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*Original Research Article*

# A Study of Tumor Associated Macrophages and their Subpopulation M1 and M2 by Immunohistochemistry in Colo-Rectal Cancer

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**Abstract**

*Background:* Tumors of the colon and rectum are one of the most common malignancies worldwide. However, its incidence was less in India compared to the developed countries. In the recent years, due to westernization, sedentary lifestyle and increased consumption of animal fats with less dietary fibre intake have increased the incidence in India in the past few decades. Family history and Microsatellite instability also predisposes the patient to Colo-rectal carcinoma. Many prognostic factors have been studied in Colo-rectal cancers and have been proved. However newer factors like macrophage infiltration in the tumor microenvironment have been studied. Many theories have been put forth to study these macrophages and their sub population M1 and M2. M1 macrophages are considered to be tumoricidal whereas M2 macrophages are considered to promote tumor growth by releasing growth factors and promoting angiogenesis. Hence, the study of these macrophage subpopulation M1 and M2 can help in assessing the prognosis in patients with Colo-rectal cancers. *Aims & Objectives:* 1. To determine the expression of CD68 and CD163 in Colorectal Cancer. 2. To correlate the expression of CD68 and CD163 with the histological grade and stage of the tumor. *Materials and Methods:* All Colorectal carcinoma specimens received in the Department of Pathology from R.L. Jalappa Hospital and Research Center attached to Sri Devaraj Urs Medical College, Tamaka, and Kolar from December 2016 to September 2018 and also the paraffin blocks taken from all cases of Colorectal cancer retrieved from Archives of Department of Pathology from the year January 2008 to November 2016 were included in the study. Data regarding the clinical details (Age, Sex, Histological grading) were collected. Hand E slides were reviewed for Histopathological types, grade and staging of the

tumor. Immunohistochemistry for CD68 and CD163 (Biocare mouse antibody) was performed on all cases of Colorectal Carcinoma using appropriate positive and negative controls by peroxidase and anti peroxidase method. *Results:* A total of 62 cases were studied of which 39 were males and 23 were females. The most common site of tumor was Rectum followed by ascending colon. Majority of the tumors were less than 5 cms. The most common grade was moderately differentiated adenocarcinoma. Maximum number of cases were in Stage III, 23 cases (37.1%) Perineural invasion was seen in 2 cases and lymphovascular invasion was seen in 3 cases. Maximum number of cases (64.5%) were in lymphnode ratio less than <0.111. Expression of CD 68 was significantly correlating with site of the tumor, Size of the tumor, Grade, and lymphnode ratio. Expression of CD 163 was correlating with T stage, N stage, TNM stage, and Lymphnode ratio. *Conclusion:* CD 68 expression was associated with better prognostic factors such as smaller size of tumor, lesser grade and lesser lymphnode ratio(LNR) and CD 163 expression was associated with poorer prognostic factors such as higher T stage, Higher N stage, and higher values of lymphnode ratio (LNR). Hence, CD 69 and CD 163 can serve as a reliable prognostic marker in colo-rectal cancers.

**Keywords:** Colo-rectal cancer; Immunohistochemistry; Prognosis.

## Introduction

Tumors of the colon and rectum are the 3rd most common malignancies in men and second most common malignancy worldwide [1]. They are the 2 nd most common cause of death from cancer [2]. They are included among the most frequently encountered malignancy in the western population and in industrialized countries. The U S SEER database showed that the incidence of colorectal adenocarcinoma was 33.7/100000 and there was an increase of 18% from 1973 to 1987 [3]. However, in the recent past, there has been a steady increase in the incidence of Colo-rectal cancers in India.

A variety of environmental and genetic factors play a vital role in the development of these tumors [3]. Tumor microenvironment consisting of leucocytes and fibroblasts are also involved in the progression of colo-rectal cancers.

The concept of macrophages differentiation and activation by classical and alternate pathway in the progression of the disease has been hypothesized and are being studied in the tumors of colon and breast [4].

The tumor associated macrophages (TAMs) are broadly classified into two types depending on their mode of activation. The M1 macrophages are activated by classical pathway and M2 macrophages are activated by alternate pathways.

