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Original Article

Prognostic impact of tumor-associated neutrophils in breast cancer

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Abstract: Objectives: Neutrophils are the most common type of leukocyte in mammals and play an essential role in the innate immune system and anti-cancer responses. However, recent studies identified the presence of tumor-associated neutrophils (TANs) as a poor prognostic factor. The present study investigated whether relationships exist between TANs and the clinicopathological factors and genetic status of breast cancer. Methods: A total of 196 breast cancer patients with sufficient biopsy, breast-conserving surgery, or mastectomy specimens between 2014 and 2021 in Hokuto Hospital were included. Results: TANs were individually counted in the tumor stroma (TS) and tumor nest (TN). A higher density of TANs in both TS and TN correlated with tumor size (TS $P = 0.010$; TN $P = 0.001$), a high histological grade (TS $P < 0.001$; TN $P < 0.001$), the histological type (TS $P = 0.009$; TN $P = 0.034$), a high ratio of lymph node metastasis (TS $P < 0.001$; TN $P < 0.001$), an advanced stage of cancer (TS $P < 0.001$; TN $P = 0.002$), intrinsic subtypes (TS $P < 0.001$; TN $P < 0.001$), *ERBB2* (TS $P < 0.001$; TN $P < 0.001$), *MAP3K1* (TS $P = 0.002$; TN $P = 0.023$), and *TP53* (TS $P < 0.001$; TN $P < 0.001$). A higher density of TANs in TS and TN also correlated with shorter disease-free survival and overall survival ($P < 0.001$). Conclusion: The present results suggest that a higher density of TANs correlates with unfavorable prognostic factors in breast cancer. Further research on clinicopathological and genetic factors associated with TANs in breast cancer is needed.

Keywords: Breast cancer, pathology, tumor microenvironment, neutrophil-associated tumor

Introduction

The prognostic importance of immune cells has been reported in breast cancer, which suggests their potential as an indicator of patient outcomes [1-5]. Immune cells, such as lymphocytes, have been suggested to indicate a favorable prognosis in breast cancer. The World Health Organization (WHO) classification describes invasive carcinomas with a medullary pattern, mostly triple-negative breast cancer (TNBC), which is one of the spectra of tumor-infiltrating lymphocyte-enriched tumors. The WHO recommends using the term invasive breast carcinoma of no special type (NST) with the medullary pattern to characterize these tumors [6].

However, lymphocytes are not the only immune cells that infiltrate the tumor microenvironment. Neutrophils are the most common type of leukocyte in mammals and are essential in the innate immune system and anti-cancer responses [7-9]. Previous studies reported that activated neutrophils suppressed cancer cells *in vitro* [10] and *in vivo* [11]. However, recent studies revealed that neutrophils also play an important role in cancer progression. The presence of tumor-associated neutrophils (TANs) indicates an unfavorable prognosis in

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various cancers, such as gastric [12], colorectal [13], liver [14], uterine cervix [15], and renal cell carcinomas [16]. Furthermore, TANs correlated with a higher tumor grade in gliomas and with more aggressive behavior in pancreatic tumors [17, 18]. A meta-analysis of 20 studies reported that TANs were associated with an unfavorable prognosis in different cancers [19]. However, only a few studies have examined TANs in breast cancer, and there are many aspects of their clinical and pathological characteristics that still need to be elucidated.

The traditional assessment of risk and treatment options for primary breast cancer relies on factors such as tumor size, the lymph node status, grading, stage, and the hormone receptor (HR) and HER2 status. Highly complex clinical laboratory testing techniques, such as next-generation sequencing (NGS), are available for the further classification of many different human cancer types and affect patient management. Since June 2019, cancer genetic panel testing using NGS has been covered by the national health insurance system in Japan, allowing patients to receive cancer genome medicine under insurance-based medical treatment. Several platforms for targeted NGS have been commercialized and used in clinical practice. The routine detection of genomic alterations potentially allows for the classification of tumors with the best chance of a good response to traditional cytotoxic chemotherapy regimens as well as more novel monoclonal antibody therapies, small molecular tyrosine kinase inhibitors, and immunotherapies. In addition, the identification of genomic abnormalities may lead to the development of drugs against new proteins involved in alternative signaling pathways [20].

Consequently, pathologists have started to reclassify tumor cells based on their molecular characteristics by NGS and numerous studies have been published on breast cancer. However, the relationship between NGS data and TANs remains unclear in breast cancer. Therefore, the present study investigated the relationships between TANs and various prognostic factors in breast cancer. The results obtained from this study may form the basis for ongoing research on TANs in breast cancer.

Materials and methods

Patients and tissue specimens

A total of 196 breast cancer patients with sufficient biopsy, breast-conserving surgery, or mastectomy specimens between 2014 and 2021 in Hokuto Hospital were included. The present study was conducted according to the Declaration of Helsinki and after approval by the Ethics Committee of Hokuto Hospital (No. 1107). The clinicopathological parameters investigated consisted of the following: age, tumor size, histological grade, histological type, lymph node status, intrinsic type, genetic status, and disease-free survival (DFS). Overall survival (OS) was recorded from the date of curative surgery to the date of breast cancer-specific death. Each patient was examined using the same procedures and standardized assays. Estrogen receptor (ER) and progesterone receptor (PR) expression was examined by immunohistochemical staining (IHC). HR-positive cases were defined as being positive for ER and/or PR, whereas HR-negative cases were negative for both ER and PR according to the Allred score [21]. Human epidermal growth factor receptor 2 (HER2) was defined as negative in cases with a score of 0 (no membrane staining) or 1+ (weak and incomplete membrane staining). Tumors were defined as HER2+ with scores of 3+ on IHC staining or amplified fluorescence *in situ* hybridization (FISH) (a ratio of HER2 to the amplification of the chromosome 17 centromere > 2.2 or an average HER2 gene copy number > 6 signals/nucleus), and HER2- with scores of 0, 1+, or 2+ on IHC staining plus negative FISH amplification [22]. Tumors were classified into three subtypes according to the receptor status: those positive for HR, but negative for HER2 were classified as HR, those positive for HER2 were classified as HER2+, and those not expressing any receptors were classified as TNBC.

