Original Research Article

Does AMACR and Cyclin D1 Immunohistochemical expression Vary on Comparing Mismatch Repair Proficient versus Deficient Colorectal Carcinomas?

Abstract

AMACR & Cyclin D1 expression in relation to different clinicopathological features was compared in MMR proficient versus deficient CRC subgroups.MLH1 & MSH2 immunostaining was used to sort studied carcinomas. AMACR, cyclin D1 & ki67 expression was evaluated in neoplastic & non-neoplastic lesions too. Of studied carcinomas, 40% were MMR proficient & 60% were MMR deficient. Low AMACR expression was detected in 50% & 66.7% of MMR proficient &deficient subgroups respectively. Cyclin D1 displayed high expression in 66.7% of MMR proficient & low expression in 53.3% of MMR deficient subgroups. AMACR is significantly related to gender, grade, extracellular mucin and dirty necrosis in MMR deficient while only to circumscription in MMR proficient subgroups. Cyclin D1 associated significantly with location, gross features, histologic type, pT, and pN in MMR proficient, and with location, histologic type, pT, pN, tumor stage, extracellular mucin, buds and dirty necrosis in MMR deficient subgroups. Correlations between AMACR and both cyclin D1 and Ki67 expression were significant in MMR deficient but were insignificant in MMR proficient subgroups. In both subgroups, the correlation between cyclin D1 and Ki67 expression was significant. AMACR and cyclin D1 seem to have a role in CRC carcinogenesis & genomic status influences their expression.

Keywords: AMACR, Cyclin D1, Mismatch repair (MMR), Colorectal carcinoma (CRC)

Key points

- Colorectal carcinoma (CRC) seems to be a heterogenous disease.
- AMACR and cyclin D1 seem to have a role in carcinogenesis of CRC.
- Genomic status of tumour cells influences pathological features & marker expression.
- Molecular classifications should be used on larger scale prospective studies to validate their predictive & prognostic value.

1. Introduction

Colorectal cancer (CRC) is the third most prevalent cancer in men and the second most common cancer in women [1]. Despite advances in the diagnostic and therapeutic approaches, local recurrence or distant metastasis occurs early in a

considerable proportion of patients [2]. The significant variability in the clinical outcome of CRC patients could be explained by tumor molecular heterogeneity. Accordingly, CRC molecular subtypes could be employed to identify patients at risk of recurrence and could aid in individualizing the treatment for better response [3].

One of the important molecular hallmarks in CRC is microsatellite instability (MSI). MSI, caused by mutations in DNA mismatch repair genes such as MLH1, MSH2, MSH6, and PMS2, is detected in 10-15% of sporadic CRCs [4]. CRC patients with MSI were reported to have better survival compared with that of microsatellite stable (MSS) CRC patients [5].

Alpha-methylacyl-CoA racemase (AMACR), an enzyme currently adopted in prostate cancer diagnosis, is a mitochondrial and peroxisomal enzyme that is implicated in the beta-oxidation of branched-chain fatty acids and cholesterol metabolites [6]. However, AMACR is not tissue specific as its expression is not confined to prostatic adenocarcinoma. AMACR was found to be highly expressed in a variety of premalignant lesions as well as other carcinomas such as hepatocellular carcinoma, renal cell carcinoma, and squamous cell carcinoma of head and neck [7].

Cyclin D1 regulates cell cycle progression via its binding partners, cyclin-dependent kinases (CDKs). Cyclin D1 overexpression induces cells to switch from G1 to S phase during mitosis [8]. Cyclin D1 has been implicated in the carcinogenesis of several cancers including CRC as one third or more of CRC displayed overexpression of cyclin D1 [9]. Ki67 is a proliferation biomarker that can be used in immunohistochemistry (IHC)-based surrogate assays to determine the need for cytotoxic therapy; however, the precise biological role of Ki67 remains uncertain [10]. Cyclin D1 is another frequently applied protein biomarker of cell proliferation in routine clinical practice. Because the role of cyclin D1 in cancer initiation and progression appears to be complex and multifaceted, more research is needed to gain a thorough understanding of therapeutic interventions targeting cyclin D1 dependent mechanisms.

So, the aim of this study was tostratify CRC cases into MMR proficient & deficient subgroups and accordingly compare AMACR & Cyclin D1 expression in relation to different clinicopathological features. Moreover, the correlations between AMACR, cyclin D1 and Ki67 expression in CRC were analysed together with evaluating their expression in precancerous & non neoplastic colorectal lesions.

Materials and Methods

1.1. Study design and data collection:

This is a retrospective cross-sectional study that included colonic tissue specimens of 12 normal colonic mucosa, 18 hyperplastic colonic lesions, 51 colonic adenomas (CAs) and 75 CRCs collected from archives of the Pathology

Department. Clinical data about patient age, gender, tumor location and gross pathologic features were retrieved from pathology reports. The study was performed in accordance with the Declaration of Helsinki. This work was approved by the institutional research ethics committee with ethical approval code number 35597/7/22.

1.2. Histopathologic evaluation:

Whole slide sections stained with hematoxylin and eosin from studied cases were examined to confirm diagnosis and report different histopathologic features to be assessed in this study. Adenoma and CRC cases were assessed for the histologic type and grade according to the World Health Organisation (WHO) 2019,5th edition [11]. Tumor staging was assigned according to the American Joint Committee on Cancer (AJCC) staging manual [12]. The evaluation of other histopathological criteria like lymphovascular (LVI), tumor circumscription, presence of extracellular mucin, tumor infiltrating lymphocytes (TILs), dirty necrosis and tumor buds were assigned as reported [13].

1.3. Tissue microarray (TMA):

Tissue Microarray (TMA) was performed using the TMA builder mould (CAT# TMA-001, Thermo Fisher Scientific, Runcorn, UK) producing recipient paraffin blocks. The areas of interest on paraffin blocks of the studied specimens were identified, then tissue cores were punched out and injected into the holes on the recipient blocks to form TMA Blocks. The selected areas were representative of tumors with good cellular preservation. Areas with necrosis, crushing artifacts, or poor cellular preservation were avoided.

1.4. Immunohistochemical staining:

Immunohistochemical staining TMA blocks were sectioned (5 µm thick) on positively charged slides and were dried for 30 min at 37°C. The slides were placed in a Dako PT Link unit for deparaffinization and antigen retrieval. EnVisionTM FLEX Target Retrieval Solution with a high pH was used at 97°C for 20 minutes. Immunohistochemistry was performed using Dako Autostainer Link 48. For 10 minutes, slides were immersed in Peroxidase-Blocking Reagent, incubated with MLH1 (clone (ESO5) and MSH2 (clone FE11), AMACR (clone 13H4), Cyclin D1 (clone EP12), Ki67 (clone MIB-1), FLEX Ready-to-Use primary antibodies from Agilent, Santa Clara, United States for 20–30 min. Following that, the slides were treated for 20 minutes with horseradish peroxidase polymer reagent and 10 minutes with diaminobenzidine chromogen. After that, the slides were counterstained with hematoxylin.

