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ORIGINAL ARTICLE

FOXP3+ regulatory T cells, mismatch repair proteins and BRAF V600E status in young-onset colorectal cancer

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Abstract

Introduction: Young onset colorectal cancer (YOCRC) is a heterogenous CRC phenotype with an increasing trend globally. This study aims to determine FOXP3+ Treg cells, Mismatch Repair (MMR) proteins, and proto-oncogene B-Raf (BRAF) V600E status among YOCRC patients at Hospital Universiti Sains Malaysia. *Materials and Methods:* This was a retrospective study of YOCRC (<50 years) over 8 years (January 2013 to December 2021). Immunohistochemistry staining of FOXP3, BRAFV600E, and MMR protein expression was performed using monoclonal antibodies. The staining intensity and percentage of positive cells were used to evaluate the staining using immunoreactive scoring. All data were analysed using descriptive and correlation statistics. A p-value of ≤ 0.05 was taken as statistically significant. *Results:* A total of 65 YOCRC patients were diagnosed, out of which 53.8% had proficient MMR (pMMR) with a mean age of 41, while 46.2% had deficient MMR (dMMR) with a mean age of 35.5. The pMMR with the BRAFV600E+ group expressed higher FOXP3+Tregs (54.2%) than the dMMR with the BRAFV600E+ group (22.9%). Patients with lower FOXP3+Tregs were observed more in dMMR with BRAFV600E- (47%) than in pMMR with BRAFV600E- (5.9%). There was a statistically significant association between the density of expressed FOXP3+Tregs with MMR and BRAFV600E status (p=0.002). *Conclusion:* While most of the YOCRC had pMMR, others exhibited dMMR with loss of one or more MMR proteins. The presence of BRAFV600E demonstrated the YOCRC's sporadic nature. A high FOXP3+Treg expression was significantly associated with MMR and BRAFV600E status. Future research must be expanded to cover other hospitals to increase the sample size and include MLH1 hypermethylation testing.

Keywords: Young-onset CRC**,** FOXP3, Treg cell, MMR proteins, BRAF V600E.

INTRODUCTION

The incidence of colorectal cancer (CRC) in people below 50 years, called young-onset CRC (YOCRC) or early-onset CRC, has been rising for several decades.[1–4] The majority of the YOCRC are sporadic (≥70%) without a family history of CRC.[5-9] The YOCRC incidence is expected to increase by 90% in colon carcinoma and 124% in rectal carcinoma worldwide by 2030.[10] Malaysia is experiencing the prevalence of YOCRC just like other countries; Ibrahim *et al*.[11] reported a YOCRC prevalence of 14.5% in Northern Malaysia from 2007 to 2017. According to data from Malaysia's National Cancer Registry, CRC is the most common cancer in males and the second most prevalent cancer in women.[12] Unfortunately, 65% of the CRC cases in Malaysia are diagnosed at advanced stages III and IV.[12-16] Among the risk factors of CRC, metabolic diseases of diabetes especially type-2 and hypertension have been strongly associated with an increased chance of developing CRC.[17-19] Kelantan is among the Malaysian States with a high prevalence of diabetes and there was an observed increase in occurrence from 11.3% in 2015 to 19.5% in 2019.[20,21]

The YOCRC is a heterogenous CRC phenotype with higher mucinous and signet ring histological types, poorer differentiation, and more aggressive behaviour.[6,22,23] Forkhead

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box P3 (FOXP3) is a transcriptional factor necessary to induce immunosuppressive functions in regulatory T-lymphocytes (Treg), mainly expressed by $CD4^+$ and $CD25^+$ cells, that is crucial in maintaining immunologic tolerance.[24,25] Since the expression of the FOXP3 is correlated with the development and functioning of the FOXP3Tregs, it is regarded to be a specific marker for these cells.[26-28] Compared to normal tissue, malignant tissue was shown to have a considerably increased density of FOXP3+Tregs.[25,26] Mutation in the *FOXP3* gene leads to Tregs deficiency, causing lethal autoaggressive lymphoproliferation and other forms of autoimmune disease such as Immune dysregulation, polyendocrinopathy, enteropathy X-linked (IPEX) syndrome in humans.[29]

Several studies have been conducted since Shimon Sakaguchi discovered Tregs in 1995 to ascertain the connection between Tregs and malignancies.[30-32] However, the outcome of the different studies conflicted on the prognostic value of increased FOXP3+Tregs in which there were favourable prognoses such as in CRC and gastric cancer as against poor prognostic values reported in carcinomas of the breast, endometrial, hepatocellular, ovarian, pancreatic and renal, among others.[28,33-35] Apart from Tregs, cancer cells, including CRC, were also reported to produce FOXP3.[36] The mechanism of FOXP3 expression by the cancer is predicted to help the tumour cells evade effector T-cell responses, resulting in tumour progression, migration, and invasion, respectively, leading to poor prognosis.[36,37] The level of FOXP3+Treg expression is also determined by the mismatch repair (MMR) status of the CRC, which can either be proficient MMR (pMMR) status or deficient MMR status (dMMR).[38]

