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Tumors Associated Macrophages (TAM) Phenotype in Colorectal Cancer Patients

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Abstract: The ability of macrophages to adapt to their environment has led to the identification of two main polarized phenotypes of macrophages. First M1 macrophage (classical) is associated with chronic inflammation, cancer initiation and promotion and the reduction of cancer risk by treatment with anti-inflammatory drugs. Second, M2 macrophage (or alternative), a high density of these tumor-associated macrophages correlates with poor prognosis and show mostly protumoral functions, promoting tumor cell survival, proliferation, and dissemination. The study aimed to detect of the distribution of different subtypes of macrophages clinical specimens of colorectal carcinoma (CRC), and how they are integrated with tumor features. Total of 47 patients with colorectal carcinoma were enrolled in this study, among of these patients 26 (55%) males and 21(45%) females, with a range age from 37 years to 72 years, mean age (54.5 year), with 1:1.2 ratio between female and male. Immunohistochemistry was used to detect nitric oxide synthase 2 (NOS2) as a marker for the M1 macrophage phenotype and the scavenger receptor CD163 as a marker for the M2 macrophage phenotype with CD68 as general marker of detection macrophage in tissue. Results recorded that NOS2 with score 3 appeared high frequency (34.04%) fallowed by 25.53% to score 2. NOS2 showed association with age groups <50 years, gender, right site, poorly differentiated, and mucinous types of cancers. As a markers of TAM1, CD163 recorded, high frequency (44.68%) for score 1 followed by score 2 in 17.02%, and association with gender, in female, left site, poorly differentiation, and mucinous cancers.

Key words: CRC, Immunohistochemistry, NOS2, CD163, CD68 **.**

علاقة نوع البلاعم الكبيرة المرتبطة سرطانيا (TAM) في مرضى سرطان القولون

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الخلاصة: قدرة البلاعم الكبيرة على التطبع في المحيط الدقيق يؤدي الى تحديد نوعين من البلاعم الكبيرة. النوع الاول التقليدي وله علاقة بالالتهابات المزمنة مع قدرتها على منع وتحديد وتقليل خطر السرطان بواسطة استخدام علاجات مضادات الالتهابات. النوع الثاني او البديل وهذا النوع متعلق ومرتبط بالسرطان وخصوصا قبل السرطان والمأل السيء له حيث تسمح لنمو وتكاثر وانتشار الخلايا السرطانية. وتهدف الدراسة الى تحديد وانتشار الانواع المختلفة من البلاعم الكبيرة في النماذج المأخوذة من مرضى سرطان القولون والمستقيم وعلاقتها مع صفات السرطان الاخرى. تم جمع حوالي 47 نموذج من مرضى مصابين بسرطان القولون والمستقيم وبمعدل اعمار يتراوح بين 37–72 سنه وبمتوسط عمر 54.5 سنه وبنسبه 1:1.2 بين الرجال والنساء. استخدمه تقنية التصبيغ المناعي الكيمونسيجي. واستخدم CD68 كعلامة لتحديد البلاعم الكبيرة في النسيج بصوره عامه. كما استخدم iNOS2 كعلامة للنمط الظاهري الاول وتم تسجيل النتائج اعتمادا على خمسه مستويات من النتائج وكان المستوى الثالث اعلى نتيجة وبنسبه 34.04% ويليه المستوى الثاني وبنسبة 25.53% . واظهرت النتائج وجود علاقة بين النمط الاول للبلاعم الكبيرة ومجموعة الاعمار الاكبر من 50 سنة وكذلك جنس النساء والجهة اليمنى للقولون والتمايز السيء وانتاج الميوسين. وتم استخدام CD163 كعلامة للنمط الظاهري الثاني وتم تسجيل النتائج بنفس الطريقة حيث اظهر المستوى الاول اعلى نتيجة بنسبة 44.68% يليها المستوى الثاني وبنسبه 17.02% مع علاقتها بجنس النساء والجزء الايسر للقولون والتمايز السيء و انتاج الميوسين.

Introduction

Colorectal cancer is one of the most frequent malignancies in the Western world. Worldwide, approximately 1, 2 million people developed colorectal cancer in year 2008 and the disease related mortality was about 36% (1,2) The disease affects slightly more men than women and sporadic colon cancer is considered to be a disease of the elderly with a median age at diagnosis of 70 years (3).More than 90% of the colorectal cancers occur sporadically, which means that affected patients do not have a family history of colon cancer (4).

Macrophages represent up to 50% of the tumor mass, and they certainly operate as fundamental actors. Macrophages constitute an extremely heterogeneous population; they originate from blood monocytes, which differentiate into distinct macrophage types, schematically identified as M1 (or classically activated) and M2 or alternatively activated (5).

(TAMs), which constitute a significant part of the tumor-infiltrating immune cells, have been linked to the growth, angiogenesis, and metastasis of variety of cancers, including breast and cervical cancers and transitional cell carcinomas most likely through polarization of

TAMs to the M2 (alternative) phenotype (6).

The role of macrophages in tumor progression has been shown to be double-edged, since these cells can both promote tumor rejection (M1 macrophages) and stimulate tumor growth (M2 macrophages). In brief, pro-inflammatory, or classically activated M1 macrophages, exerting important functions in host defense as well as bactericidal and tumoricidal activity, are held against anti-inflammatory, or alternatively activated M2 macrophages, playing important functions in immune regulation, tissue remodeling and tumor progression (7).

