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Original article

Peripheral cytotoxic T lymphocyte predicts first-line progression free survival in HER2-positive advanced breast cancer

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ABSTRACT

Background: The role of peripheral blood lymphocyte (pBL) in breast cancer has long been studied. However, the predictive role of pBL in advanced breast cancer (ABC) is poorly understood.
Methods: A total of 303 patients with ABC were consecutively recruited at our center between January 2015 and September 2019. At baseline, pBL subtypes were detected in all patients with 229 blood samples available for circulating tumor DNA (ctDNA) detection. pBL was analyzed through flow cytometry. ctDNA-based gene mutations were detected using next generation sequencing. The cutoff value of pCTL was estimated by X-tile software. Progression free survival (PFS) was estimated by Kaplan-Meier curve and Cox hazard proportion regression model, with difference detection by log-rank test.
Results: Median follow-up time of the study was 21.0 months. The median age of diagnosis was 52.0 years. Among the pBL subtypes, only pCTL level was found predictive for PFS in the HER2+ patients whom received anti-HER2 therapy (13.1 vs. 5.6 months, $P = 0.001$). However, the predictive role of pCTL was not found in HR-positive ($P = 0.716$) and TNBC ($P = 0.202$). pCTL high associated with suppressive immune indictors including lower CD4/CD8 ratio ($P = 0.004$) and high level of Treg cell ($P = 0.004$). High occurrence of FGFR1 amplification which has been reported as immune suppressor was also found in HER2+ patients with pCTL high (22.2% vs. 4.3%, $P = 0.048$).
Conclusions: Higher pCTLs level associated with shorter PFS and FGFR1 mutation in HER2+ ABC patients.

1. Introduction

Breast cancer is the leading cause of malignancy related death in women [1]. Various factors contribute to breast cancer patients' outcome including age, molecular subtype, tumor grade and stage, and treatment. The clinical relevance of the host immune system in breast cancer has long been studied, especially the role of tumor-infiltrating lymphocytes (TILs). Previous studies showed that TILs can be used as a predictor of response to neo-adjuvant chemotherapy in breast cancer [2,3]. Moreover, several randomized controlled trials revealed that TIL levels have a different prognostic efficacy in various breast cancer subtypes [4,5]. Despite the clinical importance of TILs, metastases from breast cancer have been shown to have a low immune infiltration which undermines the potential prognostic and predictive role of TILs in advanced breast cancer (ABC) patients [6]. Another limitation of TILs in predicting clinical outcome is the spatial heterogeneity, which is currently a main obstacle for manual TIL assessment [3]. Distribution of TILs in a tumor can be affected by many factors such as tumor growth patterns or histological type [7]. For example, the TILs score can be completely different between a tumor with solid growth pattern and a tumor with dissociative growth pattern [4]. Although the

Abbreviations: ABCs, advance breast cancer patients; cfDNA, cell free DNA; ctDNA, circulating tumor DNA; DFS, disease free survival; gDNA, genomic DNA; HR, hormone receptor-positive; IHC, immunohistochemistry; OS, overall survival; pBL, peripheral blood lymphocyte; pCTLs, peripheral $CD8^+$ cytotoxic T lymphocytes; PFS, progression free survival; RECIST, Response Evaluation Criteria in Solid Tumors; TNBC, triple-negative breast cancer; TILs, tumor-infiltrating lymphocytes.

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international guidelines for the evaluation of TILs were published in 2014, a standardized TILs scoring system with no controversy has not been well developed. Finally, the use of TILs as a predictor is limited for inaccessible metastases such as those in marrow and brain.

Peripheral blood lymphocytes (pBLs) have shown a great potential as a predictor of outcome because of the advantages in minimal invasiveness, dynamic monitoring and high homogeneity. The correlation between pBL count and clinical outcome in ABC patients was first reported in 1976 [8] and later confirmed by two studies showing an associated risk of recurrence [9,10]. Moreover, certain peripheral lymphocyte subtypes such as $CD3^+$ and $CD4^+$ were found to have predictive and prognostic value regarding the survival of patients with ABC [11]. A recent study showed that in patients with ABC who received adoptive T-cell therapy, high level of peripheral suppressor T cells (CD8+/CD28-) was associated with shorter progression-free survival (PFS) and overall survival (OS) [12]. Although, studies have been exploring the predictive value of pBLs in patients with ABC, the progress in this area is still limited. The main obstacle to understand the predictive mechanism of pBL lies in the lack of molecular evidence.

The primary aim of this study was the assessment of the association between different pBL subtypes and PFS in patients with ABC who received first-line of therapy. The molecular features along with the predictive value of certain pBL subtypes were also explored by detecting circulating tumor DNA (ctDNA).

2. Material and methods

2.1. Patient enrollment

All procedures involving human participants met with the criterion of the Peking University Cancer Hospital ethical committee (Approval No. 2016KT47). First-line ABC patients were recruited at Peking University Cancer Hospital between January 2016 and September 2019. The criteria for enrollment were as followed: (1) patient aged 18 years or older; (2) pathologically confirmed recurrent and metastatic breast cancer; (3) no anti-tumor therapy prior to enrollment; (4) eligible to receive standard chemotherapy or target therapy; (5) expected survival time of 3 months or beyond; and (6) provision of written and informed consent. Exclusion criteria included the following: (1) any microbial infection especially for cytomegalovirus; (2) autoimmune disease; (3) concurrent hematological disorders and other malignancies; and (4) patients who demanded withdrawal from the study. After recruitment, patients were contacted for telephone interviews to collect data on progression and survival status until September 30, 2019. Clinical response was evaluated after two or three therapeutic cycles according to Response Evaluation Criteria in Solid Tumors (RECIST) criteria. Estrogen Receptor (ER) and Progesterone Receptor (PgR) positivity was evaluated by immunohistochemistry (IHC), using antibodies against ER (Immunotech, Marseilles, France) and PgR (Novocastra Laboratories, Newcastle, UK). IHC staining against HER2 (Abcam PLC, Cambridge, UK) was scored according to intensity as 0, 1+, 2+ or 3+; sample with a scores of 0–1+ was considered HER2−, while sample with a score of 3+ as HER2+. HER2 amplification of 2+ samples were confirmed by FISH.

2.2. Patients follow-up

For each enrolled patients, one post-treatment follow-up and then routine outpatient follow-up every 3 months for imaging based medical assessments to monitor disease progression. The primary endpoint of this study was PFS. PFS was defined from start of therapy to progression of disease or last date of follow-up. Patients alive without an event as of the PD date were censored at last study follow-up date (September 1, 2019). Treatment efficacy was evaluated by diagnostic imaging per Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 [13].

