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The Relationship Between the Expression of B7-H1/PD-1+in Breast Cancer and Micro-environment TILs and the Prognosis and the Molecular Typing

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ABSTRACT

AimTo analyze the differences of the distribution patterns and the density of immune cells in the different parts of the breast cancer tissues, and to evaluate the relationship between the expression of the immunosuppressive molecule B7-H1 and the infiltration of cell Foxp3+Treg, thereby to investigate the prognostic significance for the breast cancer patients, therefore to screen out the immune cells and immune factors which possess prognostic significance in order to provide new theoretical bases for individual immunological therapy for breast cancer. MethodThe immunohistochemical methods were separately applied to study those breast cancer patients of two groups who had never been implemented any anti-cancer treatments after the prognosis and before the operation. Result Proved by many factors, the expression of tumor cell B7-H1 could be used as the independent predictive index of disease-free survival and overall survival for the patients. Conclusion The tumor cell B7-H1 is expected to be a target for the immunotherapy of breast cancer, and to provide a new theoretical basis for the immunotherapy of breast cancer.

Keywords: *Tumor infiltrating lymphocytes; cytotoxicity; T cells;prognosis; breast cancer*

INTRODUCTION

More and more researches show that the tumor micro-environment plays a significant part in the genesis and evolution of the tumor[1]. Some researches deem that the immune state in the micro-environment can directly influence the prognosis of the tumor. For instance, in the tumor micro-environment of colorectal cancer, the species, densities, distributions and functional status and other comprehensive immune factors are accurate independent prognostic factors so far[2,3]. The body tissues barely produce inmmune cellular infiltration in the normal condition, but there will be plentiful inmuunue cellular infiltration occurring when the tumor is formed.

The immune cells that have infiltrated include T lymphocytes, B lymphocytes, natural killer cells(NK), macrophages, dendritic cells(DC) and so on. The lymphocytes are defined as tumor infiltrating lymphocytes(TILs), the macrophages are defined as tumor associated macrophages(TAMs). Give priority to with immune The anti-tumor immune cells play the priority in the immune effect. The T cells and cell nucleus TAMs of NK cells are the most primary cells that play anti-tumor effect in the humon body. Cytotoxic T lymphocytes(CTLs) are the important anti-tumor effector cells for the body. Most CTLs are CD8+T cells. That CD4+T helper cells(Th) secrete the Th cells cytokines can activate CTLs. CD4+Th is one kind of vital immune modulated cells. Although CD4+Thcan not directly identify and eliminate tumor cells, it plays assistance and amplification effects in the generation of antibody and the excitation for CTLs, thereby CD4+Th can play the role of resisting the tumor. On the basis

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of the cytokines, CD4+Th is divided into two kinds cells: Th1 and Th2[4,5]. Th1 can express specific transcription factor T - bet and secrete IFN-γ and IL-2; Th2 can express specific transcription factor GATA-3 and secrete IL-4, IL-5, IL-10 andIL-13. T-bet can activate initial Th0 and promote Th0 to differentiate and form Th1. By means of suppressing cells from differentiating and forming Th2, T-bet can accelerate the phenotype of Th1, thereby T-bet can enhance the immune response to resist tumors for the body[6,10].

By means of detecting few functional immune cells infiltrated in breast cancer, this study observed and analyzed the different distribution patterns and the differences of densities of immune cells in different parts of breast cancer tissues, and screened out the immune cells associated with clinical prognosis and tried to find new targets for immunological therapy. Besides, by means of massive samples, this study detected the expression in the breast cancer and the relationship with clinical prognosis and molecular typing of the coordinated stimulus molecule B7 - H1 and the receptor PD-2, and discussed the relationship between the expression of B7-H1 and Foxp3+Treg cells.

METHODS

Experiment reagents

Mouse anti-human Cav-1 monoclonal antibody IgG (Abeam, England, 1:200). Rabbit anti-human MCT4 poly-clonal antibody IgG (Santa cruz Biotechnology, America,1:100). Mouse anti-human a-SMA monoclonal antibody IgG (mouse IgQZymed, USA, 1:150). Rabbit anti-human Estrogen receptor(ER) monoclonal antibody IgG(clone SP1, Zymed, America). Rabbit anti-human Progesterone receptor (PR)monoclonal antibody IgG(clone SP2, Zymed, America). Mouse anti-human HER-2 / neumonoclonal antibody IgG (DAKO Hercep Test TM, Denmark). Mouse anti-human EMA (epithelial membrane antigen) monoclonal antibody IgG (DAKO, Denmark, 1:100). PBS phosphate buffer (0.01MpH 7.2-7.4XZLI-9062, Beijing ZhongshanJinqiao Biotechnology Co., Ltd). Citric Acid Repair Solution (PH6.0, 0.01M) (ZLI-9065, Beijing ZhongshanJinqiao Biotechnology Co., Ltd). EDTA Repair Solution (linMPh9.0) (ZLI-9067, Beijing ZhongshanJinqiao Biotechnology Co., Ltd). Enclosed normal goat serum working solution (SP-9000, Beijing ZhongshanJinqiao Biotechnology Co., Ltd). Antibody Diluent (ZLI-9030, Beijing ZhongshanJinqiao Biotechnology Co., Ltd). Biotin-labeled secondary antibodies (SP-9000, Beijing ZhongshanJinqiao Biotechnology Co., Ltd). Horseradish peroxidase-labeled chain avidin working solution (SP-9000, Beijing ZhongshanJinqiao Biotechnology Co., Ltd). Endogenous peroxidase blocking agent (SP-9000, Beijing Zhongshan-Jinqiao Biotechnology Co., Ltd). DAB Chromogenic reagent kit (ZLI-9032, Beijing ZhongshanJinqiao Biotechnology Co. , Ltd)