The MMR system ensures the maintenance of genomic stability in proliferating cells by repairing errors involving base-base mismatch and unmatched insertion-deletion loops during cellular replication.[39-42] The primary MMR proteins are MLH1, PMS2, MSH2, and MSH6; lacking these MMR proteins results in DNA replication errors that may accumulate to cause microsatellite instability (MSI).[43-45] YOCRC can be grouped into pMMR with positivity in all 4 MMR proteins and dMMR with deficiency of 1 or more MMR proteins.[46–48] The dMMR can be sporadic due to BRAFV600E mutant and epigenetic MLH1 promoter hypermethylation or hereditary dMMR due to germline mutations without BRAFV600E mutant.[49–51] The BRAFV600E mutation in CRC triggers the mitogen-activated protein kinase (MAPK) signalling pathway, thereby promoting cell proliferation and preventing apoptosis, which supports the growth and spread of the tumour.[52–55] Clinical investigations have demonstrated that CRC patients with the BRAFV600E mutation had a poor prognosis and a median overall survival of approximately 12 months, particularly in pMMR status, compared to CRC patients with the wild-type BRAF.[56,57] In clinical settings, BRAFV600E and MMR status of CRC can be identified at the protein level using immunohistochemistry (IHC) and at the genetic level using polymerase chain reaction (PCR) or next-generation sequencing (NGS).[52,58] The IHC has evolved into a standard procedure in most pathology laboratories as a reliable method to identify dMMR.[59]

There is a lack of study on FOXP3+Tregs expression and its relationship to BRAF V600E and MMR protein status among the YOCRC patients in Hospital Universiti Sains Malaysia (HUSM). We aim to identify the FOXP3+Tregs expression in YOCRC at HUSM using FOXP3 as a specific marker of Tregs and correlate it with the MMR and BRAF V600E status. In addition, we also aim to study the association of FOXP3+Tregs expression to the clinicopathological characteristics of the YOCRC patients.

MATERIALS AND METHODS

This is a cross-sectional study of YOCRC cases in HUSM for 9 years (2013-2021). This work was assessed by the universiti ethical committee and approved with the JEPeM Code: USM/JEPeM/20120676. The patient clinical data in the Medical Record Unit and Laboratory Information System (LIS) were used to retrieve clinicodemographic data of the YOCRC patients. Age, gender, ethnicity, tumour site, initial symptom, comorbidities, and carcinoembryonic antigen (CEA) levels (elevated as $\geq$5.2 ng/mL vs normal as <5.2 ng/mL) were all considered. The CRC tissues were grouped into the proximal colon, distal colon, and rectal tumours. The proximal colon tumours include the caecum, ascending colon, hepatic flexure, and transverse colon. On the other hand, those tumours at the splenic flexure, descending colon, and sigmoid colon were categorised as distal colon tumours.

Additional information extracted from the Laboratory Information System (LIS)

histopathology report includes the CRC histological type, grading, and tumour, node and metastasis (TNM) staging upon diagnosis (Stage I, II, III, and IV). The degree of the tumour-infiltrating lymphocytes within the stromal area of the invasive tumour was assessed and categorised into absent, mild (0-10%), moderate (11%-50%), and marked (51%-100%) semiquantitatively.[60] Patients with complete medical data and resected CRC tissue histologically confirmed were included in the study, while those with incomplete records were excluded.

Immunohistochemical Staining

IHC staining was performed on formalin-fixed paraffin-embedded (FFPE) tissues cut at 5 μm using monoclonal antibodies. The antibodies used were anti-FOXP3 (Abcam UK, Cat. Ab20034), anti-BRAF V600E (Abcam UK, Cat. Ab22846), anti-MLH 1 (Dako Germany, Cat. M3640), anti-MSH 2 (Dako Germany, Cat. M3639), anti-MSH 6 (Dako Germany, Cat. M3646) and anti-PMS 2 (Dako Germany, Cat. M3647) respectively. Each tissue section was mounted on a Poly-L-Lysine microslide, dried on a hot plate, and then dewaxed in two changes of xylene. The tissues were hydrated by gradually passing the tissue sections into decreased alcohol concentrations, followed by a water rinse. Dako PT Link and Envision Flex retrieval solutions were used to extract the antigen epitope at 97^0C for 20 minutes. The tissues were then stained using the primary antibodies dilution and incubation time as Anti-FOXP3 (1:300, 1 hour), Anti-BRAF V600E (1:100, overnight), Anti-MLH1 (1:50, overnight), Anti-MSH2 (1:50, 1 hour), Anti-MSH6 (1:100, 1 hour) and Anti-PMS2 (1:50, overnight).

Each slide received 100 μL of the primary antibody dilution to begin the staining process, followed by an hour or overnight incubation accordingly. The slides were then blocked with Envision Flex hydrogen peroxide (Dako, Germany) at a concentration of 200 μL for each slide after being rinsed with TBS buffer. The slides then underwent an incubation period of 5 minutes. The slides were treated with 150 μL of Envision Flex monoclonal mouse or rabbit linker as instructed by the manufacturer and allowed to act for 15 minutes. After that, 200 μL of horseradish peroxidase (HRP) from Dako (Germany) was applied to the slides, and they were left to react for 20 minutes. The slides were then exposed to diaminobenzidine (DAB) chromogen from Dako (Germany), which was incubated for 5 minutes before the slides were cleaned with distilled water.

After a 10-second counterstaining with Harris haematoxylin solution, the stained tissue sections were washed with distilled water. The sections were dehydrated in increasing concentrations of alcohols starting at 70%, 80%, 95%, two changes of 100%, then cleared in two changes of xylene and finally mounted with a coverslip using cytoseal mountant. Two (2) pathologists used a multi-headed Olympus microscope with x10 and x40 objectives to examine the IHC slides to determine the staining's intensity, the proportion of cells that stained positively, and the clarity of the background staining. The IHC staining images were captured using a camera at a magnification of x200.