1.4.1. Evaluation of MMR proteins immunostaining:

MMR proteins (MLH1 and MSH2) immunostaining was detected as brownish nuclear staining. Tumors that lacked nuclear MLH1 or MSH2 expression were labelled MLH1 or MSH2 negative. Internal positive controls included nuclear immunostaining of normal epithelial cells, lymphocytes, and stromal cells [14].

Considering the importance of MSI, we classified CRCs cases according to MMR proteins immune staining results into two groups: pMMR group; CRCs positive for both MLH1 and MSH2 immune staining and dMMR group; CRCs with loss of nuclear staining of MLH1 and/or MSH2.

1.4.2. Evaluation of AMACR immunostaining:

AMACR expression was detected as brownish staining in the cytoplasm of tumor cells. Both the intensity and the percentage of positively stained cells were evaluated. The intensity of positivity was scored as follows: 0, negative; 1, weak; 2, moderate; and 3, strong intensity. The percentage of positive cells was scored as follows: 0, 0-5%; 1, 6-20%; 2, 21-40%; 3, 41-60%; 4, 61-80% and 5, 81-100%. The final score was obtained by multiplying the intensity and the percentage of positivity scores, which yielded a range score from 0 to 15. The scores of AMACR expression were graded as negative (Score 0), poor (Score 1-5), moderate (Score 6-10), strong (Score 11-15). For statistical purpose, negative and poor were considered as low expression whereas moderate and strong were regarded as high expression [7].

1.4.3. Evaluation of Cyclin D1 immunostaining:

Cyclin D1 immunostaining was detected as nuclear brownish staining. Both intensity of staining and percentage of positive tumor cells were considered. The intensity of staining was scored as follows: 0, no staining; 1, mild; 2, moderate; and 3, marked staining. The percentage of positive cells was reported as follows: 0, less than 5%; 1, 5–25%; 2, 26–50%; 3, 51–75%; and 4, more than 75%. Both scores were added to yield the final score from 0 to 7. Scores 0-2 indicated low expression, whereas scores 3-7 indicated high expression [9].

1.4.4. Evaluation of ki67 immunostaining:

Ki67 immunostaining was detected as nuclear brownish staining. For statistical analyses, the staining results were categorised into two groups (low and high) according to the percentage of Ki67 positive tumor cells as follows: low, 0% reaching up to 25% and high, 25% and more [15].

1.5. Statistical analysis:

Statistical analysis was carried out using Statistical Package for Social Science (SPSS version 23.0). Frequencies were used to present categorical variables, whereas numerical variables were presented as mean±standard deviation (SD).

Comparing categorical variables was performed using Chi-square test. Fisher exact and Monte-Carlo tests were used when indicated. Student's t-test was applied to compare means of two groups. Correlations between variables were evaluated using Spearman's rank coefficient. P values of <0.05 were considered statistically significant.

2. Results

2.1. Clinicopathological features of studied cases:

The current study was carried out on 156 colorectal tissue specimens that included 12 normal colonic mucosa, 18 hyperplastic colonic lesions, 51 adenomas and 75 CRCs. Table 1 summarises the clinicopathologic characteristics of the studied CRC cases.

Figures 1 and 2 demonstrate MLH1 and MSH2 expression in the studied cases. CRC cases were classified according to MLH1 and MSH2 immunostaining results, 30 cases (40%) were pMMR whereas the remaining 45 cases (60%) were dMMR.

2.2. MMR status, AMACR, cyclin D1 and Ki67 expression in normal colonic tissue, hyperplastic lesions, adenoma and CRC

As regards MMR status, pMMR could be identified in all normal tissue, and hyperplastic lesions, 36 out of 51 adenoma cases (70.6%) and 30 out of 75 CRC cases (40%). Whereas dMMR cases included 15 out of 51 adenoma cases (29.4%) and 45 out of 75 CRC cases (60%) as demonstrated in table 2.

Analysing AMACR expression revealed significant differences between groups ($p<0.001$) (Table 2, Fig 3). All normal colonic tissue and hyperplastic lesions displayed low AMACR expression. Adenoma cases showed low expression in 45 cases (88.2%) and high expression in the remaining 6 cases (11.8%). CRC cases displayed low AMACR expression in 45 cases (60%) and high expression in the remaining 30 cases (40%). On pairwise comparison, AMACR expression in CRC cases was significantly different compared to normal tissue, hyperplastic lesions and adenomas ($p=0.007$, <0.001 and 0.001 respectively).

Similarly, cyclin D1 expression was significantly different among the studied groups ($p<0.001$). All normal colonic tissue and hyperplastic lesions displayed low cyclin D1 expression. The majority of adenomas (36 cases; 70.6%) showed low expression of cyclin D1 whereas the remaining cases showed cyclin D1 high expression. CRCs cases revealed low cyclin D1 expression in 34 cases (45.3%) and high expression in the remaining 41 cases (54.7%). On pairwise comparison, cyclin D1 expression in CRC cases was significantly different compared to normal tissue, hyperplastic lesions and adenomas ($p<0.001$, <0.001 and 0.006 respectively as shown in table 2. Representative images of cyclin D1 expression in the studied cases are demonstrated in Figure 4

As regards Ki67 expression, low Ki67 expression was detected in all normal colonic tissue, 6 cases (33.3%) of hyperplastic lesions, 21 cases (41.2%) of adenoma cases and 30 cases (40%) of CRC cases whereas the remaining cases displayed high expression of Ki67 (Table 2 and Fig 5). On pairwise comparison, Ki67 expression in CRC cases was significantly different compared to normal tissue ($p<0.001$).

2.3. AMACR, cyclin D1 and Ki67 expression in pMMR and dMMR CRC groups

No significant difference could be detected in AMACR, cyclin D1 and Ki67 expression between pMMR and dMMR groups ($p=0.149$, 0,088 and 1 respectively). Low AMACR expression was detected in half of cases in the pMMR group and in 30 cases (66.7%) of dMMR group. Cyclin D1 displayed high expression in 20 cases (66.7%) of pMMR group whereas 24 cases (53.3%) of dMMR group showed cyclin D1 low expression. Ki67 was similarly expressed in both groups as 60% of pMMR and dMMR groups displayed high Ki67 expression as illustrated in table 3.

2.4. AMACR expression in relation to clinicopathologic parameters in pMMR and dMMR CRC groups

As demonstrated in table 4, the relation between AMACR expression and clinicopathologic parameters in the pMMR group were all insignificant except for tumor circumscription as all circumscribed tumors displayed high AMACR expression ($p=0.001$). On the other side, in the dMMR group, significant relations were identified between AMACR expression and gender, tumor grade, extracellular mucin and dirty necrosis ($p=0.014$, <0.001, 0.020 and 0.001 respectively). Low AMACR expression was associated with male gender, high tumor grade, the presence of extracellular mucin and the absence of dirty necrosis.

