Immunoflourecent Staining Detect the Prognostic significance of M2 macrophage.

❖ **Aseel AbdulAlla Hussain.**(Master student)
❖ **Ibrahim Abdulmajeed Altamemi** (Ph.D /Immunology) Department of Microbiology /College of Medicine/ Al-Qadisyia University/Iraq
❖ **Thair Wali Ali** (Ph.D/Pathology) Department of Pathology /College of Medicine/ Al-Qadisyia University/Iraq

الخلاصة

الخلايا السرطانية تستخدم آليات متعددة لغزو المكونات الخارج الخلية وتنتشر إلى أعضاء بعيدة. التفاعل بين الخلايا السرطانية خلايا النسيج الاخرى في بيئه الورم تلعب دورا هاما في نمو الورم والانتشار. الخلايا البلعميه المرتبطه بالاورام (TAM) هي خلايا انسجة بارزة في هذا التفاعل. حيث انها تنتج مجموعة متنوعة من عوامل النمو، السيتوكينات ، والإنزيمات التي تنظم نمو الورم،وتكوين اوعية الدموية جديده، والغزو، والانتشار. تظهر الخلايا البلعميه نوعين من الاستقطاب في استجابة للإشارات الدقيقة المختلفه لبيئه الورم. الخلايا البلعميه نوع M1, هي التي تلعب دور المحفزللمناعه و للالتهابات واباده الجراثيم، كما وتعمل كخلايا عارضة للمستضد. بينما النوع الاخر للخلايا البلعميه M2 لديها النمط الظاهري المثبطة للمناعة.

وبالتالي فإن الهدف من الدراسة الحالية هو لمعرفة ما إذا كان هناك اهميه للخلايا البلعميه نوع M2 المخترقه او المنتشره في نسيج الورم القولوني المستقيمي في التأثير على او تحديد مأل الورم من خلال ربط مستوى التعبير الموضعي لهذه الخلايا مع المتغيرات المتغيرات النسيجيه المرضيه باستخدام تقنية الوميض المناعي. كذالك من خلال قياس نمو الخلايا السرطانيه باستخدام معلم النمو Ki67.

اوجدت الدراسة الحاليه مستوى التعبير لـ Ki67 كانت أعلى بكثير في المرضى من الذين من المجموعة الضابطة، (P<0.001)، وعلاوة على ذلك كان معدل اخلايا البلعميه العامه (CD68) ، والخلايا البلعميه المثبطه للمناعه M2(CD163) هوه أعلى بكثير في المرضى من المجموعة الضابطة (p<0.001، p<0.001) على التوالي. بالاضافه الى ذالك أظهرت الدراسة الحاليه ان الخلايا البلعميه M2 (CD163) له علاقه معنويه او ارتباط كبير مع كل من درجة تمايزالورم (P<0.001)، والانتشار في العقد اللمفاويه LN (P<0.001)، ومرحلة الورم ،كما اظهرت هذه الدراسه ان معدل الخلايا البلعميه المثبطه للمناعهM2 له علاقه مع نمو وتكاثر الخلايا السرطانيه من خلال العلاقه المعنويه مع الـ Ki67 (P<0.001)، عندما ـؤخذ نسبة CD163 / CD68 بعين الاعتبار، تم الحصول على النتائج التالية: لم يكن هناك ارتباط كبير بين نسبة CD163 / CD68 وكل من مرحلة الورم والانتشار للعقد اللمفيه LN (p>0.219، p> 0.468على التوالي). في حين كان هناك ارتباط كبير بين CD68 / نسبة CD163 ودرجة تمايز الورم (p<0.03). من النتائج أعلاه يمكن أن نستنتج أن العلاقه المترابطه للخلايا البلعميه المثبطه للمناعه نوع M2 (CD163) مع مأل المرض الشيئ الطقس يمكن ان تستخدم كهدف للعلاج المناعي لخفض نمو سرطان القولون والمستقيم.

