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Epstein-barr virus may not be associated with breast cancer in Iranian patients

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Abstract

Background: Epstein-Barr Virus (EBV) is a human herpes virus which infects over 90% of the world's population. This virus has been implicated in the pathogenesis of many types of cancers. Breast cancer is the most common cancer among women worldwide. EBV has been studied as a possible cause of human breast cancer. The data related to the possible association of EBV with breast cancer are contradictory and therefore inconclusive. Here we searched for EBV in Iranian patients with breast cancer.

Methods: Paraffin embedded formalin fixed specimens were prepared from malignant tumor tissue from eighteen Iranian patients with clinically proven breast cancer. EBV as well as human cells were detected and quantified by PCR and real-time PCR, respectively. The ratio of EBV/human cells was calculated in specimens from all patients.

Results: EBV was not detected in any of breast cancer tissues in our study. Quantitative analysis showed that EBV is not present in any specimens at level of one virus per each human cell.

Conclusion: Our results showed that EBV may not be associated with breast cancer in our patients suggesting that EBV might not have a significant role in this disease in Iran.

Keywords: Epstein-Barr virus, breast cancer, paraffin embedding, real-time polymerase chain reaction

Introduction

Epstein-Barr Virus (EBV) is a human herpes virus which infects over 90% of the world's population in the childhood and remains in the body for life [1]. Although most humans live with the virus without overt disease, a small proportion will develop tumors. EBV was the first human virus clearly involved in development of human cancer [2]. EBV is important in the pathogenesis of many cancers such as Burkitt's lymphoma and nasopharyngeal carcinoma [2]. The cancers associated with EBV vary markedly in viral prevalence, from 100% of nasopharyngeal carcinoma to about 10% of gastric carcinoma [3,4]. Although EBV infection is important in carcinogenesis, it is not sufficient and other risk factors such as epidemiological ones may play an additional critical role in cancer development [5]. Normal host populations can have large differences in susceptibility to EBV-related tumors and this may be shown by differences in geographical prevalence and different immunological presentation of these cancers [2]. In cancers for which epidemiologic and molecular virological data support a causal link with EBV, majority of tumor cells present DNA and other products of this virus [1].

Breast cancer is the most common cancer among women worldwide [6]. The etiology of breast cancer is not completely understood and the known breast cancer risk factors explain only a small fraction of this disease [7].

Epstein-Barr Virus (EBV) has been studied as possible cause of human breast cancer. However, the published data remain preliminary, and do not justify a conclusion that EBV is causally associated with breast cancer [8]. The proportion of EBV-positive malignant samples ranged from 0% to 100% [8]. A recent meta-analysis failed to resolve this controversy, although suggested a significant association between EBV infection and breast cancer [9].

The available data regarding the possible association of EBV with breast cancer are not concordant and therefore inconclusive. These contradictions may originate from different sensitivities of the assay used, and various definitions of "EBV positive" [10]. Differences in sample types (paraffin-embedded tissue or fresh tissues) and different populations studied (Asian, European, American, etc.) may contribute to these discrepancies [9].

Some authors defined "EBV positive" to mean that the majority of tumor cells each have at least one copy of EBV DNA [10]. This definition seems to be valid because it is true for all human tumors with established EBV associations [10].

The first step for showing the association of a cancer with a virus is to demonstrate the virus in the affected tissue [8]. Molecular techniques are the most definitive assays in establishing viral presence in comparison to other tests based on host antibody assessment and immunohistochemistry

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Table 1. Histopathologic data and EBV per cell for Iranian breast cancer patients studied.

Patients No.	Age	Diagnosis	Grade	Size (cm)	ER	PR	LNM	Human cell/µl extracted DNA	EBV copy/Cell
1	41	IDC	3	3.5	NA	NA	+	8,635.99	< 0.029
2	40	IDC	2	2.5	NA	NA	+	6,556.02	< 0.038
3	55	IDC	2	2.3	NA	NA	-	4,649.64	< 0.054
4	65	IDC	3	4	+	-	+	6,895.11	< 0.036
5	64	ILC	NA	2.5	+	+	-	6,278.75	< 0.040
6	65	IDC	3	2.5	+	+	+	4,170.07	< 0.060
7	53	IDC	1	1.5	+	+	-	2,215.78	< 0.113
8	54	IDC	2	2	+	+	NA	3,140.43	< 0.080
9	51	IDC	2	1.8	+	+	+	660.60	< 0.378
10	66	IDC	2	1.6	+	+	-	861.78	< 0.290
11	62	IDC	2	3	+	+	+	4,456.13	< 0.056
12	46	IDC	2	1.5	+	+	+	250.91	< 0.996
13	48	IDC	NA	3.5	NA	NA	+	452.90	< 0.552
14	32	ILC	NA	3.5	+	+	+	1,187.51	< 0.210
15	58	IDC	2	1.5	+	+	-	1,902.21	< 0.131
16	41	IDC	3	NA	NA	NA	+	2,630.46	< 0.095
17	44	IDC	3	3	NA	NA	+	1,148.71	< 0.218
18	52	IDC	2	2	+	+	+	824.36	< 0.303
Raji cell	--	- -	- -	- -	--	--	--	3,989.83	65.09

