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# Outcome Based Validation of Tumour Budding as A Prognostic Marker of Breast Cancer

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## ABSTRACT

***Introduction:*** *Breast carcinoma (BC) is the commonest cancer in Sri Lanka. It is a heterogenous disease with wide variation in prognosis. Prognostic factors play key roles in selecting the most appropriate treatment. Tumour budding (TB) has emerged as a new prognostic marker for some cancers. TB is defined as isolated single cancer cells or clusters of up to four cancer cells located at the invasive tumour front. TB still has no recommended grading system for BC and yet to find its place in BC management guidelines. Hence, this study was aimed to determine the prognostic value of TB in invasive BC in a cohort of patients.*

***Method:*** *All patients fulfilling the inclusion criteria of the current study were selected from the BC data base of the Department of Pathology, Faculty of Medicine, University of Ruhuna which included all BC patients who sought the services of the immunohistochemistry laboratory from 2006-2015. Tumour buds in hotspots were counted in histology slides and graded according to the recommended grading for TB in colorectal cancers. Kaplan Meier model and log rank test were used for recurrence free and BC specific survival (RFS, BCSS) analysis.*

***Results:*** *A total of 231 BC patients were studied. Majority were 36-60 years of age (63.9%) and had lymph node metastasis (61.3%). Most tumours were 2-5 cm (77.2%) in size and Nottingham grade 2 (n=227, 50.6%) without lympho-vascular invasion (n=221, 64.3%). Most were hormone receptor negative (estrogen receptor-57.1%, progesterone receptor-56.7%) and HER2 negative (n=230, 80.9%). Low-grade (0-4TBs), intermediate-grade (5-9TBs) and high-grade TB(≥10TB) was present in 40.3%, 22.5% and 37.2% BCs respectively. Five-year RFS and BCSS of the study cohort were 77.2% and 86.5% respectively. TB grade had an independent effect on RFS and BCSS (p=0.027) imparting a poor survival when the tumour had high grade TB.*

***Conclusion:*** *TB has a significant negative effect on RFS and BCSS of invasive BC patients independent of established prognostic factors.*

## Keywords
Tumour budding, Prognostic marker, Breast cancer, Survival.

## Introduction
Female breast cancer (BC) is the most commonly diagnosed cancer in the world with an estimated 2.3 million new cases diagnosed in 2020 (11.7% of all cancers) [1)]. It accounts for the fifth cause of cancer related deaths in the world and is the leading cause of cancer death among women [1]. In Sri Lanka, BC is the commonest cancer and accounts for 23.9% cancers of females. It is the leading cause of cancer mortality in women in Sri Lanka [2].

The characteristics of BC is different in Asian and western countries. The overall incidence of BC is lower in Asian countries compared to western countries but the mortality is higher. The peak incidence is between 40-50 years in Asian females while it is

60-70 years in western females. The tumour stage at presentation is higher in Asian women leading to poor prognosis and higher treatment cost [3].

Breast carcinoma is a heterogenous disease with wide variations in prognosis. Prognosis is the likely outcome of a disease. The knowledge on prognosis of BC is important for the patient, the clinician, researchers and policy makers. Prognostic factors are important to optimize the BC treatment. If the outcome is predicted more precisely, the patient will receive the most appropriate treatment minimizing the effects of over and under treatment [4]. The standard prognostic factors of BC are age, histological type, disease stage, tumour grade, margin status, lympho-vascular invasion and hormone receptor expression and HER2 status [5]. Although new molecular and genetic markers have emerged, the standard histopathological prognostic factors still play a major role in the prognosis estimation and decision making in the treatment of BCs, especially in resource-poor settings [6,7]. Therefore, search for new prognostic markers based on routine histopathological evaluation remains important to improve the current evaluation system [6].

Tumour budding (TB) is an emerging prognostic biomarker. It has been suggested as an important factor to determine the tumour progression and survival in solid cancers in different organs such as colorectum, oesophagus, pancreas, head and neck, lung and breast [8-11]. It was first recognized as a prognostic biomarker for colorectal cancers and the standardization of TB assessment has been done at the International Tumor Budding Consensus Conference (ITBCC) held in 2016 enabling inclusion of TB assessment in reporting data sets for colorectal carcinoma.

ITBCC 2016 defined TB as a single tumour cell or a cell cluster of up to four tumour cells scattered in the stroma at the invasive tumour margin. The ITBCC group recommends to assess TB in one hotspot (in a field measuring 0.785 $mm^2$) at the invasive tumour front on a haematoxylin and eosin (H&E) stained slide [12].

