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Detection of Cyclin D1 Immunohistochemical Expression among Sudanese Patients with Colorectal Cancer

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Abstract: Objectives: *This study was aimed to evaluate the prognostic value of cyclin D1 in colorectal cancer and to correlate cyclin D1 expression with the different clinicopathological parameters.* ***Methods:*** *Tissues microarray paraffin block with 48 colorectal cancer samples were retrieved from the archives of Elrahma Medical Center. The immunostaining of cyclin D1 was performed and analyzed.* ***Results:*** *Cyclin D1 did not show association with clinicopathological features. There was no statistically significant correlation between Cyclin D1 immunoexpression and tumor grade, with p value > 0.05 that's considered as statistically insignificant.* ***Conclusion:*** *Our study indicated that; cyclin D1 immunoexpression cannot be used as a predictor of survival in colorectal cancer. It also showed no significant correlation with clinicopathological features.*

Keywords: Cyclin D1, Colorectal, Cancer, IHC, Sudan.

Introduction:

Colorectal cancer (CRC) is a cancer that begin in the colon or the rectum. These cancers named colon cancer or rectal cancer, looking on where they start. Colon cancer and rectal cancer are often grouped together because they share a lot of features. Malignancy begins when cells in the body start to grow uncontrollably. Cells in nearly any part of the body can become malignant and can spread to other parts of the body. [(1)] CRC begins in the mucosa (innermost layer) and can grow outward through some or all of the other layers. When cancer cells are in the wall, they can then grow into blood vessels or lymph vessels (tiny channels that carry away waste and fluid). From there, they can travel to nearby lymph nodes or to too far parts of the body. The stage of a colorectal cancer rely up on how deeply it grows into the wall and if it has spread outside the colon or rectum. [(1)] CRC is one of the most common cancers in the world, with over 1.2 million new cases diagnosed each year. Despite improvements in screening for early diagnosis, CRC remains one of the biggest cancer killers in the world and is responsible for over 600,000 deaths each year. [(2,3)] Early detection, adequate surgical excision and optimal adjuvant treatment are of crucial importance if a favorable outcome is to be achieved. Currently, tumor stage at diagnosis is the most important prognostic factor in CRC, and although many efforts have been made to find molecular markers to identify high-risk disease and to select patients for adjuvant treatment, none has proven sufficiently good for use in clinical routine.

Cyclin D1(CCND1), also called BCL1 - "b cell lymphoma, responsible for transition to S phase by phosphorylating the retinoblastoma gene product, which releases transcription factors to initiate DNA replication. Overexpression promotes transformation to a malignant phenotype; overexpressed in several tumors. [(1)] Cyclin D1 is an important cell-cycle regulating protein that, together with its binding partner's cyclin-dependent kinase (CDK) 4 and CDK6, forms active complexes that promote G1- to S-phase progression by phosphorylating and inactivating the retinoblastoma protein. [(4)]Cyclin D1 is activated by WNT/β-catenin signaling after mutation of the adenomatous polyposis coli gene (*APC*), an important event in the initiation of colorectal neoplasia.[(5,6)]Cyclin D1 overexpression is common in CRC, but the findings regarding its prognostic value are conflicting.[(7-19)] However, the largest study to date, comprising an analysis of 602 tumors from two independent, prospective cohort studies, found an association between cyclin D1 overexpression and a prolonged survival from colon cancer.[(19)]

This study conducted on Sudanese patients with CRC, aimed to detect cyclin D1 biomarker and to correlate its expression with tumor grade and patients age, also this study performed to evaluate the prognostic and predictive value of cyclin D1 in determination of tumor prognosis.

Materials and methods:

This was a retrospective cross sectional study aimed to detect the expression of cyclin D1 in colorectal cancer among Sudanese patients using immunohistochemistry technique.

The study samples were collected and processed in Elrahama Medical Center- Khartoum North (Sudan). The stained slides were examined at University of Medical Sciences and Technology (UMST)-Sudan and then transferred to Sharq Elneil College where they confirmed by well expertise Medical Laboratory Histopathologists.

Forty-eight formalin fixed paraffin embedded blocks (FFPE), previously diagnosed with colorectal carcinoma were selected in this study.