2.5. Cyclin D1 expression in relation to clinicopathologic parameters in pMMR and dMMR CRC groups

Studying cyclin D1 expression in pMMR group revealed significant associations with tumor location, gross features, histologic type, pT, and pN ($p=0.030$, 0.009, 0.034, 0.03 and 0.038 respectively). Whereas, in dMMR group, cyclin D1 was significantly associated with tumor location, histologic type, pT, pN, tumor stage, extracellular mucin, tumor buds and dirty necrosis ($p=0.047$, 0.010, <0.001, 0.001, 0.004, 0.002, 0.041 and 0.014 respectively) as summarized in table 5. As regards histologic type, high cyclin D1 was detected in 70.8% of conventional adenocarcinoma cases in pMMR group and half of conventional adenocarcinoma cases in dMMR group. All mucinous cases, both in pMMR and dMMR groups, displayed low cyclin D1 expression. Dealing with pT, all pT2 cases in pMMR group displayed low cyclin D1 expression while all pT2 cases in dMMR groups showed cyclin D1 high expression. In addition, 75% of pN2 cases in the pMMR group showed cyclin D1 high expression whereas all pN2 cases in the dMMR group displayed cyclin D1 low expression.

2.6. Correlation between AMACR, cyclin D1 and Ki67 expression in pMMR and dMMR CRC groups

A significant positive weak correlation was detected between AMACR and cyclin D1 expression in dMMR group (r=0.376, p=0.011) whereas their correlation in pMMR group was weak, negative and insignificant (r=-0.247, p=0.189). The correlation between AMACR and ki67 expression was significant, moderate and negative (r=-0.441, p<0.002) in dMMR group, while in pMMR group this correlation was insignificant, very weak positive correlation (r=0.105, p=0.581). As regards the correlations between cyclin D1 and Ki67 expression, they showed significant positive correlations in both groups. However, these correlations were weak in the dMMR group and moderate in the pMMR group as illustrated in Fig 6.

3. Discussion

Colorectal carcinoma is the third commonest cancer representing about 10% of global cancer incidence. To personalize treatment for patients with CRC, it is essential to understand its nature and carcinogenesis mechanisms that can promote tumor progression. This unique biological signature of CRC can be investigated by identifying the involved biomarkers and evaluating their expression [16].

In this study, the expression of AMACR and cyclin D1 was investigated in non-neoplastic and neoplastic colorectal lesions using TMA. Both AMACR and cyclin D1 expression varied significantly among the studied groups (p<0.001 for both); where they showed low expression in all normal and hyperplastic cases as well as in the majority of adenoma cases (88.2%, 70.6% respectively), while their expression was high in 40% and 54.7% of CRC cases respectively.

These findings for AMACR go with others [7, 17-19]. Jiang et al. [17] proposed a link between AMACR expression in colonic adenomas and high red meat intake and explained it by possible attribution of AMACR in oxidation of branched fatty acids present in red meat. Because adenomas are antecedents of CRCs and have higher AMACR expression than non-neoplastic tissue, Shukla et al. [7] reported that this suggests the role AMACR may play in colorectal carcinogenesis. This even shows that AMACR could be used as a diagnostic marker for neoplastic transformation.

Cyclin D1 showed sequential overexpression from normal to adenoma to carcinoma indicates an oncogenic role of cyclin D1 in colorectal carcinogenesis. This was similar to others [20-24]. Further accordant results were reported in the studies of Toru et al. [25] and Nassrat et al. [26] on colonic adenomas and CRCs which had stated that cyclin D1 is responsible for the pathological changes in the mucosa, adjunctive indicator for the risk of malignancy in adenomas as cyclin D1 overexpression disrupts the cell cycle and is associated with progression to cancer.

Müller et al. [27] reported that CRC is not a homogeneous disease but can be classified into different subtypes distinguished by specific molecular and morphological alterations. CRC is characterised by genetic instability, which can occur through at least two different mechanisms: Microsatellite instability and chromosomal instability.

Ten Hoorn et al. [28] recommended inclusion of molecular subtyping in future studies to validate prognostic and predictive associations. They added that refining the subtype classification with prognostic biomarkers could lead to a more in-depth understanding of the various subtypes. That is what the current study attempted to do.

Some CRCs are accompanied by microsatellite instability. This is caused by a defect in the DNA mismatch repair mechanism as a consequence of a germline/somatic mutation in the MMR genes [4]. MMR genesstatus, whether deficient or proficient, is considered as a useful prognostic indicator [29]. However, the effect of MMR genes on clinicopathological features and its relation to AMACR and cyclin D1 expression remains unclear.

Accordingly, CRC cases included in this study were sorted into pMMR (30 cases) and dMMR (45 cases) molecular subgroups according to their MLH1 and MSH2 expression. There was no statistically significant difference in frequency of AMACR (p= 0.149) nor in cyclin D1 (p=0.088) expression when comparing both molecular subgroups. In contrast, Chen et al. [30] observed a statistically significant difference in the expression of AMACR between MSI versus non-MSI colon carcinomas. Nosho et al. [31] reported that the identification of molecular correlates of cyclin D1 activation is critical for understanding carcinogenic pathways in different molecular subtypes of CRC. Few studies, however, have thoroughly investigated the relationship between cyclin D1, CDK inhibitors, and MSI in colorectal cancer [31].

The expression of the studied markers in each molecular subgroup was furtherly investigated in relation to clinicopathologic parameters included in this study. dMMR carcinomas showed statistically significant lower AMACR expression in male gender (p=0.014), high tumor grade (p<0.001), mucinous phenotype (p=0.020) and the absence of dirty necrosis (p=0.001). On the other hand, pMMR carcinomas showed no significant associations between AMACR expression and different clinicopathological parameters except for significant higher expression in well circumscribed tumors (p=0.001).

These findings suggest that AMACR can be considered a good indicator for early carcinogenesis. The decrease of AMACR expression in higher grade poorly differentiated CRCs can be attributed to the involvement of AMACR in providing energy for neoplastic cells via degradation of branched chained fatty acids. When the tumors become dedifferentiated, they require no more of these sources of energy. Poorly differentiated tumors may employ other mechanisms to achieve the same impact as branched fatty acid oxidation [7].

In approval with our results, Chen et al [30]. and Lin et al. [19] reported significant correlations between absent or weak AMACR staining and mucinous histology, poorer differentiation, and lymphovascular invasion. Other factors including age, sex, tumor location, and staging were not significantly related to AMACR expression in their studies. Their

results also suggested the involvement of AMACR in early tumorigenesis and also led to the speculation of a link between AMACR expression and MSI status.