Main Instruments

RM2235 Paraffin slicing machine (LEICA, Germany). Medical centrifuge, Shanghai Medical Instrument(Group) Co., Ltd. BX60 Optical microscope(Olympus, Japan). TK-C1381 Image acquisition system, JVC, Japan

Objects of Study

40 breast cancer patients of Year 2005 were collected as the samples from the tumor pathology laboratory of the First Affiliated Hospital of Zhengzhou University. 20 patients occurred lymphatic metastasis with three stages and died within 3 years. The other 20 patients did not occur lymphatic metastasis and survived during the 6 years' following-up visits without the relapse of metastasis. All the 40 cases were tested under the condition that there was much lymphocytes infiltration in the tumor micro-environment. And the differences between the immune makers of all the patients were analyzed.

501 excision samples of breast cancer that were collected from the tumor pathology laboratory of the First Affiliated Hospital of Zhengzhou University from January, 2003 to December, 2005 were chosen for large sample analysis. All samples were fixed by 10% formalin neutral fixative and then embedded by regular paraffin. The follow-up periods were 1 to 78 months, the median follow-up periods were 64 months. By means of telephone interviews, mail interviews and looking up the illness records, the living state of patients was collected and divided into two statuses: being dead and cancer relapsing. The tumor relapsing mainly focused on the local recurrence(which included the homolateral clavicular lymph node metastasis, chest wall relapsing and internal mammary lymph node metastasis), contralateral occurring and distant metastasis(which included contralateral clavicular lymph node metastasis, cervical lymph node metastasis, liver, bones, lung, brain and other distant organs metastasis). The data of cases that died of the breast cancer were defined as the complete data information, and the data of cases that survived or died of other causes were defined as the censoring data information. The PFS periods of patients were defined from the operation day to the first relapsing day of tumor or the last follow-up visit. The OS periods were defined from the operation day to the death date of patients or the last follow-up visit.

Researching Method

The following were observed by S-P method in the breast cancer tissues: (1)the quantities and distributions of T lymphocyte infiltrated in the intraepithelial parts of tumor, tumor stroma and peritumoral zone and macrophage associated with tumor; (2) the expression of Foxp3+ in the tumor and around the tumor (3) the expression of coordinated stimulus molecule in the tumor cells; (4) the expression of ER, PR, HER-2, Ki-67, CK5/6 and EGFR in tumor cells. PBS buffer solution replaced the primary antibodies and was chosen as the negative control; the known positive slice was chosen as the positive control, and the coloration results were colorated by DAB.

The basic principle of S-P method.

In this paper, the adopted method of immunohistochemistry is a highly sensitive affinity histochemistry technique, that is, the labeled streptavidin biotin technology (LSAB technology). Also known as Vitamin H, biotin is a kind of vitamin with small molecule and its molecular weight is 244; the stretavidin (SA) is a protein isolated from streptomyces with 60 KD molecular weight. There are 4 biotin binding sites in one molecule of stretavidin and the structures of strtavidinisare similar to antibiotin in ovalbumin. But the feature of the nonspecific binding between antibiotin in ovalbumin and endogenous substances in tissues can be excluded in SA. Therefore, the background color of SA is light and SA can obtain higher signal to noise ratio (See in Figure 1).

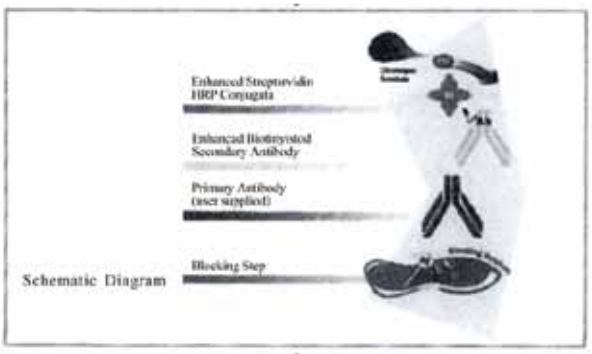

Fig 1. Schematic diagram of SP method

2. Experimental procedure

Processing for glass slides: The slides were soaked in soapy water overnight and washed with running water, then soaked with 95% alcohol overnight and dried. Put the clean slides into the APES acetone working fluid (1:50) for 20-60s, by being taken out for a moment, the slides were put into the acetone solution or rinse water to wash the unbond APES, then dried and saved in box for standby application. Paraffin

embedding tissues were sliced into pieces, the thickness was about 4μm. Then the tissues were toasted for 2 hours on the slice warmer and placed in the incubator of 60°C for one night for the sake of reducing skin flick. Paraffin were sliced into pieces and dewaxed into water with routine procedures. Washed with distilled water and soaked into PBS for 5min.