2.3. Peripheral blood T-lymphocyte subtype detection

Peripheral blood (4 ml) was obtained from each patient. Whole blood (200 μl) was incubated with primary antibody in the dark for 10 min at room temperature, and then samples went through hemolysis for another 10 min. Samples were then centrifuged for 5 min at 1300 g at room temperature, and the supernatant was removed. Finally, samples were re-suspended in 500 μl PBS and subjected to flow-cytometric analysis. Primary antibodies included CD3-PC5/CD4-FITC/CD8-PE (IM1650), CD3-FITC/(CD16+/CD56)-PE (A07735), CD (14 + 16)-FITC/CD85k (ILT3)-PE/CD33-PC5 (A23413), CD4-FITC (A007750), CD8-FITC (A07756), CD19-PC5 (A07771), and CD25-PE (A07774) (all from Beckman Coulter). Level of T lymphocyte subtype was expressed as percentage of the total lymphocytes. Total lymphocytes were selected according to physical characteristics including volume size and transmissivity. Flow cytometry was performed using Beckman-Coulter FC500 (Beckman Coulter, Inc., CA., US) and CXP analysis software (Beckman Coulter, Inc., CA., US). Peripheral cytotoxic T lymphocytes (pCTLs) were identified as $CD8^+$ and $CD28^+$. Each analysis included 10000 gated events.

2.4. Tumor infiltrating lymphocytes (TILs) evaluation

Qualified pathologists performed the evaluation of TILs on full whole slide sections of the hematoxylin & eosin stain of needle biopsies obtained from HER2-positive ABCs. TILs were assessed by use of the guidelines of the international TIL working group [4]. Briefly, stromal TILs were measured as percentage of immune cells in stromal tissue within the tumor that showed a mononuclear immunological infiltrate. The number of TILs was analyzed as a continuous measurement; additionally, three predefined categories were used: low TILs (0–10%), intermediate TILs (11–59%), or high TILs (60–100%).

2.5. Circulating cell-free DNA (cfDNA) and genomic DNA (gDNA) extraction

Out of 303 patients, 229 had peripheral blood sample available for ctDNA detection at baseline. Blood samples collected in EDTA tubes were processed within 1 h after collection and stored at −20 °C until analysis. Briefly, blood was centrifuged at 820 g for 10 min. The supernatant was transferred to sterile tubes and, centrifuged at 16000 g for 10 min; the supernatant was stored at −80 °C. cfDNA is extracted by QIAamp circulating nucleic acid kit (Qiagen, Germantown, MD) from plasma samples. Quantity and quality of the purified cfDNA were checked using a Qubit fluorimeter and Bioanalyzer 2100 (UC Davis Genome Center, CA. US). For samples with severe genomic contamination from peripheral blood cells, a bead-based size selection is performed to remove large genomic fragments. gDNA were extracted from matched peripheral blood mononuclear cells available for a subset of the patients. Up to 250 ng gDNAs were enzymatically fragmented, and purified. cfDNA and gDNA were stored at −20 °C and quantified using the LINE1 real-time PCR assay.

2.6. Library preparation, capture and sequencing

Extracted cfDNA (5–30 ng) or fragmented gDNA (40 ng) were subjected to library construction including end-repair dA-tailing and adapter ligation. Ligated library fragments with appropriate

adapters were amplified via PCR. The amplified DNA libraries were then further checked using Bioanalyzer 2100 (UC Davis Genome Center, CA. US) and samples with sufficient yield proceeded to hybrid capture.

Library capture was conducted using biotin labelled DNA probes. In brief, the library was hybridized overnight with the PredicineCARE™ panel (Predicine, Inc., CA, USA) (Supplementary Fig. 1) and bead-captured beads. The unbound fragments were washed away, and the enriched fragments were amplified via PCR amplifications. Similar to library preparation, the purified product was checked on Bioanalyzer 2100 and then loaded into Illumina HiSeq X Ten (Illumina, Inc., San Diego, CA) for next generation sequencing with paired-end 2 × 150bp sequencing kits (Illumina, Inc., San Diego, CA).

2.7. Statistical analysis

The primary objective of the study was to assess the predictive impact of peripheral T lymphocyte subtype detection at baseline regarding PFS in ABC. Secondary objectives were to evaluate the relationship between peripheral T lymphocytes detection at baseline and ctDNA detection at baseline. Clinical data were obtained from the patient electronic medical recoding system. Relationships between ctDNA derived mutational status, Cytotoxic T Cells (CTL) level and clinical characteristics were assessed using the Chi-square test or Fisher exact test, Student t-test or Mann-Whitney *U* test, and Pearson correlation tests accordingly. All analyses were conducted by SPSS 19.0 version software (IBM Inc., NY, US). Missing data were not included into the analysis. The cutoff value of CTL regarding PFS was calculated using the software of X-tile 3.6.1 version reported by Camp RL et al. [14] (Supplementary Fig. 2). Kaplan-Meier survival analysis and log-rank test was used to compare PFS between different patient cohorts. The Cox proportional hazard regression model was used to estimate the hazard ratio (HR) and 95% confidence interval (CI) of the proportion of CTLs in peripheral blood with confounder adjustment. All tests were two-sided with a significance level of 0.05.

3. Results

3.1. Patient characteristics

A total of 303 recurrent and initial stage IV breast cancer patients were enrolled in this study. The median follow-up time was 21.0 months (range: 2.0–46.0 months). The lost to follow-up rate was 14.7%. The clinical characteristics of the patients were presented in Table 1. The median age of diagnosis of present cohort was 52.0 years (range: 27–82). Forty-eight patients were stage IV breast cancer at initial treatment and 255 patients showed recurrent breast cancer. All patients received first-line therapy. Out of 303 ABCs, 109 (36.0%) were triple-negative breast cancers (TNBCs), 97 (32.0%) were hormone receptor positive/HER-2 negative (HR+) and 97 (32.0%) were HER2-positive. Hormonal therapy was used for most HR+/HER2- ABCs (65/97, 67.0%), in which six patients were

Table 1
Clinical characteristics of the study cohort (n = 303).

