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Research Paper

Associations of Epstein-Barr Virus DNA in PBMCs and the Subtypes with Breast Cancer Risk

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Abstract

Objective: Epstein-Barr virus (EBV) has been found to be implicated in the development of breast cancer. The purpose of the present study was to identify the associations of EBV DNA and the subtypes in peripheral blood mononuclear cells (PBMCs) with the risk of breast cancer.

Material and Methods: A case-control study with 671 breast cancer cases and 859 age-matched controls was conducted in Guangzhou, China. Face-to-face interviews were performed and blood samples were collected immediately after admission to the hospital for patients or after the interview for controls. EBV DNA in PBMCs and the subtypes were detected using Polymerase Chain Reaction (PCR) and restricted fragment length polymorphisms (RFLP). IgA antibodies against EBV VCA-p18 and EBNA-1 were examined using commercial enzyme-linked immunosorbent assay kits. Unconditional logistic regression analysis was applied to evaluate the associations of the DNA positivity and subtypes of EBV with the risk of breast cancer.

Results: Among the 1530 subjects, 164 cases (24.4 %) and 206 controls (24.0 %) were positive for EBV DNA in PBMCs and no significant difference occurred between cases and controls. The presence of EBV DNA was related to the positivity of EBV IgA antibodies. Of the DNA positive samples, 71 cases and 109 controls for F/f subtype and 58 cases and 112 controls for C/D subtype were successfully obtained. The D subtype was associated with an increased breast cancer risk compared with the C subtype [OR (95% CI): 2.86 (1.25~6.53)]. We did not find an association of the F/f polymorphism with breast cancer risk.

Conclusions: The present study suggested that the presence of EBV DNA in PBMCs may not be an appropriate biomarker for breast cancer risk. The subtype D of EBV was likely to be related to breast tumorigenesis.

Key words: EBV DNA; Subtypes; PBMCs; Breast cancer.

Introduction

Epstein-Barr virus (EBV) is a ubiquitous virus latently infecting more than 90% of the global population [1]. It has been found to be implicated in the development of some malignancies [2], such as immunodeficiency-related B cell lymphoma, Burkitt and Hodgkin lymphoma, nasopharyngeal carcinoma, and gastric carcinomas [3]. In the past two decades, the association of EBV with breast cancer has also been proposed [2, 4, 5]. It has been recognized that the mechanisms of these malignancies development

involve in EBV reactivation rather than the infection itself [6]. We have found that EBV-IgA antibodies, an indicator of EBV reactivation, may associate with the risk of breast cancer [7]. However, the IgA antibodies are delayed and indirect biomarkers for the reactivation of EBV. Considering that EBV primarily infects B lymphocytes and eventually resides latently in these cells [8], the DNA of EBV in peripheral blood mononuclear cells (PBMCs) may be regarded as a direct indicator of EBV presence. There were also other studies which had explored EBV DNA in PBMCs of patients with EBV-associated diseases [9-12]. One of the aims for the present study is to evaluate the association of EBV DNA in PBMCs with breast cancer risk.

In addition, we have also found that not all individuals with reactivation of EBV would develop breast cancer [7]. EBV subtypes may be one of the main reasons, although host genetic and environmental factors can't be excluded. Actually, previous studies have found that specific EBV subtypes may associate with certain tumors [13]. For example, the f variant appears to be more frequent among nasopharyngeal carcinoma (NPC) patients than healthy individuals in Southern China [14]; subtype D was found to be associated with gastric carcinoma [15], suggesting the pathogenic roles of EBV may be distinct in different EBV subtypes [16]. However, there has been no study to explore the association between EBV subtypes and breast cancer risk. Therefore, another aim of the present study is to analyze the distribution differences of EBV subtypes in breast cancer patients and healthy controls.

Materials and Methods

Study population

A total of 1530 women (671 cases and 859 controls) participated in this study. From October 2008 to March 2012, women with newly histologically diagnosed breast cancer were consecutively recruited in the First and Second Affiliated Hospitals and the Cancer Center of Sun Yat-sen University in Guangzhou, China. Age frequency-matched (within 5 years) controls were recruited from women who attended a health check-up in the same hospitals during the same period, excluding those who self-reported a history of cancer and known mental illness. All subjects must have resided in the Guangzhou area for at least 5 years and were provided written informed consent for the interviews and the specimen collections. The study was approved by the Ethical Committee of the School of Public Health at Sun Yat-sen University.