Processed with antigen hot repair (citrate, PH 6.0, high pressure repair for 2 min) and cooled to indoor temperature. Washed for 3 times with PBS and 5 minutes for each time. Incubated in H_2O_2 of 3% for 10 min at indoor temperature to eliminate endogenous peroxidase activity. Washed for 3 times with PBS and 5 minutes for each time. Sealed with the normal serum of rabbit/rat common type and incubated for 10 min at room temperature, and poured without washing. Respectively drop primary antibody (Cav-1 monoclonal antibody diluted in 1:200, MCT4 polyclonal antibody diluted in 1:100) at 4°C for one night. Used the PBS as a negative control group instead of primary antibody.

Washed with PBS for 3 times and 5 minutes for each time. Dropt second antibody working fluid labeled with biotin and incubated for 20 min at 37°C. Washed with PBS for 3 times and each for 5 min. Dropt the streptavidin third antibody work fluid labeled with horseradish peroxidase and incubated for 20 min at 37°C.

Washed with PBS 3 times and each for 5 min. Dyed with new DAB agent for 3-10 min at indoor temperature, observed under the microscope and washed with water to end the reaction. Counter stained with hematoxylin, dehydrated and sealed with neutral gum.

The determination for the immuno-histochemical coloration results

The coloration of GrB, T-bet and PD-1 positive lymphocyte cells: Thecytomembrane of GrB positivecellwascoloratedtoclaybank.Thecaryon of T-bet positive cell was colorated to claybank. And the cytomembrane of PD-1 positive cell was colorated to claybank. The cytoplasm and/or caryon of CD68 positive macrophage was colorated to claybank. The following methods were used to count the quantities of GrB+ lymphocyte, T-bet+lymphocyte, PD-1+lymphocyte, Foxp3+lymphocyte and CD68+macrophage: Firstly, the parts with the most lymphocytes were chosen under the low-power field(×100)(12 parts that included 5 intraepithelial parts, 5 tumor stroma parts and 5 peritumoral parts of tumor). Then the cells of above mentioned parts were calculated under high-power field(×400). The result was the average value of cells in 5 intraepithelial parts, 5 tumor stroma parts and 5 peritumoral parts of tumor(Meancell/0.0625mm^2). B7-H1 and PD-L2 mainly pitched in cytomembrane and cytoplasm of tumor cells and presented as claybank granules. With regard to the determination for the expression of B7-H1 and PD-L2 in tumor cells, the coloration intensity and proportion were taken into consideration.

Table 1: The dilution concentr ation of primary antibody and antigen repair conditions

antibody	producer	dilution	antigen repair conditions
Cav-1	Abcam	1:200	PH6.0 citrate high-pressure repair
MCT4	Abcam	1:100	PH6.0 citrate high-pressure repair
EMA	DAKO	1:100	PH6.0 citrate high-pressure repair
α-SMA	Zymed	1:150	PH6.0 citrate high-pressure repair
ER	Zymed	1:100	PH6.0 citrate high-pressure repair
PR	Zmyed	1:150	PH6.0 citrate high-pressure repair
HER-2	DAKO	1:400	PH6.0 citrate high-pressure repair

The positive intensities were divided into three situations: 0 score for non-colorization, 1 score for light colorization, 2 scores for medium colorization and 3 scores for strong colorization. The final standard for evaluation applied H standard, namely H-score=∑:(the percentage of positive cells×the intensity of colorization). In the end, the median of H value was chosen as the critical value to cut off the groups. The expression of Ki-67 mainly pitched in the caryon of tumor cells. Ki-67 was defined as the positive one when tumor cells that were equal or more than 14% were colorated. The expression of CK5/6 mainly pitched in the cytoplasm and cytomembrane of tumor cells. CK5/6 was defined as the positive one when tumor cells that were equal or more than 10% were colorated.

The expression of EGFR mainly pitched in the cytoplasm and cytomembrane of tumor cells. EGFR was defined as the positive one when tumor cells that were equal or more than 10% were colorated. The determination of the colorization results for ER and PR. The expression of ER and PR mainly pitched in the caryon and presented as the diffuse claybank granules. The ER and PR were defined as the positive ones when the percentage that the quantities of positive cells accounted for the quantities of tumor cells in the slices was more than 1%, negative ones when less than 1%. The determination of the colorization results for HER-2. The colorization of positive cells mainly pitched in the cytomembrane. Non-colorization in cytomembrane of infiltrated tumor cells was defined as (-); That the cytomembrane of the infiltrated tumor cells in any proportion presented light colorization and incomplete colorization and the cytomembrane of tumor cells of less 10% presented light and incomplete colorization was defined as (+); That the cytomembrane of equal or more than 10% infiltrated tumor cells presented light or inconsistent and complete colorization and the cytomembrane of equal or less than 30% infiltrated tumor cells presented strong and complete colorization was defined as (++); That the cytomembrane of more than 30% infiltrated tumor cells presented strong and complete colorization was defined as (+++). On the basis of the clinical physical application, this experiment defined 0 and (+) as HER-2 negative and defined (+++) as HER-2 positive. And this experiment would further detected the cases of (++) group by Fluorescence in situ hybridization(FISH).

