Int J Clin Exp Med 2017;10(1):1251-1258
www.ijcem.com /ISSN:1940-5901/IJCEM0037617

Original Article

The peripheral lymphocyte subsets correlates with clinicopathological characteristics of breast cancer patients

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Received August 10, 2016; Accepted October 15, 2016; Epub January 15, 2017; Published January 30, 2017

Abstract: This study aims to investigate correlation between clinicopathological characteristics and lymphocyte subsets or immuno-histochemical molecular subtypes. Peripheral blood was selected from 246 breast cancer patients. Flow cytometry assay was used to examine CD3+, CD4+, CD8+, CD4+/CD8+, CD19+ and NK cells. ER, Her-2, and Ki67 expressions were detected. We evaluated association between lymphocyte subsets and breast cancer phenotypes. The results indicated that CD3+, CD4+ cell expression were lower, and CD19+ expression was higher in malignant breast cancer patients compared to benign breast tumor patients ($P < 0.05$). Clinical pathologic factors, including age, postmenopausal, birth number (≥2), are closely related with CD4+/CD8+ ($P < 0.05$). NK cells are closely related with years (>50 years), postmenopausal, tumor size (≥2 cm) and infiltrating carcinoma patients ($P < 0.05$). Ki67 ≥14%, Her-2-, TNBC are associated with lower CD3+ levels compared with Ki67 <14%, Her-2+ and Luminal B ($P < 0.05$). ER-, Ki67 ≥14%, Her-2+ and TNBC were associated with low levels of CD4+ compared with ER+, Ki67 <14%, Her-2-, and luminal B ($P < 0.05$). Patients with highest level of CD8+ cells mainly exist in ER-, Her-2+, Ki67 ≥14% and TNBC patients compared with ER+, Her-2-, Ki67 <14% and luminal A patients. Higher levels of NK cells were correlated with increased risk of ER- and TNBC compared with ER+ and Luminal B (OR >1, $P < 0.05$). In conclusion, there is no significant differences of immune function among different immune phenotype of breast cancer. Meanwhile, ER- patients and TNBC patients always exhibit more higher tumor malignant degree.

Keywords: Breast cancer, lymphocyte subsets, triple-negative breast cancer, immune function

Introduction

Breast cancer is the most prevalent malignancy in the women worldwide globally. About 235000 women have been diagnosed with breast cancer in 2014 [1, 2]. Meanwhile, epidemiological research illustrated that about 400000 patients in the world die from breast cancer each year [3]. In Europe, the breast cancer accounts for about 10% overall cancer costs [4], therefore, this disease places a significant burden on the healthcare system and the patients themselves. In China, breast cancer is the most diagnosed and occurred malignancy, and is the sixth reason for cancer caused death [5]. The breast cancer is a heterogeneous disorder which was usually divided into 5 different molecular subtypes, including luminal A (ER+ and/or PR+, and Ki67 <14%), luminal B (ER+ and/or PR+, and Ki67 ≥14%), human epidermal growth factor receptor-2 (HER-2) overexpression (ER-, PR-, and HER-2+), HER-2 basal-like (ER+ and/or PR+, and HER-2+) and HER-2 normal like (ER-, PR-, and HER-2-, triple-negative breast cancer) subtype [6].

The pathology and progressive mechanism are also elusive. The previous studies reported that the immune system plays an important in the tumor occurrence and development [7], and the lymphocyte subpopulations are critical for the function of immune system. CD3+ T cells represent the total T cells, and which could be divided into several subpopulations according

to the different functions, including CD4+ T cells, CD8+ T cells, CD16 T cells, CD56 T cells, and etc. All of the about T cells could play the anti-tumor functions by triggering the humoral immune system. Wang et al. [8] reported that the examination of the lymphocytes and the killer cells (such NK cell) could be assistant to the disease diagnosis in clinical. The recent studies [9-11] have been reported that comparing to the normal individuals, the peripheral lymphocyte CD3+, CD4+ percentage were decreased, CD8+ percentage was increased, and the ratio of CD4+/CD8+ was decreased in the breast cancer patients. This finding illustrates that the immune system is dysfunctional in the breast cancer patients. Therefore, we speculated that the different breast cancer subtype may correlates with lymphocyte subpopulation.