Marx et al. [32] noticed AMACR positivity in 81.7% of their studied cases. Reduced AMACR expression was substantially related to high tumour grade and stage but had no association to nodal status. In contrast to our findings, AMACR positivity was more common in tubular carcinoma than in other histological subtypes such as mucinous or signet cell carcinoma. AMACR expression was substantially higher in the left-sided CRCs in their study than in the right-sided CRCs. They hypothesised that in patients with hereditary non-polyposis colon cancer syndromes or sporadic colorectal cancers with MSI, which are more commonly seen in the right colon, pathways leading to elevated AMACR expression are less frequently activated [33].

These previous studies were in accordance with Kuefer et al. [34], Atef and Bedeer [35] andAdil et al. [36] who demonstrated high AMACR expression in well to moderately differentiated CRCs and weak expression in poorly/anaplastic CRCs. Their data also suggested that AMACR expression can be a marker of tumor differentiation.

Shukla et al. [7] reported AMACR positivity in 65.9% of their CRC cases with significant difference of expression in different tumor grades, tumor stage and nodal status. Their study showed no significant distinction between adenoma and carcinoma cases, but their AMACR expression was higher than in normal colonic epithelium, suggesting that AMACR may play a role in colorectal carcinogenesis.

On the other hand, Bagheri and Ghafghazi [37] observed that all patients with colorectal cancer highly expressed AMACR. The staining intensity was more than +2 in more than half of the cases, and more than 90% of the patients showed a strong positive reaction to the AMACR marker with no significant relation to tumor grade.

The variability in results between our study and some of other involved studies can be attributed to different antibodies, different scoring systems, different techniques and different ethnicities and races.

Regarding the relation between cyclin D1 expression and clinicopathologic parameters, the results of the present study showed that tumor location was significantly associated with cyclin D1 expression in both pMMR and dMMR groups (p=0.03, =0.047 respectively) This was consistent with the work of Sharma et al. [38] which noted that Cyclin D1 expression was shown to be higher in tumours on the colon's left side, particularly in the sigmoid and descending colon. On the contrary, Jiang et al. [22], Al-Maghrabi et al. [9] and Albasri et al. [24] found such a relation was not statistically significant.

As regards histologic type, cyclin D1 varied significantly in both pMMR and dMMR molecular subgroups (p=0.034, =0.01 respectively). High cyclin D1 was detected in 70.8% of conventional adenocarcinoma cases in pMMR subgroup and half of conventional adenocarcinoma cases in dMMR subgroup, while all mucinous cases, both in pMMR and dMMR

subgroups, displayed low cyclin D1 expression. Sharma et al. [38] showed almost the same results with conventional adenocarcinoma while only 30% cases of their included mucinous adenocarcinoma showed high cyclin D1 expression. However, Albasri et al. [24] reported no significant relation found between cyclin D1 expression and histologic type of CRCs.

The results of the present study addressing cyclin D1 expression with regard to staging parameters were significantly different according to the MMR studied group, where all pT2 cases in pMMR subgroup displayed low cyclin D1 expression while all pT2 cases in dMMR subgroup showed cyclin D1 high expression. In addition, 75% of pN2 cases in the pMMR subgroup showed cyclin D1 high expression whereas all pN2 cases in the dMMR subgroup displayed cyclin D1 low expression.

Our results were concordant with Albasri et al. [24] that found cyclin D1 to be significantly correlated with lymph node metastasis, lymphovascular invasion, distant metastasis, and AJCC stage reporting. Their study was also concordant with previous studies of Assaad et al. [39], Belcerczak et al. [40] and Almaghrabi et al. [9] that showed similar correlation between pathological tumor stage and cyclin D1 over-expression. On the contrary, the study of Sharma et al. [38] reported that cyclin D1 expression was almost equal with the number of lymph nodes that showed metastatic or reactive pathology, hence no statistical significance was found.

These heterogenic results could be explained by the molecular signature of the studied CRCs cases which we aimed to spotlight on its significance when evaluating the prognostic influence of studied markers' expression. It worth mentioning that Kawakami et al. [3] demonstrated that when compared to pMMR tumours, dMMR CRCs have distinct clinical and pathologic features, such as poor differentiation and/or mucinous histology, proximal colon location, and increased tumour infiltrating lymphocytes. In their study, CRC with dMMR was more common in stage II than in stage III and was relatively uncommon in metastatic tumours. This emphasises the significance of MSI testing in early-stage tumours where patients can be cured with surgery alone or in combination with adjuvant chemotherapy.

MSI status reflects genetic abnormalities in tumour cells and influences CRC molecular characteristics and phenotypes. The genetic status of tumour cells is anticipated to influence both pathogenic features and cyclin D1 expression [31]. Moreno-Bueno et al. [41]previously reported a link between cyclin D1 overexpression and MSI in endometrial carcinoma. In terms of colorectal cancer, Ortega et al. [42] has reported that cyclin D1 expression was generally low in 11 MSI-high tumours, but higher expression levels were observed in 37 MSS/MSI-low tumors. This disparity could be attributed to differences in sample sizes as well as methods and criteria for interpreting cyclin D1.

In addition to our previous findings, the relation between AMACR expression and tumor proliferation, represented by Ki67 expression, in dMMR cases showed statistically significant negative correlation (r= -0.441, p=0.002). AMACR expression was lower in CRCs with higher tumor proliferation which was in support of the low AMACR expression in high grade tumors. Meanwhile, AMACR expression in pMMR cases positively correlated to Ki67 expression but didn't reach statistical significance. This was in line with results observed by Takagi et al. [43] who found that Ki67 expression was significantly higher in the MSI-positive tumors than that in the MSI-negative ones.

The correlation between cyclin D1 and Ki67 expression in the present study showed significant positive correlations in both molecular subgroups. However, this correlation was weak in the dMMR group and moderate in the pMMR group. Similarly, there was a significant correlation between cyclin D1 levels and Ki67 expression in the study of Kouraklis et al. [44] and Ayerden et al. [45] on CRCs suggesting Ki67 role in oncogenesis and the role contributed cyclin D1 for tumor proliferation suggesting that both can be used to predict the prognosis in CRCs.

The results of this study showed a significant positive weak correlation between AMACR and cyclin D1 expression in dMMR group (r=0.376, p=0.011) whereas their correlation in pMMR group was insignificant, weak, negative and (r=-0.247, p=0.189). This might be attributed to the effect of microsatellite status on the expression of AMACR and or cyclin D1. However, further studies should address such influence in order to reach a consensus.

Conclusion:

CRC is a heterogeneous disease. AMACR and cyclin D1 seem to have attribution in carcinogenesis of CRC, however they as well as pathological features are influenced by genomic status of tumour cells. Further prospective larger scale studies that use molecular classifications should be done to validate these classifications and evaluate their prognostic and predictive value.

