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Original Article

Human papillomavirus infection correlates with inflammatory Stat3 signaling activity and IL-17 expression in patients with breast cancer

Nan Zhang[1,2,7*], Zhi Ping Ma[1,4*], Ju Wang[3*], Hui Li Bai[1,5*], Yi Xin Li[1,2], Qin Sun[6], Lan Yang[1,2], Lin Tao[1,2], Jin Zhao[1,2], Yu Wen Cao[1,2], Feng Li[1,2], Wen Jie Zhang[1,2]

[1]Department of Pathology and The Key Laboratories for Xinjiang Endemic and Ethnic Diseases, Shihezi University School of Medicine, Shihezi 832002, Xinjiang, China; [2]Department of Pathology, The First Affiliated University Hospital, Shihezi University School of Medicine, Shihezi 832002, Xinjiang, China; [3]Department of Histology and Embryology, Shihezi University School of Medicine, Shihezi 832002, Xinjiang, China; [4]Department of Pathology, The First Affiliated University Hospital, Xinjiang Medical University, Urumqi 830054, Xinjiang, China; [5]Department of Pathology, Zhengzhou Central Hospital, Zhengzhou 450007, Henan, China; [6]Department of Mental Health Center, Hubei General Hospital, Wuhan University School of Medicine, Wuhan 430060, Hubei, China; [7]Current Address: Zhengzhou Central Hospital, Zhengzhou 450007, Henan, China. [*]Equal contributors.

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Abstract: Objectives: Microbiota has been suggested in promoting chronic inflammation in human tissues which, in turn, promotes tumor development. This study tests a hypothesis that high-risk human papillomavirus (HR-HPV) infection may correlate with proinflammatory Stat3 signaling activities and IL-17 levels in breast cancer (BC) patients. Materials and methods: This study examined HPV infection by GenChip technology, constitutively active Stat3 (p-Stat3) and IL-17 levels by immunohistochemistry (IHC) using specific antibodies in 379 BC patients, together with 245 paired adjacent breast adenosis (ABA) tissues and 100 unrelated breast adenosis (BA) tissues. Results: We obtained four major findings: (1) HR-HPV16/18 infections existed in 10.5% (34/325) of BC issues, higher than control BA tissues (4%, 4/100, $P = 0.047$). (2) Using IHC methodology, BC tissues showed more overactive p-Stat3 (2+/3+, 38.5%, 146/379) than ABA tissues (27.3%, 67/245, $P < 0.001$); similarly, BC also had more tissues overexpressing IL-17 (2+/3+, 61.5%, 233/379) than ABA tissues (51.8%, 127/245, $P < 0.001$). (3) High levels (2+/3+) of both active p-Stat3 and IL-17 correlated with poor differentiation and lymph nodal metastasis in BC (both with $P < 0.05$), but not with patients' prognosis. (4) HR-HPV infections correlated with both active p-Stat3 ($P = 0.018$) and its downstream IL-17 levels ($P = 0.021$) in BC tissues. Conclusion: There may be a possible tri-lateral relationship among HPV infection, constitutive Stat3 activity and IL-17 level, whose collaborations could orchestrate a proinflammatory microenvironment in breast tissues by which promote carcinogenesis and/or facilitate progression of breast cancer.

Keywords: Inflammation, breast cancer, high-risk HPV, IL-17, p-Stat3, prognosis

Introduction

Breast cancer (BC) is the second most common cancer worldwide following lung cancer, with incidence, mortality and 5-year prevalence accounting for 11.9%, 6.4% and 19.2%, respectively, among all cancers according to GLOBOCAN 2012 [1]. For the last few decades in China, the incidence of breast cancer has increased with a trend of more younger patients being diagnosed [2, 3]. Despite improved overall outcomes seen in BC attributable to early detection and optimal treatment, about 30% of women with BC are experiencing recurrent diseases or distant metastases [4].

The current paradigm is that most solid tumors are associated with chronic inflammation, directly or indirectly [5]. Infectious factors are responsible for approximately 18% of human cancers and it is well accepted that human breast cancer is highly associated with environmental factors, such as virus, diet, source of water, radiation, among others [6, 7]. Among many microorganisms studied, viral infections are suggested in cancer development especial-

ly those cancers caused by human papillomavirus (HPV). Recently, high-risk HPV (HR-HPV) subtype infections, such as HPV16/18, have been reported to be associated with oropharyngeal and colorectal cancers [8]. We have also found that HPV16 infection exists in almost a half (48.4%) of Chinese patients with colorectal cancer [9]. Moreover, another study has identified HR-HPV and other viruses in BC warranting investigations of underlying mechanism(s) of HPV infection in breast cancer [10].

Due to various inflammatory cells and cytokines present in tumor microenvironment, tumors have been referred to as "wounds that do not heal" [11]. Signal transducer and activator of transcription 3 (Stat3) is a critical signaling pathway that is suggested in the formation of tumor microenvironment through regulating downstream proinflammatory cytokines and factors promoting tumor growth and progression [5, 12]. Stat3 signaling not only promotes cell survival and neoplastic progression, but also stimulates metastasis and invasion [13]. Phosphorylated Stat3 (p-Stat3) is the active form of Stat3 that can be detected in various cancers including breast cancer [14]. In the cytoplasm, phosphorylated p-Stat3 dimerizes and then translocates into the nucleus where it regulates the expression of genes involved in cell proliferation, differentiation, and apoptosis [15, 16]. Among the downstream molecules induced by Stat3 signaling, interleukin-17 (IL-17) is an essential proinflammatory cytokine mainly produced by T-helper type 17 (Th17) cells, a distinct CD4+ T-helper cell subset [17], and plays a dual role in serving either as a promoter or antitumor factor depending upon cancer models [18]. Constitutively active p-Stat3 can regulate the differentiation and maturation of Th cells to secret IL-17, which, in turn, positively feeds back to promote Stat3 signaling, by which induces more expression of IL-17 [5, 19].

