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Full Length Research Paper

In situ hybridization and polymerase chain reaction methods for the detection of Epstein-Barr virus RNA in breast cancer specimens

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The role of Epstein-Barr virus (EBV) as a cofactor in breast cancer is controversial and its association with breast cancer varies. In this study, EBV was detected by using *in situ* hybridization technique (ISH) to detect Epstein-barr virus encoded RNA1 (EBER1) transcripts. Archival formalin-fixed paraffin embedded breast cancer tissue samples (n = 139) and normal breast tissue (n = 20) obtained from Hospital Tuanku Ja'afar were sectioned, stained and examined microscopically for nuclear staining and by DNA amplification of the same gene. By ISH, 83/139 (59.7%) and 12/20 (60%) were EBV positives in the breast cancer tissues and normal tissues, respectively. On the other hand, confirmation by polymerase chain reaction (PCR) found that additional six samples (89/139 or 64%) breast cancer tissues were positive for EBER1 gene. To further confirm the identity of these amplified products, two samples (UiTM-53 and UiTM-73) were sequenced, BLAST, analyzed phylogenetically and was found to be 100% similar to the EBV EBER1 gene sequences already deposited in the GenBank (accession numbers AB065135, FN545286, EF187853 and DQ883818). These preliminary findings suggest that there is a correlation between EBV and breast cancer but need further testing with more samples to confirm the role of EBV.

Key words: Breast cancer, Epstein-Barr virus, EBER1 transcripts, *in situ* hybridization, polymerase chain reaction (PCR), tumour grading.

INTRODUCTION

Epstein-Barr virus (EBV) is a double-stranded DNA virus (184-kb long) belonging to *Herpesviridae* family (Rickinson and Kieff, 2001). The virus genome is composed of up to 100 genes: Epstein Barr Nuclear Antigen (EBNA) gene, Epstein Barr Encoded RNA (EBER) gene, Latent Membrane Protein (LMP) gene, and BZLF1 (also known as active gene) and so the list goes on (Manet et al., 1998). EBV is usually found in tumor cells of Burkitt's lymphoma and it is an example of EBV-associated tumorigenesis (Magarath and Bhatia, 1999). Since EBV is classified as a primary carcinogen in human, the potential role of EBV in breast cancer would shape clinical diagnosis and patient's outcome (National Cancer Registry, 2003).

The first report on the role of EBV in a multi-step disease in breast cancer has been described by Labrecque et al. (1999). However, its role in breast cancer is an issue since there are reports that showed the presence of EBV in breast cancer (Labrecque et al., 1999; Bonnet et al., 1999; Hemminki and Dong, 1999; Fina et al., 2001; Trablesi et al., 2008; Glaser et al.,

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2004), while others have failed to identify EBV particles in their samples (Glaser et al., 2004; Chu et al., 1998; Hermann and Niedobitek, 2003; Deshpande et al., 2002; Murray and Young, 2001; Perirogue et al., 2005). These inconsistent findings may be due to the technical challenges of localizing EBV in tumor cells and the different methods used (Glaser et al., 2004).

The most abundant EBV viral transcripts that can be found in cells latently infected with EBV are EBER1 (Magarath and Bhatia, 1999; Ryan et al., 2004; Gulley, 2001). Previous review of EBV-encoded RNA by *in situ* hybridization (EBER-ISH) has found that it is a very sensitive and specific assay and is often considered the gold standard for detecting latent EBV (Labreque et al., 1999; Glaser et al., 2004; Chu et al., 1998).

The Bloom-Richardson histological grade has been developed for grading of breast cancer cases since 1996 (California Cancer Registry, 2007). This grading system is based on three morphological features: degree of tumor tubule formation, tumor mitotic activity and nuclear pleomorphism of the tumor cells (Peart, 2005). The classifications are divided into three grades: low grade (histological grade I with well differentiated cells and minimal nuclear enlargement), intermediate grade (histological grade II with moderately differentiated cells with moderate enlargement of the nucleus) and high grade (histological grade III with significantly enlarged nucleus with high nucleus: cytoplasm ratio) (Peart, 2005).

Many studies found that EBV are found in large portions of high grade breast cancer samples (Bonnet et al., 1999; Gulley, 2001; Murray and Young, 2001) with 66, 44 and 27% in grades III, II and I, respectively ($p = 0.03$) (Bonnet et al., 1999). This concurs with report which suggested EBV as a cofactor in breast cancer development (Amarante and Watanabe, 2009).

