Tumor-infiltrating lymphocytes are associated with β-catenin overexpression in breast cancer

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Abstract.

BACKGROUND: Inhibition of lymphocytes infiltration and activity may impair antitumor immune response and limit treatment responsiveness. Wnt/β-catenin pathway has been suggested to contribute to immune evasion in tumor by suppressing the function of immune cells and excluding T cell infiltration. However, the effects of Wnt/β-catenin on TILs recruitment remain controversial.

OBJECTIVE: We aimed to investigate whether intratumoral Wnt/β-catenin signaling could affect the lymphocyte infiltration in breast cancer.

METHODS: The distribution of stromal TILs, CD8$^+$ and FOXP3$^+$ TIL subsets, and the expression of β-catenin were separately assessed on consecutive sections of 96 breast cancer specimens.

RESULTS: Both stromal infiltrated TILs and β-catenin expression were upregulated in hormone receptor negative HER2-enriched and TNBC subtypes. Furthermore, high levels of stromal TILs as well as CD8$^+$ or FOXP3$^+$ TIL subsets were associated with β-catenin overexpression by breast cancer, respectively.

CONCLUSIONS: For the first time, we demonstrated that rather than excluding lymphocytes infiltration as reported in melanoma, high levels of TILs were associated with β-catenin overexpression in BC. Wnt/β-catenin signaling may play a critical role in BC immunity, particularly in HER2-enriched and triple negative BC, and may serve as a potential target for regulating immune infiltrates in breast cancer.

Keywords: Tumor infiltrating lymphocytes, Wnt/β-catenin signaling, CD8$^+$ TILs, FOXP3$^+$ TILs, breast cancer

1. Introduction

Despite the improvement of its overall 5-year survival rate in the past 3 decades, breast cancer is still the most prevalent cancer among females worldwide [1]. It is also the leading cause of cancer death among women in less developed regions and the second cause of cancer death in more developed regions [2], posing great threat to women's health. The interaction between tumor cells and immune system is critical for the development and progression of cancer [3]. Although breast cancer (BC) is not traditionally considered as immunogenic, an increasing number of retrospective and prospective studies have shown that high level of lymphocytes infiltration in BC is associated with improved outcome of patients, particularly in triple negative and HER2-enriched early breast cancer [4–6]. Moreover, the presence of TILs could favorably influence treatment responsiveness to neoadju-

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vant chemotherapy [7]. An association between higher levels of TILs and increased trastuzumab benefit in HER2$^+$ breast cancer has also been reported [8]. Accordingly, inhibition of lymphocytes infiltration and activity in the tumor microenvironment may impair antitumor immune response and limit treatment responsiveness in breast cancer.

Wnt pathway plays a key role in the development and function of hematopoietic system [9–11]. In peripheral immunity, canonical Wnt/β-catenin signaling is critical for the polarization, differentiation and survival of mature T lymphocytes [12]. It has been reported that the activation of Wnt/β-catenin pathway could negatively regulate the naive-to-effector T cell differentiation in human T lymphocytes [13, 14]. Wnt/β-catenin signaling also inhibits the proinflammatory cytokine induction of dendritic cells (DCs) in response to toll like receptor ligands and promotes DC generation of FOXP3$^+$ (forkhead box P3) regulatory T cells, thus reprogramming DC responses for immune tolerance [15]. Wnt signaling is frequently activated in various cancers [16], and their immune suppressive and tolerogenic effects on T cells and DCs may contribute to tumor immune evasion and cancer progression. In melanoma, accumulation of β-catenin in tumor cells and tissues upregulated IL-10 expression by directly binding to the IL-10 promoter, which impaired DC maturation and induced regulatory phenotype among DCs. Moreover, the anti-tumor effect of melanoma-specific cytotoxic CD8$^+$ T cells was inhibited by Wnt/β-catenin activity *in vitro* and *in vivo* [17]. Emerging evidence from clinical data suggest that immunotherapeutic strategies, including checkpoint blockade, are predominantly effective in patients with a pre-existing T cell-inflamed tumor microenvironment [18–20]. Spranger and colleagues [21] reported that melanoma intrinsic Wnt/β-catenin signaling prevented T-cell infiltration in tumor microenvironment and resulted in the resistance to anti-PD-L1/anti-CTLA-4 monoclonal antibody therapy. However, the effects of Wnt/β-catenin on tumor immunity remain controversial. Wnt/β-catenin signaling is proved to be required for the reconstitution and quiescence of hematopoietic stem cells (HSC) [22,23]. In a mouse model, activation of the Wnt pathway prevented CD8$^+$ T cells from differentiating into effector T cells, and generated a population of CD8$^+$ T cell subset showing a young/memory phenotype similar to stem cell memory T cells (T$_{SCM}$), which showed higher proliferation and antitumor activity than central memory T cells (T$_{CM}$) or effector memory T cells (T$_{CM}$). Fur-

thermore, Wnt/β-catenin signaling improved the persistence of human CD8$^+$ T cells by overexpression of Bcl-2 and absence of active caspase-3 [24]. These findings revealed that Wnt/β-catenin pathway might contribute to the maintenance of stemness in mature CD8$^+$ T cells, indicating the potential value of Wnt/β-catenin pathway in adoptive T cell therapy for cancer. Taken together, whether Wnt/β-catenin pathway plays a positive or negative role in anti-tumor immunity is open to question and requires further investigation.