Assessment of the IHC staining and scoring

The staining intensity and percentage of positive cells on the stained CRC tissues were assessed using the Immunoreactive Scoring (IRS).[47,61] The staining intensity was rated as 0 for no colour reaction, +1 for mild nuclear staining, +2 for moderate nuclear staining, and +3 for strong nuclear staining. The percentage of positive cells was graded as 0 for no staining, +1 for 1%- 10%, +2 for 11%- 50%, +3 for 51%-80%, and +4 for 81%-100% of labelled positive tumour cells, respectively. The staining in non-neoplastic colonic tissues and other stromal cells was used as an internal control.

An Immunoreactive Scoring (IRS) of 0–12 was determined by multiplying the scoring of positive cells with the staining intensity to produce a scale of 0-12.[47,61] The scale was interpreted as 0–1 (negative), indicating that the protein was not expressed; 2-3 as mild expression; 4–8 as moderate expression; and 9–12 as a strong expression.[61] Positive anti-BRAF V600E is recorded at an immunoreactive score of moderate to strong intensity with ≥80% of malignant cells.[47,62] Based on the MMR result, the MMR status was determined as proficient MMR when all four tested MMR proteins were present or deficient MMR when one or more of the MMR proteins were absent. The FOXP3 expression was assessed using the staining on the mononuclear infiltrates of the TILs in the stromal area within the invasive tumour. The YOCRC patients were finally grouped based on the density of the FOXP3 expression into FOXP3 low expression (<4 on the IRS scale) and FOXP3 high expression (≥4 on the IRS scale).

Statistical Analysis

All the data obtained were analysed using IBM Corporation's SPSS Statistics version 27. The mean and standard deviation (SD) were used to calculate continuous variables, whereas proportions and percentages were used to summarise categorical variables. The Pearson Chi-squared and Fisher's tests were performed to associate FOXP3 expression with the clinic-demographic characteristics of the patients, as well as BRAF V600E and MMR protein status. A *p*-value of ≤0.05 was taken as statistically significant.

RESULTS

A total of sixty-five (65) patients were diagnosed as YOCRC at HUSM from January 2013 to December 2021. MLH1 was expressed in 49 patients (75.4%) and negative in 16 patients (24.6%), while PMS2 was expressed in 54 patients (83.1%) and negative in 11 patients (16.9%). MSH2 was expressed in 54 patients (83.1%) and negative in 11 patients (16.9%), while MSH6 was expressed in 62 patients (95.5%) and negative in 3 patients (4.6%). Figures 1 and 2 showed the positivity and negativity of MLH1, MSH2, MSH6 and PMS2, respectively. A total of 35 patients (53.8%) with a mean age of 41.0 years (SD: 6.65) had pMMR, with positivity in all 4 MMR tests. In contrast, 30 patients (46.2%) with a mean age of 35.5 years (SD: 8.38) had dMMR deficient in one or more MMR proteins. BRAFV600E was expressed in 45 patients (69.2%) and negative in 20 patients (30.8%), as indicated by Figure 3.