With the advent of Polymerase chain reaction (PCR) method, the results of the EBER ISH can be confirmed by amplification of the EBV EBER latent gene (Bonnet et al., 1999). The use of PCR for EBER1 gene amplification have been reviewed from 1993 to 2004 with results ranging from 32 to 51% positives (Glaser et al., 2004).

This present study aim to detect the presence of EBV by both ISH method and amplification of EBER1 transcripts and to investigate the correlation of EBV and tumour grade in breast cancer tissue specimens.

MATERIALS AND METHODS

Sample collection

Archival formalin-fixed paraffin embedded (FFPE) breast carcinoma tissue samples (n = 139) and normal breast tissue (n = 20) were obtained from the Pathology Department, Hospital Tuanku Ja'afar, Seremban, Negeri Sembilan. The diagnosis of breast carcinoma and the Bloom and Richardson classification were obtained from the patient's histopathology report and has been approved by the Ethical Committee of Universiti Teknologi MARA (UiTM), and the National Medical Research Register (NMRR), Ministry of Health Malaysia (MOH).

Sample preparation for EBER1 *in situ* hybridization sample preparation

Tissue blocks were sectioned into 5 µm thick using a rotary microtome (Microm, HM335 model) and were attached to glass slides (Aazmi, 2008) and proceeded for ISH. Briefly, the tissue sections were dewaxed in xylene (R and M Chemicals, U.K.) for 3 min twice. The tissue sections were hydrated in 99% v/v ethanol (R and M Chemicals, U.K.) for 3 min, repeated twice and followed by hydration in 95% v/v ethanol for 3 min. Next, the tissue sections were immersed in water for 3 min and also repeated twice. The slides were placed in an incubation tray (Dako, U.S.A.) and were covered with 100 µL of proteinase K in 50 mM Tris-HCl buffer (pH 7.6) and incubated for 30 min at 37°C. After the incubation period, the tissue sections were immersed in two changes of distilled water for 3 min each, followed by dehydration in 95% v/v ethanol for 3 min. Finally the tissue sections were dehydrated in 99% v/v ethanol for 3 min before finally being air dried (Leica, 2008).

In situ hybridization (ISH) with commercially prepared probe

ISH was performed using Epstein - Barr virus Probe ISH Kit (Novocastra Laboratories, United Kingdom) following the manufacturer's instructions (Perrigoue et al., 2005). EBV in the sections was examined microscopically at 10 and 40X magnifications. The interpretation of the results was based on the presence of blue or black staining of the nucleus against a clear background (Aazmi, 2008). EBV/EBER positive control slides (PanPath, Netherland) were included as well.

Amplifications of EBV EBER1 gene by PCR

Prior to PCR, paraffin were removed from the tissue ribbons using the QIAamp DNA FFPE Tissue Kit (QIAGEN, 2007). The extracted DNA was used as template for PCR. Each reaction mixture consisted of 12.5 µl of 2X *Taq* Master Mix (QIAGEN, Germany), 0.5 µl of EBER1A Primer (10 µm), 0.5 µl EBER1B Primer (10 µm) (Limaye et al., 1999), 1.0 µl of $MgCl_2$ (50 mM), 1.0 µl of EBV DNA template and 9.5 µl of nuclease-free water for 25 µl total volume and the mixture was short spinned. Amplification was carried out in the Px2 Thermal Cycler (Thermo Electron's USA) with pre-denaturation step at 95ºC for 2 min (1 cycle) and for 36 cycles of PCR (denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min) and final extension at 72°C for 10 min. Electrophoresis of the PCR product was done using 1.5% agarose gel in TBE buffer at 95 V for 1 h.

Statistical analysis

Chi-square tested for relatedness or independence was used to look for relatedness between EBV and breast cancer and also to look for relatedness between EBV positivity (SPSS software version 17.0).