TAN evaluation

TANs were evaluated using the following method. Each hematoxylin-eosin slide was screened at a low magnification (×100), and the four areas with the highest number of neutrophils (a hot spot area) were selected for further examination. The mean neutrophil count in these areas was assessed under a high magnification

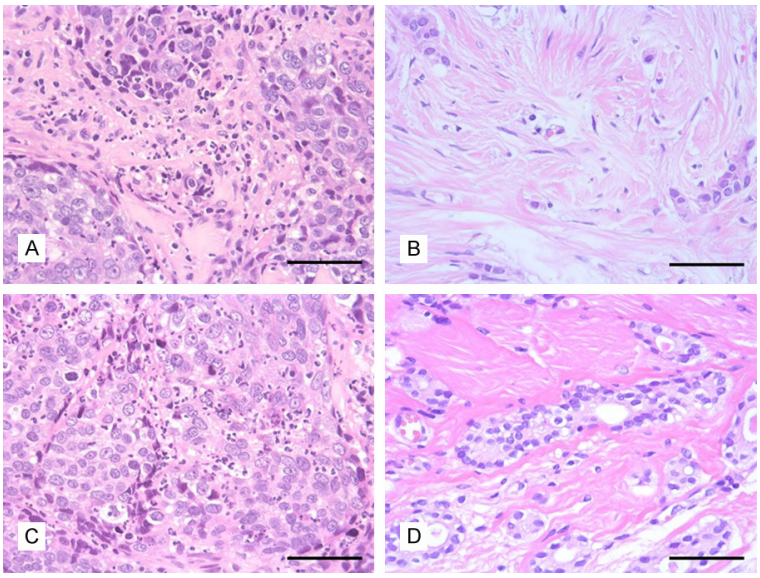

Figure 1. Carcinoma of the breast. A. Representative H&E staining images in the tumor stroma (TS). Under a high magnification, the stroma contains TANs, which was considered to be a high group. Original magnification: ×400. B. Under a high magnification, the TS contains TANs, which was considered to be a low group. Original magnification: ×400. C. H&E staining images in the tumor nest (TN). Under a high magnification, the tumor contains TANs, which was considered to be a high group. Original magnification: ×400. D. Under a high magnification, the TN contains TANs, which was considered to be a low group. Original magnification: ×400. Bar = 50 μm.

(×400) [23]. Neutrophils were individually counted in the tumor stroma (TS) and tumor nest (TN) (**Figure 1A-D**). The definition of TS in the present study was stromal tissue surrounding TN. TANs in TN were defined as intraepithelial tumor-infiltrating neutrophils. In statistical analyses, the number of neutrophils was classified into lower and higher groups according to cut-off points based on the mean. Therefore, the cut-off for neutrophils was 3.2 in TS and 1.5 in TN. Two pathologists (AK and HK) performed observations and were blinded to any clinical information.

Genetic status

The Department of Pathology, Laboratory of Cancer Medical Science, Hokuto Hospital conducted NGS according to the manufacturer's instructions [24-26]. They extracted total DNA from 5-μm-thick formalin-fixed paraffin-embedded (FFPE) tumor tissue sections using a Maxwell 16 FFPE Plus LEV DNA purification kit (Promega, Madison, WI). The quality of genomic DNA was evaluated using a Qubit dsDNA BR assay kit (Life Technologies, Carlsbad, CA) and GeneRead DNA QuantiMIZE kit (Qiagen, Valencia, CA). We selected 160 cancer-related genes using a GeneRead DNA seq Targeted Panel V2 human comprehensive cancer panel (NGHS-501X; Qiagen, Valencia, CA). The quality of the library was assessed with an Agilent 2100 bioanalyzer (Agilent, Santa Clara, CA) and GeneRead Library Quant kit (Qiagen). Libraries were sequenced on an Illumina MiSeq sequencer (Illumina, San Diego, CA). Each variant was compared with known alterations using online analytical resources from the catalogue of somatic alterations in the cancer databases ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), COSMIC (https://cancer.sanger.ac.uk/cosmic), and OncoKB (https://www.oncokb.org/).

Statistical analysis

Statistical analyses were performed using SPSS 26.0 (IBM Corporation, Armonk, NY, USA). A p-value < 0.05 was regarded as significant and all statistical tests were two-sided. Correlation analyses of TANs and clinicopathological and genetic parameters were performed using Pearson's chi-square test and Fisher's exact test. DFS was assayed from the date of the histological confirmation of a tumor to the date of the first relapse or the date of the last follow-up. DFS was analyzed using the Kaplan-Meier method and compared using the Log-rank test.

Results

The relationships between TAN densities and clinicopathological features are shown in **Table 1**. Age ranged between 28 and 90 years with a median of 62 years. Tumor sizes ranged between 1.2 and 21 cm with a median of 2.0 cm. Histological grade I or II was observed in 137 patients, while 59 were classified as histological grade III. Histological types included IBC-NST (n = 155), lobular carcinoma (n = 11), mixed invasive cribriform (n = 18), mucinous

Table 1. Clinicopathological features of breast cancer and the status of TANs

Clinicopathological features	TANs					
	Tumor stroma			Tumor nest		
	Low	High	*P* value	Low	High	*P* value
Age (years)			0.907			0.974
< 50	37	20		38	19	
≥ 50	89	50		93	46	
Tumor size (cm)			*0.010*			*0.001*
≤ 2	76	25		76	25	
> 2	50	45		55	40	
Histological grade			*< 0.001*			*< 0.001*
I, II	108	29		107	30	
III	18	41		24	35	
Histological type			*0.009*			*0.034*
IBC-NST	90	65		96	59	
Invasive lobular carcinoma	9	2		8	3	
Mixed invasive cribriform	17	1		17	1	
Mucinous carcinoma	3	1		3	1	
Solid papillary carcinoma	6	0		6	0	
Adenoid cystic carcinoma	0	1		0	1	
Neuroendocrine carcinoma	1	0		1	0	
Lymph node status			*< 0.001*			*< 0.001*
Absent	86	32		91	27	
Present	28	16		26	18	
N/A	12	22		14	20	
Stage			*0.002*			*< 0.001*
I, II	112	50		118	44	
III, IV	14	20		13	21	
Intrinsic subtypes			*< 0.001*			*< 0.001*
HR+	123	31		122	32	
HER2+	2	12		4	10	
TNC	1	27		5	23	

TANs: tumor-associated neutrophils, IBC-NST: invasive breast carcinoma of no special type, N/A: not applicable.

carcinoma (n = 4), solid papillary carcinoma (n = 6), adenoid cystic carcinoma (n = 1), and neuroendocrine carcinoma (n = 1). Axial and sentinel lymph node metastasis was detected in 44 patients. Patients were in the clinical stage of I or II (n = 162) and III or IV (n = 34). Intrinsic types included the HR+ (n = 154), HER2+ (n = 14), and TNBC (n = 28) subtypes. A higher density of TANs in both TS and TN correlated with tumor size (TS $P = 0.010$; TN $P = 0.001$), a high histological grade (TS $P < 0.001$; TN $P < 0.001$), the histological type (TS $P = 0.009$; TN $P = 0.034$), a high ratio of lymph node metastasis (TS $P < 0.001$; TN $P < 0.001$), an advanced stage of cancer (TS $P < 0.001$; TN $P = 0.002$), and intrinsic subtypes (TS $P < 0.001$; TN $P < 0.001$) (**Table 1**). No correlations were observed between the densities of TANs and age in TS or TN.

