Establishment of ultrasensitive PCR assay targeting *cell-free* EBV DNA for early detection of nasopharyngeal carcinoma

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Abstract:

In Vietnam, nasopharyngeal carcinoma (NPC) is the eighth most common cause of death from cancer. *Cell-free* Epstein Barr virus DNA (*cf*-EBV DNA) was reported to be present in almost all NPC patients. However, currently available assays in Vietnam can detect *cf*-EBV DNA in only 67.6% of NPC patients, thus leaving 32.4% of cancer cases undetected. Therefore, in this study, we aim to develop a highly sensitive quantitative PCR (qPCR) assay that measures the load of *cf*-EBV DNA for the purpose of early detection of NPC, and then evaluate the sensitivity and the specificity of the developed qPCR assay on the clinical samples. The major methods used in this study include primer/TaqMan probe design, *cf*-DNA extraction, optimization of qPCR assay and statistical analysis. Using an international standard panel from the Chinese University of HongKong, the linear range of developed qPCR assay is from 50-150,000 copies/ml (R^2 = 0.99613) and the detection limit has been shown to be 25 copies/ml. The developed assay could detect *cf*-EBV DNA with a sensitivity of 96.9% (31/32 NPC patients) and *cf*-EBV DNA has not been detected in 103 out of 105 healthy controls, which corresponds to a specificity of 98%. Consequently, the performance of the optimal assay has achieved remarkably high sensitivity and specificity. Moreover, the detection limit of our optimal qPCR assay is 25 copies/ml of plasma, which is at least ten times better than other assays tested in recent studies in Vietnam. This developed qPCR assay will also form the basis for further studies in Vietnam and will open many new applications in management of NPC.

Keywords: ***cell-free Epstein-Barr virus DNA, nasopharyngeal carcinoma, quantitative PCR.***

Classification numbers: ***3.2, 3.5***

Introduction

Nasopharyngeal carcinoma (NPC) is malignant tumors that arise from epithelium cells of the nasopharynx - the upper part of the throat that is situated behind the nasal cavity and near the base of the skull [1, 2]. Globally, there were an estimated 84,400 cases of NPC and 51,600 deaths in 2008. In 2012, it was reported that there were 86,700 cases and 50,800 deaths respectively [3, 4]. However, the prevalence and the distribution of NPC varies throughout the world. NPC shows a low prevalence, and is even rare in some parts of the world such as Western Europe, North America and Japan, where it accounts for as little as 1% of total cases per year. Conversely, Southern China (especially Guangzhou) and Hong Kong are the regions with the highest incidence across the globe; while some parts of Southeast Asia and Asian countries show intermediate rates of this cancer [5-7]. In Vietnam, NPC is the eighth most common cancer with an annual incidence rate of about 7 per 100,000 people [5].

Due to the fact the patients often lack specific symptoms, NPC is often diagnosed at a late stage, leading to a 5-year survival rate of only 41%. However, if this disease is diagnosed at an early stage, this survival rate can be improved to more than 95% [8]. Unfortunately, owing to the hidden anatomic area of the nasopharynx, the occurrence of occult primary tumors in this area increases the difficulty of diagnosing the disease early and accurately. Meanwhile, imageological examinations are not effective for early detection of NPC tumors [9]. For these reasons, the development of reliable methods to detect NPC at an early stage is critical to improve the survival rate of patients.

Epstein-Barr virus (EBV) is known as human ubiquitous γ-herpesvirus 4 (HHV-4) and is one of eight known viruses in

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the herpesviridae family. This virus is one of the most common viruses in humans with greater than 90-95% of worldwide adult human population latently infected [10, 11]. Morphologically, EBV has the same structure of virion as other members of the herpesviridae family [12]. The genome of the EBV virus is a linear, double-stranded DNA molecule, varying in size from 168 kilo base pairs (kb) to 192 kb, that encodes more than 85 genes, most of these protein-encoding genes [13, 14].

A strong association between NPC and the Epstein-Barr virus has been documented in many studies. *Cf*-EBV DNA has been detected in the plasma and serum of patients with NPC and correlated with EBV status in tumors. In contrast, *cf*-EBV DNA has not been detected in healthy control subjects. In 1998, Mutirangura, et al. detected *cf*-EBV DNA in peripheral blood of NPC patients while no viral load was detected in healthy controls. Although this study only had a sensitivity of 31% [15], it was still an important breakthrough in early detection of NPC patients using a *cf*-EBV DNA assay. Later, in 1999, Lo, et al. developed a qPCR assay that could detect *cf*-EBV DNA in 96% of NPC patients (55 of 57) and 7% of controls (3 of 43), showing a high sensitivity and specificity [16]. Realtime qPCR for detecting *cf*-EBV DNA is a highly sensitive, specific and quantitative diagnostic approach. Since its advent, it has become a promising tool for early detection of NPC.