Collection of data and specimens

All subjects were asked to complete face-to-face interviews by trained interviewers using a structured questionnaire. The following information was obtained from the interview: demographic factors, menstrual and reproductive history, lifestyle, family history of cancer. Menopausal status was defined as the date of last menses followed by 12 months of no menses. Height and weight were measured by the nurses upon admission to the hospital. For all breast cancer cases, statuses of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER-2) were determined by immunohistochemistry (IHC). The definitions of ER, PR, and HER2 status were previously described in detail [7]. Blood samples were collected immediately after admission to the hospital for patients or after the interview for controls. The PBMCs were separated from the blood by centrifugal and stored at – 80°C.

DNA amplification

Genomic DNA was extracted from PBMCs using the TIANamp Genomic DNA Kit (TianGen Biotech Co. Ltd., Beijing, China) according to the manufacturer's instructions. Real-time PCR was used to detect the presence of EBV DNA with the primers and probes for *Bam*H1-K of EBV: 5′-CCG GTG TGT TCG TAT ATG GAG-3′ (F), 5′-GGG AGA CGA CTC AAT GGT GTA-3′ (R), 5′-TGC CCT TGC TAT TCC ACA ATG TCG TCT T-3′ (SEB). In addition, C-reactive protein (CRP) as a housekeeping gene was also included for each parallel sample of EBV DNA detection. Its 5′ primer sequence was 5′-CTT GAC CAG CCT CTC TCA TGC-3′, the 3′ primer was 5′-TGC AGT CTT AGA CCC CAC CC-3′, and the probe was 5′-TTT GGC CAG ACA GGT AAG GGC CAC C-3′. EBV DNA amplifications were carried out in a 384-well reaction plate with a reaction volume of 5.0 μl, including 5μl of 10ng/μl DNA template (dried before adding the PCR reactant), 2.5 μl PCR Master Mix (2×), 0.45 μl of each 10μM forward primer and 10μM reverse primer, 0.125μl of 10μM probe and 1.475 μl purified water. Each run included multiple control samples that contained purified water as the negative control and Raji DNA as the positive control. Thermal cycling conditions were as follows: 50°C Uracil-DNA Glycosylase (UDG) incubation for 2 min and 95°C for 10 min, followed by an initial denaturation step for 15 seconds at 95°C, primer annealing step for 1 min at 60°C, and amplified for 40 cycles. In this study, EBV DNA was defined as positive if the threshold cycle (Ct) value was between 16 and 36, as well as the CRP was positive, while negative if there was no curve or Ct value was more than 36, as well as the CRP was positive. In the

present study, the CRP was able to be detected in all of the 1530 samples.

EBV subtyping

EBV can be classified as subtypes F/f and C/D according to the polymorphisms of virus isolates in the *Bam* HI F and *Bam* HI W1/I1 region of the EBV genome [14, 17]. Types F and C lack *Bam* HI site in the *Bam* HI F region and *Bam* HI W1/I1 region, respectively, whereas subtypes f and D has an extra *Bam* HI site in the corresponding region [18]. In the present study, EBV typing was performed among the EBV DNA positive samples, using PCR and restricted fragment length polymorphisms (RFLP). The details of the primers for DNA amplification were as follows: 5′-TCC CAC CTG TTA CCA CAT TC-3′ (F), 5′- GGC AAT GGG ACG TCT TGT AA-3′ (R) for *Bam* HI F region and 5′-ACC TGC TAC TCT TCG GAA AC-3′ (F), 5′- TCT GTC ACA ACC TCA CTG TC-3′ (R) for *Bam* HI W1/I1 region. PCR reaction conditions were similar for F/f and C/D subtypes, performing in a reaction volume of 25 μl containing 22.0 μl 1×PCR Master Mix, 1 μl forward primer (0.5 μM), 1μl reverse primer (0.5μM), and 10 ng/μl DNA template. Thermal cycling parameters were initial denaturation for 5 min at 94°C, followed by a denaturation step for 30 seconds at 94°C, primer annealing step for 30 seconds at 55°C; then, 40 cycles at 72°C for 45 seconds, 75°C for 10 min and a final extension step of 72°C for 10 min. Each run included purified double-distilled water as the negative control and the DNA of Raji cells as the positive control.