ABSTRACT

Tumor cells use multiple mechanisms to invade extracellular matrix and metastasize to distant organs. The interaction between the tumor cells and stromal cells in the tumor microenvironment plays an important role in tumor growth and metastasis. Tumor associated macrophage (TAM) are prominent stromal cells in this interaction. They secret a variety of growth factors, cytokines, chemokines, and enzymes that regulate tumor growth, angiogenesis, invasion, and metastasis**.** Macrophages show two polarization states in response to different micro-environmental signals. M1macrophages are pro-inflammatory and function as bactericidal and antigen-presenting cells. M2 macrophages have an immunosuppressive phenotype.

Thus the aim of current study is to find out whether there is prognostic value for M2 macrophage that infiltrating colorectal tumor tissue through linking its expression level with tumor histopathological variables by using immunoflourecent technique. Further more to find if there an association for M2 macrophage with tumor cell proliferation by using Ki67 as a proliferation marker.

Current study found that the Mean Ki67 score was significantly higher in patients than in control group, (P<0.001), moreover the common macrophage mean $CD68^{+}$ macrophages,

and M2 macrophage ($CD163^+$) count was significantly higher in patients than in control group ($P<0.001$, $P<0.001$), respectively. I addition present study demonstrated that the M2 macrophage ($CD163^+$) count showed significant correlation with tumor grade ($P<0.001$), LN involvement ($P<0.001$), stage ($P<0.001$), and Ki67% ($P<0.001$). However, when the ratio of CD68/CD163was considered, the following results were obtained: there was no significant correlation between CD68/CD163 ratio and both of tumor stage and LN involvement ($P>0.219$, $P>0.468$ respectively). While there was significant correlation between CD68/CD163 ratio and tumor grade ($p<0.03$).

From above results one can conclude that high expression of CD163 macrophages is associated with poor prognosis and from other hand could be targeted by immune therapy to lower the progression of colorectal cancer.

Introduction

Colorectal cancer is one of common malignancies in the world. The incidence of colorectal cancer ranks 3rd among malignancies, and the mortality is only inferior to that of lung cancer, gastric cancer and liver cancer [1]**.**Tumor cells use multiple mechanisms to invade extracellular matrix and metastasize to distant organs. The interaction between the tumor cells and stromal cells in the tumor microenvironment plays an important role in tumor growth and metastasis. Macrophages are prominent stromal cells in this interaction. They secret a variety of growth factors, cytokines, chemokines, and enzymes that regulate tumor growth, angiogenesis, invasion, and metastasis [2**].** Monocytes are actively recruited to the tumor stroma, and tumor-associated macrophages (TAM) are common in the stromal compartment of several malignancies. Macrophages show two polarization states in response to different micro-environmental signals [3]. M1macrophages are pro-inflammatory and function as bactericidal and antigen-presenting cells. M2 macrophages have an immunosuppressive phenotype, stimulate Th2 cell differentiation and are activated by apoptotic cells and anti-inflammatory molecules such as IL-4, IL-13, and IL-10 [4].

In addition, different markers are used to distinguish M1 and M2 macrophages. But the expression of CD163 is the most recently described, [5] Despite the macrophage classification schemes, little is known about the complexity of the microenvironment populations of macrophages and their associations with clinical cancer progression [6].

TAMs represent the M2-type macrophages and play an important role in tumor cell migration, invasion and metastasis. They also support angiogenesis and tissue repair [7]. Experimental studies show that TAMs promote tumor progression, and high infiltration in many tumor types is correlated with lymph node involvement and distant metastasis [8]. The presence of macrophages directes tumours towards a histologically more malignant phenotype. Inhibition of macrophage infiltration in tumors may inhibit metastasis and progression of secondary tumors [9, 10]. However, the clinical significance of macrophage infiltration in tumor stroma, remains unclear. High infiltration of TAMs is correlated with poor prognosis in, prostatic, ovarian and cervical carcinoma [11]. In colorectal cancer, there are conflicting data on the clinical significance of macrophage infiltration, but several studies show that low macrophage density in tumor stroma is associated with an unfavorable prognosis [11,12].