FFPE tissue blocks with CRC from Sudanese patients were included in this study as a case group. Other tissues with a colon and rectum diseases and benign lesions were excluded from this study. Race, tribe, age and residence were not considered in this study.

The patients and specimen's identification data and the obtained results were recorded using data sheet. Cyclin D1 immunoexpression was detected using IHC method.

Sampling technique

Simple random sampling technique was used for collecting data from target study area.

Sample processing

The collected samples were assembled in one tissue microarray (TMA) block using conventional mechanical pencil tips; a hollow needle was used to remove tissue cores as small as 1mm in diameter from regions of interest in FFPE tissue samples. These tissue cores were then inserted in a recipient paraffin block in a precisely spaced array pattern. Then the TMA block was sectioned (3 microns), using rotary microtome (MR22150-K2258-1124 Histoline – Italy). The cut section was initially floated in 70% ethanol, and then floated in water bath (LAB TECK, 009222 -India) at $45c^0$, after floatation the slide was dried in dry oven at $50c^0$ for 12-24 hours. After flotation TMA sections were contained in forested end positive charge slide. Then slide was stained immunohistochemically. For analysis of data, Statistical Package for Social Sciences software, version 21.0 (IBM SPSSInc., Chicago, IL) and STATA 11were used. Initially, all information gathered via data master sheet then coded into variables. Descriptive and inferential involving Fisher exact Test and binary logistic regression were used to present results. A P-value<0.05 considered as statistically significant. Other variables and frequencies were calculated and presented as figure and tables.

Ethical consideration:

Written Approval from UMST, Khartoum Ministry of Health and Elrahma medical center was taken. Research purpose benefits and objectives were explained to hospital and laboratory administration in clear simple words with assurance on confidentiality.

Cyclin D1 Ab was applied in formalin fixed paraffin embedded tissue section already diagnosed as colorectal cancer sample and after got permission from hospital administration.

Method of staining

Immunohistochemistry staining was done as followed; following deparaffinization in xylene, slide was rehydrated through a graded series of alcohol and then washed in distilled water (D.W). Then slide was steamed using high Tris buffer (pH 9) in water bath at $95C^0$ for 40 min. After that, slide was washed in phosphate buffer saline (PBS) for 3 min. Then the endogenous peroxides activity was blocked using 3% hydrogen peroxide in methanol for 10 min, then slide was washed in PBS for 3 min. Then incubated with 100 µ L of mouse monoclonal antibody (Dako-USA), specific against cyclin D1 for 30 min at room temperature in a moisture chamber. After that slide was washed in PBS for 3 min, binding of antibodies was detected by incubating for 20 min with dextran labeled polymer (Dako-USA). Finally, the slide was washed in three changes of PBS, followed by adding 3, 3 di amino benzidine tetra hydrochloride (DAB) as a chromogen to produce the characteristic brown stain for the visualization of the antibody/enzyme complex for up to 5 min. After that, slide was washed in D.W for 3 min. Slide then counterstained with hematoxylin (Mayer's) for one min, then blued in running tap water for 7-10. After bluing slide was dehydrate in alcohol, cleared in xylene and mounted in Dixteren a Plasticizer and Xylene (DPX).

Interpretation of result

We scored the intensity of nuclear cyclin D1 expression as negative (scored zero) when no brown nuclear stain was observed in (0.00 cells or 1% of cells), weak positive expression was scored as score one when pale brown nuclear stain was observed (2-25% of cells). Moderate expression was scored 2 when 26-50% of cells were with brown nuclear stain while strong expression was scored when 75% or more of cells showed brown nuclear stain. [(20)]

Quality Control

Quality control measures have been taken in all steps to avoid contamination and curry over by using microtome knife for each case, cleaning thoroughly the microtome and water bath and examining the slide. Manufactural instruction was followed precisely.

Results

The age of patients ranged from 30 to 75 years old with average mean of age is 50 years old. The age of patient's sub grouped into 2 groups, group one included patients with less than 50 years old, the second age group included those with age equal or more than 50 years old.

The age group one included 20 (42%) patient samples, while the second age group included 28(58%) patient samples, as indicated in figure (4.1 and 4.2).

Regarding the gender of patients, males were 36 (75%) while females represented 12 (25%) as revealed in figure (3).