High β-catenin activity has been reported to correlate with poor prognosis and tumor progression in breast cancer [25,26]. Our previous findings also suggest that Wnt/β-catenin pathway is abnormally activated in breast cancer, and may be of mechanistic and pharmatheutical significance in the disease [27]. However, the involvement of Wnt/β-catenin signaling in tumor immune microenvironment has not been clearly established.

In this study, we seek to further explore the clinical-pathological factors that affect T lymphocytes infiltration in breast cancer and the correlation between components of tumor inflammatory infiltrates and Wnt/β-catenin pathway activity, as measured by β-catenin expression in tumor tissues. Various reports have suggested that infiltration of CD8$^+$ T cells in breast cancer has antitumor activity as judged by their favorable effect on patients' survival, while high levels of FOXP3$^+$ Tregs predict reduced survival in overall breast cancer with exception for TNBC [28–32], indicating clinical significance for these two subsets. Thus we further assessed the infiltration of these two TIL subsets and their association with β-catenin expression in breast cancer. Here, we provide the novel findings that rather than excluding TILs from tumor microenvironment, activation of intrinsic Wnt/β-catenin signaling in breast cancer is positively associated with high levels of total lymphocyte infiltration as well as the number of CD8$^+$ or FOXP3$^+$ TIL subsets.

2. Materials and methods

2.1. Patients' specimen and clinicopathological data

Female patients with first diagnosed, primary operable breast carcinoma, without clinical distant metastasis, no history of receiving neoadjuvant chemotherapy or preoperative radiation therapy were considered for inclusion in this study. A total number of 96 paraffin-embedded tumor samples were obtained from primary

breast cancer patients. Ninety-six tumor samples were reported as infiltrating ductal or lobular breast carcinoma. Median age of the patients is 52 years old (range 28–77). Detailed clinicopathological data of the tumor samples were retrieved from the routine reports of Department of Pathology, the Second Affiliated Hospital of Xi'an Jiaotong University. Immunohistochemical analyses were performed by qualified pathologists, and the cutoffs for ER, PR were 1% [33]. The criteria used to determine the HER2 status was in accordance with the 2013 ASCO/CAP guideline [34]. In samples showing IHC 2^+ (equivocal), fluorescent in situ hybridization (FISH) amplification was applied to determine HER2 status. This study was reviewed and approved by the ethics committee of Xi'an Jiaotong University. All the participants were informed and signed consent for the use of their biological tissues.

2.2. Histopathologic analysis of TILs

Full-face breast cancer slides (5 μm thickness) were stained with hematoxylin-eosin (H&E) using standard procedures. TILs in the stromal compartment were defined and analyzed according to the standardization and guidelines for TILs assessment described by Salgado et al. [35]. Histopathologic evaluation of Str-TILs was performed separately by two experienced pathologists in the Department of Pathology of our hospital. They both were blinded to the clinicopathological information collected in this study. Results would be discussed if substantial discrepancies occur. The results were reported as three categories: TILs Low (%Str-TIL < 10%), TILs Intermediate (%Str-TIL = 10–40%) and TILs High (%Str-TIL ⩾ 40%). These cutoffs were also adopted from the same guidelines mentioned previously [30].

2.3. Immunohistochemical staining

The expression of β-catenin protein by tumor tissue and populations of $CD8^+$ T cells and $FOXP3^+$ Tregs dispersed in the cancer stroma were determined by immunohistochemical staining with the IHC Detection Kit (CWBiotech, Beijing, China) on 5 μm consecutive paraffin-embedded tissue slides, respectively. Antigen retrieval with citrate buffer was included. Primary monoclonal antibodies against β-catenin (ab32572, clone E247, Abcam, Cambridge, MA; 1:500 dilution), CD8α (#70306, clone C8/144B, CST, Danvers, MA; 1:200 dilution) and FOXP3 (ab20034, clone 236A/E7, Abcam, Cambridge, MA; 1:100 dilution) were used

according to the protocol of the manufacturer. Slides that were incubated with phosphate-buffered saline (PBS) instead of primary antibody served as negative control. Images of the full-face slides were taken using the automatic digital slide scanner (Zeiss Axio Scan .Z1, Jena, Germany).

2.4. Immunohistochemistry evaluation

β-catenin expression and the number of $CD8^+$ or $FOXP3^+$ T cells were evaluated independently by two qualified pathologists, who were blinded to the clinicopathological data of the slides. Joint re-assessment would be made for the final consensus. For each slide, ten non-overlapping high-power fields (x400 magnification) in the tumor stroma were randomly chosen for evaluation.

All tumors slides were assessed for both intensity (score 1 to 3, Fig. 1), and the proportion of cells staining positive in the nucleus and/or cytoplasm. Score of 0 if < 5% of morphologically unequivocal tumor cells discretely expressed cytoplasmic/nuclear β-catenin; score 1 = 6–25% positive cells; score 2 = 26–50% positive cells; score 3 = 51–75% positive cells; score 4 = > 75% of tumor cells expressed cytoplasmic/nuclear β-catenin. A histoscore (H) was calculated by multiplying the scores of percentage of positively stained cells with the score of staining intensity [36]. The final staining score was defined according to the H score: score of 1 if H = 0–1; score of 2 if H = 2–4; score of 3 if H = 6 or 8; score of 4 if H = 9 or 12. Final staining score 0–2 was considered as cytoplasmic/nuclear β-catenin negative, and score 3–4 was defined as cytoplasmic/nuclear β-catenin positive.

