

The Accuracy of Plasma EBV-DNA Quantification Using LMP2 as Primer to Detect Distance Metastasis After Radiation of Nasopharyngeal Cancer in “Dharmais” National Cancer Center

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ABSTRACT

Nasopharyngeal carcinoma (NPC) is common in Indonesia and is associated with advanced disease. The presence of Epstein-Bar virus deoxyribonucleic acid (EBV-DNA) has been proposed for early detection of distant failure after radiation therapy. The latent membrane protein-2 (LMP-2) is a potentially cost effective primer to be applied in Indonesia, which had not been studied.

This study was aimed to test the accuracy of plasma EBV-DNA quantification using LMP2 as primer to detect metastasis after radiation therapy in NPC patients.

Plasma sample were obtained from Nasopharyngeal cancer patients at least six month after the last radiation therapy. DNA was extracted and analyzed by a real-time polymerase chain reaction (RT-PCR) (Light Cycler, Roche Diagnostics) using primers specific to latent membrane protein (LMP2). The results were compared to the standard quantitative RT-PCR using primers specific to Epstein-Bar Virus nuclear antigen 1 (EBNA-1) and DNA segment in the BamHI-W fragment region of the EBV Genome. Mean differences of EBV-DNA level were compared using the analysis of variance (ANOVA) test. The sensitivity and specificity were calculated using the receiver-operating characteristic (ROC) curve.

Twenty-three non-metastatic NPC cases were enrolled during the study period, consisting 17 (73.9%) men and 6 (26.1%) women. Patients' median age was 48 years (range 21-67%). Majority of cases were stage III (56.5%) followed by stage IVA-B (26.1%). All patients received radiation for 6000-7000 cGy. Ten (43.5%) patients had distant metastasis at least six months after radiation therapy. No signs of local recurrence at the primary site. There was no difference of mean EBV-DNA level using EBNA-1 as the primer in patients with metastasis and without metastasis (5135 copies/mL vs 7827 copies/mL; $p = 0.245$). There was a significant difference of mean EBV-DNA level between patients with metastasis and without metastasis using LMP2 as primer (1565 copies/mL vs. 0 copies/mL; $p=0.044$) and using BamHI-W as the primer (455.078 copies/mL vs 0 copies/mL; $p=0.007$). BamHI-W primer gave 100% sensitivity and 100% specificity at EBV-DNA level of 1080 copies/mL with an area under curve (AUC) of 1.0. The LMP2 primer gave 89% sensitivity and 100% specificity at 17 copies/mL with an AUC of 0.9

Detection of EBV-DNA using LMP2 as a primer is useful to detect early metastatic event after radiation in NPC patients with no metastasis at diagnosis. This primer had sensitivity and specificity almost as high as the BamHI-W primer.

Key words: Metastasis, Radiation, Nasopharyngeal

ABSTRAK

Kanker nasofaring (NPC) biasa ditemukan di Indonesia dan berkaitan dengan penyakit tingkat lanjut (*advanced disease*). Keberadaan virus Epstein-Bar deoxyribonucleic acid (EBV-DNA) sudah lama ditawarkan untuk deteksi awal terhadap kegagalan jarak (*distant failure*) setelah pemberian radiasi (*radiation therapy*). Selaput laten protein-2 (LMP-2) sangat potensial untuk diterapkan di Indonesia, karena efektif dalam menekan biaya. Namun, hal ini belum pernah di pelajari sebelumnya.

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Penelitian ini bertujuan untuk mengetes penghitungan akurasi plasma EBV-DNA menggunakan LMP2 sebagai cara awal untuk mendeteksi metastasis setelah pemberian radiasi pada pasien NPC.

Contoh plasma diambil dari pasien penderita kanker nasofaring paling tidak enam bulan setelah pemberian radiasi. DNA diekstrak dan dianalisis dari reaksi rantai polimerase berdasarkan hitungan waktu (*real-time polymerase chain reaction* –RT-PCR) (*Light Cycler, Roche Diagnostics*) menggunakan spesifikasi dasar LMP-2. Hasilnya kemudian dibandingkan dengan standar kuantitatif RT-PCR antara EBNA-1 dan segmen DNA dalam area BamHI-W dari genome EBV.

