ORIGINAL ARTICLE

Increased CD3 Immunostaining Associated with High Grade and Tumor Size in Colorectal Carcinoma

[1]Oula S. Alalwany, [1]Dhafer A. Alghezi*, [2]Rasha Q. Aljawher, [3]Ali Harb

[1]Department of Medical Microbiology, College of Medicine, University of Thi-Qar, Thi-Qar, 64001, Iraq

[2]Department of Histopathology and Forensic Medicine, College of Medicine, University of Thi-Qar, Thi- Qar, 64001, Iraq

[3]Department of Community Health Technologies, College of Health and Medical Technologies, National University of Science and Technology, Thi-Qar, Iraq

Key words:
Colorectal Carcinoma, CD3, Immunohistochemistry, Tumor grades, Tumor invasion

Corresponding Author:
Dhafer A. Alghezi, PhD
Department of Medical Microbiology, College of Medicine
University of Thi-Qar, Nassiryha, Thi-Qar, 64001,Iraq.
Tel: +9647825347701
Dr.daf79@utq.edu.iq

ABSTRACT

Background: *Colorectal cancer lacks reliable prognostic biomarkers, complicating the differentiation between aggressive and indolent tumors. While CD3+ T lymphocyte infiltration generally correlates with improved prognosis and response to immunotherapy, its specific role in tumor aggressiveness is not fully understood.* ***Objective****: This study aims to assess CD3+ T cell infiltration in benign and malignant colorectal tissues and explore its potential association with clinical outcomes.* ***Methodology****: Immunohistochemistry was used to assess CD3+ T cell staining in the stromal tissue of benign (n=24) and malignant (n=80) colorectal samples. CD3+ T cell expression was then correlated with clinic pathological data such as grade and stage.* ***Results****: CD3+ T cell infiltration was significantly higher in colorectal carcinoma than benign tissues (p=0.007). A positive association was observed between CD3+ T cell immunostaining and high tumor grade (p=0.002), while a negative correlation emerged between CD3+ T cell immunostaining and tumor size (p=0.005). No significant relationship was identified between CD3+ immunostaining and lymph node involvement.* ***Conclusion****: CD3+ T cells might be involved in the aggressiveness of malignancy. Further research is needed to determine the significance of CD3+ T cells in colorectal carcinoma and its potential as a biomarker for cancer progression.*

INTRODUCTION

Colorectal carcinoma (CRC) remains a major global health burden, ranking as the fourth most frequently diagnosed cancer worldwide and standing as the third leading cause of cancer-related deaths [1, 2]. The incidence of CRC is comparable in both men and women [3]. In the United States, CRC represents the second leading cause of cancer mortality, contributing to over 600,000 deaths annually[4]. In Iraq, CRC constitutes a significant proportion of gastrointestinal malignancies, standing as the third most common cancer with 2,210 new cases reported [5]. Histologically, approximately 95% of CRCs are adenocarcinomas[6], while only 4% present as medullary-type CRC [7].

One of the most used histopathological grading schemes for assessing the progression of CRC is the grading system [8,9]. This system is divided into three distinct grades (1–3) according to the guidelines provided by the American Joint Commission on Cancer tumors [10]. The low grade means that the cancer cells are more likely to spread less readily and develop more slowly. High grade contains undifferentiated cancer cells, develop more quickly, and have a greater potential to spread [9,11]. However, this system cannot distinguish between aggressive and non-aggressive cancers[11].

In addition, another system known as the Tumor-Node-Metastasis (TNM) system is used to diagnose and progress CRC. This system makes it possible to choose the best treatment course and evaluate their prognosis [12, 13]. It represents a process utilized to assess the extent of cancer metastasis from the colon or rectum to other areas of the body and can be classified into four stages as stage (I, II, III, or IV) [14, 15]. Unfortunately, this system is insufficient on its own to predict prognosis because clinical outcomes might vary across patients at the same cancer's histopathological stage.

There are clinical challenges in differentiation between malignant versus benign and localized versus metastasized CRC, and finding novel possible CRC biomarkers becomes a priority [15].

One of these potential biomarkers identified is Cluster of Differentiation 3 (CD3). The recent studies have shown that immune cell infiltration in tumors has a positive prognostic influence[16]. Tumor-infiltrating lymphocytes (TILs), including CD3 + T cells, have been shown to penetrate tumors. They may serve as biomarkers of the host immune response to the tumor and be a potent independent positive predictor of overall

survival and recurrence [17]. CD3 is a protein complex composed of several subunits, also known as the T3 complex [18, 19]. The T cell receptor (TCR) and CD3 combine to create a TCR/CD3 complex, which is essential for transmitting stimulation signals in T cells and recognizing antigens [18]. CD3 is found in all T-lymphocyte maturation stages and is detected on cytotoxic T lymphocytes (CTLs), Treg, and Th cells, serving as a precise pan-T-cell marker in normal tissue samples [20]. Several studies have highlighted the clinical importance and predictive value of its expression in immune cells that infiltrate CRC. Lee *et al.* discovered that 59% of lymphocytes was found in CRC [21]. Despite limited global studies and a lack of local research in Iraq, the association between CD3+ T cell expression and colorectal cancer aggressiveness using immunohistochemistry remains underexplored.

