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Research Article

Expression of PD-L1 in Relation to Intrinsic Molecular Subtypes of Breast Cancer in Hainan Free Trade Port, China

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Abstract

Background: Intrinsic molecular subtype and histological grade are closely related to clinical prognosis in breast cancer. However, their relationship with Programmed Cell Death-Ligand 1 (PD-L1) expression is not very clear, particularly for Hainan Aboriginal patients. Herein, this research aims to reveal the relationship between PD-L1 expression and intrinsic molecular subtypes of breast cancer.

Methods: 225 breast tumor samples from female patients were analyzed for PD-L1 expression using the Immunohistochemistry (IHC) method. The PD-L1 expression level was detected by IHC and the relationship between the expression and clinical parameters was analyzed statistically.

Results: Positive staining of PD-L1 was mainly found in the plasma membrane. In all cases, the positive rate was 12.0% (27/225). The PD-L1 expression level was significantly reduced in Luminal A subtype (the corrected ratio OR=0.15, p=0.04) whereas increased in HER2-positive subtype (OR=4.2, p=0.01). PD-L1 was significantly related to HER2-positive subtype ($p<0.05$) and histological grade 3 ($p<0.05$). There was statistically significant association between PD-L1 expression and metastasis (p=0.046), but not with the patient's age, the tumor stage and menstruation ($p>0.05$). Moreover, there was a significant difference in the frequency of intrinsic subtypes between patients with positive and negative PD-L1 expression ($p<0.001$) among patients with metastasis.

Conclusions: PD-L1 expression in breast cancer was positively correlated with HER2-positive subtype, higher pathological grade and metastasis of breast cancer, while negatively correlated with Luminal A in female patients in Hainan, China. PD-L1 may be a new independent marker to predict the prognostic factor in HER2-positive subtype breast cancer.

Keywords: PD-L1 expression; Breast cancer; HER2-positive subtype; Histological grade; Immunohistochemistry

Abbreviations

PD-1: Programmed Death 1 Protein; PD-L1: Programmed Cell Death-Ligand 1; IHC: Immunohistochemistry; OR: Odds Ratio; TILs: Tumor-Infiltrating Lymphocytes; TNBC: Triple Negative Breast Cancer

Introduction

Breast cancer is now the most common malignant tumor in global women [1,2] , including Chinese women [3]. With the continuous improvement of diagnosis and comprehensive treatment, most breast cancers are curable. However, the recurrence and distant metastasis of breast cancer are still challenging issues that threaten women's lives and health.

Breast cancer is a highly heterogeneous malignant tumor. For intrinsic HER2 subtype breast cancer with high malignancy, it is of great scientific significance and clinical value to understand whether immunotherapy can be applied and when is the interfering timing while surgery, chemotherapy combined with targeted therapy, or radiotherapy is performed.

Inhibiting the PD-1/PD-L1 pathway is a promising immunotherapy to enhance the body's anti-tumor immunity. In recent years, blocking immune checkpoints with antibody drugs, such as Programmed Death 1 Protein (PD-1) and its ligand (PD-L1), has achieved promise results in the treatment of various solid tumors, such as melanoma and lung cancer [4,5]. Targeting the T cell PD-1 has also shown clinical efficacy against cancer.

PD-L1 (also known as CD274 and B7-H1) is a 40kDa transmembrane protein that is expressed on a wide variety of normal tissues, including natural killer cells, macrophages, myeloid dendritic cells, B cells, epithelial cells, and vascular endothelial cells [6]. It is one of the ligands for Programmed Cell Death Protein 1 (PD-1). PD-L1 is a negative costimulatory molecule that binds to its receptor PD-1 on the T cell surface and inhibits the proliferation and activation of CD^{4+} and CD^{8+} T cells. As consequence, PD-L1 often leads to immune

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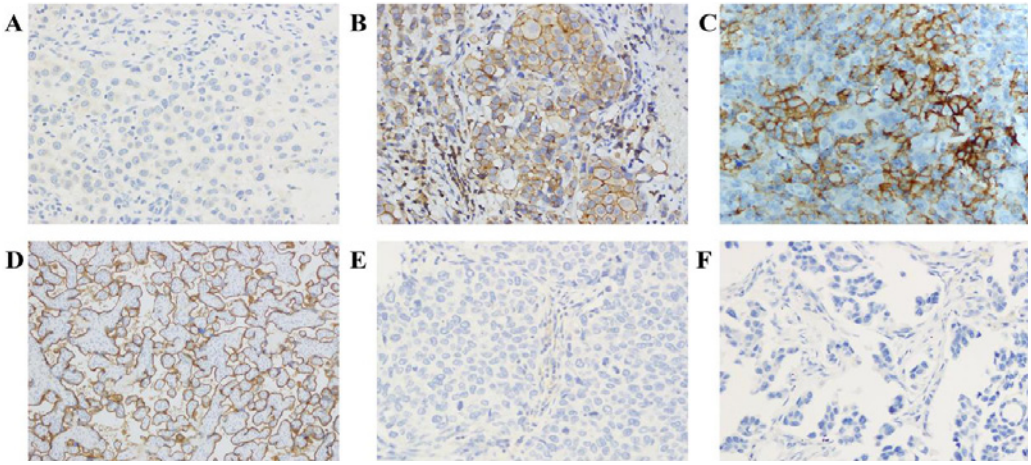

