Original Article

DOI: 10.7860/JCDR/2022/49491.15900

Pathology Section

A Laboratory-based Exploratory Study of Tumour-associated Macrophages and their Subpopulation M1 and M2 by Immunohistochemistry in Primary Breast Carcinoma

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ABSTRACT

Introduction: Breast carcinoma is one of the common causes of mortality in women. The macrophage is the primary immune cell present in the tumour microenvironment. They are, therefore, also called Tumour-Associated Macrophages (TAMs). CD68 has a proinflammatory and anti-tumour response. CD (cluster of differentiation)163 has an anti-inflammatory response. The collection of TAMs correlates with adverse clinical outcomes. Tumour microenvironment targeting helps in reducing tumour burden and improving prognosis.

Aim: To study the density of expression of TAMs in primary breast carcinoma and study the association of TAMs with stage and grade of primary breast carcinoma.

Materials and Methods: This was a laboratory-based exploratory study conducted in the Kolar district of Karnataka, from December 2018 to May 2020 and data of 55 primary breast carcinoma cases were included. Cases with metastatic tumours from other sites, recurrent lesions, and patients subjected to chemotherapy and radiotherapy were excluded. Haematoxylin and Eosin (H&E) slides were reviewed. Immunostaining for CD68 and CD163 was performed. The cases were distributed into low and high groups based on cut-off points according to the median. Statistical Package for Social Sciences (SPSS) version 22.0 was used for statistical analysis and a p-value of <0.05 was considered statistically significant.

Results: Out of the total 55 cases, the maximum number of cases were between 50-59 years and maximum patients lump sized between 2-5 cm. The study demonstrated that the density of CD68 macrophages in the peritumoural area increased as the pathological stage increased (p-value 0.037) and the density of CD68 macrophages in the intratumoural area decreased as the tumour grade increased (p-value 0.023). Cancer tissue showed higher CD163 TAMs density than those in normal tissues, but the association with pathological stage, grade, and lymph node metastasis was not significant (p-value >0.05).

Conclusion: The CD68 targeted therapy can be used to treat breast carcinoma as it inhibits the tumourigenic factors at the interface between tumour and stroma.

Keywords: Immunotherapy, Intratumoural, Peritumoural, Targeted therapy, Tumour microenvironment

INTRODUCTION

One of several major causes of cancer-related deaths in women is breast carcinoma [1]. Worldwide incidence of breast malignancy varies from 19.3-89.7/100,000 populations [2]. The tumour microenvironment comprises both malignant and non malignant populations of cells. Non malignant populations include leukocyte infiltrate, proliferating blood vessels, and stromal cells. Stromal cells of the tumour microenvironment are cancer-associated fibroblasts, adipocytes, pericytes, lymphatic, and vascular endothelial cells. Leukocytes comprising the tumour microenvironment are macrophages, T-lymphocytes, B-lymphocytes, natural killer cells. The macrophage is the primary immune cell present in the tumour microenvironment. They are, therefore, also called TAMs. TAMs classify as M1 and M2 depending on their activation mechanism, namely, classical and alternative activation [3].

The CD68 is a pan macrophage marker and plays a crucial role in proinflammatory and anti-tumour response by activating type 1 T cell response, and by producing free radicals that can damage DNA, it has tumouricidal activity [4]. CD163 is highly specific for M2 macrophages. It has an anti-inflammatory response and leads to hypoxia-induced angiogenesis upregulated as the carcinoma progresses to promote metastasis and proliferation [5]. TAMs have been studied, thoroughly in hepatocellular carcinoma, lung carcinoma, and gastric carcinoma [6-8].

Many studies have postulated that the collection of intratumoural TAMs correlates with adverse clinical outcomes [9-11]. But still, the prognostic importance of localisation and densities of both the TAMs is not well evaluated. Previous studies concluded that TAMs are associated with Oestrogen Receptor (ER), Progesterone Receptor (PR), Her2neu status, stage, grade, and lymph node status. So in breast cancer, TAMs in different locations and densities may have different prognostic values [9-11].

Therapies available for breast cancer targets tumour cells only. Many studies concluded that targeting the tumour microenvironment helps in reducing tumour burden and improves prognosis [3,12,13]. In breast cancer, TAMs may increase invasion, modulate tumourigenesis by stimulating tumour angiogenesis through Vascular Endothelial Growth Factor (VEGF), degrade the extracellular matrix by generating proteases, and lead to repression of the function of CD8+ T cells, which inhibits the tumour growth resulting in poor prognosis [14].

