= 0.001
Negative	149	30	
Positive	97	48	
Distant metastasis			= 0.006
Negative	221	60	
Positive	25	18	
AJCC stage			= 0.001
I	31	3	
II	118	27	
III	72	30	
IV	25	18	

various crucial signaling pathways of the cell cycle in the development and progression of CRC. One such pathway involves the over-expression of *cyclin D1*. *Cyclin D1* is an important checkpoint regulator protein at the G1 to S phase transition (Bali et al., 2004; Chen et al., 2007). *Cyclin D1* can act as an oncogene during tumorigenesis by modulating certain processes such as excessive growth, invasion, angiogenesis, and resistance to apoptosis (Holland et al., 2001). The carcinogenic role of *cyclin D1* over-expression has been reported in the cancers of various organs, includingCRC, and this over-expression appears to be associated with aggressive behavior and worse prognosis (Elkablawy and Albasri, 2015).

Recently, an immunohistochemistry-based meta-analytical study of *cyclin D1* expression in squamous cell carcinoma of the esophagus revealed a significant association between *cyclin D1* expression and aggressive tumor histology, lymph node metastasis, and higher AJCC stages (Hur et al., 2000). Asimilar study on estrogen-positive (ER) breast cancers showed that *cyclin D1* over-expression was associated with the worst clinicopathological features and prognosis (Ibrahim et al., 2008). Moreover, *cyclin D1* over-expression is also reportedly associated with poor survival outcomes in patients with serous ovarian cancers and non-small-cell carcinoma of the lung (International Association of Cancer Registries, 2017; Jang et al., 2012).

The role of *cyclin D1* over-expression in CRC is somewhat controversial.While some reports have observed a significant association between its expression and poor survival outcomes, others have reported it as a marker of a good prognosis (Jares et al., 1994; Li et al., 2014), and still others have reported no significant association between *cyclin D1* expression and prognosis (Mate et al., 1996; Maeda et al., 1997). In view of these controversial reports, we evaluated the *cyclin D1*over-expression pattern by immunohistochemistry on TmA blocks in CRC cases. Three patterns of *cyclin D1* over-expression by immunohistochemistry were demonstrated in previous studies, i.e., nuclear, cytoplasmic, and nuclear plus cytoplasmic. However, for CRC, only the nuclear staining pattern was considered to be significant (McKay et al.,

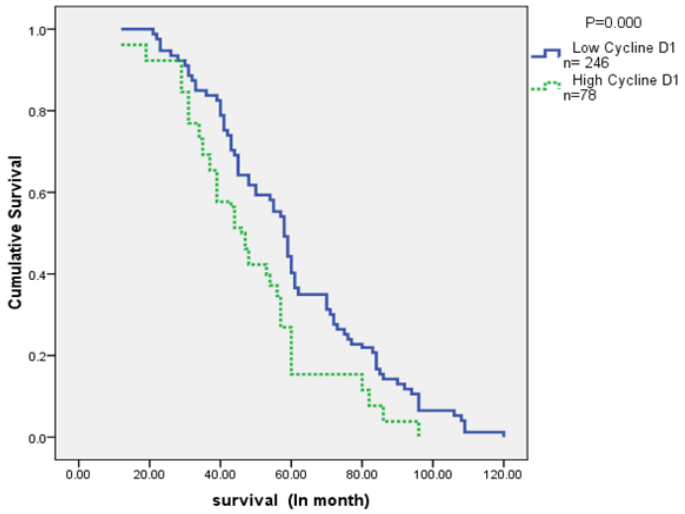

Figure 2. Shows Kaplan-Meier Curves for Significant Overall Survival Functions for Cylin D1 Expression in CRC

Table 3. Test Statistics for Equality of Survival Distribution for Prognostic Factors Examined in 324 Colorectal Carcinomas. A Univariate Approach to Cancer-specific Mortality

Factor	Univariate X^2 for the log-rank	Df.	P value
Clinicopathological factors:			
AJCC stage	772.81	3	<0.001
Lymph node stage	400.28	1	<0.001
Metastasis stage	507.58	1	<0.001
Lymphovascular invasion	400.28	1	<0.001
Histological tumour grade	170.27	2	<0.001
Tumour size	2.141	1	0.133
Tumour site	0.366	1	0.545
Tumour type	1.153	1	0.283
Age	0.308	1	0.579
Sex	0.064	1	0.800
Immunostaining			
Cycline D1 immunostaining	15.96	1	= 0.000

2002; Li et al., 2014).