M1 macrophages (classically activated cells) originate upon encounter with IFN-_ and microbial stimuli such as LPS and are characterized by IL-12high and IL-23 production and consequent activation of polarized type I T cell response (5). In general, M1 macrophages act as soldiers: they defend the host from viral and microbial infections, fight against tumors, produce high amounts of inflammatory cytokines, and activate the immune response.

M2 macrophages are diverse, but in general are involved in T helper 2 (Th2) response, have an immunoregulatory function, and orchestrate encapsulation and containment of parasites and promote tissue repair, remodeling, and tumor progression (8).In case of M2 polarization, several mechanisms for a worse prognosis have been proposed. A positive feedback loop has been identified between tumor cells and macrophages that propagates the growth and promotes the survival of colon cancer cells (9). Based on the M1 versus M2 paradigm of macrophage polarization (10).TAM express low levels of the major histocompatibility complex class II and reduced antimicrobial and anti-tumor activity, while increasing production of mediators that promote angiogenesis, such as vascular endothelial growth factor and cyclooxygenase-2 (COX-2)–derived prostaglandin E2, as well as the anti-inflammatory cytokine IL-10 (11).On the other hand, distinct types of M2 cells differentiate when monocytes are stimulated with IL-4 and IL-13 (M2a), with immune complexes/TLR ligands (M2b), or with IL-10 and glucocorticoids (M2c) (5). M2 cells are workers of the host: they promote scavenging of debris, angiogenesis, remodeling and repair of wounded/damaged tissues. Of note, M2 cells control the inflammatory response by down-regulating M1-mediated functions.

NOS2.

However, NOS2, which is a Ca+2-independent isoform, is predominantly regulated through gene expression during a variety of stress conditions including inflammation and generates a larger quantity of NO for a sustained period (12). The two facets of nitric oxide (NO.) action, one as an important regulator of cellular function and the other as its toxic response, after inflammation toward invading pathogens, are well-established (13). However, there is considerable controversy and confusion in understanding its role in cancer biology. It is said to have both tumoricidal as well as tumor promoting effects which depend on its timing, location(4).For the above data we tried to evaluate the phenotypes of TAM in cancer tissues using molecules expressed by them.

Materials and methods

This study enrolled 47 patients that attended the GIT center, Baghdad hospital and private hospitals, from April,2013 to February,2014. The procedures to obtain human colon cancer tissues and follow-up information are in accordance with the Ethical Principles for Medical college ethical council.Biopsies and colon cancer tissues were collected from surgeries performed from April, 2013 to February, 2014. The forty seven patients with age range from 37 to 72, mean age 54.5 years with female to male ratio 1:1.2. The inclusion criteria was, without pre-operative chemotherapy or radiotherapy. All of the cases that satisfied the inclusion criteria were included in this study. Histological evaluation was based on the World Health Organization criteria. Tumor stage was evaluated according to the International Union against Cancer TNM classification system.Samples were fixed with 10% buffered formalinized saline, for preparation the paraffin embedded tissue blocks to histological evaluation and immunohistochemical staining tests.

Histological evaluation specimens, slides from fixed paraffin embedded tissue blocks were stained with haematoxylin – eosin stain and subsequently evaluated by an experienced pathologist. Four-μm thick tissue sections were de-waxed in xylene and rehydrated through graded alcohols. Antigen retrieval was carried out using microwave at middle-to-high temperature for 8 min, low-to-high temperature for 5 min, and then cooled down at room temperature for 20 min. mouse anti-human CD163 antigen monoclonal antibodies ,Rabbit anti-human nitic oxide synthase2(NOS2) monoclonal antibodies(recognizing M1 macrophages) ,and rabbit anti-human CD68 antigen monoclonal antibodies (recognizing M2 macrophages) were obtained from (Abcam-UK).

Immunohistochemical staining of individual marker was performed using the HRP/DAB detection IHC kit and test was performed according to the manufacturer's instructions. Development of brown color was scored according to (15). Sections were then counterstained with hematoxylin and mounted in an aqueous mounting medium. Five representative high-power fields (× 400 magnification) per tissue section were selected using an Olympus microscope (Olympus, Tokyo, Japan). The number of nucleated cells with positive staining for the phenotypic marker in each area was counted. Scoring system was;score 1;no or weak stain,score 2;moderate,score 3 strong/robust and score 4; massive infiteration.

Results

Sex and Age Distribution

From the 47 patients with colorectal carcinoma 26(55%) were males and 21(45%) were females ,Figure (1), with a range age from 37 years to 72 years, mean age (54.5 year), with 1:1.2 ratio between female and male. The patients' age were classified into two group first 50≥ years (38.29%), second group 50≤ years 29 (61.70%) Figure (2).