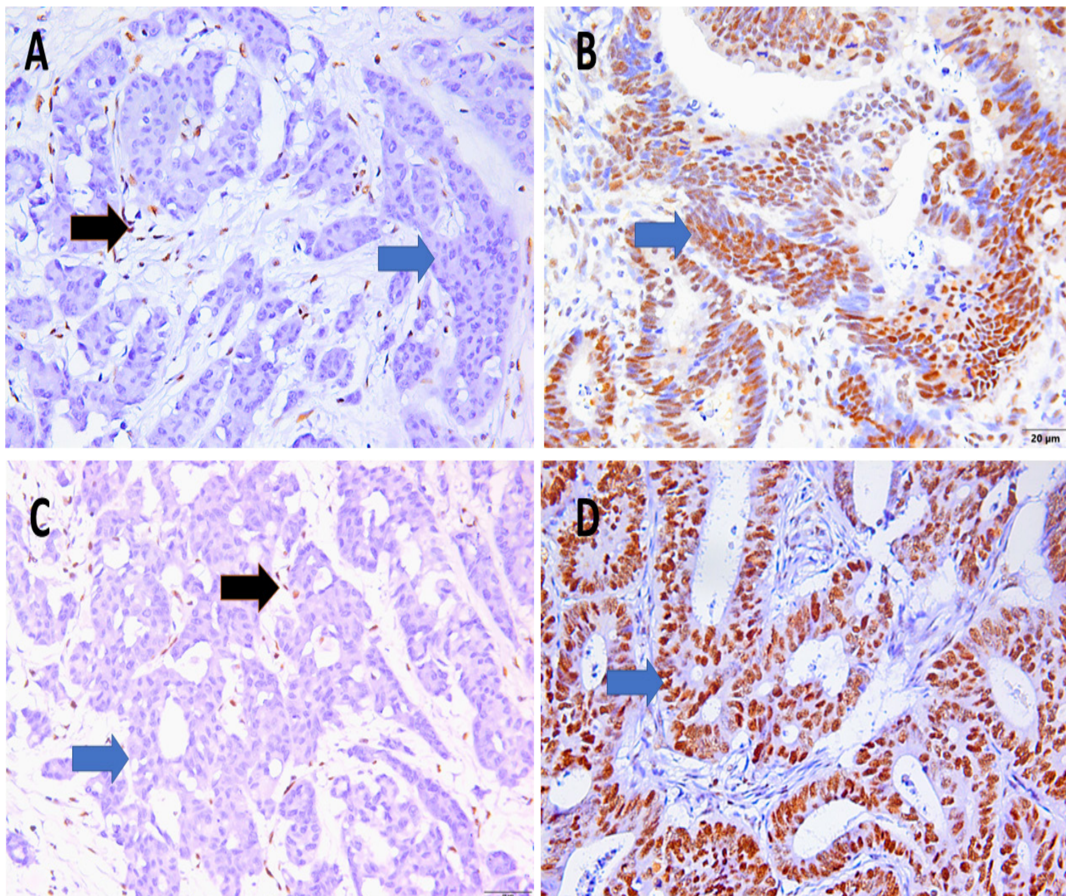

Figure 1: Immunohistochemical staining of MLH1 and PMS2 ×200 magnification. (A) Negative expression of MLH1 by the CRC cells (blue arrow) in the presence of stained lymphocytes serving as an internal control (black arrow). (B) Nuclear positive expression of MLH1 by the CRC cells (blue arrow) (C) Negative expression of PMS2 by the CRC cells (blue arrow) in the presence of stained lymphocytes serving as an internal control (black arrow). (D) Nuclear positive expression of PMS2 by the CRC cells (blue arrow).

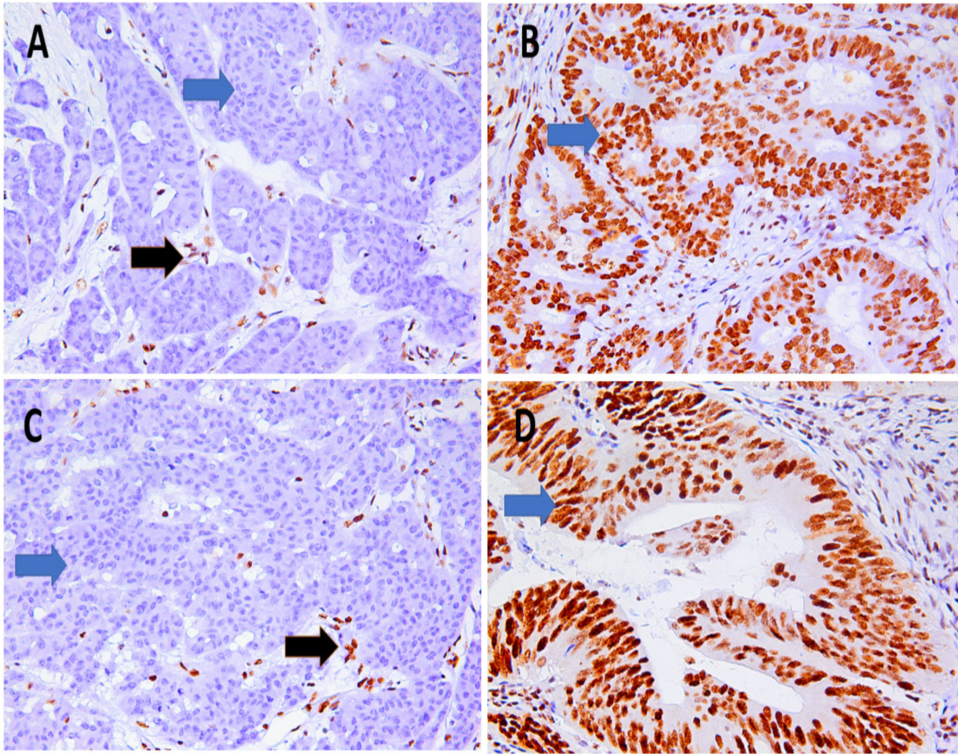

Figure 2: Immunohistochemical staining of MSH2 and MSH6 ×200 magnification. (A) Negative expression of MSH2 by the CRC cells (blue arrow) in the presence of stained lymphocytes serving as an internal control (black arrow). (B) Nuclear positive expression of MSH2 by the CRC cells (blue arrow). (C) Negative expression of MSH6 (blue arrow) in the presence of stained lymphocytes serving as an internal control (black arrow). (D) Nuclear positive expression of MSH6 by the CRC cells (blue arrow).