M1 macrophages cause good inflammatory response by releasing pro inflammatory cytokines such as TNF alpha, IL Beta and IL 6 thus fight against the tumor cells and are considered tumoricidal.

The M2 macrophages secrete anti-inflammatory cytokines such as TGF Beta, IL 10 and IL 3 and may help in tumor progression [5].

CD 68 and CD 163 are the proteins expressed by the circulating macrophages, monocyte derived macrophages and tissue macrophages. CD 68 stains cytoplasm of the M1 macrophages that are considered to be tumor suppressive and CD 163 stains the cytoplasm of M2 macrophages that are considered to help in tumor progression.

The patients with Colo-rectal cancer have better prognosis when there is increase density of macrophages at the tumor front which exhibit M1 phenotype, despite the parallel increase of M2 phenotype [6].

On H and E section, it is difficult to differentiate M1 and M2 phenotypes. Hence Immunostaining is used to identify M1 and M2 sub population of macrophages. CD68 is been taken as a marker for M1 macrophage and CD163 is been taken as a marker for M2 macrophage.

Only few studies determining expression of CD68 and CD163 have been done on Colorectal Cancers and published in Indian Literature so far.

Hence the study is undertaken to determine the expression of CD68 and CD163 in Colorectal Carcinomas.

*Aims of the study*

1. To determine the expression of CD68 and CD163 in Colorectal Cancer
2. To Correlate the expression of CD68 and CD163 with the histological grade and stage of the tumor

## Materials and Methods

After obtaining clearance from the institutional ethics committee, all Colorectal carcinoma specimens received in the Department of Pathology from R.L. Jalappa Hospital and Research Center attached to Sri Devaraj Urs Medical College, Tamaka, and Kolar from December 2016 to September 2018 and also the paraffin blocks taken from all cases of Colorectal cancer retrieved from Archives of Department of Pathology from the year January 2008 to November 2016 were included in the study. Complete data regarding the clinical details (Age, Sex, Histological grading) were collected. Hand E slides were reviewed for Histopathological types, grade and stage of the tumor. The exclusion criteria were metastatic tumor to Colo-rectal region, recurrent lesions and patient subjected for chemotherapy and Radiotherapy.

Sample size was estimated by using the proportion of CD163 marker positivity in Colo-rectal cancers in study done by Ivan Shabo et al. [7] and a total of 62 subjects with primary colorectal cancers were included in the study.

Immunohistochemical staining for CD68 and CD163 (Biocare mouse antibody) was performed on all cases of Colorectal Carcinoma using appropriate controls by peroxidase and anti peroxidase method. Tonsil tissue containing macrophages were taken as positive control.

*Selection of Hot spots and Grading of IHC*

The CD 68 and CD 163 immuno stained smears were examined under low magnification (10X) and was looked for areas with maximum expression of CD 68 and CD 163 by two observers and were called as "Hot spots". These hotspots were then viewed under higher magnification (40X) and CD 68 and CD 163 positive cells were counted and the mean was taken. Expression of macrophages antigen CD 68 and CD 163 were graded with the proportion of macrophages staining positive in the tumor stroma. Out of hundred cells counted and the grading is a follows [8,9].

GRADE+1: Less than 10% cells positive

GRADE+2: More than 10% & less than 50% cells positive

GRADE+3: More than 50% cells positive

The tumor size was divided two groups. I.e tumors with size less than 5 cms (< 5) and more than 5 cms (>5) according to the study done by Ohnishi K et al. [10] on Prognostic role of CD 169 positive macrophages in Colo-rectal cancers.

Lymph node ratio is the ratio of number of Lymph node with metastasis to the number of Lymph nodes harvested. In the study, LNR was divided into 4 groups according to the study done by Ren JQ et al. [11].

LNR 1 - ≤ 0.111

LNR 2 - 0.111 0 to ≤ 0.200

LNR 3 - 0.200 to ≤ 0.429

LNR 4 - > 0.429

*Statistical Analysis*

Data was entered into Microsoft excel data sheet and was analysed using SPSS 22 version software. Categorical data was represented in the form of Frequencies and proportions. Chi-square test was used as test of significance for qualitative data. p Value of <0.05 was considered as statistically significant.

