УДК 618.19-006.6-008.93:577.122
DOI: https://doi.org/10.17816/PAVLOVJ108478

Экспрессия белка Forkhead box P3 (FOXP3) инфильтрирующих опухоль лимфоцитов при инвазивном раке молочной железы: связь с гистопатологическими параметрами и общей выживаемостью

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АННОТАЦИЯ

Обоснование. Белок *Forkhead box P3* (FOXP3) экспрессируется как в опухолевых клетках, так и в инфильтрирующих опухоль лимфоцитах (TILs) и, как сообщается, ассоциирован с различиями в клинических исходах. Актуальные литературные данные свидетельствуют о том, что FOXP3 позитивные (FOXP3+) Т-регуляторные клетки (Tregs) влияют на противоопухолевый иммунитет при солидных опухолях.

Цель. Определить экспрессию FOXP3+ в Tregs, связанную с различными прогностическими факторами, при карциноме молочной железы (КМЖ) в популяции Центральной Индии, а также взаимосвязь FOXP3+ Tregs с выживаемостью при инвазивной протоковой КМЖ с различными гистопатологическими проявлениями.

Материалы и методы. Это ретроспективное и проспективное обсервационное исследование, в котором FOXP3+ Tregs были подсчитаны в околоопухолевой области методом иммуногистохимии в 47 последовательных случаях КМЖ, прооперированных и подтвержденных. Пациенты находились под наблюдением в течение 48–69 месяцев на предмет прогрессирования заболевания.

Результаты. В исследуемой области преобладают (n = 30) опухоли высокой степени злокачественности независимо от стадии клинической картины. Пациенты, которые могли придерживаться своего плана лечения, оставались без неблагоприятных исходов до конца периода наблюдения, длившегося 69 месяцев (p = 0,001). Ни один молекулярный подтип в нашем исследовании не выявил специфической склонности к высокому количеству Tregs в околопухолевой области. Никакие другие клинические или патологические параметры существенно не коррелировали с количеством FOXP3+ Tregs, в том числе общая и безрецидивная выживаемость.

Заключение. Исследование продемонстрировало, что люминальная КМЖ, негативная по рецепторам эпидермального фактора роста человека 2, и КМП, положительная по рецепторам эпидермального фактора роста человека 2, демонстрируют сравнительно высокое количество FOXP3+ Tregs. При этом не выявлено ассоциации со степенью опухоли, стадией TNM, важными иммунными маркерами, общей и безрецидивной выживаемостью.

Ключевые слова: *FOXP3; инфильтрирующие опухоль лимфоциты; карцинома молочной железы*

Для цитирования:
Hagone S., Bharat P.U., Gupta A., Nitin G. Экспрессия белка Forkhead box P3 (FOXP3) инфильтрирующих опухоль лимфоцитов при инвазивном раке молочной железы: связь с гистопатологическими параметрами и общей выживаемостью // Российский медико-биологический вестник имени академика И. П. Павлова. 2023. Т. 31, № 2. С. 265–272. DOI: https://doi.org/10.17816/PAVLOVJ108478

Рукопись получена: 02.06.2022 **Рукопись одобрена:** 16.09.2022 **Опубликована:** 30.06.2023

ЭКО•ВЕКТОР

DOI: https://doi.org/10.17816/PAVLOVJ108478

Expression of FOXP3 of Tumor-Infiltrating Lymphocytes in Invasive Breast Cancer: Its Relationship to Histopathological Parameters and Overall Survival

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ABSTRACT

BACKGROUND: Forkhead box P3 protein (FOXP3) is expressed in both tumor cells and tumor-infiltrating lymphocytes (TILs) and is reported to be associated with differences in clinical outcomes. Recent literature shows that FOXP3 positive (FOXP3+) T regulator cells (Tregs) influence anti-tumor immunity in solid tumors.

AIM: To explore FOXP3+ Tregs expression related to various prognostic factors in breast carcinoma (BC) in the central Indian population. Our study is also helpful in correlating the role of FOXP3+ Tregs in the survival of invasive ductal BC with different histopathological presentations.