Abbreviations

AJCC: American Joint Committee on Cancer; AMACR: Alpha-methylacyl-CoA racemase; Cas: colonic adenomas; CDKs: cyclin-dependent kinases; CRCs: colorectal carcinomas; dMMR: MMR deficient; IHC: Immunohistochemistry; LVI: lymphovascular invasion; MSI: microsatellite instability; MSS: microsatellite stable; pMMR: MMR proficient; SD: Standard deviation; SPSS: Statistical Package for Social Science; TILs: tumor infiltrating lymphocytes; TMA: Tissue Microarray; WHO: world health organization

Statements and Declarations:

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate:

The study was performed in accordance with the Declaration of Helsinki & has been approved by the institutional research ethics committee with ethical approval code number 35597/7/22.

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Table 1: Clinicopathologic characteristics of colorectal carcinoma cases

	Total N (%)
Age (years)	
mean ± SD	58.79±10.54
Gender	
Male	39 (52)
Female	36 (48)
Location	
Right side	18 (24)
Left side	54 (72)
Multicentric	3 (4)
Gross picture	
Endophytic/Ulcerative	30 (40)
Exophytic /Cauliflower	36 (48)
Endophytic /Annular	9 (12)
Histologic type	
Conventional adenocarcinoma	60 (80)
Mucinous	9 (12)
Signet ring cell	3 (4)
Medullary carcinoma	3 (4)
Pathologic grade	
Low	45 (60)
High	30 (40)
pT	
pT2	12 (16)
pT3	54 (72)
pT4	9 (12)

pN	
pN0	30 (40)
pN1	27 (36)
pN2	18 (24)
Stage	
II	30 (40)
III	45 (60)
Lymphovascular invasion	
No	6 (8)
Yes	69 (92)
Circumscription	
No	36 (48)
Yes	39 (52)
Extracellular mucin	
No	48 (64)
Yes	27 (36)
Tumor buds	
No	24 (32)
Yes	51 (68)
TILs	
No	24 (32)
Yes	51 (68)
Dirty necrosis	
No	30 (40)
Yes	45 (60)

TILs: Tumor infiltrating lymphocytes

Table 2: MMR status, AMACR, cyclin D1 and Ki-67 expression in normal colonic tissue, hyperplastic lesions, adenoma and carcinoma

		Normal N=12 (%) A	Hyperplastic N=18 (%) B	Adenoma N=51 (%) C	Carcinoma N=75 (%) D	P value
MMR status						
Proficient	**81**	12 (100)	18 (100)	36 (70.6)	30 (40)	**<0.001***
Deficient	**60**	0 (0)	0 (0)	15 (29.4)	45 (60)	
Pairwise comparison		**AC=0.031*, AD<0.001*, BC<0.007*, BD<0.001*, CD=0.001***				
AMACR						
Low	**120**	12 (100)	18 (100)	45 (88.2)	45 (60)	**<0.001***
High	**36**	0 (0)	0 (0)	6 (11.8)	30 (40)	
Pairwise comparison		AC=0.585, **AD=0.007*,** BC=0.328**, BD<0.001*, CD=0.001***				
Cyclin D1						
Low	**100**	12 (100)	18 (100)	36 (70.6)	34 (45.3)	**<0.001***
High	**56**	0 (0)	0 (0)	15 (29.4)	41 (54.7)	
Pairwise comparison		**AC=0.031*, AD<0.001*, BC<0.007*, BD<0.001*, CD=0.006***				
Ki-67						
Low	**69**	12 (100)	6 (33.3)	21 (41.2)	30 (40)	**<0.001***
High	**87**	0 (0)	12 (66.7)	30 (58.8)	45 (60)	

Pairwise comparison	**AC<0.001*, AD<0.001*,** BC=0.779, BD=0.789, CD=1	

*significant (p value<0.05), MMR: Mismatch repair

Table 3: AMACR, cyclin D1 and Ki-67 expression in MMR proficient and MMR deficient colorectal carcinoma groups

		MMR proficient N= 30 (%)	MMR deficient N = 45 (%)	P value
AMACR				
Low	**45**	15 (50)	30 (66.7)	0.149
High	**30**	15 (50)	15 (33.3)	
Cyclin D1				
Low	**34**	10 (33.3)	24 (53.3)	0.088
High	**41**	20 (66.7)	21 (46.7)	
Ki-67				
Low	**30**	12 (40)	18 (40)	1
High	**45**	18 (60)	27 (60)	

Table 4: Relation between AMACR expression and clinicopathologic parameters in MMR proficient and MMR deficient colorectal carcinoma groups

	AMACR							
	Proficient				Deficient			
	Total	AMACR Low N=15 (%)	AMACR High N=15 (%)	P value	Total	AMACR Low N=30 (%)	AMACR High N=15 (%)	P value
Age (years)								
mean ± SD		55.53±12.40	55.20±12.43	0.942		60.60±9.99	62.00±5.36	0.616
Gender								
Male	**18**	6 (33.3)	12 (66.7)	0.060	**21**	18 (85.7)	3 (14.3)	**0.014***
Female	**12**	9 (75)	3 (25)		**24**	12 (50)	12 (50)	
Location								
Right side	**9**	3 (33.3)	6 (66.7)	0.427	**9**	6 (66.7)	3 (33.3)	0.644
Left side	**21**	12 (57.1)	9 (42.9)		**33**	21 (63.6)	12 (36.4)	
Multicentric	**0**				**3**	3 (100)	0 (0)	
Gross picture								
Endophytic/Ulcerative	**15**	9 (60)	6 (40)	0.258	**15**	9 (60)	6 (40)	0.451
Exophytic/Cauliflower	**12**	6 (50)	6 (50)		**24**	18 (75)	6 (25)	
Endophytic /Annular	**3**	0 (0)	3 (100)		**6**	3 (50)	3 (50)	
Histologic type								
Conventional adenocarcinoma	**24**	12 (50)	12 (50)	0.050	**36**	21 (58.3)	15 (41.7)	0.075
Mucinous	**3**	3 (100)	0 (0)		**6**	6 (100)	0 (0)	
Signet ring cell	**3**	0 (0)	3 (100)		**0**			
Medullary carcinoma	**0**				**3**	3 (100)	0 (0)	
Pathologic grade								
Low	**21**	12 (57.1)	9 (42.9)	0.427	**24**	9 (37.5)	15 (62.5)	**<0.001***
High	**9**	3 (33.3)	6 (66.7)		**21**	21 (100)	0 (0)	
pT								
pT2	**3**	3 (100)	0 (0)	0.224	**9**	3 (33.3)	6 (66.7)	0.077
pT3	**27**	12 (44.4)	15 (55.6)		**27**	21 (77.8)	6 (22.2)	
pT4	**0**				**9**	6 (66.7)	3 (33.3)	
pN								
pN0	**15**	9 (60)	6 (40)	0.201	**15**	9 (60)	6 (40)	0.261
pN1	**3**	0 (0)	3 (100)		**24**	15 (62.5)	9 (37.5)	
pN2	**12**	6 (50)	6 (50)		**6**	6 (100)	0 (0)	
Stage								
II	**15**	9 (60)	6 (40)	0.273	**15**	9 (60)	6 (40)	0.502
III	**15**	6 (40)	9 (60)		**30**	21 (70)	9 (30)	
Lymphovascular invasion								
No	**6**	3 (50)	3 (50)	1	**0**	0 (0)	0 (0)	
Yes	**24**	12 (50)	12 (50)		**45**	30 (66.7)	15 (33.3)	
Circumscription								
No	**21**	15 (71.4)	6 (28.6)	**0.001***	**15**	9 (60)	6 (40)	0.502
Yes	**9**	0 (0)	9 (100)		**30**	21 (70)	9 (30)	
Extracellular mucin								
No	**12**	3 (25)	9 (75)	0.060	**36**	21 (58.3)	15 (41.7)	**0.020***
Yes	**18**	12 (66.7)	6 (33.3)		**9**	9 (100)	0 (0)	
Tumor Buds								
No	**12**	6 (50)	6 (50)	1	**12**	6 (50)	6 (50)	0.153
Yes	**18**	9 (50)	9 (50)		**33**	24 (72.7)	9 (27.3)	
TILs								
No	**12**	6 (50)	6 (50)	1	**12**	9 (75)	3 (25)	0.722
Yes	**18**	9 (50)	9 (50)		**33**	21 (63.6)	12 (36.4)	
Dirty necrosis								
Absent	**15**	9 (60)	6 (40)	0.273	**15**	15 (100)	0 (0)	**0.001***
Present	**15**	6 (40)	9 (60)		**30**	15 (50)	15 (50)	

