

Association study of LMP-1 expression and promoter methylation status of tumor suppressor gene RASSF1A in nasopharyngeal carcinoma

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ABSTRACT

Nasopharyngeal carcinoma (NPC) is a cancer originating from nasopharyngeal epithelial tissue. Genetic susceptibility, exposure to carcinogens, and Epstein-Barr virus (EBV) infection are the main factors in NPC development. Latent membrane protein 1 (LMP-1) is a product of EBV genome, which is able to interact with various intracellular signaling pathways that leads to expression of many proteins, e.g DNA methyltransferase. The increase expression of DNA methyltransferase could induce hypermethylation of tumor suppressor genes (TSG). Ras-association domain family 1A (RASSF1A) is one of TSG that frequently hypermethylated in NPC cases. The aim of this study is to determine the association between LMP-1 expression and promoter methylation status of RASSF1A in NPC patients. The research subjects were 36 NPC patients of the Dr. Sardjito Hospital, Yogyakarta, Indonesia. Latent membrane protein 1 was stained immunohistochemically using monoclonal antibody OT21C. Ras-association domain family 1A methylation status was examined by methylation specific PCR (MSP) of DNA isolated from nasopharyngeal brushing. Chi-square analysis was conducted to examine the association between LMP-1 expression and methylation status of RASSF1A with 95% confidence interval. Latent membrane protein 1 was expressed in 44.4% subjects. The scores of LMP-1 expression were ranged from 0-8 (average of 1.56 ± 2.16). Ras-association domain family 1A methylated in 66.7% of subjects. Statistical analysis showed that there was a relationship between LMP-1 expression and methylation status of RASSF1A ($p < 0.05$). Statistical analysis also showed association between LMP-1 expression score and RASSF1A methylation status ($p < 0.05$). It can be concluded that there was an association between the expression of LMP-1 and RASSF1A methylation status in NPC patients.

Keywords: LMP1 - RASSF1A – NPC – hypermethylation - DNA methyltransferase - methylation specific PCR

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is cancer originated from nasopharyngeal squamous epithelial cells. The incidence of NPC is scattered in distinct populations in several areas world wide, such as China, East Asia, Southeast Asia, Artic, Alaska, North Africa and North America.¹ This incidence pattern indicates the contributions of genetic and environmental factors in the development of this cancer.¹ However 95% NPC cases are related to Epstein-Barr virus (EBV) infection which indicates that EBV is a primary factor in NPC development.^{2,3}

Epstein-Barr virus is a DNA virus that infects more than 90% of the adult population, and the infection lasts for lifetime. Several diseases are known as EBV associated diseases e.g infectious mononucleosis, Hodgkin's disease, Burkitt's lymphoma, NPC, oral hairy leukoplakia, and undifferentiated gastric carcinoma.⁴ Epstein-Barr virus is potential to be considered as anorganic virus due to the expression of EBV latent genes such as, latent membrane proteins (LMP-1, LMP-2A and LMP-2B) and core antigens (EBNA 1 and EBNA2). LMP-1 is the main oncogene that play a role in

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NPC cells immortalization. Latent and expressed in 80-90% of NPC. Latent membrane protein 1 allegedly associated with inhibition of transcription and inactivation of TSG through hypermethylation.^{5,6} Hypermethylation of several TSGs in NPC cases have been reported, including hypermethylation of RASSF1A (66.7% -84%), death-associated protein kinase (DAP-kinase) (76%), p16 (46%), tumor suppressor in lung cancer 1 (TSLC1), retinoic acid receptor beta (RAR β) (80%), methylguanine DNA methyltransferase (MGMT) (20%) and glutathione S-transferases (GSTP) (13%).⁷⁻¹⁰

Ras-association domain family 1A inactivation has been reported in various types of tumors.¹¹ It has a major role in intracellular signal transduction pathways, such as apoptosis, cell cycle arrest and cell migration.^{3,11} When RASSF1A is hypermethylated, the RASSF1A expression is silenced. The lack of RASSF1A expression in a cell will develop unregulated proliferation. The loss of RASSF1A expression also causes the cell to migrate easily, leading to tissue invasion or metastasis.¹¹ Therefore, to increase further understanding about NPC carcinogenesis, this study aims to determine whether there is any association between LMP-1 expression with methylation status of RASSF1A in NPC patients.