MATERIALS AND METHODS: This is a retrospective and prospective observational study in which FOXP3+ Tregs was counted in the peritumoral area by immunohistochemistry in 47 consecutive cases of BC operated on and diagnosed already. The patients were followed for 48 to 69 months for disease progression.

RESULTS: High-grade tumors are prevalent (n = 30) in the study area irrespective of the stage of clinical presentation. Patients who could adhere to their treatment plan remained free of adverse outcomes until the end of our follow-up period of 69 months (p = 0.001). No molecular subtype in our study showed specific predilection towards a high Tregs count in the peri-tumoral area. No other clinical or pathological parameters significantly correlated with FOXP3 +Treg count, including overall survival and disease-free survival.

CONCLUSION: The study shows that luminal human epidermal growth factor receptor 2 negativ and human epidermal growth factor receptor 2 enriched BC show comparatively high FOXP3+ Tregs count. There is no relation with tumor grade, TNM stage, important immune markers, or overall survival and disease-free survival.

Keywords: *FOXP3; tumor-infiltrating lymphocytes; carcinoma breast*

For citation:
Hagone S, Bharat PU, Gupta A, Nitin G. Expression of FOXP3 of Tumor-Infiltrating Lymphocytes in Invasive Breast Cancer: Its Relationship to Histopathological Parameters and Overall Survival. *I. P. Pavlov Russian Medical Biological Herald.* 2023;31(2):265–272. DOI: https://doi.org/10.17816/PAVLOVJ108478

Received: 02.06.2022 **Accepted:** 16.09.2022 **Published:** 30.06.2023

ECO•VECTOR

LIST OF ABBREVIATIONS

BC — breast carcinoma
DFS — disease-free survival
ER — estrogen-receptor
FOXP3 — forkhead box P3 protein
FOXP3+ — forkhead box P3 protein positive
HEBC — human epidermal growth factor receptor 2 enriched breast carcinoma
HER2 — human epidermal growth factor receptor 2
HPFs — high-power fields
HR — hormone receptor
IHC — immunohistochemistry
IL — interleukin
Lum — luminal
MGIMS — Mahatma Gandhi Institute of Medical Sciences
MRM — modified radical mastectomy
NGS — Nottingham grading system
OS — overall survival
PR — progesterone-receptor
TILs — tumor-infiltrating lymphocytes
TNBC — triple-negative breast cancer
Tregs — regulatory T cells

BACKGROUND

Breast carcinoma (BC) is the most prevalent cancer in women worldwide, with approximately 2,261,419 new cases diagnosed in females in 2020, accounting for about 24.53% of all cancer cases worldwide and 6.91% of all cancer deaths [1]. In 2020, there will be 19.3 million cancer cases and 10 million cancer deaths worldwide. Cancer incidence among Indian women is expected to reach 712,758 by 2020 (104 per 100,000) [2]. The origin of BC is unclear. However, age, genetics, genes, radiation exposure, obesity, delayed pregnancy, and alcohol are all established risk factors for this disease [3]. BC metastasis is a multi-step biological process that various genes and substances can regulate [4]. As a result, in-depth research of the molecular and signal transduction pathways that play significant roles in BC metastasis is critical for understanding the mechanism of BC metastasis, both theoretically and clinically.

BC is a complex disease whose development involves various factors. Factors that can affect its risk include heredity and genetics and the factors affecting endogenous hormone production, like a lifestyle change, reproductive factors, ingestion of exogenous hormones, anthropometric features, mammography showing increasing breast density, and benign breast diseases [5]. The number of positive axillary nodes, tumor size, tumor grade (histologic or nuclear), lymphatic and vascular invasion, and estrogen-receptor (ER) and progesterone-receptor (PR) positivity are all traditional prognostic markers. They predict the likelihood of recurrence or mortality from BC.