Results of studies done on breast carcinoma using TAMS are quite variable. They used various markers to assess macrophages. Some of them used only CD68, while others combined both CD68 and CD163 [15,16]. Some studies were done on both the tumour stroma and the tumour proper, while others only counted the total TAMs within the tumour proper [15,16].

The presence of total tumour-infiltrating lymphocytes and specific CD8+ cytotoxic T cells associated with a successful response to chemotherapy and a significant reduction in the relative risk of death. However, the ability of TAMs to suppress T-cell responses at the interface between tumour and stroma represents a significant obstacle to successful immunotherapy. Macrophages have emerged as an independent co-factor in breast cancer progression and represent an attractive target for breast cancer therapy. TAMs can be targeted for therapy as it inhibits the tumourigenic factors such as Epithelial Growth Factor (EGF) mediated metastasis and Cancer Stem Cells (CSC) support which provides a novel mechanism to treat breast cancer.

This study analysed the density of expression of CD68 TAMs and CD163 TAMs with intratumoural and peritumoural distribution in primary breast carcinoma and study the association of CD68 TAMs and CD163 TAMs with stage and grade of primary breast carcinoma.

MATERIALS AND METHODS

This was a laboratory-based exploratory study done on primary breast carcinoma specimens received from December 2018 to May 2020, in Sri Devaraj Urs Medical College, Kolar district of Karnataka, India. Also, the paraffin blocks were retrieved from the archives of the department from the year January 2016 to November 2018 were included in the study. Approval was taken from the Institutional Ethics Committee (DMC/KLR/IEC/704/2020-21).

Sample size calculation: It was done by using the proportion of CD163 marker positivity in primary breast carcinomas, which was 9%[12]. Using the confidence level of 95%, a sample size of 55 subjects with primary breast cancer was selected for the study.

Inclusion criteria: All primary breast carcinoma specimens, confirmed using histopathological examination, were included in the study.

Exclusion criteria: Cases with metastatic tumours from other sites, recurrent lesions, patients subjected to chemotherapy and radiotherapy were excluded from the study.

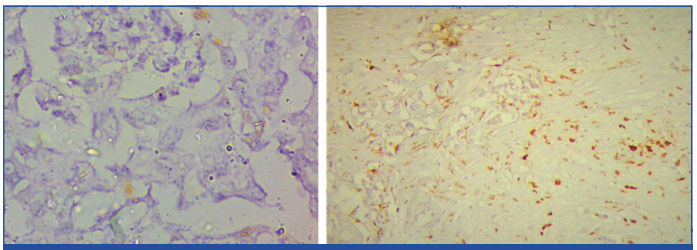
Data regarding the clinical details (age, sex, histological grading) was collected from the Medical Records Department (MRD). Haematoxylin and Eosin (H&E) slides were reviewed for histopathological types and grading and staging of the tumour. Immunostaining for CD68 and CD163 was performed on all breast carcinoma cases using appropriate positive and negative controls by peroxidase and anti-peroxidase method. CD68 was used in a prediluted form obtained from a mouse with a KP1 clone, which shows cytoplasmic and membranous staining. For CD163, EP324 clone was used in the prediluted form obtained from rabbit, which also shows membranous and cytoplasmic staining. Tonsil was taken as a positive control for CD68, and the spleen was used as a positive control for CD163.

According to the definition, intratumoural macrophages are intraepithelial tumour-infiltrating macrophages. Peritumoural macrophages are macrophages in the stromal tissue surrounding the tumour nest. A hotspot is an area with the highest level of TAMs. CD68 and CD163 were identified by their macrophage morphology and cytoplasmic staining with strong cell membrane positivity. For screening, low magnification (10x) was used, and ten hotspot areas were selected with the maximum density of cells showing positivity. At high magnification (40x), the total number of positively stained cells were counted in both peritumoural and intratumoural areas separately without access to any clinical information [17].