MATERIALS AND METHODS

Subjects

The subjects of this study were NPC patients at Dr. Sardjito Hospital, Yogyakarta, Indonesia in 2003-2009. The subjects were diagnosed by otolaryngologist based on result of CT-scan and biopsy examination. Based on Estimated Difference Proportion calculation,¹² the minimum number of subjects for this study was 20 subjects. Stages of cancer were classified by TMN method based on classification of the American Joint Committee on Cancer (AJCC).¹³ Stage I and II were classified as early stage, while stage III and IV were classified as late stage.^{14,15} Paraffin blocks of nasopharyngeal biopsy were prepared for histopathological and immunohistochemical examination. Nasopharyngeal brushing was performed and DNA extracted with Boom's method.¹⁶ This study has been approved

by The Ethics Research Committee of Faculty of Medicine, Gadjah Mada University, Yogyakarta.

LMP-1 immunohistochemistry

Nasopharyngeal carcinoma paraffin blocks were sliced in 5 μ m thickness, then placed on object glass. The specimens were stained with Streptavidin-Biotin labeled method to determine LMP-1 expression. 140Antibody of OT21C kindly provided by Professor Jaap Middeldorp (Vrije Universiteit EBV Group Medisch Centrum/VUMC, Amsterdam). LMP-1 positive control was obtained from tissue that was known expressing LMP-1. Whereas the negative control was samples without OT21C monoclonal antibody. Positive LMP-1 expression was marked by the appearance of brown color in the cytoplasm. LMP-1 expression examination was carried out on five visual field by three examiners, and the scoring expression score was calculated quantitatively based on color intensity and percentage of immunopositive cells per visual field with a scale of 0-12 according to the previous studies.^{8,17} For analysis, LMP-1 expression scores were grouped into low expression, middle expression and high expression groups. Cut point was determined by Binning method using SPSS. Latent membrane protein 1 expression score of less than 1 was grouped in low expression group, and LMP1 expression score of 1-3 was grouped in middle expression. While the expression score more than 3 was grouped in the high expression group.

RASSF1A methylation specific PCR

The isolated DNA samples were modified using the Zymo EZ DNA methylation kit (Zymo Cat. No: D5001). Methylated specific PCR (MSP) primer sequences were GTGTTAACGCGTTGCGTATC (F) and AACCCCGCGAAC TAAAACGA (R) with product size of 95 base pairs (bp). Unmethylated specific PCR (USP) primary sequence used were TTTGGTTGGAGTGTGTTAATGTG (F) and CAAACCCACAAACTAAAACAA (R) with product size of 105 bp. Methylated specific PCR amplification was performed using PE-9600. PCR was performed for 45 cycles, with annealing temperature at 60 °C for one minute. PCR products were analyzed by gel electrophoresis, using 2% agarose gel. DNA of patients with known positive

NPC MSP and USP was used as positive control for MSP and USP. While the negative control used PCR mix without template. To analyze product of PCR, Fermentas FastRuler™ Low Range DNA Ladder (Fermentas Cat. No: # SM1103) was used as DNA ladder.

Statistical analysis

Association between methylation status of tumor suppressor gene RASSF1A and LMP-1 expression was analyzed by Chi-square with 95% confidence interval. Chi square analysis was also conducted to determine the association between RASSF1A methylation status with age and stage of the subject.

RESULTS

Subjects characteristics

The subjects in this study were 36 subjects aged 26-72 years with an average age of 47.55 years.

Most of the subjects was male (72.2%). The results showed that 88.9% of research subjects were at an advanced stage (stage III and IV).

LMP-1 expression examination

The LMP-1 immunohistochemistry staining showed the variation of LMP-1 expression (FIGURE 1). The LMP-1 expression intensity varied in each subject. The weak, medium and strong intensities were found in the subjects being examined. In some subjects, LMP-1 was expressed uniformly at the entire field examined. While on the other subject, LMP1 was expressed in a scattered or expressed in a group at a particular location. Sixteen subjects (44,4%) expressed LMP-1 (expression score ≥ 1). Among them, 14 subjects were in advanced stage. The LMP-1 expression score obtained was ranged between 0-8, with an average value of 1.56 ± 2.16 .