In this study, we examined the peripheral blood CD3+, CD4+, CD8+, CD4/CD8, CD19+ and NK (CD56+) levels by using the flow cytometry assay. Meanwhile, the levels of ER, Ki-67 and HER-2 were also detected by using the pathological examination methods. This study aims to analyze correlation between these immune indexes and the clinicopathological characteristics.

Materials and methods

Patients

A total of 246 patients with breast cancer were retrospectively selected from the Tianjin Medical University Cancer Institute and Hospital from May, 2014 to December, 2014. For all of these patients, the clinicopathological materials, fresh whole blood pre-operation, pathological tissue post-operation were conserved and analyzed. Exclusive criteria: distant metastasis of tumor, serious infection, autoimmune diseases and the other affecting immune function disorders. Among the 246 patients, total correction 31 cases, Imitation radical cure 111 cases, segmental resection 59 cases, breast conserving surgery 23 cases and simple excision 19 cases. The age is range from 19 years to 82 years, with the average age of 49.11.

Blood sample collection

The blood samples were collected at the room temperature and processed within one hour on the day performing the blood collection. The whole blood was collected aseptically into potassium ethylenediaminetetra-acetic acid-containing tubes. The complete blood counts and the differential counts were performed by using the automated hematology analyzer and the flow cytometry. The parameters analyzed in this study were listed as the followings, including the white blood cells (WBC) and the percentage of the lymphocytes, such as CD3+ cells, CD4+ cells, CD3+ CD4+ cells, CD3+ CD19+ cells, CD3+ CD56+ cells and CD3+ CD8+ cells. Moreover, the ratio of CD4+/CD8+ was evaluated in this study.

Flow cytometry analysis

Aliquots of the whole blood were added to the capped poly-propylene test tubes, which contain the pre-mixed monoclonal antibodies. The mouse anti-human CD3+, CD4+, CD19+, CD8+, CD56+ antibodies were purchased from Santa Cruz Inc. CA, USA (catalogue No. sc-1175, sc-514746, sc-373897, sc-1177, sc-3929, respectively). The fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD3+ monoclonal antibody (mAb), phycoerythrin (PE)-conjugated mouse anti-human CD8+ mAb, allophycocyanin (APC)-conjugated mouse anti-human CD45 mAb were mixed in one test tube. The FITC-conjugated mouse anti-human CD3+ mAb, PE-conjugated mouse anti-human CD56+ mAb, APC-conjugated mouse anti-human CD45 mAb and PE-conjugated mouse anti-human CDE19 mAb were mixed in another test tube. Blood was incubated with the above antibodies in the dark room at room temperature for 20 min. The red blood cells were lysed by treating with FACS lysing solution (BD Biosciences, San Jose, CA, USA) at temperature for 15 min. Subsequently, the Count Bright absolute counting beads was added to the test tube, and was evaluated by using the flow cytometer (BD, FACSCalibur, San Jose, CA, USA). The obtained data were analyzed by using the Cell Quest pro software (BD, San Jose, CA, USA).

Pathological examination

The stage of tumors was based on the American Joint Committee on Cancer pathological cancer staging classification (7th edition) [12]. If the pathological tumor size was missing, the clinical stage was defined based on the imaging studies and clinical examination. The hematox-

Table 1. Clinicopathological characteristics of patients

Clinicopathological characteristics		Case number
Age	<50	129
	≥50	117
Menopausal status	No	143
	Yes	103
Abortion	No	89
	Yes	157
Number of birth	≤1	182
	≥2	64
Clinical staging classification	I/II	207
	III/IV	39
Tissue grading classification	I	9
	II	128
	III	42
Tumor size	<2 cm	45
	≥2 cm	201
lymph node metastasis	Yes	68
	No	178
Tumor type	Invasive breast cancer	179
	Benign breast cancer	67

ylin-eosin staining (HE) was used to identify the clinical stage of the breast cancer. Immunohistochemical findings regarding hormone receptor status, Ki-67 expression was available from previous study [13]. The status of Ki-67 was evaluated according to the following criteria. Immunostaining for Ki-67 was considered positive when <14% of cells indicated staining [14]. Definition of estrogen receptor (ER) negatively followed current Swedish clinical guidelines (<10% positive nuclei). Human epidermal growth factor receptor 2 (HER2) was considered as weakly expression when scores range from o to 2, and considered as strongly when scores above 3 [15].