Abbreviations: IDC, infiltrating ductal carcinoma; ILC, infiltrating lobular carcinoma; ER, estrogen receptor; PR, progesterone receptor; LNM, lymph node metastasis; NA, not assayed.

[8]. Up to now, no standard method has been generally accepted for EBV detection in cancer tissues [11]. PCR and Real-Time PCR have been considered as the most sensitive methods and have been used frequently to detect EBV [10,12-16]. It is necessary to measure viral load by real-time PCR and report the ratio of viral load to an endogenous gene so as to adjust for the quantity and quality of DNA extraction in the sample [5]. Here we determined the numbers of EBV as well as the human cells in paraffin-embedded malignant tissues in Iranian patients with breast cancer for finding the relationship between EBV and breast cancer.

Materials and methods

Patients and samples

Eighteen patients with clinically proven breast cancer were selected from Pars General Hospital in Tehran, Iran. Biological characteristics of these patients are shown in **Table 1**. Paraffin embedded formalin fixed specimen were prepared from malignant tumor tissue of each patient. DNA was extracted from malignant tissue within 3-7 days of their collection from patients.

DNA extraction

Three sections (8 µm thickness each) were prepared from paraffin embedded formalin fixed malignant tissue of each patient. DNA extraction was performed by QIAamp® DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). The DNA extraction method was mainly according to procedure provided by the company with few modifications as follows: sections were placed in a 1.5 ml centrifuge tubes, 1 ml xylene (Merck, Darmstadt, Germany) was added immediately and centrifuged (16000 × g, 2 min, 25°C) to remove paraffin. The supernatant was discarded and the pellet was resuspended in 1 ml pure ethanol (Merck, Darmstadt, Germany) and centrifuged for 2 min, 16000 × g at 25°C. The supernatant was discarded; the pellet was resuspended in 180 µl ATL buffer (QIAamp® DNA FFPE Tissue Kit) and 20 µl proteinase K (Vivantis, Malaysia), and incubated at 56°C overnight. DNA was eluted in 50 µl ATE buffer (QIAamp® DNA FFPE Tissue Kit) and stored at -70°C until use.

Raji cell

Raji cell line, as EBV-positive control, was purchased from Cell Bank of Pasteur Institute of Iran, cultured in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine (SIGMA, USA) , Penicillin (100 IU/ml) and Streptomycin (100 µg/ml). Cells were cultured at starting concentration of 10^5 cell/ml and were subcultured at concentration of 2×10^6 cell/ml at about 3-day intervals. The cells were counted using Neubauer Chamber hemocytometer (Marienfeld, Harsewinkel, Germany) and aliquots of 2×10^5 cells were prepared in 0.2 ml centrifuge tubes by centrifugation (400 × g, 6 min, at 25°C). Finally the supernatants were discarded and pellets were

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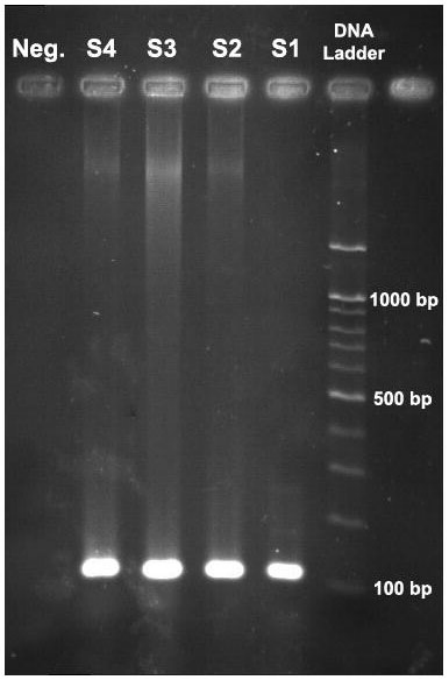

Figure 1. **Quality of the extracted DNA from specimens was acceptable for PCR because beta-Globin was successfully amplified from the extracted DNA**.
Sharp single bands (110 bp) corresponding to beta-Globin are observed in the lanes of the samples (S1 to S4), while no bands is seen in negative control lane (Neg.).

stored at -70°C until use. DNA extraction from aliquots of Raji cell was performed exactly as mentioned for tissue samples from patients using the same kit and procedure.