The grading of BC is determined using several scoring methods. The Nottingham Histological Grading System is being used, along with immunohistochemistry. Tumors were only classified according to their clinical stage until the late 1950s, which ignored the accepted range of biological behavior of BCs. According to H. J. Bloom and W. W. Richardson, clinical staging '*fails to signal the likelihood of occult lymphatic and blood-borne metastases being present in what appears to be an early illness, nor the speed with which such metastases may grow*'. This inspired them to design a method of histologic grading, which was updated in 1991 by C. W. Elston and I. O. Ellis. They used semiquantitative criteria to improve objectivity and reproducibility [6]. The Elston–Ellis modification of the Bloom and Richardson grading classification (Nottingham grading system, NGS) is now widely used to guide the management of invasive BC worldwide [7].

The transcription factor forkhead box P3 (FOXP3), which belongs to the forkhead/pterygoid family, was initially thought to be a molecular marker for regulatory T cells (Tregs) [8]. Many studies have recently revealed that FOXP3 is also a tumor suppressor gene in BC [9]. FOXP3 expression was initially thought to be limited to hematopoietic tissues only. From 2007 onwards, an increase in the FOXP3 gene has been noted not in Tregs in the tumor but also in epithelial cells, acting as tumor epithelial suppressor gene. The precise mechanism by which FOXP3+ tumor-infiltrating lymphocytes (TILs)/Tregs suppress immune cell function remains unclear. However, this is thought to be through a combination of contact with each other and cytokine production, with a role for interleukin (IL) 10 and transforming growth factor-β [10]. It leads to T cell function down-regulation and prevents autoimmune disease in normal individuals [11]. Wild-type FOXP3 in normal tissue cells represses human epidermal growth factor receptor 2 (HER2) and c-myc oncogenes in mammary and prostate, respectively. FOXP3 overexpression in the human carcinoma cell line also suppresses tumor growth. However, mutated FOXP3 helps in Tregs proliferation and escape of tumor cells from immune surveillance. FOXP3 protein is expressed in both tumor cells (nuclear or cytoplasmic) and TILs and is reported to be associated with differences in clinical outcomes [12]. The expression of FOXP3 in tumor cells suggests the possibility that tumor-infiltrating Tregs influence antitumor immunity and modulate T-cell function through FOXP3, raising hopes for a potential prognostic marker [11]. FOXP3+ regulatory T cells showed poor prognosis in hormone receptor (HR) positive BC. However, it showed of favorable prognosis in HR-/HER2+ tumors [13]. Few studies demonstrated that CD3+, CD8+, and FOXP3+ lymphocyte densities did not add any new prognostic information to stromal TILs assessed on hematoxylin and eosin in early intermediate/high-risk BC treated with adjuvant chemotherapy [14]. Our study may fill the gap and explain the potential prognostic values of FOXP3 in BC.

DOI: https://doi.org/10.17816/PAVLOVJ108478

This is one of the rare limited studies done on the role of FOXP3+ Tregs in our country's prognosis of BC. This study **aimed** to determine FOXP3+ Tregs expression related to various prognostic factors in BC in the central Indian population. Our study is also helpful in correlating the role of FOXP3+ Tregs in the survival of invasive ductal carcinoma of the breast with different histopathological presentations.

MATERIALS AND METHODS

Study designs and patient selection criteria. The study was carried out at The Mahatma Gandhi Institute of Medical Sciences (MGIMS), a medical school in Sevagram in Central India. It is attached to Kasturba Hospital, a nearly 1000 bedded tertiary care rural hospital. The Histopathology section of the Department of Pathology receives approximately 6,500 specimens each year. The present prospective observational study was conducted from May 2018 to August 2020 in the Histopathology section of the Department of Pathology. Forty-seven diagnosed and operated women with invasive duct carcinoma with reported ER, PR, and HER2 neu status with complete demographic records such as age, sex, diagnosis, stage, treatment, and medical records such as type and stage of cancer were included. They were diagnosed two to four years back from the initiating point of the study. Patients who refused to give consent or patients lost to follow up after initial diagnosis, cases with trucut biopsy and or wedge biopsy have been performed, male patients with BC and history of recurrence and neoadjuvant therapy cases were excluded. The tissue was obtained during modified radical mastectomy (MRM) and lumpectomy specimens.