Physicians face challenges with CRC grading and staging systems, including the difficulty in differentiating between aggressive and non-aggressive tumor, which often requires specialized knowledge and equipment, compounded by the limited availability of prognostic biomarkers [22]. Therefore, this study aims to evaluate stromal infiltrating CD3+ T cells in benign and malignant colorectal tissues and determine if its expression is associated with its clinical data such as grade and stage.

METHODOLOGY

Patients and ethics statement

The cross sectional study was carried out in Thi-Qar City, Iraq, and was approved by the ethics board of Al Hussein Teaching Hospital (No. 162 on 7/8/2023). The study included 104 colon and rectum tissue samples, consisting of 80 formalin-fixed, paraffin-embedded samples obtained from colonoscopy and resection of CRC samples. In addition, twenty-four benign colon tissues obtained from non-cancerous individuals visiting the gastrointestinal center needed for endoscopy were used as a control group. The study excluded the patients under the age of 20 years old complaining of serious gastrointestinal diseases or bleeding such as Crohns or ulcerative colitis, as diagnosed by gastroenterologist. Each patient confirms that they have been adequately informed about the study's objectives, procedures, potential risks, and benefits.

These samples were examined using the immunohistochemistry (IHC) method to test the expression of anti-CD3 in both groups. Positive (Lymph node) and negative (no primary antibody added) tissues were also used in our study. Haematoxylin and Eosin staining as a routine method was used according to Chan, 2014 to understand the normal architecture of the tissues as well as to detect the pathological parameters of samples such as grade and stage [23].

Immunohistochemistry

The benign and malignant colorectal tissue sections were stained using IHC with an independent anti-CD3 antibody (Rabbit polyclonal, dilution 1:100, Pathn Situ, cat. number Pp160-6mlRTU, India). This study used the immunoperoxidase secondary detection kit purchased from Pathn Situ, cat number: PEH002-50ml, India. The procedure of this method was as follows, according to Alghezi, *et al.* [11].

Three pretreatment steps were carried out before IHC. The tissue samples were cut (4µm), deparaffinized to remove the paraffin from tissue samples using xylene, and then rehydrated using graduated alcohols (100%, 95%, and 70%, respectively). After permeabilizing the tissue sections with 0.5% triton X-100 in phosphate buffer saline (PBS), they were heated to 90°C for 30 minutes to induce epitope retrieval in a Tris/EDTA buffer (pH 9) with 0.05% Tween 20. These sections were then allowed to cool for 20 minutes at room temperature. 3% H_2O_2 drops were applied to the tissue sections in a humid chamber to inhibit the activity of endogenous peroxidase. In addition, a solution of 1ml normal goat serum with 18 µ bovine serum albumin was produced in PBS. Subsequently, drops of this solution were applied to the tissue sections. At this point, the pretreatment steps were done, and the treated tissue sections became ready for staining.

The first step of staining was carried out using an anti-CD3 antibody, which was previously diluted using primary antibody diluent (Pathn Situ, cat number: PS010-100mlEly, USA); drops of this diluted antibody were applied on tissues and incubated overnight at a temperature of 4°C. The next day, these sections were washed three times for 10 minutes each. For 30 minutes, the secondary antibody was then applied to these tissue sections and incubated in a humid chamber at room temperature, followed by washing with PBS solution three times for 5 minutes each. Drops of Poly Excel Poly HRP were applied to the tissues section. After 15 minutes of room temperature incubation in a humid chamber, the tissues were washed three times with washing buffer for five minutes each time. Drops of DAB chromogenic solution were then added to these slides for 5 minutes in a dark place at room temperature, followed by washing with distal water for 10 minutes to terminate the chromogenic reactions. After that, drops of Vector hematoxylin solution as a counterstain were added to these sections for a minute at room temperature. These sections were then rinsed for three minutes with tap water. The mounting media which is used to adhere the coverslip on these sections was DPX (Cat number: 06522-100mL, Sigma-Aldrich, Gillingham, UK). Five random images were captured using a Nikon Digital Camera (DS-U1 CCD) connected to a Nikon Eclipse E800 microscope.