$CD8^+$ T cells are mainly stained in the membrane and the nuclear (Fig. 2A). For FOXP3 detection, dark-brown colored nuclear staining was defined as positive (Fig. 2B). The average counts of stromal CD8 positive or FOXP3 positive lymphocytes were recorded and accepted as the infiltration of $CD8^+$ cytotoxic T cells and $FOXP3^+$ Tregs, respectively [37].

2.5. Statistical analyses

Associations between TILs, $CD8^+$ or $FOXP3^+$ T cells counts, cytoplasmic/nuclear β-catenin expression and clinicopathological parameters were evaluated using Pearson Chi-square (χ^2) test or Fisher's exact tests. Kruskal-Wallis test and Pearson Correlation test were applied for evaluating the correlation between TILs, $CD8^+$ or $FOXP3^+$ T cells counts and β-catenin ex-
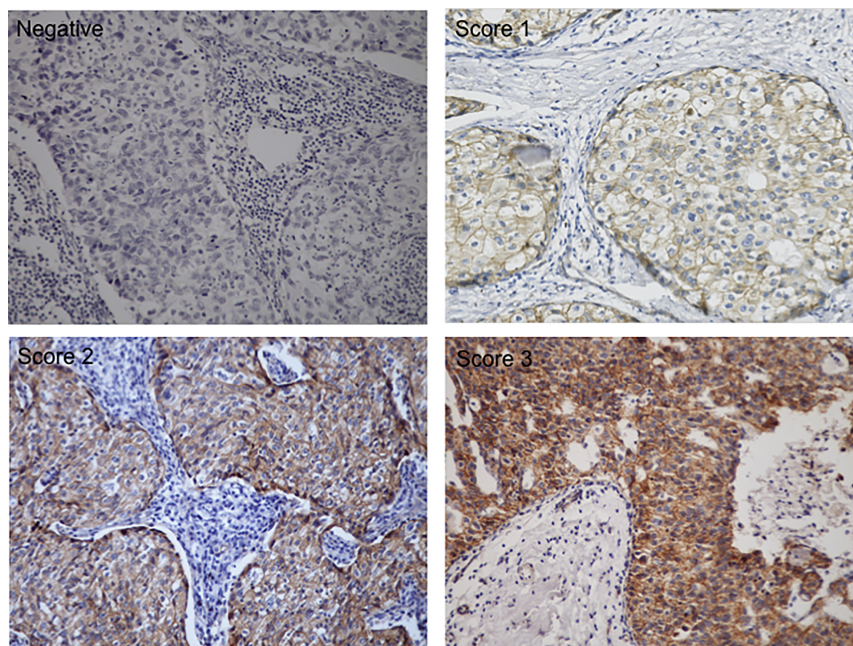
Fig. 1. Representative images of β-catenin expression in breast cancer sections (magnification, x200). Scores of 1 to 3 were defined according to the immunohistochemical intensity of β-catenin.
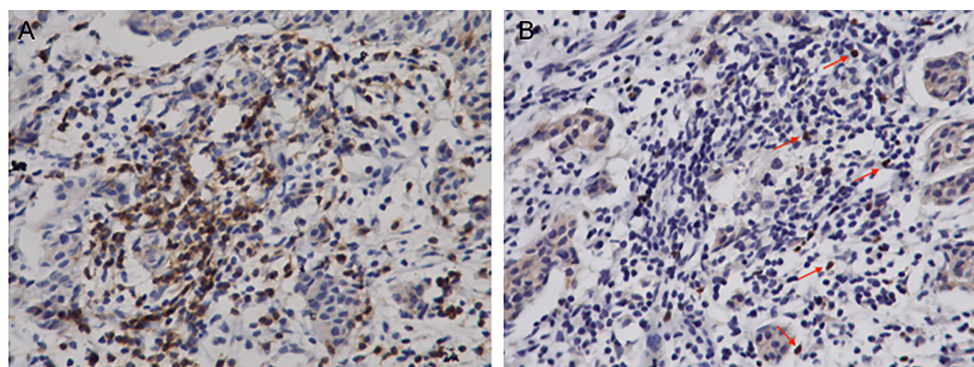

Fig. 2. Representative immunohistochemistry staining of stromal CD8$^+$ and FOXP3$^+$ T cell infiltrates in breast cancer (magnification, x400). Consecutive sections were used for immunohistochemical detection of FOXP3$^+$ (A) and CD8$^+$ cells (B).

pression, respectively. All statistical analysis was performed using SPSS 20.0 (IBM Corporation, Armonk, NY, USA) statistics software. The significance level was taken to be 0.05, and all p-values were two-sided.