Clinical characteristics	Number of patients (%)
Age at diagnosis (Range:27–82, median = 52.0)	
≤45 years	114 (37.6)
> 45 years	189 (62.4)
Histological type at primary diagnosis	
Ductal	261 (86.1)
Lobular	19 (6.3)
Others	23 (7.6)
Immunohistochemistry at primary diagnosis	
HR positive & HER2 negative	97 (32.0)
HER2 positive	97 (32.0)
TNBC	109 (36.0)
Tumor grade at primary diagnosis	
I	15 (5.0)
II	200 (66.0)
III	86 (28.4)
Unknown	2 (0.6)
Tumor stage at primary diagnosis	
I	36 (11.9)
II	116 (38.3)
III	96 (31.7)
IV	48 (15.8)
Unknown	7 (2.3)
Visceral metastasis[a]	
Yes	182 (60.1)
No	121 (39.9)
Number of metastatic sites[b]	
Single metastatic site	128 (42.2)
Multiple metastatic site	175 (57.8)
Disease free survival	
≤36.0 months	146 (57.3)
> 36.0 months	109 (42.7)
Regimens of first-line therapy	
Taxane single agent or Taxane based chemotherapy	109 (36.0)
Trastuzumab and/or pertuzumab plus chemotherapy	86 (28.4)
Trastuzumab and/or pertuzumab plus hormonal therapy	11 (3.6)
Hormonal therapy	65 (21.4)
Others	32 (10.6)

[a] Visceral metastasis including brain, liver and lung.
[b] Multiple lesions occurred in the same organ only count once.

also combined with Palbociclib (6/97, 6.1%). All HER2-positive ABCs (97/97, 100.0%) received trastuzumab and/or pertuzumab based therapy. Among them, five patients (5/97, 5.2%) received double-targeted therapy of trastuzumab and pertuzumab. Taxane monotherapy or taxane-based chemotherapy was commonly applied for TNBCs (89/109, 81.7%).

3.2. Peripheral CTL was an independent predictive factor of PFS

We first evaluated the association of conventional clinical characteristics with first-line PFS. In our cohort, DFS (HR: 0.627, 95% CI: 0.471–0.833), IHC of primary tumor (HR: 1.189, 95%CI: 1.016–1.393) and tumor grade at primary diagnosis (HR: 1.920, 95% CI: 1.501–2.455) was associated with PFS. Other factors such as age of diagnosis (HR: 0.966, 95%CI: 0.742–1.258), primary tumor stage (HR: 1.138, 95%CI: 0.989–1.310) and visceral metastasis status (HR: 0.816, 95%CI: 0.628–1.061) were not associated with PFS (Table 2).

We next screened out the association of each pBL subtypes with PFS using the X-tile software. Only peripheral CTL was found to be predictive for PFS with a cutoff value of 13.9% ($\chi^2 = 5.093$, $P = 0.024$) (Supplementary Fig. 2). We set the cutoff value of pCTL as 14.0%, and divided the 303 ABCs into the pCTL low group (≤14.0%) and pCTL high group (>14.0%). Univariate survival analysis showed that the peripheral CTL level at baseline successfully predicted PFS (HR = 0.667, 95%CI: 0.496–0.924, $P = 0.023$) (Fig. 1A). After making adjustment for Disease Free Survival (DFS), IHC and tumor grade, multivariate analysis further confirmed that peripheral CTL at baseline was an independent predictive factor of PFS (9.7 months *vs.* 7.9 months, $P = 0.022$) (Fig. 1B). The gating parameters for pCTL selection are showed in Fig. 1 C.

3.3. Peripheral CT L selectively predicts PFS of HER2-positive subtype

It has been reported that different molecular subtypes of breast cancer possess varying immune features. Thus, we sought to evaluate the predictive value of peripheral CTL in each molecular subtype. Univariate survival analysis revealed that no association between pCTL and PFS was observed in HR-positive (10.9 months *vs.* 13.7 months, $P = 0.716$) and TNBC subgroup (5.9 months *vs.* 6.4 months, $P = 0.202$) (Fig. 2A and B). However, we found that high pCTL (>14.0%) predicted a shorter PFS in HER2-positive patients (13.1 months *vs.* 5.6 months, $P = 0.001$) (Fig. 2C). Multivariate analysis adjusted for DFS and tumor grade also showed that the high pCTL level is a worse predictor for PFS in HER2-positive patients (HR = 0.712, 95%CI: 0.532–0.953, $P = 0.004$) (Fig. 2D). Considering that the possibility of uneven distribution of clinical characteristics might perturb the predictive value of pCTL, we performed a comparison according to pCTL level in the HER2-positive subgroup. The data showed that no significant distributional variation of clinical characteristics, especially for those associated with PFS, was found between pCTL low and pCTL high groups (Table 3).

3.4. Suppressive immune status of pCTL high group

In view of the predictive role of peripheral CTL in HER2-positive ABC, we compared the distribution pattern of different pBL subtypes according to pCTL level. Higher levels of $CD3^+$ T cells ($P = 0.005$), T8 cells ($P = 0.002$) and regulatory T-cells (Treg cell) ($P = 0.004$) were found in the pCTL high group (> 14.0%), and lower

Table 2
Univariate analysis of conventional clinical features at baseline regarding first-line PFS.

Baseline Clinical Characteristics	n	Median PFS±SD	*P* value[a]	HR (95%CI)
Age of diagnosis			0.798	0.966 (0.742–1.258)
≤45 years	114	9.000 ± 1.023		
> 45 years	189	9.000 ± 0.557		
Primary tumor stage			0.070	1.138 (0.989–1.310)
I	36	8.300 ± 2.274		
II	116	10.400 ± 1.528		
III	96	8.200 ± 1.046		
IV	48	8.700 ± 0.872		
Unknown	7			
Primary tumor grades			< 0.001	1.920 (1.501–2.455)
I	15	18.800 ± 2.068		
II	200	10.100 ± 0.700		
III	86	5.800 ± 0.545		
Unknown	2			
Primary immunohistochemistry			0.030	1.189 (1.016–1.393)
HR positive & HER2 negative	97	11.000 ± 0.966		
HER2 positive	97	10.100 ± 0.736		
Triple-negative	109	6.400 ± 1.010		
Disease free survival			0.001	0.627 (0.471–0.833)
≤36.0 months	146	6.900 ± 1.023		
> 36.0 months	109	12.200 ± 1.732		
Visceral metastasis[b]			0.126	0.816 (0.628–1.061)
Yes	121	9.500 ± 0.804		
No	182	8.800 ± 0.613		
Number of metastatic sites[c]			0.390	0.893 (0.689–1.157)
Single metastatic site	128	9.200 ± 0.700		
Multiple metastatic sites	175	8.800 ± 0.563		

[a] *P* value of K-M survival analysis was calculated by log-rank test.
[b] Visceral metastasis includes liver, lung and brain metastasis.
[c] Mutiple lesions occurred in the same organ only count once.