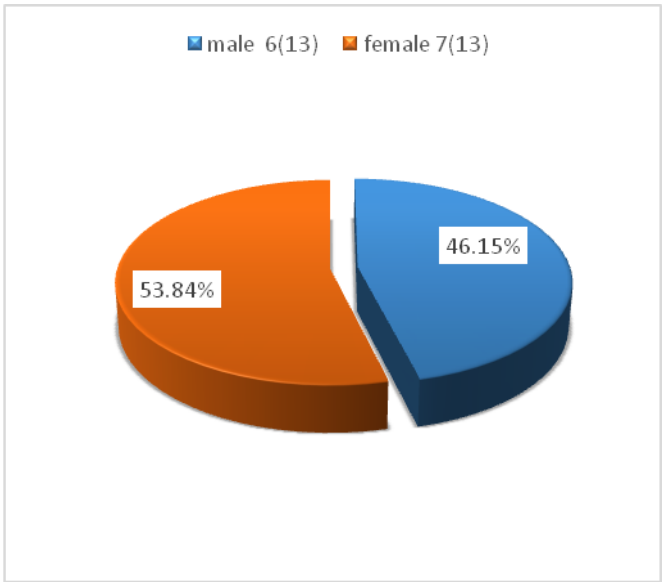


Figure 1: The sex distribution of CRC

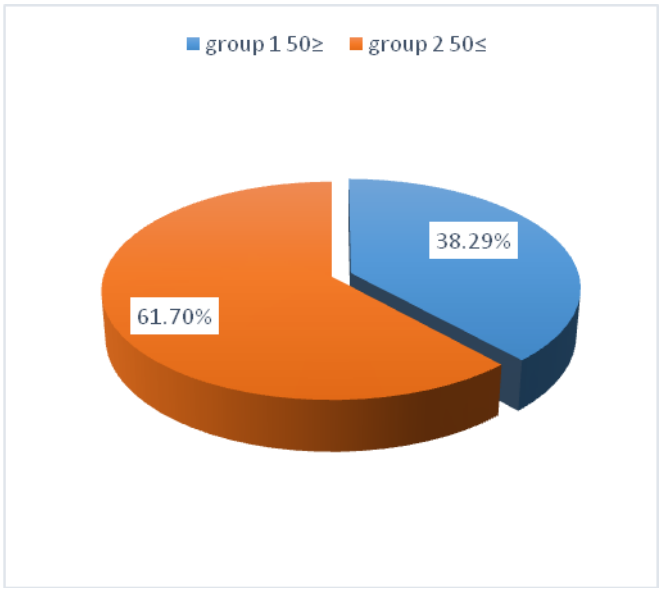


Figure 2: The age group frequency in CRC

Morphological differentiation

As regards with grades of colorectal carcinoma, it was observed that (14.89%) cases of well differentiated, (53.19%), moderate differentiated and (53.19%), and (31.91%) poorly differentiated Figure 3.

Site of tumor

Patients with CRC were classified according to the site of tumor location as shown in Figure (4), right site consistent (51.06 %) of all cases and patients, with left site (36.17 %) and (12.76 %) in the rectum from all cases.

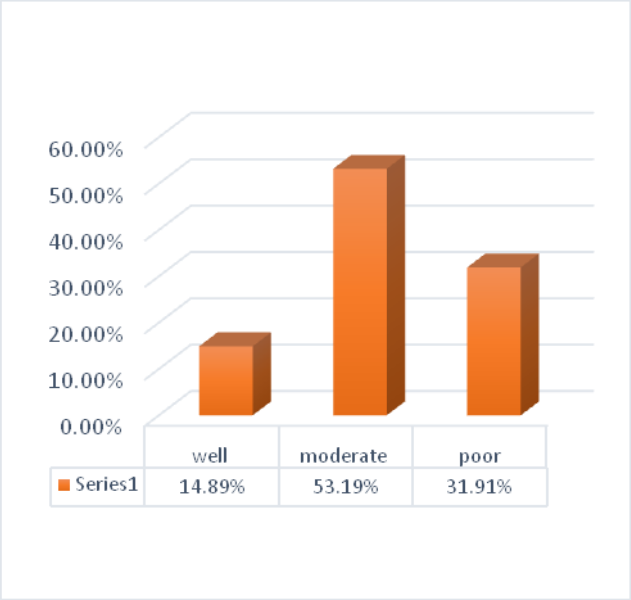


Figure 3:The morphological distribution of CRC

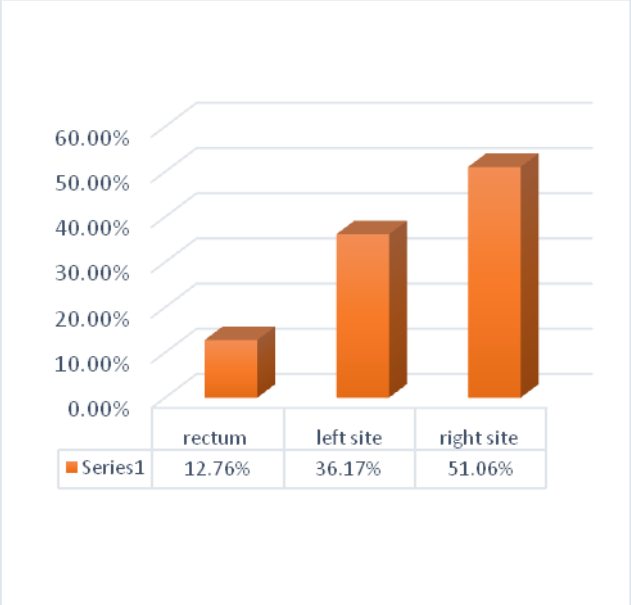


Figure 4: Site of tumor distribution in CRC

Tumor Associated Macrophage TAM1

In general CD68 used as marker for detection macrophage in tissue figure 5(A,B), As a markers of TAM1 used iNOS2Figure 6 (A,B), the results recorded depended five scores, score 3 appeared high frequency 34.04% compared with 25.53% to score 2 Figure 7.