The enzymatic reactions were carried out in a 20μl reaction mixture containing 10 μl of PCR products, 2 μl (10 ×) reaction buffer, 0.5 μl *Bam* HI endonucleases (20000 U/ml). After incubation at 37°C for 3 h, each enzyme-digested products for F/f subtype and C/D subtype were electrophoresed on a 2% agarose gel containing 0.5μg/ml ethidium bromide respectively and visualized by gel imaging analysis system to determine the subtypes.

The PCR product for *Bam* HI F region was 198bp. After digestion by *Bam* HI enzyme, the size of 198bp was considered as subtype F, whereas the presence of two bands of 127bp and 71bp indicated subtype f. For the region of *Bam* HI W1/I1, the PCR products was 206bp, the presence of 206bp *Bam* HI enzyme-digested product was defined as subtype C, the presence of 130bp and 76bp *Bam* HI enzyme-digested product indicated subtype D.

Serological tests

We further examined 349 cases and 500 controls with IgA antibodies against EBV VCA-p18 and EBNA-1 using commercial enzyme-linked immunosorbent assay kits (Zhongshan Bio-Tech, Zhongshan, China). The serological tests were performed strictly according to the manufacturer's instructions and a blind method was used to detect the cases and controls. The definitions of seropositivity for VCA IgA and EBNA-1 IgA were previously described in detail [19].

Statistical Analysis

Statistical analyses were performed using SPSS 20.0 for Windows. Student's *t* test for continuous variables and Chi-squared (χ^2) test for categorical variables were used to compare the distributions of major demographic variables between cases and controls. Unconditional logistic regression analysis was used to evaluate the associations between DNA and the subtypes of EBV and the risk of breast cancer by estimating the odds ratios (ORs) and 95 % confidence intervals (CIs), with adjustment for age and potential confounders, such as education, marital status, BMI, age at menarche, menopausal status, parity, and family history of breast cancer. Stratified analyses were performed by menopausal status, BMI, and clinical characteristics. We also tested for multiplicative interaction between menopausal status, BMI, clinical characteristics, and EBV DNA and subtypes on breast cancer risk, respectively.

Results

Characteristics of the cases and controls are presented in Table 1. Cases were more likely to be less educated and premenopausal than controls. However, there were no significant differences between cancer cases and controls with respect to age, breast-feeding, BMI, age at menarche, marital status, parity, and family history of breast cancer.

Table 2 presents the association between the presence of EBV DNA in PBMCs and breast cancer risk. Among the 1530 subjects, 164 cases (24.4 %) and 206 controls (24.0 %) were positive for EBV DNA. Overall, no significant association was observed between EBV DNA positivity and breast cancer risk (OR=1.05, 95% CI: 0.81-1.37), even after stratified by menopausal status, BMI, or clinical characteristics.

Among the 370 EBV DNA positive samples, 71 cases and 109 controls for F/f subtype and 58 cases and 112 controls for C/D subtype were successful typed (Supplemental Table 1). We further compared the characteristics between successful and unsuccessful typed subjects. No differences were observed between them for F/f subtype in age, education, marital status, BMI, age at menarche, breast-feeding, parity, and family history of breast cancer. For C/D subtype, only menopausal status is different between the two groups. Therefore, we

considered that the characteristics of successfully and unsuccessfully typed subjects were basically not different, suggesting that the successfully typed subjects was representative for the whole study population.

Table 1. Characteristics of breast cancer cases and controls.