TB is believed to provide the histological evidence for invasion and metastases in cancers. It was also suggested that TB represents the epithelial mesenchymal transition (EMT) histologically [9,13]. In EMT, epithelial cells partially or completely lose their epithelial phenotype and acquire a mesenchymal phenotype, which is important for the invasive and metastatic properties of cancer cells [8,14]. TB has the potential to be used as a prognostic marker for treatment with specific anti-metastatic treatments because of its association with EMT, which is paramount in metastasis of invasive cancers [8].

The ITBCC group has recognized TB as an independent predictor of lymph node metastasis and survival in pT1 and stage II colorectal cancers respectively. They recommend to include TB as a prognostic factor in the reporting protocols of colorectal cancers [12]. Furthermore, TB was introduced as a second major grading criteria in the 2019 World Health Organization (WHO) classification of colorectal cancer [15].

Most of the studies done on the prognostic significance of TB in breast carcinoma have demonstrated it as a poor prognostic factor. However, the lack of a validated scoring system for breast carcinoma precludes wider adoption of tumor budding as a prognostic biomarker that guides therapeutic decisions. In the literature, there were no studies reported on the prognostic significance of TB in breast carcinomas in Sri Lankan patients. Therefore, the aim of this study was to assess the prognostic value of TB in breast carcinoma in a cohort of patients in Southern Sri Lanka and to validate ITBCC TB grading of colorectal carcinoma for BC.

## Methods

The current study was a retrospective cohort study conducted in the Department of Pathology, Faculty of Medicine, University of Ruhuna, Galle, Sri Lanka. All BC patients who fulfilled the inclusion criteria of the current study were selected from the BC data base of our department which included all BC patients who sought the services of the Immunohistochemistry laboratory for hormone receptor assessment from 2006 to 2015. The inclusion criteria included; patients treated with surgical resection as the initial treatment and patients in whom invasive tumour front is included in the representative H&E slide of the BC data base. Patients who received neoadjuvant therapy and of whom Tru cut biopsies were submitted for immunohistochemical assessment of hormone receptors were excluded from the study. The clinicopathological data were extracted from the data base. Informed written consent has been obtained from all patients included in the study. Ethical approval to conduct the study has been granted by the Ethics Review Committee of the Faculty of Medicine, University of Ruhuna, Sri Lanka.

The recommendations of ITBCC/2016 for reporting TB in colorectal cancers were followed in evaluating TB [12]. Tumour buds were defined as a single tumour cell or a cell cluster of up to 4 tumour cells at the invasive tumour front. First, ten individual fields along the invasive front of the H&E-stained slide was scanned under medium power (x10 objective) to identify the hotspot. With a permanent marker, this field area was marked on the slide. Then the tumour buds in the selected hotspot were counted using x20 objective. Finally, the budding category was assigned using the ITBCC three-tier system (Table 1).

**Table 1:** Tumour budding categories.

| Tumour budding category | Number of tumour buds |
|---|---|
| Low | 0-4 |
| Intermediate | 5-9 |
| High | >/=10 |

Chi-square test was used to determine the association between TB and other clinicopathological factors. Kaplan Meier model and log rank test were used for estimation of recurrence free survival (RFS) and BC specific survival (BCSS) analysis. Cox-regression model with backward stepwise factor retention method was used for multivariate analysis to identify whether TB has an independent effect on the RFS and BCSS.

## Follow up and outcomes

The study subjects have been followed up for recurrence or death at six months intervals from enrollment to the database till 31st December 2015.

The RFS was calculated from the date of surgery to the date of diagnosis of recurrence (local/distant metastasis) [16]. Radiological and histopathological data were used to confirm the recurrence. The date on which the said investigation done was considered the date of recurrence. Patients who have not experienced the relevant end point were censored at the last follow-up. Death was not considered as an event for RFS [16]. The recurrence free interval was measured from the date of first therapeutic intervention to the date of confirmation of the first recurrence in months [16].

The BCSS time was calculated from the date of diagnosis of the disease to the date of death. Patients who died of BC or who died with BC (progression/metastasis) were included [16]. Patients died of other causes or from unknown causes were censored to the date of death. The cause of death of the patient was obtained from the death certificate issued by the Department of Registrar General, Sri Lanka.