Immunohistochemical analysis

For quantification of CD3+ T cell immunostaining, tissue sections were manually reviewed by histopathologist. Five fields of view were used to score the CD3 immunostaining in the stromal tissue of colon using a semi-quantitative scoring system under a microscope objective for analysis. The proportion and intensity of CD3 staining scored as follows: The percentage of positive cells was used the following scoring system: 0 (less than 10%), 1 (11-25%), 2 (26-50%), and 3 (more than 51%). A score of negative (0), mild (+1), moderate (+2), or strong (+3) was assigned to the stromal CD3 staining. The total intensity and percentage ratings, which vary from 0 to 7, are the ultimate score for every case according to Trabelsi, *et al*. [12].

Statistical analysis

To calculate the data's mean, standard error, and standard deviation, GraphPad Prism version 8.00 for Windows was used (GraphPad Software, La Jolla, California, USA, www.graphpad.com). Tukey's multiple comparisons tests, the one-way ANOVA, and the unpaired t-test were the statistical analyses used in this investigation. For statistical significance, a significance level of $p<0.05$ was used.

RESULTS

Clinical Data of Study Population

The present study involved 80 CRCs (77%) and 24 benign colon tissues (23%). The age range was increased significantly in cancerous patients compared to non-cancerous people (p= 0.001). Increased CRC cases were also reported in males compared to females. However, this difference was not significant (p=0.776). people who reside in cities have been found to have more CRC cases than those who live in rural areas. (p=0.003). In addition, the majority of CRC patients were in grade II (75%) compared to those with grade 1 (7.5%) and grade III (17.5) (p=<0.001). Stage III was detected in almost 38.5% of CRC patients. In contrast, stages I and II were observed in 15.4% and 11.5% of CRC cases, respectively, and the stage was unknowable in the remaining cancer cases (p=<0.001). The distribution of CRC cases with T3-4 was significantly higher that those with T1-2 (p=0.001). In addition, the CRC cases with lymph nodes involved was significantly lower than those without lymph node involved (p=<0.001).The clinical data of benign and malignant colon tissues is summarized in Table 1.

Table 1: The distribution of malignant and benign patients according to age, sex, residency, grade, and stage

The clinical data			Number	%	P value
Number of samples	Benign		23	22.3	0.001*
	Malignant		80	77.7	
Age range	Benign	23-32	6	25%	0.001*
		33-42	5	20.8	
		43-52	7	29.2	
		53-62	6	25%	
	CRC	23-32	4	5	
		33-42	16	20	
		43-52	22	27.5	
		53-62	18	22.5	
		63-73	14	17.5	
		<73	6	7.5	
Sex	CRC	Male	٤٤	55	0.776
		Female	36	٤٥	
	Benign	Male	13	56.5	
		Female	10	43.5	
Residency	CRC	City	58	72.5	0.003*
		Rural	22	27.5	
	Benign	City	12	52.2	
		Rural	11	47.8	
Grade	Grade 1		6	7.5	<0.001*
	Grade2		60	75	
	Grade 3		14	17.5	
Stage	Stage 1		8	15.38	0.001*
	Stage II		6	11.54	
	Stage III		20	38.46	
	N/A		18	34.62	
T category	T1-T2		7	8.75	<0.001*
	T3-T4		20	25	
	N/A		53	66.25	
N category	N0		18	22.5	<0.001*
	N1		8	10	
	N/A		54	67.5	
M category	M0		0	0	n/a
	M1		0	0	
	N/A		80	100	

***** means a significant result

CD3 Immunostaining in Benign and Malignant Colorectal Tissues

IHC was carried out on benign and malignant colorectal tissues to examine the expression of CD3. The result showed that both groups had stromal CD3 immunostaining with varying signal strength, ranging from strong to moderate. Few stromal cells in the benign colon tissue showed strong CD3 immunostaining (Figure 1 A, yellow arrow). The CRC tissue and the normal adjacent area had strong stromal CD3 staining (Figure 1, B, yellow arrow). Strong stromal CD3 immunostaining was observed in CRC tissue (Figure 1, C, yellow arrow). Moderate stromal CD3 immunostaining was found in the malignant colon tissue (Figure 1, D, yellow arrow). In this study, Lymph node tissues were used as positive control for CD3 expression [24], and IHC revealed strong CD3 staining in lymph node tissue, as predicted (Figure 1, E, yellow arrow). Negative control showed no significant background staining (Figure 1, F, yellow arrow).

Increased CD3 expression is associated with CRC high grade and tumor size.

IHC staining quantification showed increased stromal CD3 immunostaining significantly in CRC patients compared to benign colon tissue (p: 0.007) (Figure 2 A; Table 2). Increased stromal CD3 immunostaining was positively associated with an increasing grade of CRC (p= 0.002) (Figure 2 B; Table 2). Multi-comparison (Tukey) test showed increased stromal CD3 immunostaining significantly in patients with high grades compared to those with low (p= 0.004) and moderate grades (p=0.022) (Figure 2 B; Table 2). In contrast, CD 3 immunostaining showed no significant difference between grade 1 and II (Table 2). In contrast, CD3 immunostaining was negatively associated with the pathological stage of CRC. Specifically, CD3 immunostaining was decreased significantly in CRC patients with T3-4 compared to those with T1-2 (p= 0.005) (Figure 2 C, Table 2), but not with other clinical-stage parameters, such as lymph nodes (p=0.893) (Figure 2 D, Table 2).