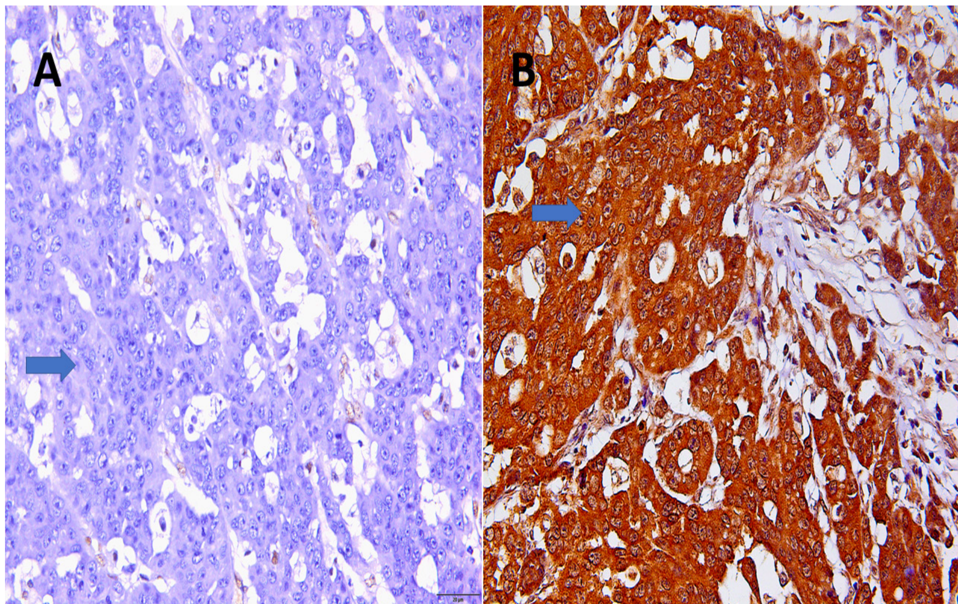

Figure 3: IHC of BRAFV600E IHC ×200 magnification (A) BRAFV600E negative malignant cells (blue arrow) (B) BRAFV600E positive malignant cells with marked cytoplasmic and nuclear expression of the BRAF V600E (blue arrow).

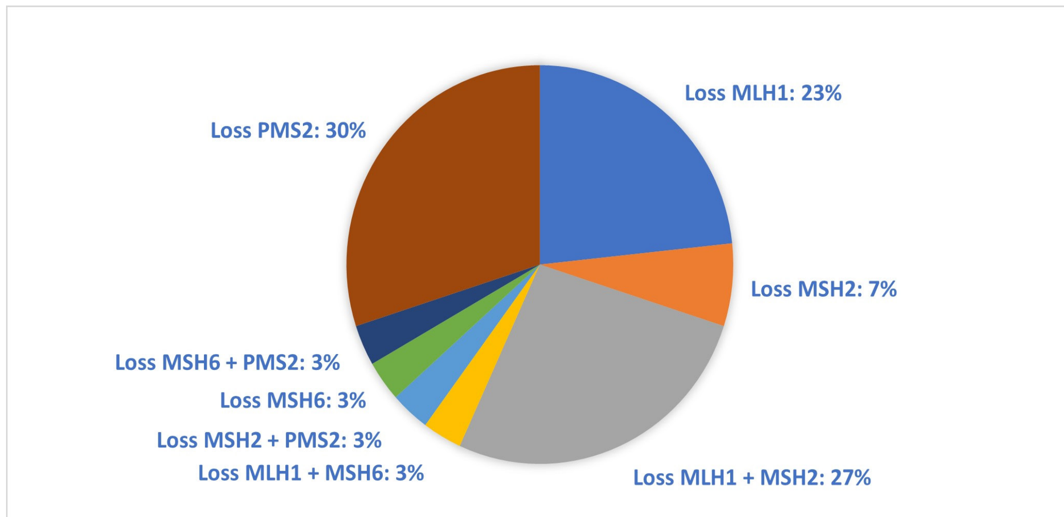

Figure 4: Pie chart showing the percentage of MMR proteins negativity.

The negativity of MMR proteins was seen occurring either alone or in combination with another protein (Figure 4). The most common loss MMR protein was PMS2 (30%), followed by MLH1+MSH2 (27%) and MLH1 (23%). There was also other less commonly occurring MMR protein loss, as indicated by Figure 4.

FOXP3 protein expression showed 61 (93.8%) were FOXP3+Tregs while 4 (6.2%) were FOXP3-Tregs. The rate of FOXP3 expression in the Tregs was further divided into low FOXP3 expression, which was observed in 17 patients (26.2%), while high FOXP3 was expressed in 48 patients (73.8%). The low and high FOXP3 expressions are indicated in Figure 5.

A high FOXP3+Treg expression was seen more in 26 (54.2%) patients with pMMR/BRAFV600E+, while high FOXP3+Treg was expressed in 11 (22.9%) patients with dMMR/BRAFV600E+. The pMMR/BRAFV600E- group also had high FOXP3+Treg in 6 patients (12.5%), while dMMR/BRAFV600E- had high FOXP3+Treg in 5 (10.4%). Patients with pMMR/BRAFV600E+ had low FOXP3+Treg expression in 2 (11.8%), while pMMR/BRAFV600E- had low FOXP3+Treg in 1 (5.9%). Many patients with low FOXP3+Treg are those with dMMR having BRAFV600E+ (35.3%) and BRAFV600E- (47%) more than those with pMMR. A significant association was observed in FOXP3 associated with MMR status (p=0.001)