Bacterial infections have been shown to collaborate with Stat3 signaling to induce BC [5]. It is not known, however, whether viral infections may also be able to act in the same fashion as bacteria. We have previously revealed a tri-lateral relationship among HPV infection, constitutive Stat3 activity and IL-17 level, whose collaborative act may orchestrate a proinflammatory microenvironment in the colorectum that, in turn, may promote carcinogenesis and possibly facilitate progression of colorectal cancer [9]. This study extends those observations found in colorectal cancer [9] and focuses on patients with breast cancer to seek possible correlations of HPV infection with Stat3 signaling activity and its downstream IL-17 cytokine. Here we have tested the hypothesis again that, similar to bacterial infections, chronic HPV infection may also be able to collaborate with Stat3 signaling, triggering an inflammatory response that may facilitate the formation of a microenvironment favorable for carcinogenesis and/or cancer progression of BC. By examining tissues from a cohort of 379 BC patients and 245 paired adjacent breast adenosis (ABA), we have obtained four major findings: (1) High-risk HPV16/18 infections exist in 10.5% of BC issues, higher than control BA tissues (4%); (2) More BC tissues show constitutively overactive p-Stat3 and overexpressed IL-17 than ABA tissues; (3) High levels of both active p-Stat3 and IL-17 correlate with poor differentiation and lymph nodal metastasis in BC; and (4) There is a tri-lateral relationship among HR-HPV infection, p-Stat3 activity and expressed IL-17 in BC patients.

Materials and methods

Ethics statement

This study was approved by the Institutional Ethics Review Board (IERB) at our First Affiliated Hospital of Shihezi University School of Medicine (IERB No. SHZ2010LL01). The IERB waived the need of patients' consent due to anonymous analyses of the data and standard university hospital guidelines in accordance with the Declaration of Helsinki including confidentiality and anonymity were followed in the handling and publication of patients' tissues and clinical data.

Patients and tissue specimens

We obtained a total of 379 surgically resected and paraffin-embedded archival tissues of human breast cancer and paired adjacent breast adenosis (ABA, 245 tissues available), diagnosed from 2000 to 2010, from the Department of Pathology at the First Affiliated Hospital of Shihezi University School of Medicine. All selected tissues, including 100 unrelated and surgically resected breast adenosis tissues (BA) as HPV test controls (see below), were based on histopathological diagnoses and reconfirmed for diagnoses before experi-

Table 1. Breast cancer (BC) tissues have a higher frequency of high-risk HPV infections than breast adenosis (BA) tissues

Disease	n	HPV16/18 infection		
		Negative n (%)	Positive n (%)	*P*
BC	325	291 (89.5)	34 (10.5)	0.047
BA	100	96 (96.0)	4 (4.0)	
IDC	246	221 (89.8)	25 (10.2)	0.856
ILC	53	47 (88.7)	6 (11.3)	
DCIS	26	23 (88.5)	3 (11.5)	

Note: BC, breast cancer; BA, breast adenosis; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; DISC, ductal carcinoma in situ. Positives were compared with negatives using chi-square method.

mentation. None of the patients had received chemotherapy and/or radiotherapy prior to surgery and no autoimmune diseases were reported. These patients were originally selected because of both their available clinical information (see **Tables 3** and **4**) and sufficient tissue size for experimental analyses (including repeats). Of 379 BC patients, 100 cases had follow-up information available (166 months or 13.8 years) until January, 2014. Survival time was defined as the interval between the date of surgery and the date of death or last follow-up. ABA tissues were taken from approximately 4 cm away from the edge of cancer tissues.

Detection of HPV infection using GeneChip technology

We examined HPV infections in 325 BCs because of sufficient tissues available for DNA extraction and 100 BA tissues from unrelated non-cancer patients as controls. Genomic DNA was extracted from tissues using a DNA extraction kit (QIAGEN, Germany) following manufacturer's protocols. The following precautions were taken to avoid possible cross-contamination during the process: (1) Single-use disposables were employed in DNA extraction and subsequent HPV testing; (2) From the first batch of extracted DNA and HPV test results, we selected one negative HPV control (a BA tissue) and one positive HPV control (a BC tissue) to be included in every batch of DNA extraction; (3) In each batch of DNA extraction, proportional numbers of BC tissues and BA tissues were included; and (4) The same batch of control DNAs and test DNAs was HPV-tested together.

DNA specimens were coded without identification of cancer or breast adenosis and sent to the Specialty HPV Genotyping Laboratory within Yaneng Bioscience Co., Ltd, Shenzhen, China (http://www.yanengbio.com/en/home2.asp). HPV Genotyping Kit is a clinical diagnostic product of Yaneng Bioscience Co. (Chinese State FDA approval no. 2008-340099) which had been chosen by the International Agency for Research on Cancer (IARC, World Health Organization) to perform the Prevalence Surveys of HPV Infection and Cervical Neoplasia in China. The quality and integrity of extracted DNA was tested by PCR using b-actin gene as an internal control (forward primer: 5'-CAGACACCATGGTGCACCTGAC-3' and reverse primer: 5'-CCAATAGGCAGAGAGAGTCAGTG-3').

PCR reactions were performed in the Specialty Laboratory and a positive HPV control DNA was placed on every hybridization blot. The reverse line blot method (RLB) was used on all specimens with amplifiable DNA and the hybridization conditions were described previously [20, 21]. PCR products were manually loaded onto the chip blots and hybridization procedure was performed by automation. The blots were initially read automatically and checked manually before reporting. The DNA chip was able to detect 23 HPV subtypes including 18 high-risk HPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 83, and MM4) and 5 low-risk HPV types (HPV 6, 11, 42, 43, and 44). Each DNA specimen was triplicated in 3 gradient concentrations on genotyping chip blots and the positive coincidence rate was 100%. The test has a sensitivity of detecting 103 copies of HPV DNA/ml and a specificity of 99% based on the company's specifications. Test results were feedback to us and decoded to match original individuals before analyses.