The quantification of *cf*-EBV DNA was demonstrated as a useful tool for early detection of NPC. However, its clinical utility is still not routinely applied in Vietnam. There are few studies following this approach in our country. Quantitative PCR assays for detection of *cf*-EBV DNA in peripheral blood in our country have not been described in detail and have low sensitivity, with a LoD of 300 copies/ml plasma [17]. Meanwhile, the procedure applied at research centers around the world has reached the LoD of 60 copies/ml plasma [18]. As a consequence, studies in Vietnam are able to detect DNA of EBV in peripheral blood in only 67.6% of NPC patients, leaving more than 30% of cancer cases undetected - a sensitivity much lower than that in other studies around the world. Consequently, we aim to develop a qPCR assay that is able to detect *cf*-EBV DNA in peripheral blood with high sensitivity in order to make early detection of NPC possible, therefore lengthening the survival rate of patients. We further aim to evaluate the sensitivity and specificity of the developed qPCR assay on clinical samples. This development would also form the basis for further studies and open many new applications to improve management of NPC patients and increase their survival rate.

Methods and materials

Study subjects

This study involved two groups of samples: clinical samples and healthy samples. Frozen and fresh human plasma samples were obtained from the archives of our clinical and research laboratories. In clinical groups, a total of 32 biopsy-proven NPC patients were recruited for this study with informed consent. In another group, plasma samples from 105 volunteers were used as healthy controls, and follow up examinations of healthy volunteers were conducted for 6-12 months.

International standard panel

In this study, we have evaluated the performance of the optimal qPCR assay using the international standard panel that was provided by Prof. Allen Chan, at the Chinese University of Hong Kong. The international standard panel includes the series of nine DNA standard samples with known concentrations including: 15,000 - 6,000 - 600 - 120 - 60 - 30 - 15 - 5 - 2.5 copies/5 µl and a standard plasma sample positive with EBV DNA.

EBV DNA extraction from plasma

Peripheral venous blood (5 ml) was taken from each patient and control, and placed in an EDTA tube. Within 6 hours of collection, all blood samples were transported to the research laboratory and were centrifuged at 1,500 g for 10 min. The plasma was then carefully removed from the EDTA tubes and transferred into 1.5 ml microtubes. The clotted blood samples and the plasma samples were stored at -80°C for further processing or gene analysis as needed.

DNA was extracted from the plasma by using ANAPURE VIRAL DNA/RNA Mini Kits (Anabio, Vietnam), with a modification regarding the volume of plasma utilized for isolating DNA, to increase the concentration of final eluted DNA by approximately 3 times (Fig. 1). This modification partly contributes to the enhancement of the sensitivity of the real-time PCR assay that measures the loads of *cf*-EBV DNA in peripheral blood.

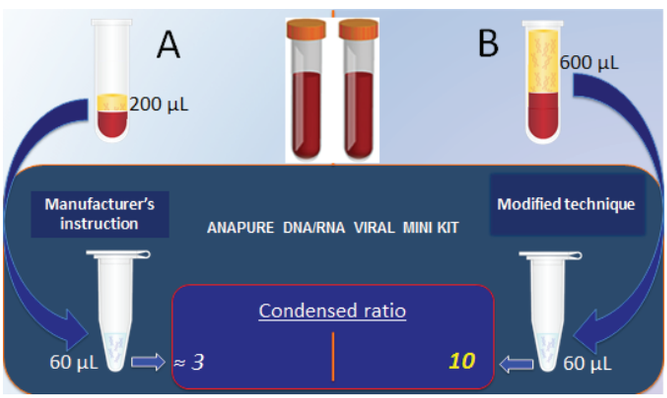

Fig. 1. Modified procedure for extracting DNA from plasma. The manufacturer's instruction **(A)** uses 200 µl of plasma and elutes 60 µl DNA, so the sample is concentrated about 3 times. Meanwhile, the eluted DNA was concentrated 10 times with the modified procedure **(B)** using 600 µl of plasma.