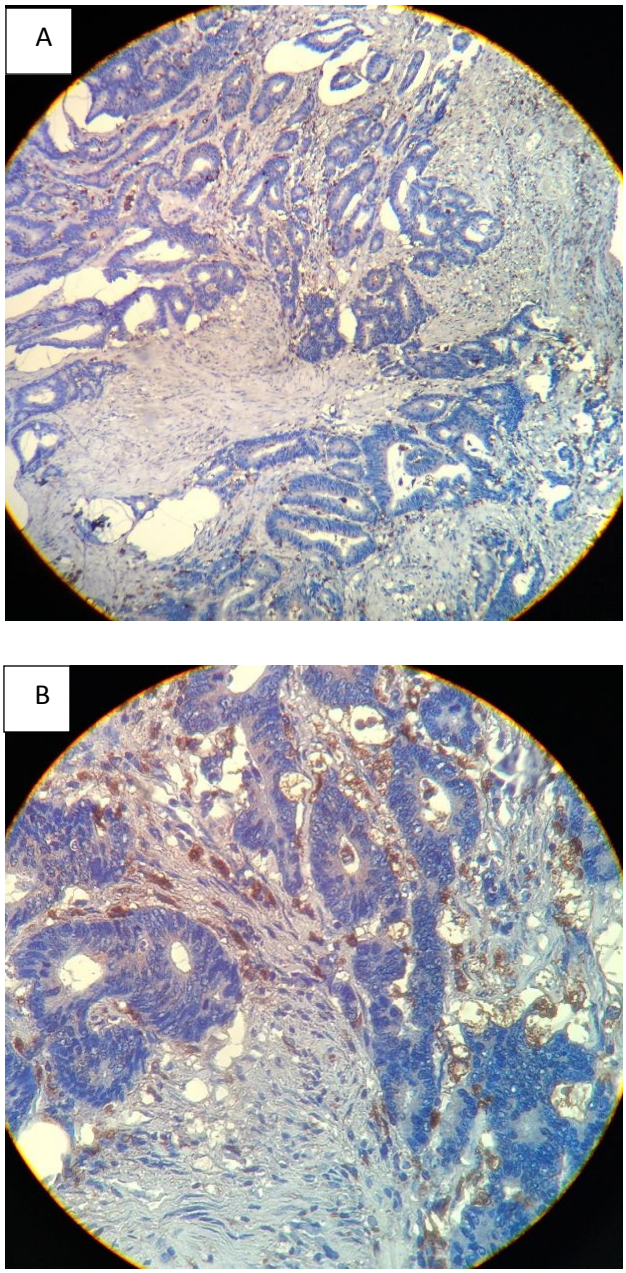


Figure 5: surface staining by immunohistochemistry with DAB chromgen and Myer's hematoxlyin detection CD68 with CRC, A:-low power, B: - high power

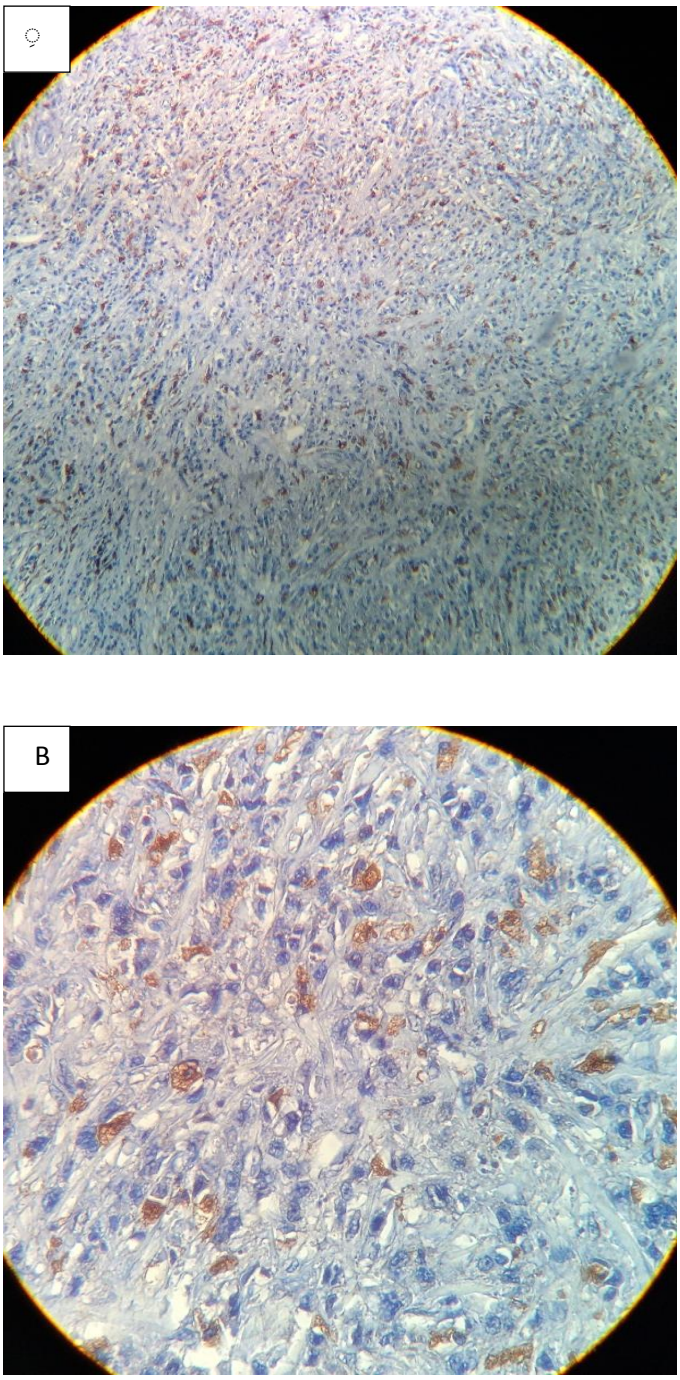


Figure 6: cytopasmic staining by immunohistochemistry with DAB chromgen and Myer's hematoxlyin detection NOS2 with CRC, A:-low power,B:- high power