Statistical analysis

All data were presented as the mean ± SD from at least three different experiments. Differences between groups were analyzed by chi-square test. The lymphocyte subpopulations were divided into 4 groups, including lower amounts group, medium amounts group and higher amounts group. The chi-square test was used to analyze the odds ratio (OR), 95% confidence intervals (95% CI) and *P* value between lower amounts (medium amounts) and immunohistochemical results or lower amounts (higher amounts) and immunohistochemical results. Statistical analyses were performed by using the SPSS software (SPSS, 19.0 version, IBM, Chicago, IL, USA). A *P* value less than 0.05 was considered as the statistically significant.

Results

Clinicopathological characteristics of patients

In this study, we retrospectively analyzed the age, menopausal status, abortion situation, number of birth, clinical stage classification, tumor size, lymph node metastasis and the tumor type. The results were listed in **Table 1**.

Relationship between lymphocyte subpopulation and clinicopathological characteristics for breast cancer patients

The result showed that CD3+ levels in >50 years, non-menopausal, abortion history, clinical III/IV stage, tumor size <2 cm sub-group was significantly higher compared to the CD3+ levels in age <50 years, menopausal, non-abortion history, clinical I/II stage and tumor size >2 cm, respectively (**Table 2**, P <0.05). CD8+ levels in age <50 years, clinical III/IV stage sub-group was higher significantly compared to levels in age >50 years and clinical I/II patients, respectively (**Table 2**, P <0.05). The CD4+/CD8+ levels in age >50 years, menopausal, abortion time more than 2 times were higher significantly compared to levels in age <50 years, non-menopausal, abortion time less then2 times (**Table 2**, P <0.05). The NK cell levels in age >50 years, menopausal, tumor size >2 cm were significantly higher compared to levels in age <50 years, non-menopausal, tumor size <2 cm (**Table 2**, P <0.05). The CD19+ levels in non-abortion patients were higher significantly compared to the abortion patients (**Table 2**, P <0.05).

Relationship between immunohistochemistrical results and clinicopathological characteristics for breast cancer patients

In this study, we also analyzed the relationship between the immunohistochemistrical results and the clinicopathological characteristics. The chi-square test results indicated that the ER expression (positive or negative) are correlated

Table 2. Analysis for the relationship between lymphocyte subpopulation and clinicopathological characteristics of breast cancer patients

Clinicopathological characteristics		CD3+ m±s	*P* value	CD8+ m±s	*P* value	CD4+ m±s	*P* value	CD4+/CD8+ m±s	*P* value	NK m±s	*P* value	CD19+ m±s	*P* value
Age	<50	70.01±8.3	0.03	26.55±7.33	0.038	39.69±7.62	0.002	1.58±0.61	0.002	14.74±6.75	0	13.98±5.72	0.56
	≥50	66.1±11.3		25.38±9.33		38.58±10.05		1.73±0.94		17.96±10		14.92±5.94	
Menopausal status	No	70.22±8.31	0	26.58±7.64	0.1	39.83±7.06	0	1.58±0.62	0.003	14.78±7.02	0	13.74±5.49	0.165
	Yes	65.23±11.46		25.1±8.1		38.23±10.37		1.76±0.96		18.36±10.11		15.41±6.2	
Abortion	No	66.37±11.02	0.037	25.13±8.28	0.819	38.01±9.16	0.414	1.69±0.87	0.591	16.38±9.49	0.105	16.14±6.33	0.029
	Yes	69.02±9.27		26.4±8.16		39.72±8.63		1.64±0.74		16.29±8.09		13.5±5.35	
Number of birth	≤1	68.2±10.22	0.361	26.2±7.87	0.39	38.75±8.5	0.049	1.58±0.65	0.001	16.21±8.73	0.426	5.67±0.42	0.192
	≥1	67.91±9.57		25.27±9.12		40.31±9.86		1.88±1.07		16.47±8.35		6.35±0.79	
Clinical staging classification	I/II	68.09±9.62	0.009	25.7±7.79	0.028	39.33±8.32	0.003	1.67±0.78	0.424	16.19±8.3	0.085	14.51±5.79	0.484
	III/IV	68.42±12.07		27.53±10.09		38.24±11.4		1.56±0.81		16.72±10.16		14±6.15	
Tumor size	<2 cm	70.95±7.42	0.016	27.72±8.78	0.806	39.7±8.77	0.859	1.64±0.8	0.952	13.73±6.08	0.018	14.21±5.93	0.89
	≥2 cm	67.5±10.44		25.59±8.03		39.04±8.9		1.66±0.79		16.86±8.99		14.48±5.83	
Lymph node metastasis	Yes	66.64±10.57	0.09	27.22±8.46	0.29	37.23±9.38	0.223	1.58±0.95	0.16	17.2±8.6	0.485	15.16±6.06	0.372
	No	68.71±9.78		25.52±8.07		39.89±8.57		1.68±0.71		15.93±8.6		14.15±5.74	
Tumor type	Invasive breast cancer	67.07±10.93	0	26.11±8.5	0.395	37.98±9.21	0.024	1.58±0.76	0.326	17.04±9.31	0.001	14.75±6.2	0.025
	Benign breast cancer	70.79±6.33		25.67±7.41		42.27±7.04		1.85±0.82		14.27±5.96		13.58±4.67	