Detection of p-Stat3 and IL-17 using immunohistochemistry (IHC) assay

Paraffin-embedded tissues were sectioned in 4-μm slices and IHC analyses were carried out using streptavidin peroxidase method (SP kit, Zhongshan Golden Bridge Co., Beijing, China). Briefly, tissue slides were dewaxed and endogenous peroxidase was blocked by 3% hydrogen peroxide. After blocking with normal goat serum, primary rabbit anti-human polyclonal antibodies (Santa Cruz, CA, USA) against IL-17 (1:200 dilution) and p-Stat3 (1:400 dilution) were applied onto the slides following the procedures as described previously [22].

Positive IHC stains were defined as yellow-brown color according to the manufacturer's demonstrative slides in tumor cells. IHC stain-

Table 2. Breast cancer tissues show more positive stainings of 1+, 2+/3+, and 1+/2+/3+ of constitutively active p-Stat3 and expressed IL-17 than ABA tissues

Characte-ristics	Total	Negative	Positive Combinations					
		0	1+		2+/3+		1+/2+/3+	
		n (%)	n (%)	*P*	n (%)	*P*	n (%)	*P*
p-Stat3								
BC	379	24 (6.3)	209 (55.1)	< 0.001	146 (38.5)	< 0.001	355 (93.6)	< 0.001
ABA	245	93 (37.9)	85 (34.7)		67 (27.3)		152 (62.0)	
IL-17								
BC	379	16 (4.2)	130 (34.3)	< 0.001	233 (61.5)	< 0.001	363 (95.8)	< 0.001
ABA	245	36 (14.7)	82 (33.4)		127 (51.8)		209 (85.3)	

Note: BC, breast cancer. ABA, adjacent breast adenosis. Positive combinations were compared with negatives (0) for pStat3 and IL-17 in each category, respectively, using chi-square method.

Table 3. Strong positive staining (2+/3+) of p-Stat3 correlates with moderate/poor differentiation and lymph node metastasis among analyzed clinicopathological characteristics of breast cancer

Characteristics of breast cancer	n	Negative	Positive Combinations			
		0	1+		2+/3+	
		n (%)	n (%)	P	n (%)	P
Differentiation						
Well+DCIS	62	5 (8.1)	39 (62.9)	0.876	18 (29.0)	0.046
Moderately	227	14 (6.2)	116 (51.1)		97 (42.7)	
Poorly	90	5 (5.6)	54 (60.0)		31 (34.4)	
Tumor size				0.333		0.152
> 2 cm	266	20 (7.5)	149 (56.0)		97 (36.5)	
≤ 2 cm	113	4 (3.5)	60 (53.1)		49 (43.4)	
Lymph node metastasis				0.701		0.044
Yes	167	8 (4.8)	78 (46.7)		81 (48.5)	
No	212	16 (7.5)	131 (61.8)		65 (30.7)	
Estrogen receptor				0.725		0.585
Positive	283	17 (6.0)	155 (54.8)		111 (39.2)	
Negative	96	7 (7.3)	54 (56.3)		35 (36.5)	
Progesterone receptor				0.923		0.913
Positive	272	17 (6.3)	150 (55.1)		105 (38.6)	
Negative	107	7 (6.5)	59 (55.1)		41 (38.3)	
Her-2				0.286		1.000
Positive	331	20 (6.0)	189 (57.1)		122 (36.9)	
Negative	48	4 (8.3)	20 (41.7)		24 (50.0)	
Clinical stage				1.000		0.758
0+I+II	312	20 (6.4)	167 (53.5)		125 (40.1)	
III+IV	67	4 (6.0)	42 (62.7)		21(31.3)	
Total	379	24	209		146	

Note: Her-2, human epidermal growth factor receptor 2. Positive combinations were compared with negatives (1+ or 2+/3+ vs. 0) for each category, respectively, using chi-square method.

ing slides were read on a Leica Inverted Confocal Microscope over yellow-brown color stains for 12 consecutive fields and scored according to two variable factors: (1) counting the number of positively stained cells; and (2) scoring the staining intensity. The final score was the product of (1) multiplying (2) for each slide (**Table 7**) as we reported previously [9], similar in principle to another published report [23].

Table 4. Strong positive staining (2+/3+) of IL-17 correlates with moderate/poor differentiation and lymph node metastasis among analyzed clinicopathological characteristics of breast cancer

Characteristics of breast cancer	n	Negative	Positive Combinations			
		0	1+		2+/3+	
		n (%)	n (%)	*P*	n (%)	*P*
Differentiation						
Well+DCIS	62	1 (1.6)	28 (45.2)	0.103	33 (53.2)	0.048
Moderately	227	7 (3.0)	69 (30.4)		151 (66.5)	
Poorly	90	8 (8.9)	33 (36.7)		49 (54.4)	
Tumor size						
> 2 cm	266	8 (3.0)	95 (35.7)	0.056	163 (61.3)	0.096
≤ 2 cm	113	8 (7.1)	35 (31.0)		70 (61.9)	
Lymph node metastasis						
Yes	167	2 (1.2)	52 (31.1)	0.052	113 (67.7)	0.008
No	212	14 (6.6)	78 (36.8)		120 (56.6)	
Estrogen receptor						
Positive	283	10 (3.5)	93 (32.9)	0.454	180 (63.6)	0.179
Negative	96	6 (6.3)	37 (38.5)		53 (55.2)	
Progesterone receptor						
Positive	272	13 (4.8)	99 (36.4)	0.764	160 (58.8)	0.404
Negative	107	3(2.8)	31 (29.0)		73 (68.2)	
Her-2						
Positive	331	13 (3.9)	114 (34.4)	0.440	204 (61.6)	0.441
Negative	48	3 (6.3)	16 (33.3)		29 (60.4)	
Clinical stage						
0+I+II	312	12 (3.8)	106 (34.0)	0.510	194 (62.2)	0.490
III+IV	67	4 (6.0)	24 (35.8)		39 (58.2)	
Total	379	16	130		233	

Note: Her-2, human epidermal growth factor receptor 2. Positive combinations were compared with negatives (1+ or 2+/3+ vs. 0) for each category, respectively, using chi-square method.