3. Results

3.1. Lymphocytes infiltration in breast cancer correlates with tumor grade and ER/PR status

The clinicopathological information of 96 breast cancer patients recruited in this study is summarized in Table 1. Fifty-two patients (54.2%) presented with clinically detectable axillary lymph node metastasis at diagnosis. Fifty-six of patients (58.3%) had moderately (grade II) or poorly differentiated (grade III) tumors, and 84 patients (87.5%) presented with stage II or III tumors. According to the criteria previously described [35], we divided the distribution of stromal TILs into three levels based on the percentage of TILs that occupied the stromal areas. Increased lymphocytes infiltration in tumor stroma was significantly associated with higher histological grade ($p = 0.020$). Sixty percent of tumors presented with low histological grade showed low density of TILs. By

Table 1
Correlation analyses between the presence of stromal TILs and clinicopathologic parameters in 96 primary breast cancer patients

	Number of patients (%)	TILs			p
		Low (< 10%)	intermediate (10–40%)	High (⩾ 40%)	
Age (years)					
⩽ 50	44 (45.8)	24 (54.5)	17 (38.6)	3 (6.8)	0.773
> 50	52 (54.2)	24 (46.2)	24 (46.2)	4 (7.6)	
Tumor size (mm)					
⩽ 20	35 (36.5)	16 (45.7)	18 (51.4)	1 (2.9)	0.372
21–50	56 (58.3)	30 (53.6)	21 (37.5)	5 (8.9)	
> 50	5 (5.20)	2 (40.0)	2 (40.0)	1 (20.0)	
Grade					
II	40 (41.7)	24 (60.0)	12 (30.0)	4 (10.0)	0.020
II	32 (33.3)	9 (28.1)	16 (50.0)	7 (21.9)	
III	24 (25.0)	6 (25.0)	11 (45.8)	7 (29.2)	
Nodal status					
Negative	44 (45.8)	23 (52.3)	17 (38.6)	4 (9.1)	0.736
Positive	52 (54.2)	25 (48.1)	24 (46.2)	3 (5.7)	
HER2					
Negative	56 (58.3)	32 (57.1)	20 (35.7)	4 (7.2)	0.216
Positive	40 (41.7)	16 (40.0)	21 (52.5)	3 (7.5)	
ER					
Negative	28 (29.2)	7 (25.0)	16 (57.1)	5 (17.9)	0.001
Positive	68 (70.8)	41 (60.3)	25 (36.8)	2 (2.9)	
PR					
Negative	42 (43.7)	15 (35.7)	22 (52.4)	5 (11.9)	0.029
Positive	54 (56.3)	33 (61.1)	19 (35.2)	2 (3.7)	
Stage					
I	12 (12.5)	5 (41.7)	7 (58.3)	0	0.346
II	63 (65.6)	30 (47.6)	26 (41.3)	7 (11.1)	
III	21 (21.9)	13 (61.9)	8 (38.1)	0	

contrast, more than 70% of the poorly differenced breast tumors (grade II and III) have higher levels of lymphocytes infiltration. Moreover, significant correlations were recorded for both ER ($p = 0.001$) and PR ($p = 0.029$) status in breast cancer. Seventy-five percent among ER negative breast tumors showed intermediate to high level of lymphocytes infiltration while in most ER positive tumors (60.3%), the percentage of stromal TILs was low (< 10%). Similar to ER status, PR positive tumors tend to have less lymphocytes infiltration, while significantly higher density of stromal TILs was observed in the PR negative breast cancer (64.3%). No significant association was identified between HER2 overexpression and stromal TILs (Table 1).

3.2. Both stromal lymphocytes infiltration and β-catenin expression differs between the molecular subtypes of breast cancer

Breast cancer has been classified into four molecular subtypes: luminal types A, luminal types B, HER2-enriched and triple-negative (TNBC) as previously described [38]. According to the immunohistochemical report, tumor specimens included in this study consist of 41 (42.7%) luminal type A, 20 (20.8%) luminal type B, 19 (19.8%) HER2-enriched subtype and 16 (16.7%) triple-negative breast cancer. As shown in Table 2, stromal TILs significantly differed between subtypes of breast cancer ($p = 0.004$). 77.8% of triple-negative breast cancer showed intermediate to high infiltration, which is close to HER2-enriched subtype (73.8%), but much higher than luminal type A (34%) and B (52.4%). These data indicated elevated stromal lymphocytes infiltration in HER2-enriched and TNBC subtypes of breast cancer, which is partly in agreement with our previous findings that the ER/PR negative breast cancers tend to have higher density of TILs.

It has been reported that Wnt/β-catenin signaling is upregulated in TNBC [39]. The results showed that cytoplasmic enrichment of β-catenin in tumor cells was significantly associated with biological subtypes of breast cancer ($p = 0.000$). Among TNBC, 81.2% of tumor specimens showed positive staining of β-catenin in the cytoplasm. 73.7% of HER2-enriched breast cancers were cytoplasmic β-catenin positive, while in luminal A and luminal B subtypes, the percentages were significantly lower (56.1% and 55.0%, respectively), indicating that oncogenic Wnt/β-catenin signaling is also upregulated in HER2-enriched breast cancer and TNBC (Table 3).

Table 2
Comparison of stromal lymphocyte infiltration among molecular subtypes of breast cancer

Subtype	Number of patients (%)	TILs			p
		Low (< 10%)	Intermediate (10–40%)	High (⩾ 40%)	
Luminal A	41 (42.7)	27 (66.0)	13 (31.9)	1 (2.1)	0.004
Luminal B	20 (20.8)	9 (47.6)	10 (47.6)	1 (4.8)	
HER2$^+$	19 (19.8)	5 (26.3)	12 (63.2)	2 (10.5)	
TNBC	16 (16.7)	4 (22.2)	7 (44.5)	5 (33.3)	

Table 3
Comparison of β-catenin expression among molecular subtypes of breast cancer