## Results

In the study, 42 (67.7%) cases were in the age group <60 years, 20 (32.3% )were in the age group >60 years with a slight male preponderance (39 cases, 62.9%). The most common site of malignancy was Rectum in 27 (43.5%) cases, followed by ascending colon and sigmoid colon in 13 cases each (27%), transverse colon in 6 cases (9.7%) and descending colon in 3 (4.8%) cases. About 39 (62.9%) cases had tumor size <50 mms and 23 (37.1%) cases had tumor size >50 mms.

Majority of the cases were moderately differentiated adenocarcinomas (30 cases, 48.4%) followed by well differentiated carcinomas (20 cases, 32.3%) and were poorly differentiated carcinomas (12 cases, 19.4%). Most of the cases were in the T3 stage (32 cases, 51.6%) N0 stage (39 cases,

62.9%). Majority of the cases were in Stage III (23 cases, 37.1%).

Perineural invasion was seen in 2 cases (3.2%) and lymphovascular invasion was seen in 3 cases(4.8%).

On comparing different histolopathological prognostic factors with CD 68 expression, there was a statistitically significant association between the size of tumor, site of the tumor and lymphnode ratio (Table 1) whereas CD 163 expression showed significant association with T stage, N stage and Overall tumor stage and lymphnode ratio (Table 2).

The overall distribution of CD 68 and CD 163 is shown in Table 3 and Table 4 respectively.

**Table 1**: Association between clinicopathological parameters and CD 68 Expression

| Parameters | <10% of Cells positive | >10% to <50% of Cells Positive | >50% of Cells Positive | Total | p value |
|---|---|---|---|---|---|
| *Age (years)* | | | | | |
| <60 | 17 (40.5%) | 15 (35.7%) | 10 (23.8%) | 42 | 0.781 |
| >60 | 7 (35%) | 9 (45%) | 4 (20%) | 20 | |
| *Gender* | | | | | |
| Male | 13 (33.3%) | 18 (46.2%) | 8 (20.5%) | 39 | 0.288 |
| Female | 11 (47.8%) | 6 (26.1%) | 6 (26.1%) | 23 | |
| *Site* | | | | | |
| Ascending colon | 4 (30.8%) | 7 (53.8%) | 2 (15.4%) | 13 | |
| Transverse colon | 2 (33.3%) | 0 (0%) | 4 (66.7%) | 6 | 0.037 |
| Descending colon | 2 (66.7%) | 1 (33.3%) | 0(0%) | 3 | |
| Sigmoid colon | 2 (15.4%) | 6 (46.2%) | 5 (38.5%) | 13 | |
| Rectum | 14 (51.9%) | 10 (37.0%) | 3 (11.1%) | 27 | |
| *Tumor growth* | | | | | |
| Proliferative | 5 (62.5%) | 2 (25%) | 1 (12.5%) | 8 | |
| Ulceroproliferative | 11 (32.4%) | 16 (47.1%) | 7 (20.6%) | 34 | 0.429 |
| Ulcerative/Infiltrative | 8 (40%) | 6 (30%) | 6 (30%) | 20 | |
| *Tumor size* | | | | | |
| <50 mm | 16 (41%) | 11 (28.2%) | 12 (30.8%) | 39 | 0.044 |
| >50 mm | 8 (34.8%) | 13 (56.5%) | 2 (8.7%) | 23 | |
| *Grading* | | | | | |
| Well | 4 (20%) | 7 (35%) | 9 (45%) | 20 | |
| Moderate | 15 (50%) | 11 (36.7%) | 4 (13.3%) | 30 | 0.041 |
| Poor | 5 (41.7%) | 6 (50%) | 1 (8.3%) | 12 | |
| *T stage* | | | | | |
| T1 | 1 (25%) | 1 (25%) | 2 (50%) | 4 | |
| T2 | 5 (31.2%) | 6 (37.5%) | 5 (31.2%) | 16 | 0.683 |
| T3 | 14 (43.8%) | 12 (37.5%) | 6 (18.8%) | 32 | |
| T4 | 4 (40%) | 5 (50%) | 1 (10%) | 10 | |
| *N stage* | | | | | |
| N0 | 13 (33.3%) | 13 (33.3%) | 13 (33.3%) | 39 | |
| N1 | 10 (47.6%) | 10 (47.6%) | 1 (4.8%) | 21 | 0.137 |
| N2 | 1 (50%) | 1 (50%) | 0 (0%) | 2 | |
| *Tumor stage* | | | | | |
| I | 5 (29.4%) | 5 (29.4%) | 7 (41.2%) | 17 | |
| II | 8 (36.4%) | 8 (36.4%) | 6 (27.3%) | 22 | 0.091 |
| III | 11 (47.8%) | 11 (47.8%) | 1 (4.3%) | 23 | |
| *Lymph node ratio* | | | | | |
| <0.111 | 12 (30%) | 15 (37.5%) | 13 (32.5%) | 40 | |
| 0.111 to 0.200 | 2 (66.7%) | 1 (33.3%) | 0 (0%) | 3 | 0.024 |
| 0.200 to 0.429 | 5 (100%) | 0 (0%) | 0 (0%) | 5 | |
| >0.429 | 5 (35.7%) | 8 (57.1%) | 1 (7.1%) | 14 | |