Statistical analyses

Statistical analyses were performed using the SPSS statistical software package (version 17.0). χ^2 test was adopted for variance analysis and correlation was analyzed using Spearman rank correlation method. Patient survival was analyzed using Kaplan-Meier Estimator. Univariate and multivariate survival analyses were used to judge the hazards of survival. Differences with $P < 0.05$ were considered statistically significant.

Results

Prevalence of HR-HPV infections in BC tissues

In BC tissues, HPV infections were detected in 38 cases, of which 31 cases were infected with HPV16, 1 case with HPV18, 2 cases with mixed HPV16/58, and 4 cases with low-risk HPV (HPV6/11). In BA tissues, however, only 4 cases were positive for HPV16, and 6 cases were HPV11. As ABA tissues were very limited in size, we instead used breast adenosis (BA) tissues from unrelated non-cancer patients as controls for DAN extraction and HPV testing. The infection rate of HR-HPV in BC tissues was 10.5% (34/325, 32 cases with HPV16 or 18, 2 cases with HPV16 and 58 mixed) higher than that in BA tissues (4.0% or 4/100, $\chi^2 = 3.92$, $P = 0.047$) (**Table 1**). There were no differences in HR-HPV infections among three BC 3 subtypes, i.e., invasive ductal carcinoma (IDC), invasive lobular carcinoma (ILC), and ductal carcinoma in situ (DCIS) ($\chi^2 = 0.31$, $P = 0.856$) (**Table 1**). However, we failed to observe any correlations of HR-HPV infection with clinicopathological characteristics among these BC patients including differentiation, lymph node metastasis, clinical staging among others.

Table 5. Cross correlation analyses reveal a tri-lateral relationship among HPV infection, p-Stat3 and IL-17 in breast cancer tissues

Characteristics	HPV16/18	p-Stat3	IL-17
HPV16/18	1		
p-Stat3	0.131*	1	
IL-17	0.128*	0.136**	1
Characteristics	HPV (all-types)	p-Stat3	IL-17
HPV (all types)	1		
p-Stat3	0.117*	1	
IL-17	0.113*	0.136**	1

Note: Numbers shown in the table are correlation coefficient r values from Spearman rank correlation analyses. *represents $P < 0.05$; **indicates $P < 0.01$.

In addition, 4.5% (17/379) of BC patients in this cohort had a family history of cancer among their first degree relatives, similar to our previous observations in Chinese BC patients [3]. The familial cancers included 3 cases of breast cancer (17.6%, 3/17) and the remaining patients (82.4%, 14/17) had other types of cancer. Of these 17 familial cancer patients, 12 were tested for HPV and 2 out of 12 (16.7%) had HR-HPV infection, a frequency higher than those without familial history (10.2%, 32/313).

Overactive p-Stat3 and overexpressed IL-17 in BC tissues

As shown in **Figure 1**, p-Stat3 antibody stained little in ABA tissue (scored as 0), however, strongly stained mostly in the nuclei of BC tissues in all three subtypes of IDC, ILC, and DCIC (scored as 3+, respectively). Similarly, IL-17 antibody had no staining in ABA tissue (scored as 0) but showed strong staining mostly in the cytoplasm of BC tissues of the three subtypes as above (scored as 3+, respectively). Overactive p-Stat3 and overexpressed IL-17 in BC were apparent when compared with ABA in the whole cohort. As tabulated in **Table 2**, three categories of positive staining combinations (1+, 2+/3+, and 1+/2+/3+, respectively) were compared with negative staining (0) for p-Stat3 and IL-17, respectively. In general, significantly more BC tissues showed active p-Stat3 than ABA tissues (1+/2+/3+ vs. 0: 93.6% vs. 62.0%, $P < 0.001$). For IL-17, on the other hand, this phenomenon (see **Table 2**) was similar to p-Stat3 in that many more BC tissues showed positive expression of IL-17 than ABA tissues (1+/2+/3+ vs. 0: 98.5% vs. 85.3%, $P < 0.001$).

To investigate general differences between BC and ABA for their Stat3 activities and IL-17 levels, we compared pooled IHC scores from all individuals tested for p-Stat3 and IL-17 using t-test. As shown in **Figure 2**, panel A, constitutive p-Stat3 levels were significantly higher in BC tissues than in ABA tissues (M ± SD, 4.4 ± 2.4 vs. 3.0 ± 3.0, $P < 0.001$). Very similarly, IL-17 levels were also higher in BC tissues than in ABA tissues (M ± SD, 6.4 ± 3.3 vs. 4.6 ± 2.9, $P < 0.001$). These observations, from a different angle, were supporting the observations as presented in **Table 2**, indicating a highly inflamed microenvironment in tumor tissues. On the other hand, in terms of the number of individuals positively stained, panel B in **Figure 2** revealed that more individual BC tissues stained for p-Stat3 ($P < 0.001$) and IL-17 ($P < 0.001$) than ABA tissues, again, in keeping with the observations as shown in **Table 2** (1+/2+/3+).

p-Stat3 and IL-17 levels correlate with tumor differentiation and lymph node metastasis

In seeking whether active p-Stat3 and/or IL-17 levels would affect patients' clinical characteristics, we investigated possible correlations of p-Stat3 and IL-17 levels with a number of clinicopathological characteristics including tumor differentiation, tumor size, lymph node metastasis, ER, PR, Her-2 and clinical staging [24] among others. The analyses revealed that high p-Stat3 levels correlated with worse tumor differentiations (moderately and poorly, 2+/3+ vs. 0, $P = 0.046$) and lymph node metastasis (2+/3+ vs. 0, $P = 0.044$) (**Table 3**). Very similar to p-Stat3, IL-17 levels also correlated with worse tumor differentiations (moderately and poorly, 2+/3+ vs. 0, $P = 0.048$) and lymph node metastasis (2+/3+ vs. 0, $P = 0.008$) (**Table 4**). The phenomenon that both Stat3 activities and the pathway's downstream IL-17 cytokine correlated with the same clinical characteristics suggested a possibility that the Stat3/IL-17 signaling may work together or act in sequence to influence the development and/or progression of breast cancer. No correlation was found between HPV infection and clinical characteristics in BC patients (data not shown).