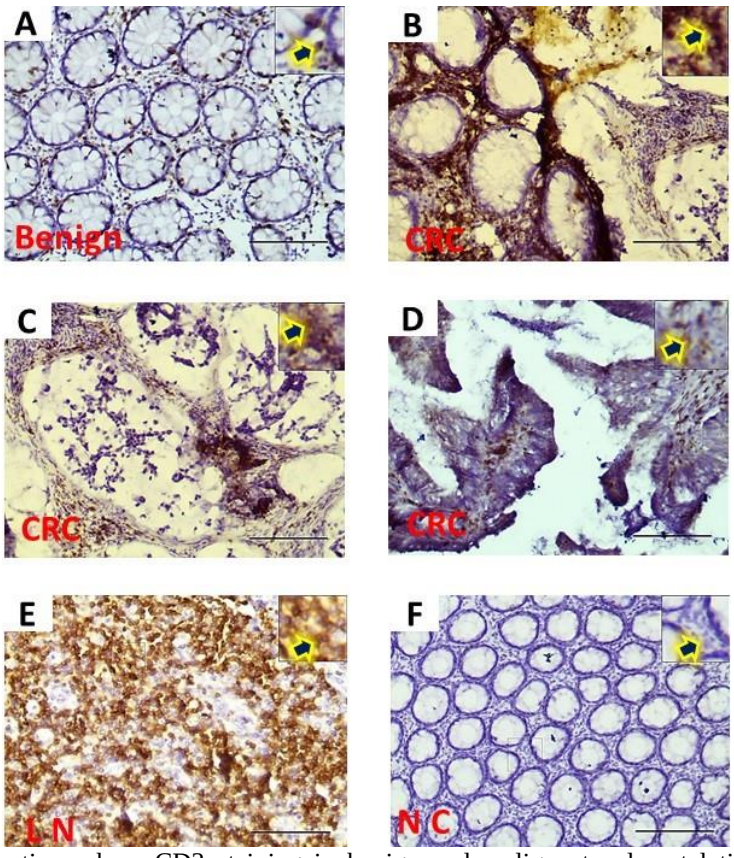

Figure 1: Histological sections show CD3 staining in benign and malignant colorectal tissue samples. A) Strong stromal CD3 immunostaining was observed in benign colon tissue. B) Strong stromal CD3 immunostaining in the adjusent normal and malignant tissues of colon. C) Increased stromal CD3 was observed in the adjacent area of CRC. D) Moderate stromal CD3 immunostaining was found in CRC tissues. E) Strong stromal CD3 immunostaining was found in lymph node tissues. F) There is no background staining in benign colon tissue as a negative control. Benign colon tissue (Benign), Colorectal carcinoma (CRC), LN (Lymph node) and Negative control (NC). Bars of scale: 100 µm. The inset images are digitally magnified using Image J program (Immunostain ×200).

Table 2: Stromal CD3 immunostaining results in Benign and malignant tissues with clinical data

Comparison	Sample Numbers	Stromal anti-CD3 immunostaining		
		Results		p. value
Benign vs. CRC	24	Increased in CRC		0.007*
	80			
Grades	G1: 6	Increased in high grade	ANOVA test	0.002*
	G2: 60		Grade 1 vs. Grade 2	0.886
	G3: 14		Grade 1 vs. Grade 3	0.004*
			Grade 2 vs. Grade 3	0.022*
Stage (T)	T1-2: 7	Increased in T1-T2		0.005*
	T3-4: 20			
Stage (N)	N0: 18 N1: 8	No significant difference		0.893