The molecular typing standards of breast cancer

This research applied the molecular typing standards of Cheang[3]: type luminal A(ER+ and/or PR+, HER-2 and Ki-67<14%), type luminal B(ER+ and/or PR+, HER-2 and Ki-67≥14%), type luminal-HER-2(ER+ and/or PR+, HER-2+), type HER-2 enriclzed(ER-, PR- and HER-2+), type Basal-like(ER-, PR-, HER-2-, EFGR and/or CK5/6+), and type TNP-

Table 2: immunohistochemical signs of breast cancer- molecular typings tandards

	luminal A	luminal B	luminal-HER-2	HER-2 enriched	Basal-like	TNP-nonBasal
ER	+	+	+	-	-	-
PR	+	+	+	-	-	-
HER-2	-	-	+	+	-	-
HER-2	-	-	+	+	-	-
CK5/6					+	-
Ki-67	<14%	≥14%				

nonBasal(ER, PR, HER-2, EGFR and CK5/6-). Table 2 showed the above mentioned molecular typing standards of breast cancer.

Statistical analysis on the experimental data

All data were counted by statistical package SPSS 15.0. The differences had statistical significance when the P value was less than 0.05($P<0.05$). The infiltrated lymphocytes and macrophages in different tissues and the comparison between the expression of B7-H1 and PD-L2 were tested by Mann-Whitney U. The expression of B7-H1, the indexes of clinical pathology and the correlation analysis between Foxp3+Tregs and PD-1+lymphocytes were tested by Spearman. Kaplan-Meier(Product limit process)was used to draw the survival curve. The survival test was examined by log-rank. The survival analysis of single factors and multi-factors was recorded by the Cox proportional hazard model.

RESULT

The distributions and amount features of infiltrated immune cells in breast cancer and the expression of related immune molecule of tumor.

The immune cells infiltrated in micro-environment of breast cancer mainly had three distribution patterns: the intraepithelial parts of tumor, tumor stroma and peritumoral zone. The density of GrB+ lymphocytes obviously presented dense tendency in the intraepithelial parts of tumor and tumor stroma but no obvious dense tendency in the peritumoral zone; The density of T-bet+ lymphocytes presented dense tendency in the intraepithelial parts of tumor and tumor stroma but diffuse distribution in the peritumoral zone; The density of CD68+ macrophage presented diffuse distribution in the whole tumor tissues of breast cancer but dense distribution in the peritumoral zone; The density of PD-1+ lymphocytes presented diffuse distribution in the intraepithelial parts of tumor and tumor stroma but dense and concentrated distribution in the peritumoral zone which presented lymphoid aggregates(See in Figure 2 to Figure 5).

Between 20 cases with better prognosis that generated bottom transfer of lymph node and 20 cases with inferior prognosis that generated staging N3 of lymph node, further analysis was taken to find the differences of infiltrated immune cells in different parts (See in Table 3). The results showed out that in the group with better prognosis, the density of GrB+lymphocytes infiltrated in the intraepithelial parts of tumor was the highest(with median of 14/0.0625mm^2), but the densities infiltrated in tumor stroma and peritumoral zone were similar(with respective medians of 4/0.0625 mm^2 and 3/0.0625mm^2); In the group with inferior prognosis, the density of GrB+lymphocytes infiltrated in the tumor stroma was the highest, but the density infiltrated in the intraepithelial parts was the lowest. The comparison about the differences of the infiltrated immune cells in the same parts of two groups showed that the differences of the quantities of GrB+lymphocytes infiltrated only in the intraepithelial parts of tumor had statistical significance ($P<0.0001$). In the cases with better prognosis, the infiltrated density of T-bet+lymphocytes was higher than the cases with inferior prognosis, and the quantities of infiltrated lymphocytes in the intraepithelial parts of tumor and tumor stroma of the two groups had statistical differences($P=0.001$,$P=0.001$), and the infiltrated densities of lymphocytes in the peritumoral zone of two groups had no obvious statistical differences.

The quantities of Foxp3+Treg cells in tumor stroma and peritumoral zone of two groups had differences ($p<0.0001$,$p<0.0001$).

In addition, the analysis on the expression levels of CD68+in the tumor and peritumoral zone revealed that in the group with better prognosis, the density of CD68+macrophage was higher than the group with inferior prognosis, and

the differences of the quantities of infiltrated cells in the peritumoral zone of two groups had statistical differences (P=0.006).

In the group with better prognosis, the infiltrated density of PD-1+lymphocytes was higher than the group with inferior prognosis, and the differences had statistical significance (P=0.044), but the quantities of infiltrated lymphocytes in the intraepithelial parts of tumor and tumor stroma of two groups had no obvious statistical differences.