We examined survival rates in consideration of the different expression profiles of TANs using the Kaplan-Meier method and Log-rank test (**Figure 2**). Follow-up data were available in 192 of the 196 patients. The follow-up period ranged between 2 and 180 months, with a median period of 42 months. A total of 179 (93.2%) patients were alive without disease. The results obtained revealed a correlation between a higher density of TANs in TS and TN and shorter DFS and OS ($P < 0.001$) (**Figure 2**).

We performed a standardized cancer gene panel based on the AmpliSeq platform to investigate our breast cancer patients. Distinct genomic variants were detected in 150 patients, while no variant was identified in 42. A total of 269 variants were identified within 15 of the 160 targeted genes, including 210 variants affecting function (VaF) and 59 variants of uncertain significance (VUS). Genomic variants included the following: 140 single nucleotide variants, 25 amplifications, 9 copy number losses, 6 insertions, and 23 deletions. Among VaFs, 83 were distinct (only in one patient), while 54 were present in two or three patients and 4 in four or more patients. Among VUS, 42 were distinct, while 9 were observed in two or three patients. We investigated that associations with the alterations of the 160 genes and the top eight genes commonly found to have reportable variants, namely: tumor protein p53 (*TP53*) (n = 56), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) (n = 57),

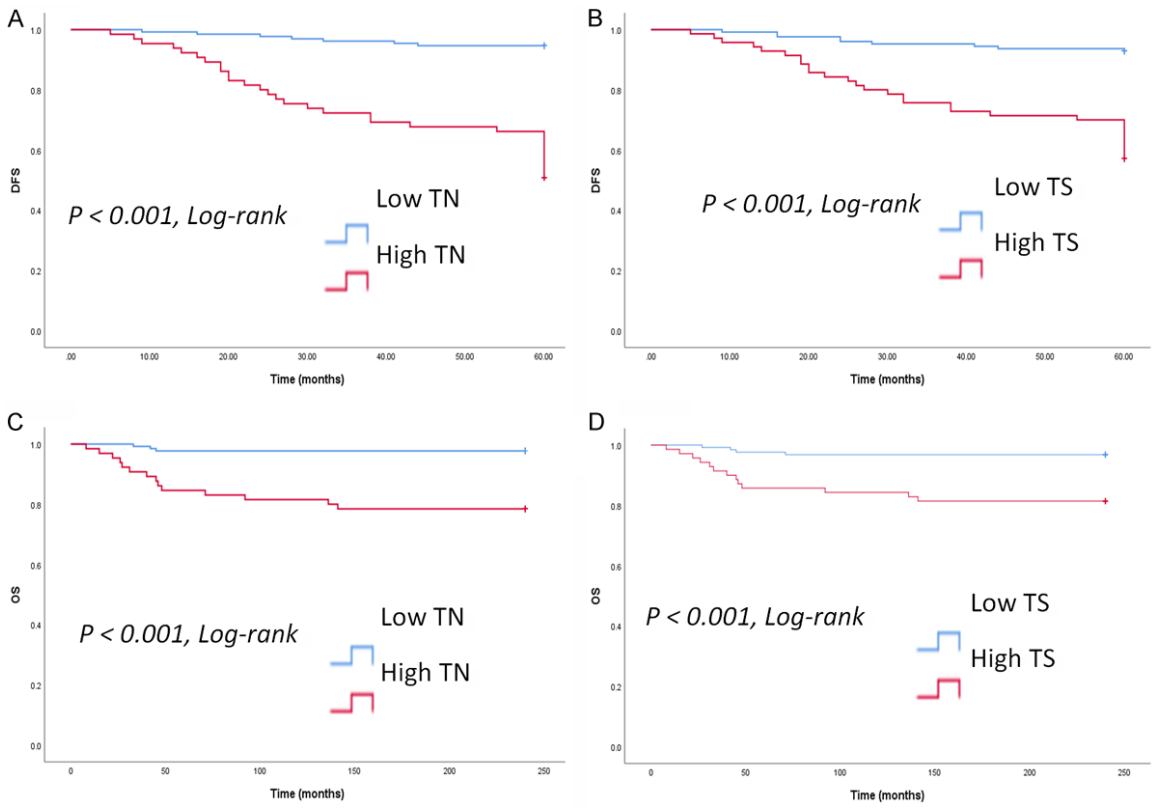

Figure 2. Kaplan-Meier estimates of disease-free survival (DFS) (A, B) and overall survival (OS) (C, D) in all patients based on TANs in TN and TS.

GATA binding protein 3 (*GATA3*) (n = 28), erythroblastic leukemia viral oncogene homolog 2 (*ERBB2*) (n = 26), mitogen-activated protein kinase 1 (*MAP3K1*) (n = 20), protein kinase B (*AKT*) (n = 15), phosphatase and tensin homolog (*PTEN*) (n = 11), and breast cancer type 2 susceptibility gene (*BRCA2*) (n = 9).

We found that a higher density of TANs in both TS and TN correlated with *ERBB2* ($P < 0.001$; $P < 0.001$), *MAP3K1* ($P = 0.002$; $P = 0.023$), and *TP53* ($P < 0.001$; $P < 0.001$) (**Table 2**). In a univariate analysis, DFS was associated with the intrinsic type (HR+ vs TNBC), stage, TS, TN, and TP53. However, in a multivariate analysis, only the intrinsic type (HR+ vs TNBC) ($P = 0.032$) and TN ($P < 0.001$) were associated with DFS (**Table 3**). In the univariate analysis, OS was associated with the histological grade, lymph node status, stage, TS, and TN. However, in the multivariate analysis, only stage ($P = 0.020$) was associated with OS (**Table 3**).