RESULTS

Overall, 83/139 (59.7%) breast cancer tissue and 12/20

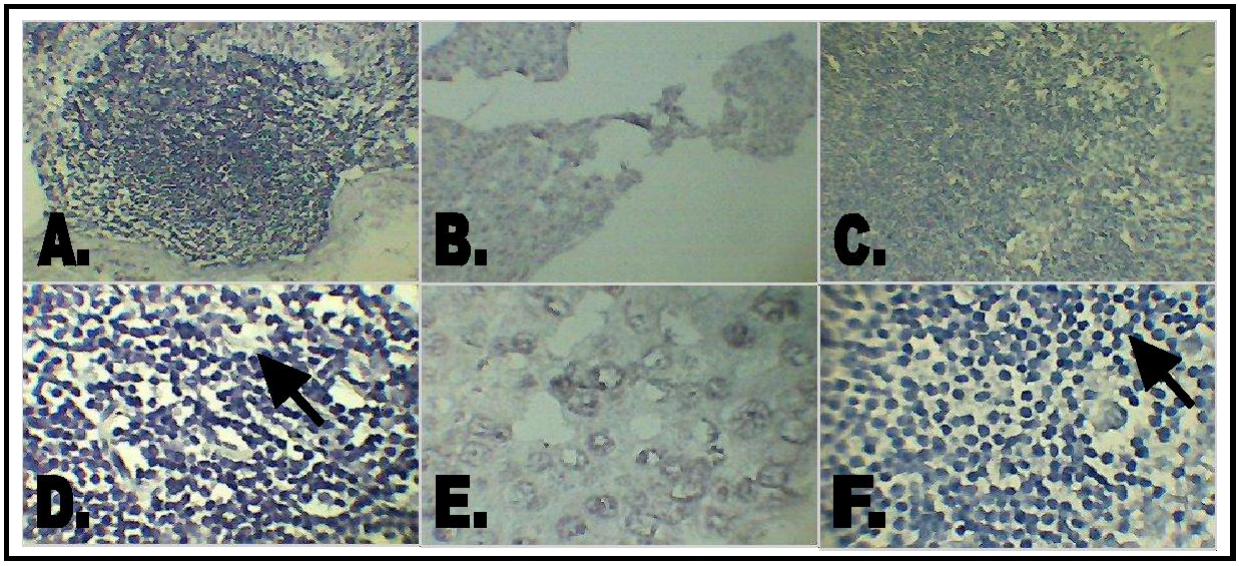

Figure 1. Results of ISH for positive control (A, D), negative control (B, E) and sample 53 (C,F). Upper rows (A-C, 10x magnification), lower rows (D-F, 40X magnification). Blue or black coloration of nucleus against clear background were classified as positive (arrow in D and F). Microscopic images of the stained tissues were taken using Premiere® Digital Microscope Eyepiece.

Table 1. Results of EBV detection by ISH and PCR methods according to type of samples.

Type of breast tissue samples Breast cancer tissue (n = 139)	Number screened	Total (%)	No. of positive samples by PCR (%)
Positive	83	83/139 (59.70)	89/139 (64.0)
Negative	56	56/139 (40.0)	50/139 (35.9)
Normal breast tissue (n = 20)			
Positive	12	12/20 (60.0)	12/20 (60.0)
Negative	8	8/20 (40.0)	8/20 (40.0)
Total	159	100	100

(60%) normal breast tissue were stained blue or black (positive) (Figure 1C and F) while 56(40%) breast tissue samples and 8(40%) normal breast tissue were negative and did not react to the probe used (Table 1).

The control slide that was processed together with test samples was used to verify the various steps during the optimization of ISH procedure. In this positive control slide (Figure 1A and B), the expected blue/black colour and staining intensity in the nucleus was achieved and was used to compare with test samples (Figure 1C and F) and negative control slide were also included (Figure 1B and E). All 95 positive samples revealed similar nuclear pattern.

Figure 2 shows the amplification results for samples, in-house positive control (IPC) and negative control (NC). The expected molecular weight of EBER1 gene was 118 bp in the IPC (lane 3) and no bands in the NC (lanes 2 and 22). Out of 159 samples tested, 89/139 (59.7%) breast cancer tissues and 12/20 (60%) normal breast tissue were found positive for EBER1 gene (Table 1). This present showed that by combining the ISH and PCR methods, a total of 89/139 (64%) were EBV positives. Statistically it was also found that there is an association between EBV and breast cancer ($p = 0.000$).

This study also found that EBV were detected majority in higher histological grade with 34.7, 29.7 and 16.8% for grade III, II and I (Bloom-Richardson histological grade), respectively. However, it was found that there was no significant association between EBV and histological grade ($p = 0.288$).