Concerning tumor grade, the tumor grade1 (low grade) comprised one tissue block (2.1%), grade 2 (moderate) comprised 23 samples (47.9%) while grade 3 (high) comprised 24 samples (50%), as summarized in figure (4).

The frequency of cyclin D1 positive expression among study populations were as followed; the negative expression was detected in 7(14.6%) samples, while positive expression was detected in 41(85.4%), as summarized in figure (5).
The cyclin D1 positive expression scored as followed; score 1 (weak expression) was detected in 18 samples (43.9%), score 2(moderate expression) was detected in 17 samples (41.5%), score 3 (strong expression) was detected in 6 samples (14.6%), as indicated in figure (6).
Regarding correlation of cyclin D1 immunoexpression with tumor grades, our results revealed that; the negative expression score (0.00) was detected in (0 out of 1=0.00%) samples in grade1, (3 out of 23=13%) samples in grade 2, and (4out of 24=16.7%) samples in grade 3. The positive expression was detected (1 out of 1=100%) samples in grade1, (20 out of 23=87%) samples in grade 2, and (20 out of 24=83.3%) samples in grade 3, the p value was 0.999, as showed in table (1).
Regarding correlation of cyclin D1 immunoexpression with age our results revealed that; the negative expression was detected in 1 sample out of 20 samples (5%) from patients with age group below 50 years old, while the positive expression in the same age group was 19 =95%. The negative expression in the second age group of 50 years old and above was 6 out of 28 samples (21.4%), while the positive expression in the same age group was 22 =78.6%, the p value was 0.214, as indicated in table (2).
Regarding cyclin D1 immunoexpression with the gender of patients, our results revealed that; the positive expression was detected in 29 out of 36 males (80.6%), the remainder 7 males (19.4%). Regarding immunoexpression of cyclin D1 among females our results showed that; all samples (100%) were present with positive expression, the p value 0.169, as listed in table (3).

Discussion

This is a retrospective cross sectional study conducted in Khartoum State-Sudan, aimed to detect immunoexpression of cyclin D1 in CRC. To achieve these aim 48 FFPE tissues with CRC were involved in this study. Our results showed that; the CRC in this study more frequently observed in advanced ages. This finding consistent with those studies conducted by Motaz *et al*., who concluded that; the mean age of the patients was 50.5 (±11.7) [(21)], and Ahmed *et al*., [(22)] who summarized that twenty-five percent of cancers occurred in patients aged with less than 40 years. On the other hand, our results regarding correlation of age with the occurrence of CRC inconsistent with those studies conducted by Alsanae., *et al*., who summarized that; CRC presents at a younger age [(23)], and Mohamed *et al*., [(24)] who found that; more than 17% of the study populations was below age of 40 years, and 43.84% was below 50 years, and Abdalla*et al*.,[(25)] who summarized that; more than 100 (34.5%) of the study populations (n=277) were below the age of 40 years, and 17.3% were below 30 years.
Regarding the association of gender with CRC, our results revealed that; the CRC among males were predominantly (3/4) than females (1/4), this result agree with Alsanea*et* al., who concluded that; most common CRC cancer among men and the third commonest among women since 2002 in Saudi Arabia [(23)]. Also agree with that study done by Saeed *et al*., who found that; Male: female ratios were 1:1.18 for adults and 1.46:1 for children and also agree with Abdalla who summarized that; the male to female ratio was 1.5:1 [(25)]. But our result regarding gender and CRC disagree with that study conducted by Ahmed *et al*., who concluded that; fifty-six percent were females [(22)], and Mohamed *et al* who summarized that; the male to female ratio was 1:1.02 [(24)]. Concerning the frequency of tumor grades in samples with CRC, our results showed that; usually patients presented with advanced stages of disease, because we observed more than 90% of patients with advanced grades, and this finding usually due to lack of systemic health care system, lack of screening program, lack of sufficient early detection method, and also due to bad culture in health and protection methods. This finding regarding tumor grade similar to results achieved by Motaz *et al*., who summarized that; 65% of the sample were advanced "Duke's stage C & D, and also agree with Alsanea, who summarized that higher proportion of advanced stage cancer at presentation [(21)].
Regarding the relationship between cyclin D1 immunoexpression and CRC, our results indicated that; near to 90% of immunohistochemically stained sections showed positive expression, this results matched with other studies done by Abeer *et al*., who concluded that; 41 out of the 60 (68.3%) CRC cases were positive [(26)]. And Sakariase*t al*., which summarized that; cyclin D1 expression could be evaluated in 527of 557 tumors (94.6%) represented in the TMA [(27)]. And also agree with Blaserczak *et al*., who summarized that; expression of *CCND1* gene was found in 54 out of 111 cases of colorectal cancers [(20)], also agree with shuji*et al*., who summarized that; cyclin D1 overexpression was observed in 330 (55%) tumors by immunohistochemistry [(28)], and other study done by von stockmar *et al*., and they summarized that; 75% of cyclin D1 immunoexpression was observed in CRC cases [(29)]. And disagree with Other studies done by Jaudah *et al*., who summarized that; there were more cases with low CCND1 immunostaining[(30)].
Our results revealed that there is insignificant correlation between cyclin D1 expression and age. Younger age participant more contributed to be positive in cyclin D1 expression. This finding is similar to previous study done by Von stockmar*et al*., [(29)] who said that; there is no correlation with gender, growth pattern, staging, grading or prognosis, and disagree with Yang Li, who found that; cyclin d1 over expression correlated with age [(32)].
In this study we revealed that cyclin D1 immunoexpression has insignificant correlation with histopathological grade. Participant with higher histological grades more contributed to be positive in cyclin D1 Expression. Previous studies have examined the relationship between tumoral cyclin D1 expression and *histopathological* stage in colorectal cancer. However, those studies have yielded inconsistent results. Although the study of, Arber *et al*., [(31)] Yang Li *et al*., [(32)], Mao *et al*., [(33)], have shown that; cyclin D1 expression has been associated with poor prognosis. Most studies have shown no