Perbedaan rata-rata (*mean*) dari level EBV-DNA kemudian dibandingkan menggunakan tes analisis varian (ANOVA). Perhitungan sensitivitas dan spesifikasi dihitung berdasarkan kurva ROC (*receiver-operating characteristics*).

Kajian ini menggunakan dua puluh tiga kasus NPC yang non-metastatik, di mana jumlah pasien laki-laki 17 orang (73,9%) dan wanita 6 orang (26,1%). Median dari umur pasien adalah 48 tahun (21-67 tahun). Kebanyakan kasus sudah mencapai stadium III (56,5%), diikuti stadium IVA-B (26,1%). Semua pasien menerima radiasi sebanyak 6000-7000 cGy. Sepuluh pasien (43,5%) telah mendapatkan metastasis (*distant metastasis*) paling tidak enam bulan setelah terapi radiasi. Tidak ada tanda-tanda kambuh pada lokasi awal (*primary site*). Tidak ada perbedaan *mean* pada tingkat EBV-DNA, yaitu antara penggunaan EBNA-1 secara dasar antara pasien dengan metastasis dan pasien tanpa metastasis (5135 copies/mL vs. 7827 copies/mL; $p=0,245$). Ada perbedaan mencolok pada *mean* EBV-DNA antara pasien dengan metastasis dan pasien tanpa metastasis saat menggunakan LMP2 sebagai dasar dan menggunakan BamHI-W sebagai dasar. BamHI-W primer memberikan tingkat sensitivitas 100% dan spesifisitas 100% pada tingkat EBV-DNA 1080 copies/mL dengan AUC 1,0. Perhitungan dengan LMP2 primer memberikan tingkat sensitivitas 89% dan spesifisitas 100% pada pemberian 17 copies/mL dengan AUC 0,944.

Kesimpulannya, deteksi EBV-DNA menggunakan LMP2 primer berguna bagi deteksi awal metastatik setelah pemberian radiasi bagi pasien NPC yang tidak memiliki metastasis pada saat proses diagnosis. Primer ini memiliki tingkat sensitivitas dan spesifisitas hampir seakurat BamHI-W primer.

Kata kunci: Metastasis, Radiasi, Nasofaring.

BACKGROUND

Nasopharyngeal carcinoma is common in Indonesia. Pathology-based cancer registry in 2000 showed that NPC was the fourth most common cancer after cervical, breast and skin cancers with a prevalence of 5.33%.¹ There were 257 NPC cases between 2000 and 2005 in "Dharmais" National Cancer Center which was associated with advanced disease. Distant failure was very common in stage-IV disease (56%) and occurred in less than 2 years after radiation therapy.²

Although NPC is radiosensitive tumor, patients survival is poor.^{3,4} With radiotherapy alone, the 5-year survival rate for stage-IV disease is only 28-35%.⁵ Failure of treatment due to locoregional recurrence occurred in 40%-80% of patients, where as distant recurrence could happen in 15 % to 50 % of patients.⁶

The association of Epstein- Barr virus (EBV) and NPC have been reported since the detection of EBV-DNA in NPC biopsies in 1970.⁷ EBV proteins were found in all NPC cells, either from primary tumors or from various metastatic sites. Detectin of circulating EBV-DNA can be done by using the polymerase chain reaction (PCR) rechique which could detect the EBNA-I, LMPI, LMP2, and the *Bam*HI-W fragment of EBV genome.⁸ Plasma EBV-DNA was found in NPC patients with median plasma level of 1461 copies/ mL, 681 copies/mL patient with distant metastasis.⁹

The presence of EBV-DNA, Epstein-Bar in the circulation has been proposed for use as early detection of distant failure after radiation therapy. It is a sensitive tumor marker which is helpful to identify residual

disease^{10,11} and have a high sensitivity when measured by a quantitative PCR system.¹² Most patients in remission have undetectable plasma EBV-DNA.¹³ The latent membrane protein-2 (LMP2) is a potentially cost effective primer to be applied in Indonesia, Which had not been studied. This study was aimed to test the accuracy of plasma EBV-DNA quantification using LMP2 as primer to detect metastasis after radiation therapy NPC patients.