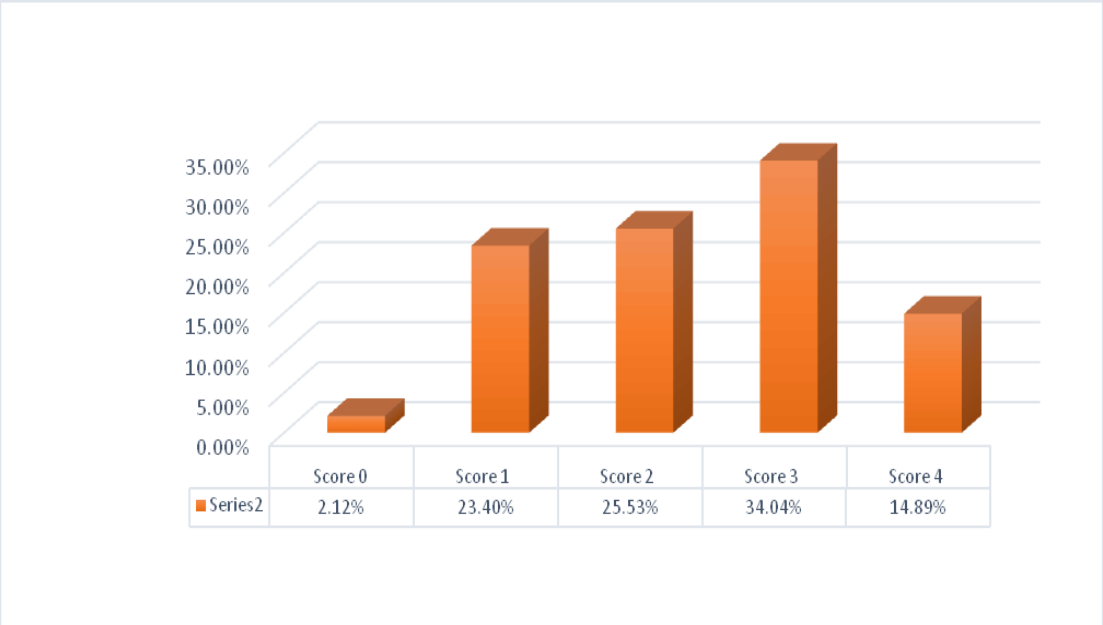


Figure 7: NOS2 frequency in patients with colorectal carcinoma

TAM1 related with gender

NOS2 showed associated with gender, in female score 2 appeared 38.09% vs 15.38% in male, while in male 38.46% score 3 compared 28.57% in female Table 1.

Table 1: NOS2 related with genders in patients with CRC

Sex	Score 0 No/%	Score 1 No/%	Score 2 No/%	Score 3 No/%	core 4 No/%	Total
Female	0	3(14.28)	8(38.09)	6(28.57)	4(19.04)	21
Male	1	8(30.76)	4(15.38)	10(38.46	3(11.53)	26
	1	11	12	16	7	47

X2=5.27, P 0.260

TAM1 related with site of tumor

NOS2 was shown close associated with the site of tumors, score 2 and 3 in left site was recorded high percentage with 35.29% and 47.05% respectively, while in right site score 3 and 1was showed high percentage with 33.33% and 37.5% respectively, while in rectum score 2 and 4 showed high percentage with 50% and 33.33% respectively, Table2.

Table 2: NOS2 related with site of tumor in patients with CRC.

Site of tumor	Score 0 No/%	Score 1 No/%	Score 2 No/%	Score 3 No/%	Score 4 No/%	Total
Right	0	9(37.5)	3(12.5)	8(33.33)	4(16.66)	24
Left	1(5.88)	1(5.88)	6(35.29)	8(47.05)	1(5.88)	17
Rectum	0	1(16.66)	3(50)	0	2(33.33)	6
	1	11	12	16	7	47

CRC. X2 =15.026, p=0.0586

TAM1 related with morphological features

poorly differentiated was appeared high associated with score 3 with 53.33%, followed by well differenced in 42.85% with same score, while score 1 appeared high percentage in moderate differentiated, Table3.

Table 3: NOS2 related with morphological feature in patients with CRC

Grade of tumor	Score 0	Score 1	Score 2	Score 3	Score 4	total
Well	0	1(14.28)	2(28.57)	3(42.85)	1(14.28)	7
Moderate	1(4)	9(36)	6(24)	5(20)	4(16)	25
Poor	0	1(6.66)	4(26.66)	8(53.33)	2(13.33)	15
	1	11	12	16	7	47

X2=7.969, P 0.436

TAM 1 related with age groups

NOS2 score 3 showed high percentage frequency with group 1 38.88% vs 31.03% in same score in group 2, while score 2 was recorded high percentage in group 2 with 34.48 compared with group1,Table 4.