**Table 2:** Association between clinicopathological parameters and CD 163 Expression

| Parameters | <10% of Cells positive | >10% to <50% of Cells Positive | >50% of Cells Positive | Total | p value |
|---|---|---|---|---|---|
| *Age(years)* | | | | | |
| <60 | 4 (9.5%) | 26 (61.9%) | 12 (28.6%) | 42 | 0.643 |
| >60 | 2 (10%) | 10 (50%) | 8 (40%) | 20 | |
| *Gender* | | | | | |
| Male | 3 (7.7%) | 23 (59%) | 13 (33.3%) | 39 | 0.786 |
| Female | 3 (13%) | 13 (56.5%) | 7 (30.4%) | 23 | |

| | | | | | |
|---|---|---|---|---|---|
| *Site* | | | | | |
| Ascending colon | 1 (7.7%) | 4 (30.8%) | 8 (61.5%) | 13 | |
| Transverse colon | 1 (16.7%) | 5 (83.3%) | 0 (0%) | 6 | 0.343 |
| Descending colon | 0 (0%) | 2 (66.7%) | 1 (33.3%) | 3 | |
| Sigmoid colon | 1 (7.7%) | 8 (61.5%) | 4 (30.8%) | 13 | |
| Rectum | 3 (11.1%) | 17 (63%) | 7 (25.9%0 | 27 | |
| *Tumor growth* | | | | | |
| Proliferative | 1 (12.5%) | 3 (37.5%) | 4 (50%) | 8 | |
| Ulceroproliferative | 4 (11.8%) | 20 (58.8%) | 10 (29.4%) | 34 | 0.677 |
| Ulcerative/Infiltrative | 1 (5%) | 13 (65%) | 6 (30%) | 20 | |
| *Tumor size* | | | | | |
| <50 mm | 5 (12.8%) | 24 (61.5%) | 10 (25.6%) | 39 | 0.257 |
| >50 mm | 1 (4.3%) | 12 (52.2%) | 10 (43.5%) | 23 | |
| *Grading* | | | | | |
| Well | 4 (20%) | 13 (65%) | 3 (15%) | 20 | |
| Moderate | 2 (6.7%) | 18 (60%) | 10 (33.3%) | 30 | 0.067 |
| Poor | 0 (0%) | 5 (41.7%) | 7 (58.3%) | 12 | |
| *T stage* | | | | | |
| T1 | 3 (75%) | 1 (25%) | 0 (0%) | 4 | |
| T2 | 2 (12.5%) | 9 (56.2%) | 5 (31.2%) | 16 | 0.0001 |
| T3 | 0 (0%) | 21 (65.6%) | 11 (34.4%) | 32 | |
| T4 | 1 (10%) | 5 (50%) | 4 (40%) | 10 | |
| *N stage* | | | | | |
| N0 | 6 (15.4%) | 27 (69.2%) | 6 (15.4%) | 39 | |
| N1 | 0 (0%) | 8 (38.1%) | 13 (61.9%) | 21 | 0.004 |
| N2 | 0 (0%) | 1 (50%) | 1 (50%) | 2 | |
| *Tumor stage* | | | | | |
| I | 5 (29.4%) | 9 (52.9%) | 3 (17.6%) | 17 | |
| II | 1 (4.5%) | 18 (81.8%) | 3 (13.6%) | 22 | <0.001 |
| III | 0 (0%) | 9 (39.1%) | 14 (60.9%) | 23 | |
| *Lymph node ratio* | | | | | |
| <0.111 | 6 (15%) | 29 (72.5%) | 5 (12.5%) | 5 | |
| 0.111 to 0.200 | 0 (0%) | 2 (66.7%) | 1 (33.3%) | 1 | 0.001 |
| 0.200 to 0.429 | 0 (0%) | 2 (40%) | 3 (60%) | 3 | |
| >0.429 | 6 (9.7%) | 3 (21.4%) | 11 (78.6%) | 11 | |