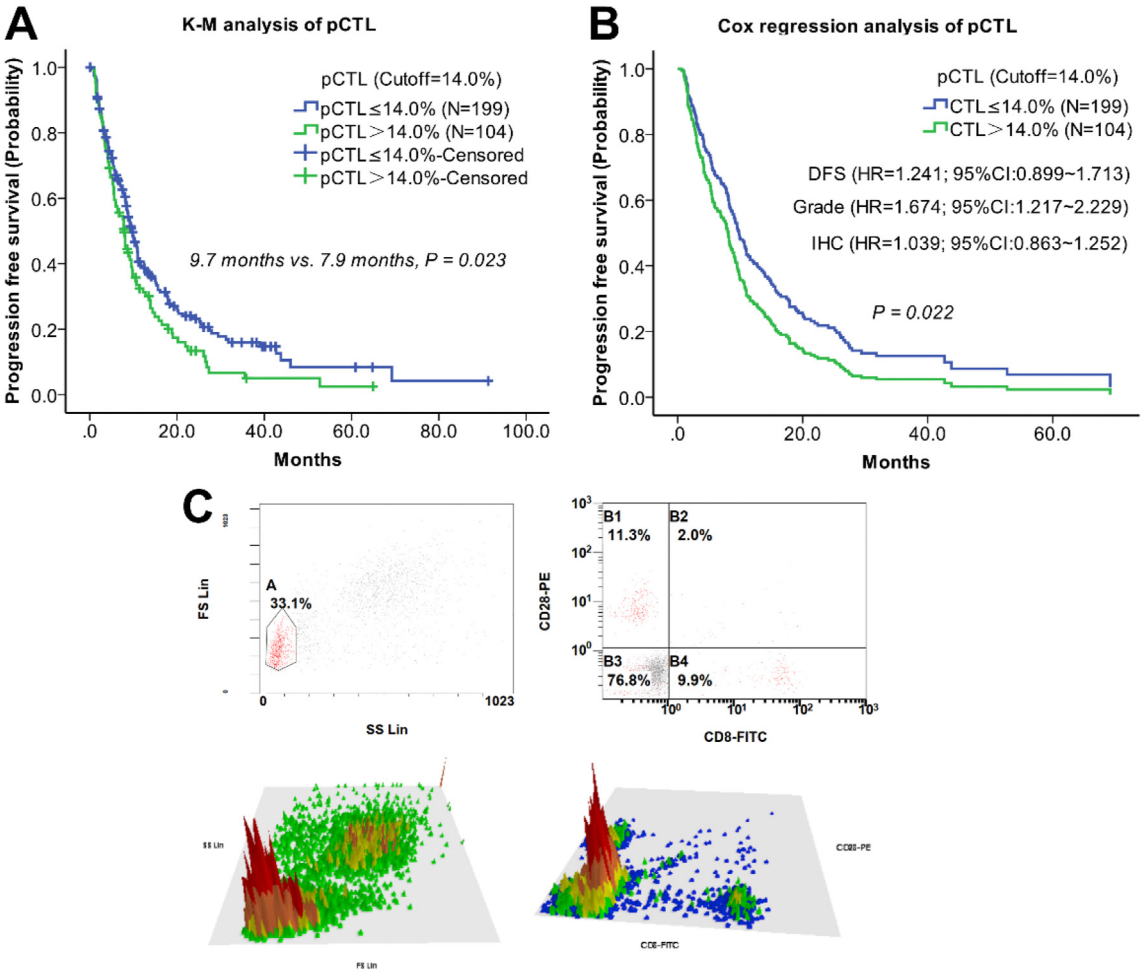

Fig. 1. Univariate and multivariate survival analysis regarding baseline CTL. (A) Kaplan-Meier plot of PFS according to baseline pCTL. (B) Cox-regression plot of PFS according to baseline pCTL. (C) Gates and thresholds for pCTL selection. DFS: Disease free survival; IHC: Immunohistochemistry of primary tumor; Grade: tumor grade at primary diagnosis; pCTL: Peripheral cytotoxic lymphocyte.

CD4+/CD8+ ratio ($P = 0.004$) and suppressor T-cell ($P = 0.003$) were also found in the pCTL high group. The rest of the pBL subgroups did not show correlation with pCTL. Detailed information was listed in Table 4. Meanwhile, we also detected the TILs level of 16 HER2-positive ABCs according to their pCTL level (Fig. 3A and B). In pCTL high group, 1 patients (1/6, 16.7%) were confirmed high TIL, 1 patients (2/6, 33.3%) were intermediate TIL, and 3 patients (3/6, 50.0%) were low TIL. In pCTL low group, 5 patients (5/10, 50.0%) were confirmed high TIL, 2 patients (2/10, 20.0%) were intermediate TIL, and 3 patients (3/10, 30.0%) were low TIL. Although we found that low TIL was more frequently observed in pCTL High group, but the result did not reach statistical significance (16.7% vs. 50.0%, $P = 0.144$) (see Fig. 4) (see Fig. 4).

3.5. Amplified FGFR1 gene and mutation of downstream targets in the pCTL high group

In the HER2-positive subgroup, 64 out of 97 patients had ctDNA detection at baseline. The common single nucleotide variants were in TP53 (66.1%), and PIK3CA (36.3%), and the common copy number variations were in ERBB2 (70.1%) (Fig. 3A). For most single nucleotide variants and copy number variations, no distributional difference was found between pCTL high and pCTL low groups. Notably, three copy number gain mutations and one missense mutation of FGFR1 gene was found in the pCTL high group with only one copy number gain mutation in the pCTL low group (22.2% vs. 4.3%, $P = 0.048$). However, similar data were not found for other FGFR family members including FGFR2 ($P = 1.000$), FGFR3 ($P = 0.553$) and FGFR4 ($P = 0.487$). Subsequently, we detected the mutational frequency of the main downstream genes of FGFR1 pathway using our gene panel. These genes include PIK3CA (35.9%), PIK3R1 (3.1%), KRAS (3.1%), BRAF (6.3%) and NF1 genes (7.8%). We defined any gain-of-function mutation of FGFR1, KRAS, BRAF, PIK3CA and PIK3R1 gene and loss-of-function mutation of NF1 gene as FGFR1 pathway hyper-activation. Mutation of the FGFR1 pathway more frequently occurred in the pCTL high group compared with pCTL low group (77.8% vs. 39.1%, $P = 0.011$). The heatmap of mutational pattern for FGFR1 pathway is shown in Fig. 3B.