The survival rates were estimated using the Kaplan-Meier model with log rank test. The univariate analysis was performed using Cox proportional hazards model with 95% CI. All covariates which had a p value <0.100 were taken for multivariate analysis. Multivariate analysis was done with Cox proportional hazards model with backward stepwise factor retention method. All p values <0.05 were considered as statistically significant.

## Results

A total of 231 BC patients including 205 mastectomies and 26 wide local excisions were studied. The clinicopathological characteristics of the study sample are summarized in Table 2. The most common age group at presentation was 36-60 years of age (64%). Majority (92.6%) of the cases were invasive carcinoma, no special type (NST) of breast. The majority of patients had tumours of 2-5 cm in size (77%), which corresponds to pT2 stage (AJCC TNM 8th edition), had Nottingham grade 2 tumours (53%), and had lymph node metastases (61.3%). In 64% of cases, lympho-vascular invasion was absent. The majority had ER negative (57.1%), PR negative (56.7%) and HER2 negative (73.0%) tumours.

TB was assessed in all cases and was graded into three categories according to ITBCC (2016) for colorectal cancers. Of them, 40.3%, had low grade TB, 22.5% had intermediate grade TB and 37.2% had high grade TB.

Five-year RFS and BCSS of the study cohort were 77.2% and 86.5% respectively. The Kaplan Meier survival curves for RFS (shown in Figure 1) and BCSS (shown in Figure 2) of BC patients in the three TB groups showed that there is a significantly poor survival in high grade TB group. Intermediate and low-grade TB groups showed a better survival but their survival curves overlap. Therefore, the low and intermediate TB groups were combined and estimated the RFS and BCSS for the two TB groups; that is low grade group with 0-9 tumour buds and high-grade group with 10 or more tumour buds. Both RFS and BCSS curves show that there is a significantly poor survival in high grade TB compared to low grade TB (p=0.010 for BCSS, p=0.008 for RFS) (shown in Figures 3, 4).

**Table 2:** Clinico-pathological parameters of the study sample.

| Features | Observation | Frequency n (%) |
|---|---|---|
| Age (years) | ≤ 35<br>36-60<br>>60 | 19 (8.2%)<br>147 (63.6%)<br>64 (27.7%) |
| Histological type | IBC, NST<br>Invasive lobular carcinoma<br>Invasive mucinous carcinoma<br>Others | 214 (92.6%)<br>12 (5.2%)<br>2 (0.9%)<br>3 (1.3%) |
| Tumour size (T stage) | < 2cm (T1)<br>2-5cm (T2)<br>>5cm (T3) | 34 (14.7%)<br>176 (76.2%)<br>18 (7.8%) |
| Lymph node stage | Node negative<br>N1 (1-3 LNs)<br>N2 (4-9 LNs)<br>N3 (≥ 10 LNs) | 82 (35.5%)<br>66 (28.6%)<br>42 (18.2%)<br>22 (9.5%) |
| Nottingham grade | Grade 1<br>Grade 2<br>Grade 3 | 27 (11.7%)<br>115 (49.8%)<br>76 (32.9%) |
| TNM (8th) Stage | Stage I<br>Stage II<br>Stage III | 17 (7.4%)<br>128 (55.4%)<br>66 (28.6%) |
| ER expression | Negative<br>Positive | 132 (57.1%)<br>99 (42.9%) |
| PR expression | Negative<br>Positive | 131 (56.7%)<br>100 (43.3%) |
| HER 2 expression | Negative<br>Borderline<br>Positive | 168 (72.7%)<br>19 (8.2%)<br>43 (18.6%) |
| Ki 67 labelling index | <14%<br>>14% | 45 (19.5%)<br>148 (64.1%) |
| TB grade | Low<br>Intermediate<br>High | 93 (40.3%)<br>52 (22.5%)<br>86 (37.2%) |

Abbreviations: ER-oestrogen receptor; Her2- Human epidermal growth factor receptor 2; IBC NST- invasive breast carcinoma of no special type; LN-lymph node; N-Node; n-number; PR-progesterone receptor; T-Tumour; TB-Tumour budding; TNM- Tumour-Node-Metastasis.

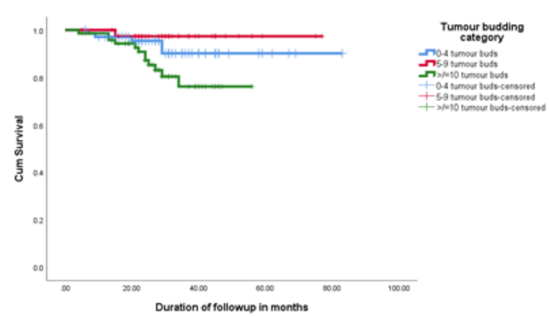

**Figure 1:** Kaplan Meier survival curves for RFS of the breast cancer patients in the three TB groups (three tier grading) (p=0.027).