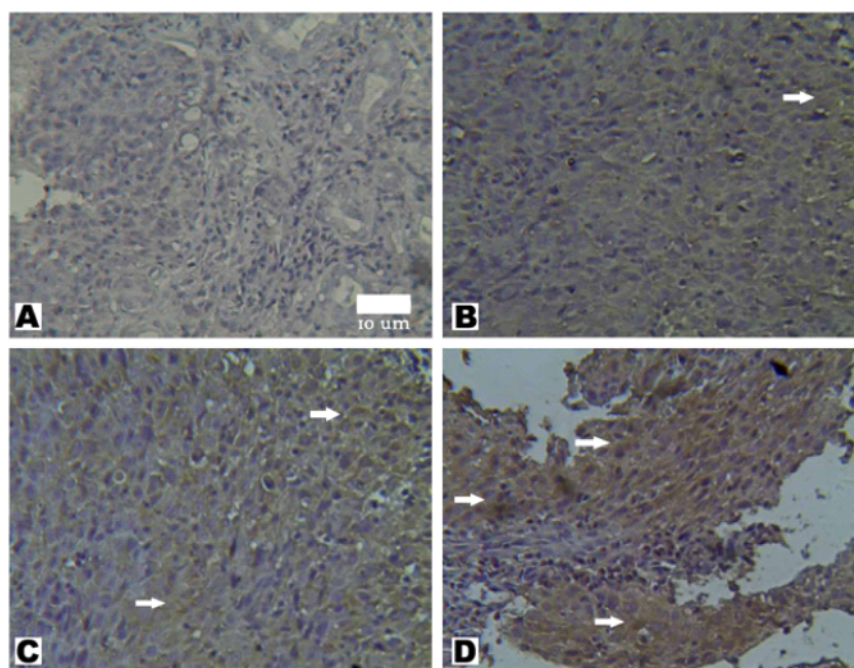


FIGURE 1. Immunohistochemistry staining of latent membrane protein 1 in NPC biopsies. Positive LMP-1 expression was marked by the appearance of brown color on the cytoplasm. Figure A showed no expression of LMP-1. B, Arrow indicating Expression of LMP-1 with weak color intensity. C, Arrow indicating expression of LMP-1 intermediate level and spread $>50\%$ of the visual field. D, expression of LMP-1 with strong intensity and spread $>75\%$ field of view. White arrow show the representative area of LMP-1 expression (magnification 400x).

RASSF1A methylation status

Methylated specific PCR results showed that hypermethylation of RASSF1A occurred in 24 subjects (66,7%). Figure 2 represented the results of RASSF1A MSP NPC patients. PCR band in MSP was an indicator that hypermethylation occurred in the sample. In case PCR band appeared in both USP and MSP, sample was considered as hypermethylated since there was possibility that the sample also contaminated cells. Figure 2 showed that RASSF1A hypermethylation occurred in the subject no 1 and 3, while the subject no 2 was unmethylated.

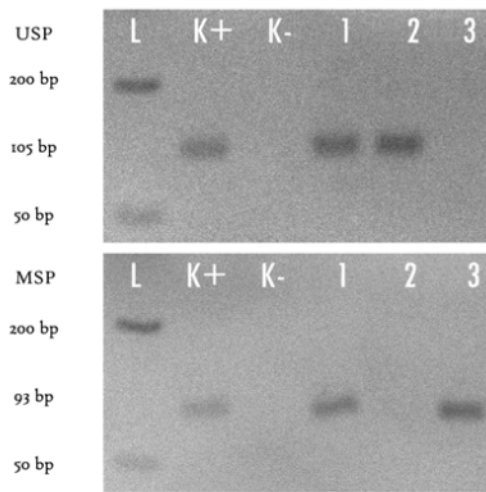


FIGURE 2. The results of RASSF1A MSP electrophoresis. RASSF1A hypermethylation occurred in subject no. 1 and 3. While subject no. 2 was unmethylated.

Association of LMP-1 expression and RASSF1A methylation status

Table 1 showed the distribution of LMP-1 expression and methylation status of RASSF1A. Latent membrane protein 1 positive subject had more RASSF1A hypermethylation compared to LMP-1 negative subject. Fourteen subjects of 16 LMP-1 positive subjects had RASSF1A hypermethylation. On the other hand, 10 out of 20 LMP-1 negative subjects had RASSF1A hypermethylation.

TABLE 1. Chi square analysis of LMP-1 expression with RASSF1A methylation status

Variable	n	MSP RASSF1A		χ^2	p
		Unmethylated	Methylated		
LMP-1 expression					
• Positive	16	2	14	5.625	0.018
• Negative	20	10	10		

Phi value = 0.395

Chi-square analysis was conducted to determine the association between LMP-1 expression with RASSF1A methylation status. The Chi-square test results showed that there was an association between expression of LMP-1 with RASSF1A methylation status (p= 0.018) and phi value was 0.395. The result indicated that there was a tendency that RASSF1A hypermethylation occurred in LMP-1 positive subjects.¹⁸ To further analyzed the association between LMP-1 expression score with RASSF1A methylation status, LMP-1 expression score were grouped into low, middle and high expressions. Data were analyzed using cross tabulation to determine the association between the score of LMP-1 expression with methylation status of RASSF1A. Table 2 showed that there was an association between the score of LMP-1 expression with RASSF1A methylation status (p=0.010).