Discussion

The present results demonstrated that a higher density of TANs in breast cancer patients was not only associated with poor DFS, but also adverse clinical indicators, such as a large tumor size, high histological grade, high ratio of lymph node metastasis, advanced stage of cancer, and intrinsic subtypes.

Neutrophils are the predominant type of leukocyte in mammals and play a crucial role in anticancer responses in the human body [7-9]. Activated neutrophils have been shown to inhibit tumor cells both *in vitro* [10] and *in vivo* [11]. Previous studies indicated that neutrophils migrate from the bloodstream into tissues where their activation leads to the release of reactive oxygen species (ROS). This activation either triggers the ROS-induced apoptosis of tumor cells or contributes to their suppression [27, 28]. However, the finding in a study by Yan et al. contradicted this perspective, suggesting that the anticancer function of neutrophils in cancer patients was insufficient relative to that in healthy individuals [29]. Moreover, neutrophils have been shown to support cancer cells in the acquisition of epithelial-mesenchymal transition, thereby promoting tumor invasion and metastasis, which leads to a poor progno-

Table 2. Correlations between genomic alterations and tumor-associated neutrophils (TANs) in breast cancer

Genetic status	TANs					
	Tumor stroma			Tumor nest		
	Low	High	*P* value	Low	High	*P* value
ATM			0.172			0.162
Positive	5	0		5	0	
Negative	121	70		126	65	
AKT1			0.580			0.558
Positive	11	4		9	6	
Negative	115	66		122	59	
BRAF			1.000			1.000
Positive	1	0		1	0	
Negative	125	70		130	65	
BRCA1			0.618			0.601
Positive	2	2		2	2	
Negative	124	68		129	63	
BRCA2			0.724			0.161
Positive	5	4		4	5	
Negative	121	66		127	60	
CDH1			0.459			0.790
Positive	4	4		5	3	
Negative	122	66		126	62	
CHECK2			1.000			1.000
Positive	1	0		1	0	
Negative	125	70		130	65	
ERBB2			*< 0.001*			*0.001*
Positive	7	19		10	16	
Negative	119	51		121	49	
GATA3			*0.088*			0.390
Positive	22	6		21	7	
Negative	104	64		110	58	
KRAS			0.618			0.601
Positive	2	2		2	2	
Negative	124	68		129	63	
MAP3K1			*0.002*			*0.023*
Positive	19	1		18	2	
Negative	107	69		113	63	
PIK3CA			0.439			0.974
Positive	39	18		38	19	
Negative	87	52		93	46	
PTEN			0.749			0.755
Positive	8	3		8	3	
Negative	118	67		123	62	
STK11			1.000			1.000
Positive	1	0		1	0	
Negative	125	70		130	65	
TP53			*< 0.001*			*< 0.001*
Positive	20	36		22	34	
Negative	106	34		109	31	

TANs: tumor-associated neutrophils.

sis [30-32]. These findings appear to support the present results showing that a higher density of TANs was associated with a poor prognosis and adverse clinical indicators. Additionally, we revealed a strong correlation between a higher density of TANs and NST. However, it is important to note that the smaller number of special types, such as invasive lobular, mucinous carcinoma, and solid papillary carcinoma, is generally reported to have a good prognosis [6].

We also investigated the relationship between tumor intrinsic subtypes classified by immunostaining and the presence of TANs. The present study aimed to elucidate the relationships between a higher density of TANs and some intrinsic subtypes of breast cancer. We confirmed the presence of a higher density of TANs in both TS and TN, particularly in the TNBC and HER2+ subtypes. A previous study reported that TNBC correlated with a higher number of circulating neutrophils. Celis et al. examined 105 breast cancer cases and revealed a higher density of TANs in TNBC cases (88% were positive for TANs in TN, $P < 0.001$) [23]. Furthermore, a genomic investigation revealed that pathways associated with leukocyte diapedesis, extravasation, and adhesion were more prominent in some TNBC cases, such as patients with mesenchymal and basal-like TNBC [33]. Although HER2+ correlated with a higher density of TANs in the present study, a previous study on HER2+ breast cancer reported a relationship with HER2 activity and a pro-trastuzumab tumor immune microenvironment. A gene expression analysis and IHC staining of 53 HER2+ breast cancer patients showed that trastuzumab-responsive tumors expressed markedly higher levels of immune system-related chemokines [34]. Similar to the present results, the higher density of TANs in specific tumor subtypes sug-

Table 3. Univariate and multivariate Cox regression analyses for disease-free survival (DFS) and overall survival (OS) in breast cancer patients