The comparison about the expression of the co-stimulatory molecular B7-H1 associated with the lymphocyte activation in the intraepithelial parts of tumor revealed that the differences between the expressions of two groups had statistical significance (P=0.029). The expressions of tumor cell PD-L2 in two groups had no obvious differences.

The relationship between the expression of B7-H1 in tumor cells and the clinical pathological features and the molecular typing of breast cancer

After screening the above-mentioned immune markers with significant differences, 501 cases were further chosen to be implemented the immumo histochemical colorization of B7-H1(See Figure 6). Among the 501 cases of

Table 3 Comparison between the infiltrated immune cells in breast cancer and associated immune molecular of tumor cells

		Survivaln=20	Death n=20	*P
		median*	median*	
GrB+	Intratumoral zone			
	intraepithelial parts	14	1	<0.0001
	Tumor stroma	4	5	0.566
	peritumoral zone	3	3	0.289
T-bet	Intratumoral zone			
	intraepithelial parts	18	1	0.001
	Tumor stroma	20	2	0.001
	peritumoral zone	18	7	0.083
Foxp3+	Intratumoral zone			
	intraepithelial parts	2	5	0.032
	Tumor stroma	8	22	<0.0001
	peritumoral zone	30	62	<0.0001
PD1	Intratumoral zone			
	intraepithelial parts	8	9	0.661
	Tumor stroma	8	9	0.828
	peritumoral zone	6	12	0.044
B7-H1/PD-L1	Low expression	13(68.4)	6(31.6)	0.029
n(%)	High expression	7(33.3)	14(66.7)	
B7DC/PD-L2	Low expression	11(57.9)	8(42.1)	0.348
n(%)	High expression	9(42.9)	12(57.1)	
CD68+	Intratumoral zone	13	6	0.201
	peritumoral zone	23	10	0.006

*Mann-Whitney U test.

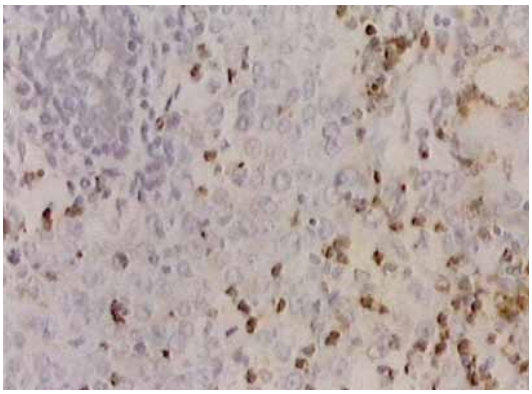

Fig. 2: The immumo histochemical colorization of GrB+lymphocytes infiltrated in tumor. The density of GrB+lymphocytes infiltrated in the intraepithelial parts of tumor presented obvious dense tendency.

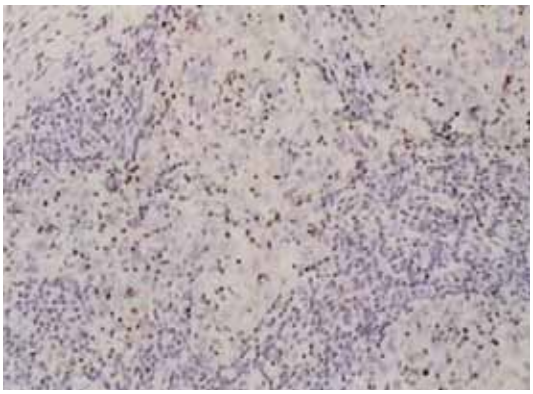

Fig. 3: The immumohistochemical colorization of T-bet+lymphocytes infiltrated in tumor. The T-bet+lymphocytes infiltrated in the intraepithelial parts of tumor presented obvious dense distribution.

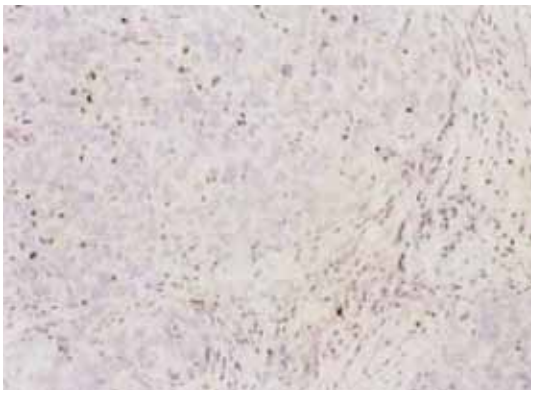

Fig. 4: The immumo histochemical colorization of CD68+macrophage infiltrated in tumor. The CD68+macrophage infiltrated in tumor presented diffuse distribution.