Quantitative PCR

Design of primer/Taqman probe for quantitative PCR assay:

The Primer and Taqman probe used for the qPCR assay in this study were automatically designed and validated by Oligo Primer Analysis Software (Molecular Biology Insights, USA). EBV genomic sequences were downloaded from the database of GenBank (www.ncbi.nlm.nih.gov, accession: V01555).

One of the most important factors determining a high detection sensitivity of the plasma/serum qPCR test for circulating EBV DNA is sensitive target selection. In our study, we have designed three sets of primers/probes targeting three different regions of the EBV genome with characteristics: *BamH1*-W, EBNA-1, and LMP-1. *BamH1*-W is the region containing a large number of short repeated sequences (W repeats) and the EBNA-1 region also has glycine alanine sequence repeats [19]. LMP-1 was reported as the major oncogenic factor of NPC development and these were detected in 80-90% of NPC tumors [13], so this region was selected as one of the target genes for designing primers/probes in the qPCR assay. Moreover, during the study, a designation of primer/probe sets targeting the BZLF1 gene of the EBV genome from University of North Carolina was referenced [20].

The procedure of qPCR assay:

The quantitative PCR assay was performed and products were detected using a Rotor-Gene Q instrument system which is essentially a combined thermal cycler/fluorescence detector with the ability to optically monitor the progress of individual PCR reactions. The DNA template volume used in each 20 µl-qPCR reaction was a 10 µl DNA sample which was extracted from plasma. The designed primer and conventional dual labelled probes conjugated with FAM fluorophore and BHQ1 non-fluorescent quencher were used as the components of real-time PCR reaction. All the data was collected and analysed using Rotor-Gene Q software.

In qPCR experiments specific error is seen due to the quality of DNA after extraction from plasma. In order to minimize this error, a housekeeping gene, in this case β-actin, was simultaneously amplified in the same run with the sample, which served as an internal control template for all DNA samples with primer and probe set which were described in previous studies [21].

Statistical analysis

For plasma samples, *cf*-EBV DNA was expressed in copies per ml of plasma. Plasma results were considered negative for EBV when the spiked internal positive control sequence was amplifiable while EBV DNA was not detected. For purposes of data analysis, samples with no measurable EBV DNA were reported as having a viral load of zero.

The concentration of *cf*-EBV DNA in a plasma sample was calculated by the equation:

$$C = Q \times (V_{DNA}/V_{PCR}) \times (1/V_{ext})$$

where:

C = target concentration in plasma (copies per milliliter);

Q = target quantity (copies) determined by sequence detector in PCR;

V_{DNA} = total volume of DNA obtained after extraction, typically 50 µl per Anabio extraction;

V_{PCR} = volume of DNA solution used for PCR, typically 8,6 µl;

V_{ext} = volume of plasma extracted, typically 600 µl.

All the collected data was used to calculate the sensitivity and the specificity of the qPCR assay on the clinical samples.

Results and discussions

Optimization of quantitive PCR assay

Design of primer/probe used for qPCR assay:

After designing three primer/Taqman probe sets, we simultaneously validated these three sets (I), (II), (III) in one run using the same DNA template which was extracted from the standard EBV DNA positive plasma sample from the Chinese University of Hong Kong. In the initial experiment, the primer/Taqman probe set (III) targeting the *BamHI*-W region showed the earliest amplification signal (the red line) and highest specificity (no amplification signal in the "no template" control) (Fig. 2). Therefore, the primer/Taqman probe set (III) was chosen for further optimization of the qPCR assay.

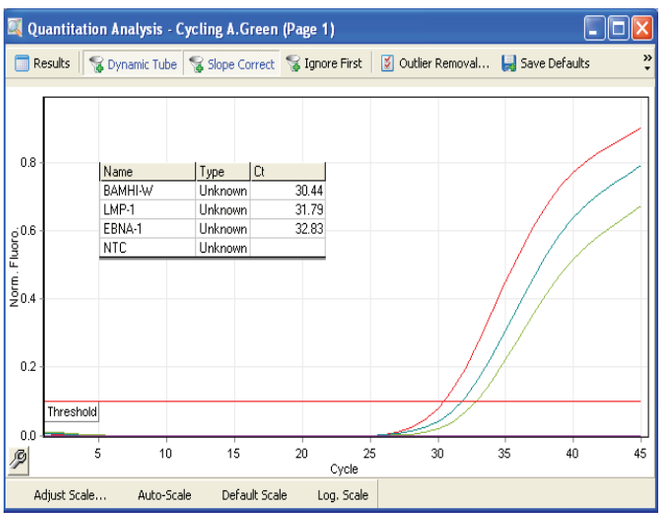

Fig. 2. The evaluation of three sets of primer/Taqman probes. EBNA-1, LMP-1 and BamHI-W indicated for three sets of primer/Taqman probes (I), (II), and (III), respectively.