DISCUSSION

In this present study, the samples were archival FFPE

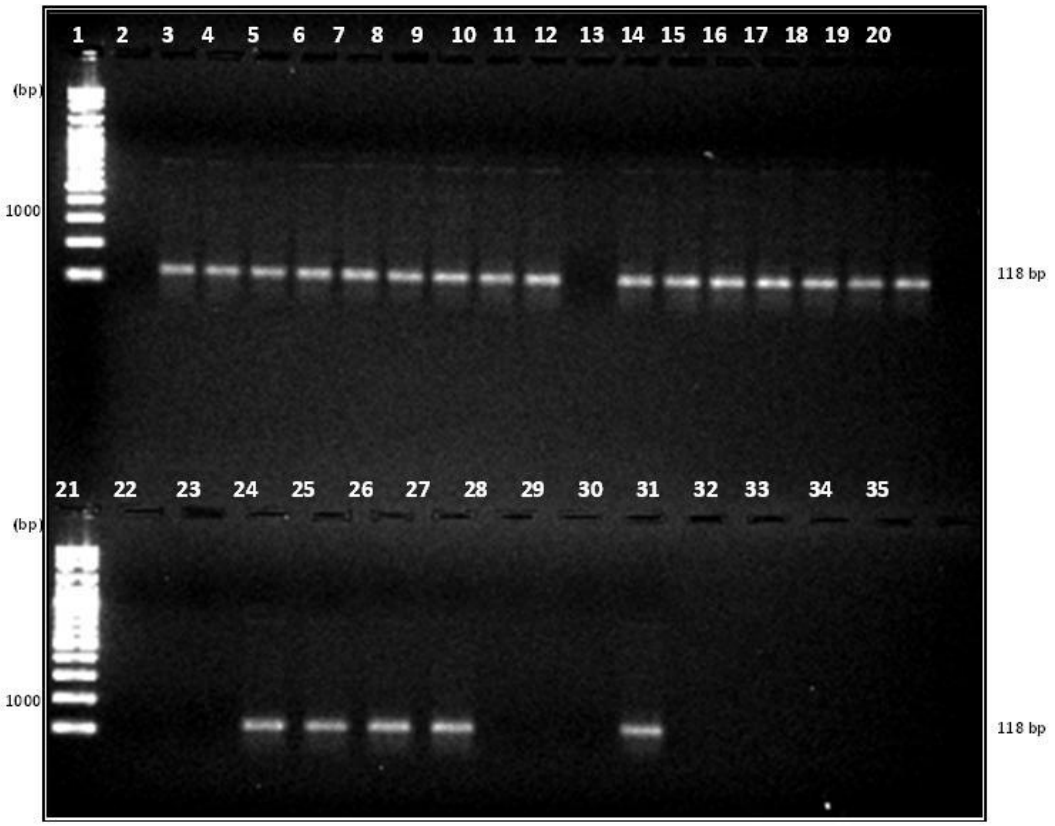

Figure 2. Amplifications of EBV EBER1 gene on various samples using 1.5% agarose gel electrophoresis and stained with ethidium bromide. Lanes 1 and 21: 100 bp DNA ladder (Vivantis Vc 100 bp); negative controls in lanes 2 and 22 (no band present); no bands or negative in lanes 12, 20, 22,23,28,29, and 31 to 35. Expected bands of 118 bp were observed in lanes 3 (IPC) and 4 to11, lanes 13 to19, lanes 24 to 27 and lane 30.

tissue blocks sampled from the year 2003 to 2009. A review by Glaser et al. (2004) reported that fresh frozen samples are preferred as compared to FFPE samples. FFPE samples contain formalin residues which interfere with PCR amplification reaction since it modifies the structure of nucleic acid. However, this problem was taken care by using ethanol (Qiagen, 2007). Other researches also used FFPE samples (Chu et al., 1998; McCall et al., 2001; Luqmani and Shousha, 1995; Preciado, 2003) while some scientists worked with fresh frozen samples (Bonnet et al., 1999; Chu et al., 2001).

A potential viral mediated cancer has become current concern in the cancer community (Ryan et al., 2004; Labreque et al., 1999; Glaser et al., 2004; Gulley, 2001). The detection of EBV in high percentage of breast cancer patients has driven similar efforts to verify the presence of this virus in the local samples. EBER-ISH which is regarded as the gold standard was used in this present study to detect EBV products in both breast cancer and normal breast tissues.

Earlier study shown that ISH of EBER1 gene were done manually by first principle using digoxigenin labeled synthetic oligonucleotide probes which has significantly increased the sensitivity of EBV detection as compared to other methods (Chu et al., 2001). In 2008, the ISH method was commercialized at Leica Microsystems, UK thus contributing to a more consistent and standard procedure for EBV detection with fluorescein-conjugated probes. The validation of the results for ISH detection of EBER1 gene transcripts by using the commercial ISH detection kit was done by including EBV/EBER1 positive control slides (PanPath, Netherland), in-house positive control as well as negative control and further verification by the pathologists in Hospital Tuanku Ja'afar, Seremban.

The results for ISH detection of EBER1 gene transcript for this study were 59.7% in breast cancer samples. Others reported 0% positive (Luqmani and Shousha, 1995; Chu et al., 1998; Glaser et al., 1998), 10% (Chu et al., 2001) and 50% (McCall et al., 2001). It was clear that in this present study, the percentage of EBV positives detected by ISH of EBER1 transcript was higher than other studies done globally since the above said commercial EBV detection kit was used.