Ethical considerations. This study was approved by the institutional ethics committee of MGIMS Sevagram (MGIMS/IEC/Path/89/2018; 15 November 2018). Informed consent was also be taken from every patient in their language regarding their willingness to participate in the study.

Histopathology and Immunohistochemistry (IHC). The selected formalin-fixed paraffin-embedded tissue block was retrieved from archives for each case. Sections of 3–5 microns in thickness were obtained and stained with hematoxylin and eosin. After case selection, an immunohistochemical stain for FOXP3 was performed on these sections. For IHC, hematoxylin and eosin-stained slides will be screened to obtain the best section and rescreened to find the best section for FOXP3 stain. 0.1ml concentrate anti-human FOXP3/NB600-246 rabbit polyclonal antibody (Novus biologicals) with preservative 0.1% sodium azide and isotype-IgG was used, and dilution made in 1:100 with antibody diluent. FOXP3 IHC was carried out on a 3–5 μm thick formalin-fixed, paraffin-embedded section mounted on positively charged glass slides. The criteria of S. Lee, et al. [15] were used to count FOXP3+ Tregs expression (The cut off values was 15 FOXP3+ TILs/10 high-power fields (HPFs) and for low was < 15 FOXP3+ TILs/10 HPFs and for high was ≥ 15 FOXP3+ TILs/10 HPFs). The FOXP3+ Tregs were counted by a consultant pathologist who was blinded to the clinico-histological profile of the cases. Under a light microscope at 400 x magnification, ten representative fields at tumor bed and stroma were chosen. The count of the positive lymphocytes on each slide was reported as a mean value of the representative ten fields. FOXP3+ Tregs were calculated only in the peritumoral region. Patient's outdoor and indoor visits to the hospital after primary management were recorded using departmental records, the hospital information system, and our population-based cancer registry, and follow-up data were collected for the disease course for 48 to 69 months.

Statistical analysis. Statistical analysis was done using descriptive and inferential statistics using the $\chi 2$ test and Kaplan Meire Survival Analysis. The software used in the study were SPSS 24.0 version and GraphPad Prism 7.0 version. $P < 0.05$ is considered a level of significance.

RESULTS

In our study, the peri-menopausal age group (40–59 years) is the most common age group accounting for 66 percent of our cases (n = 31), with a mean age of 49 years. We classified our study cases into four molecular subgroups based on the expression of three immune markers in the tumor cells — ER, PR, and HER2/neu. Since the Ki-67 immune marker expression was not available for the study, we could not categorize our cases into other molecular classes.

Adverse outcomes were present in all 100% of cases where patients could not comply with their treatment plan for various personal and socio-economic reasons. 76.33% of patients who could adhere to their treatment plan remained free of adverse outcomes until a follow-up period of 69 months (p = 0.001, Table 1). Treatment plan: I — Surgery + Adjuvant chemotherapy; II — Surgery + Adjuvant chemotherapy + Radiotherapy; III — Neoadjuvant chemotherapy + Surgery + Radiotherapy.

The findings in our study indicate that high-grade tumors are prevalent (63.78%) in the study area irrespective of the stage of clinical presentation. Most distant metastases were found in the liver, with 57.00% of distant metastasis and 44.41% of all adverse outcomes. Since luminal (Lum) HER2- and HEBC constitute maximum cases of both low and high count of FOXP3+. This finding is statistically insignificant and suggests no molecular subtype in our study with specific predilection towards high Tregs count in the peri-tumoral area (Table 2). With FOXP3+ Tregs count groups, no definite relationship is appreciated between histological grading and TNM staging.