Optimization of the primer concentration:

To optimize the qPCR assay, we optimized several components in the real-time quantitative PCR reaction, starting with primer concentration. The range of primer concentrations to test in the primer optimization as well as the concentrations of the probe to use needed to be determined separately. In our study, we utilized SYBR® Green dye-based assays to optimize the primer concentration without using a Taqman probe.

Five different concentrations of primer pairs in set (III) which was chosen in previous experiments were investigated, from 0.1 to 0.5 µM, leaving all the other reaction conditions unchanged. A primer concentration was considered to be optimal when the amplification resulted in an amplicon of the expected size, and when it showed a better performance than other concentrations.

As it was shown in Fig. 3, the real-time PCR analytical result revealed no amplification signal in reactions with primer concentration of 0.1 µM and "no-template" control, which means no contamination occurs in this experiment. The amplification signals of the remaining reactions with the primer concentration ranging from 0.2 to 0.5 µM were equivalent with the oscillation interval of Ct value from 30.89 to 31.90. The result from melting curve analysis (only for SYBR® Green dye-based assays) indicated the specificity of the amplification products.

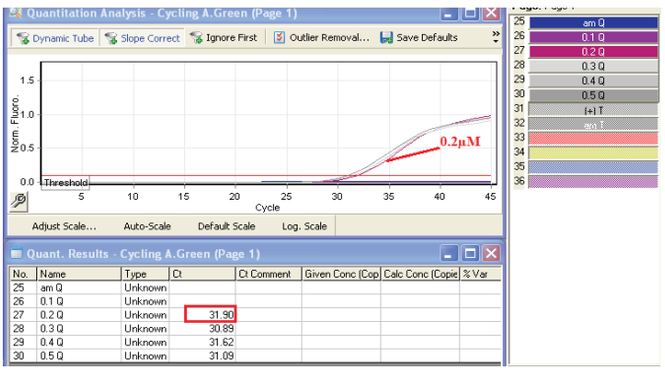

Fig. 3. Optimization of the primer concentrations for qPCR assay. 0.1Q, 0.2Q, 0.3Q, 0.4Q, 0.5Q indicated for the qPCR reactions with the same volume of positive DNA template and different primer concentrations, 0.1 µM, 0.2 µM, 0.3 µM, 0.4 µM and 0.5 µM, respectively. The primer concentration of the negative control or "no-template" control qPCR reaction (am Q) was 0.2 µM. As a result, the optimal primer concentration for the qPCR assay was determined to be 0.2 µM.

Optimization of the Taqman probe concentration:

Using the optimal primer concentration of 0.2 µM, we performed an experiment to determine the optimal concentration for the Taqman probe used in the qPCR assay among 3 different concentrations: 0.05, 0.1 and 0.2 µM.

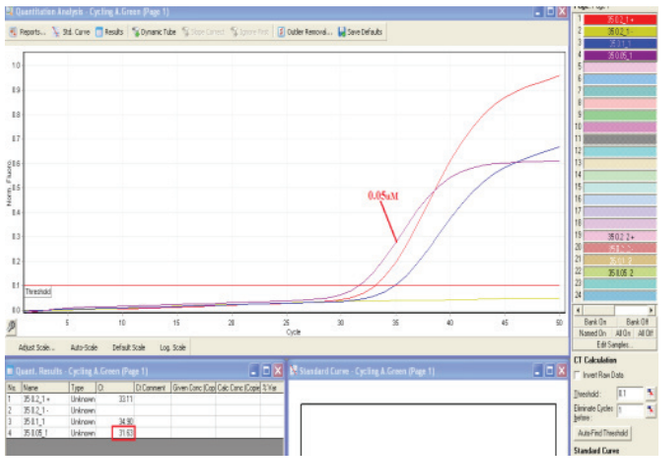

Fig. 4. Optimization of the Taqman probe concentration. (35 0.2_1+), (35 0.1_1), and (35 0.05_1) indicated for the qPCR reactions with the same volume of DNA template, optimal concentration of primer (0.2 µM), and of the Taqman probe at three different titres - 0.2, 0.1 and 0.05 µM, respectively. (35 0.2_1-) was the "no-template" control reaction with a primer concentration of 0.2 µM and probe concentration of 0.2 µM .