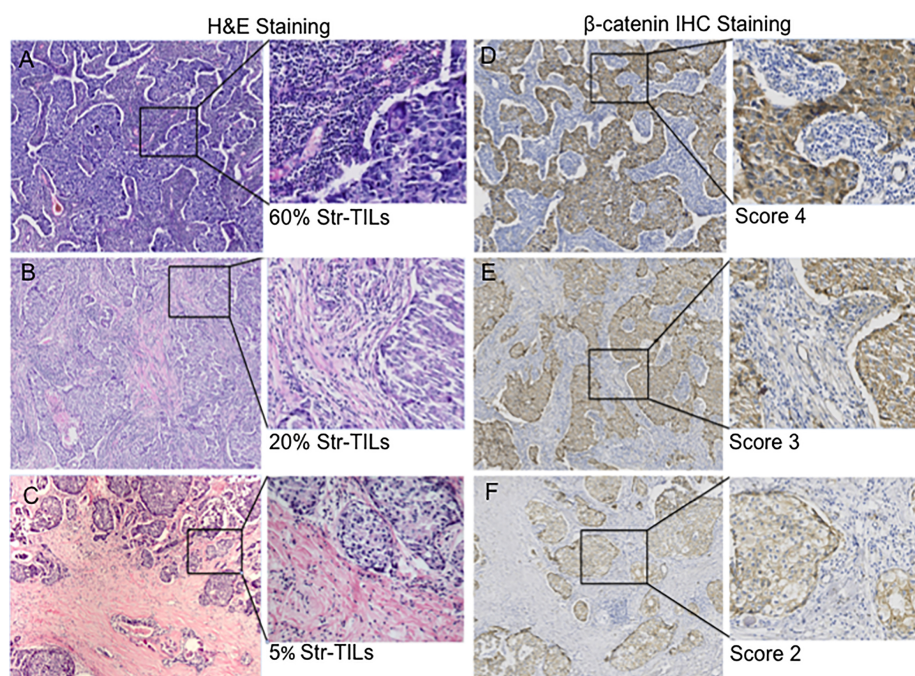| Subtype	Number of patients (%)	Nuclear β-catenin		p	Cytoplasm β-catenin		p
		Negative	Positive		Negative	Positive	
Luminal A	41 (42.7)	18 (43.9)	23 (56.1)	0.051	18 (43.9)	23 (56.1)	0.000
Luminal B	20 (20.8)	10 (50.0)	10 (50.0)		9 (45.0)	11 (55.0)	
HER2$^+$	19 (19.8)	8 (42.1)	11 (57.9)		5 (26.3)	14 (73.7)	
TNBC	16 (16.7)	5 (31.3)	11 (68.7)		3 (18.8)	13 (81.2)	

Fig. 3. Correlation between TILs and β-catenin in breast cancer. Consecutive sections were used for immunohistochemical detection of TILs distribution and β-catenin expression in breast cancer specimens, and cells were evaluated in the same chosen area of the serial sections. Representative images (A–C) show different levels of lymphocyte infiltration in tumor stroma by H&E staining. TILs are reported as the percentage of area occupied by mononuclear inflammatory cells over total intratumoral stromal area, within the borders of the invasive tumor. Corresponding sections were stained with β-catenin antibody by standard IHC techniques (D–F). Scores shown are final staining scores defined according to the H score of cytoplasmic β-catenin expression.

3.3. High levels of TILs are associated with β-catenin overexpression in breast cancer

In our study, consecutive sections were used for immunohistochemical detection of TILs distribution and β-catenin expression in breast cancer specimens, and cells were evaluated in the same chosen area of the serial sections. Representative figures (Fig. 3) showed that tumors with higher lymphocytes infiltration also overexpressed β-catenin, while in TILs low tumors, β-catenin was low as well, indicating a positive association between TILs and β-catenin. We then performed Kruskal-Wallis test to address correlation between β-catenin expression and stromal lymphocytes

Table 4
Correlation analyses between presence of stromal TILs and β-catenin expression in breast cancer