***** means a significant result

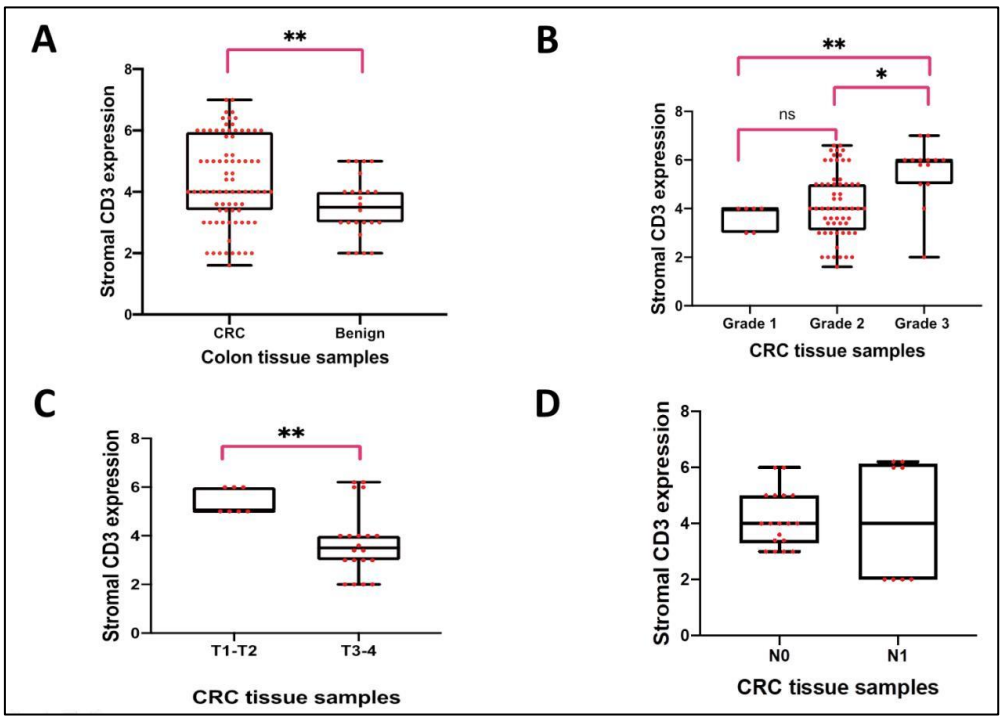

Fig. 2: Quantification of anti-CD3 immunostaining in benign and malignant colorectal tissue samples. (A) Increased stromal CD3 staining was found in the stroma of CRC compared to benign tissues (p=0.007). (B) Increased stromal CD3 immunostaining was associated significantly with increasing CRC grades (p=0.002). Increased CD3 staining was seen using Tukey's tests when comparing high grades (G3) to either moderate (G2) (p= 0.022) or low grades (G1) (p = 0.004). (C) Stromal CD3 staining was increased significantly in T1-2 (p=0.005). (D) There was no significant association between stromal CD3 staining and lymph node invasion (p:0.893). Statistical significance was determined with an unpaired T-test or a nova test for each set of conditions. Benign (n=24), CRC (n=80), grade 1 (n=6), grade 2 (n=60), grade 3 (n=14), T1-2 (n=7), T3-4(n=20), N0(N=18), N1(n=8). Corlorectal carcinoma (CRC), Lymph node status (N), Tumor size (T).

DISCUSSION

This study investigated the stromal immunostaining of CD3 as a possible biomarker for diagnosis, prognosis, and treatment of benign and malignant tissues of colon using IHC. This study found increased CRC cases in males compared to females. This data is agreed with a previous study [25], but disagreed with another study, which reported the frequency of male equal to that of females [26]. These differences may be because of the sample size. In addition, compared to males, females have noticeably more significant amounts of estrogen, which is found to play an essential role in preventing CRC development [27]. They also found that the protective impact is likely to be facilitated by estrogen receptor β (ERB) [27]. In addition, this result found that increased CRC is reported in older adults and

is consistent with the previous report, which found CRC development is associated significantly with significant age [28]. The reason for that is that the immune system becomes weaker in elderly people compared to young people, and the risk of CRC development is higher. The incidence of CRC was found to be higher in urban areas than in rural areas. The current data agrees with the previous CRC studies [29]. However, it was inconsistent with other data [30, 31]. The reason for this differences may be that living in the city exposes people to many risk factors such as pollutants and factory and car waste as well as the type of food which is usually a home grow made food in the rural areas.

The present study showed also increased CD3 immunostaining in the stromal tissue of CRC compared to the benign. The current data agrees with the previous CRC studies [16, 31, 32] as well as with earlier studies on other cancers such as breast [33], ovarian [34], and gastric tumors [35]. Taking together, CD3 may have a role in tumor development and might be used as a biomarker to evaluate the infiltration of T lymphocytes (T cells) within the tumor microenvironment [36].

Moreover, this study determined if stromal CD3 immunostaining and CRC grade were related. This data showed a positive association between stromal CD3 staining and the CRC grades. our data was consistent with the previous study done by Kasurinen *et al* [37]. It is also in agreement with previous nasopharyngeal cancer study [22]. However, it was inconsistent with previous breast [33] and colon cancer studies [38]. This discrepancy could result from the use of different primary antibodies and scoring system, as well as a different sample size. Together with previously published data, CD3 seems to be associated with the differentiation of tumors, increased CD3 infiltration on colon tissues may promote the advancement of CRC, and elevated CD3 staining in tumors may serve as a bad prognostic biomarker for CRC patients.