4. Discussion

The host immune system has a great impact on the disease course of breast cancer. Dysregulation of cellular and/or humoral immunity gives rise to oncogenesis, tumor metastasis and treatment failure [15]. Several studies in ABC have focused on the relationship between clinical outcome and immune factors such as TILs [3]. However, few studies have paid attention to the predictive value of pBL, and the related mechanism has been unclear. To the best of our knowledge, the present study is the first to report the

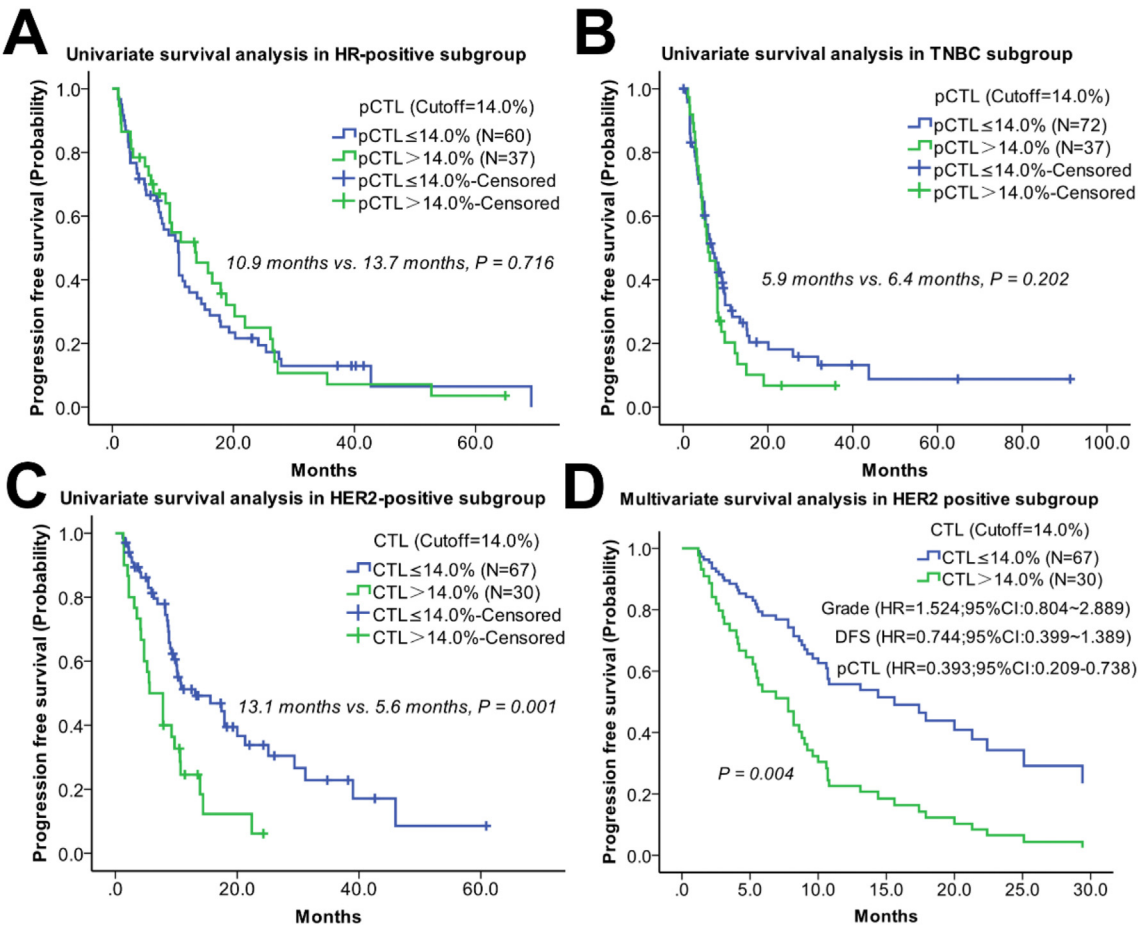

Fig. 2. Predictive value of pCTL in different IHC subgroups. (A) Kaplan-Meier plot of PFS according to baseline pCTL in HR-positive subgroup. (B) Kaplan-Meier plot of PFS according to baseline pCTL in TNBC subgroup. (C) Kaplan-Meier plot of PFS according to baseline pCTL in HER2-positive subgroup. (D) Cox-regression plot of PFS according to baseline pCTL in HER2-positive subgroup. DFS: disease free survival; Grade: tumor grade at primary diagnosis; pCTL: peripheral cytotoxic lymphocyte.

predictive value of peripheral CTLs regarding PFS, especially in HER2-positive patients received anti-HER2 therapy. For TILs, the high level of infiltrating CTLs is usually associated with better survival [16]. Unexpectedly, our data showed that a high level of peripheral CTLs indicated shorter PFS in the HER2-positive subgroup (13.1 months *vs.* 5.6 months, $P = 0.001$) (Fig. 2C). Therefore, we examined why peripheral CTLs were a negative predictor of PFS.

Previous studies found that a low level of CD4/CD8 ratio at baseline was associated with no therapeutic response or tumor progression in breast cancer patients who received neoadjuvant chemotherapy [17,18]. In addition, a low level of CD4/CD8 ratio was also associated with impaired cellular immunity [19] and lymph node involvement [20,21]. Other studies showed that high level of Treg cells contributes to immunosuppressive and poor response to chemotherapy and poor clinical outcomes in ABCs [22–25]. Our data showed that pCTL level (cutoff = 14.0%) was negatively associated with CD4/CD8 ratio and positively associated with Treg cells. Furthermore, we also found that lower TIL level was more frequently observed in patients with pCTL high, although this result did not reach statistical level ($P = 0.144$). This result was consistent with what we found in peripheral blood. Together, these results indicate a possible suppressive immune status in HER2-positive patients with high level of pCTL, which could be part of the reason for the shorter PFS.