Figure 1: Immunohistochemical staining for PD-L1 in breast cancer and non-cancerous tissues (×100). (A) PD-L1-negative staining in breast cancer. (B-C) PD-L1-positive staining in breast cancer. (D) Placental tissue as a positive control. (E) PD-L1-negative staining in normal breast tissue. (F) PD-L1-negative staining in para-cancerous tissue.

evasion and promotes the proliferation of tumor cells [7]. Many studies have reported that PD-L1 is highly expressed in tumor-related diseases, and higher PD-L1 expression is often associated with the poor prognosis in breast cancer [8-11]. Clinical trials targeting the PD-1/PD-L1 signaling pathway have achieved very positive results. For example, Opdivo, a PD-1 inhibitor for the treatment of non-small cell lung cancer approved in Japan in July 2014 significantly increased the treatment efficacy [12].

There are some studies reporting expression of PD-L1 in Chinese breast cancer patients [13-15], mainly in mainland China. However, the characteristics of aboriginal Chinese breast cancer patients in Hainan Free Trade Port, a rural island in the south part of China, have not been investigated and reported yet. This study thus aimed to reveal expression of PD-L1 in breast cancer female patients from Hainan Island and its association with clinicopathologic features and the intrinsic molecular subtypes of breast cancer, especially the HER2 subtype and the histological grade of breast cancer.

Materials and Methods

Study design

This retrospective study used an anti-PD-L1 Immunohistochemistry (IHC) antibody optimized for staining of formalin-fixed paraffin-embedded tissue samples of breast cancer patients. The breast cancer tissues were collected from patients enrolled in the First Affiliated Hospital of Hainan Medical University from June 30, 2007 to June 30, 2016 who were aboriginals in Hainan Free Trade Port and did not receive any neoadjuvant chemotherapy or neoendocrine therapy. There were 1830 samples collected initially. Then we performed the random sampling, and a total of 225 cases were included in our study. We randomly selected para-cancerous tissues in 20 cases with the tumor tissue greater than 5cm as the control group. The clinical data of the selected cases were complete, containing patients' age, tumor stage, lymph node involvement, tumor grade, intrinsic subtype, postoperative chemotherapy regimen, the location of tumor metastasis and metastasis time. The location of metastasis was diagnosed by biopsy, CT or bone scan. All cases were followed up by telephone. The basic clinical data are listed in Table 1.

Immunohistochemistry

Immunohistochemical staining, dewaxing and hydration were performed with 4μm continuous tissue sections, followed by incubation with 3% hydrogen peroxide for 5 min to eliminate endogenous peroxidases. To validate PD-L1 expression, the slides were stained with PD-L1 monoclonal antibody (1:200; CST) at 4^0C overnight after heat-induced antigen retrieval. Afterwards, the slides were incubated with anti-rabbit antibody (1:500; Abcam) for 20min at room temperature. Standard DAB technique was performed for visualization. PD-L1 expression was quantified for quantitative analysis. The staining intensity and percentage of PD-L1 positive tumor cells for each sample were assessed. Dyeing intensity scores were defined as follows. 0: no expression; 1: low expression; 2: moderate expression; and 3: high expression. The percentage of PD-L1 positive tumor cells in each sample was rated as 0-100%, and the four-point scale was defined as follows. 0: <5%; 1: 5-25 %; 2: 26-50 %; and 3: >50% positive cells. Scores were assessed by two independent pathologists and reported as the mean. Multiplied by the scores, the overall results of staining intensity were recorded as negative (≤1) and positive (>1) [16,17].

Statistical analysis

Descriptive statistics were performed to summarize the distribution of clinical parameters of interest for whole sample and in terms of PD-L1 expression. Chi-square test or Fisher's exact method was used to explore the unadjusted correlations between PD-L1 expression and clinical characteristics. We also conducted univariate and multivariate regression analysis to estimate the relationship between PD-L1 expression and clinical parameters. All analyses were performed using SPSS 19.0.