*significant (p value <0.05), TILs: tumor infiltrating lymphocytes

Table 5: Relation between cyclin D1 expression and clinicopathologic parameters in MMR proficient and MMR deficient colorectal carcinoma groups

	Cyclin D1							
	Proficient				Deficient			
	Total	Cyclin D1 Low N=10 (%)	Cyclin D1 High N=20 (%)	P value	Total	Cyclin D1 Low N=24 (%)	Cyclin D1 High N=21 (%)	P value
Age (years)								
mean ± SD		51.50±13.47	57.30±11.38	0.226		62.62±8.30	59.29±8.97	0.202
Gender								
Male	**18**	4 (22.2)	14 (77.8)	0.235	**21**	12 (57.1)	9 (42.9)	0.632
Female	**12**	6 (50)	6 (50)		**24**	12 (50)	12 (50)	
Location								
Right side	**9**	0 (0)	9 (100)	**0.030***	**9**	3 (33.3)	6 (66.7)	**0.047***
Left side	**21**	10 (47.6)	11 (52.4)		**33**	21 (63.6)	12 (36.4)	
Multicentric	**0**				**3**	0 (0)	3 (100)	
Gross picture								
Endophytic/Ulcerative	**15**	6 (40)	9 (60)	**0.009***	**15**	6 (40)	9 (60)	0.415
Exophytic/Cauliflower	**12**	1 (8.3)	11 (91.7)		**24**	15 (62.5)	9 (37.5)	
Endophytic /Annular	**3**	3 (100)	0 (0)		**6**	3 (50)	3 (50)	
Histologic type								
Conventional adenocarcinoma	**24**	7 (29.2)	17 (70.8)	**0.034***	**36**	18 (50)	18 (50)	**0.010***
Mucinous	**3**	3 (100)	0 (0)		**6**	6 (100)	0 (0)	
Signet ring cell	**3**	0 (0)	3 (100)		**0**			
Medullary carcinoma	**0**				**3**	0 (0)	3 (100)	
Pathologic grade								
Low	**21**	7 (33.3)	14 (66.7)	1	**24**	12 (50)	12 (50)	0.632
High	**9**	3 (33.3)	6 (66.7)		**21**	12 (57.1)	9 (42.9)	
pT								
pT2	**3**	3 (100)	0 (0)	**0.030***	**9**	0 (0)	9 (100)	**<0.001***
pT3	**27**	7 (25.9)	20 (74.1)		**27**	15 (55.6)	12 (44.4)	
pT4	**0**				**9**	9 (100)	0 (0)	
pN								
pN0	**15**	4 (26.7)	11 (73.3)	**0.038***	**15**	3 (20)	12 (80)	**0.001***
pN1	**3**	3 (100)	0 (0)		**24**	15 (62.5)	9 (37.5)	
pN2	**12**	3 (25)	9 (75)		**6**	6 (100)	0 (0)	
Stage								
II	**15**	4 (26.7)	11 (73.3)	0.700	**15**	3 (20)	12 (80)	**0.004***
III	**15**	6 (40)	9 (60)		**30**	21 (70)	9 (30)	
Lymphovascular invasion								
No	**6**	3 (50)	3 (50)	0.372	**0**	0 (0)	0 (0)	
Yes	**24**	7 (29.2)	17 (70.8)		**45**	24 (53.3)	21 (46.7)	
Circumscription								
No	**21**	7 (33.3)	14 (66.7)	1	**15**	6 (40)	9 (60)	0.205
Yes	**9**	3 (33.3)	6 (66.7)		**30**	18 (60)	12 (40)	
Extracellular mucin								
No	**12**	3 (25)	9 (75)	0.694	**36**	15 (41.7)	21 (58.3)	**0.002***
Yes	**18**	7 (38.9)	11 (61.1)		**9**	9 (100)	0 (0)	
Tumor Buds								
No	**12**	3 (25)	9 (75)	0.694	**12**	3 (25)	9 (75)	**0.041***
Yes	**18**	7 (38.9)	11 (61.1)		**33**	21 (63.6)	12 (36.4)	
TILs								
No	**12**	4 (33.3)	8 (66.7)	1	**12**	6 (50)	6 (50)	0.787

Yes	**18**	6 (33.3)	12 (66.7)		**33**	18 (54.5)	15 (45.5)	
Dirty necrosis								
Absent	**15**	7 (46.7)	8 (53.3)	0.245	**15**	12 (80)	3 (20)	**0.014***
Present	**15**	3 (20)	12 (80)		**30**	12 (40)	18 (60)	

*significant (p value <0.05), TILs: tumor infiltrating lymphocytes

Figure Legends:

Figure 1:	**Expression of MLH1 in colorectal tissues detected by immunohistochemistry (DAB staining). MLH1 positivity in: Normal mucosa (A), Hyperplastic Polyp (B), Adenoma with Low grade dysplasia (C), Adenoma with High grade dysplasia (D&E), Adenocarcinoma (F&G). MLH1 negativity in Adenocarcinoma (H-J), with maintained expression is in overlying mucosa in upper right corner of H (A-E, G & J X400; F,I X200; H X100).**
Figure 2:	**Expression of MSH2 in colorectal tissues detected by immunohistochemistry (DAB staining). MSH2 positivity in: Normal mucosa (A), Hyperplastic Polyp (B), Adenoma with Low grade dysplasia (C), Adenoma with High grade dysplasia (D), Adenocarcinoma (F-I). MSH2 negativity in: Adenoma with High grade dysplasia (E), Adenocarcinoma (J). (A, C, D, E, I , J X400; B, F-H X200).**
Figure 3:	**Expression of AMACR in colorectal tissues detected by immunohistochemistry (DAB staining). AMACR Low expression in: Normal mucosa (A), Hyperplastic Polyp (B), Adenoma with Low grade dysplasia (C), Adenocarcinoma (F). AMACR High expression in: Adenoma with High grade dysplasia (D, E), Adenocarcinoma (G-J). (A, C-E, G, H, J X400; B, F X200; I X100).**
Figure 4:	**Expression of CCDN1 in colorectal tissues detected by immunohistochemistry (DAB staining). CCDN1 Low expression in: Normal mucosa (A), Hyperplastic Polyp (B), Adenoma with Low grade dysplasia (C), Adenocarcinoma (E, F). CCDN1 High expression in: Adenoma with High grade dysplasia (D), Adenocarcinoma (G, H). (A, D, F-H X400; B, C, E, X200).**
Figure 5:	**Expression of Ki67 in colorectal tissues detected by immunohistochemistry (DAB staining). Ki67 Low expression in: Normal mucosa (A). Ki67 High expression in: Hyperplastic Polyp (B), Adenoma with Low grade dysplasia (C), Adenoma with High grade dysplasia (D), Adenocarcinoma (E-H). (A, C, D, F, G X400; B, E, H X200).**

Figure 6:	**Correlations between AMACR, cyclin D1 and Ki67 expression in MMR proficient group (A, C, E) and MMR deficient group (B, D, F).**

Figure 1:

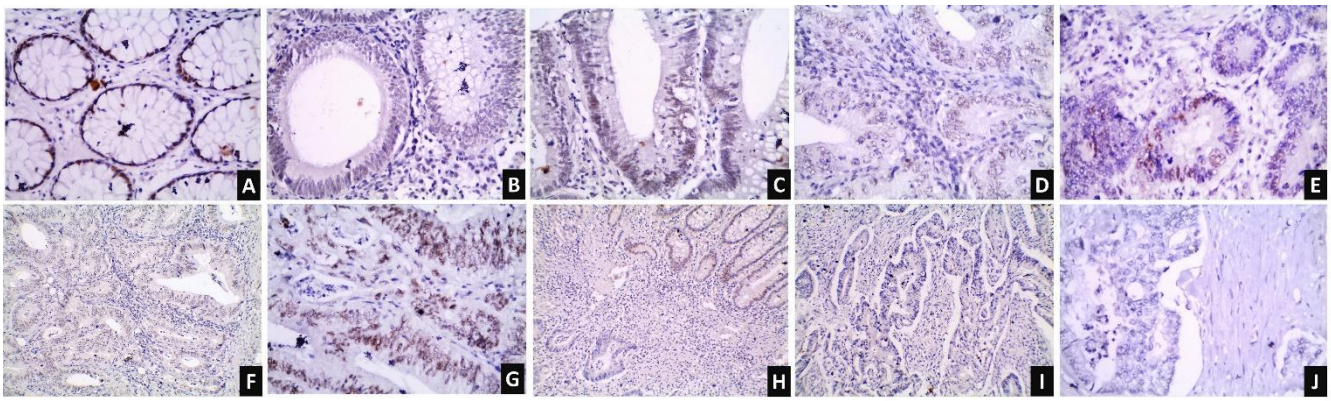


Figure 2:

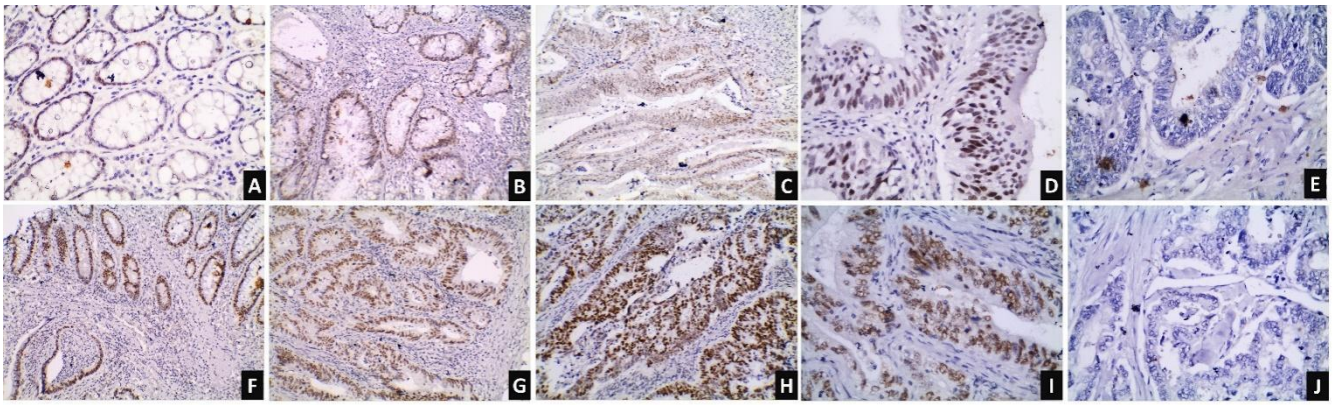


Figure 3:

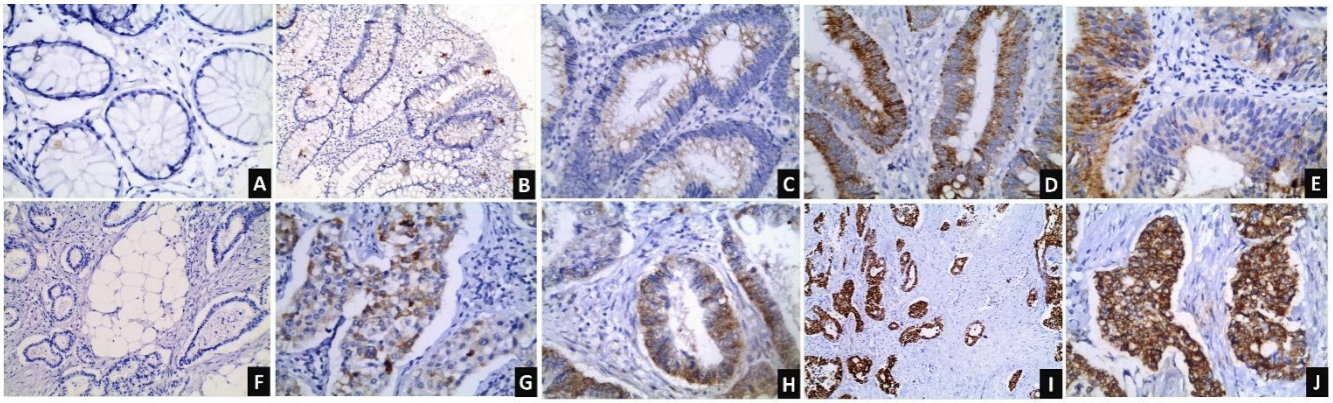


Figure 4:

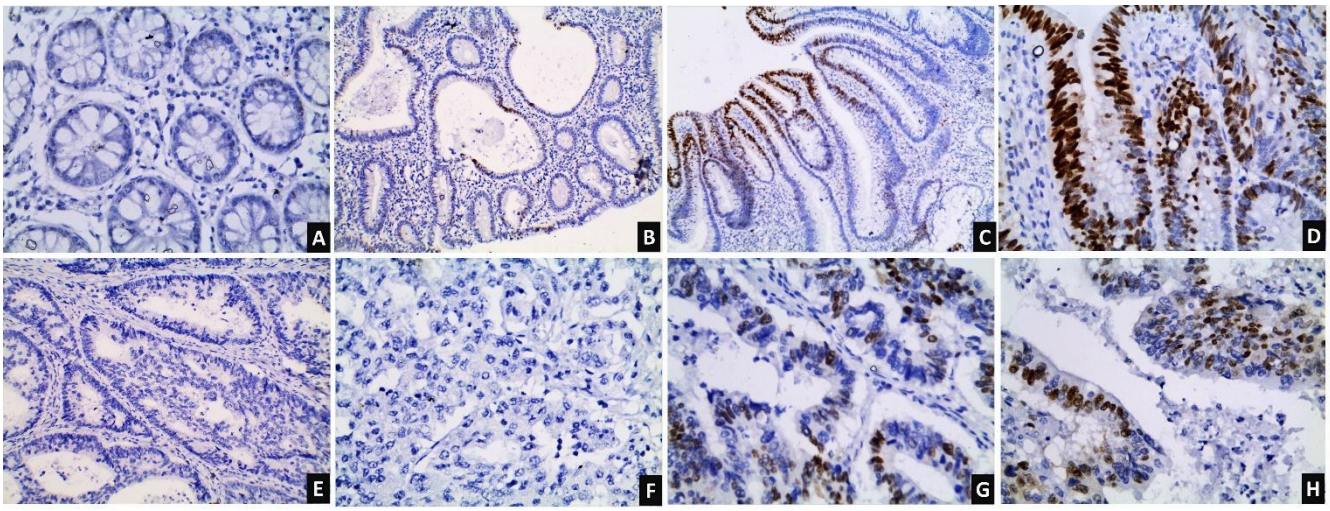


Figure 5:

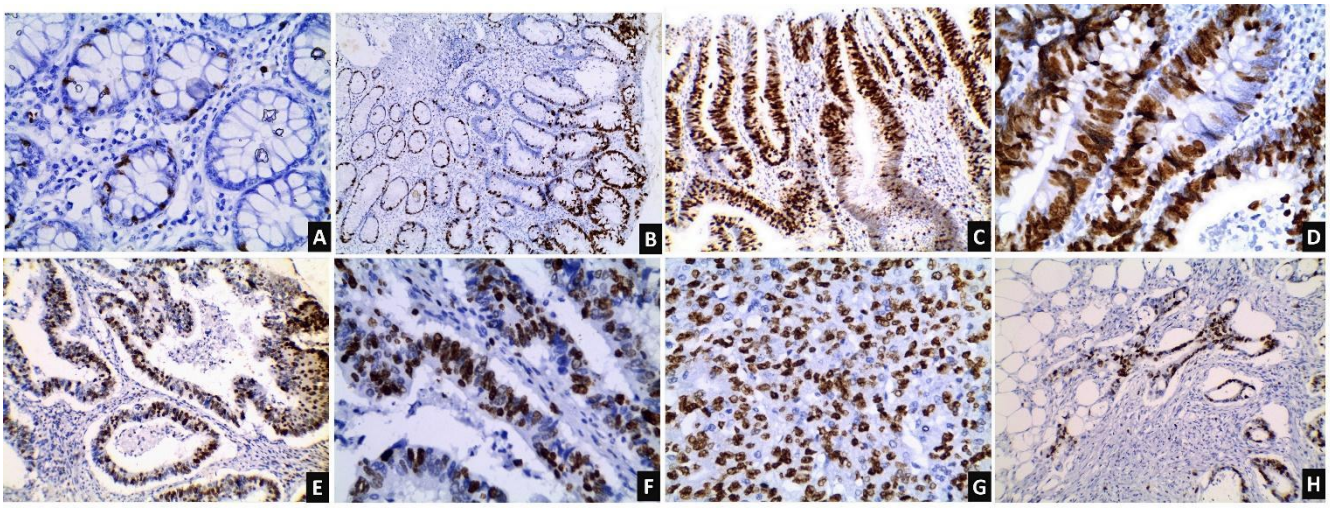
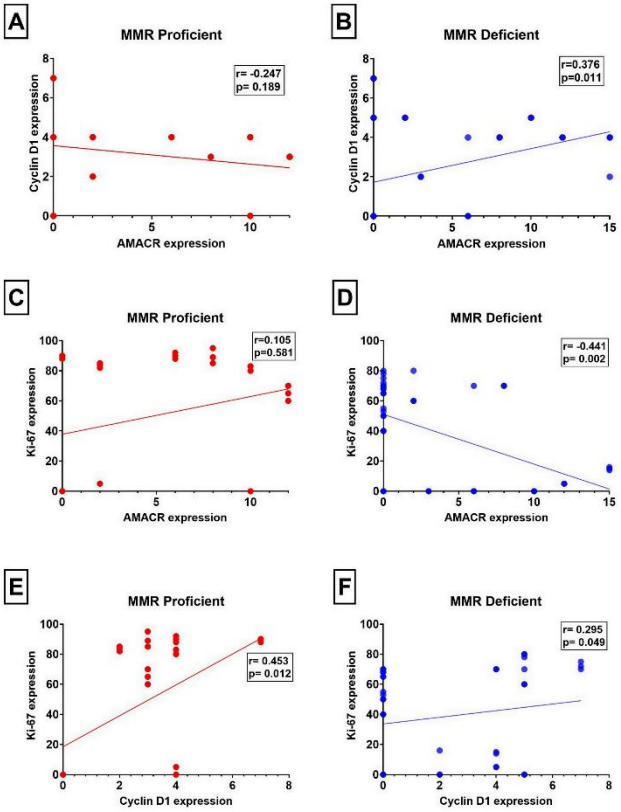


Figure 6:


A
MMR Proficient
r= -0.247
p= 0.189
Cyclin D1 expression
AMACR expression
B
MMR Deficient
r=0.376
p=0.011
Cyclin D1 expression
AMACR expression
C
MMR Proficient
r=0.105
p=0.581
Ki-67 expression
AMACR expression
D
MMR Deficient
r= -0.441
p= 0.002
Ki-67 expression
AMACR expression
E
MMR Proficient
r= 0.453
p= 0.012
Ki-67 expression
Cyclin D1 expression
F
MMR Deficient
r= 0.295
p= 0.049
Ki-67 expression
Cyclin D1 expression