TAMs have been associated with a decreased survival in patients with e.g.melanoma [13], breast [14], kidney [15] and bladder cancer [16]. However, this is not true for all cancers. Others have previously shown that an increased density of macrophages in CRC is correlated to a better prognosis [17]. It is becoming increasingly evident that macrophages can play different roles in tumorigenesis dependent on tissue

and cancer type. It is interesting to speculate that the different roles played by macrophages in various cancers could involve variations in the balance between M1 and M2 phenotypes (tumor prevention vs. tumor promotion), driven by factors in the tumor microenvironment of individual cancers [18].

The aim of the present study was to analyze colorectal cancer infiltration by macrophages of various phenotypes *in situ* and to test whether there is a prognostic significance for M2 macrophage through linking its expression level with tumor histopathological variables which include (tumor stage, grade, LN involvement, and tumor growth by utilizing Ki67 as a proliferation indicator). By using immunofourecent technique.

Patients And Methods.

Patients and Sampling*:* Forty two patients with colorectal adenocarcinoma, who were confirmed histopathologicaly, were included in this study. Their age were ranged from 32- 78 years. Paraffin embedded blocks of tumor and resection margins were retrieved along with the histopathological report of each patient from histopathological laboratory. For staging of the cancer, astler-coller staging system was adopted in this study [19]. In addition, resection margins were confirmed again to be free of malignancy. Adequate thin paraffin embedded sections (5µm thick) of tumor and resection margins were prepared on positively charged slides for the immunoflourecent Technique (IF).

Direct Immunoflourecent Detection of CD 163, CD68,and ki67 posative cells in Paraffin Embedded Sections.

Immunofluorescence is an antigen-antibody reaction where the antibodies are tagged (labelled) with a fluorescent dye and the antigen-antibody complex is visualized using ultra-violet (fluorescent) microscope. Fluorochromes are dyes that absorb ultra-violet rays and emit visible light. This process is called fluorescence. the fluorochromes used in this study, is fluorescein isothiocyanate (FITC), which is commonly used flourochrome. When fluorescein (FITC) is excited by a blue (wavelength 488nm) light, it will emit a green (520nm) colour.

The steps involved are: Fixation of smear on the slide, antigen unmasking by heat epitope retreval methods by using water bath and epitope retrival solution with 6pH. Fallowed by treating the samples with flourecene labeled antibody (Santa cruz, USA), incubation, washing to remove unbound excess labeled antibody and visualization under fluorescent microscope. When viewed under fluorescent microscope, the field is dark and areas with bound antibody fluoresce green.

Results:

Table 1 showed the mean age and pathologic characteristics of the study group. Mean age of study group was 58.60±11.43 with an age range of (32-78) years. According to grade there were 7 patients (16.7%) with grade I tumor, 23 patients (54.8%) with grade II tumor and 12 patients (28.6%) with grade III tumor. Regarding Lymph node involvement, 24 patients out of 42 (57.1%) had positive lymph node involvement while the rest of patients (42.9%) had no lymph node involvement. Stage I was reported in only 8 patients (19.0 %), stage II in 12 patients (28.6 %), stage III in 12 patients (28.6 %) and stage IV in 10 patients (23.8 %).

Table 1: Mean age and pathologic characteristics of patients with colorectal carcinoma

Characteristic	Mean ±SD; N (%)
Age (years)	58.60±11.43 (32-78)
Grade	
I	7 (16.7%)
II	23 (54.8%)
III	12 (28.6%)
Lymph node	
Involved	24 (57.1%)
Not involved	18 (42.9%)
Stage	
I	8 (19.0 %)
II	12 (28.6 %)
III	12 (28.6 %)
IV	10 (23.8 %)

Table 2 showed the mean score of Ki67, mean count of CD68 macrophages and mean count of CD 163 macrophages in colorectal tissues of patients and control group. Mean Ki67 score was significantly higher in patients than in control group, 57.02±19.66 % versus 17.10±4.58 %, (P<0.001), moreover mean CD68 macrophages count was significantly higher in patients than in control group 67.31±19.13 cell/HPF versus 17.65±4.82 cell/HPF (P<0.001), in addition to the finding that CD163 macrophages count was significantly higher in patients than in control group, 59.10±18.97 cell/HPF versus 32.35±6.21(P<0.001). The immunoflourecent cellular staining of CD68, CD163, and Ki67 are shown in figure 1.