Results and Discussion

PD-L1 expressed in breast cancer tissues in Hainan patients

The staining data demonstrated that PD-L1 was mainly expressed on the membrane of breast cancer cells (Figure 1A-1C). As a positive control, the placental tissue [18] showed high expression of PD-L1 (Figure 1D), while the normal breast tissue showed no expression

Table 1: The comparative analysis of positive and negative PD-L1 expression in breast cancer patients.

	n	Percentage (%)	PD-L1-negative		PD-L1-positive		*p*-value
			Number(n)	Percentage (%)	Number(n)	Percentage (%)	
Age at Diagnosis							0.539
<50	130	57.8	116	58.6	14	51.8	
≥50	95	42.2	82	41.4	13	48.2	
Tumor stage							0.969
pT1	57	25.3	50	25.3	7	25.9	
pT2	138	61.3	121	61.1	17	63	
pT3	23	10.2	21	10.6	2	7.4	
pT4	7	3.1	6	3	1	3.7	
Lymph Node Involvement							0.056
pN0	150	66.7	135	68.2	15	55.5	
pN1	40	17.8	37	18.7	3	11.1	
pN2	27	12	20	10.1	7	26	
pN3	8	3.5	6	3	2	7.4	
Tumor Grade							<0.001
1	41	18.2	40	20.2	1	3.7	
2	142	63.1	131	66.2	11	40.7	
3	42	18.7	27	13.6	15	55.6	
Menstrual Situation							0.754
Menopause	123	54.7	109	55.1	14	51.9	
Menstruation	102	45.3	89	44.9	13	48.1	
Intrinsic Subtype							<0.001
Luminal A	80	35.6	79	39.9	1	3.7	
Luminal B	76	33.8	68	34.3	8	29.6	
TNBC	38	16.8	26	13.1	12	44.4	
HER2	31	13.8	25	12.6	6	22.2	

Luminal A: ER positive and/or PR positive, HER2 negative, Ki-67 <20%; Luminal B: ER positive and/or PR positive, HER2 negative/or $HER2^{+}$, Ki-67 ≥20 %; TNBC: ER negative, PR negative, HER2 negative; HER2: HRE2 positive, ER negative and PR negative.

(Figure 1E). There was no expression in the para-cancerous tissue (0/20) (Figure 1F). Collectively, PD-L1 was positive in 27 of 225 patients, accounting for 12.0% of the total patients. There were 198 cases with negative expression of PD-L1, accounting for 88.0%.

PD-L1 expression associated with intrinsic molecular subtype and tumor grade

To compare the differences between positive and negative PD-L1 expression groups in Hainan Island patients, chi-square test was performed to test whether PD-L1 expression is associated with the age, the tumor stage, the molecular classification, the histological grade and the menstrual status (Table 1). Noteworthily, PD-L1 expression was statistically significant in the tumor grade ($p<0.001$) and the intrinsic molecular subtype ($p<0.001$). The analysis data also show that there was no correlation between PD-L1 expression and the patient's age ($p=0.539$), the tumor stage ($p=0.969$), the lymph node involvement ($p=0.056$), or the menstrual status ($p=0.754$).

HRE2 subtype and histological grade were related to PD-L1 expression

We conducted multivariate analysis on the main clinicopathologic characters, containing the tumor stage, the lymph node involvement, the tumor grade, the menstrual situation and the intrinsic molecular subtype. The analysis data show that intrinsic molecular subtype was very much related to PD-L1 expression in breast cancer patients in Hainan Island (Table 2). Further analysis indicates that the PD-L1 expression level was significantly reduced in Luminal A subtype (the corrected ratio OR=0.15, $p=0.04$) whereas it was significantly increased in HER2-positive subtype (OR=4.2, $p=0.01$) (Table 2). Additional comparative analysis indicates that the PD-L1 expression level was enhanced in HER2-positive subtype patients in comparison with non-HER2-positive subtype patients (OR=5.07, $p<0.001$) (Table 3). These analyses imply that the PD-L1 expression level is negatively associated with the Luminal A subtype, but positively correlated to the HER2-positive subtype of breast cancer patients in Hainan Island.

Previous investigations have reported that the high tumor histological grade (i.e. grade 3), but not the low grade (i.e. grade 1 and 2), is related to PD-L1 expression in breast cancer patients [18-20]. Similarly, our data demonstrated no significant difference in the PD-L1 expression level in patients with grade 2 and 1 (OR=2.84, $p=0.34$). Consistently, grade 3 patients had significantly higher expression of

Table 2: Multivariate logistic regression for the relationship between PD-L1 expression and clinicopathologic characteristics.