The current study also determined if stromal CD3 immunostaining and CRC stage were correlated. The data showed that the clinical stage and CD3 staining were negatively associated. In particular, decreased stromal CD3 staining was observed in patients with tumor size T3-4 compared to those with T1-2. This data agreed with previous colon cancer studies [12,39]. It is also in agreement with previous studies [37, 40]. On the other hand, these data revealed an inconsistent outcome with another colon cancer study [34] as well as other cancer types such as nasopharyngeal [22] and breast cancers [33]. This discrepancy may be attributed to variations in IHC scoring methodologies, the specific antibodies used, the techniques utilized for antigen retrieval, the size of the sample populations, and the distinct tumor types examined.Tumor infiltration by T lymphocytes, namely CD3-positive cells, is typically correlated with a more favorable prognosis and enhanced responsiveness to immunotherapy. In addition, The previous study demonstrated a direct correlation between tumor-infiltrating lymphocytes, particularly those expressing CD3, and micro-invasive status. The presence of CD3+ T-cells in the tumor center indicates their crucial involvement in the immune response and the resulting disease outcome [39]. However, the current study showed no significant differences between CD3 staining and lymph node status. This finding agreed with a previous finding[33]. In contrast, the recent data disagreement with previous findings demonstrated a substantial correlation between higher CD3 levels and the spread of cancer to the lymph nodes [12, 34]. These variations might result from using various antibodies, antigen retrieval techniques, scoring methods, or a range of research populations. These results imply that increased CD3 staining may be a useful biomarker for CRC, as it is linked to the disease's progression and prognosis.

The primary limitation of this study is the small sample size of benign prostate tissue. Additionally, no clinical data on metastasis were available, as the tissue samples were obtained via endoscopy. Another drawback is the limited validation of the antibodies used; further research is needed to confirm these findings, either by employing RNAscope to measure CD3 mRNA levels in the tissues or by using a second independent antibody with a larger cohort. Moreover, investigating the functional role of CD3 in colon cell lines through tissue culture would be highly beneficial.

CONCLUSION

Our study reported an increased CD3 immunostaining in CRC compared to benign and a positive correlation was shown between the CD3 immunostaining and CRC grade. In contrast, a negative association was demonstrated between stromal CD3 staining and tumor size. Based on available data, it appears that CD3 might be involved in aggressiveness and treatment of CRC. Some limitations of the current study should be taken into account in follow-up research that aims to expand on these findings.

Acknowledgement

The University of Thi-Qar's College of Medicine's Cancer Research Unit is appreciated by the authors for allowing them to utilize their laboratories. The Al-Hussein Teaching Hospital staff members who collected the samples are all thanked by the authors.

Author Contributions

Oula and Dhafer designed the study, carried out the experimental procedures, wrote the first manuscripts, and performed the statistical analysis. Dhafer and Rash handled the data collection. Oula, Dhafer, Rasha and Ali wrote, reviewed, and edited the material. All authors reviewed and approved the final manuscript.

Declarations:
Consent for publication: Not applicable
Availability of data and material: Data are available upon request.
Competing interests: The author(s) declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.
This manuscript has not been previously published and is not under consideration in another journal.
Funding: Authors did not receive any grants from funding agencies.