A tri-lateral relationship among HPV infection, p-Stat3 activity and IL-17 level in BC patients

To investigate whether high-risk HPV16/18 infection would be associated with heightened

Table 6. Lymph node metastasis is an independent predictor for breast cancer prognosis

Variables	Univariate analysis		Multivariate analysis	
	HR (95.0% CI)	*P*	HR (95.0% CI)	*P*
Differentiation (Moderately+Poorly vs. DCIS+Well)	3.97 (0.53-29.76)	0.197	1.85 (0.22-15.79)	0.575
Tumor size (> 2 cm vs. ≤ 2 cm)	1.62 (0.54-4.89)	0.390	2.03 (0.48-8.56)	0.336
Lymph node metastasis (Yes vs. No)	5.70 (1.89-17.18)	0.002	4.96 (1.47-16.68)	0.010
Distant metastasis (Yes vs. No)	7.51 (2.13-26.45)	0.002	6.98 (0.96-50.99)	0.056
Clinical stages (III/IV vs. 0/I/II)	2.50 (0.95-6.59)	0.063	0.98 (0.25-3.91)	0.980
Estrogen receptor (+ vs. neg-)	0.96 (0.32-2.90)	0.954	1.20 (0.38-3.82)	0.757
Progesterone receptor (+ vs. neg-)	0.59 (0.23-1.51)	0.273	0.73 (0.28-1.89)	0.511
Her-2 (1+/2+ vs. neg-)	0.77 (0.18-3.32)	0.724	1.18 (0.23-6.06)	0.845
Active p-Stat3 (2+/3+ vs. 0/1+)	1.03 (0.41-2.55)	0.957	1.14 (0.36-3.67)	0.824
IL-17 expression (2+/3+ vs. 1+)	1.89 (0.76-4.71)	0.170	2.25 (0.80-6.33)	0.124

Note: HR, hazard ratio; CI, confidence interval; neg-, negative; 0/1+ and 2+/3+ are staining combinations. Cox regression test was used in comparisons.

Table 7. Scoring Criteria of Immunohistochemistry (IHC) Assay with Specific Antibodies Used in this Study [9]

Staining positive cells		Staining intensity		Final score product	
Percent (%)	Score 1	Intensity	Score 2	Score 1 × Score 2	= Score 3
≤ 5%	0	Absent	0	0-1	0 (-)
6%-25%	1	Weak	1	2-4	1+ (+)
26%-50%	2	Moderate	2	5-8	2+ (++)
51%-75%	3	Strong	3	9-12	3+ (+++)
76%-100%	4				

Note: Scoring results are based on screening 12 consecutive microscopic fields. Percent positive cells (score 1) multiply staining intensity (score 2) equals to final product score (score 3). Either 0 or (-) depicts negative staining. For example, an individual slide had < 5% of staining cells (= 0) with a staining intensity of 1 (= weak) which would generate a final product score of 0 × 1 = 0; another slide had 80% of staining cells (= 4) with a staining intensity of 3 (= strong) which would give a final product score of 4 × 3 = 12 (3+) (see ref. 9).

Stat3 activities or IL-17 levels or both, we conducted bivariate correlational analyses. In BC tissues, as shown in **Table 5**, positive correlations were revealed between HPV16/18 infection and active Stat3 levels (r = 0.131, *P* = 0.018); between HPV infection and IL-17 levels (r = 0.128, *P* = 0.021); and a strong positive correlation between p-Stat3 and IL-17 levels (r = 0.136, *P* = 0.008), respectively. This tri-lateral relationship, again, suggests a possibility that HPV infection may indeed be able to react with the Stat3/IL-17 signaling to orchestrate an inflammatory microenvironment favored by development and/or progression of breast cancer.

Lymph node metastasis and distant metastasis affect survival of BC patients

Among 100 BC patients with available follow-up information, 81 were alive and 19 were dead by January 2014. The median overall survival time was 70 months and the longest survival time was 161 months. As shown in **Figure 3**, Kaplan-Meier survival curves indicated that BC patients' survival was affected by distant metastasis (*P* < 0.001), lymph node metastasis (*P* < 0.001), and the number of metastatic lymph nodes (*P* < 0.001). No influence was observed between survival and p-Stat3 or IL-17 levels (*P* = 0.986 and *P* = 0.162, respectively). Further analyses using univariate analysis indicated that lymph nodal metastasis and distant metastasis were significant factors affecting survival (*P* = 0.002), but only lymph nodal metastasis was suggested to be an independent predictor for survival using multivariate analysis (*P* = 0.01) (**Table 6**). HPV infection did not affect survival among BC patients (data not shown).