Characteristics	Cases, n (%) (n = 671)	Controls, n (%) (n = 859)	*P* value [a]
Age			
≤ 40	170 (25.3)	225 (26.2)	
41– 60	398 (59.3)	489 (56.9)	
≥61	103 (15.4)	145 (16.9)	0.600
Mean ± SD	48.95 ± 11.60	49.21 ± 11.76	0.662
Education			
Junior middle school or below	315 (46.9)	312 (36.3)	
Senior middle school	173 (25.8)	326 (38.0)	
College or above	128 (19.1)	188 (21.9)	<0.001*
Unknown	55 (8.2)	33 (3.8)	
Marital status			
Never married	33 (4.9)	33 (3.8)	
Married/living as married	569 (84.8)	716 (83.4)	
Separated/widow	33 (4.9)	63 (7.3)	0.100
Unknown	36 (5.4)	47 (5.5)	
BMI (kg/m^2)			
< 22	283 (42.2)	339 (39.5)	
22~	196 (29.2)	270 (31.4)	
25~	161 (24.0)	195 (22.7)	0.489
Unknown	31 (4.6)	55 (6.4)	
Age at menarche (years)			
≤12	87 (13.0)	134 (15.6)	
>12	539 (80.3)	686 (79.9)	0.201
Unknown	45 (6.7)	39 (4.5)	
Menopausal status			
Premenopausal	387 (57.7)	400 (46.6)	
Postmenopausal	263 (39.2)	429 (49.9)	<0.001*
Unknown	21 (3.1)	30 (3.5)	
Age at menopausal (years)			
≤45	52 (19.8)	72 (16.8)	
46–50	103 (39.2)	182 (42.4)	
≥51	73 (27.8)	154 (35.9)	0.187
Unknown	35 (13.3)	21 (4.9)	
Parity			
0	56 (8.3)	59 (6.9)	
≥1	582 (86.7)	769 (89.5)	0.244
Unknown	33 (4.9)	31 (3.6)	
History of breastfeeding			
Never	89 (13.3)	145 (16.9)	
Ever	448 (66.8)	642(74.7)	0.386
Unknown	134 (20.0)	72 (8.4)	
Family history of breast cancer			
Absent	625 (93.1)	798 (92.9)	
Present	18 (2.7)	27 (3.1)	0.602
Unknown	28 (4.2)	34 (4.0)	

[a] Student's *t*-test for continuous variables; χ^2 test for categorical variables;
* Statistical significance
[b] Postmenopausal women only.

The associations of EBV subtypes with breast cancer risk were shown in Table 3. For F/f subtype, no significant association was observed with breast cancer risk [OR (95% CI):1.05 (0.46~2.38)]. For C/D subtype, however, a significant increased risk was found for D subtype [2.86 (1.25~6.53)].

We further performed stratified analyses to assess whether the associations between F/f subtype or C/D subtype and the risk of breast cancer were modified by menopausal status, BMI, and clinical-pathological characteristics. For F/f subtype, no differential association was observed after stratified by any above factors (Supplemental Table 2). For C/D subtype, a similar increased risk of breast cancer (D vs. C) occurred among every strata of menopausal status, BMI, ER, PR, and HER2 as among the whole subjects, and no interaction was observed (Supplemental Table 3).

Table 2. Association between EBV DNA and breast cancer risk, and stratified by menopausal status, BMI, and clinical characteristics.