HEBC (36.00%) and Lum HER2- (36.0%) were predominant molecular subtypes with high FOXP3+ Tregs count but a statistically insignificant p-value of 0.74. Also, there was no significant relationship between FOXP3+ Tregs count and individual ER, PR, and HER2 status as almost equal positive and negative study cases shows low and high count (p = 0.86, 0.86, and 0.89, respectively, Table 3). During

Table 1. Adverse outcomes in the context of compliance with a treatment plan

Adverse Outcome	Completion of Treatment (%)		Total
	Completed	Not completed	
Absent	29 (76.34)	0	29 (61.70)
Present	***9 (23.72)***	***9 (100)***	***18 (38.30)***
Total	38 (80.85)	9 (19.15)	47 (100)
χ2 Test	19.01, p = 0.001		

Note: Adverse Outcome = Patients who had a recurrence or distant metastases or died during the follow-up

Table 2. Correlation of FOXP3 with histological parameters and molecular subtypes

FOXP3+ Tregs		Low, n (%)	High, n (%)	Total, n (%)	χ2 Test
Histological Grading	Grade 1	0	1 (4.00)	1 (2.13)	1.06, p = 0.58
	Grade 2	7 (31.82)	9 (36.00)	16 (34.04)	
	Grade 3	15 (68.18)	15 (60.00)	30 (63.83	
	Total	22 (46.81)	25 (53.18)	47 (100.00)	
TNM Staging	Stage I	0	3 (12.00)	3 (6.38)	3.86, p = 0.27
	Stage II	9 (40.91)	8 (32.00)	17 (36.17)	
	Stage III	8 (36.36)	6 (24.00)	14 (29.79)	
	Stage IV	5 (22.73)	8 (32.00)	13 (27.66)	
	Total	22 (46.81)	25 (53.18)	47 (100.00)	
Molecular Subtype	Lum HER2+	3 (13.64)	3 (12.00)	6 (12.77)	0.11, p=0.74
	Lum HER2-	7 (31.82)	9 (36.00)	16 (34.04)	
	TNBC	4 (18.18)	4 (16.00)	8 (17.02)	
	HEBC	8 (36.36)	9 (36.00)	17 (36.17)	

Notes: HEBC — human epidermal growth factor receptor 2 enriched breast carcinoma, Lum — luminal, TNBC — triple-negative breast cancer

Table 3. Correlation of FOXP3+ Tregs count with ER/PR/Her2 neu status

	FOXP3+ Tregs		Total, n (%)	χ2 Test
	Low, n (%)	High, n (%)		
ER Status				
Negative	12 (48.00)%)	13 (52.00)%)	25 (53.19%)	0.03 p = 0.86
Positive	10 (45.45%)	12 (54.55%)	22 (46.81%)	
PR Status				
Negative	12 (48.00)%)	13 (52.00)%)	25 (53.19%)	0.03 p = 0.86
Positive	10 (45.45%)	12 (54.55%)	22 (46.81%)	
HER2 New				
Negative	11 (45.83%)	13 (54.17%)	24 (51.06%)	0.01 p = 0.89
Positive	11 (47.83%)	12 (52.17%)	23 (48.97%)	

Notes: ER — estrogen-receptor, FOXP3 — forkhead box P3, HER2 — human epidermal growth factor receptor 2, PR — progesterone-receptor, Tregs — regulatory T cells

the study period, 12 patients died, and 35 patients survived, all of which responded well to treatment, irrespective of whether they had a low or high FOXP3+ Tregs. The data also indicates that out of 12 death cases, 66.68% belong to the low FOXP3+ Treg group and 33.32% to the high FOXP3+ Treg group, suggesting that high FOXP3 expression is associated with a better prognosis. Mean overall survival (OS) with high FOXP3+ Treg was 1427.60 ± 409.52 days, while with low FOXP3+ Treg was 1373.31 ± 361.09 days, and it is statistically insignificant. Similar findings were found with disease-free survival (DFS). Mean DFS with high FOXP3+ Treg was 1176.80 ± 516.16 days while low FOXP3+ Treg was 1151.36 ± 487.82 days and are also statistically not significant (Table 4, Figure 1).