**Table 3:** CD 68 distribution among subjects

| | | Count | Percent (%) |
|---|---|---|---|
| CD 68 | <10% of Cells | 24 | 38.7% |
| | >10% to <50% of Cells Positive | 24 | 38.7% |
| | >50% of Cells Positive | 14 | 22.6% |

**Table 4:** CD 163 distribution among subjects

| | | Count | Percent (%) |
|---|---|---|---|
| CD 163 | <10% of Cells | 6 | 9.7% |
| | >10% to <50% of Cells Positive | 36 | 58.1% |
| | >50% of Cells Positive | 20 | 32.3% |

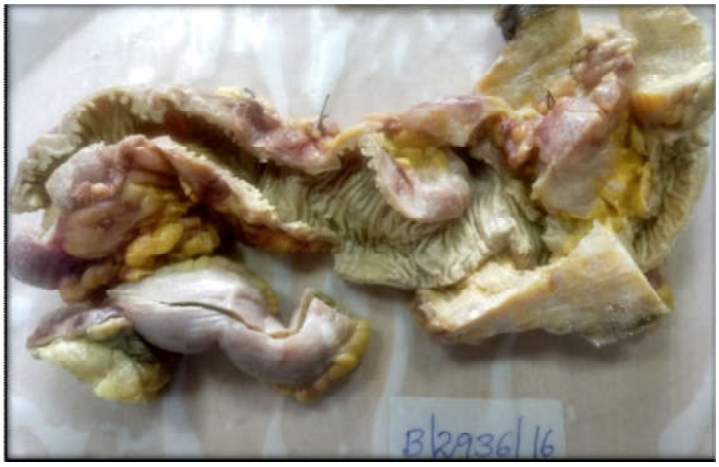


**Fig. 1:** Cut section showing Grey white tumor measuring 4.5x3x2 cms. Serosa was involved by the tumor in this case

Immunohistochemical staining and grading of the tumor associated macrophages CD 68 and CD 163 in the tissue sections are shown in Figures 2,3,4 and Figures 5,6,7 respectively.