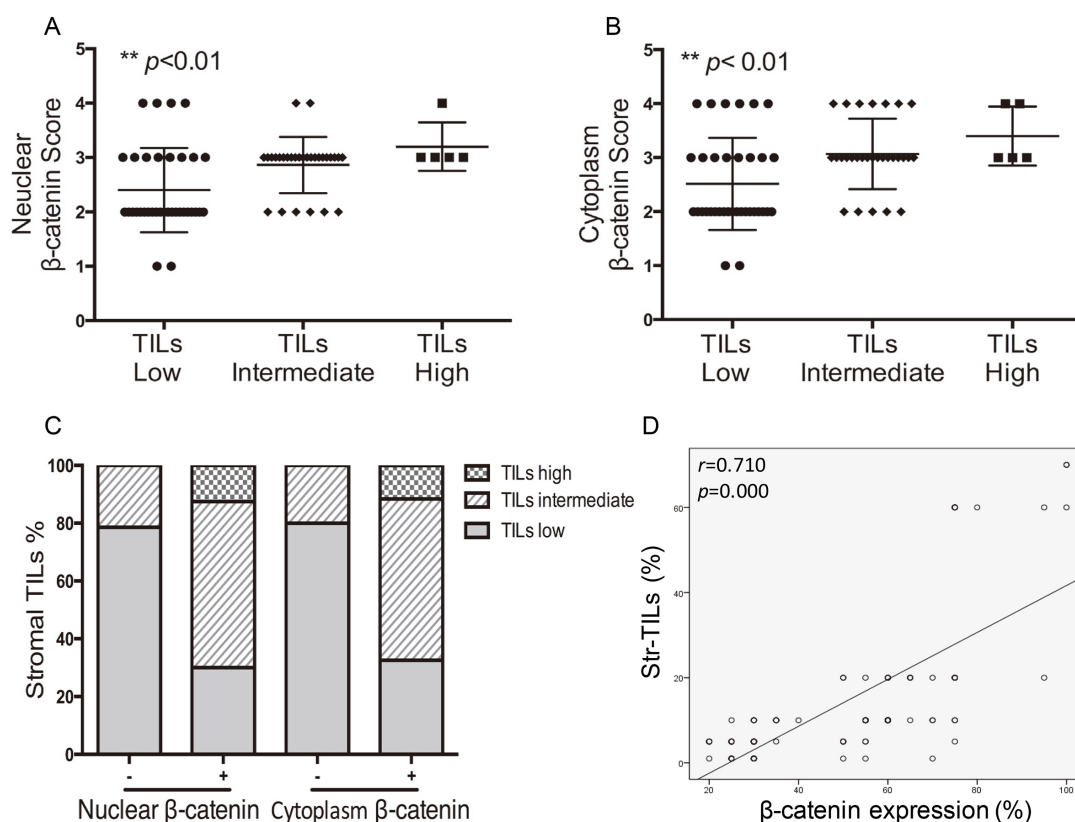|	Nuclear β-catenin		p	Cytoplasm β-catenin		p
	Negative	Positive		Negative	Positive	
TILs Low (< 10%)	33 (68.7)	15 (31.3)	0.000	28 (58.3)	20 (41.7)	0.000
TILs Intermediate (10–40%)	9 (22.0)	32 (78.0)		7 (17.1)	34 (82.9)	
TILs High (⩾ 40%)	0	7 (100)		0	7 (100)	

Fig. 4. Correlation of stromal TILs with β-catenin expression in 96 breast cancer patients. Patients were grouped into 3 main categories, TILs Low (< 10%), TILs Intermediate (10–40%) and TILs High (⩾ 40%) based on the percentage of TILs in tumor stroma. $p < 0.01$ represents significant correlations between the level of stromal lymphocytes infiltration and both nuclear β-catenin (A) and cytoplasm β-catenin (B) expression. Correlation analysis between stromal TILs with β-catenin expression by using the positive/negative cutoff of β-catenin expression instead of β-catenin expression scores (C). Pearson Correlation test were applied for evaluating the correlation between stromal TILs and β-catenin expression (D).

infiltration in breast cancer. As shown in Fig. 4A and B, both nuclear and cytoplasm β-catenin expressions were significantly associated with the level of lymphocytes infiltration in breast cancer ($p < 0.01$). When we applied the- positive/negative cutoff of β-catenin instead of β-catenin scores, the results again confirmed that tumors with β-catenin overexpression tend to have higher level of lymphocytes infiltration (Fig. 4C and Table 4). Moreover, there was a linear correlation between β-catenin expression and TILs level ($r = 0.710$, $p < 0.001$) as shown by Pearson correlation analysis (Fig. 4D).

3.4. Association of CD8+ and FOXP3+ TIL subsets infiltration with β-catenin expression in breast cancer

We further assessed the association of two TIL subsets, CD8+ and FOXP3+ T cells with β-catenin expression in all the 96 breast cancer samples. Both CD8+ T cells ($r = 0,682$, $p = 0.000$) and FOXP3+ Tregs ($r = 0.450$, $p = 0.000$) infiltration were associated significantly with stromal TILs in breast cancer (Fig. 5A and B). Pearson correlation revealed a linear correlation between CD8+ T cells ($r = 0.577$, $p =$