Table 2: Mean Ki67 score, CD68 macrophages and CD138 macrophages count in patients and control groups

Parameter	Colorectal Carcinoma Mean ±SD	Control Mean± SD	P-value
ki67	57.02±19.66	17.10±4.58	<0.001
CD68	67.31±19.13	17.65±4.82	<0.001
CD163	59.10±18.97	32.35±6.21	<0.001

Table 3 showed the correlation between CD68 macrophage count and other variables and also the correlation between CD163 count and other variables in patient group. CD 68 macrophage count showed significant positive correlation with CD163 count (P<0.001), grade (P<0.001), LN (P<0.001), stage (P<0.001), and Ki67% (P<0.001). On the other hand CD163 macrophage count also showed significant correlation with grade (P<0.001), LN (P<0.001), stage (P<0.001), and Ki67% (P<0.001).

When the ratio of CD68/CD163was considered the following results were obtained: there was no significant correlation between CD68/CD163 ratio and LN and between this ratio and stage, while there was significant correlation between CD68/CD163 ratio and grade. Moreover, there was a significant correlation between CD68/CD163 ratio and Ki67.

Table 3: Correlations among various markers in patient group

Parameter		CD163	grade	LN	Stage	ki67
CD68	r	0.927	0.638	0.849	0.957	0.945
	P	<0.001	<0.001	<0.001	<0.001	<0.001
CD163	r		0.679	0.811	0.930	0.917
	P		<0.001	<0.001	<0.001	<0.001
Ratio	r		0.327	0.115	0.194	0.571
	P		0.034	0.468	0.219	<0.001