Table 4: related of age groups with NOS2 in CRC patients

Age groups	Score 0 No/%	Score 1 No/%	Score 2 No/%	Score 3 No/%	Score 4 No/%	total
50≥	0	6(33.33)	2(11.11)	7(38.88)	3(16.66)	18
50≤	1(3.44)	5(17.24)	10(34.48)	9(31.03)	4(13.79)	29
	1	11	12	16	7	47

X2=4.488. p=0.34397274

TAM1 related with mucinous

NOS2 appeared close associated with mucinous, score 3 and 4 high percentage recorded in mucinous, while score 3and 2 shown in 30.23% and 27.90% in non-mucinous Table 5.

Table5: related NOS2 with mucinous and non-mucinous in CRC patients

Site of tumor	Score 0 No/%	Score 1 No/%	Score 2 No/%	Score 3 No/%	Score 4 No/%	Total
Mucinous	0	0	0	3(75)	1(25)	4
Non mucinous	1(2.32)	11(25.58)	12(27.90)	13(30.23)	6(13.95)	43
	1	11	12	16	7	47

X2=4.68P=0.32094

Tumor Associated Macrophage2 TAM2

As a markers of TAM2 we used CD163, the results recorded depended five scores, score 1 appeared high frequency 44.68% followed by score 2 in 17.02% to Figure8 , 9.

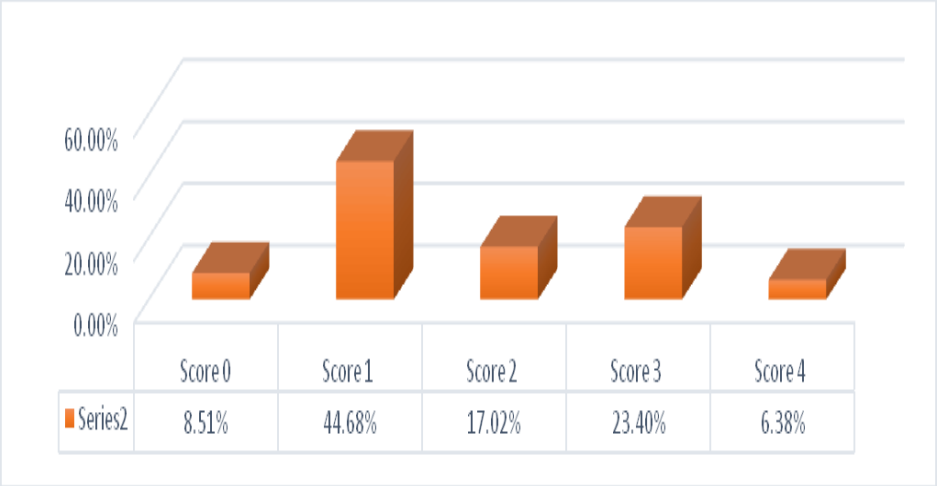
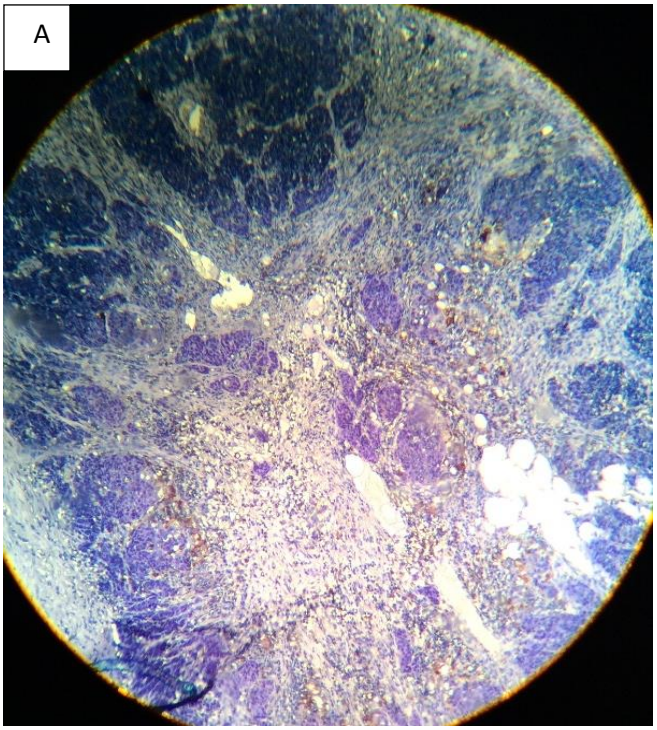