Clinicopathological features	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	DFS HR (95% CI)	*P* value	DFS HR (95% CI)	*P* value	OS HR (95% CI)	*P* value	OS HR (95% CI)	*P* value
Age (50 vs. > 50)	1.377 (0.654-2.900)	0.400			1.331 (0.434-4.083)	0.617		
Tumor size (2 cm vs. > 2 cm)	1.539 (0.803-2.950)	0.194			2.031 (0.681-6.060)	0.204		
Histological grade (I, II vs. III)	1.369 (0.712-2.634)	0.347			2.787 (1.075-7.225)	*0.035*	0.893 (0.303-2.634)	0.838
Histological type (IBC-NST vs. other types)	0.671 (0.281-1.602)	0.369			1.177 (0.384-3.608)	0.776		
Intrinsic types (HR+ vs. HER2+)	1.246 (0.377-4.118)	0.718			0.043 (0.00-311.552)	0.489		
Intrinsic types (HR+ vs. TNC)	0.430 (0.207-0.891)	*0.023*	2.516 (1.085-6.211)	*0.032*	0.412 (0.145-1.170)	0.096		
Intrinsic types (HER2+ vs. TNC)	0.729 (0.382-1.391)	0.338			0.158 (0.003-7.287)	0.345		
Lymph node status (absent vs. present)	1.000 (0.999-1.001)	0.727			1.002 (1.001-1.003)	*< 0.001*	1.001 (1.000-1.002)	0.075
Stage (I, II vs. III, IV)	3.015 (1.547-5.876)	*0.001*	1.755 (0.842-3.657)	0.133	7.952 (3.023-20.917)	*< 0.001*	3.487 (1.223-9.947)	*0.020*
TS (low vs. high)	7.148 (3.392-15.065)	*< 0.001*	2.471 (0.897-6.805)	0.080	6.347 (2.069-19.469)	*0.001*	1.316 (0.307-5.629)	0.712
TN (low vs. high)	11.544 (5.091-26.177)	*< 0.001*	7.156 (0.842-3.657)	*< 0.001*	10.372 (2.980-36.102)	*< 0.001*	4.869 (0.928-25.53)	0.061
ATM (pos vs. neg)	0.048 (0.00-218.533)	0.479			0.048 (0.00-0.00)	0.651		
AKT1 (pos vs. neg)	1.041 (0.321-3.382)	0.946			2.782 (0.799-9.685)	0.108		
BRAF (pos vs. neg)	0.049 (0.00-0.000)	0.753			0.049 (0.00-0.00)	0.840		
BRCA1 (pos vs. neg)	3.164 (0.762-13.130)	0.113			0.048 (0.00-0.00)	0.686		
BRCA2 (pos vs. neg)	1.871 (0.576-6.076)	0.297			1.355 (0.180-10.220)	0.768		
CDH1 (pos vs. neg)	2.255 (0.694-7.327)	0.176			1.464 (0.194-11.038)	0.712		
CHECK2 (pos vs. neg)	0.049 (0.00-0.00)	0.753			0.049 (0.00-0.00)	0.840		
ERBB2 (pos vs. neg)	1.463 (0.646-3.315)	0.362			1.409 (0.405-4.905)	0.590		
GATA3 (pos vs. neg)	0.863 (0.338-2.207)	0.758			0.771 (0.176-3.374)	0.730		
KRAS (pos vs. neg)	0.048 (0.00-586.485)	0.527			0.048 (0.00-0.00)	0.686		
MAP3K1 (pos vs. neg)	0.216 (0.030-1.571)	0.130			0.547 (0.072-4.121)	0.558		
PIK3CA (pos vs. neg)	1.068 (0.541-2.109)	0.849			1.336 (0.494-3.613)	0.568		
PTEN (pos vs. neg)	0.934 (0.225-3.876)	0.925			1.036 (0.137-7.809)	0.973		
STK11 (pos vs. neg)	0.049 (0.00-0.00)	*0.753*			0.049 (0.00-0.00)	0.840		
TP53 (pos vs. neg)	2.784 (1.485-5.218)	*0.001*	1.776 (0.821-3.841)	0.144	2.399 (0.925-6.217)	0.072		

Multivariate Cox regression analyses of all potential variables that correlated with survival in the univariate analysis were performed. DFS disease-free survival. HR: hazard ratio, CI: confidence interval, IBC-NST: invasive breast carcinoma of no special type, TS: tumor stroma, TN: tumor nest.

gested that tumor-related factors within some intrinsic types of breast cancer exert either direct or indirect effects on neutrophil production in bone marrow and their subsequent migration into cancerous tissues.

The present study revealed that the number of alterations in *ERBB2* genes, which were detected by NGS, was increased in breast cancer with a higher density of TANs in TS and TN. *ERBB2* is a receptor tyrosine kinase associated with HER2, which induces uncontrolled cell proliferation and contributes to tumorigenesis through diverse mechanisms [35]. *ERBB2* is a vital cancer marker, lacks a known ligand, forms a heterodimer with another *ERBB* family member to form a more stable and stronger signaling function, and is considered to be important for predictions of patient outcomes and as a therapeutic target for cancer [36-40]. The oncogenic significance of the amplification of *ERBB2* has been demonstrated in breast and gastric cancers. This has led to the clinical practice of a well-established combination of IHC and kinase inhibitors targeting *ERBB2* kinase. However, a notable fraction of patients fails to derive benefits from this therapeutic approach [41, 42]. Therefore, a number of studies have investigated the possibility of identifying biomarkers to distinguish patients sensitive to inhibitors targeting *ERBB2* kinase from those suited for novel targeted approaches [43]. In accordance with this and based on increasing evidence for the role of both innate and adaptive immunities in the mechanism of action of trastuzumab, immune-related factors, such as tumor-infiltrating lymphocyte counts or immune-associated signatures, have been examined and provided predictive insights into the efficacy of trastuzumab [44-46]. Therefore, similar to some of the oncogenes implicated in the pathogenesis of human cancers, such as *RAS, BRAF, RET/PTC1*, and *MYC*, a direct relationship may exist between *ERBB2* activity and immune cell infiltration within tumors [47].

Liu et al. previously reported that *ERBB2* and *ERBB3* expression levels correlated with lymphocytes and neutrophils in the context of cutaneous melanoma. This finding suggests that *ERBB2* affects the presentation of immune cells, potentially impacting the prognosis of melanoma [48]. However, the relationship between TANs and *ERBB2* gene alterations identified through NGS in breast cancer remains unknown. In the present study, we observed a correlation between TANs and *ERBB2* gene alterations, which suggests that TANs and *ERBB2* gene alterations correlate and both may serve as adverse prognostic indicators in breast cancer.

In this present study, alterations in the *MAP3K1* and *TP53* genes correlated with a higher density of TANs in TS and TN. Razavi et al. previously reported on the role of MAPK pathway alterations in mediating resistance to ET in human HR+ HER2- breast cancer [49]. Ferrando et al. also demonstrated that *MAP3K13* mutations were present in metastatic breast cancer patients, but not in their early breast cancer counterparts [50]. Only *MAP3K* mutations were associated with significantly shorter DFS and OS. The relationship between *MAP3K* mutations and worse clinical outcomes remained significant after adjustments for the patient menopausal status, primary tumor size, nodal involvement at diagnosis, the tumor grade, and the percentage of the Ki-67 labeling index [50]. These findings are consistent with the present results, indicating that a higher density of TANs correlates with a poor prognosis.