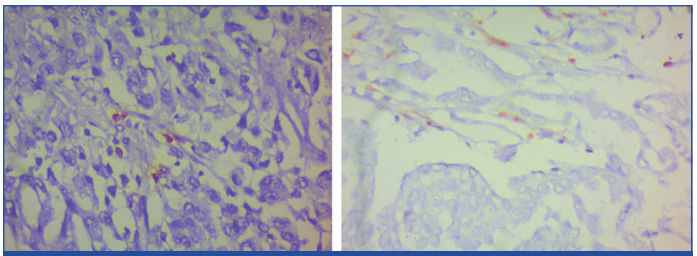
For statistical analysis, positive cells were categorised into two groups of low and high, based on cut-off points according to the median [Table/Fig-1]. The cases were distributed into low and high groups using cut-off values of 3/high power field (hpf) for CD68 TAMs intratumoural distribution [Table/Fig-2], 10/hpf for CD68 TAMs peritumoural distribution [Table/Fig-3], and 3/hpf for CD163 TAMs for intratumoural distribution [Table/Fig-4], and 8/hpf for CD163 TAMs for peritumoural distribution [Table/Fig-5].

Variable	Mean	Median	Range
CD68 TAMs			
Intratumoural	3.05	3.00	2-5
Peritumoural	10.47	10.00	2-15
CD163 TAMs			
Intratumoural	3.69	3.00	3-8
Peritumoural	10.8	8.00	3-20
The ratio of CD163 and CD68			
Intratumoural	1.2	1	1-1.11
Peritumoural	1.03	0.8	1-1.25

[Table/Fig-1]: Distribution of CD68 and CD163 macrophages.
TAMs: Tumour Associated Macrophages; CD: Cluster of differentiation



[Table/Fig-2]: Immunohistochemical (IHC) staining with CD68 showing low {<3/high power field (hpf)} intratumoural distribution (40X). **[Table/Fig-3]:** IHC staining with CD68 showing high (>10/hpf) peritumoural distribution (10X). (Images from left to right)



[Table/Fig-4]: IHC staining showing low (<3/hpf) intratumoural distribution of CD163 (40X). **[Table/Fig-5]:** IHC staining with CD163 showing high (>8/hpf) peritumoural distribution (40X). (Images from left to right)

STATISTICAL ANALYSIS

Microsoft Excel datasheet was used for data collection and entry and then analysed using the software's SPSS 22.0 version. Categorical data were represented in frequencies and proportions, and Chi-square was calculated as a test of significance. Continuous data are represented as a mean and standard deviation, and an independent t-test was used as a test of significance to identify the mean difference. A p-value of <0.05 is considered statistically significant.

RESULTS

Out of the total 55 cases, the maximum number of cases were between 50-59 years, Most of the patients lump sized between 2-5 cm, i.e., in 30 cases. Infiltrating ductal carcinoma was the most prevalent form and was seen in 51 participants [Table/Fig-6]. In 31 patients, metastasis was found in lymph nodes.

The density CD68 and CD163 expression were determined for all 55 cases. CD68 and CD163 macrophages were detected in intratumoural and peritumoural areas [Table/Fig-7,8]. The study demonstrated that the density of CD68 macrophages in the peritumoural area increased as the pathological stage increased (p=0.037). In the study, the density of expression of CD68

Parameters		Frequency (n)	Percentage (%)
Age	≤50 years	21	38.1
	>50 years	34	61.9
Tumour size	≤5 cm	34	61.8
	>5 cm	21	38.2
Histopathological diagnosis	Infiltrating ductal carcinoma	51	92.72
	Mucinous carcinoma+Infiltrating ductal carcinoma	1	1.82
	Papillary carcinoma	1	1.82
	Squamous cell carcinoma	1	1.82
	Mucinous carcinoma	1	1.82
Stage	I	3	5.4
	II	26	47.2
	III	26	47.2
Grade	Grade I	32	58.2
	Grade II	15	27.3
	Grade III	8	14.5
T category	T1	9	16.3
	T2	30	54.6
	T3	11	20
	T4	5	9.1
N category	N0	22	40
	N1	15	27.2
	N2	11	20
	N3	5	9
	NX	2	3.8