Table 3. Analysis for the relationship between immunohistochemistrical data and clinicopathological characteristics of breast cancer patients

Clinicopatholgical characteristics		Case number	ER			Ki67			Her-2		
			ER- (N=43)	ER+ (N=99)	*P* value	<14% (N=19)	≥14% (N=115)	*P* value	Her-2- (N=20)	Her-2+ (N=65)	*P* value
Age	<50	64	38	26	0.015	5	58	0.899	11	32	0.836
	≥50	78	61	16		14	57		9	33	
Menopausal status	No	72	48	24	0.455	10	59	0.973	10	37	0.913
	Yes	69	50	19		9	55		10	28	
Abortion	No	49	34	15	0.253	15	81	0.953	4	21	0.492
	Yes	93	65	28		4	33		16	44	
Number of birth	≤1	102	77	26	0.174	14	83	0.963	17	43	0.261
	≥2	39	22	17		5	31		3	22	
Clinical staging classification	I/II	114	79	35	0.826	16	86	0.617	16	53	0.469
	III/IV	28	20	8		3	19		4	12	
Tissue grading classification	I	5	4	1	0.489	2	3	0.051	1	3	0.307
	II	105	75	30		16	83		18	46	
	III	32	20	12		1	29		1	16	
Tumor size	<2 cm	23	19	4	0.264	4	18	0.374	1	3	0.587
	>2, <5 cm	107	73	34		17	87		18	46	
	>5 cm	12	7	5		0	10		1	16	
Lymph node metastasis	No	104	74	30	0.934	16	82	0.209	16	43	0.871
	Yes	38	25	13		3	33		4	22	

Table 4. Lymphocyte subpopulation reference range

Lymphocyte subpopulations	Range		
	Lower value	Medium value	Higher value
CD3+	<60.8	60.8-75.4	>75.4
CD8+	<18.2	18.2-32.8	>32.8
CD4+	<29.4	29.4-45.8	>45.8
CD4+/CD8+ ratio	<0.98	0.98-1.94	>1.94
NK	<9.5	9.5-23.5	>23.5
CD19+	<6.8	6.8-15.8	>15.8

closely with the age of the patients (>50 years or <50 years) (**Table 3**, P=0.015). However, the expression of the Ki67, HER-2 are not correlated with all of the clinicopathological characteristics (P >0.05).

Correlation between clinicopathological characteristics and breast cancer molecular subtypes

The correlation between age, menopausal status, abortion situation, number of birth, clinical stage classification, tumor size, lymph node metastasis and the breast cancer sub-types have also been analyzed. The results proved that there are not significant correlation between clinicopathological characteristics and the molecular subtypes (P >0.05, data not shown).