REFRENCES

1. Fidler MM, Gupta S, Soerjomataram I, Ferlay J, Steliarova-Foucher E, Bray F. Cancer incidence and mortality among young adults aged 20-39 years worldwide in 2012: a population-based study . Lancet Oncol. 2017;18(12):1579-89. DOI: 10.1016/S1470-2045(17)30677-0.
2. Alalwany OS, Alghezi DA ,Aljawher RQ. Identifying Colibactin-Producing Escherichia coli as Candidate Biomarker for Aggressive Colorectal Cancer. Egyptian Journal of Medical Microbiology. 2024;33(4):59-66.
3. Al-Harbi SA, Al-Judaibi AA. Relationship between Escherichia coli and colon cancer. GSC Biological and Pharmaceutical Sciences. 2020;12(2):188-89. DOI: 10.30574/gscbps.2020.12.2.0101 .
4. Li S, Liu J, Zheng X, Ren L, Yang Y, Li W, et al. Tumorigenic bacteria in colorectal cancer: mechanisms and treatments. Cancer Biol Med. 2021;19(2):147-62.
5. Jawad MA. Overexpression of tumor suppressor gen (TP 53)in relation to C19-9 in colorectal and gastric cancer (IHC STUDY): Kufa University 2010.
6. Mullangi S, Lekkala MR. Adenocarcinoma. [Updated 2023 Jun 18]. In: StatPearls [Internet .[Treasure Island (FL): StatPearls Publishing; 2024 Jan-. Available from : //:www.ncbi.nlm.nih.gov/books/NBK562137 ./
7. Nagtegaal ID, Hugen N. The Increasing Relevance of Tumour Histology in Determining Oncological Outcomes in Colorectal Cancer. Curr Colorectal Cancer Rep. 2015;11(5):259-66 .DOI: 10.1007/s11888-015-0280-7
8. Hagen CE, Farooq A. Histologic Evaluation of Malignant Polyps and Low-Stage Colorectal Carcinoma. Arch Pathol Lab Med. 2019;143(12):1450-4. DOI: 10.5858/arpa.2019-0291-RA
9. Alhassani OJ, Atiyah, S. and Musa, H. The Role Of Salmonella In Progression Of Colorectal Cancer . Annals of the Romanian Society for Cell Biology. 2010; 25(4):20037-20047.
10. Olawaiye AB, Baker TP, Washington MK, Mutch DG. The new (Version 9) American Joint Committee on Cancer tumor, node, metastasis staging for cervical cancer. CA Cancer J Clin. 2021;98-287(4). DOI: 10.3322/caac.21663
11. Alghezi D, Aljawher R, Musawi S. Increased CD73 expression is associated with poorly differentiated Gleason score and tumor size in prostate cancer. J Adv Biotechnol Exp Ther.2023; 6 (1):161. doi: 10.5455/jabet.2023.d115 .
12. Trabelsi M, Farah F, Zouari B, Jaafoura MH, Kharrat M. An Immunoscore System Based On CD3(+) And CD8(+) Infiltrating Lymphocytes Densities To Predict The Outcome Of Patients With Colorectal Adenocarcinoma. Onco Targets Ther. 2019;12:8663-73. DOI: 10.2147/OTT.S211048
13. Chen K, Collins G, Wang H, Toh JWT. Pathological Features and Prognostication in Colorectal Cancer. Curr Oncol. 2021;28(6):5356-83. DOI: 10.3390/curroncol28060447
14. Weiser MR. AJCC 8th Edition: Colorectal Cancer. Ann Surg Oncol. 2018;25(6):1454-5 . doi.10.1245/s10434-018-6462-1.
15. Alghezi DA, Whitley P, Beresford M, Bowen R, Mitchard J, Chalmers AD. Decreased ABCG2 Expression in Prostate Cancer and Negatively Associated with Poorly Differentiated Grade and Biochemical Recurrence. TQMJ. 2020;20 (2):242-56.
16. Dahlin AM, Henriksson ML, Van Guelpen B, Stenling R, Oberg A, Rutegård J, et al. Colorectal cancer prognosis depends on T-cell infiltration and molecular characteristics of the tumor. Mod Pathol. 2011;24(5):671-82. DOI: 10.1038/modpathol.2010.234
17. Tavana S, Mokhtari Z, Sanei MH, Heidari Z, Dehghanian AR, Faghih Z, et al . Clinicopathological significance and prognostic role of LAG3 + tumor-infiltrating lymphocytes in colorectal cancer; relationship with sidedness. Cancer Cell Int.2023; .23(1): DOI: 10.1186/s12935-023-02864-3
18. Liu J, Li J, Luo F, Wu S, Li B, Liu K. The Predictive Value of CD3+/CD8+ Lymphocyte Infiltration and PD-L1 Expression in Colorectal Cancer. Curr Oncol. 2023;30(11):9647.٥٩. DOI: 10.3390/curroncol30110699
19. Smith-Garvin JE, Koretzky GA, Jordan MS. T cell activation. Annu Rev Immunol. 2009;27: 591-619. doi: 10.1146/annurev.immunol.021908.132706.
20. Fridman WH, Pagès F, Sautès-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. Nat Rev Cancer. 2012;12(4):298-306. DOI: 10.1038/nrc3245