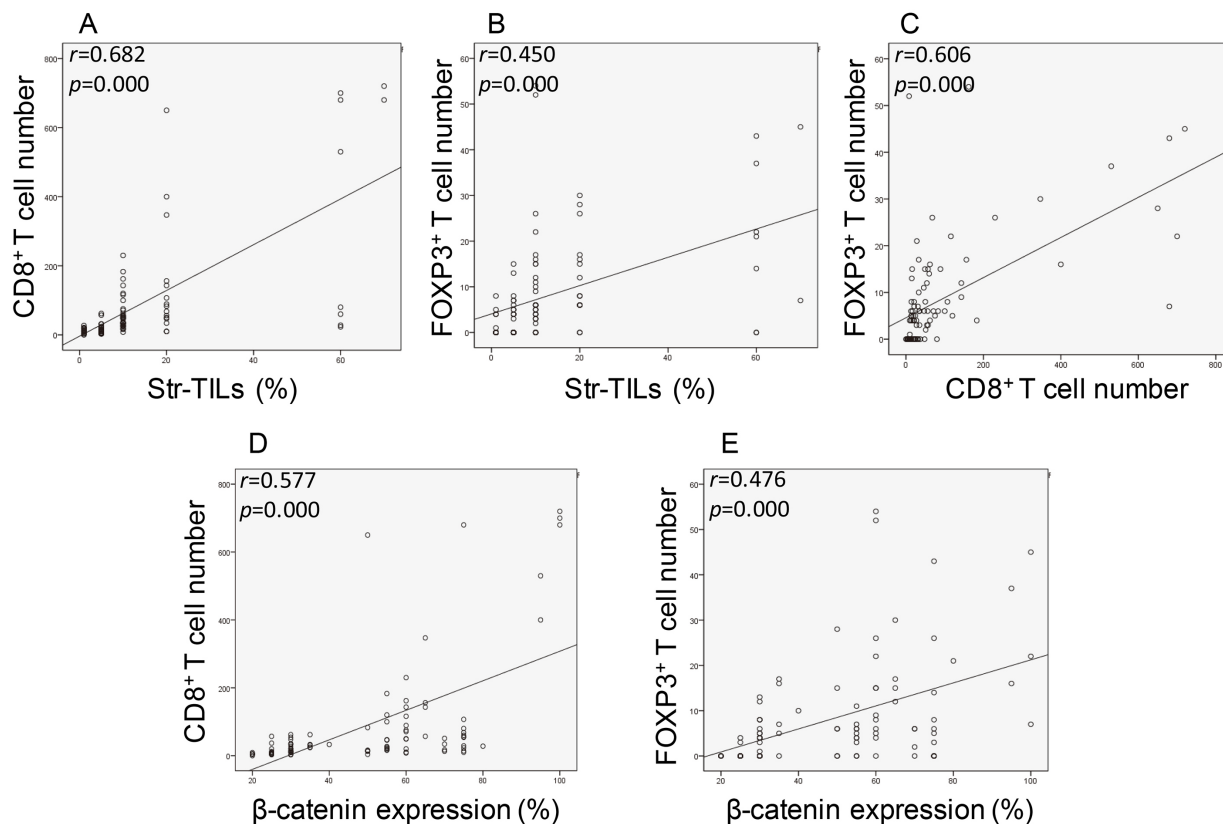Fig. 5. Association of TIL subsets infiltration with β-catenin expression in breast cancer. Correlation of stroma TILs with CD8$^+$ T cells (A) or FOXP3$^+$ T cell (B) infiltration; correlation between CD8$^+$ T cells and FOXP3$^+$ T cell infiltration (C) and the correlation of β-catenin expression with CD8$^+$ T cells (D) or FOXP3$^+$ T cell (E) were analyzed by Pearson Correlation test.

0.000) /FOXP3$^+$ Tregs ($r = 0.476$, $p = 0.000$) and the expression of β-catenin by tumor cells (Fig. 5C and D). Intriguingly, the presence of FOXP3$^+$ Tregs in tumor stroma was positively associated with the infiltration by CD8$^+$ T cells ($r = 0.606$, $p = 0.000$) (Fig. 5E).

4. Discussion

TILs that interact with other immune infiltrates in the tumor microenvironment are mainly responsible for intratumoral immune responses. Detection of TILs by immunohistochemical techniques may be an indicator of immune responses to cancer development. In addition, presence of TILs shows predictive value for patients' responses to neoadjuvant chemotherapy and can potentially help oncologists to identify the subgroup of patients that will receive greater benefit from the therapy [40,41]. Rather than showing clinical significance among overall breast cancer population, the prognostic or predictive values of TILs seems to be restricted

for certain molecular subtypes of breast cancer, mainly TNBC and HER2-enriched subtypes [42,43]. Our clinicopathological data revealed significant correlations between ER/PR status and stromal TILs. ER/PR negative BC i.e. TNBC or HER2-positive BC tends to have higher level of lymphocytes infiltration, while in luminal type A and B, the percentage is much lower. These results are partly consistent with previous data form other studies [5,8] showing that the clinical value of TILs should be emphasized in hormone receptor negative BC, i.e. TNBC and HER2$^-$ enriched BCs. We suggest that not only using hormone receptors for defining cancer subtypes, further research into how hormone activity may affect lymphocytes recruitment in BC is warranted.

β-catenin is the central player in canonical Wnt/β-catenin pathway. It is mainly involved in regulation of cell-cell adhesion and transcription for a wide rage of genes. In the absence of Wnt signaling, the β-catenin protein is constitutively degraded by the β-catenin destruction complex. When activated by Wnt

ligands, co-activation of the Frizzled and low-density lipoprotein receptor-related proteins (LRP) receptors induces the inhibition of the destruction complex and the consequent stabilization of β-catenin in the cytoplasm. β-catenin eventually translocates in nuclear, where it modulates expression of a broad range of target genes [44]. β-catenin accumulates in various malignant tumors, which is related to abnormal activation of the Wnt/β-catenin pathway during carcinogenesis and tumor progression [45,46]. Alterations of Wnt/β-catenin signaling also present in more than 60% of breast cancer and may predict unfavorable disease outcome [47].

Wnt/β-catenin pathway provides important proliferative and maintenance signals, which regulate self-renewal of hematopoietic stem cells and the development of lymphocytes [11,48–51]. Later on, increasing studies indicated that Wnt/β-catenin signaling exerts significant effects on tumor immune system. It induces immune suppression and resistance by regulating some key cytokines [17] and the function of immune cells [52]. In melanoma, tumour-intrinsic activation of β-catenin signalling results in T-cell exclusion and resistance to anti-PD-L1/anti-CTLA-4 monoclonal antibody immunotherapy [21]. These findings suggested that deregulation of Wnt/β-catenin pathway in malignant tumors may contribute to cancer immune evasion and resistance to immunotherapies.