As it is shown in Fig. 4, the concentration that showed the earliest amplification signal was 0.05 µM. Consequently, 0.05 µM was determined as the optimal concentration of the Taqman probe for the qPCR assay.

Optimzation of the DMSO concentration:

Following optimization of the primer and probe concentration in several previous experiments, we realized that non-specific products appeared in some real-time quantitative PCR reactions. Therefore, we tried to add to the qPCR reaction a small amount of DMSO as an additive to decrease the non-specific products generated in the qPCR reactions.

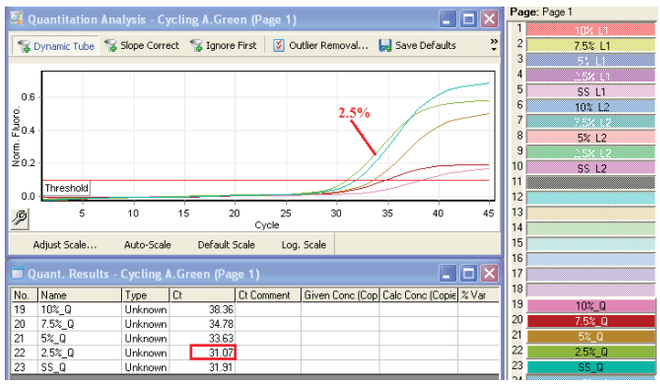

Fig. 5. Optimization of the DMSO concentration. (10%_Q), (7.5%_Q), (5%_Q), (2.5%_Q) indicated for the qPCR reactions with the same conditions of primer, probe, DNA template but different DMSO concentrations - 10%, 7.5%, 5% and 2.5%, respectively. (SS_Q) was the positive control reaction without DMSO addition (0% DMSO).

The concentrations of DMSO that were used in this experiment were: 2.5%, 5%, 7.5%, 10% and 0% (no addition of DMSO). The reaction with 0% DMSO was used as positive control to compare with other concentrations. As it is shown in the Fig. 5, the (2.5%_Q) sample gave the earliest amplification signal, even earlier than the positive control (SS_Q). The (2.5%_Q) sample was proven to amplify specific PCR products by agarose electrophoresis. Consequently, the DMSO concentration of 2.5% is optimal for the qPCR assay.

Evaluation of qPCR assay on international standard panel

Upon achieving an optimal qPCR assay, we evaluated this assay using an international standard panel from the Chinese University of Hong Kong. Nine DNA standard samples with concentrations of 150,000 - 60,000 - 6,000 - 3,000 - 1,200 - 600 - 300 - 150 - 50 - 25 copies/ml (equivalent to 15,000 - 6,000 - 600 - 300 - 120 - 60 - 30 - 15 - 5 - 2.5 copies/5 µl) were used as a template of an optimal qPCR assay to investigate the sensitivity of the optimal qPCR assay. Reactions with high and medium concentrations of the template were run in duplicate and those reactions with low titers were run in triplicate (Fig. 6).

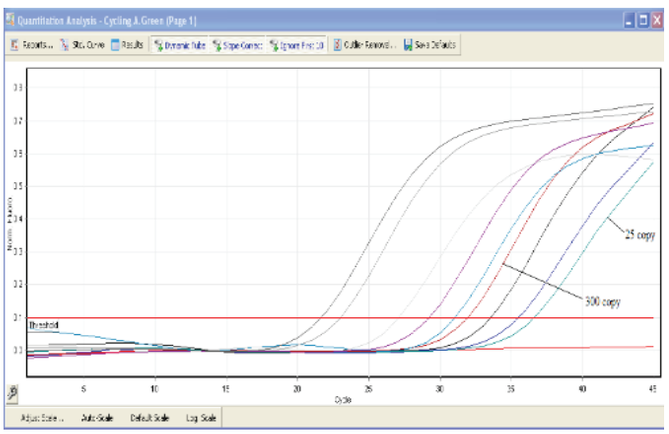

Fig. 6. The analytical sensitivity of the qPCR assay on standard panels. Amplification signals have been detected in all reactions (9/9) with standard DNA samples at different titers as template signals. The lowest concentration of DNA template corresponds to 25 copies/ml, which is ten times lower than the current detection limit in Vietnam. The negative control without a DNA template (red line below the threshold) did not show an amplification signal, indicating high specificity of the qPCR assay.