TP53 is a commonly mutated gene in human cancer, suggesting its critical role as a tumor suppressor [51]. The *TP53* gene encodes the P53 protein, which plays a pivotal role in preserving genomic stability [52]. The P53 protein is involved in the activation of DNA repair mechanisms, cell responses to stress signals, and the regulation of stem cell production [53]. Recent studies reported the clinical characteristics of *TP53* alterations using DNA sequencing in breast cancer. Rossner et al. showed that *TP53* mutations were associated with a negative HR status and an unfavorable prognosis in a cohort of 859 breast cancer patients [54]. Furthermore, Olivier et al. demonstrated that *TP53* mutations analyzed by gene sequencing were linked to an elevated risk of breast cancer-specific mortality regardless of tumor size, the lymph node status, and HR expression in 1,794 patients [55]. *TP53* mutations correlated with poor OS in 442 patients with HR-positive breast cancer [56]. Park et al. recently reviewed the targeted NGS data of 219 patients with breast cancer. Among the various genetic alterations examined, only *TP53* mutations were

associated with reduced short-term DFS in patients with breast cancer. Moreover, they reported that *TP53* mutations were associated with poor prognostic factors, such as a high histological grade, a high ki-67 index, and the non-luminal subtype. Their multivariable analysis demonstrated that *TP53* mutations independently predicted the risk of recurrence [57]. Recent studies revealed a relationship between TANs and a poor prognosis, which provides support for the correlation observed between a higher density of TANs and *TP53* gene alterations in the present study.

To the best of our knowledge, the relationship between TANs and *TP53* gene alterations in breast cancer remains unclear. However, a few studies investigated similar relationships in other organs. Wang et al. reported that the presence of CD15+ neutrophils in pancreatic cancer was associated with a poor prognosis; however, they did not find a correlation with *TP53* gene mutations [58]. In contrast, Yan et al. developed a ten-gene signature, including *TP53*, to evaluate the prognostic potential of the tumor immune microenvironment. Through multivariate Cox regression and nomogram analyses of urinary bladder cancer, they found that higher percentages of neutrophils and *TP53* mutations were associated with a high-risk group [59]. These findings support the present results showing that a higher density of TANs was associated with *TP53* gene alterations.

In the present study, *ERBB2, MAP3K1,* and *TP53* gene alterations were increased in breast cancer with a higher density of TANs in TS and TN. However, in multiple analyses for DFS and OS, *ERBB2, MAP3K1*, and *TP53* were not significant. Therefore, *ERBB2, MAP3K1*, and *TP53* may correlate with TANs; however, their role in breast cancer with TANs remains unclear. Further studies are necessary to obtain a more comprehensive understanding of these relationships.

Conclusion

We found that a higher density of TANs in both TS and TN correlated with a large tumor size, high histological grade, the histological type, a high ratio of lymph node metastasis, an advanced stage of cancer, intrinsic subtypes (TNBC and HER2+), and *MAP3K1, ERBB2,* and *TP53*. Furthermore, the present results revealed a correlation between a higher density of TANs in TS and TN and shorter DFS and OS. These results suggest that a higher density of TANs is associated with poor prognostic factors in breast cancer. Further research into the clinicopathological and genomic factors associated with TANs in breast cancer is necessary.

Disclosure of conflict of interest

None.

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References

[1] Kuroda H, Jamiyan T, Yamaguchi R, Kakumoto A, Abe A, Harada O and Masunaga A. Tumor-infiltrating B cells and T cells correlate with postoperative prognosis in triple-negative carcinoma of the breast. BMC Cancer 2021; 21: 286.

[2] Jamiyan T, Kuroda H, Yamaguchi R, Nakazato Y, Noda S, Onozaki M, Abe A and Hayashi M. Prognostic impact of a tumor-infiltrating lymphocyte subtype in triple negative cancer of the breast. Breast Cancer 2020; 27: 880-892.

[3] Heimes AS, Riedel N, Almstedt K, Krajnak S, Schwab R, Stewen K, Lebrecht A, Battista MJ, Brenner W, Hasenburg A and Schmidt M. Prognostic impact of CD38- and IgkappaC-positive tumor-infiltrating plasma cells in triple-negative breast cancer. Int J Mol Sci 2023; 24: 15219.

[4] Huertas-Caro CA, Ramirez MA, Rey-Vargas L, Bejarano-Rivera LM, Ballen DF, Nunez M, Mejia JC, Sua-Villegas LF, Cock-Rada A, Zabaleta J, Fejerman L, Sanabria-Salas MC and Serrano-Gomez SJ. Tumor infiltrating lymphocytes (TILs) are a prognosis biomarker in Colombian patients with triple negative breast cancer. Sci Rep 2023; 13: 21324.

[5] Qiu X, Zhao T, Luo R, Qiu R and Li Z. Tumor-associated macrophages: key players in triple-negative breast cancer. Front Oncol 2022; 12: 772615.

[6] Tan PH, Ellis I, Allison K, Brogi E, Fox SB, Lakhani S, Lazar AJ, Morris EA, Sahin A, Salgado R, Sapino A, Sasano H, Schnitt S, Sotiriou C, van Diest P, White VA, Lokuhetty D and Cree IA; WHO Classification of Tumours Editorial Board. The 2019 World Health Organization