Correlation between immunohistochemistrical data and lymphocyte subpopulations

According to the classification for the lymphocyte subpopulations in our hospital (**Table 4**), the lymphocyte subpopulations were divided into 3 groups, including lower amounts group, medium amounts group and higher amounts group. The chi-square test was used to analyze the odds ratio (OR), 95% confidence intervals (95% CI) and *P* value between lower amounts (medium amounts) and immunohistochemical results or lower amounts (higher amounts) and immunohistochemical results. The results indicated that compared to the Ki67 <14% group, possibility of higher CD3+ levels were decreased significantly in the Ki67 >14% group (even decreased 5-fold, OR 0.195, 95% CI (0.162-0.235) (**Table 5**). The possibility of higher CD3+ levels were increased significantly in HER-2+ group compared to HER-2- group (more than 1.5-fold, OR=1.478, 95% CI (1.237-1.765)) (**Tables 5**, **6**). The possibility of higher CD8+ levels in ER- group, Ki67 <14% group, HER-2- were significantly increased compared to ER+, Ki67 >14% group, HER-2+ group, respectively (more than 1 fold, OR >1) (**Tables 5**, **6**). The possibility

Table 5. Correlation analysis for correlation between immunohistochemistrical data and lymphocyte subpopulations

	ER- v.s. ER+		Ki67 ≥14% v s. Ki67 <14%	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value
CD3				
1	1.08 (0.961, 1.214)	0.194	0.885 (0.729, 1.076)	0.220
2	0.995 (0.876, 1.130)	0.936	0.195 (0.162, 0.235)	0.000
CD8				
1	1.909 (1.504, 2.423)	0.000	0.917 (0.647, 1.301)	0.627
2	1.483 (1.149, 1.912)	0.002	1.693 (1.133, 2.531)	0.010
CD4				
1	0.597 (0.492, 0.725)	0.000	0.415 (0.261, 0.659)	0.000
2	0.571 (0461, 0.689)	0.000	0.066 (0.042, 0.105)	0.000
CD4/CD8				
1	0.588 (0.207, 1.699)	0.315	0.518 (0.063, 4.251)	0.533
2	0.525 (0.654, 1.529)	0.233	0.171 (0.021, 1.370)	0.064
NK				
1	1.107 (0.818, 1.498)	0.511	1.059 (0.693, 1.616)	0.792
2	1.039 (0.753, 1.433)	0.817	1.288 (0.815, 2.037)	0.278
CD19				
1	3.562 (2.022, 6.277)	0.000	1.632 (0.734, 3.630)	0.225
2	2.040 (1.168, 3.652)	0.011	2.123 (0.958, 4.706)	0.058

Number "1" represents comparison between medium value and lower value. Number "2" represent comparison between higher value and lower value.

Table 6. Analysis for correlation between breast cancer molecular subtypes and lymphocyte subpopulations

	Her-2+ v.s. Her-2-		TNBC v.s. LuminalB	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value
CD3				
1	0.870 (0.744, 1.017)	0.081	0.674 (0.556, 0.817)	0.000
2	1.478 (1.237, 1.765)	0.000	0.389 (0.317, 0.478)	0.000
CD8				
1	1.288 (0.953, 1.740)	0.099	1.614 (1.134, 2.297)	0.007
2	2.050 (1.474, 2.853)	0.000	1.792 (1.219, 2.633)	0.003
CD4				
1	0.362 (0.283, 0.455)	0.000	0.564 (0.440, 0.722)	0.000
2	0.344 (0.260, 0.455)	0.000	0.278 (0.191, 0.404)	0.000
CD4/CD8				
1	0.327 (0.079, 1.342)	0.108	0.630 (0.387, 6.502)	0.519
2	0.468 (0.116, 1.886)	0.278	0.630 (0.137, 2.890)	0.551
NK				
1	0.799 (0.541, 1.182)	0.261	0.790 (0.484, 1.291)	0.346
2	1.463 (0.959, 2.232)	0.076	0.629 (0.375, 1.055)	0.077
CD19				
1	0.831 (0.337, 2.049)	0.687	2.902 (1.050, 8.022)	0.032
2	0.439 (0.182, 1.060)	0.060	3.368 (1.220, 9.294)	0.013

Number "1" represents comparison between medium value and lower value. Number "2" represent comparison between higher value and lower value.

of higher CD19+ levels in ER- group were significantly increased compared to ER+ group (more than 1 fold, OR >1) (**Table 5**). The possibility of higher CD4+ levels in ER- group, Ki67 <14%, HER-2- group were significantly decreased compared to ER+, Ki67 >14% group, HER-2+, respectively (less than 1 fold, OR <1) (**Tables 5**, **6**).