independent prognostic value of cyclin D1, Abeer *et al.,* [26], Sakeryas*et al.,* [27], Jaudah*et al.,* [30], Bukholm *et al.,* [34], Lyal*et al.,* [35], Von Stockmar*et al.,* [29]. Also there are other studies of shuji*et al*., Schernhammer*et al*., Wang *et al*., has shown good prognosis associated with cyclin D1 expression [28, 36,37].

The variability in results could be due to the use different anti-CCND1 antibody clones and using different cut of points for immune staining 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 previous study done by Jauda*et al*., [30], and Von stocmar*et al*., [29], supporting the observations that there was no statistically significant correlation between CCND1 expression and overall survival probability or clinical pathological features.

In this study we found that; cyclin D1 positive expression was frequently observed in female samples than male samples, but there is no significant correlation of cyclin D1 expression with patient gender, this finding was agree with Von Stockmar[29], who concluded that; there is no significant correlation of cyclin D1 expression with the patient gender. Our results did not match with the findings of Sakarias*et al*., [27] who found that; there is strongly accentuated prognostic effect of cyclin D1 in male, and Schernhammer*et al*., who found that; it is good prognostic in woman [36]

Conclusion:

According to the obtained results we concluded that that;

- Cyclin D1 immunoexpression was higher among high tumor grades, but with no significant correlations.
- Cyclin D1 expression was higher in females than males, but also with no significant correlation.
- Cyclin D1 expression was frequently observed in elderly patients, but also with no significant correlation.

Table (1): Comparison between cyclin D1 expression with tumor grades.

Tumor grade	Cyclin D1 expression		p value
	Positive	Negative	
Grade I	1 (2.10%)	0.00 (0.00%)	
Grade II	20 (41.70%)	3 (6.30%)	
Grade III	20 (41.7%)	4 (8.3%)	0.999
Total	41(85.4%)	7 (14.6%)	
N=48			

- p value <0.05 that's considered as statistically significant.

Table (2): Comparison between cyclin D1 expression with patients age.

Patient age	Cyclin D1 expression		p value
	Positive	Negative	
Less than 50 years	19 (39.60%)	1 (2.1%)	
50 years and above	22(45.80%)	6 (12.50%)	0.214
Total	41(85.4%)	7 (14.6%)	
N=48			

- p value <0.05 that's considered as statistically significant.

Table (3): Comparison between cyclin D1 expression and patient gender

Patient gender	Cyclin D1 expression		p value
	Positive	Negative	
Males	29 (60.40%)	7 (14.60%)	
Females	12 (25%)	0.00 (0.00%)	0.169
Total	41(85.4%)	7 (14.6%)	
N=48			

- p value <0.05 that's considered as statistically significant.