classification of tumours of the breast. Histopathology 2020; 77: 181-185.
[7] Opsha Y and Brophy A. Blood, blood components, plasma, and plasma products. Side Effects of Drugs Annual 2015; 37: 403-418.
[8] Kruger P, Saffarzadeh M, Weber AN, Rieber N, Radsak M, von Bernuth H, Benarafa C, Roos D, Skokowa J and Hartl D. Neutrophils: between host defence, immune modulation, and tissue injury. PLoS Pathog 2015; 11: e1004651.
[9] Malech HL, DeLeo FR and Quinn MT. The role of neutrophils in the immune system: an overview. Methods Mol Biol 2020; 2087: 3-10.
[10] Gerrard TL, Cohen DJ and Kaplan AM. Human neutrophil-mediated cytotoxicity to tumor cells. J Natl Cancer Inst 1981; 66: 483-488.
[11] Katano M and Torisu M. Neutrophil-mediated tumor cell destruction in cancer ascites. Cancer 1982; 50: 62-68.
[12] Zhao JJ, Pan K, Wang W, Chen JG, Wu YH, Lv L, Li JJ, Chen YB, Wang DD, Pan QZ, Li XD and Xia JC. The prognostic value of tumor-infiltrating neutrophils in gastric adenocarcinoma after resection. PLoS One 2012; 7: e33655.
[13] Rao HL, Chen JW, Li M, Xiao YB, Fu J, Zeng YX, Cai MY and Xie D. Increased intratumoral neutrophil in colorectal carcinomas correlates closely with malignant phenotype and predicts patients' adverse prognosis. PLoS One 2012; 7: e30806.
[14] Li YW, Qiu SJ, Fan J, Zhou J, Gao Q, Xiao YS and Xu YF. Intratumoral neutrophils: a poor prognostic factor for hepatocellular carcinoma following resection. J Hepatol 2011; 54: 497-505.
[15] Carus A, Ladekarl M, Hager H, Nedergaard BS and Donskov F. Tumour-associated CD66b+ neutrophil count is an independent prognostic factor for recurrence in localised cervical cancer. Br J Cancer 2013; 108: 2116-2122.
[16] Jensen HK, Donskov F, Marcussen N, Nordsmark M, Lundbeck F and von der Maase H. Presence of intratumoral neutrophils is an independent prognostic factor in localized renal cell carcinoma. J Clin Oncol 2009; 27: 4709-4717.
[17] Fossati G, Ricevuti G, Edwards SW, Walker C, Dalton A and Rossi ML. Neutrophil infiltration into human gliomas. Acta Neuropathol 1999; 98: 349-354.
[18] Reid MD, Basturk O, Thirabanjasak D, Hruban RH, Klimstra DS, Bagci P, Altinel D and Adsay V. Tumor-infiltrating neutrophils in pancreatic neoplasia. Mod Pathol 2011; 24: 1612-1619.
[19] Shen M, Hu P, Donskov F, Wang G, Liu Q and Du J. Tumor-associated neutrophils as a new prognostic factor in cancer: a systematic review and meta-analysis. PLoS One 2014; 9: e98259.
[20] Marotti JD, de Abreu FB, Wells WA and Tsongalis GJ. Triple-negative breast cancer: next-generation sequencing for target identification. Am J Pathol 2017; 187: 2133-2138.
[21] Allison KH, Hammond MEH, Dowsett M, McKernin SE, Carey LA, Fitzgibbons PL, Hayes DF, Lakhani SR, Chavez-MacGregor M, Perlmutter J, Perou CM, Regan MM, Rimm DL, Symmans WF, Torlakovic EE, Varella L, Viale G, Weisberg TF, McShane LM and Wolff AC. Estrogen and progesterone receptor testing in breast cancer: ASCO/CAP guideline update. J Clin Oncol 2020; 38: 1346-1366.
[22] Wolff AC, Somerfield MR, Dowsett M, Hammond MEH, Hayes DF, McShane LM, Saphner TJ, Spears PA and Allison KH. Human epidermal growth factor receptor 2 testing in breast cancer. Arch Pathol Lab Med 2023; 147: 993-1000.
[23] Soto-Perez-de-Celis E, Chavarri-Guerra Y, Leon-Rodriguez E and Gamboa-Dominguez A. Tumor-associated neutrophils in breast cancer subtypes. Asian Pac J Cancer Prev 2017; 18: 2689-2693.
[24] Ellison G, Huang S, Carr H, Wallace A, Ahdesmaki M, Bhaskar S and Mills J. A reliable method for the detection of BRCA1 and BRCA2 mutations in fixed tumour tissue utilising multiplex PCR-based targeted next generation sequencing. BMC Clin Pathol 2015; 15: 5.
[25] Gremel G, Lee RJ, Girotti MR, Mandal AK, Valpione S, Garner G, Ayub M, Wood S, Rothwell DG, Fusi A, Wallace A, Brady G, Dive C, Dhomen N, Lorigan P and Marais R. Distinct subclonal tumour responses to therapy revealed by circulating cell-free DNA. Ann Oncol 2016; 27: 1959-1965.
[26] Bandoh N, Akahane T, Goto T, Kono M, Ichikawa H, Sawada T, Yamaguchi T, Nakano H, Kawase Y, Kato Y, Kamada H, Harabuchi Y, Shimizu K and Nishihara H. Targeted next-generation sequencing of cancer-related genes in thyroid carcinoma: a single institution's experience. Oncol Lett 2018; 16: 7278-7286.
[27] Grivennikov SI, Greten FR and Karin M. Immunity, inflammation, and cancer. Cell 2010; 140: 883-899.
[28] Greten FR and Grivennikov SI. Inflammation and cancer: triggers, mechanisms, and consequences. Immunity 2019; 51: 27-41.
[29] Yan J, Kloecker G, Fleming C, Bousamra M 2nd, Hansen R, Hu X, Ding C, Cai Y, Xiang D, Donninger H, Eaton JW and Clark GJ. Human polymorphonuclear neutrophils specifically recognize and kill cancerous cells. Oncoimmunology 2014; 3: e950163.
[30] Grosse-Steffen T, Giese T, Giese N, Longerich T, Schirmacher P, Hansch GM and Gaida MM. Epithelial-to-mesenchymal transition in pan-

creatic ductal adenocarcinoma and pancreatic tumor cell lines: the role of neutrophils and neutrophil-derived elastase. Clin Dev Immunol 2012; 2012: 720768.

[31] Lopez-Lago MA, Posner S, Thodima VJ, Molina AM, Motzer RJ and Chaganti RS. Neutrophil chemokines secreted by tumor cells mount a lung antimetastatic response during renal cell carcinoma progression. Oncogene 2013; 32: 1752-1760.

[32] Mayer C, Darb-Esfahani S, Meyer AS, Hubner K, Rom J, Sohn C, Braicu I, Sehouli J, Hansch GM and Gaida MM. Neutrophil granulocytes in ovarian cancer - induction of epithelial-to-mesenchymal-transition and tumor cell migration. J Cancer 2016; 7: 546-554.

[33] Burstein MD, Tsimelzon A, Poage GM, Covington KR, Contreras A, Fuqua SA, Savage MI, Osborne CK, Hilsenbeck SG, Chang JC, Mills GB, Lau CC and Brown PH. Comprehensive genomic analysis identifies novel subtypes and targets of triple-negative breast cancer. Clin Cancer Res 2015; 21: 1688-1698.

[34] Triulzi T, Forte L, Regondi V, Di Modica M, Ghirelli C, Carcangiu ML, Sfondrini L, Balsari A and Tagliabue E. HER2 signaling regulates the tumor immune microenvironment and trastuzumab efficacy. Oncoimmunology 2018; 8: e1512942.

[35] Hynes NE and Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. Nat Rev Cancer 2005; 5: 341-354.

[36] Giroux S. Overcoming acquired resistance to kinase inhibition: the cases of EGFR, ALK and BRAF. Bioorg Med Chem Lett 2013; 23: 394-401.

[37] Milik SN, Lasheen DS, Serya RAT and Abouzid KAM. How to train your inhibitor: design strategies to overcome resistance to epidermal growth factor receptor inhibitors. Eur J Med Chem 2017; 142: 131-151.