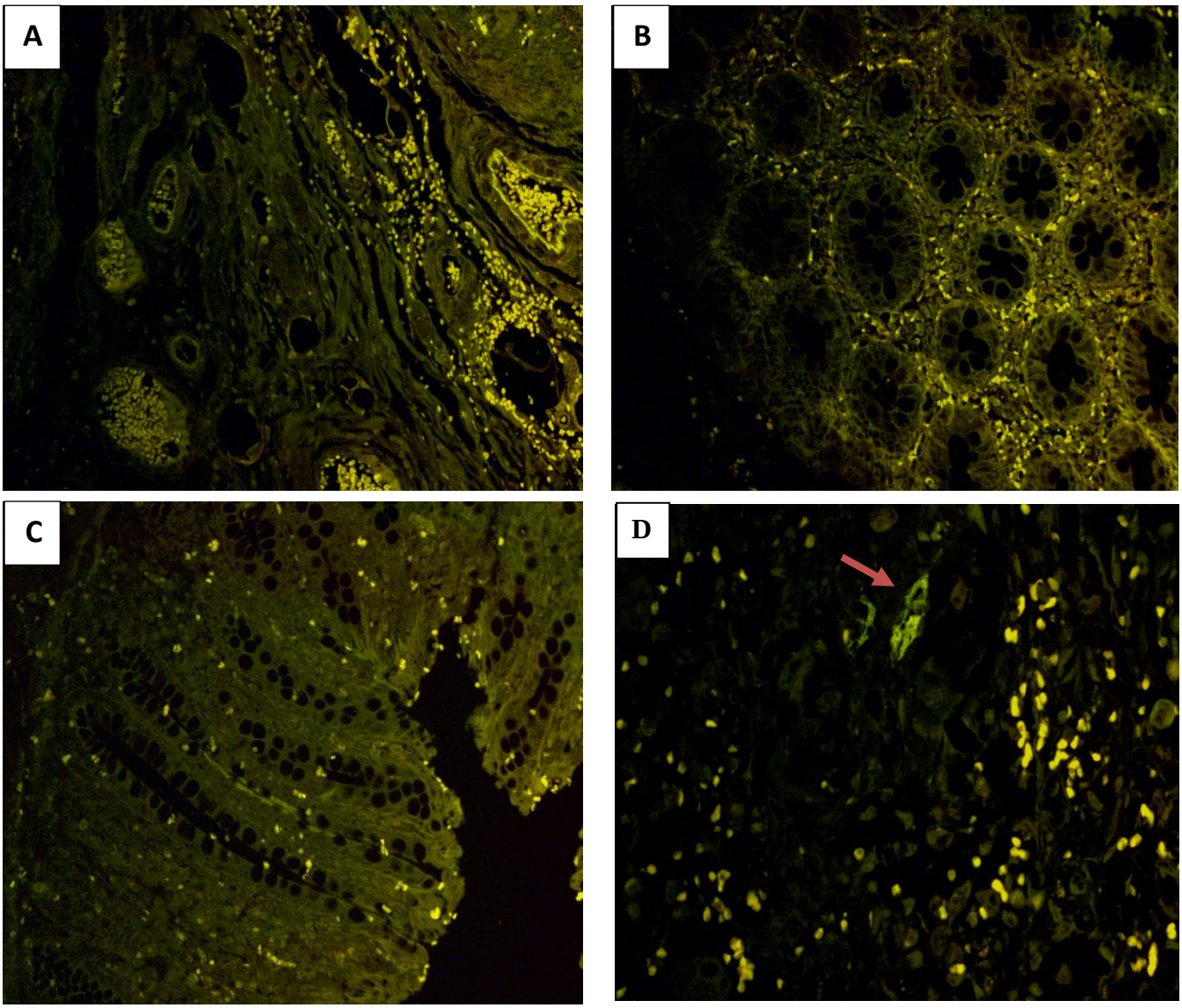

Figure1. Immunoflourecent staining of tumor infiltrating macrophage CD68, CD163, and K-i67 in colorectal adenocarcinoma section by FITC flourochrome (Green color) with dark background. (A)Common tumor infiltrating macrophage (CD68) expression within tumor tissue which. (B)M2 macrophage (CD163) expression within tumor tissue. (C) Ki-67 nuclear staining of actively proliferating tumor cells. ***(D)*** Positive CD163 expression in cancer cells as a result of cell fusion with CD163 TAM. Magnification power (20X).

Discussion

The result of the present pointed to a mean age of 58.60±11.43 in patients with colorectal carcinoma. According to Iraqi cancer registry (2009) the mean age of patients with colorectal carcinoma was 53.98+14.71 years and the median age was 55 years. These findings are comparable to the findings of the present study and the minor differences are clearly due to the difference in sample size which was 701 in Iraqi cancer registry while it was only 42 cases in the present study [20].