METHOD

Study design and subjects

This was a comparative study of plasma EBV-DNA quantification using three primers in selected cases of NPC patients. Subject were new NPC patients in 2004 who show no distant metastasis at the time of diagnosis. All patients received standard treatment with radiation of 6000-7000 cGY. Plasma samples were obtained from nasopharyngeal cancer patients at least six month after the last radiation therapy.

Plasma EBV-DNA Quantification

DNA was extracted and analyzed by a real time polymerase chain reaction (RT-PCR) (Light Cyclear, Roche Diagnostics) using primers specific to latent membrane protein (LMP2) in the laboratory of clinical Pathology Dharmais National Cancer Center. The results were compared to the standard quantitative RT-PCR using primer specific to a DNA segment in the BamHI-W fragment region of the EBV genome in laboratory of

Table 1: Characteristics of the study subjects (n=23)

Characteristics	N	%
Sex		
• Men	17	73.9
• Women	6	26.1
Age group		
• 21- 30 years	2	8.7
• 31-40 years	13	13.0
• 41-50 years	11	47.8
• 51-60 years	6	26.1
• 61-70 years	1	4.3
Tumor size		
• T 1	3	13.0
• T 2	11	47.8
• T 3	7	30.4
• T 4	2	8.7
Nodal status		
• N0	3	13.0
• N1	6	26.1
• N2	12	52.2
• N3	2	8.7
Stage		
• Stage I	1	4.3
• Stage II	4	17.4
• Stage III	15	65.2
• Stage IV	3	13.0
Histopathological type		
• Undifferentiated carcinoma	21	91.3
• Squamous cell carcinoma	2	8.7

Table 2: Mean difference of EBV-DNA level measured with three primers

Primer	Mean level (copies/mL)		P value (ANOVA)
	Metastasis (+)	Metastasis (-)	
EBNA-1	5134 ± 3246.8	7828 ± 6494.0	0.245
BamHI-W	455,078 ± 552,160.4	1.9 ± 6.93	0.007
LMP2	1564 ± 2649.4	0	0.044

prince of Wales Hospital, Hong Kong. Later, the same specimens were also tested by using primer specific to the Epstein-Barr virus nuclear antigen 1 (EBNA-1) in the Laboratory of *Vrije Universiteit medisch centrum, Amsterdam*. The Netherlands.

DNA Extraction

Peripheral blood (5ml) was taken from each patients by venipuncture and placed into an EDTA tube. Blood were centrifuged at 1600 g and plasma was removed and transferred into polypropylene tubes. Samples were