[Table/Fig-6]: Distribution of cases in the study group.
T: Tumour; N: Necrosis

Histopathological feature	Intratumoural CD68 macrophages			Peritumoural CD68 macrophages		
	Low (≤3/hpf)	High (>3/hpf)	p-value	Low (≤10/hpf)	High (>10/hpf)	p-value
Staging						
Stage I	2 (3.6%)	1 (1.8%)	0.402	1 (1.8%)	2 (3.6%)	**0.037***
Stage II	11 (20%)	15 (27.2%)		5 (9.1%)	21 (38.1%)	
Stage III	8 (14.5%)	18 (32.7%)		0	26 (47.2%)	
Grading						
Grade I	16 (29.1%)	16 (29.1%)	**0.023***	4 (7.2%)	28 (51%)	0.826
Grade II	4 (7.2%)	11 (20%)		1 (1.8%)	14 (25.4%)	
Grade III	1 (1.8%)	7 (12.7%)		1 (1.8%)	7 (12.7%)	
Tumour size						
Tumour size ≤5 cm	17 (31%)	22 (40%)	0.197	5 (9.1%)	34 (61.8%)	0.478
Tumour size >5 cm	4 (7.2%)	12 (21.8%)		1 (1.8%)	15 (27.2%)	
Node metastasis						
Node metastasis-absent	10 (18%)	14 (25.4%)	0.640	5 (9.1%)	19 (34.5%)	**0.038***
Node metastasis-present	11 (20%)	20 (36.3%)		1 (1.8%)	30 (54.5%)	

[Table/Fig-7]: Comparison of the density of expression of intratumoural and peritumoural CD68 macrophages. Chi-square test and independent t-test were used as a test of significance.

macrophages in the intratumoural area decreased as the grade increased (p=0.023).

A high density of CD68 macrophage expression was seen peritumoural if lymph node metastasis was present (p=0.038). The relation of the density of expression of CD163 macrophages with the pathological stage, tumour grade, tumour size, and lymph node metastasis is not statistically significant.

Histopathological feature	Intratumoural CD163 macrophages			Peritumoural CD163 macrophages		
	Low (≤3/hpf)	High (>3/hpf)	p-value	Low (≤8/hpf)	High (>8/hpf)	p-value
Staging						
Stage I	1 (1.8%)	2 (3.6%)	0.338	0	3 (5.4%)	0.883
Stage II	19 (34.5%)	7 (12.7%)		2 (3.6%)	24 (43.6%)	
Stage III	19 (34.5%)	7 (12.7%)		2 (3.6%)	24 (43.6%)	
Grading						
Grade I	22 (40%)	10 (18.1%)	0.914	2 (3.6%)	30 (54.5%)	0.474
Grade II	11 (20%)	4 (7.2%)		2 (3.6%)	13 (23.6%)	
Grade III	6 (11%)	2 (3.6%)		0	8 (14.5%)	
Tumour size						
Tumour size ≤5 cm	27 (49.1%)	12 (21.8%)	0.669	4 (7.2%)	35 (63.6%)	0.183
Tumour size >5 cm	12 (21.8%)	4 (7.2%)		0	16 (29.1%)	
Node metastasis						
Node metastasis-absent	17 (31%)	7 (12.7%)	0.991	2 (3.6%)	22 (40%)	0.790
Node metastasis-present	22 (40%)	9 (16.3%)		2 (3.6%)	29 (52.7%)	

[Table/Fig-8]: Comparison of the density of expression of intratumoural and peritumoural CD163 macrophages. Chi-square test and independent t-test were used as a test of significance.

DISCUSSION

The TAMs are a part of the tumour microenvironment and can elicit tumour cell transformation, induce destructive tumour reactions, and positively or negatively affect tumour progression depending on the subset, i.e., CD68 or CD163. Areas of hypoxia occur in tumours more than 2 mm, and factors like Monocyte Chemotactic Protein (MCP-1) and granulocyte-macrophage-colony stimulating factors are produced to recruit monocytes within the tumour microenvironment [9]. Studies are currently concentrating on targeting the tumour microenvironment to improve prognosis and decrease the resistance against the treatment [9].

The CD68 has M1 and M2 subtypes of macrophages. M1 is tumouricidal, and M2 has protumour activity, but a study [10] shows that CD68 macrophages increase vascularity and lymph node metastasis. and CD163, a specific biomarker for M2 are associated with poor clinicopathological characters. TAMs were observed in all the cases in the present study, and more TAMs infiltration was seen in intratumoural and peritumoural areas.

The study demonstrated that the density of CD68 macrophages in the peritumoural area increased as the pathological stage increased. No association was found in a study done by Jamiyan T et al., between high density of CD68 TAMs infiltration with any clinicopathological parameters [18]; this may be due to CD68 expressed by both M1 which is tumouricidal and M2 which has tumour-promoting activity.