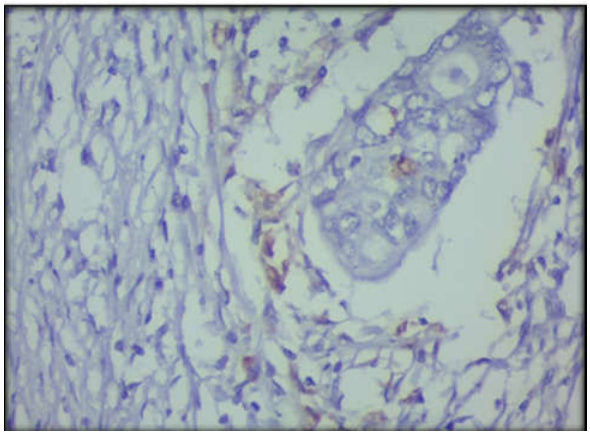

**Fig. 2:** IHC staining with CD 68 showing Less than 10% cells positive (x 40X)

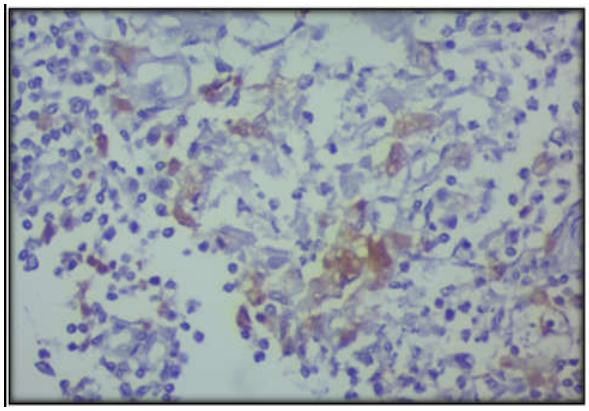

**Fig. 3:** IHC staining with CD 68 showing More than 10% & less than 50% cells positive (x 40X)

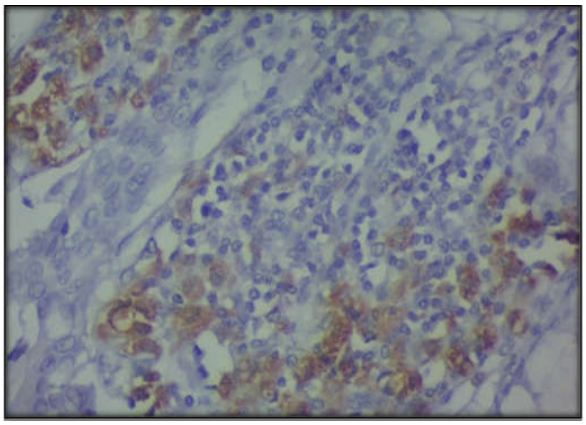

**Fig. 4:** IHC staining with CD 68 showing More than 50% cells positive (x 40X)

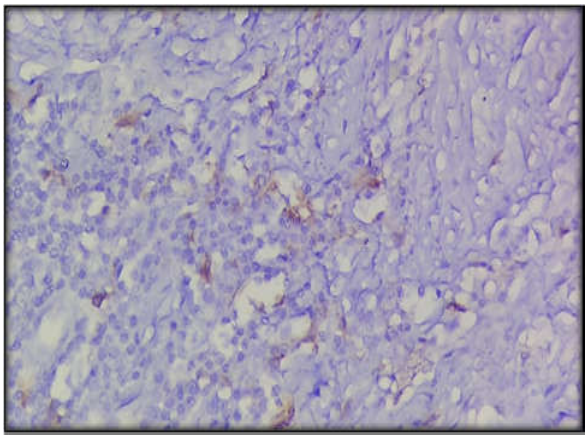

**Fig. 5:** IHC staining with CD 163 showing Less than 10% cells positive (x 40X)

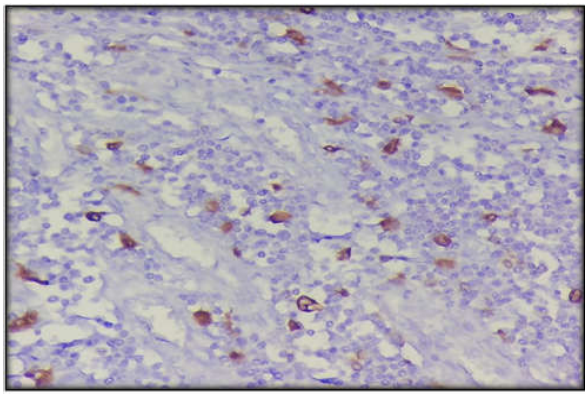

**Fig. 6:** IHC staining with CD 163 showing More than 10% & less than 50% cells positive (x 40X)

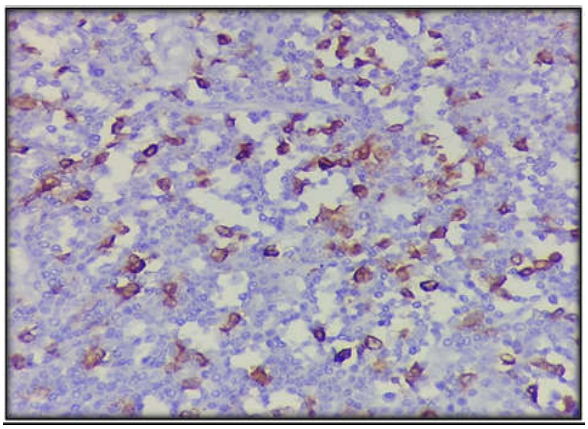

**Fig. 7:** IHC with CD 163 showing More than 50% cells positive (x 40X)

## Discussion

In the present study, the majority of the cases (42 cases, 67.7%) were less than 60 years of age and (20 cases, 32.3%) cases were over the age of 60 years. Other studies done by Gulubova M et al. [12] and Forssell et al. [13] observed that majority of the cases, i.e. 137 (67.5%) and 137 (67.5%) were above the age of 60 years respectively and maximum number of cases of colo-rectal cancers were in male population similar to the observations made by Gulubova M et al. [12] and Majek O et al. [14] showing that incidence of colo-rectal cancers are more common in men than in women.

The maximum number of cases were rectal carcinomas which was similar to the study done by Pirzada MT et al. [15] and Patra T et al. [16]. Tumor size is a well proven prognostic factor in Colo-rectal cancers as smaller tumours are associated with better prognosis. In the study, as there was a significant correlation between CD 68 and size of the tumor, it supports the hypothesis that CD 68 positive M1 macrophages in the tumor stroma are a good prognostic markers and is associated with lesser T stage and better survival.

The moderately differentiated adenocarcinomas and well differentiated adenocarcinomas which are the majority in the present study group are associated with better prognosis compared to poorly differentiated adenocarcinomas and M1 macrophages posses antigen presenting molecules, which is co-stimulatory receptor for lymphocytes and many pro inflammatory cytokines on their surface. This immune mechanism is considered as body's response against the tumor cells and helps in tumor differentiation and further supports the hypothesis that CD 68 expression is a good prognostic marker in colo-rectal cancers.