21. Lee WS, Park S ,Lee WY, Yun SH, Chun HK. Clinical impact of tumor-infiltrating lymphocytes for survival in stage II colon cancer. Cancer. 2010;116(22):5188-99. DOI: 10.1002/cncr.25293.
22. Al-Rajhi N, Soudy H, Ahmed SA, Elhassan T, Mohammed SF, Khoja HA, et al. CD3+Tlymphocyte infiltration is an independent prognostic factor for advanced nasopharyngeal carcinoma .BMC Cancer. 2020;20(1):240. DOI: 10.1186/s12885-020-06757-w.
23. Chan JKC. The Wonderful Colors of the Hematoxylin–Eosin Stain in Diagnostic Surgical Pathology. International Journal of Surgical Pathology. 2014;22(1):12-32. doi:10.1177/1066896913517939
24. Cho J. Basic immunohistochemistry for lymphoma diagnosis. Blood Res. 2022;57(S1):55-61. doi: 10.5045/br.2022.2022037
25. Wang P, Liang J, Wang Z, Hou H, Shi L, Zhou Z. The prognostic value of p53 positive in colorectal cancer: A retrospective cohort study. Tumour Biol. 2017; 39 (5). 10.1177/1010428317703651
26. Hartman J, Gustafsson JA. Estrogen receptors in colorectal cancer: goalkeepers, strikers, or bystanders? Cancer Prev Res (Phila). 2010;3(8):897-9. DOI: 10.1158/1940-6207.CAPR-10-0132
27. Cheng K, Nair NU, Lee JS, Ruppin E. Synthetic lethality across normal tissues is strongly associated with cancer risk, onset, and tumor suppressor specificity. Sci Adv .2021; 7(1). doi 10.1126/sciadv.abc2100
28. Aboagye JK, Kaiser HE, Hayanga AJ. Rural-Urban Differences in Access to Specialist Providers of Colorectal Cancer Care in the United States: A Physician Workforce Issue. JAMA surg. 2014; 149 (6). 10.1001/jamasurg.2013.5062
29. Rogers CR, Blackburn BE, Huntington M, Curtin K, Thorpe RJ, Jr., Rowe K, et al. Rural-urban disparities in colorectal cancer survival and risk among men in Utah: a statewide population-based study. Cancer Causes Control. 2020;31(3):241-53. DOI: 10.1007/s10552-020-01268-2
30. Jassim HM, Laf SA, Majeed YH. Escherichia coli Biomarker Types in Colorectal Cancer Patients. IJDDT. 2022;12(2). DOI:10.25258/ijddt.12.2.27
31. Herstad KMV, Gunnes G, Rørtveit R, Kolbjørnsen Ø, Tran L, Skancke E. Immunohistochemical expression of β-catenin, Ki67, CD3 and CD18 in canine colorectal adenomas and adenocarcinomas . BMC Vet Res. 2021;17(1):119. DOI: 10.1186/s12917-021-02829-6.
32. Barbosa AM, Martinho O, Nogueira R, Campos J, Lobo L, Pinto H, et al .Increased CD3(+), CD8(+), or FoxP3(+) T Lymphocyte Infiltrations Are Associated with the Pathogenesis of Colorectal Cancer but Not with the Overall Survival of Patients. Biology (Basel). 2021;10(8). DOI: 10.3390/biology10080808
33. Rathore AS ,Kumar S, Konwar R, Makker A, Negi MP, Goel MM. CD3+, CD4+ & CD8+ tumour infiltrating lymphocytes (TILs) are predictors of favourable survival outcome in infiltrating ductal carcinoma of breast. Indian J Med Res. 2014;140(3):361-9.
34. Han LY, Fletcher MS ,Urbauer DL, Mueller P, Landen CN, Kamat AA, et al. HLA class I antigen processing machinery component expression and intratumoral T-Cell infiltrate as independent prognostic markers in ovarian carcinoma. Clin Cancer Res. 2008;14(11):3372-9. DOI: 10.1158/1078-0432.CCR-07-4433
35. Lee HE,Chae SW, Lee YJ, Kim MA, Lee HS, Lee BL, et al. Prognostic implications of type and density of tumour-infiltrating lymphocytes in gastric cancer. Br J Cancer. 2008;99(10):1704-11. Doi: 10.1038/sj.bjc.6604738
36. Yang Y, Zang Y, Zheng C, Li Z, Gu X, Zhou M, et al. CD3D is associated with immune checkpoints and predicts favorable clinical outcome in colon cancer. Immunotherapy. 2020;12(1):25-33. DOI: 10.2217/imt-2019-0145
37. Kasurinen J, Hagström J, Kaprio T, Beilmann-Lehtonen I, Haglund C, Böckelman C. Tumorassociated CD3- and CD8-positive immune cells in colorectal cancer: The additional prognostic of CD8+-to-CD3+ ratio remains debatable. Tumour Biol. 2022;44(1):37-52. DOI: 10.3233/TUB٢١١٥٧١
38. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pagès C, et al. Type ,density, and location of immune cells within human colorectal tumors predict clinical outcome .Science. 2006; 313(5795): 1960–1964. DOI: 10.1126/science.1129139
39. Lea D, Watson M, Skaland I, Hagland HR, Lillesand M, Gudlaugsson E, et al. A template to quantify the location and density of CD3 + and CD8 + tumor-infiltrating lymphocytes in colon cancer by digital pathology on whole slides for an objective, standardized immune score assessment . Cancer Immunol Immunother. 2021;70(7):2049-57. DOI: 10.1007/s00262-020-02834-y.
40. Wirta EV, Seppälä T, Friman M, Väyrynen J, Ahtiainen M, Kautiainen H, et al. Immunoscore in mismatch repair-proficient and -deficient colon cancer. J Pathol Clin Res. 2017;3(3):203-13.