Table 4. Survival analysis of study cases with the context of FOXP3+ Tregs counts

FOXP3+ Tregs	Mean DFS, days	Mean OS, days	Total cases, n	Alive cases, n	Censored
					Death, n (%)
Low	1151.36 ± 487.82	1373.31 ± 361.09	22	14	8 (36.44)
High	1176.80 ± 516.16	1427.60 ± 409.52	25	21	4 (16.00)
Overall	1164.89 ± 497.80	1402.19 ± 384.41	47	35	12 (25.5.2)
Log rank test: OS — 0.47, p = 0.63 and DFS — 0.06, p = 0.86)					

Notes: DFS — disease-free survival, FOXP3 — forkhead box P3, OS — overall survival

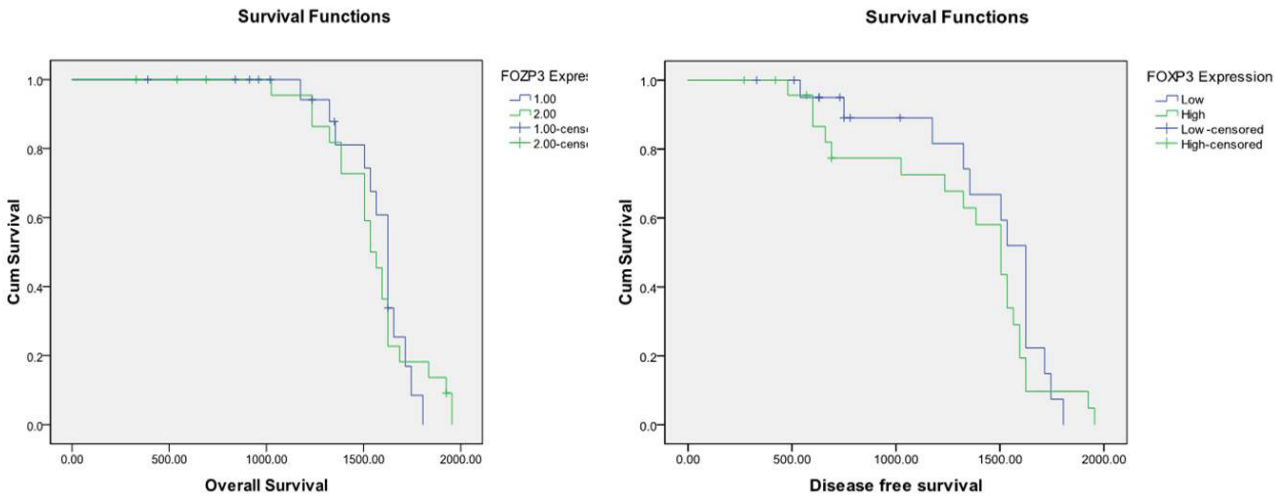

Fig. 1. Overall and disease-free survival.

DISCUSSION

FOXP3 protein is expressed in both breast tumor cells (nuclear or cytoplasmic) and TILs and is reported to be associated with differences in clinical outcomes [12]. In the present study, we evaluated the role of FOXP3+ Tregs present in the tumor area in context to the disease presentation and progression. We did not find any significant association of FOX P3+ Tregs with any of these aspects of BC. The available literature is also highly divided on the role of FOXP3+ Tregs in carcinoma BC. Some previous studies supported our findings, while few other data sets could not be correlated.

In the present study with a small sample size (n = 47), approximately half cases (n = 25) showed high, and in the rest of the 22 patients, a low FOXP3+ Tregs count was seen. Of these half cases (53.19%), HEBC and Lum HER2 are predominant molecular subtypes with high FOXP3+ Tregs count. But these findings are statistically insignificant (p = 0.74), and these findings are supported by E. Papaioannou, et al. [16]. They determined that FOXP3+ (Tregs) and CD8+ TILs show a high level in early-stage triple-negative breast cancer (TNBC) and the HER2+ BCs and decrease with disease progression to the advanced stages. Similar to the study of M. Mohamed, et al. [17], we also could not find any correlation of FOXP3+ Tregs count with the three essential IHC markers (ER, PR, HER2) expression.