PCR

Amplification of BALF5 sequence of 90 base pairs by polymerase chain reaction (PCR) [17,18] was used for detection of EBV. PCR was performed using following primers: forward: 5′-CGGAAGCCCTCTGGACTTC-3′ and reverse: 5′-CCCTGTTTATCCGATGGAATG-3′ [17,18]. BALF5 amplification contains 10 µl 2 X Taq DNA Poly Mix (Fermentas, Sankt Leon-Rot, Germany), 0.5 µM of each primer, 2 µl of extracted DNA, and distilled water up to 20 µl. The PCR program was 94°C for 5 min, 35 cycles of 94°C for 30 sec, 59°C for 30 sec, and 72°C for 45 sec. Final extension was 72°C for 5 min. Suitability of DNA for PCR amplifications was assessed by amplifying the constitutive gene of beta-Globin. A 110- base pair fragment of beta-Globin gene was amplified using PCR by following primers: forward: 5′-ACACAACTGTGTTCACTAGC-3′ and reverse: 5′-CAACTTCATCCACGTTCACC-3′. The reaction mixture for beta-Globin PCR was: 10 µl PCR Master Mix (Fermentas, Sankt Leon-Rot, Germany), 0.4 µM of each primer, 2 µl of extracted DNA, and distilled water up to 20 µl. The PCR program for beta-Globin was: 95°C for 5 min, 35 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, final extension 72°C for 7 min. No-template control, as a negative control, was included in all PCR runs as indicator of potential DNA contamination throughout the PCR process.

Gel electrophoresis

One microliter DNA sample buffer (Vivantis, Malaysia) was added to 5 µl of PCR product of each sample, loaded on 2% agarose gel and electrophoresed with 120 volt for 40 min. Green Viewer (Pars Tous, Iran) at 1% v/v was added to agarose gel to visualize the bands before loading of samples.

Real-time PCR

Real-time PCR was performed by using Corbett Rotor-Gene 6000 (QIAGEN, Valencia, USA). Real-time PCR amplifying of BALF5 was performed according to the method described for BALF5-PCR with some modifications: 5 µl SYBR Green Master Mix (GenetBio, South Korea), 0.5 µM of each primer, 1 µl of extracted DNA, and 3 µl distilled water (10 µl final volume), 40 cycles of annealing was applied and the final extension was omitted. Different 2-fold serial dilutions of Raji cell DNA were used to prepare standard curve.

In order to calculate the ratio of EBV/human cell in specimens from each patient, the cell number of paraffin-embedded tissues was determined. Beta-Globin gene was used for quantitation of cells in each sample. A 110 base pair sequence of beta-Globin gene was amplified by Real-Time PCR with the same primers mentioned above for beta-Globin-PCR. The reaction mixture for beta-Globin Real-Time PCR was: 5 µl SYBR Green Master Mix, 0.5 µM of each primer, 1 µl of extracted DNA, and 3 µl distilled water (final volume of 10 µl). The amplification program for beta-Globin was same as mentioned above for beta-Globin-PCR except that 40 cycles of annealing was applied and the final extension was omitted. Standard curve was prepared by running three consecutive 5 fold dilutions of Raji DNA corresponding to 4000, 800, and 160 cells/µl of extracted DNA. Concentration of Raji cells were determined through counting by hemocytometer. Standard curve was drawn in each run, *i.e.,* standards were run parallel to specimen in each assay.

Specificity of amplification for target sequences (beta-Globin and BALF5) were checked in real-time PCR through melt curve analysis and the results were accepted if non-specific amplification were not present. All samples and standards were assayed in duplicate in all Real-Time PCR tests, and the data were analyzed using Corbett Rotor-Gene 6000 software version 1.7.87.