No amplification signal was detected in the "no-template" control, which indicated high specificity of the optimal qPCR assay. All the concentrations of DNA templates in the standard panels were detected in six out of the six replicates except the lowest standard concentration (25 copies/ml) that was detected in 17/18 replicates (95%). Therefore, 25 copies/ml was determined as the LoD of the optimal qPCR assay. This LoD is more than ten times lower than that of qPCR assays published so far in Vietnam with an LoD of 300 copies/ml [17].

Simultaneously, the performance of the optimal qPCR assay for quantification of DNA EBV was also assessed on the international standard panels with a high correlation coefficient of $R^2 = 0.99613$ (>0.99) (Fig. 7). Thus, the optimal qPCR assay has a linear range from 50 to 150,000 copies/ml with an LoD of 25 copies/ml and highly reliable quantification of the *cf*-EBV DNA concentration.

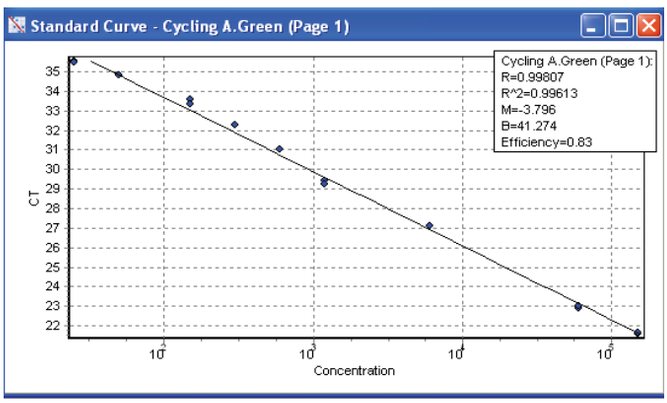

Fig. 7. Standard curve of qPCR assay based on international standard panel. The log of each known concentration in the dilution series of the standard panel (x-axis) is plotted against the corresponding Ct value for that concentration (y-axis).

Evaluation of qPCR assay on clinical samples

Using the optimal qPCR assay and the standard curve established using an international standard panel, we evaluated the qPCR assay on clinical samples to determine its sensitivity and specificity. The plasma samples from NPC patients (n=32) and healthy controls (n=105) were collected and quantified using the optimal qPCR assay to measure the *cf*-EBV DNA load in each sample (Fig. 8).

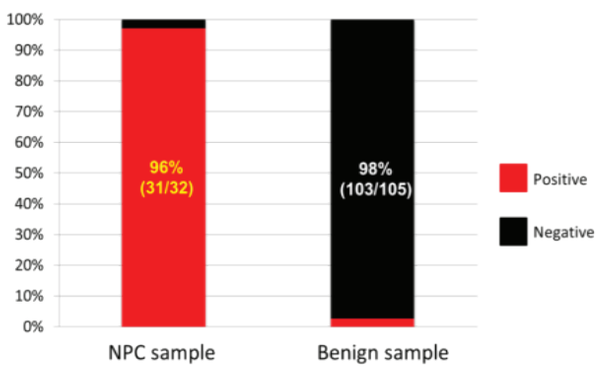

Fig. 8. The sensitivity and the specificity of the qPCR assay based on clinical samples.

Figure 8 illustrates the sensitivity and the specificity of the optimal qPCR assay based on two clinical sample groups: blood samples of NPC patients and those of healthy controls. In the first group, the optimal qPCR assay detected *cf*-EBV DNA in 31 of 32 NPC patients, which corresponds to a sensitivity of 96.9%. The concentration of *cf*-EBV DNA in these samples varied from 53 to 3.8 x 10^5 copies/ml. In the group of healthy controls, *cf*-EBV DNA was detected in only 2 cases and was not detected in 103 out of 105 healthy controls which corresponds to a specificity of 98% (103/105). The *cf*-EBV DNA loads detected in two blood samples derived from healthy controls were lower than 40 copies/ml. However, no *cf*-EBV DNA was detected by the optimal qPCR assay with the blood drawn from these two healthy controls after two weeks. All the extracted DNA samples have been shown to be amplifiable with the internal control qPCR assay (β-actin) (Fig. 9).