Variables	EBV DNA	Cases, n(%)	Controls, n(%)	OR[a] (95%CI)	*P* value
Total					
	Negative	507 (75.6)	653 (76.0)	1.00 (reference)	
	Positive	164 (24.4)	206 (24.0)	1.05 (0.81~1.37)	
Menopausal status					
Premenopausal	Negative	305 (78.8)	328 (82.0)	1.00 (reference)	
	Positive	82 (21.2)	72 (18.0)	1.13 (0.77~1.67)	
Postmenopausal	Negative	184 (70.0)	306 (71.3)	1.00 (reference)	
	Positive	79 (30.0)	123 (28.7)	0.91 (0.62~1.32)	0.440[b]
BMI (kg/m^2)					
<23	Negative	276 (76.2)	345 (78.9)	1.00 (reference)	
	Positive	86 (23.8)	92 (21.1)	1.25 (0.87~1.80)	
≥23	Negative	208 (74.8)	269 (73.3)	1.00 (reference)	
	Positive	70 (25.2)	98 (26.7)	0.86 (0.58~1.27)	0.085[b]
ER					
Negative	Negative	132 (74.2)	653 (76.0)	1.00 (reference)	
	Positive	46 (25.8)	206 (24.0)	1.03 (0.68~1.56)	
Positive	Negative	337 (77.5)	653 (76.0)	1.00 (reference)	
	Positive	98 (22.5)	206 (24.0)	0.99 (0.73~1.35)	0.593[c]
PR					
Negative	Negative	173 (72.7)	653 (76.0)	1.00 (reference)	
	Positive	65 (27.3)	206 (24.0)	1.07 (0.74~1.54)	
Positive	Negative	296 (78.9)	653 (76.0)	1.00 (reference)	
	Positive	79 (21.1)	206 (24.0)	0.94 (0.68~1.31)	0.296[c]
HER2					
Negative	Negative	322 (77.8)	653 (76.0)	1.00 (reference)	
	Positive	92 (22.2)	206 (24.0)	0.93 (0.68~1.28)	
Positive	Negative	141 (73.8)	653 (76.0)	1.00 (reference)	
	Positive	50 (26.2)	206 (24.0)	1.13 (0.76~1.69)	0.216[c]
Clinical stage					
I/ II	Negative	338 (77.0)	653 (76.0)	1.00 (reference)	
	Positive	101 (23.0)	206 (24.0)	0.96 (0.71~1.30)	
III/IV	Negative	106 (78.5)	653 (76.0)	1.00 (reference)	
	Positive	29 (21.5)	206 (24.0)	0.85 (0.51~1.42)	0.994[c]

[a] Adjusted for age, education, marital status, BMI, age at menarche, menopausal status, parity, and family history of breast cancer.
[b] *P* for multiplicative interaction.
[c] *P* for heterogeneity.

Results of the relationship between EBV DNA and EBV IgA was shown in Table 4. We discovered positive correlation between EBV DNA and VCA IgA and between EBV DNA and combined IgA (P<0.05).

No significant correlation was found between EBV subtypes and EBV IgA (Supplemental Table 4-5).

Table 3. Association between EBV subtypes and breast cancer risk.

Subtypes	Cases, n (%)	Controls, n (%)	OR[a] (95%CI)	OR[b] (95%CI)
F/f subtype				
F	57 (80.3)	83 (76.1)	1.00 (reference)	1.00 (reference)
f	14 (19.7)	26 (23.9)	0.78 (0.37~1.62)	1.05 (0.46~2.38)
C/D subtype				
C	33 (56.9)	88 (78.6)	1.00 (reference)	1.00 (reference)
D	25 (43.1)	24 (21.4)	2.86 (1.42~5.75)*	2.86 (1.25~6.53)*

[a] Adjusted for age.

[b] Adjusted for age, education, marital status, BMI, age at menarche, menopausal status, parity, and family history of breast cancer.

*Statistical significance

Table 4. Correlation between EBV DNA and EBV IgA.

EBV IgA	EBV DNA		r_s	*P* value
	Negative, n (%)	Positive, n (%)		
VCA IgA				
Negative	557 (86.1)	161 (80.1)		
Positive	90 (13.9)	40 (19.9)	0.071	0.040[a]*
Median (25^{th}-75^{th})	0.08 (0.02~0.21)	0.09 (0.02~0.26)		0.019[b]*
EBNA-1 IgA				
Negative	628 (97.1)	190 (94.5)		
Positive	19 (2.9)	11 (5.5)	0.058	0.089[a]
Median (25^{th}-75^{th})	0.07 (0.03~0.14)	0.06 (0.02~0.12)		0.602[b]
Combined EBV IgA				
Negative	544 (84.1)	154 (76.6)		
Positive	103 (15.9)	47 (23.4)	0.083	0.015[a]*

[a] *P* for spearman rank correlation.

[b] *P* for rank test.

*Statistical significance

Discussion

Our results demonstrated that the EBV DNA in PBMCs was not significantly associated with the risk of breast cancer, although it was shown to be related to the positivity of EBV IgA antibodies. In addition, we detected EBV subtypes of F/f and C/D in PBMCs and found that C/D subtype was associated with the risk of breast cancer.