We found that FOXP3+ Tregs count is not significantly associated with any histological grade or TNM stage of BC in our 47 study cases. S. Lee, et al. also found the same in their 86 cases [15]. However, few other studies did find a significant positive correlation between high FOXP3+ TILs with a higher grade of BC [12]. Regarding mean OS and DFS, again, we were unable to find any significant relationship at all with FOXP3+ Tregs (p = 0.47).

Our findings showed partial correlation with G. Peng, et al. studies reported that FOXP3 expression in tumor stroma was not significantly associated with OS [18]. However, when the entire tumor was taken into account, patients with low FOXP3 expression had a higher OS than those with high FOXP3 expression. Studies show better OS with high FOXP3+ Tregs count but only with TNBC and HEBC [15, 19]. Other subtypes with high FOXP3+ Tregs are associated with worse OS [12]. Only one study [20] found that the overall 5-year DFS rate was 85.7% in the high FOXP3 Tregs group versus 98.5% in the low FOXP3 Tregs group (p = 0.042). This shows a lack of consensus between various studies on the relation of Tregs with DFS and OS.

Findings of the present study regarding the grade, stage, or OS/DFS in BC cases may be explained because most of our patients presented themselves in advanced stage (stage III + IV), possibly due to socio-economic constraints.

So, we couldn't have enough cases in the early stages of the disease to analyze these parameters satisfactorily. But the insignificance may also be either due to the counter-effect of FOXP3 expression in carcinoma cells or the effect of the TILs (CD4, CD8, and CD25), which we didn't study or due to the zonal difference in the presence of FOXP3+ Tregs as various studies counted Tregs in different tumor zones. The different cut-off values for FOXP3+ Treg used in other studies may also contribute to this difference. Less likely, the discrepancy in various studies may be due to actual differences in the genetic constitution of the study population.

CONCLUSION

The study shows that luminal HER2-negative breast cancer and human epidermal growth factor receptor 2 enriched breast carcinoma show comparatively high regulatory T cells fork head box P3 count. There is no relation with tumor grade, TNM stage, important immune markers, or overall survival/disease-free survival. More studies with large sample size and standardized methodology are needed to confirm our findings. We recommend standardization of methods for future studies in this domain to be compared more reliably and robustly.

ADDITIONALLY

Funding. Financially aided by the Research Grant Committee of the institute Mahatma Gandhi Institute of Medical Science.
Conflict of interests. The authors declare no conflicts of interests.
Acknowledgment. This project was financially aided by the Research Grant Committee of the institute Mahatma Gandhi Institute of Medical Science.
Contribution of the authors: *S. Hagone* — data collection, statistical analysis, and interpretation, text writing; *P. U. Bharat* — statistical analysis, and interpretation, text writing; *A. Gupta* — study concept and design, data collection, statistical analysis, and interpretation, text writing, editing; *G. M. Nitin* — text writing, editing. The authors confirm the correspondence of their authorship to the ICMJE International Criteria. All authors made a substantial contribution to the conception of the work, acquisition, analysis, interpretation of data for the work, drafting and revising the work, final approval of the version to be published and agree to be accountable for all aspects of the work.

Финансирование. При финансовой поддержке Комитета по исследовательским грантам Mahatma Gandhi Institute of Medical Science.
Конфликт интересов. Авторы заявляют об отсутствии конфликта интересов.
Благодарность. Этому проекту оказал финансовую поддержку Комитет по исследовательским грантам Mahatma Gandhi Institute of Medical Science.
Вклад авторов: *Hagone S.* — сбор данных, статистический анализ и интерпретация, написание текста; *Bharat P. U.* — статистический анализ и интерпретация, написание текста; *Gupta A.* — концепция и дизайн исследования, сбор данных, статистический анализ и интерпретация, написание текста, редактирование; *Nitin G. M.* — написание текста, редактирование. Авторы подтверждают соответствие своего авторства международным критериям ICMJE (все авторы внесли существенный вклад в разработку концепции и подготовку статьи, прочли и одобрили финальную версию перед публикацией).

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