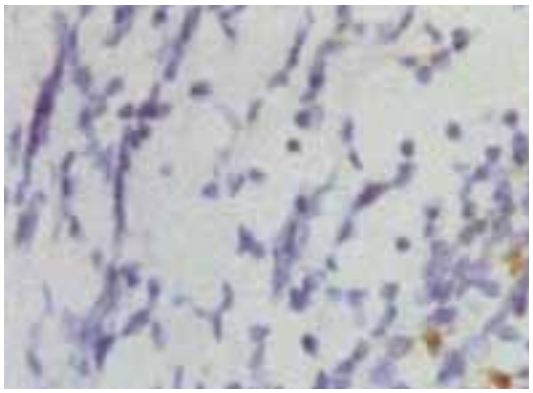

Fig. 5: The immumohistochemical colorization of PD-1+lymphocytes infiltrated in tumor. The density of PD-1+lymphocytes infiltrated in peritumoral zone presented obvious dense tendency.

infiltrated breast cancer, 270 cases(53.9%) presented low expression and 231 cases(46.1%) presented high expression. The expression of tumor cell B7-H1 associated with many kinds of undesirable features of clinical pathology that included histological grading (rs=0.114,P=0.012), high lymph node staging (rs=0.102, P=0.027), ER negative status (rs=-0.110, P=0.014) and PR negative status (rs=-0.141, P=0.002). But the expression of B7-H1 was independent of the age of onset and the size of tumor of patients(See Figure 4).

In addition, the relationship between the expression of B7-H1 and the molecular typing of breast cancer was also analyzed. The expression of B7-H1 obviously related with the molecular typing of breast cancer, and different kinds of molecular typing had differences in the expression. What was noteworthy was that especially in the breast cancer of Basal-like type, the expression of B7-H1 tended to high expression and was superior to the breast cancer of Luminal subtype.

Table 4: The relationship between the expression of tumor cell B7-H1 and the features of clinical pathology of breast cancer and the molecular typing.

Features of clinical pathology	B7-H1		rs	*P
	Low expression	High expression		
Age				
<50	144(58.1)	104(41.9)	0.083	0.064
≥50	126(49.8)	127(50.2)		
Size of tumor(cm)				
≤2	68(52.7)	61(47.3)	-0.006	0.893
>2	180(53.4)	157(46.6)		
histological grading				
	19(54.3)	16(45.7)	0.114	0.012
	191(58.4)	136(41.6)		
III	56(43.1)	74(56.9)		
lymph node staging				
N0	110(56.7)	84(43.3)	0.102	0.027
N1	67(59.8)	45(40.20		
N2	35(42.5)	50(57.5)		
N3	37(42.5)	50(57.5)		
ER status				
negative	84(46.4)	97(53.6)	-0.110	0.014
positive	184(57.9)	134(42.1)		
PR status				
negative	105(46.1)	123(53.9)	-0.141	0.002
positive	163(60.1)	108(39.9)		
HER-2 status				
negative	218(55.5)	175(44.5)	0.056	0.213
positive	48(48.5)	51(51.5)		
molecular typing				
Luminal A	116(63.0)	68(37.0)	0.163	<0.001
Luminal B	64(54.2)	54(45.8)		
Luminal HER-2	13(41.9)	18(58.1)		
HER-2-enriched	13(41.9)	18(58.1)		
Basal-like	38(41.8)	53(58.2)		

*Spearman's Rank-Correlation test.

The relationship between the expression of B7-H1 and the prognostic of breast cancer

The features of clinical pathology of breast cancer patients were proceeded survival analysis with single factor. The traditional features of clinical pathology of breast cancer such as histological grading, lymph node staging, ER status, PR status and HER-2 status were significantly related to the progression-free survival(PFS) and ovearall survival(OS). The analysis on the relationship between the expression of tumor cell B7-H1 and the PFS

Table 5: Single factor and double factors analysis on the PFS of 501 cases of breast cancer

Variable	Single factor analysis			Double factors analysis		
	HR	95%CI	P	HR	95%CI	P
Age(<50 vs≥50)	0.984	0.664-1.460	0.937	1.105	0.728-1.667	0.639
Size of tumor, cm(≤2vs.>2)	1.076	0.682-1.695	0.754	0.911	0.556-1.492	0.821
histological grading (□vs□vsIII)	2.075	1.437-2.997	<0.001	1.543	1.049-2.269	0.028
lymph node staging(N0vs.N1vs. N2vs.N3)	2.045	1.709-2.446	<0.001	2.003	1.656-2.422	<0.001
ER status(negative vs. positive)	0.482	0.325-0.716	<0.001	0.598	0.366-0.977	0.040
PR status(negative vs. positive)	0.542	0.363-0.808	0.003	0.852	0.527-1.379	0.852
HER-2 status(negative vs. positive)	1.753	1.135-2.709	0.11	1.733	1.092-2.478	0.020
B7-H1(low expression vs. high expression)	2.262	1.498-3.416	<0.001	1.833	1.181-2.844	0.007

Table 6: Single factor and double factors analysis on the OS of 501 cases of breast cancer