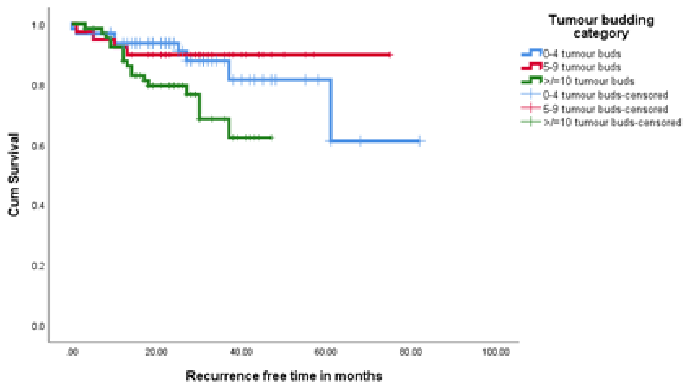


**Figure 2:** Kaplan Meier survival curves for BCSS of the breast cancer patients in the three TB groups (three tier grading) (p=0.027).

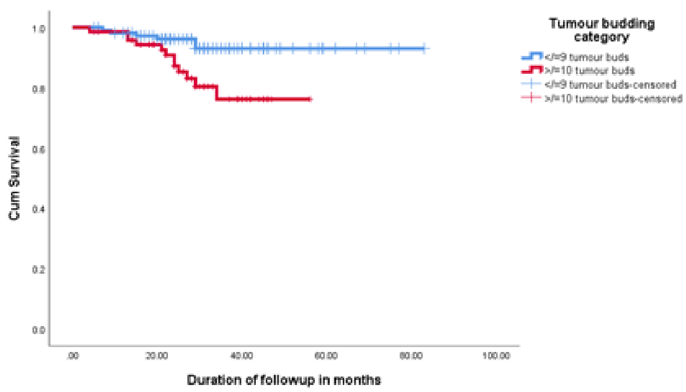


**Figure 3:** Kaplan Meier survival curves of RFS of the breast cancer patients in the two TB groups (two tier grading) (p=0.008)

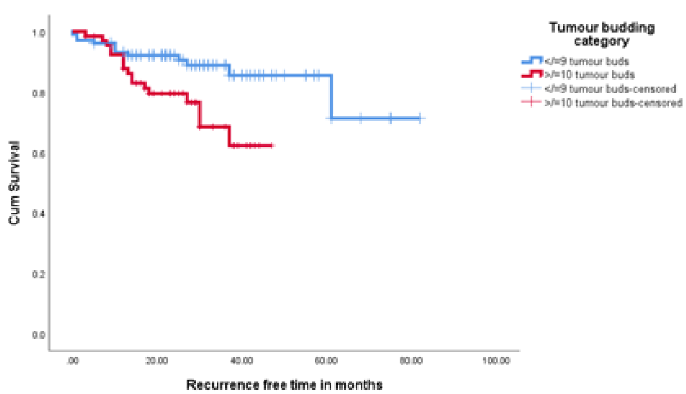


**Figure 4:** Kaplan Meier survival curves for BCSS of the breast cancer patients in the two TB groups (two tier grading) (p=0.010).

In the univariate analysis, it was found that BCSS is significantly associated with age at presentation (p=0.001), lymph node stage (p=0.001), expression of ER (p=0.025) and PR (p=0.018) and TB (p=0.027) (Table 3). Then, multivariate analysis was done using these factors with a significant association and found that lymph node stage (p=0.005) and TB (p=0.048) had a significant independent negative effect on BCSS (Table 3). Age at presentation (p=0.001) & TB (p=0.027) showed significant association with RFS in univariate analysis. Both factors had a significant independent negative effect on RFS in multivariate analysis (p=0.002 and p=0.024 respectively) (Table 3).

## Discussion

Breast carcinoma is a heterogenous disease with wide variations in prognosis. Although different treatment protocols are available, recurrences and metastases are known to occur in patients with BC [17]. Therefore, more efficient prognostic factors are needed to predict the outcome precisely, thus the patient will receive the most appropriate treatment minimizing the effects of over and under treatment.