Jeong H et al., and Gwak JM et al., observed that the high density of TAMs was related to high tumour grade in both locations (intratumour and peritumour) [16,19]. Similarly, Sousa S et al., and Ni C et al., showed high CD68 TAMs with a high histological grade but did not specify the location [5,17]. Ch'ng ES et al., and Yang M al., observed that increased TAMs in the peritumoural area and not within the tumour were associated with high tumour grades [20,21]. On the contrary, Yuan ZY et al., did

not find any correlation between CD68 TAMs and tumour grade [22]. Medrek C et al., found that the high density of CD68 TAMs in both intratumoural and peritumoural locations was related to poor prognostic factors [11].

In a study done by Jeong H et al., and Ahmed I et al., the higher density of TAMs showed statistical association with more tumour size, a higher grade, which can be explained by the protumoural M2 phenotype of TAM [16,23]. TAMs have been shown to enhance the growth of breast cancer cells. These genes are responsible for enhanced tumourigenicity and resistance to chemotherapy in breast cancer cells. They also showed that higher nodal metastasis is statistically associated with a higher density of TAMs. The epithelial-mesenchymal transition of tumour cells is necessary to initiate the invasion promoted by TAMs release by downregulating β-catenin and E-cadherins. In the present study, the density of expression of CD68 macrophages in the intratumoural area decreases as the grade increases and is statistically significant (p=0.023). More density of expression of CD68 macrophages is seen in both intratumour and peritumour areas if the tumour size is < 5 cm compared to the tumour size of > 5 cm, however statistically, this is not significant.

Jamiyan T et al., found no significant difference in CD163 TAMs density among TNM stages in lung, breast, or thyroid cancer [18]. However, cancer tissue showed higher CD163 TAMs density than those in normal tissues, similar to the present study. Medrek C et al., concluded that dense infiltration of CD163 TAMs in the peritumoural area was associated with ER and PR negativity, grade, tumour size, but there was no such association in the intratumour area [11]. Several studies have reported that TAMs densities are associated with a good prognosis; such contradictory results might be due to differences in grade, the number of cases, or tumour size.

Studies were done on relapse-free survival and overall survival. Sousa S et al., revealed that CD163 cells in primary breast tumours are associated with poor prognosis, correlated with ER negativity, poor differentiation (grade 3), and ductal type [5]. Yang M et al., also found that increased CD163 TAMs in the peritumoural area were correlated with poor prognostic factors, but they also did not find any statistical difference in the intratumoural area [21]. Jamiyan T et al., included only triple-negative cancer and observed that CD163 does not affect prognosis statistically, but more TAMs density was found, especially CD163 [18]. Shourouk E and El-Guindy DM reported a high density of CD163 TAMs was associated with larger tumour size, vascular invasion, nodal metastasis, and stage in both intratumoural and peritumoural areas [24].

Ni C et al., also concluded that CD163 TAMs are significantly associated with poor prognosis and advanced histologic grades in early breast cancer [17]. However, they included only basal-like breast cancer cells because they are more likely to express a broader range of receptors for macrophage type of cytokines, which could recruit macrophages into the microenvironment and promote monocyte differentiation into M2-like macrophages.

Limitation(s)

Firstly, the sample size is less. Secondly, as immunohistochemistry can only measure one or two markers per sample, it may not fully reflect the complex factors involved.

CONCLUSION(S)

This study conclusively demonstrates the density of expression of CD68. TAMs progressively increased in concordance with the pathological stage of breast cancers. Simultaneously, the density of CD68 TAMs in the intratumoural area exhibited progressive reduction as the grade of breast cancer increased. Also, breast carcinomas with lymph node metastasis exhibited a high density of CD68 TAMs in the peritumoural area. However, more such studies are needed along with molecular subtyping to study the role of CD68 and CD163 TAMs expression in primary breast carcinoma.

More advanced studies using different technologies are expected, and further studies are required to determine the cross-interaction between diverse TAMs and the tumour microenvironment.

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PLAGIARISM CHECKING METHODS: [Jain H et al.]
- Plagiarism X-checker: Apr 19, 2021
- Manual Googling: May 20, 2021
- iThenticate Software: Nov 30, 2021 (12%)

ETYMOLOGY: Author Origin

AUTHOR DECLARATION:
- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? NA
- For any images presented appropriate consent has been obtained from the subjects. NA

Date of Submission: **Mar 31, 2021**
Date of Peer Review: **May 19, 2021**
Date of Acceptance: **Dec 02, 2021**
Date of Publishing: **Jan 01, 2022**