Figure 8: CD163 frequency in patients with CRC patients



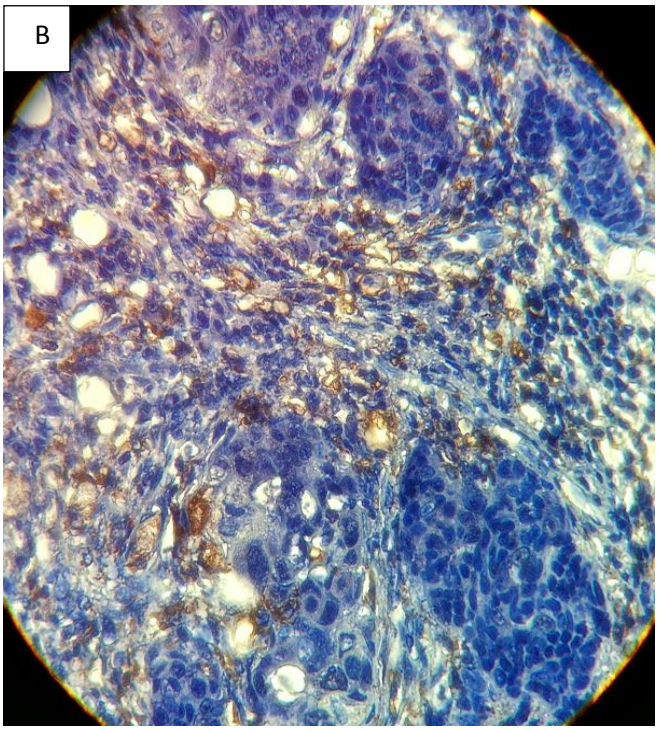


Figure 9: surface staining by immunohistochemistry with DAB chromgen and Myer's hematoxlyin detection CD163 with CRC, A:-low power, B:- high power

TAM2 related with gender

CD163 showed associated with gender, in female score 1 appeared 52.38% while in male high percentage 38.46% recorded in same score, Table 6.

Table 6: CD163 related with genders in patients with CRC

Gender	Score 0	Score 1	Score 2	Score 3	Score 4	Total
Female	2(9.52)	11(52.38)	2(9.52)	5(23.80)	1(4.76)	21
Male	2(7.69)	10(38.46)	6(23.07)	6(23.07)	2(7.69)	26
Total	4	21	8	11	3	47

X2=1.962 p=0.7427

TAM2 related with site of tumor

CD163 was shown close associated with the site of tumors, score 1 was recorded high percentage in left and right site with 35.29% and 50%, followed by score 3, 20.83% and 23.52% respectively, while in rectum score 3 was showed high percentage with 33.33% table 7.

Table 7: CD163 related with site of tumor in patients with CRC

Site of tumor	Score 0	Score 1	Score 2	Score 3	Score 4	Total
Right	2(8.33)	12(50)	4(16.66)	5(20.83)	1(4.14)	24
Left	1(5.88)	8(47.05)	3(17.64)	4(23.52)	1(5.88)	17
Rectum	1(16.66)	1(16.66)	1(16.66)	2(33.33)	1(16.66)	6
	4	21	8	11	3	47

X2=3.347 P=0.91073

TAM2 related with morphological feature

All types of differentiated was appeared high associated with score 1 with 60%, 42.85% and 36% in poorly, well and moderate differenced respectively table 8.

Table 8: CD163 related with morphological feature in patients with CRC

Grade of tumor	Score 0	Score 1	Score 2	Score 3	Score 4	total
Well	0	3(42.85)	1(14.28)	2(28.57)	1(14.28)	7
Moderate	4(16)	9(36)	2(8)	9(36)	1(4)	25
Poor	0	9(60)	5(33.33)	0	1(6.66)	15
	4	21	8	11	3	47

X2=14.5, p=0.06962

TAM2 related with age groups

CD163 score 1 was shown high percentage frequency in both groups, 44.44% and 44.82% respectively, followed by score 3 with 22.22% and 24.13 in group1 and 2 respectively table 9.

Table 9: related of age groups with CD163 in CRC patients

Site of tumor	Score 0	Score 1	Score 2	Score 3	Score 4	total
50≥	1(5.55)	8(44.44)	3(16.66)	4(22.22)	2(11.11)	18
50≤	3(10.34)	13(44.82)	5(17.24)	7(24.13)	1(3.44)	29
	4	21	8	11	3	47

X2=1.341, p 0.85438

TAM2 related with mucinous

CD163 was appeared close associated with mucinous, score 1 was shown high percentage recorded in mucinous with 100%, while score 1 and 2 was shown high percentage with 39.30% and 18.60% in non-mucinous respectively table 10.

Table 10: relation of CD163 with mucinous and non-mucinous in CRC

Type of tumor	Score 0 No/%	Score 1 No/%	Score 2 No/%	Score 3 No/%	Score 4 No/%	Total
Mucinous	0	4(100)	0	0	0	4
Non mucinous	4(9.30)	17(39.53)	8(18.60)	11(25.58)	3(6.97)	43
	4	21	8	11	3	47

X2=5.413**, p=**0.24748

Discussion

TAM Associated with CRC:A CRC specimens were stained byusing immunohistochemistry for nitric oxide synthase 2 (NOS2) as a marker for the M1 macrophage phenotype and the scavenger receptor CD163 as a marker for the M2 macrophage phenotype with using CD68 as general marker of detection macrophage in tissue,to determine the degree of infiltrating macrophages with a M1 or M2 phenotype in these specimens.

The average infiltration of NOS2 and CD163 expressing macrophages along the invasive tumor front was semi-quantitatively evaluated using a four-graded scale, according toForssellet al.,(2007)(15).

High macrophage infiltration has been correlated to improved survival in colorectal cancer (CRC). Tumor associated macrophages (TAMs) play complex roles in tumorigenesis since they are believed to hold both tumor preventing (M1 macrophages) and tumor promoting (M2 macrophages) activities (16).

An inflammatory tumor microenvironment has been suggested as the hallmark of cancer progression (17). Analysis of the immune contexture, the location, density and functional orientation of immune cells and how it is integrated with tumor molecular features can provide important information on patient prognosis as well as prediction of the response to various treatment (18, 19).