[38] Khanjani F, Sajedi RH and Hasannia S. Rapid screening of drug candidates against EGFR/HER2 signaling pathway using fluorescence assay. Anal Bioanal Chem 2018; 410: 7827-7835.

[39] Gilcrease MZ, Woodward WA, Nicolas MM, Corley LJ, Fuller GN, Esteva FJ, Tucker SL and Buchholz TA. Even low-level HER2 expression may be associated with worse outcome in node-positive breast cancer. Am J Surg Pathol 2009; 33: 759-767.

[40] Ding Q, Chen H, Lim B, Damodaran S, Chen W, Tripathy D, Piha-Paul S, Luthra R, Meric-Bernstam F and Sahin AA. HER2 somatic mutation analysis in breast cancer: correlation with clinicopathological features. Hum Pathol 2019; 92: 32-38.

[41] Valachis A, Mauri D, Polyzos NP, Chlouverakis G, Mavroudis D and Georgoulias V. Trastuzumab combined to neoadjuvant chemotherapy in patients with HER2-positive breast cancer: a systematic review and meta-analysis. Breast 2011; 20: 485-490.

[42] Dahabreh IJ, Linardou H, Siannis F, Fountzilas G and Murray S. Trastuzumab in the adjuvant treatment of early-stage breast cancer: a systematic review and meta-analysis of randomized controlled trials. Oncologist 2008; 13: 620-630.

[43] Triulzi T, Bianchi GV and Tagliabue E. Predictive biomarkers in the treatment of HER2-positive breast cancer: an ongoing challenge. Future Oncol 2016; 12: 1413-1428.

[44] Bianchini G and Gianni L. The immune system and response to HER2-targeted treatment in breast cancer. Lancet Oncol 2014; 15: e58-68.

[45] Kroemer G, Senovilla L, Galluzzi L, Andre F and Zitvogel L. Natural and therapy-induced immunosurveillance in breast cancer. Nat Med 2015; 21: 1128-1138.

[46] Di Modica M, Tagliabue E and Triulzi T. Predicting the efficacy of HER2-targeted therapies: a look at the host. Dis Markers 2017; 2017: 7849108.

[47] Borrello MG, Degl'Innocenti D and Pierotti MA. Inflammation and cancer: the oncogene-driven connection. Cancer Lett 2008; 267: 262-270.

[48] Liu S, Geng R, Lin E, Zhao P and Chen Y. ERBB1/2/3 expression, prognosis, and immune infiltration in cutaneous melanoma. Front Genet 2021; 12: 602160.

[49] Razavi P, Chang MT, Xu G, Bandlamudi C, Ross DS, Vasan N, Cai Y, Bielski CM, Donoghue MTA, Jonsson P, Penson A, Shen R, Pareja F, Kundra R, Middha S, Cheng ML, Zehir A, Kandoth C, Patel R, Huberman K, Smyth LM, Jhaveri K, Modi S, Traina TA, Dang C, Zhang W, Weigelt B, Li BT, Ladanyi M, Hyman DM, Schultz N, Robson ME, Hudis C, Brogi E, Viale A, Norton L, Dickler MN, Berger MF, Iacobuzio-Donahue CA, Chandarlapaty S, Scaltriti M, Reis-Filho JS, Solit DB, Taylor BS and Baselga J. The genomic landscape of endocrine-resistant advanced breast cancers. Cancer Cell 2018; 34: 427-438, e426.

[50] Ferrando L, Vingiani A, Garuti A, Vernieri C, Belfiore A, Agnelli L, Dagrada G, Ivanoiu D, Bonizzi G, Munzone E, Lippolis L, Dameri M, Ravera F, Colleoni M, Viale G, Magnani L, Ballestrero A, Zoppoli G and Pruneri G. ESR1 gene amplification and MAP3K mutations are selected during adjuvant endocrine therapies in relapsing hormone receptor-positive, HER2-negative breast cancer (HR+ HER2- BC). PLoS Genet 2023; 19: e1010563.

[51] Surget S, Khoury MP and Bourdon JC. Uncovering the role of p53 splice variants in human malignancy: a clinical perspective. Onco Targets Ther 2013; 7: 57-68.
[52] Lane DP. Cancer. p53, guardian of the genome. Nature 1992; 358: 15-16.
[53] Lane D and Levine A. p53 research: the past thirty years and the next thirty years. Cold Spring Harb Perspect Biol 2010; 2: a000893.
[54] Rossner P Jr, Gammon MD, Zhang YJ, Terry MB, Hibshoosh H, Memeo L, Mansukhani M, Long CM, Garbowski G, Agrawal M, Kalra TS, Gaudet MM, Teitelbaum SL, Neugut AI and Santella RM. Mutations in p53, p53 protein overexpression and breast cancer survival. J Cell Mol Med 2009; 13: 3847-3857.
[55] Olivier M, Langerod A, Carrieri P, Bergh J, Klaar S, Eyfjord J, Theillet C, Rodriguez C, Lidereau R, Bieche I, Varley J, Bignon Y, Uhrhammer N, Winqvist R, Jukkola-Vuorinen A, Niederacher D, Kato S, Ishioka C, Hainaut P and Borresen-Dale AL. The clinical value of somatic TP53 gene mutations in 1,794 patients with breast cancer. Clin Cancer Res 2006; 12: 1157-1167.
[56] Blaszyk H, Hartmann A, Cunningham JM, Schaid D, Wold LE, Kovach JS and Sommer SS. A prospective trial of midwest breast cancer patients: a p53 gene mutation is the most important predictor of adverse outcome. Int J Cancer 2000; 89: 32-38.
[57] Park JH, Kwon MJ, Seo J, Kim HY, Min SK and Kim LS. Somatic mutations of TP53 Identified by targeted next-generation sequencing are poor prognostic factors for primary operable breast cancer: a single-center study. J Breast Cancer 2022; 25: 379-386.
[58] Wang WQ, Liu L, Xu HX, Wu CT, Xiang JF, Xu J, Liu C, Long J, Ni QX and Yu XJ. Infiltrating immune cells and gene mutations in pancreatic ductal adenocarcinoma. Br J Surg 2016; 103: 1189-1199.
[59] Yan Y, Huang Z, Cai J, Tang P, Zhang F, Tan M and Shen B. Identification of a novel immune microenvironment signature predicting survival and therapeutic options for bladder cancer. Aging (Albany NY) 2020; 13: 2780-2802.