The FGFR pathway initiates several downstream pathways that regulate cell proliferation, angiogenesis, migration, and survival in breast cancer [26,27]. FGFR1 is a crucial member of the FGFR family, and FGFR1 mutation was found in approximately 14% of all breast cancer and is associated with worse prognosis among breast cancer patients [28]. We observed a frequent occurrence of FGFR1 mutation in the pCTL high group compared with the pCTL low group (22.2% vs. 4.3%, $P = 0.048$), but no differences were observed in FGFR2 ($P = 1.000$), FGFR3 ($P = 0.553$) and FGFR4 mutations ($P = 0.487$). This result indicated a driver mutation role of FGFR1 in HER2-positive patients with high level pCTL. Notably, dysregulation of FGFR pathway also contributes to immune evasion of breast cancer. Previous studies found that myeloid-derived suppressor cells (MDSCs) could activate Treg cells and inhibit CD8 T cells and nature killer cells, leading to immune evasion in the tumor microenvironment [29–31]. MDSC infiltration and tumor angiogenesis are significantly enhanced during mammary tumorigenesis in MMTV-Wnt1/iFGFR1 bi-genic mice in comparison with MMTV-Wnt1 transgenic mice. Tumor regression and disappearance of MDSCs from the residual mammary gland was found in MMTV-Wnt1/iFGFR1 bi-genic mice treated with BGJ398, an FGFR inhibitor [32]. Similarly, AZD4547, another FGFR inhibitor, also successfully reduced MDSCs in the tumor microenvironment and systemic circulation [33]. Thus, hyper-activation of the FGFR pathway will lead to accumulation of MDSCs in the tumor microenvironment and facilitate the immune evasion of breast cancer cells. In our cohort, mutations were found in FGFR1 pathway genes including FGFR1, PIK3CA, PIK3R1, KRAS, BRAF and NF1 genes. When taking

Table 3
Comparison of clinical characteristics according to pCTL level in HER2 positive subgroup (n = 97).

Clinical characteristics	n	Peripheral CTL		*P* value[c]
		≤14.0% (n = 67)	> 14.0% (n = 30)	
Age at diagnosis				0.479
≤45.0 years	30	19 (63.3%)	11 (36.7%)	
> 45.0 years	67	48 (71.6%)	19 (28.4%)	
Tumor grade at primary diganosis				0.126
I	2	1 (50.0%)	1 (50.0%)	
II	69	52 (75.4%)	17 (24.6%)	
III	24	13 (54.2%)	11 (45.8%)	
Tumor stage at primary diganosis				0.907
I	9	6 (66.7%)	3 (33.3%)	
II	36	25 (69.4%)	11 (30.6%)	
III	28	20 (71.4%)	8 (28.6%)	
IV	21	14 (61.9%)	8 (38.1%)	
Visceral metastasis[a]				0.819
Yes	66	45 (68.2%)	21 (31.8%)	
No	31	22 (71.0%)	9 (29.0%)	
Number of metastatic sites[b]				0.371
Single metastatic site	36	27 (75.0%)	9 (25.0%)	
Multiple metastatic site	61	40 (65.6%)	21 (34.4%)	
Disease free survival				0.616
≤36.0 months	44	30 (68.2%)	14 (31.8%)	
> 36.0 months	31	23 (74.2%)	8 (25.8%)	
Regimens of first-line therapy				0.761
Trastuzumab and/or pertuzumab plus chemotherapy	86	60 (69.8%)	26 (30.2%)	
Trastuzumab and/or pertuzumab plus hormonal therapy	11	8 (72.7%)	2 (27.3%)	
Optimal response of first-line therapy				0.082
CR/PR	38	27 (71.1%)	11 (28.9%)	
SD	34	26 (76.5%)	8 (23.5%)	
PD	19	9 (47.4%)	10 (52.6%)	

[a] Visceral metastasis includes brain, liver and lung.
[b] Mutiple lesions occurred in the same organ only count once.
[c] *P* value was calculated using Chi-square test or Fisher exact test.

Table 4
Peripheral lymphocyte subtypes distribution according to pTCL level in HER2+.

Peripheral lymphocyte subtypes (Phenotype)	pCTL (mean ± SD)		*P*-value[a]
	≤14.0%	> 14.0%	
$CD3^+$ T-cells ($CD3^+$)	60.31 ± 12.59	66.69 ± 8.62	0.005
T4 cell ($CD3^+$ & $CD4^+$)	33.44 ± 9.70	34.26 ± 8.35	0.689
T8 cell ($CD3^+$ & $CD8^+$)	24.30 ± 9.51	30.73 ± 8.21	0.002
$CD4^+/CD8^+$ ratio ($CD4^+/CD8^+$)	1.62 ± 0.81	1.22 ± 0.50	0.004
Natural killer cell ($CD3^-$ & $CD56^+$ & $CD16^+$)	16.88 ± 10.33	13.75 ± 8.69	0.151
Natural killer T-cell ($CD3^+$ & $CD56^+$ & $CD16^+$)	6.70 ± 6.47	8.34 ± 8.14	0.289
B cells ($CD3^-$ & $CD19^+$)	14.80 ± 8.22	12.65 ± 4.73	0.108
Regulatory T-cell ($CD4^+$ & $CD25^+$)	3.03 ± 1.66	4.35 ± 2.11	0.004
Suppressor T-cell ($CD8^+$ & $CD28^-$)	21.46 ± 9.24	15.66 ± 6.92	0.003

[a] *P* value was calculated using Student t-test or Mann-Whitney *U* test.

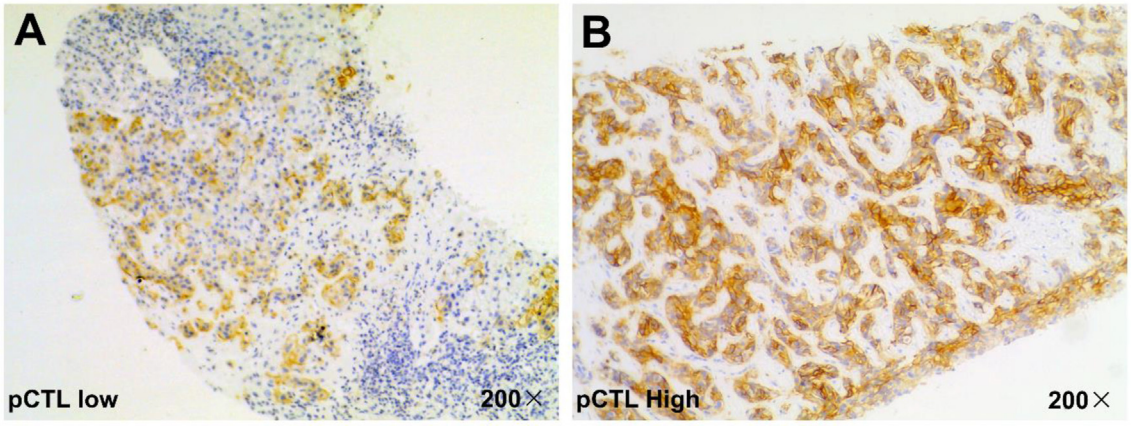

Fig. 3. Immunohistochemistry of ErbB2 in HER2-positive patients with different pCTL level. (A) HER2-positive patient with low level of pCTL. (B) HER2-positive patient with high level of pCTL. Magnification times: 200.