Table 2. Chi square analysis of LMP-1 expression score with RASSF1A methylation status.

Variable	n	MSP RASSF1A		χ^2	p
		Unmethylated	Methylated		
LMP-1 expression score					
• Low (score <1)	20	10	10	8.100	0.017
• Mid (score 1-3)	5	2	3		
• Negative (score ≥3)	11	0	11		
Fisher's exact test				8.95	0.10

Phi value = 0.474

DISCUSSION

Examination of LMP-1 Expression

Latent membrane protein 1 expression was detected in 44.4% research subjects. This frequency was lower than the range of frequencies that have

been reported which ranged from 50-100%.^{8,17,19} Latent membrane protein 1 expression score in this study ranged between 0-8 of the maximum score (12) that can be obtained. This score was lower than previous studies. By using the same method, Hariwiyanto¹⁷ reported that LMP-1 expression score ranged from 2-12 with an average of 7.6 (\pm SD = 2.6). While other studies reported that LMP-1 score ranged from 3-11.⁸

The different LMP-1 expression score in this study, possibly caused by the differences in types of antibody used. The antibody used to detect the expression of LMP-1 in this study was a monoclonal antibody that recognized a conformational epitope at residues 290 to 318, overlapped with 11 amino acid repeat in LMP-1.²⁰ Khabir *et al.*,⁸ and Hariwiyanto,¹⁷ used antibodies CS1-4. The CS1-4 was a pool of four types of monoclonal antibodies that recognized the C-terminus of LMP-1. The target was the variable region epitopes on 11 amino acids between residues 205 to 308, and also at 386 the end residue of C-terminus.^{21,20} Different target antigen epitopes influenced the sensitivity and specificity of antibodies, it was influential on the final assessment of LMP-1 expression.⁷

Jiwa *et al.*,⁷ reported that there were different immunoreactivity between CS1-4 monoclonal antibody with the S12 (OT17). The S12 monoclonal antibody was known to be more sensitive in detecting LMP-1 compared with CS1-4. The S12 also provided better color and contrast than CS1-4. Based on author's knowledge, research on immunoreactivity comparison between antibody CS1-4 with OT21C has never been done, therefore the level of sensitivity between the two antibodies can not be compared.

RASSF1A methylation status

In this study, RASSF1A hypermethylated in 66.7% subjects. This was in line with previous studies which reported that the frequency of RASSF1A hypermethylation in NPC ranged from 66.7%-84%.^{2,7,12} Statistical analysis showed that there was no relationship between RASSF1A methylation status with age of study subjects ($p = 0.637$). The RASSF1A methylation status was also

not associated with stage NPC subjects ($p = 0.708$). The result was in contrast with previous studies which stated that the incidence of TSG promoter methylation increased with the increasing age of subjects.^{4,11,21-23}

Association of LMP-1 expression and RASSF1A methylation status

The result successfully demonstrated the relationship between LMP-1 expression in NPC biopsy with hypermethylation of RASSF1A promoter in DNA that was isolated from nasopharyngeal brushing. The result showed a tendency of hypermethylation of RASSF1A in subject with high LMP-1 expression score. This result indicated that LMP-1 expression can be used as marker of RASSF1A hypermethylation incidence. However, intracellular mechanism of LMP-1 in inducing RASSF1A hypermethylation still need further study.

Latent membrane protein 1 is known to induce tumor suppressor genes hypermethylation through DNA methyltransferase (DNMT1, and DNMT3b DNMT3a) activities.^{24,25} There was a report of phosphorylation of DNA methyltransferase by LMP-1 signaling via c-Jun NH2-terminal kinase (JNK).²⁶ The LMP-1 activates JNK, and phosphorylates transcription factor c-Jun. The phosphorylated c-Jun binds the activator protein 1 (AP-1) and transactivates the DNMT1 promoter. Elevated DNMT1 expression leads to hypermethylation of TSG.⁶ In addition, LMP-1 may inhibit expression of RASSF1A through the NF- κ B.²⁰ If the mechanism of LMP-1 in inducing hypermethylation RASSF1A can be recognized, it will be very useful in the development of therapeutic agents. This will open the possibility of application of 3,4-dihydroxybenzalacetone (DBL) for inhibiting NF- κ B,²⁷ or application of azacytidine to inhibit DNA methyltransferase²⁸ activity as one of the NPC therapies.

CONCLUSION

There was an association between expressions of LMP-1 with RASSF1A methylation status in NPC patients.

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