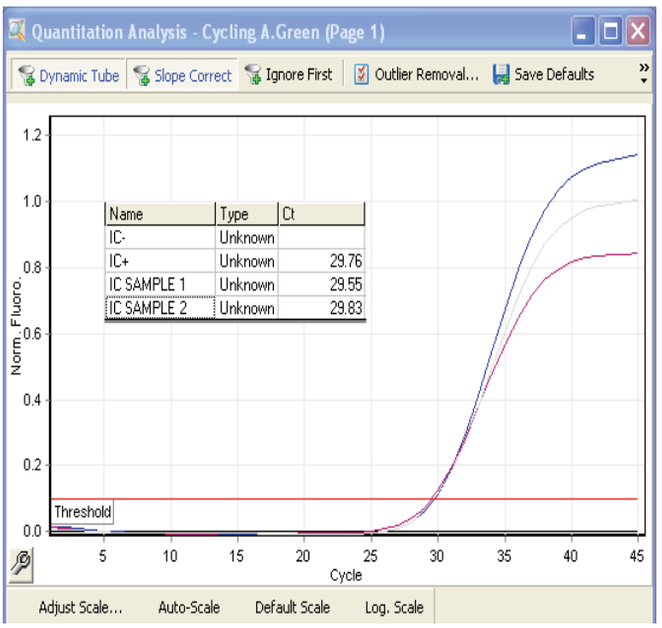

Fig. 9. Illustration of internal control qPCR assay. IC+ indicated for positive control of internal control, IC- indicated for no template control and IC SAMPLE 1 and IC SAMPLE 2 indicated for the evaluation of extracted DNA samples using internal control qPCR assay targeting β-actin gene.

Compare to recent studies in Vietnam

Based on the data we have so far, optimal qPCR assay targeting cf-EBV DNA for early detection of NPC has achieved high sensitivity of 96.9% and specificity of 98%. Currently available qPCR assays in studies published so far in Vietnam (2015) have a sensitivity of 68% at most, and unknown specificity, which is not sufficient for early detection of NPC. Moreover, the LoD of our optimal qPCR assay is 25 copies/ml plasma, which is ten times lower than other qPCR published in recent studies in Vietnam.

Conclusions

This study has shown the successful establishment of an ultrasensitive qPCR assay for detection of *cf*-EBV DNA with the detection limit of 25 copies/ml, which is more than ten times better than currently available assays in our country (300 copies/ml).

Evaluation on clinical samples has proven a remarkably high sensitivity of 96.9% and high specificity of 98%.

Furthermore, the optimal qPCR assay was evaluated using the international standard panel from the Chinese University of Hong Kong, thus resulting in high reliability.

Our novel qPCR assay enables detection of NPC at an early stage, thus contributing to an improved survival rate of patients. It will also form a basis for further studies in Vietnam and open many new applications in the management of NPC.

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REFERENCES

[1] American Cancer Society (2015), *Nasopharyngeal Cancer.*

[2] P. Busson, C. Keryer, T. Ooka, M. Corbex (2004), "EBV-associated nasopharyngeal carcinomas: from epidemiology to virus-targeting strategies", *Trends Microbiol.*, **12(8)**, pp.356-360.

[3] L.M. Sri Ganesh Muthiah (2016), "Epstein-barr virus (EBV) and its association with nasopharyngeal carcinoma (NPC): a systematic review", *International Journal of Public Health and Clinical Sciences*, **3(4)**, p.149.

[4] L.A. Torre , F. Bray, R.L. Siegel, J. Ferlay, J. Lortet-Tieulent, A. Jemal (2015), "Global cancer statistics, 2012", *CA Cancer J. Clin.*, **65(2)**, pp.87-108.

[5] M.D. Cheng Her (2001), "Nasopharyngeal Cancer and the Southeast Asia Patient", *American Family Physician*, **63(9)**, pp.1776-1783.

[6] Everett E. Vokes, David N. Liebowitz, Ralph R. Weichselbaum (1997), "Nasopharyngeal carcinoma", *Lancet*, **350**, pp.1087-1091.

[7] K.F.T. Kwok Wai Lo, Dolly P. Huang (2004), "Focus on nasopharyngeal carcinoma", *Cancer Cell*, **5(5)**, pp.423-428.

[8] S.J. Chai, K.C. Pua, A. Saleh, Y.Y. Yap, P.V. Lim, S.K. Subramaniam, C.L. Lum, G. Krishnan, W.R. Mahiyuddin, N.P.C.S.G. Malaysian, S.H. Teo, A.S. Khoo, L.F. Yap (2012), "Clinical significance of plasma Epstein-Barr Virus DNA loads in a large cohort of Malaysian patients with nasopharyngeal carcinoma", *J. Clin. Virol.*, **55(1)**, pp.34-39.