In this study no relation of NOS2 + or CD163 + macrophage infiltration was found to gender, age, grade, site of tumor and mucinous histology. These results were in agreement with (16).

Furthermore, patients harboring tumors highly infiltrated by NOS2+ cells may had a significantly better prognosis than those infiltrated by few NOS2+ cells.

The relation between infiltrating macrophages of M2 phenotype and prognosis in CRC has been previously analyzed in a few studies. According to (20), like results in our study, stromal infiltration of CD163 + M2 macrophages in CRC may correlated to a significantly improved survival .

In many clinical studies it has been observed that a high infiltration of TAMs correlates to a poor prognosis and TAMs are thus suggested to be of M2 phenotype that promote tumor progression. However, we and others have recently seen that high amounts of TAMs in CRC (20-22).

That high infiltration of M1 macrophages is correlated to a better prognosis in CRC in a stage dependent manner. However, in CRC the increased infiltration of M1 macrophages at the tumor front was found to be accompanied by a concomitant increase in M2 macrophages, therefore, we suggest that the presence of M1 macrophages is favorable for survival in patients with CRC, despite the parallel presence of M2 macrophages. Finally we concluded that macrophages play an important role at the tumor front, secreting factors that in many ways might affect both the tumor and surrounding stromal cells, including other cells of the immune system.

References

1- Cunningham, D., Atkin, W., Lenz, H J., et al (2010). Colorectal cancer. Lancet 375:1030-47.

2- Jemal A., Bray, F., Center, MM., et al (2011). Global cancer statistics. CA Cancer J Clin, 2011.

3- Van Steenbergen, LN., Elferink, MA.,Krijnen, P. et al. (2010). Improved survival of colon cancer due to improved treatment and detection: a nation wide population-based study in The Netherlands 1989-2006. Ann Oncol; 21: 2206–2212.

4- Weiss LM. , Arber DA. , Chang KL. (1994). CD68: a review. Appl Immunohistochem.;2:2-8.

5- Martinez F. O., Helming L., Gordon S. (2009). Alternative activation of macrophages: an immunologic functional perspective. Annu. Rev. Immunol.27, 451–483.

6- Bingle L., Brown NJ. , Lewis CE. (2002).The role of tumourassociated macrophages in tumour progression: implications for new anticancer therapies. J Pathol 3:254–265

7- Sica A. ,Schioppa T. , Mantovani A. , et al (2006). Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. Eur J Cancer 42: 717–727.

8- Zhou Q. ,Peng RQ. , Wu XJ. , et al. (2010). The density of macrophages in the invasive front is inversely correlated to liver metastasis in colon cancer. J Transl Med 8: 13.

9- Kaler P., Galea V., Augenlicht L., et al. (2010). Tumor associated macrophages protect colon cancer cells from TRAIL induced apoptosis through IL-1beta-dependent stabilization of Snail in tumor cells. PLoS One 7:e11700.

10- Gordon, S. and Taylor, P. R. (2005) Monocyte and macrophage heterogeneity. Nat. Rev. Immunol. 5, 953–964.

11- Pollard J. W. (2004) Tumor-educated macrophages promote tumor progression and metastasis. Nat. Rev. Cancer 4, 71–78.

12- Zamora R. ,Vodovotz Y. , Billiar TR. (2000). Inducible nitric oxide synthase and inflammatory diseases. Mol Med;6:347–73.

13- Murad F. Shattuck Lecture. (2006). Nitric oxide and cyclic GMP in cell signaling and drug development. N Engl J Med;355: P:2003–2011.

14- Sheetal K., Minal C. ,Sachin B. , et al (2013). Nitric oxide and cancer: a review. World Journal of Surgical Oncology 2013, 11:118.

15- Forssell J., Oberg A., Henriksson ML., et al. (2007) High macrophage infiltration along the tumor front correlates with improved survival in colon cancer. Clin Cancer Res 13: 1472–1479.

16- Edin S., WikbergM.andDahlin A. et al., (2012). The Distribution of Macrophages with a M1 or M2 Phenotype in Relation to Prognosis and the Molecular Characteristics of Colorectal Cancer. PLOS ONE | | Volume 7 Issue 10 e47045.

17- Colotta F., Allavena P., Sica A., et al. (2009) Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability.Carcinogenesis; 30(7):1073-81.

18- Ogino S. ,Galon J. , Fuchs CS. , et al (2011). Cancer immunology–analysis of host and tumor factors for personalized medicine. Nat Rev ClinOncol 8: 711–719.

19- Fridman WH., Pages F., Sautes-Fridman C., et al (2012). The immune contexture in human tumours: impact on clinical outcome. Nat Rev Cancer 12: 298–306.

20- Nagorsen D. , Voigt S. , Berg E. , et al. (2007). Tumor-infiltrating macrophages and dendritic cells in human colorectal cancer: relation to local regulatory T cells, systemic T-cell response against tumor-associated antigens and survival. J Transl Med 5: 62.

21- Zhou Q. ,Peng RQ. , Wu XJ., et al (2010). The density of macrophages in the invasive front is inversely correlated to liver metastasis in colon cancer. J Transl Med 8: 13.

22- Algars A., Irjala H., Vaittinen S., et al (2012). Type and location of tumor-infiltrating macrophages and lymphatic vessels predict survival of colorectal cancer patients. Int J Cancer 131: 864–873.