Discussion

The current paradigm is that the immune system and the microbiota mutually interact to maintain homeostasis in vivo. However, components of the microbiota may alter this balance and promote chronic inflammation, further fueling tumor development [5]. Among many microorganisms, HPV infection, particularly HPV types 16 and 18, is found most frequently in human cancers. For example, high-risk HPV infection is the major cause for cervical cancer

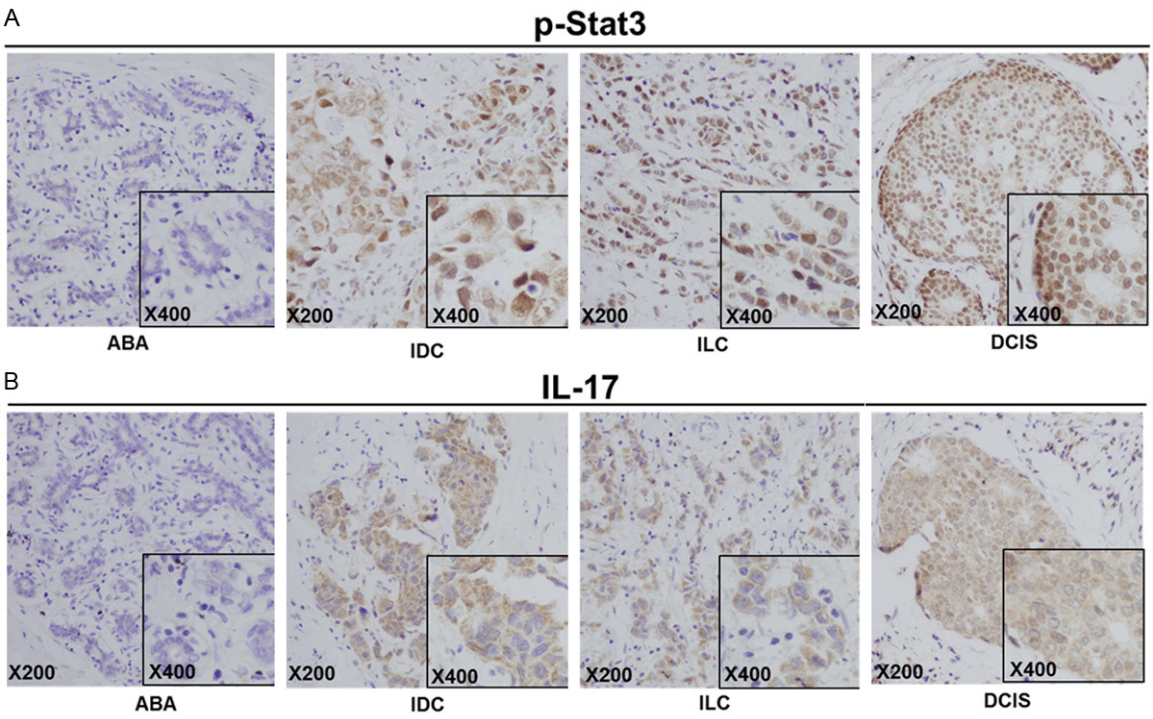

Figure 1. BC tissues show constitutively over-phosphorylated Stat3 (p-Stat3) and overexpressed IL-17 than adjacent breast adenosis (ABA) tissues using IHC with specific antibodies. Positively stained cells are shown in yellow-brown. As expected, p-Stat3 is mainly localized in the nucleus (A), while IL-17 is primarily in the cytoplasm (B). It can be seen that p-Stat3 is strongly stained in BC tissues of subtypes IDC, ILC, and DCIS (scored as 3+) as compared with ABA tissue (scored as negative or 0). Similarly for IL-17 staining, BC tissues show strong staining for subtypes of IDC, ILC, and DCIS (scored as 3+) but ABA tissue shows little or no staining (scored as negative or 0). Microscopic magnification is × 200 with inserts of × 400. ABA = adjacent breast adenosis; IDC = invasive ductal carcinoma; ILC = invasive lobular carcinoma; and DCIS = ductal carcinoma in situ.

and HPV vaccination is effective in primary prevention of cervical cancer [25]. Interestingly, HPV infection has also been found in many non-cervical cancers. One meta-analysis of adenomas and adenocarcinomas has reported that, among 1,184 cases of breast carcinoma stratified by geographical regions, HPV infections are found to be the highest in patients from Oceania (42.11%) and Asia (32.42%) [26]. Other meta-analyses have also reported HPV infections in cancers of the ovary [27], bladder [28], lung [29], and colorectum [30].

The frequency of HR-HPV infection in breast cancer patients varies dramatically ranging from 10% to 86% depending on regions where studies were performed [31]. We have recently found HR-HPV infections in 48.4% of patients with colorectal cancer, the highest ever reported [9] and 90.4% of patients with cervical cancer (manuscript in preparation). Our observed frequencies of HPV infection in these cancers are in agreement with most published reports, suggesting the reliability of the HPV genotyping method used here. In this study, we have observed a frequency of 11.7% (38/325) of BC patients infected by HPV, of whom 10.5% (34/325) were positive for high-risk HPV16/18, much higher than control BA patients (**Table 1**).

In search for collaborators of HPV infection that may be involved in orchestrating an inflammatory microenvironment [5] favoring tumor development and progression, we have investigated correlations of HPV infection with constitutively active p-Stat3 and its downstream proinflammatory IL-17 cytokine. As shown in **Table 2**, p-Stat3 is significantly increased in BC tissues than in ABA tissues (93.6% vs. 62%). At the same time, expressed IL-17 follows the step of p-Stat3 to show a higher level in BC than in ABA (95.8% vs. 85.3%). The stronger p-Stat3 activities and higher IL-17 levels in BC than in ABA are confirmed by two additional comparison strategies: one is the difference in pooled IHC scores (**Figure 2A**), and the other, the differ-