[9] J. Zhang, C. Shu, Y. Song, Q. Li, J. Huang, X. Ma (2016), "Epstein-Barr virus DNA level as a novel prognostic factor in nasopharyngeal carcinoma: A meta-analysis", *Medicine (Baltimore)*, **95(40)**, p.e5130.

[10] M.S. Chang and W.H. Kim (2005), "Epstein-Barr virus in human malignancy: a special reference to Epstein-Barr virus associated gastric

carcinoma", *Cancer Res. Treat.*, **37(5)**, pp.257-67.

[11] I. Anagnostopoulos, M. Hummel, Cornelia Kreschel, Harald Stein (1995), "Morphology, immunophenotype, and distribution of latently and/or productively Epstein-Barr virus-infected cells in acute infectious mononucleosis: implications for the interindividual infection route of Epstein-Barr virus", *Blood*, **85(3)**, p.744-750.

[12] O.A. Odumade, K.A. Hogquist, H.H. Balfour Jr. (2011), "Progress and problems in understanding and managing primary Epstein-Barr virus infections", *Clin. Microbiol. Rev.*, **24(1)**, pp.193-209.

[13] A.S. Ali, M. Al-Shraim, A.M. Al-Hakami, I.M. Jones (2015), "Epstein- Barr virus: clinical and epidemiological revisits and genetic basis of oncogenesis", *Open Virol. J.*, **9**, pp.7-28.

[14] S.D. Hudnall (2014), "Epstein-Barr virus: pathogenesis and host immune response", *Viruses and Human Cancer*, pp.7-24.

[15] A. Mutirangura, W. Pornthanakasem, A. Theamboonlers, V. Sriuranpong, P. Lertsanguansinchi, S. Yenrudi, N. Voravud, P. Supiyaphun, Y. Poovorawan (1998), "Epstein-Barr viral DNA in serum of patients with nasopharyngeal carcinoma", *Clin. Cancer Res.*, **4(3)**, pp.665-669.

[16] Y.M. Lo, L.Y. Chan, K.W. Lo, S.F. Leung, J. Zhang, A.T. Chan, J.C. Lee, N.M. Hjelm, P.J. Johnson, D.P. Huang (1999), "Quantitative analysis of cell-free Epstein-Barr virus DNA in plasma of patients with nasopharyngeal carcinoma", *Cancer Res.*, **59(6)**, pp.1188-1191.

[17] Huy Tan Pham, Huy Thinh Tran, Thi Thuy Hang Tran, Dinh Phuc Nguyen, Van Khanh Tran (2015), "Quantitative analysis of *cell - free* EBV-DNA in plasma of patients with nasopharynx cancer and correlation to Tumor Nodes Metastasis diagnosis", *Journal of Medical Research*, **95(3)**, pp.24-31.

[18] Q.T. Le, Q. Zhang, H. Cao, A.J. Cheng, B.A. Pinsky, R.L. Hong, J.T. Chang, C.W. Wang, K.C. Tsao, Y.D. Lo, N. Lee, K.K. Ang, A.T. Chan, K.C. Chan (2013), "An international collaboration to harmonize the quantitative plasma Epstein-Barr virus DNA assay for future biomarker-guided trials in nasopharyngeal carcinoma", *Clin. Cancer Res.*, **19(8)**, pp.2208-2215.

[19] T.T. Yip, R.K. Ngan, A.H. Fong, S.C. Law (2014), "Application of circulating plasma/serum EBV DNA in the clinical management of nasopharyngeal carcinoma", *Oral Oncol.*, **50(6)**, pp.527-538.

[20] J.L. Ryan, H. Fan, S.L. Glaser, S.A. Schichman, N. Raab-Traub, M.L. Gulley (2004), "Epstein-Barr virus quantitation by real-time PCR targeting multiple gene segments: a novel approach to screen for the virus in paraffin-embedded tissue and plasma", *J. Mol. Diagn.*, **6(4)**, pp.378-385.

[21] Y.M. Lo, M.S. Tein, T.K. Lau, C.J. Haines, T.N. Leung, P.M. Poon, J.S. Wainscoat, P.J. Johnson, A.M. Chang, N.M. Hjelm (1998), "Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis", *Am. J. Hum. Genet.*, **62(4)**, pp.768-775.