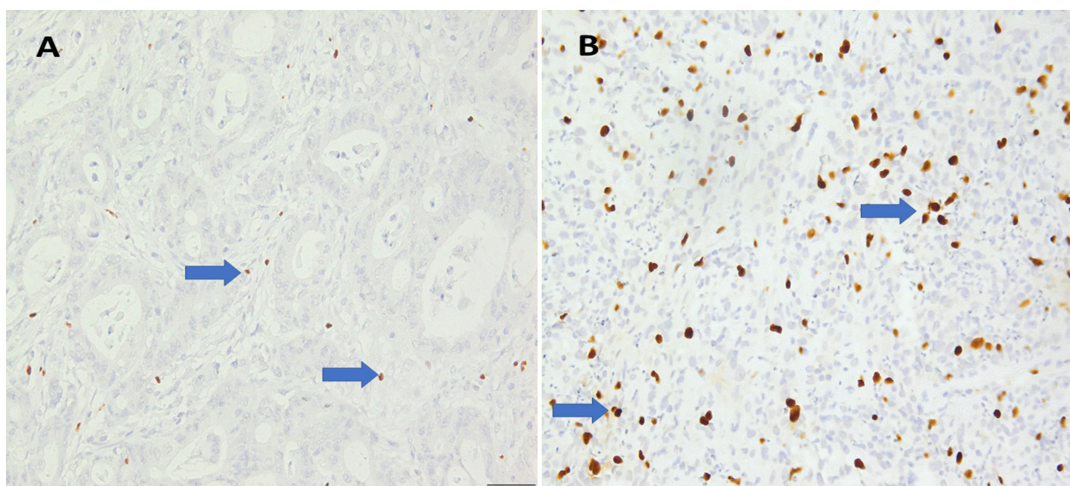

Figure 5: IHC of FOXP3 IHC ×200 magnification (A). Low expression of FOXP3 by the intratumoral lymphocytic infiltrates (blue arrow) (B.) High expression of FOXP3 by the intratumoral infiltrates (blue arrow).

and the MMR and BRAFV600E status with the FOXP3 expression (p=0.002), as indicated in Table 1.

There was no statistically significant association between the level of FOXP3+Treg expression and most of the demographic or clinicopathological parameters of the patients. However, the histological types in this study showed a significant relation (p=0.003) with the level of FOXP3+Treg expression. Adenocarcinoma was the most histological type, having the highest number of YOCRC cases with high FOXP3 expression (42 patients, 87.5%). In contrast, mucinous with 6 patients (35.3%) and signet ring with 3 patients (17.7%) histology showed a lower expression of FOXP3. Histological grading of the YOCRC also has a statistically significant association (p=0.046) with density of the FOXP3 expressed. Moderately differentiated adenocarcinoma had the highest number of patients (40 patients, 83.8%) expression of a high density of FOXP3 (Table 2).

DISCUSSION

The National Institute for Health and Care Excellence (NICE) 2017 recommended universal screening for CRC using IHC to identify dMMR or MSI testing by PCR on all newly diagnosed patients.[58] The IHC has 100% specificity and 92% sensitivity in classifying CRC into pMMR and dMMR.[63,64] It also guides the selection of patients to be further subjected to gene sequencing and profiling for full MSI assessment.[42,49,53] Our study showed that 53.8% of the YOCRC patients in this study had intact MMR proteins with positivity in all 4 MMR tested, while 46.2% were dMMR with loss of one or more of the MMR proteins. Ballester *et al.* [65] reported that the percentage of dMMR tumours among YOCRC ranges from 19.7% to 41.0%. YOCRC patients with dMMR had a lower mean age of 35.5 years compared to pMMR patients with a mean age of 41.0 years. The risk of developing dMMR decreases as people age, especially those related to germline mutation such as Lynch Syndrome (LS).[46,66] This finding is consistent with Vos *et al.*, who reported the prevalence of LS was 18% in CRCs under 40 years, 1.7% in CRCs between 40 and 64 years, and 0.7% in CRCs between 65 and 69 years.[67]

The loss of MMR proteins in dMMR differs depending on whether the CRC is sporadic in origin or hereditary due to germline mutation.[68,69] The loss of the MMR protein(s) will result in the inactivation of the MMR system, leading to the accumulation of errors that serve as the

Table 1a: Association of FOXP3 Expression with MMR and BRAFV600E Status

Parameter	Total n (%)	Low FOXP3 n (%)	High FOXP3 n (%)	p-value
MMR status				
Proficient MMR	35 (53.8)	3 (17.6)	32 (66.7)	**0.001**[b*]
Deficient MMR	30 (46.2)	14 (82.4)	16 (33.3)	
BRAF V600E status				
Positive	45 (69.2)	8 (47.1)	37 (77.1)	**0.032**[a*]
Negative	20 (30.8)	9 (52.9)	11 (22.9)	

[a]: Chi-square analysis; [b]: Fisher's analysis; [*]: Significant p-value

Table 1b: FOXP3+Treg expression in YOCRC cases with MMR and BRAF V600E status

MMR and BRAF V600E status	n (%)	Low FOXP3+Treg n (%)	High FOXP3+Treg n (%)	p-value
pMMR/ BRAFV600E+	28 (43.1)	2 (11.8)	26 (54.2)	**0.002*[a]**
pMMR/ BRAFV600E-	7 (10.8)	1 (5.9)	6 (12.5)	
dMMR/ BRAFV600E+	17 (26.2)	6 (35.3)	11 (22.9)	
dMMR/ BRAFV600E-	13 (20)	8 (47)	5 (10.4)	

[a]: Chi-square analysis; [*]: Significant p-value

Table 2: Comparison of demographic and clinicopathological data between the low and high FOXP3 Expression