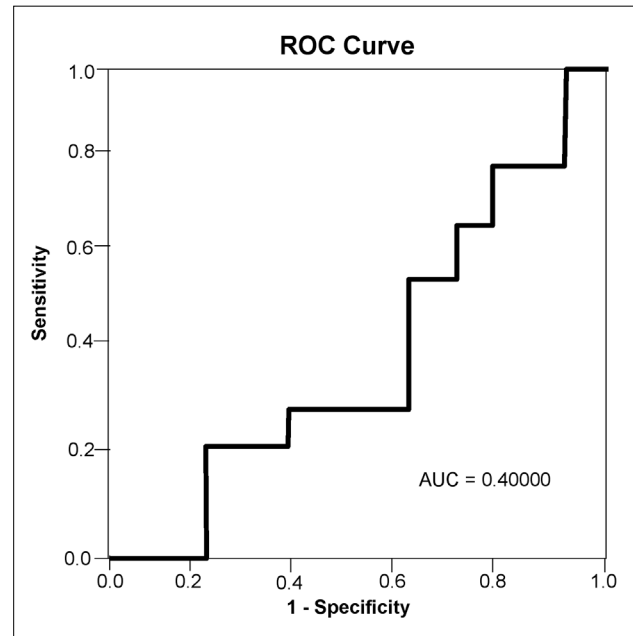


Figure 1: ROC curve of EBV-DNA level using EBNA-1 as primer

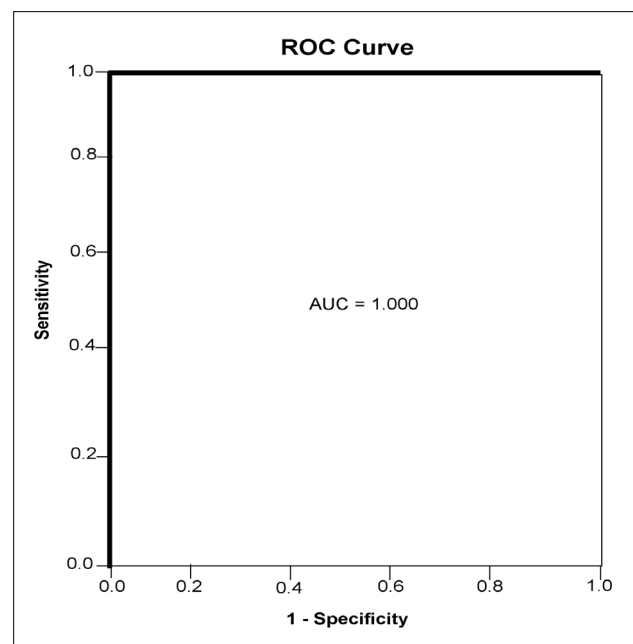


Figure 2: ROC curve of EBV-DNA level using BamHI-W fragment as primer

stored frozen at 20°C until further analysis. DNA was extracted from plasma samples using a QIAamp Blood Kit (Qiagen, Hilden, Germany) Using the protocols as recommended by the manufacturer. Plasma samples (130-800 uL/column) were for DNA extraction. The exact amount was documented for the calculation of the target DNA concentration. A final elution volume of 50 uL was used.

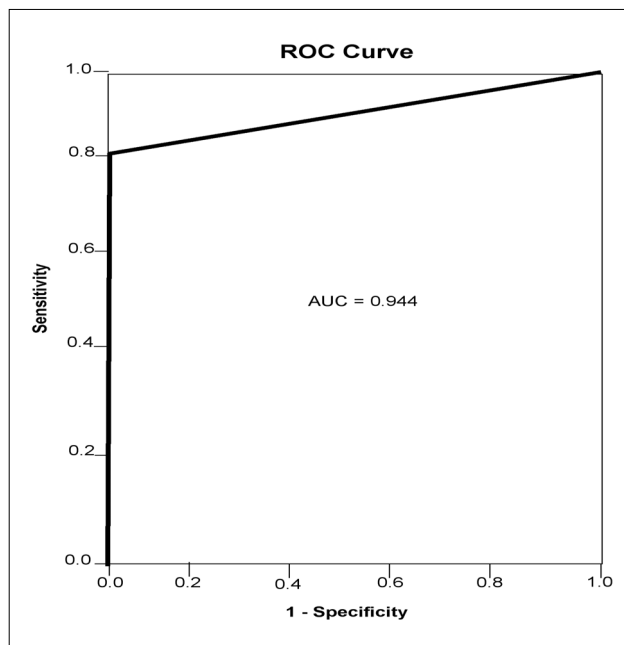


Figure 3: ROC curve of EBV-DNA level using LMP-2 as primer

Real-Time Quantitative PCR

Real-time quantitative PCR system were developed for EBV-DNA detection toward the LMP-2 segment and *Bam*HI-W regions. The third system was done later toward the 213-bp region of EBNA-1 LMP2 DNA was applied in duplicate by real-time PCR on the Light Cycler version 1.5. Amplification curve consists of log concentration (x axis) versus crossing point (y axis) across five standards. The Viral load for each plasma sample was extrapolated and automatically calculated by the Light Cycler software. The duplicate values were averaged and then manually multiplied by dilution factor of 100 to obtain and EBV viral load in copies of EBV DNA per ml plasma. Undetectable EBV DNA was reported as zero copies.