The aim of the current study was to determine the prognostic value of TB in BC. TB is a complex histological process that provides histological basis for tumour invasion and metastases [18]. It was first recognized as a prognostic marker for colorectal cancers following the standardization of TB assessment by the ITBCC grading in 2016. In subsequent years, it has also been recognized as a novel prognostic indicator in esophageal, gastric, breast, bladder, and pancreatic tumors [15]. However, there is no standardized system for the assessment of TB in solid cancers other than for colorectal cancer. Different studies have used different methods for counting, staining and grading tumour buds. In our study, we assessed tumour buds in a field measuring 0.785 $mm^2$ using X20 objective, at the invasive tumour margin, as recommended by the ITBCC grading 2016 for colorectal cancer. The same method was used by Silva et al. [19]. Some studies have used pancytokeratin immunostains to highlight the TB [7,15,20,21], but in the current study we used H&E slides as recommended by ITBCC grading 2016. Although pancytokeratin stain can highlight TB from its mimickers, usage of H&E is cost effective for a developing country like ours. In the current study, TB was not significantly associated with standard prognostic factors; patient age, tumour size, lymph node stage, Nottingham grade, TNM stage, ER/PR/HER2 status and Ki67 labelling index. Silva et al. who used the same assessment method, found that a higher number of tumor buds was associated with occurrence of lymphovascular and perineural invasion ($p < 0.001$), tumor size ($p = 0.012$), higher nuclear grading ($p < 0.001$), molecular subtype ($p = 0.019$), Ki-67 index ($p = 0.011$) [19]. But there was no statistically significant difference between the TB groups and nodal status, estrogen, or progesterone receptor expression.

Gujam et al. also didn't find a significant association between TB and age, size, grade, and Ki67. But ER positive status, lymph node stage, presence of lymphovascular invasion and high tumour stroma percentage were positively associated with high TB in their study [22]. In the study of Ozer et al., TB was not significantly associated with age, molecular subtype, histological grade, and multicentricity. However, it was statistically associated with tumour size, pTstage, lymphovascular invasion, perineural invasion, number of metastatic axillary lymph nodes and extra nodal extension [17]. Therefore, the association of TB with currently used histopathological prognostic features is not consistent across studies which could be due to variations in the sample size and composition and the methodology used in assessing TB.

**Table 3:** Cox proportional hazards regression models of variables associated with survival outcomes.

| Factor | Recurrence Free Survival | | | | | | Breast Cancer Specific Survival | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Univariate analysis | | | Multivariate analysis | | | Univariate analysis | | | Multivariate analysis | | |
| | HR | 95% CI | *P* value | HR | 95% CI | *P* value | HR | 95% CI | *P* value | HR | 95% CI | *P* value |
| Age at presentation | | | 0.001 | | | 0.002 | | | 0.004 | | | 0.458 |
| ≤35 years | Reference | | | Reference | | | Reference | | | Reference | | |
| 36-60 years | 0.228 | 0.089-0.585 | | 5.582 | 1.770-17.599 | | 0.218 | 0.076-0.630 | | 2.952 | 0.507-17.184 | |
| >60 years | 0.194 | 0.062-0.608 | | 1.115 | 0.439-2.836 | | 0.088 | 0.17-0.455 | | 2.410 | 0.507-11.463 | |
| Tumour size | | | 0.210 | | | | | | 0.490 | | | |
| T1 (≤20 mm) | Reference | | | | | | Reference | | | | | |
| T2 (>20-50 mm) | 2.907 | 0.685-12.337 | | | | | 80705.38 | | | | | |
| T3 (>50 mm) | 4.531 | 0.752-27.304 | | | | | 172016.68 | | | | | |
| Nottingham grade | | | 0.288 | | | | | | 0.662 | | | |
| Grade 1 | Reference | | | | | | Reference | | | | | |
| Grade 2 | 0.899 | 0.250-3.237 | | | | | 23266.08 | | | | | |
| Grade 3 | 1.636 | 0.472-5.668 | | | | | 35719.96 | | | | | |
| Lymph node stage | | | 0.060 | | | | | | 0.001 | | | 0.005 |
| 0 (Negative) | Reference | | | | | | Reference | | | Reference | | |
| 1 (1-3 LNs positive) | 0.806 | 0.296-2.194 | | | | | 0.406 | 0.042-3.899 | | 0.492 | 0.050-4.870 | |
| 2 (4-9 LNs positive) | 1.160 | 0.397-3.388 | | | | | 4.400 | 1.098-17.631 | | 4.521 | 1.095-18.673 | |
| 3 (>/=10 LNs positive) | 3.131 | 1.136-8.633 | | | | | 10.526 | 2.710-40.885 | | 8.483 | 2.037-35.339 | |
| Expression of ER | | | 0.067 | | | | | | 0.038 | | | 0.944 |
| Presence | Reference | | | | | | Reference | | | | | |
| Absence | 2.107 | 0.932-4.764 | | | | | 3.717 | 1.076-12.844 | | | | |
| Expression of PR | | | 0.476 | | | | | | 0.030 | | | 0.233 |
| Presence | Reference | | | | | | Reference | | | | | |
| Absence | 1.313 | 0.620-2.781 | | | | | 0.253 | 0.073-0.873 | | | | |
| Over expression of Her2 | | | 0.983 | | | | | | 0.506 | | | |
| Yes | 0.990 | 0.371-2.640 | | | | | 0.798 | 0.410-1.552 | | | | |
| No | Reference | | | | | | Reference | | | | | |
| Expression of Ki67 | | | 0.701 | | | | | | 0.340 | | | |
| <14% | Reference | | | | | | Reference | | | | | |
| >14% | 1.212 | 0.454-3.234 | | | | | 0.601 | 0.208-1.733 | | | | |
| Presence of TB | | | 0.028 | | | 0.024 | | | 0.028 | | | 0.048 |
| 0-4 tumour buds | Reference | | | Reference | | | Reference | | | Reference | | |
| 5-9 tumour buds | 0.750 | 0.225-2.495 | | 0.617 | 0.181-2.108 | | 0.323 | 0.038-2.765 | | 0.253 | 0.029-2.205 | |
| >/= 10 tumour buds | 2.412 | 1.019-5.709 | | 2.388 | 1.019-5.597 | | 2.476 | 0.871-7.033 | | 2.418 | 0.804-7.274 | |