In the recent years, the use of PCR in the detection of EBV (Gulley, 2001) has become more relevant by applying the amplification of nucleic acids (Saiki et al., 1986). In 1999, Bonnet et al. detected EBV by PCR with 51% EBER gene positive. Most clinical tissue samples are routinely fixed in formalin and embedded in paraffin wax for archiving purposes and to maintain excellent cell morphology (Grinstein et al., 2002). Formalin fixed, paraffin wax embedded clinical samples can be used to detect EBER1 gene by amplification of extracted DNA from breast tissues.

When EBER1 gene was screened by PCR, an additional six samples which were ISH EBV negatives were detected as EBV positives by PCR: 64% in breast cancer tissue and 60% in normal breast tissue and statistically associated with breast cancer ($p = 0.000$). Others have reported variation in EBV positivity in breast cancer tissue: 32% (Fina et al., 2001), 35% (Preciado, 2003), 42% (Grinstein et al., 2002), 50% (Labreque et al., 1999) and 51% (Bonnet et al., 1999). This again shows the present study found a higher percentage of EBV positives in breast cancer, thus confirmed that PCR is more sensitive compared to ISH. To validate the PCR results, the in-house positive control was checked by sequencing and phylogenetic method. It was found that the in-house positive control was 100% similar to EBER1 gene deposited in the GenBank. However, although PCR is the most sensitive method, it could not localize the exact EBV location, therefore, the PCR has actually covers the weakness of ISH which is less sensitive, while ISH has covers the weakness of PCR being unable to detect the exact location of EBV.

With respect to EBV in normal breast tissue, this present study found that 60% normal breast tissue was EBV positive by both ISH and PCR. In 1999, Bonnet et al. found 10% of the samples screened were EBV positive for normal breast tissue whilst Chu et al. (2001) found that 15% of the samples were EBV positive for normal breast tissue. Bonnet et al. (1999) also reported that statistically EBV is restricted to tumour cells only although EBV was detected in 10% of the normal samples ($p = 0.000$). In addition, it was reported that the presence of EBV in normal tissue may be due to early invasion of EBV from lymphocytes in the circulation or from the adjacent infected tumour cells (Bonnet et al., 1999). Therefore, the presence of EBV in 60% of the samples (normal breast tissue) in this study signals that the patients may have a higher risk of getting breast cancer and early treatment needs to be done to stop the carcinogenesis caused by EBV. Patients can be treated with antiviral drugs like acyclovir or ganciclovir upon lytic infection thus reducing the risk (Murray and Young, 2001).

Furthermore, when the association of EBV, breast cancer and histological grade were analysed. EBV were detected majority in higher histological grade with 34.8, 29.2 and 16.8% for grade III, II and I respectively, however it was found that there was no significant association between EBV and histological grade ($p = 0.288$). As compared to another study done by Bonnet et al., they also found EBV in higher histological grade with 66, 44 and 27% for grade III, II and I, respectively ($p = 0.03$) (Bonnet et al., 1999). This present findings on the association between EBV and histological grade therefore does not concur with Bonnet et al. in terms of percentages however both studies reported highest EBV in grade III. EBV was found to be present in breast cancer thus suggesting that EBV may be a cofactor in breast cancer development (Bonnet et al., 1999; Gulley, 2001; Murray and Young, 2001; Amarante and Watanabe, 2009).

In terms of the presence of EBV in normal tissue, this signals that the patients may have higher risk of developing cancer and further studies need to be done immediately to alert the physicians. Early treatment of EBV infection can reduce the risk of developing cancers since EBV is found in 90% of the population and therefore the bigger picture is that it could reduce the incidence rate of cancers in Malaysia.

Therefore this study provides a preliminary data for the Malaysian population however due to sampling from one hospital; the result could not be generalized to the whole population. The other limitation was in getting a matched numbers of normal breast tissue and breast cancer tissue, as it is unethical to perform biopsy on normal patients. However, this study is a preliminary finding which shows that there is breast cancer cases that are EBV associated. This finding is in line with results from other parts of the world that support the argument that EBV is a cofactor in breast cancer.

The same study should be performed on other hospitals in Malaysia in order to obtain a more meaningful data. Since this study is done on limited samples, the results could not be generalized for the whole country. However, this study is relevant as breast cancer is the number one cause of death for women in Malaysia and across the globe. In conclusion, the use of two detection methods (ISH and PCR) to increase the detection rate is recommended and therefore, more effective treatment can be initiated at the early stage of infection and thus reducing the risk of cancers.

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