Patient gender	Cyclin D1 expression	p value	Patient gender	Cyclin D1 expression
	Positive	Negative		Positive
Males	29 (60.40%)	7 (14.60%)	Males	29 (60.40%)
Females	12 (25%)	0.00 (0.00%)	Females	12 (25%)
Total	41(85.4%)	7 (14.6%)	Total	41(85.4%)
N=48			N=48	

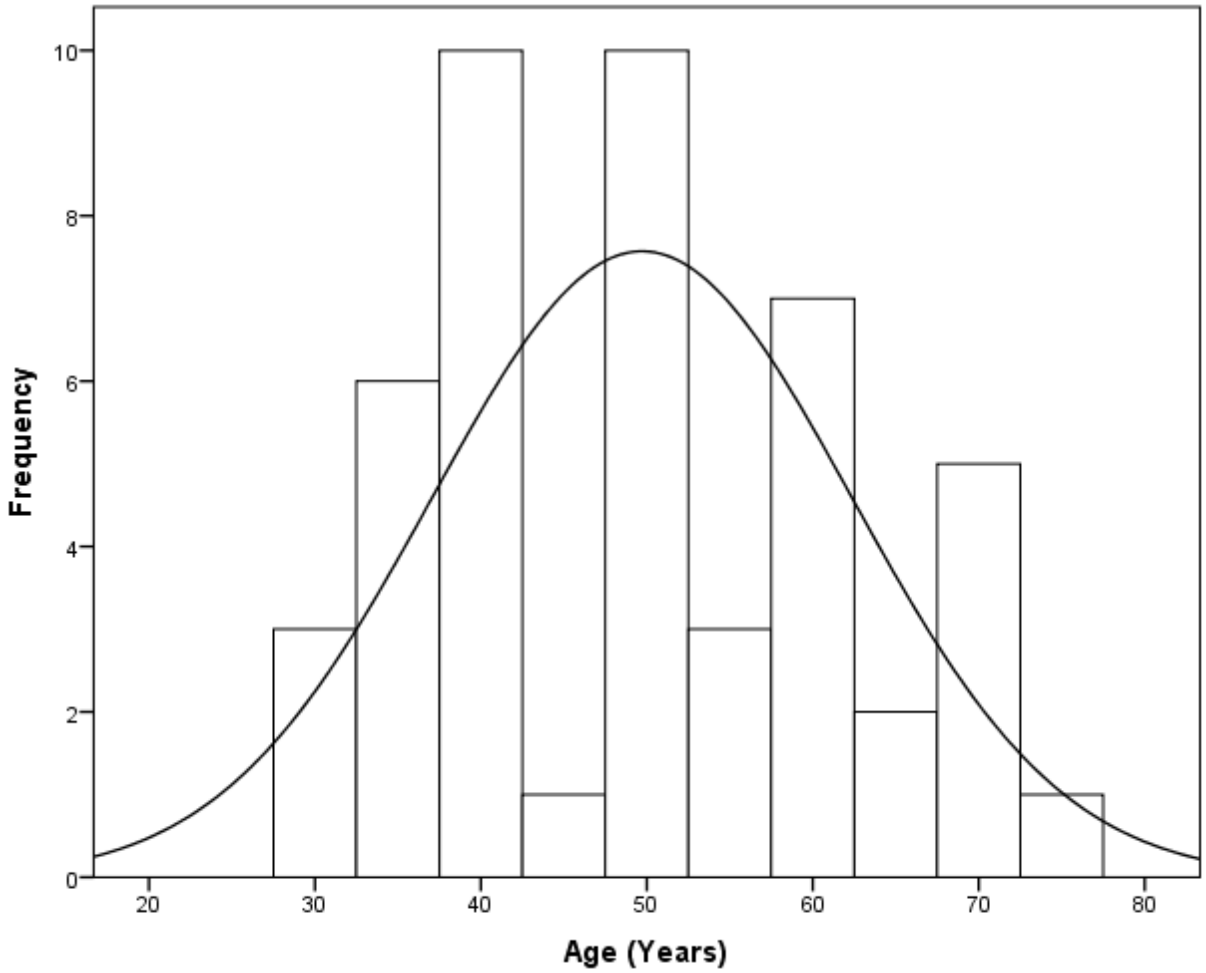

Figure (1): Shows distribution of age groups among study population

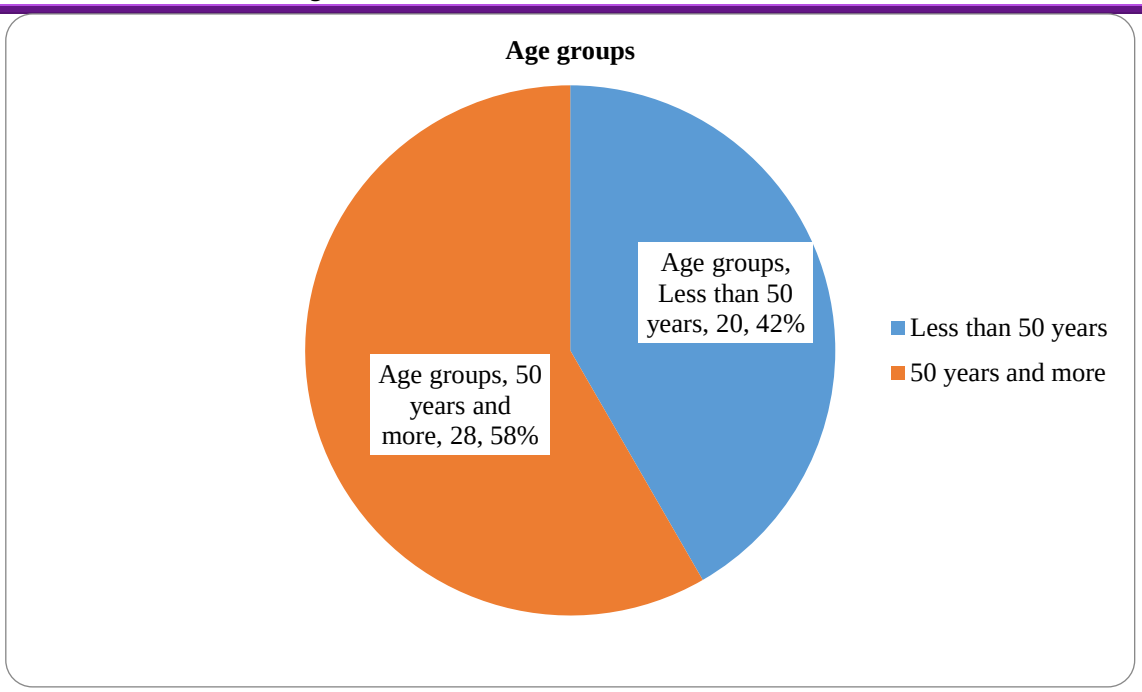

Figure (2): Summarizes the frequency of age according to the age sub groups.