Results

Quality of the extracted DNA

Beta-Globin is a constitutive gene in human genome, so PCR amplification of beta-Globin shows acceptable quality of the extracted human DNA as far as PCR is concerned. As shown in **Figure 1**, PCR amplification of DNA extracted from

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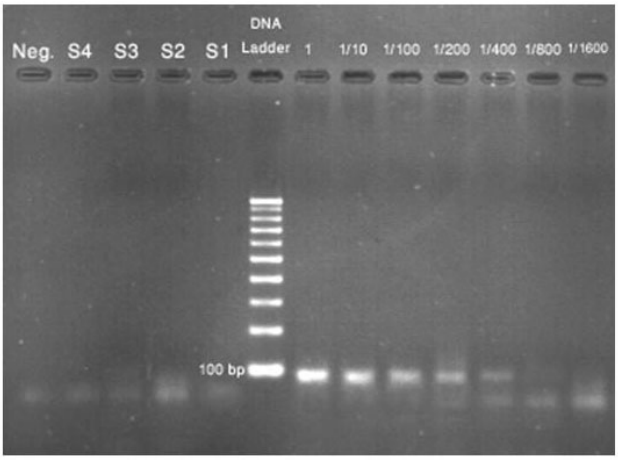

Figure 2. PCR amplification of EBV BALF5 sequence. Serial dilutions of Raji DNA (1-1/1600) were applied to find the lowest number of EBV (*i.e.,* BALF5 sequence) copy that can be detected by PCR. The right panel shows that up to 1/400 dilution of Raji DNA (which is equal to 500 copies of EBV) can be detected in our PCR setting. In the left panel, PCR amplification of BALF5 sequence was performed to find EBV in DNA extracted from tumor specimens (S1, S2, S3, and S4). No band was observed in any tumor specimens suggesting that no EBV genome is detectable in the specimens.
Abbreviations: S1 - S4: sample 1 - sample 4; Neg.: negative control.

patients tumor tissues resulted in a sharp single band of 110 base pair compatible with beta-Globin. These data indicated that the DNA extracted from paraffin embedded formalin fixed specimen had acceptable quality for PCR.

Sensitivity of the EBV detection

Raji cell line contains ~50 EBV copies/cell [19]. Each EBV virus contains one BALF copy [20,21]. In order to determine the sensitivity of our BALF5-PCR and Real-Time PCR assay, the extracted DNA from 2×10^5 Raji cells (eluted in 50 µl volume) was serially diluted, from undiluted DNA (equal to 2×10^5 copy of BALF5/µl of extracted DNA) to dilution of 1/1600 (equal to 125 copy of BALF5/µl of extracted DNA), and used as templates in BALF5-PCR as well as in Real-Time PCR assays. Serial dilutions of Raji genome were applied to find the lowest copy number of EBV (*i.e.,* BALF5 sequence) that can be detected by PCR and Real-Time PCR.

The result of BALF5-PCR (**Figure 2**) showed that the lowest level of detection in our BALF5-PCR assay is about 10 Raji genome/µl of extracted DNA (equivalent to 500 copies of BALF5 sequence/µl of extracted DNA). This assumption is based on the following calculation: DNA of 2×10^5 Raji cells were extracted in 50 µl volume, 2 µl of the extracted DNA was used in 20 µl-PCR mix, and 5 µl of this reaction mix was loaded in each well during electrophoresis (2×10^5 Raji cells DNA / 50 µl × 2 µl / 20 µl × 5 µl / dilution of 400 = 5 Raji cells/ 2 µl of extracted DNA which equals to 10 Raji cell or 500 BALF5 sequence/µl of extracted DNA).

The result of Real-time PCR for BALF5 showed that about 250 copies of BALF5 sequence can be detected in each microliter of extracted DNA. This result is calculated as follows: 2×10^5 Raji cells DNA / 50 µl / dilution of 800 = 5 Raji cells or 250 EBV copies.

We measured the number of human cells in each patient's specimen in order to calculate EBV/human cell ratio in each patient. Numbers of human cell were determined by quantifying beta-Globin copy number in each specimen. Beta-Globin was amplified by real-time PCR in consecutive 5 fold serial dilutions of Raji cells with defined cell numbers. Concentration of Raji cells were determined through counting by hemocytometer. A standard curve was drawn by plotting cell numbers in one axis versus Ct values of amplified beta-Globin sequence on the other axis. Hence the relationship between numbers of human cell and copy numbers of beta-Globin were obtained. The standard curve (R^2: 0.99 and Efficiency: 1.01) was used for calculation of human cell numbers in each patient's specimen. The numbers of human cells in each specimen are presented in **Table 1**. Specimens contained 250 to 8635 (mean 2302) human cells/µl of extracted DNA.

No EBV was detected in all specimens

Since BALF5 is a non-repetitive and constitutive sequence in EBV genome [20,21], presence of this sequence was studied by PCR and its copy number was determined by real-time PCR in patient's specimen. The PCR were negative for all the patients' specimens (**Figure 2**) showing that there is no EBV genome detectable in any of them. The results of Real-Time PCR confirmed the PCR results, *i.e.,* all the specimen were negative.