Statically Analysis

Characteristic of the patients were presented descriptively. Mean differences of EBV-DNA level were compared using analysis of variance (ANOVA) test. The sensitivity and specificity were calculated using the receiver-operating characteristics (ROC) curve. Analysis was done by using computer software SPSS version 11.5 for windows PC.

RESULTS

Twenty-three non metastasis NPC were enrolled during the study period, consisting 17 (79.9%) men and 6 (26.1%) women. Patients median age was 48 years, ranging from 21 to 67 years. The peak incidence was found among patients GED 41-50 years. Majority of cases

were stage III (56.5) followed by stage IV-B (26.1 %).

Ten (43.5 %) patients had distant metastasis at least six months after radiation therapy. No signs of local recurrence at the primary site. There was no difference of mean EBV-DNA level using EBNA-1 as the primer in patients with metastasis and without metastasis (5135 copies/mL vs 7827 copies/mL; $p = 0.245$). There was a significant difference of mean EBV-DNA level between patients with metastasis and without metastasis using LMP2 as primer (455.078 copie/mL vs 0 copies/mL; $p = 0.007$). *Bam*HI-W primer gave 100% sensitivity and 100% specificity at EBV-DNA level of 1080 copies/ mL with an area under curve (AUC) of 1.0. The LMP-2 primer gave 89% sensitivity and 100% specificity at 17 copies/mL with an AUC of 0.944.

DISCUSSION

The study results showed that there was a high plasma level of EBV-DNA in patients with distant metastasis after radiation therapy by using *Bam*HI-W and LMP2 as primers. However, detection using the EBNA-1 primer showed no difference of EBDNA concentration. EBNA-1 protein is an important protein for maintenance of the EBV genome inside the host. To maintain viral chromosome in the proliferating cells, the chromosome should be duplicate during each cell cycle to be passed to the nuclear of progeny cells during mitosis. There is a region of 1800 bp in the EBV genome called oriP which needs only EBNA-1 for its activity.¹⁴ Experiment on B cells showed that EBNA-1 is required for stable and efficient latent EBV infection.¹⁵ EBNA-1 is also needed for EBV replication and function as a transcriptional regulator essential for the expression of EBV latent genes.¹⁶ Therefore, the expression of EBNA -1 latent gene is sign of EBV infection.

Our results support the use of LMP2 as primer for detection of distant metastasis after radiation therapy. This implies that patients with positive plasma EBV-DNA after therapy who clinically show no distant metastasis should be further explored. Since patients with no metastasis showed no clinically detectable EBV- DNA, therefore a low plasma EBV-DNA level with no evidence of recurrence should be repeated after 3 to 6 months.

A recent study in Malaysia supported the use of EBV-DNA to asses the prognosis of NPC. The mean of plasma EBV-DNA level was significantly higher in patients without therapy (11.533 copies/mL, median 2.043 copies/mL) compared to patients after radiotherapy (2.000 copies/mL, mean 0 copy/mL) compared to patients after radiotherapy (2.000 copies/mL, mean 0 copy/mL). The high concentration of patients after therapy was due to patient with EBV-DNA level of 325.000 copies/mL which then was proved to have local recurrence and

distant metastasis during follow-up.¹⁷

In several case, low level of EBV-DNA could be detected in the plasma although the patients show no distant metastasis after radiation. The positive results could not be regarded as false positive, since EBV DNA could detected several months before the recurrence if tested 3-6 months after radiation.¹⁸

There was one case with metastasis which showed negative results by using LMP2 as primer but positive by using BamHI-W as primer. This indicated that the selection of primers could affect the diagnosis. It is also possible to have positive result when the PCR cycle is increased. In this study, measurement was done with 45 cycles.

CONCLUSION

Detection of EBV-DNA using LMP2 as a primer is useful to detect early metastasis event after radiation in NPC patients with no metastasis at diagnosis. This primer had sensitivity and specificity almost as high as the BamHI-W primer, ♦

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