Liang et al. identified that TB grade, tumour size, nodal status and lympho-vascular invasion status as independent prognostic factors in invasive duct carcinoma, not otherwise specified, based on a survival analysis [6]. The association between TB and survival of BC patients have been assessed in various other studies as well. The survival has been assessed as overall survival (OS), BCSS, disease free survival (DFS) or RFS.

In our study, we identified that TB has a significant negative effect on the RFS and BCSS of invasive BC patients independent of the established prognostic factors. Lymph node stage and age at presentation also found to have a significant negative effect on the BCSS and RFS respectively in the multivariate analysis ($p=0.005$ and 0.002 respectively). Similar to our study, Gujam et al. also have shown TB as an independent prognostic factor of BCSS in invasive duct carcinoma of breast [22].

Li et al. showed that TB was significantly associated with decreased OS in triple negative BCs (TNBC] but not in ER+/HER2- cases. DFS was not significantly associated with TB neither in ER+/HER2- nor in TNBC, in their study [23]. Silva et al. found significant correlation between TB and DFS, but no statistically significant association between TB and OS was demonstrated [19]. Xiang et al. demonstrated worse DFS associated with high grade TB [15] while Liang et al. found worse OS with high grade TB in BC [6].

In our study we have demonstrated that TB is associated with poor prognosis and reduced survival. There is a significant difference in the survival (BCSS and RFS) between the low and high grade TB (shown in Figure 3 and 4). This study validates the usefulness of two-tiered grading against three-tiered grading recommended by the ITBCC for colorectal carcinoma. However, we have used all other recommendations made by ITBCC in identifying TB in the histology sections for this study on BC. Therefore, the current study validates the ITBCC recommended grading for colorectal carcinoma with the modification to a two-tiered grading for BC. However, external validation has to be done on a different cohort of patients before it is recommended for clinical use. External validation is the testing of the developed grading system in a set of new patients to determine whether the model works to a satisfactory degree [24].

Small sample size and retrospective design were the limitations of the current study where missing data can be more frequent than in prospective studies. However, the current cohort of patients has been managed at a single center and their management can be considered uniform which improves the validity of the study. Clinicians caring for the patients were unaware of the TB status of the BC patients at the time of decision making. Therefore, blinding required in prognostic factor studies was achieved automatically due to the retrospective nature of the study.

## Conclusions

TB has a significant negative effect on the RFS and BCSS of invasive BC patients independent of the established prognostic factors. High grade TB gives a significant survival disadvantage to BC patients. The current study substantiates the validity of two tiered grading of TB as a prognostic parameter for breast carcinoma and recommends to include it in routine reporting of BC as a simple, cost-effective less time consuming histological prognostic parameter following external validation.

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