The maximum number of cases were in the T3 stage similar to the studies by Kim SM et al. [17] and Ladeira KM [18]. The expression of CD 163 by M2 macrophages is considered to be a bad prognostic factor as they are hypothesized to promote tumor growth by releasing growth factors and promoting angiogenesis [12]. The significant association between CD 163 expression and higher T stage supports this hypothesis.

According to the observations made by Liu Q et al. [19] Soylu L et al. [20] and Moug SJ et al. [21], maximum number of cases were in N0 category with 66.98%, 58.9% and 61.5% respectively and the least number of cases were seen on N2 category with 10.01%, 17.4% and 13.3% respectively. Similar trend was seen in the present study with maximum number of cases in N0 category (62.9%) and least number of cases in N2 category (3.22%).

There was a statistically significant correlation between N stage and CD 163 expression whereas CD 68 did not show any correlation.

Shabo I et al. [7] studied the expression of CD 163 in colo-rectal cancers and concluded that its expression is a bad prognostic factor, but the N stage was not included in his study. In the present study, majority of cases were in N0 stage and the observation in the N stage showed that CD 163 is a good prognostic factor which is in contrast to the study done by Shabo I et al. [7]. This could be due to the more number of cases in N0 category and a larger sample size could provide a better understanding in this regard.

Lymph node ratio is one of the important, newer factors in determining the prognosis of Colo rectal cancers. It has also been studied in tumors of stomach, pancreas, bladder and breast [24]. Lymph node ratio is the proportion of the number of Lymph node with tumor deposits to the number of Lymph nodes examined [22]. It has also been studied that increased harvesting of Lymph nodes during surgery in colorectal cancers is associated with better outcomes [23]. Different cut-off values have been studied by various authors for determining Lymph node ratio. In general, higher Lymph node ratio is associated with poor 3 year relapse free survival, higher tumor stage, perineural invasion and overall survival [25].

## Conclusion

CD 68 expression was associated with better prognostic factors such as smaller size of tumor, lesser grade and lesser Lymph node ratio(LNR).

CD 163 expression was associated with poorer prognostic factors such as higher T stage, Higher N stage, and higher values of Lymph node ratio (LNR).

Hence, CD 68 and CD 163 can be used as novel biomarkers in assessing the prognosis in Colo-rectal cancer patients. Further studies may help in improving the therapeutic modalities by targeted therapies.

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