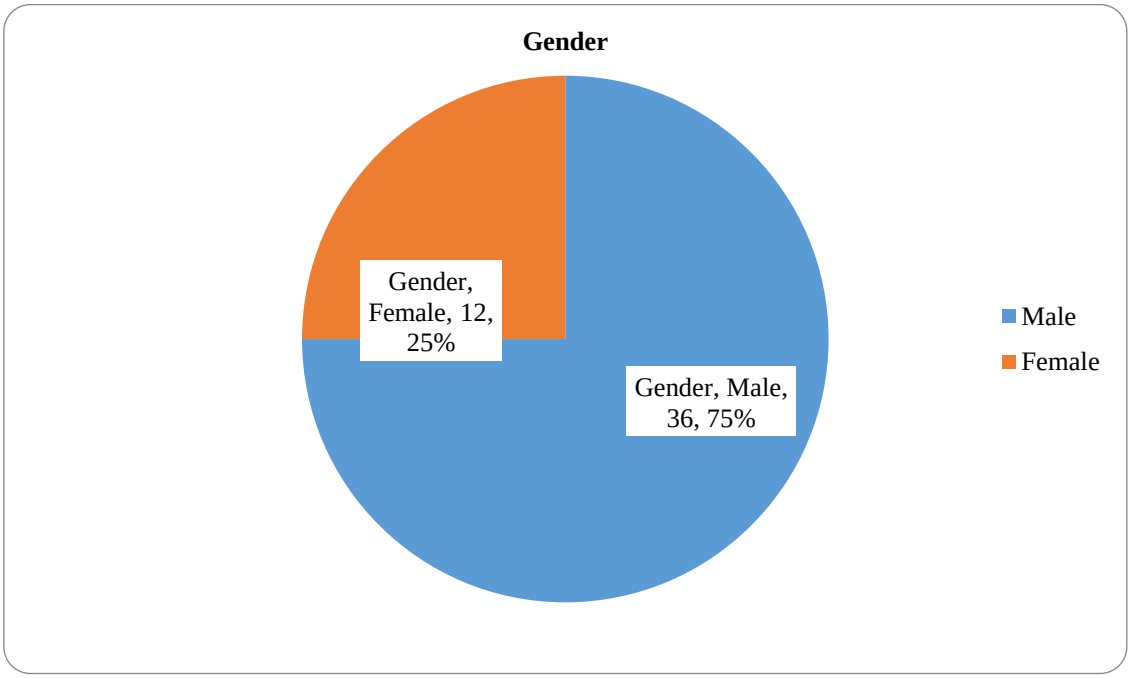

Figure (3): The distribution of gender among study populations.

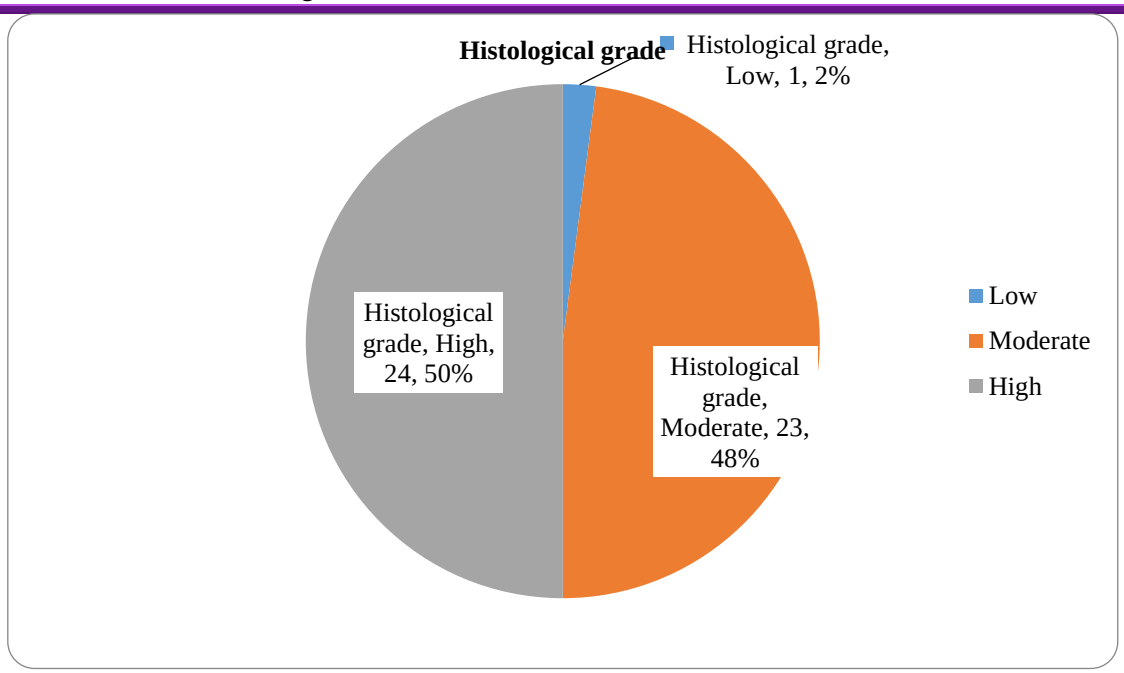

Figure (4): Indicates frequency of histological grade among study samples.