Variable	Single factor analysis			Double factor analysis		
	HR	95%CI	P	HR	95%CI	P
Age(<50 vs≥50)	1.017	0.661-1.564	0.939	1.196	0.754-1.897	0.447
Size of tumor, cm(≤2vs.>2)	1.188	0.718-1.967	0.502	1.075	0.617-1.873	0.789
histological grading (□vs□vsIII)	2.116	1.413-3.171	<0.001	1.583	1.031-2.431	0.036
lymph node staging(N0vs.N1vs. N2vs.N3)	2.103	1.725-2.563	<0.001	2.084	1.689-2.572	<0.001
ER status(negative vs.positive)	0.389	0.251-0.600	<0.001	0.504	0.293-0.867	0.013
PR status(negative vs.positive)	0.437	0.279-0.684	<0.001	0.790	0.462-1.351	0.389
HER-2 status(negative vs.positive)	2.087	1.315-3.314	<0.001	1.987	1.213-3.256	0.006
B7-H1(low expression vs.highexpression)	2.544	1.607-4.028	<0.001	1.985	1.194-3.213	0.008

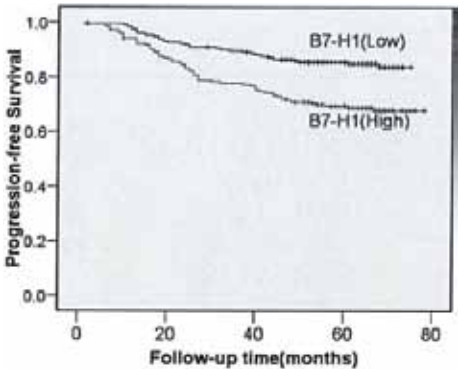

Fig. 6: The analysis of survival curve Kaplan-Meier showed that the expression of B7-H1 related to the prognosis of breast cancer. The expression of B7-H1 had negative correlation to the PFS of patients.

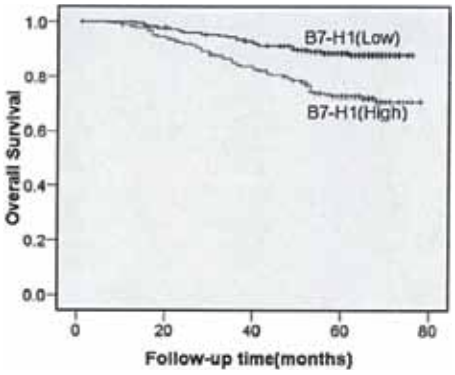

Fig. 7: The analysis of survival curve Kaplan-Meier showed that the expression of B7-H1 related to the prognosis of breast cancer. The expression of B7-H1 had negative correlation to the OS of patients.

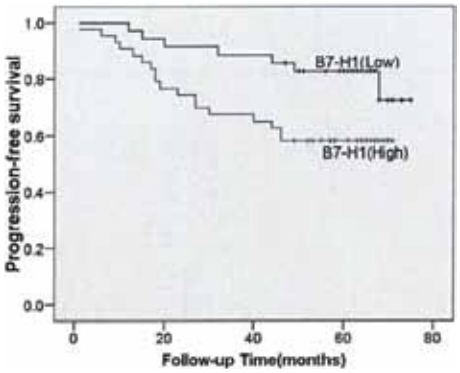

Fig. 8: The analysis of survival curve Kaplan-Meier showed that the expression of B7-H1 in breast cancer of Basal-like type related to the prognosis of breast cancer. The expression of B7-H1 had negative correlation to the PFS of patients(p=0.003).

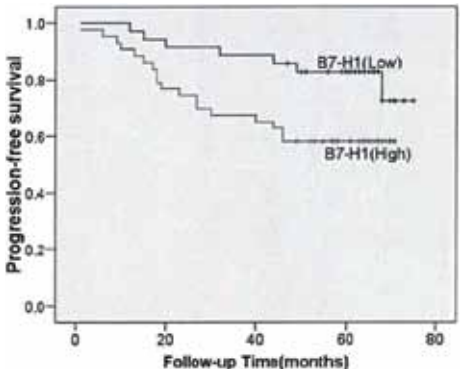

Fig. 9: The analysis of survival curve Kaplan-Meier showed that the expression of B7-H1 in breast cancer of Basal-like type related to the prognosis of breast cancer. The expression of B7-H1 had negative correlation to the OS of patients(p=0.020).

and OS revealed that the expression of B7-H1 had a significant negative correlation to the PFS ans OS(PFS,P<0.001;OS,P<0.001)(See Figure 6,7). At the same time, the survival analysis with Cox double factors was proceeded, which verified that B7-H1 could be regarded as the independent predictive index for the poor prognosis of breast cancer(See Figure 5, 6).

The relationship between the expression of B7-H1in breast cancer of Basal-like type and the clinical prognostic

Among 501 cases of breast cancer, 91 cases were breast cancer of Basal-liketype, then further analysis on the prognostic significance of the expression of B7-H1 in Basal-like type was proceeded. In survival curve Kaplan-Meier, it was found that the expression of B7-H1 related to the PFS and OS of patients(PFS, P=0.030;OS, p=0.020), and the expression of B7-H1 in Basal-like type still had predictive value of clinical prognosis(See Figure8,9).