PD-L1 Expression, All Cases	Odds Ratio (95% CI)	*p*-value
Tumor Stage		
pT1	Reference	
pT2	0.65 (0.21, 2.02)	0.46
pT3	0.40 (0.06, 2.57)	0.33
pT4	0.60 (0.51, 7.03)	0.6
Lymph Node Involvement		
pN0	Reference	
pN1	2.31 (0.53, 5.31)	0.31
pN2	1.23 (0.22, 6.15)	0.26
pN3	6.32 (0.42, 7.81)	0.53
Tumor Grade		
1	Reference	
2	2.84 (0.34, 23.87)	0.34
3	11.0 (1.28, 94.83)	0.03
Menstrual Situation		
Menopause	Reference	
Menstruation	0.53 (0.20, 1.40)	0.28
Intrinsic Subtype		
Luminal B	Reference	
Luminal A	0.15 (0.02, 1.29)	0.04
TNBC	1.73 (0.51, 5.84)	0.38
HER2	4.20 (1.36, 12.99)	0.01

Table 3: The relationship between PD-L1 expression and HER2 subtype in breast cancer patients.

PD-L1 Expression, All Cases	Odds Ratio (95% CI)	*p*-value
Intrinsic Molecular Subtype		
No HER2	Reference	
HER2	5.07 (1.90, 13.52)	<0.001

PD-L1 compared with grade 1 patients (OR=11.0, p=0.03). These data suggest that PD-L1 expression was highly correlated with grade 3 patients, which normally have poor histological differentiation compared to grade 1 patients with breast cancer (Table 2).

PD-L1 expression was associated with metastasis

Very interestingly, there was statistically meaningful relationship between PD-L1 expression and tumor metastasis of breast cancer patients in Hainan Island, as revealed in the univariate and multivariate metastatic analysis. The unadjusted hazard of having metastatic conditions in patients with positive PD-L1 was 90% higher than that with negative PD-L1 (OR = 1.9, 95% CI (1.01, 3.55), p=0.046). The adjusted hazard of having metastatic conditions in patients with positive PD-L1 was 90% higher than that with negative PD-L1 (OR = 1.9, 95% CI (0.99, 3.52), p=0.05) (Table 4). Furthermore, among patients with metastasis, there was a statistically significant difference in the frequency of intrinsic subtype between patients with positive and negative PD-L1 expression (p<0.001) (Table 5). Previous reports reveal that the higher the expression level of PD-L1, the stronger

Table 4: The relationship between PD-L1 expression and tumor metastasis in breast cancer patients.

Variable	Univariate Results		Multivariate Results	
	Hazard Ratio (95% CI)	*p*-value	Odds Ratio (95% CI)	*p*-value
PD-L1				
Negative	Reference	-	Reference	
Positive	1.90 (1.01, 3.55)	0.046	1.88 (0.99, 3.52)	0.05
Age	--	--	1.002 (0.97, 1.03)	0.88
Menopause				
No	--	--	Reference	
Yes	--	--	0.82 (0.42, 1.62)	0.57

the invasiveness and metastasis of breast cancer, and high PD-L1 expression was correlated with the prevalence of Tumor-Infiltrating Lymphocytes (TILs) and distant metastasis [17,21]. These data are consistent with the current research results.

Discussion

In this research, we collected 225 breast tissue samples and examined PD-L1 expression in these Hainan aboriginal patients to investigate the correlations between PD-L1 expression and clinical characteristics. Our data indicate that PD-L1-positive tumors were statistically significant in patients with HER2-positive subtype, histological grade 3 and metastasis. Conversely, PD-L1 expression was negatively correlated with Luminal A subtype in female breast cancer patients. It is worth mentioning that PD-L1 expression was not associated with the patients' age, the tumor stage and menstruation.

With the deep understanding of the pathogenesis of breast cancer and the development of molecular biotechnology, combined with the development of breast cancer clinical individualization and precision medicine, molecular targeted therapy has been often used in the treatment of breast cancer. The clinical characteristics of different breast cancer subtypes vary greatly in response to treatment and prognosis [22]. Reports show that Luminal A subtype breast cancer is the most common molecular subtype, sensitive to endocrine therapy, with a low risk of recurrence and a better prognosis. Luminal B type is commonly seen in elderly patients and has a variable sensitivity to chemotherapy [23].