Parameter	Total n (%)	Low FOXP3 n (%)	High FOXP3 n (%)	p-value
Gender				0.783[a]
Female	33 (50.8)	8 (47.1)	25 (52.1)	
Male	32 (49.2)	9 (52.9)	23 (47.9)	
Race				0.458[b]
Chinese	1 (1.5)	1 (5.9)	0 (0.0)	
Malay	63 (97)	16 (94.1)	47 (97.9)	
Others	1 (1.5)	0 (0.0)	1 (2.1)	
Family history of CRC				0.512[b]
Absent	50 (76.9)	12 (70.6)	38 (79.2)	
Present	15 (23.1)	5 (29.4)	10 (20.8)	
Initial symptom				0.088[b]
Abdominal distension	4 (6.2)	2 (11.7)	2 (4.1)	
Abdominal pain	27 (41.5)	7 (41.2)	20 (41.7)	
Altered bowel habit	18 (27.7)	7 (41.2)	11 (22.9)	
Per-rectal bleeding	16 (24.6)	1 (5.9)	15 (31.3)	
Smoking status				0.263[b]
Non-smoker	54 (83.1)	16 (94.1)	38 (79.2)	
Smoker	11 (16.9)	1 (5.9)	10 (20.8)	
Comorbidity				0.467[b]
Absent	58 (89.2)	14 (82.4)	44 (91.7)	
Diabetes	2 (3.1)	1 (5.9)	1 (2.1)	
Hypertension	5 (7.7)	2 (11.7)	3 (6.2)	
CEA level				0.451[a]
Elevated (≥5.2ng/mL)	37 (56.9)	11 (64.7)	26 (54.2)	
Normal (<5.2ng/mL)	28 (43.1)	6 (35.3)	22 (45.8)	
Tumour site				>.99[b]
Left	54 (83.1)	14 (82.3)	40 (83.3)	
Right	11 (16.9)	3 (17.7)	8 (16.7)	
Type of specimen				>.99[b]
Distal colon	33 (50.8)	9 (52.9)	24 (50)	
Proximal colon	11 (16.9)	3 (17.7)	8 (16.7)	
Rectum	21 (32.3)	5 (29.4)	16 (33.3)	
Histological types				**0.003[b*]**
Adenocarcinoma	49 (75.4)	7 (41.2)	42 (87.5)	
Mucinous adenocarcinoma	10 (15.4)	6 (35.3)	4 (8.3)	
Signet ring carcinoma	4 (6.1)	3 (17.6)	1 (2.1)	
Neuroendocrine carcinoma	2 (3.1)	1 (5.9)	1 (2.1)	
Grading				**0.046[b]**
Well-differentiated	6 (9.2)	0 (0.0)	6 (12.5)	
Moderately differentiated	54 (83.1)	14 (82.4)	40 (83.3)	
Poorly differentiated	5 (7.7)	3 (17.6)	2 (4.2)	

Staging				0.414[b]
I	3 (4.6)	0 (0.0)	3 (6.2)	
II	15 (23.1)	2 (11.7)	13 (27.1)	
III	24 (36.9)	7 (41.2)	17 (35.4)	
IV	23 (35.4)	8 (47.1)	15 (31.3)	
Tumour infiltrating lymphocytes				0.689[b]
Absent	7 (10.8)	1 (5.9)	6 (12.5)	
Mild	30 (46.2)	7 (41.2)	23 (47.9)	
Moderate	19 (29.2)	7 (41.2)	12 (25)	
Marked	9 (13.8)	2 (11.7)	7 (14.6)	

[a]: Chi-square; [b]: Fisher's; CEA: Carcinoembryonic Antigen, [*]: Significant p-value

basis for the MSI.[65] The MSI may be able to cause CRC primarily if it occurs in the coding regions of genes involved in several vital cellular processes and pathways.[46,70] Most dMMR in sporadic CRC results from MLH1 loss through hypermethylation of CpG Islands in the promoter region, thereby causing transcriptional silencing of MLH1.[68] We observed the loss of MLH1 as the most frequent MMR protein loss, alone or in conjunction with other MMR proteins. The dual loss of MLH1 and PMS2 was also seen in this study; this is because the loss of MLH1 affected the stability of PMS2, thereby causing the accompanying loss of PMS2.[71-73] Similar findings of loss of MLH1, as the most frequently observed aberration in MMR protein IHC, were also reported in previous studies.[58,67]

Out of 16 patients that lost MLH1, 56.3% tested positive for BRAFV600E, indicating sporadic CRC, and it is suggested that these patients not be further tested for the CRC germline mutation.[49,74] In comparison, the remaining 43.7% that lost MLH1 tested negative for BRAFV600E, and they need to be further subjected to MLH1 hypermethylation to confirm their sporadic status.[75,76] We also observed the loss of PMS2 alone in 9 patients (30%), which, despite forming a heterodimer combination with MLH1 in the MMR system, did not affect the presence of MLH1 in those patients.[42,58] Sugano *et al*.[77] reported the loss of PMS2 alone is associated with the germline mutation. MSH2 alone or combined with other MMR proteins was lost in 28.6% of the patients, while the loss of MSH6 alone was seen in 3.3%. The loss of MSH2 and MSH6, MSH6 alone, PMS2 alone, PMS2, and MLH1 with a negative BRAFV600E are assumed to belong to germline mutation, thereby need to be referred for further testing with genetic testing.[78]