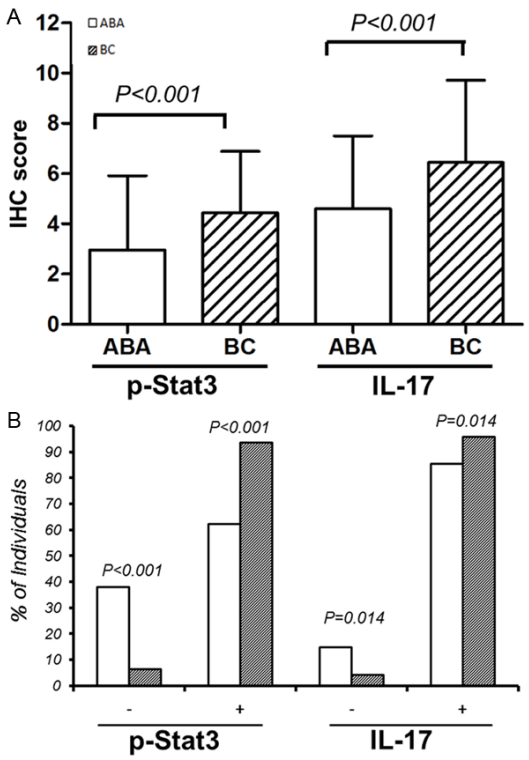

Figure 2. BC tissues show over-phosphorylation of Stat3 (p-Stat3) and overexpression of IL-17 compared with ABA. A. Based on the scoring criteria (**Table 7**), pooled scores for p-Stat3 or IL-17 are compared between BC and ABA individuals using t-test and the data are expressed as mean ± standard deviation (M ± SD). As seen, p-Stat3 level is higher in BC than in ABA (M ± SD, 4.4 ± 2.4 vs 3.0 ± 3.0, $P < 0.001$). Similarly, IL-17 level is also higher in BC than in ABA (M ± SD, 6.4 ± 3.3 vs 4.6 ± 2.9, $P < 0.001$). B. Comparison between BC and ABA for the number of individuals (%) with varying staining intensities using χ^2 test. As shown for p-Stat3, BC category has less individuals scored as 0 or negative than ABA category (6.3% vs 37.9%, $P < 0.001$). To the contrary, BC has many more individuals scored as positives (+) than ABA (93.6% vs 62.0%, $P < 0.001$). A striking similarity is true for IL-17, of which BC category has less individuals scored as 0 or negative than ABA category (4.2% vs 14.7%, $P = 0.014$) but again, BC has many more individuals scored as strong positives than ABA (95.8% vs 85.3%, $P = 0.014$). ABA indicates adjacent breast adenosis, and BC depicts breast cancer, respectively.

ence in the percentage of individuals (**Figure 2B**). In support of our above observations are studies in other cancers such as ovarian, esophageal, gastric cancers and cutaneous T-cell lymphoma [32-36]. It may not be coincidental that both strong p-Stat3 activities and high IL-17 levels are correlated with tumor cell differentiation and lymph node metastasis in BC patients (**Tables 3** and **4**), suggesting a possible collaborative action between Stat3 and IL-17 in BC.

Taken the above together, in a virus-mediated inflammation, Stat3 signaling pathway may also play a role as bacterium-mediated inflammation does in carcinogenesis [5, 37]. Furthermore, Stat3 pathway's downstream proinflammatory cytokine IL-17 is likely to act as an accomplice in orchestrating such a carcinogenic inflammation that may lead to cancer development. Importantly, IL-17 serves as a positive feedback agent to Stat3 signaling and therefore, can amplify the magnitude of the inflammation that may further facilitate the development/progression of BC.

It has been increasingly realized that breast cancers are much more heterogeneous diseases than what is determined by clinical subtypes, and that better prediction of prognosis is needed early on for better personalized treatment and management [38]. Previous studies have reported IL-17 to affect survival in patients with ovarian cancer and small cell lung cancer [39, 40]. In this study, both IL-17 and p-Stat3 have failed to affect patients' prognosis although their levels are high in BC patients (**Figure 3**). We are, however, able to show correlations of BC patients' survival with distant metastasis and lymph node metastasis (**Figure 3**). When the number of metastatic lymph nodes is analyzed against survival, it is evident that the more metastatic lymph nodes the patients carry, the poorer survival the patients are, in keeping with previous observations [41]. Furthermore, multivariate analyses indicate lymph node metastasis to be an independent predictor for survival (**Table 6**).

As mentioned previously, microorganisms may manipulate and/or collaborate with cellular signaling pathways to promote inflammatory microenvironment, facilitating cancer development in vivo. Indeed in BC patients, we have observed positive correlations of HR-HPV infection with active p-Stat3 as well as IL-17 levels (**Table 5**). Furthermore, it is relevant that a correlation also exists between p-Stat3 and IL-17. Therefore, this tri-lateral relationship may reveal a possible conspiracy among the three agents, i.e., HR-HPV, Stat3, and IL-17 in promoting tumor development. We therefore favor the hypothesis that HR-HPV virus may initiate