EBV/human cell ratio

Beta-Globin copy numbers were determined in specimens from all the breast cancer patients (**Table 1**) in order to calculate the number of human cells of each specimen. The ratio of EBV virus to human cells was then calculated for every specimen. The number of human cells of each specimen was about 250 to 8600. The ratio of EBV/human cells was between <0.03 to <0.99 in all the specimens studied.

Discussion

Choosing a reliable method is a critical step in EBV detection studies. PCR and Real-Time PCR are considered as sensitive methods and have been used frequently in EBV detection [10,12-16,22-24], so we used these techniques to detect the virus in tumor tissues. Beta-Globin is a constitutive gene present in human DNA, so amplifying beta-Globin with PCR can be used for assessing the quality and quantity of the extracted DNA. Therefore we applied beta-Globin PCR for all specimens and confirmed that the extracted DNA had acceptable quality and quantity for amplification through PCR.

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On the other hand, formalin which is used for preparation of formalin-fixed paraffin-embedded tissues may fragmentize the DNA and interfere with PCR amplification of large sequence of DNA templates. A simple way for reducing the effect of DNA degradation on the EBV detection in specimens is targeting of short DNA sequences (<200 base pair) in PCR [25]. Thus here we amplified a 90 base pair sequence (BALF5) of EBV genome, encoding EBV DNA polymerase [20,21] for EBV detection in tissue specimens.

A previous study reported no association between EBV and breast cancer in Iranian patients [26]. However this study suffers from major shortcomings. Firstly, no EBV-positive control specimen is included in the study, thus their negative results may be due to many variables including unsuitable PCR condition. Secondly, sensitivity of the EBV detection assay was not defined in the study. So it is not clear that how many EBV viruses were left un-detected in the study. Hence the reported negative results cannot be compared with other reports. Therefore in the present study we used Raji cell line as an EBV-positive control to eliminate false negative results and also to define sensitivity of our assays.

Our results showed that all the 18 patients in our study did not have any detectable EBV in real time PCR. The ratios of EBV/human cells were determined in specimens from all breast cancer patients in our study in order to compare our data with those of other reports. The ratio of EBV/human cells in all the breast cancer patients were below one EBV per each human cell. This ratio is much lower than the ratio of EBV/human cells in Raji cell (about 50), which is a Burkit lymphoma cell [19]. Hence all the breast cancer tissue samples were negative in comparison to a well defined EBV positive cancer cell. The definition of one or more EBV per each human cell is considered to be true for all human cancers for which EBV was associated with the malignancy definitely [10]. Considering this definition, all the breast cancer tissues of our study were negative, because the ratio of EBV/human cell was less than 1 in all patients.

EBV was not detected in any of specimen in our study. On the other hand, it is well known that EBV infects most of humans and remains in the body for life. No EBV was detected in any specimens in our study. This finding may be explained by the low sensitivity of our EBV detection assay. Our assay was able to detect only the high EBV load present in pathological cases like Raji cell and not the low EBV load present in most of humans. Our assay will become positive if there is at least 250-500 copy of EBV/μl of extracted DNA. Therefore this assay has not been sufficiently sensitive to detect the low copy number of EBV present in most humans. This assumption is based on the following calculation: the frequency of EBV infected cells in infected individuals is about 1 infected cell for every 10^4 B cells [27]. Our specimen contain maximum of 8635 human cells/μl of extracted DNA and B cells may be a small fraction of these cells. Assuming that all cells in our specimen are B cell, about 0.86 (≈ 1) EBV infected cell should have been present in the specimen containing the highest numbers of cells (8635/10000 = 0.86). Assuming that the copy number of EBV in each B cell and Raji cell (as a definite EBV positive control) are equal, there should have been about 43 (0.86 × 50 = 43) EBV copy/μl of the extracted DNA from our specimen containing highest human cells. The 43 copy is lower than the detection limit of 250 EBV/μl of extracted DNA in our assay. In other words the low level of EBV present in most of humans is not detected in our assay, while the assay can detect higher levels of EBV that are present in pathologic "EBV positive" cells like Raji cell. We did not study women with no history of breast cancer as controls because no pathological levels of EBV were found in malignant specimens.

In summary, our results showed that no pathological levels of EBV were detected in breast caner tissues of Iranian patients we examined, suggesting that EBV may not have a significant role or at least is uncommon in this disease in Iran. Similar suggestion has also been reported [10,28]. It should be also noted that finding the definite relationship between EBV and breast cancer in Iranian patients needs further studies on an increased number of patients.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
HM and PF conceived idea of the research. HM, PF, BS, MR, and AA developed design of the research. PF and BS provided the patients' specimens and their clinical and pathological data. HM and MR performed the experiments. All authors have read and corrected the manuscript.

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