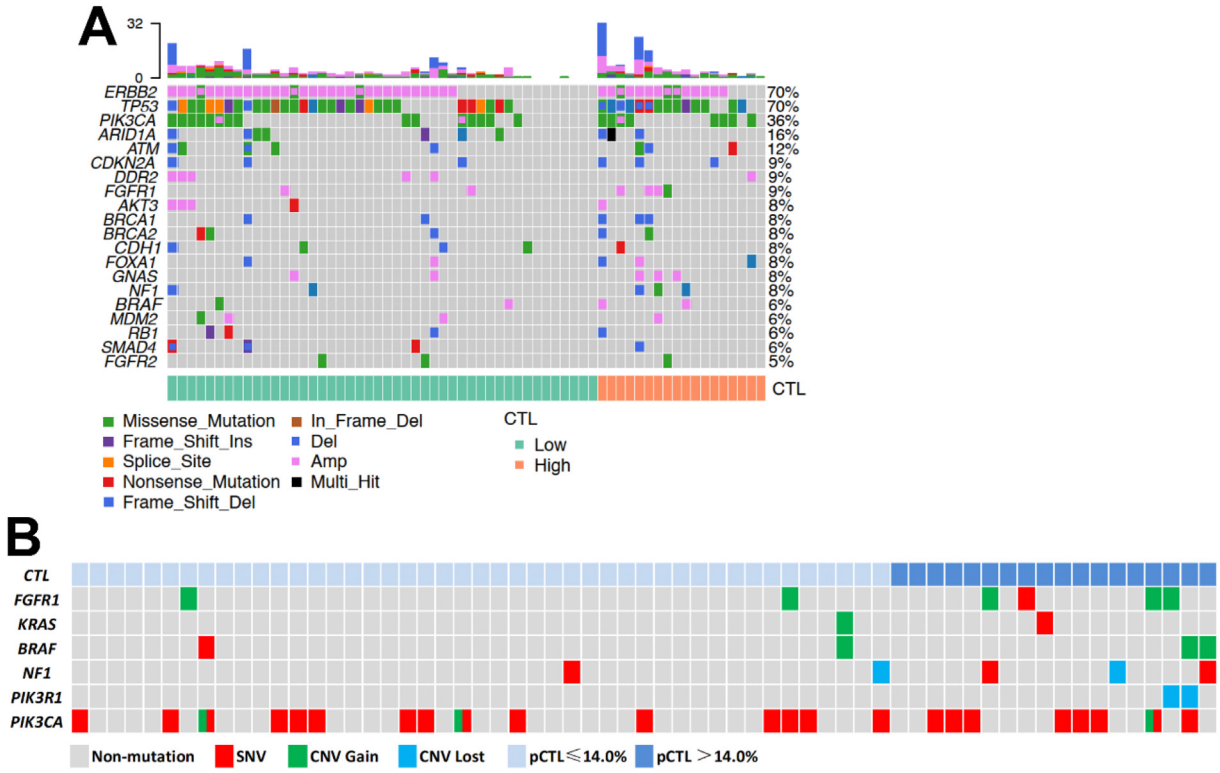

Fig. 4. Mutational landscape of 158 tumor related genes in HER2-positive patients. (A) Mutational distribution of SNVs and CNVs according to pCTL level. (B) Mutational distribution of FGFR1 pathway genes according to pCTL level. SNV: single nucleotide variant; CNVs: copy number variation; pCTL low: ≤14.0%; pCTL high: > 14.0%.

their mutational status as a whole into consideration, we observed a high mutational occurrence of FGFR1 pathway genes in the pCTL high group (77.8% vs. 39.1%, $P = 0.011$). This result indicates a possible immunosuppressive status of the tumor microenvironment in HER2-positive patients with high level of pCTL. This could be, at least, one of the reasons underlying the shorter PFS of patients with high pCTL level. Future work should evaluate the feasibility of combined FGFR inhibitor with anti-HER2 therapy according to pCTL level in HER2-positive patients.

There are several limitations of our study. First, present study was performed in single center using retrospective design and analysis. The records on some clinical parameters were incomplete. Currently, trastuzumab plus pertuzumab is recommended as standard treatment for first line anti-HER2 therapy. However, due to the current medical situation in China, only 6.1% HER2-positive patients of present study cohort received the trastuzumab plus pertuzumab. Thus, a larger prospective study using standard anti-HER2 therapy which focused on the predictive role of pCTL and the mechanism behind was needed in future works. Second, due to the limited number of enrolled patients, an external validation cohort regarding the cutoff value of pCTL in HER2-positive ABCs was not performed in present study. Finally, the dynamic monitoring of pCTL should be considered to better understanding the predictive role of pCTL.

5. Conclusions

Together, our results show that higher pCTL levels were associated with shorter first-line PFS in HER2-positive ABC patients who received anti-HER2 therapy, but no associations were observed with HR-positive and TNBC patients. High levels of pCTL were also associated with FGFR1 mutations in HER2-positive ABC patients. As a minimal invasive approach, evaluation of pCTL level exhibit a great clinical potential in HER2-positive ABC patients.

Declaration of competing interest

The authors declare no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.breast.2020.11.006.