By IHC staining of β-catenin in breast cancer specimens, we first report an association between Wnt/β-catenin signaling and lymphatic infiltration in human breast cancer. Cytoplasmic and/or nuclear accumulation of β-catenin in tumor nest was observed in all the 96 invasive breast cancer samples. By consecutive section staining, we noticed that rather than preventing T cell infiltration into tumor site, overexpression of β-catenin in cancer cells was associated with higher TILs density in the stroma. Moreover, a linear correlation between β-catenin expression and TILs level was identified ($r = 0.710$, $p < 0.001$). These results are surprisingly inconsistent with current knowledge reported in melanoma [21] . This can be partly explained by the fact that Wnt-β-catenin pathway may function differently depending on the distinct biological backgrounds of cancers. Besides, comparative gene expression profiling instead of IHC staining for TILs was used in their study, which may lead to different results as well. However, our observations again raised the question whether tumor intrinsic Wnt-β-catenin signaling only displays unfavorable effects on anti-tumor immune response. According to studies of Forget et al. [24] and

Gattinoni et al. [49], Wnt/β-catenin signaling plays a key role in the maintenance of stemness and promoted T_{SCM} subset in mature memory CD8$^+$ T cells from both TILs and the periphery, indicating that the function of Wnt/β-catenin pathway in vivo may be beneficial for the improvement of adoptive immunotherapies. Since the H&E staining method only locates mononuclear TILs but is limited in revealing the functional status of lymphocytes, further investigations concerning the biology characteristics of human TILs and its correlation with Wnt/β-catenin activity are required.

Different subgroups of the immune complex within tumor site may exert diverse functions. For example, DCs, Th1 CD4$^+$ T cells and cytotoxic CD8$^+$ T cells are found to be involved in anticancer immunosurveillance. In contrast, Th17 CD4$^+$ T cells and FOXP3$^+$ CD4$^+$ regulatory T cells (Tregs) function in the context of protumorigenic inflammation [54]. In recent years, the bulk of studies suggested that distinct infiltrating cell types have distinct prognostic and potentially predictive significance [54]. In BC, high level of CD8$^+$ lymphocyte infiltration indicates better prognosis of patients, while high density of FOXP3$^+$ Tregs are often associated with poor survival outcomes [42]. These findings suggest the possibility that Wnt/β-catenin pathway may function differently on these two TIL subsets. We found that in all the 96 specimens, densities of CD8$^+$ T cells are much higher than FOXP3$^+$ lymphocytes. Both CD8$^+$ and FOXP3$^+$ TILs densities were positively correlated with total stromal TILs ($r = 0.682$, $P < 0.001$; $r = 0.450$, $P < 0.001$). West et al. [55] argued that despite the well-accepted fact that FOXP3$^+$ and CD8$^+$ T cells function contradictorily during anti-tumour immune responses, the prognostic value of FOXP3$^+$ TILs for breast cancer is dependent of the presence of CD8$^+$ TILs. Notably, our results indicated that FOXP3$^+$ TILs number is significantly associated with the total CD8$^+$ infiltrates in tumor stroma ($r = 0.606$, $P < 0.001$), which is consistent with the previous report [52]. It is unclear at present why CD8$^+$ TILs still exert anti-tumor effects and are associated with better prognosis when co-infiltrating with a large number of FOXP3$^+$ Tregs. According to some in vitro models, Tregs require close contact with target cells to induce immune suppression [56]. Besides, the interactions of TILs with tumor cells and stromal cells within tumor microenvironment are extremely complicated. When using CD25 as the molecular marker of Tregs, Prat et al. [57] demonstrated that markers of dendritic cell activation, inflammatory cytokine production and CD8$^+$ T-cell cytotox-

icity were enriched in CD25-high breast tumors, suggesting a complex of anti-tumour immunity can be active in breast tumors despite the presence of Tregs. In our case, the analysis of Tregs-CD8$^+$ TILs interaction within tumor site is demanded in order to interpret the functional status of TILs. And looking into multiple components of TILs instead of focusing on certain lymphocytes should be considered. At last, we analyzed the correlation between CD8$^+$/FOXP3$^+$ TIL subsets and tumor intrinsic β-catenin over-expression. Based on our findings, it can not be concluded whether Wnt/β-catenin pathway functions differently on these two TIL subgroups, since both the number of CD8$^+$ T cells ($r = 0.577$, $P < 0.001$) and FOXP3$^+$ T cells ($r = 0.476$, $P < 0.001$) are positively associated with abnormal β-catenin expression in breast cancer. Moreover, our results did not support the report that activation of Wnt/β-catenin signaling excluded T-cell infiltration in tumor [21]. Nevertheless, our findings demonstrated a significant association of Wnt/β-catenin pathway with total TILs and two TIL subgroups in breast cancer, indicating that Wnt/β-catenin pathway is potentially involved in tumor immunosurveillance and anti-tumor immunity. Further studies are demanded to verify these findings and to explore the underlined mechanisms.

To sum up, our results demonstrated that lymphocytes infiltration in tumor stroma varies with hormone receptor status, tumor grade and different molecular subtypes of breast cancer. Both stromal lymphocytes infiltration and β-catenin expression were more enriched in TNBC and HER2 positive BCs. Rather than excluding lymphocytes infiltration as reported in melanoma, we found that high levels of TILs were associated with β-catenin overexpression in BC. And both the number of CD8$^+$ CTLs and FOXP3$^+$ T cells were positively associated with abnormal β-catenin expression. Further studies to explore the effects of Wnt/β-catenin pathway on breast cancer immunity and the functional status of CTLs and Tregs in different breast cancer subtypes will lead us to better understand tumor-TILs interaction mechanisms and provide therapeutic targets for the improvement of immunotherapy of breast cancer.

Acknowledgments

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