The proportion of HER2-positive subtype in breast cancer is about 30%, with the characteristics of axillary lymph node metastasis tendency, high degree of malignancy, sensitivity to chemotherapy combined with anti-HER2 targeted therapy and poor prognosis [24]. TNBC is common in the late stage, young and premenopausal women, with the characteristics of chemosensitivity and ready recurrence and metastasis. Carey et al. [25,26] reported that the overall survival rate of breast cancer patients was 80%, and the four subtypes had a prognosis of 84% (Luminal A subtype), 80% (Luminal B subtype), 75% (TNBC subtype), and 52% (HER2-positive subtype), respectively. Similar results were found by the Onitilo team [27], revealing that the survival rate of breast cancer with different molecular classification is significantly different. Moreover, the malignant degree of tumors gradually increases, and the tendency of tumor recurrence and metastasis goes up with the histological grade in breast cancer patients [28].

Table 5: The relationship between PD-L1 expression and intrinsic subtypes in patients with tumor metastasis.

Clinicopathological Features	n	Percentage (%)	PD-L1-negative		PD-L1-positive		*p*-value
			Number (n)	Percentage (%)	Number (n)	Percentage (%)	
Intrinsic Subtype							<0.001
Luminal A	30	40	30	47.6	0	0	
Luminal B	21	28	18	28.6	3	25	
TNBC	9	12	6	9.5	3	25	
HER2	15	20	9	14.3	6	50	

In recent years, a series of studies have demonstrated that PD-L1 expression in malignant tumors can be used as an important indicator for prognosis [29-31]. Kim et al. [9] evaluated expression of PD-L1 in tumor cells and TILs, confirming that PD-L1 expression was associated with the high histological grade and the high density of TILs, suggesting that PD-L1 expression might be a positive prognostic factor in HER2-positive subtype breast cancer. Soliman et al. [32] showed that basal subtype breast cancer constitutively expresses a higher level of PD-L1, and targeting PD-L1 eradicates invasive breast cancer cells from the immune system. In consistence with these findings, our data indicate that PD-L1 expression was statistically significantly correlated with the HER2-positive subtype and the higher pathological level, suggesting that high PD-L1 expression is a vital factor to poor prognosis. At the same time, we found that PD-L1 was negatively correlated with Luminal A subtype breast cancer in Hainan patients. Unfortunately, we did not group basal subtype breast cancer, which may have an impact on the outcome of TNBC. Our analyses show that PD-L1 expression was more readily expressed in relatively high histopathological grades, suggesting that PD-L1 is an immune suppressor, leading to a lack of normal immune surveillance and causing the deterioration of these types of breast cancer. PD-L1 inactivates effecter T cells by binding to its receptor PD-1, thereby inhibiting anti-tumor immunity, deteriorating the situations in histological grade 3 and HER2-positive subtype breast cancer patients. Previous studies have been concentrated on PD-L1 expression of breast cancer in mainland China patients. As reported by Qin et al. [33], the positive PD-L1 expression rate was 21.7% (189/870) in breast cancer patients, much higher than that (12.0%) found in our study for Hainan aboriginal females. A study of breast cancer in Mainland China emphasizes epidemiology and the diversity of epidemiology and socioeconomics. The incidence and mortality are higher in urban areas, which may be related to the high economic level (high life pressure), the low fertility rate, the increase of the first birth age and the limited breast-feeding. Nearly 2/3 of breast cancer patients in Mainland China are diagnosed with the advanced stage [34]. In this study, the selected cases were local aboriginal women in Hainan Island, which is an economically underdeveloped area with more peaceful life, and the detection and diagnosis of breast cancer are relatively delayed. However, whether the clinical manifestations and histopathological characteristics of this disease are the same as that in the mainland region is not fully confirmed yet by this research, which warrants a further research.

Conclusion

This investigation has clearly revealed that high PD-L1 expression was positively correlated with HER2-positive subtype, higher pathology grade and metastasis, whereas negatively correlated with Luminal A subtype in female Hainan patients. PD-L1 expression may be a useful indicator for the progress of breast cancer and immune therapy targets. These data are very helpful for patients with high risk and poor prognosis, such as HER2-positive subtype. The prophylaxis and treatment measures can be taken as early as possible by detecting the PD-L1 expression level to prevent disease from deteriorating. Furthermore, PD-L1 can be an important target for breast cancer immunotherapy using PD-L1 antagonist to block the PD-1/PD-L1 signaling pathway.

Declaration

Ethical approval: This study was conducted according to the ethical standards of CUHK-NETC CREC, the Helsinki declaration of 1964 and its later amendments ethical standards.

Informed consent: Since only the archival data in the pathological database were retrieved after diagnosis, the patients could not be identified, so informed consent could not be obtained. Thus, studies were conducted without informed consent.

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