The relationship between the expression of B7-H1 and Foxp3+Treg cells and PD1+T lymphocytes

The previous research proved that the Foxp3+Treg cells infiltrated in breast cancer related to the survival prognosis of patients

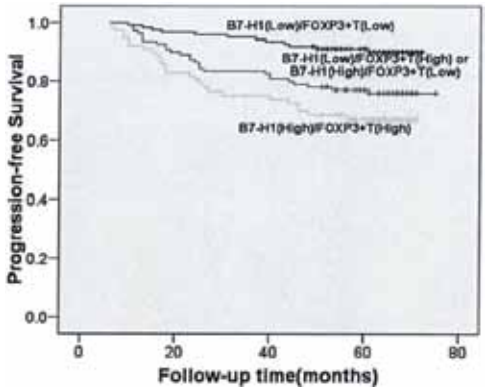

Fig. 10: The PFS analysis on combining the expression of B7-H1 and the infiltrated density of Foxp3+Treg cells in tumor. The PFS rate was the lowest when the expression of B7-H1 was at high level or the infiltrated density of Foxp3+Treg was at high level(p<0.001).

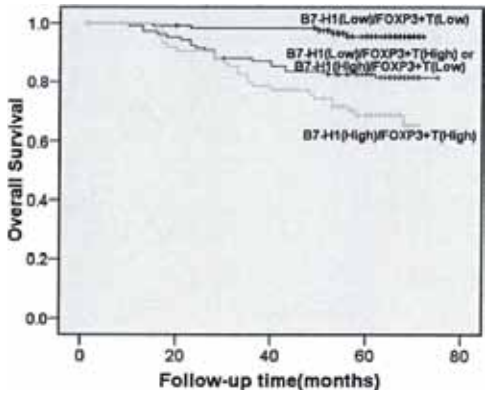

Fig. 11: The OS analysis on combining the expression of B7-H1 and the infiltrated density of Foxp3+Treg cells in tumor. The OS rate was the lowest when the expression of B7-H1 was positive or the infiltrated density of Foxp3+Treg was at high level(p<0.001).

and had negative correlation to the PFS and OS of patients. Therefore, further analysis on the relationship between the expression of B7-H1 and Foxp3+Treg cells was proceeded(See Figure 7). The results of the experiment showed that the expression of B7-H1 had positive correlation to the quantities of the Foxp3+Treg cells infiltrated in the tumor(rs=0.287,p<0.001). The survival analysis on combining the expression of B7-H1 and the infiltrated density of Foxp3+Treg cells was taken, and the cases of breast cancer were divided into three groups. Then it turns out that the prognosis of cases with high-level B7-H1 and Foxp3+Treg at one time was inferior to the prognosis of cases with low-level B7-H1 and Foxp3+Treg at one time or cases with high-level B7-H1 and low-level Foxp3+Treg or low-level B7-H1 and high-level Foxp3+Treg(PFS, p<0.001;OS, p<0.001) (See Figure 10,11).

Table 7: The relationship between the quantities of Foxp3+Treg and PD-1+T lymphocytes and the expression of B7-H1 in tumor cells

	B7-H1(H value, median)0.9	rs	*P
FOXP3+ (median a)	10	0.287	<0.001
PD-1(median a)	3	0.092	0.465

[a]Unit: quantities of cells/0.0625mm^2
*Spearman's Rank-Correlations test.

Otherwise, the relationship between the expression of B7-H1 and PD1+T lymphocytes infiltration was analyzed, and no obvious correlation between them was found(rs=0.092, p=0.465).

DISCUSSION

The occurrence, development, invasion and metastasis of tumor related to the immune state of the body. In the pathogenesis of breast cancer, the imbalance of immune played an important role in the pathogenic process. In the tumor micro-environment, there were plenty of antigen presenting cells, regulatory T cell Treg, immune suppressor cells, various negative immune factors excreted by cells with functional limitations, the interactions between rejection capability ligrands and receptors and the negative regulation of the metabolic activity of T cells in micro-environment. And all the above-mentioned factors were the important factors in the tumor immune escape mechanism and could help the tumor cells escape from

the collective tumor immune monitoring and cause the occurrence of the immune escaping of the tumor cells. In the different distribution spaces and different stages of one same tumor, the same immune cells may play different roles. Therefore, the research aiming to simplex or selected immune cells and immune factors could hardly generally reflect or explain the complicated immune status in the tumor micro-environment. Consequently, this research analyzed the immune functions status of infiltrated immune cells in breast cancer and related immune factors and selected immune cells or immune factors with significance of prognosis to find the possible individual immune treatment targets of breast cancer.

CONCLUSION

The expression of tumor cell B7-H1can be the independent predictive index of the OS and PFS of the breast cancer patients and is counted to become the target for the immune treatment of the breast cancer. And it is likely that the expression of tumor cell B7-H1 can provide potential immune targets for the treatment of Basal-like type such as Fairy which is short of the effective treatment strategies. The molecules of B7-H1 may regulate and control the adaptive immunity in negative direction under the cooperation with Treg cells, which makes significant contributions to the immune escape of tumor. In addition, the selected infiltrated cells with differences in the density and distribution provide new theoretical foundation for the immune treatment of breast cancer.

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