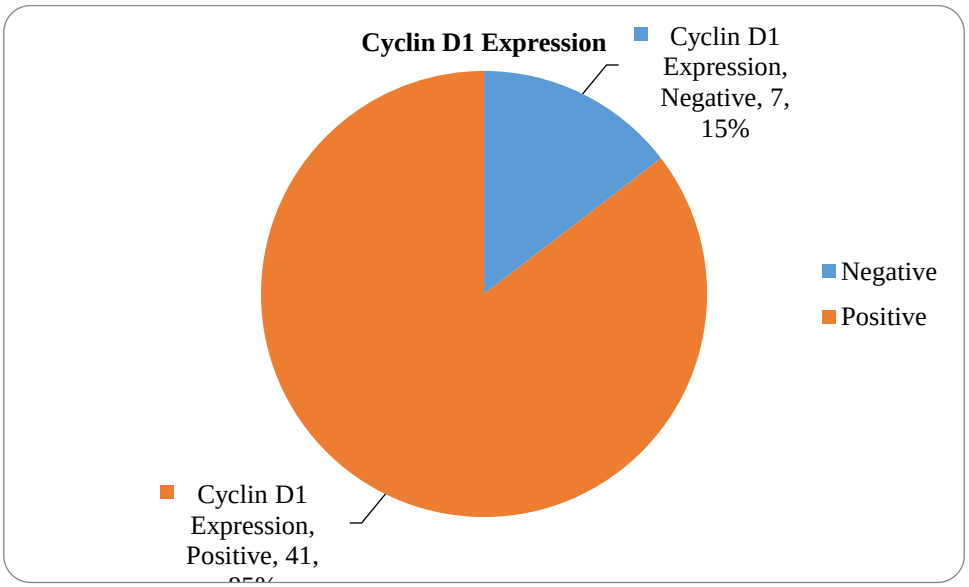

Figure (5): Represents the result of cyclin D1 immune expression.

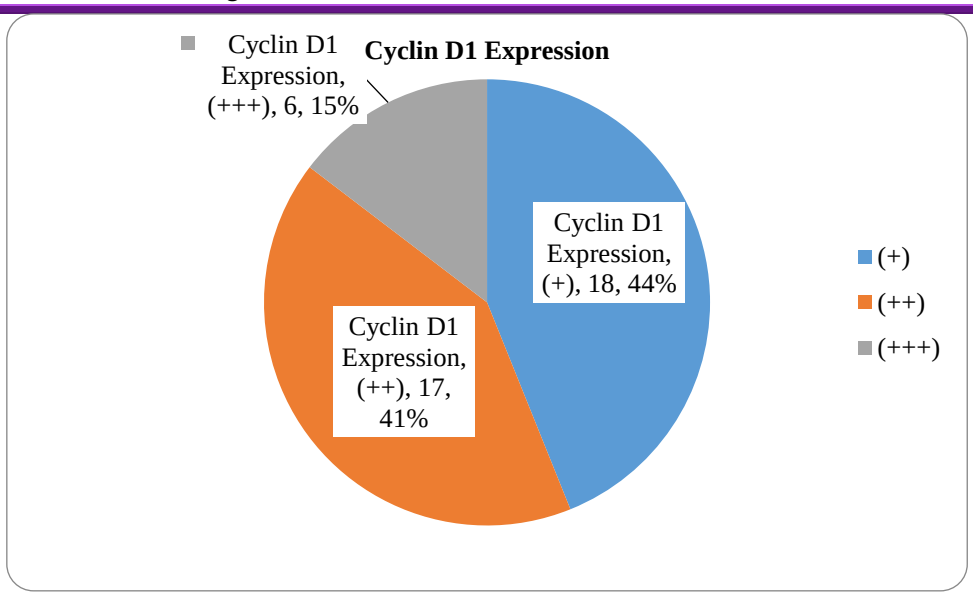

Figure (6): Detects the expression score of cyclin D1 among study samples

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Conflict of interest

Authors declare no conflict of interest

Ethics approval and consent to participate

Written Approval from UMST Ethical Research Committee in accordance with the Declaration of Helsinki Principles, similar approval took from Khartoum Ministry of Health and Elrahma medical center was taken. Research purpose benefits and objectives were explained to hospital and laboratory administration in clear simple words with assurance on confidentiality.

Cyclin D1 Ab was applied in formalin fixed paraffin embedded tissue section already diagnosed as colorectal cancer sample and after got permission from hospital administration.

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Authors' contributions

HISM and MAE conceived the design and carried out the experiments. AAI obtained, analyzed and interpreted the data. MAE and AAI wrote and revised the manuscript. HISM provides financial support for all experiments. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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Availability of data and materials

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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