In our study, evaluation of *cyclin D1* over-expression on TmA sections containing normal mucosa, colorectal adenomas, and colorectal carcinomas revealed a negative expression pattern in normal colonic mucosa and a gradual increase in its detection in the normal to adenoma to carcinoma sequence. This suggested an oncogenic role of *cyclin D1* over-expression in colorectal carcinogenesis. Arber et al., (1996) reported *cyclin D1* over-expression in 30% of adenomas, and concluded this phenomenon was possibly an early event during tumorigenesis. This sequential up-regulation of *cyclin D1* over-expression from normal to adenoma to carcinoma in our study is in accordance with the findings of previous authors, indicating an oncogenic role of *cyclin D1* in colorectal carcinogenesis (Bahnassy et al., 2004; Jiang et al., 2006; Li et al., 2014).

In contrast to normal mucosa and adenoma, *cyclin D1* over-expression was observed as a low score in 75.9% of cases and a high score in 24.1% of cases. Several previous studies on CRC reported *cyclin D1* positivity in a range as low as 23.1% to as high as 100% (Myklebust et al., 2012; Ogino et al., 2009). This wide range of expression may be attributed largely to factors such as cohort size, type of antibody used, antigen retrieval techniques, and the scoring system. In the present study, the level of expression was more intense or high in the poorly differentiated carcinomas. The correlation between high histopathological grade and *cyclin D1* over-expression has also been reported in other tumors, such as non-small-cell lung carcinomas (Pasz-Walczak et al., 2001), squamous cell carcinomas of the larynx (Sterlacci et al., 2010), squamous cell carcinomas of the esophagus (Hur et al., 2000), and breast carcinomas (Ibrahim et al., 2008).

In our study, no significant correlation was observed between the *cyclin D1* over-expression and patients' gender, tumor size, tumor histologic type, and tumor location.Similar findings of no significant correlation with these clinicopathological parameters were reported by various previous authors (Bahnassy et al., 2004; Jiang et al., 2006; Li et al., 2014). However, a significant correlation was seen with the patient's age, lymph node metastasis, lymphovascular invasion, distant metastasis, and AJCC stage. Previous studies by Assaad et al., (2000), Belcerczak et al., (2005), and Almaghrabi et al., (2015) from different parts of the world found a similar strong correlation between pathological tumor stage and *cyclin D1* over-expression. According to Belcerczak et al., (2005) and Almaghrabi et al., (2015), among all clinicopathological parameters, lymph node metastasis and lymphovascular invasion showed the strongest correlation with *cyclin D1* over-expression, as observed also in our study. Hence, our observations of a significant association betweencyclin D1 over-expression and different clinicopathological parameters are in agreement with previous studies from the region and around the world.

In univariate and multivariate analysis, contrasting results have been documented on the prognostic and predictive value of *cyclin D1* over-expression. Our findings in the present study support that high *cyclin D1* over-expression is associated with a shorter survival time and poor patient outcomes, as compared to low levels of *cyclin D1* over-expression. Several previous studies have yielded a controversial result relating to the prognostic significance of *cyclin D1* over-expression in CRC patients. Similar to our findings of a significant association, Bahnassy et al., (2004), Mykelbust et al., (2012), Ogino et al., (2009), and Li et al., (2014) reported *cyclin D1* over-expression as a poor prognostic indicator and associated with a short survival period. By contrast, McKay et al., (2002) observed that *cyclin D1* expression in CRC is associated with a survival advantage for patients. However, Assaad et al., (2000), Von Stockmar-Von Wangenheim et al., (2008), and Almaghrabi et al., (2015) found no association between *cyclin D1* expression and patients' survival. In the present study, *cyclin D1* turned out to be an independent prognostic marker for CRC, similar to the findings of Bahnassy et al., (2004).

In conclusion, our findings of an increasing percentage of *cyclin D1* over-expression in the sequence from normal to adenoma to carcinoma suggest an oncogenic role for *cyclin D1* in CRC patients. We observed a significant correlation with lymph node metastasis, lymphovascular invasion, distant metastasis, and advanced AJCC stages. Moreover, a significant short survival period was noted in patients with high *cyclin D1* over-expression. Lymph node metastasis, lymphovascular invasion, distant metastasis, and advanced AJCC stages were the independent prognostic indicators, as calculated by multivariate analysis usingthe Cox regression model. Our findings confirmed the important role of *cyclin D1* in CRC carcinogenesis and can be used as a reliable prognostic indicator in CRC patients.

Acknowledgements

The authors thank the Deanship of Scientific Research, Taibah University, Almadinah Almonawwarah, Saudi Arabia, for their encouragement and support. The authors

thank Dr. Alla Khadri (pathology Department, Taibah University, KSA) for revising the grammer and language. The authors thank Mrs. Faten Bokhary (Lab. technician, Taibah University, KSA) for technical advice.

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