The EBV DNA in PBMCs was supposed to be an immediate and direct biomarker of EBV reactivation compared with EBV IgA antibody. However, it was not found to be associated with breast cancer risk as EBV IgA antibody. These different associations with breast cancer risk may attribute to various sensitivities of the assays for detection of EBV DNA and IgA antibodies to some extent. Another probable reason is that EBV IgA antibody occurs in serum fairly stable in a definite time, while the increase of EBV DNA load in PBMCs occurs in a pattern of short episodes and EBV DNA load changes more frequently as opposed to EBV IgA antibody among non-immunodeficiency subjects during EBV replication circles [20]. EBV usually harbors latently in memory B cells and reactivates periodically [20]. In response to internal and external signals, EBV-carrying memory cells differentiate into plasma cells which enters lymphoid tissues and infects new naive B lymphocytes [21]. After a phase of EBV-driven transformation and differentiation, the infected B cells are released into the peripheral blood as resting memory B cells, resulting in the episode of an increase of peripheral EBV DNA load [20]. Therefore, the capture of the increase episode of EBV DNA load is not as easy and stable as EBV IgA antibody.

In addition, during the above replication circle, a feedback mechanism may play roles. Once the memory B cells have initiated viral replication, immune system would produce antibodies to kill these cells and prevent the spread [21], while the immune system is unable to eliminate the virus completely. As a consequence, the virus would establish a latent infection in B lymphocytes and shut down the expression of viral proteins [22]. This feedback mechanism explains to some extent that the EBV DNA in PBMCs was not associated with the risk of breast cancer while it was related to the positivity of IgA against EBV.

The present study showed that subtype D of EBV was associated with an increased risk of breast cancer compared with the C type at the locus, suggesting that subtype D may be involved in the pathogenesis of breast cancer. A similar association was found between the type D and gastric cancer risk: the D frequency was significantly higher in the tumor tissues of gastric cancer (62/64, 96.9%) than the throat washings cells of healthy donors (170/255, 66.7%) in Latin America [15]. Four studies in China and other four studies have detected the C/D subtype in gastric cancer and NPC tissues [16, 18, 23-28]. However, they had no healthy controls to be compared, resulting that the association between the C/D subtype and cancer risk could not be concluded, although the C type was the predominant isolate in the patients. There was molecular evidence to support our finding that the C/D subtype of EBV may be disease related: some particular EBV genes located in the vicinity of the C/D locus, such as Bam H1-A Rightward Frame-1 (BARF1) and Latent Membrane Protein 2A (LMP2A), which have functions of transformation and immortalization in tumorigenic process [15]. Nevertheless, the exact mechanisms remain to be explored.

The frequency of successfully typed subjects among EBV positive individuals was low in the present study and the representative of the subjects may be questioned. However, the baseline characteristics between successfully and unsuccessfully typed subjects were basically balanced, except menopausal status. The frequencies

of the two subtypes of EBV were similar to that from other studies performed in the same areas. It has been reported that EBV type F is predominant throughout the world [29], while f subtype mainly exists in Southern China [30]. In the present study, the frequency of subtype f was 23.9% (26/109), which was similar to that among a southern Chinese healthy population in a previous study (18.9%) [14]. As for EBV C/D subtype, C variant was the minor stain in Europe and North Africa [28, 31], while it was a dominant strain in Asia [30]. For example, the frequency of C type in a southern Chinese population was 84.6% [30], similar to that in the present study [78.6%, 88/112]. In addition, the positive rate of EBV DNA in our study was also was similar to that [18.8%, 3/16] in healthy control individuals of Portugal [32].

In conclusion, the presence of EBV DNA in PBMCs may not be an appropriate biomarker for breast cancer risk. The subtype D of EBV is likely to be related to the malignant pathologies of breast, but the mechanism remains unclear. Further study is required to confirm the causal association and the mechanisms of EBV in the tumorigenesis of breast.

Supplementary Material

Supplementary tables.
http://www.jcancer.org/v08p2944s1.pdf

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Competing Interests

The authors have declared that no competing interest exists.

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