The BRAFV600E was expressed by 69.2% of the YOCRC patients in this study, while 30.8% were negative for this BRAF mutant type. Of the patients harbouring the mutant BRAFV600E, 62.2% belonged to pMMR, while 37.8% were dMMR patients. Previous studies have identified the BRAFV600E mutant pMMR as a unique CRC subtype arising from sessile serrated adenomas (SSA) methylating an epigenetic target other than MLH1.[79–81] Although this CRC subtype has epigenetic methylation that did not affect MLH1, the MLH1 is then retained, giving rise to CRC of pMMR phenotype with features of SSA and traditional sessile adenoma.[79] Mesteri *et al*.[82] detected BRAFV600E in 95% of CRC arising from the traditional sessile adenoma pathway belonging to pMMR. The pMMR/BRAFV600E+ CRCs are similar to BRAF wild-type cancers in terms of the younger age of onset and equal gender distribution, and they are more commonly present at advanced stages.[79,83] Other specific characteristics of pMMR/BRAFV600E CRC were high-grade histology, more distally located, and a poor prognosis than that of BRAFV600E mutant dMMR.[69,84] Compared to BRAF-mutant pMMR CRC, the BRAF-mutated dMMR CRC has a less aggressive clinical phenotype and a better overall survival.[55] The BRAFV600E mutation in CRC also helped differentiate sporadic CRC with loss of MLH1 from those harbouring germline mutation or hereditary form.[47] The BRAFV600E is rarely seen in hereditary CRC (1.6%), and the presence of this mutant BRAF is being used as exclusion criteria for germline testing.[64,85]

There was 93.8% positive nuclear expression of the FOXP3 protein by the Tregs, while 6.2% were FOXP3 negative lymphocytes. The expression of FOXP3 is different from other types of T-cells which is nuclear rather than

cytoplasmic staining. Our result was consistent with Tanja *et al*.,[86] which also recorded 92.3% FOXP3+Tregs, and Eliane *et al*.[87], with 85.5% FOXP3+Tregs results in their studies, respectively. Malignant tissues have more expression of FOXP3Tregs when compared to the normal tissues, and increased FOXP3+Tregs in CRC were associated with a good prognosis by Martin *et al*.[25] The high expression of FOXP3+ Tregs in our study may likely suggest good prognosis and overall survival in CRC as they are known in their immunosuppressive functions and promotion of tolerance through halting of autoimmune reaction by preventing cytotoxic T-cells from attacking self-cells or healthy cells.[88–90] While a good prognosis was reported in CRC by the high expression of FOXP3+ Tregs, there are reports of poor prognosis in some carcinomas, such as hepatocellular carcinoma (HCC) and breast carcinoma.[91,92] Wang *et al*.[91] reported that increased FOXP3+ overexpression by the HCC cells is an unfavourable indicator of poor prognosis indicating pro-cancer effects.[91] According to Wang *et al*.,[91] increased FOXP3 expression by the HCC cells may promote the tumour cells to mimic characteristics of Tregs in helping them to evade immune system attacks in the tumour microenvironment. The conflicting findings is as a result of our study and others that worked on Tregs were looking at the expression of the FOXP3 by the Tregs while Wang *et al* was looking at the expression of the FOXP3 by the cancer cells (pro-cancer effects). The pMMR with the presence of BRAFV600E showed a higher expression of FOXP3 than the dMMR with the absence of BRAFV600E, which has a lower expression of FOXP3. The high FOXP3+Tregs expression by the pMMR in our study agreed with Gouvello *et al*.[93], which recorded higher FOXP3+Tregs in pMMR than dMMR using IHC and RT-qPCR techniques in their research. Similarly, Bai *et al*.[38] also observed a significantly higher expression of FOXP3+Tregs in pMMR.

In contrast to our findings on having more FOXP3+Tregs in pMMR, Michel *et al*.[94] had a significantly higher density of FOXP3+Tregs in dMMR than in pMMR using IHC and immunofluorescence double staining techniques. The discrepancies between studies on the density of FOXP3Tregs expression between pMMR and dMMR could be related to the differences in sample size, methodology used, the Treg phenotype, and the stage of the CRC diagnosis.[28] According to Kuwahara *et al*.[95], another reason for the low density of FOXP3Tregs in dMMR was it has a significant association with high infiltration of effector cytotoxic CD3 and CD8 but not with a high density of CD4Tregs or other FOXP3+ cells. We observed no significant association ($p>0.005$) between most of the demographic and clinicopathological variables of the YOCRC patients in this study with the density of FOXP3+Tregs. Xiuwei *et al*.[28] also observed no significant correlation between cases with FOXP3+Tregs expression and patients' variables, including gender, age, primary cancer site, and distant metastasis. However, a significant association between the density of FOXP3 and histological subtypes ($p= 0.003$) and histological grading ($p=0.046$) was seen in this study. The adenocarcinomas have higher expression of FOXP3 than mucinous and signet ring carcinomas, most of which expressed low FOXP3. According to Kuwahara *et al*.[95], the low FOXP3+Tregs expression in the mucinous and signet ring histology could be attributed to the high immunogenicity of dMMR leading to infiltration of these tumours by effector CD3 and CD8 cytotoxic lymphocytes more than the CD4 and CD25 FOXP3Tregs.

This study has some limitations; one is the sporadic CRCs due to the loss of MLH1 and BRAFV600E negative, which were not further tested for MLH1 hypermethylation or germline mutation due to limited funding. Additionally, the study was a single institutional-based research study with a small sample size. We recommend that future research cover multiple centres treating YOCRC in Kelantan and Malaysia to increase the sample size and include MLH1 methylation testing to confirm sporadic CRC.

CONCLUSION

While most of the YOCRC had pMMR, others exhibited dMMR with loss of one or more MMR proteins. The presence of BRAFV600E demonstrated the YOCRC's sporadic nature. A high FOXP3Treg expression was significantly associated with MMR and BRAFV600E status. Future research must be expanded to cover other hospitals to increase the sample size and include MLH1 hypermethylation testing.

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