It was published in the Iraqi Cancer registry (2009) that about 82 % of colorectal carcinoma patients are above 40 years of age and that around 18% of patients are bellow the age of 40 years. This result is clearly similar to the finding of the present study[20]. Talib *et. al*. stated that despite the clear relationship with aging, colorectal carcinoma is not strictly a disease of elderly and 6-8 % of cases occur in patients below 40 years of age [21]. **A**l-Humadi (2008) reviewed the records of 511 patients with colorectal carcinoma who were diagnosed in the period extended from 1965 through 1994 in Baghdad and stated that the median age was 50 years , a finding which strongly supported the finding of the present study. He also stated that 21.1% of cases occurred before the age of 40 years in accordance with finding of the present study[22]. In another Iraqi study done in Kirkuk city, it was mentioned that the mean age of patients was 51.4 years [23], which is again similar to mean age obtained in the present study. Moreover another study done in Iraq stated that the mean age of patients with colorectal carcinoma was 54.5 years, a finding that solidified the result of the present study [24]. Another Iraqi study done in Al-Kadhimiya teaching hospital on 94 colorectal cancer patients stated that about 24% of cases were under the age 40 years, a finding which is slightly higher than the finding of the present study which was 20%, but again it is in accordance with result of the present study [25]. In another study done in Iraq, the mean age of patients with colorectal carcinoma was 52.4±16.3 years and an age range of 21-81 years [26]; again these findings are similar to the findings of the present study. Additionally an Iraqi study was done in Duhok and stated that the mean age of colorectal cancer patients was 50.1 years and the age range was 20-80 years [27]; these results again are in accordance to the results of the present study. Qasim *et. al*. (2012) stated, in a study done in Baghdad, that the mean age of patients with colorectal carcinoma was 56.03 and the age range 27-80 years; again these figures are approximately similar to the findings of the present study[28]. Also Hashim *et. al.* (2010) stated, in a study done in Al-Najaf city that the mean age of patients with colorectal carcinoma was 58.1 years; this finding is higher than that of the present study but it is still in the sixth decade[29].

The finding of the present study showed that the majority of patients had well to moderately differentiated tumor. According to Ackerman surgical pathology textbook (2011), the usual malignant tumor of the large bowel is a well-to-moderately differentiated adenocarcinoma secreting variable amounts of mucin [30]. Rahim et.al. in (2012) reported that majority of colorectal carcinoma cases (64%) were well differentiated grade I lesions [31]. This result is similar to the result of the present study. Well differentiated morphology was the major histological grade reported by previous studies done by Talib et.al.,; Summer and Osama 2013; and Qasim et.al., [21,23,28]

The present study showed that a minority of patients had a stage I disease. This can be attributed to the fact that early stage (carcinoma in situ and stage I disease) tumors are often not diagnosed due to lack of proper screening programs like colonoscopy and imaging techniques which can be applied on high risk groups. As it is difficult to cure patients with advanced stage tumors, it is mandatory to apply screening programs for early detection of these common tumors in Iraq.

The finding of the present study that that majority of patients had stage II disease is in agreement with many authors: Summer and Osama; Qasim et.al; Ali et.al; Rola et.al; and Munir et.al [23,28,29,32,33].

The present study showed that tumor associated macrophages count was significantly higher in patient group. There was an over expression of CD163 macrophages in correlation with advanced stage and this was in accordance with other studies done by Cui, et al. and Shabo et al. [34,35]. The explanation for this correlation between CD163 macrophages and advanced stage might be due to certain cytokines produced by tumor cells that favour the differentiation of already present tissue macrophages to acquire the phenotypic characteristics of CD163. The advantage for tumor cell of such behavior might be explained by the observation thatCD163 macrophages will get fused with primary tumor cells leading to acquisition by tumor cells to hybrid characteristics of CD163 and tumor cells (36-42). This fused has been observed in the present study as shown in figure 1.D. This fusion will help tumor cell to metastasize by degredation of extracellular matrix by lytic enzymes and by facilitated locomotion[43]. On the other hand CD163 can offer the production of growth factors like EGF, FBGF1, TGFB1 , VEGFA and matrix remodeling factors like FGF1 and fibrin and matrix metalloproteinases and also production of immune regulatory factors like IL-10, TGFB1 that dump the response and by this way the tumor progression will get facilitated [44]. CD163 has been shown to be correlated with higher grade and high proliferation index in the present study. This might be due to the ability of CD163 cells to affect the differentiation of tumor cells by a mechanism which is still not identified. Regarding high proliferative activity the best explanation is through the theory of fusion and production of growth factors by CD163 macrophages. The ratio of CD163/CD68 was also correlated with higher grade and high proliferative activity. From above discussion one can conclude that high expression of CD163 macrophages is associated with poor prognosis and from other hand could be targeted by immune therapy to lower the progression of colorectal cancer.

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