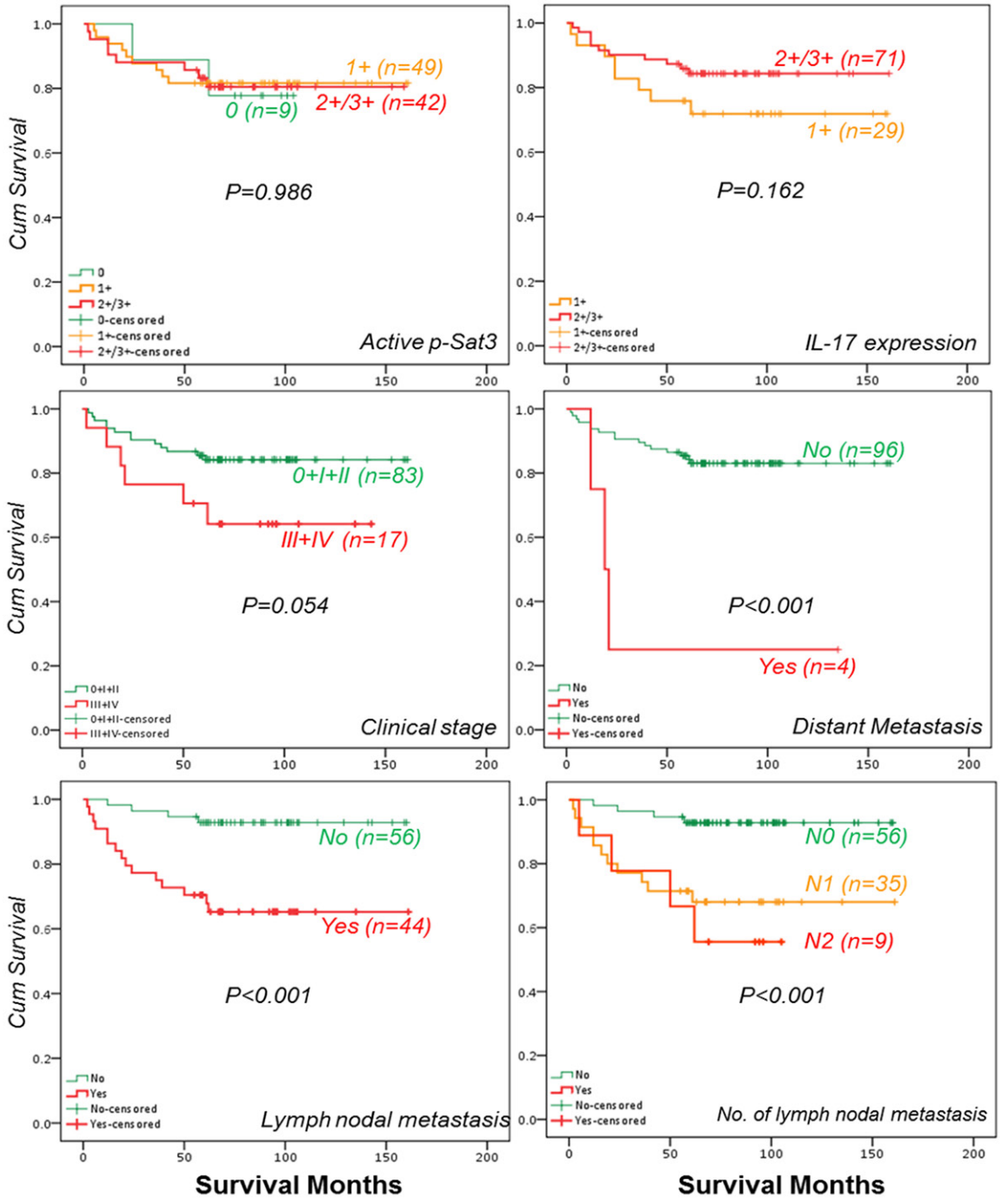

Figure 3. Distant metastasis and lymph node metastasis, not p-Stat3 or IL-17 levels, correlate with poor prognosis. Factors that may affect overall survival of 100 BC patients, 81 alive and 19 dead, are analyzed by Kaplan-Meier Estimator. As shown, p-Stat3 (P = 0.986) and IL-17 (P = 0.162) are not correlated with patients' survival. The same is true for clinical stage (P = 0.054) although a trend is present. However, distant metastasis and lymph node metastasis are correlated with poor prognosis (both $P < 0.001$). When the number of metastatic lymph nodes is analyzed against survival, it is evident that the more metastatic lymph nodes patients carry, the poorer prognosis patients have ($P < 0.001$). N0 = no lymph nodal metastasis, N1 = 1-5 metastatic lymph nodes, and N2 ≥ 5 metastatic lymph nodes, respectively. Survival Months are the months survived after surgery.

inflammation through promoting Stat3 activities (and Stat3-related molecules), which is then sustained by involving Stat3's downstream cytokines, such as IL-17, by which cre-

ates an inflammatory microenvironment that facilitates the development of BC.

The above notion is echoed by animal studies. For example, in HPV16 E7-infected mice, bacteria can induce a higher level of IL-17 expression than those without E7-infection [42]. Similarly, HPV16 E6 can up-regulate the expression of IL-17 in non-small cell lung cancer [43]. In cervical exfoliated cells, the content of IL-17 in HPV-positive patients with cervical cancer is higher than that in HPV-negative patients [44]. Additional studies have demonstrated that Stat3 activation is increased in HPV16-positive patients with cervical cancer [45, 46] and colorectal cancer [9]. However, the current correlative studies cannot rule out a possibility that, during cancer progression, immunologically and metabolically compromised BC patients with advanced stages (**Tables 3** and **4**) may induce opportunistic viral replication blast in otherwise latent HPV infection, which may lead to increased copy number of HPV detected on the tumor site. This opportunistic HPV infection may further trigger the activation of Stat3 signaling and the subsequent expression of proinflammatory cytokines such as IL-17 in the tumor microenvironment which, in turn, promotes cancer progression [9].

To the contrary, Stat3 and IL-17 may also possess anti-tumor functions. For example, IL-17 may trigger anti-tumor responses through producing helper and cytotoxic T cells during tumor development [42, 47]. Th17 cells display a late plasticity which is necessary for anti-tumor activity of Th17 cells [48]. Furthermore, in a murine mammary cancer model, doxorubicin efficiently combines with Th1 or Th17 lymphocytes to suppress tumor development and metastatic disease [49]. Therefore, in terms of cancer development and progression, Stat3 signaling may serve as a “double agent” depending upon a number of conditions including, but not limited to, cancer types, cancer models, and types of dominant immune response (Th1 vs. Th2) among others. Nevertheless, in the case of HPV infection as shown here, Stat3 signaling and IL-17 expression may serve as a promotional agent in the development/progression of BC.

In summary, this study has demonstrated four major observations: (1) High-risk HPV16/18 infections exist in 10.5% of BC issues. (2) More BC tissues show constitutively overactive p-Stat3 and overexpressed IL-17 than ABA tissues. (3) High levels of both active p-Stat3 and IL-17 correlate with tumor cell differentiation and lymph nodal metastasis in BC. (4) There is a tri-lateral relationship among HR-HPV infection, p-Stat3 activity and IL-17 level in BC patients. These findings are implicative of a possible underlying HPV-Stat3-IL-17 mechanism in the pathogenesis and progression of breast cancer, which strongly warrants further investigations.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Wen Jie Zhang, Department of Pathology, Shihezi University School of Medicine, 59 North 2nd Road, Shihezi 832002, Xinjiang, China. Tel: 86 187 99280902; Fax: 86 993 2057136; E-mail: zhangwj82@yahoo.com

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