Correlation between breast cancer molecular subtypes and lymphocyte subpopulations

The chi-square test was also used to analyze the odds ratio (OR), 95% confidence intervals (95% CI) and *P* value between lower amounts (medium amounts) and molecular subtypes or lower amounts (higher amounts) and molecular subtypes. The results indicated that Compared to the Luminal B, the possibility of higher CD3+ levels were decreased significantly in TNBC group (more than 2.5-fold, OR=0.389, 95% CI (0.317-0.478)) (**Table 6**). The possibility of higher CD8+ levels in TNBC group were significantly increased compared to Luminal B group (more than 1 fold, OR >1) (**Table 6**). The possibility of higher CD4+ levels in TNBC were significantly decreased compared to Luminal B group (less than 1 fold, OR <1) (**Table 6**). The possibility of higher CD19+ levels in TNBC were significantly increased compared to Luminal B group (more than 1 fold, OR >1) (**Table 6**).

Discussion

The immune dysfunction always exist in the process of the breast cancer. The distri-

bution of lymphocyte subpopulation is critical for the functions of the lymphocytes [16]. Wang et al. reported that the decreased Th/Tr cells ratio indicates impaired immune function, suggesting that the stage IV breast cancer and the Her-2/VEGF positive breast cancer patients have lower immune function [17]. Therefore, examination for the lymphocyte subpopulation is helpful to acknowledge the immune function, assist the disease diagnosis and investigate the pathological mechanism of breast cancer.

The present study collected the peripheral blood and examined the lymphocyte subpopulation to reflect the changes of the immune function. The CD3+, CD4+, CD4+/CD8+, CD19+ and NK levels in the peripheral blood were also detected by using the Flow cytometry analysis. The T lymphocyte could recognize the tumor cells, and could kill the tumor cells when activated by the tumor stimuli. The immune function closely related to the tumorigenesis, tumor development and tumor prognosis [18]. This study showed that the CD3+ and CD4+ levels in the malignant breast cancer patients were lower significantly compared to the benign breast cancer patients. Meanwhile, the NK and CD19+ cells in malignant breast cancer patients were higher significantly compared to the benign breast cancer.

In the development of the tumor, the tumor cell could secret many kinds of immunosuppressive factors, and induced the production of the suppressive T lymphocyte. Subsequently, the immunosuppressive factors and the suppressive T cells induce the abnormal of the CD4+ and CD8+ cells, and trigger the out-balance of CD4+/CD8+ ratio, and cause the dysfunction finally [19]. In this study, we found that the age (50 years old), menopausal status and the number of birth could affect the CD4+/CD8+ ratio, and the CD4+/CD8+ ratio was decreased according to the aggravation of breast cancer, all of which are consistent with the previous study [20]. This study also proved that the NK cells were related with the age of patients, menopausal status and tumor size in the malignant breast cancer patients. These suggest that the later of the tumor progression, the more burden of tumor development, the more obvious for the immune immuno-suppression.

The breast cancer is a kind of clinical diversity and molecular diversity diseases, which was classified according to the expression of the biomarkers, such as ER, HER-2, Ki67, and ect [21]. The present study proved that the lower levels of CD3+ closely related with the Ki67 levels >14% compared to Ki67 level <14%, and also CD3+ closely associated with the TNBC compared to Luminal B. The levels of CD3+ in HER-2+ patients were significantly higher compared to HER-2- patients (even achieves to 1.5-fold). Lower CD4+ levels and higher CD8+ levels were correlated with ER-, Ki67 >14%, HER-2- and TNBC patients. The possibility of higher levels of CD19+ in ER+ and TNBC patients were increased significantly compared to ER- and Luminal B patients (OR >1, P <0.05). Therefore, our findings suggest that the CD8+ is the risk factor for the ER-, Ki67 >14%, HER-2+ and TNBC (OR >1), and CD19+ is the risk factor for the ER- and TNBC (OR >1).

In conclusion, there is no significant differences of immune function among the different immune phenotype of breast cancer. Meanwhile, the ER- patients and the TNBC patients always exhibit the more higher tumor malignant degree.

Acknowledgements

This study was granted by Tianjin Medical University Cancer Institute and Hospital Level Program (Grant No: Y1411).

Disclosure of conflict of interest

None.

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