Additional information

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References

[1] Coughlin SS. Epidemiology of breast cancer in women. Adv Exp Med Biol 2019;1152:9–29.
[2] Denkert C, Loibl S, Noske A, Roller M, Muller BM, Komor M, et al. Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. J Clin Oncol 2010;28:105–13.
[3] Savas P, Salgado R, Denkert C, Sotiriou C, Darcy PK, Smyth MJ, et al. Clinical relevance of host immunity in breast cancer: from TILs to the clinic. Nat Rev Clin Oncol 2016;13:228–41.
[4] Salgado R, Denkert C, Demaria S, Sirtaine N, Klauschen F, Pruneri G, et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer:

recommendations by an International TILs Working Group 2014. Ann Oncol 2015;26:259–71.
[5] Dieci MV, Griguolo G, Miglietta F, Guarneri V. The immune system and hormone-receptor positive breast cancer: is it really a dead end? Canc Treat Rev 2016;46:9–19.
[6] Solinas C, Carbognin L, De Silva P, Criscitiello C, Lambertini M. Tumor-infiltrating lymphocytes in breast cancer according to tumor subtype: current state of the art. Breast 2017;35:142–50.
[7] Nawaz S, Heindl A, Koelble K, Yuan Y. Beyond immune density: critical role of spatial heterogeneity in estrogen receptor-negative breast cancer. Mod Pathol 2015;28:766–77.
[8] Franks CR, Williams Y. Prognostic value of peripheral lymphocyte count in hormone therapy of advanced breast cancer. Br J Canc 1976;34:641–4.
[9] Pattison CW, Woods KL, Morrison JM. Lymphocytopenia as an independent predictor of early recurrence in breast cancer. Br J Canc 1987;55:75–6.
[10] Vicente Conesa MA, Garcia-Martinez E, Gonzalez Billalabeitia E, Chaves Benito A, Garcia Garcia T, Vicente Garcia V, et al. Predictive value of peripheral blood lymphocyte count in breast cancer patients treated with primary chemotherapy. Breast 2012;21:468–74.
[11] Yang J, Xu J, E Y, Sun T. Predictive and prognostic value of circulating blood lymphocyte subsets in metastatic breast cancer. Cancer medicine 2019;8:492–500.
[12] Song Q, Ren J, Zhou X, Wang X, Song G, Hobeika A, et al. Circulating CD8(+) CD28(-) suppressor T cells tied to poorer prognosis among metastatic breast cancer patients receiving adoptive T-cell therapy: a cohort study. Cytotherapy 2018;20:126–33.
[13] Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur J Canc 2009;45:228–47.
[14] Camp RL, Dolled-Filhart M, Rimm DL. X-tile: a new bio-informatics tool for biomarker assessment and outcome-based cut-point optimization. Clin Canc Res 2004;10:7252–9.
[15] Stambrook PJ, Maher J, Farzaneh F. Cancer immunotherapy: whence and whither. Mol Canc Res 2017;15:635–50.
[16] Mahmoud SM, Paish EC, Powe DG, Macmillan RD, Grainge MJ, Lee AH, et al. Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. J Clin Oncol 2011;29:1949–55.
[17] Murta EF, de Andrade JM, Falcao RP, Bighetti S. Lymphocyte subpopulations in patients with advanced breast cancer submitted to neoadjuvant chemotherapy. Tumori 2000;86:403–7.
[18] Garcia-Martinez E, Gil GL, Benito AC, Gonzalez-Billalabeitia E, Conesa MA, Garcia Garcia T, et al. Tumor-infiltrating immune cell profiles and their change after neoadjuvant chemotherapy predict response and prognosis of breast cancer. Breast Cancer Res 2014;16:488.
[19] Wang K, Shen T, Siegal GP, Wei S. The CD4/CD8 ratio of tumor-infiltrating lymphocytes at the tumor-host interface has prognostic value in triple-negative breast cancer. Hum Pathol 2017;69:110–7.
[20] Jafarian AH, Tasbandi A, Gilan H, Sheikhi M, Roshan NM. Evaluation of CD30/CD4/CD8 in triple-negative invasive ductal carcinoma of breast in association with clinicopathological prognostic factors. Indian J Pathol Microbiol 2018;61:500–4.
[21] Alam SM, Clark JS, George WD, Campbell AM. Altered lymphocyte populations in tumour invaded nodes of breast cancer patients. Immunol Lett 1993;35:229–34.
[22] Wang Y, Sun J, Zheng R, Shao Q, Gao W, Song B, et al. Regulatory T cells are an important prognostic factor in breast cancer: a systematic review and meta-analysis. Neoplasma 2016;63:789–98.
[23] Perez SA, Karamouzis MV, Skarlos DV, Ardavanis A, Sotiriadou NN, Iliopoulou EG, et al. CD4+CD25+ regulatory T-cell frequency in HER-2/neu (HER)-positive and HER-negative advanced-stage breast cancer patients. Clin Canc Res 2007;13:2714–21.
[24] Banin-Hirata BK, de Oliveira CEC, Losi-Guembarovski R, Ozawa PMM, Vitiello GAF, de Almeida FC, et al. The prognostic value of regulatory T cells infiltration in HER2-enriched breast cancer microenvironment. Int Rev Immunol 2018;37:144–50.
[25] Hashemi V, Maleki LA, Esmaily M, Masjedi A, Ghalamfarsa G, Namdar A, et al. Regulatory T cells in breast cancer as a potent anti-cancer therapeutic target. Int Immunopharm 2020;78:106087.
[26] Lieu C, Heymach J, Overman M, Tran H, Kopetz S. Beyond VEGF: inhibition of the fibroblast growth factor pathway and antiangiogenesis. Clin Canc Res 2011;17:6130–9.
[27] Turner N, Grose R. Fibroblast growth factor signalling: from development to cancer. Nat Rev Canc 2010;10:116–29.
[28] Jafarian AH, Kooshkiforooshani M, Farzad F, Mohamadian Roshan N. The relationship between fibroblastic growth factor receptor-1 (FGFR1) gene amplification in triple negative breast carcinomas and clinicopathological prognostic factors. Iran J Pathol 2019;14:299–304.
[29] Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science 2011;331:1565–70.
[30] Talmadge JE, Gabrilovich DI. History of myeloid-derived suppressor cells. Nat Rev Canc 2013;13:739–52.
[31] Mantovani A, Sica A. Macrophages, innate immunity and cancer: balance, tolerance, and diversity. Curr Opin Immunol 2010;22:231–7.
[32] Holdman XB, Welte T, Rajapakshe K, Pond A, Coarfa C, Mo Q, et al. Upregulation of EGFR signaling is correlated with tumor stroma remodeling and tumor recurrence in FGFR1-driven breast cancer. Breast Cancer Res 2015;17:141.
[33] Liu L, Ye TH, Han YP, Song H, Zhang YK, Xia Y, et al. Reductions in myeloid-derived suppressor cells and lung metastases using AZD4547 treatment of a metastatic murine breast tumor model. Cell Physiol Biochem 2014;33:633–45.