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SIMULTANEOUSLY BOTH EXPRESSION OF *LMP-1* AND METHYLATION OF *E-CADHERIN*: MOLECULAR BIOMARKER IN STAGE IV OF NASOPHARYNGEAL CARCINOMA PATIENTS

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ABSTRACT

The phenome of *E-cadherin* gene methylation and the expression of latent membrane protein 1 (*LMP-1*) gene are associated with nasopharyngeal carcinoma (NPC). In order to determine whether cooperative *LMP-1* expression or methylation of *E-cadherin* could serve as the potential molecule biomarker target for diagnosis and therapy of NPC, a case-control study including 93 NPC biopsy samples and 100 non cancerous nasopharyngeal swab samples were examined, as well as the strength of association among them by the quantitative polymerase chain reaction (qPCR) and nested-methylation-specific PCR methods. The significantly higher frequency of *LMP-1* expression and *E-cadherin* methylation in NPC biopsy samples, accounting for 76.34 and 73.12%, respectively, compared to non cancerous samples, accounting for 0.00 and 30.00%, respectively, were observed. The significant correlation between the *LMP-1* expression and *E-cadherin* methylation in NPC samples was reported. In detail, in the stage IV of NPC, in case of *LMP-1*-positive samples, 35 of 37 samples (accounting for 94.60%) were positive for methylation of *E-cadherin*. It was demonstrated that cooperative *LMP-1* expression and *E-cadherin* gene methylation could serve as a molecular biomarker in NPC.

Keywords: Biomarker; Diagnosis; *E-cadherin* gene; Latent membrane protein-1 (*LMP-1*) gene; Nasopharyngeal carcinoma (NPC); Therapy.

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INTRODUCTION

A nasopharyngeal carcinoma (NPC) is a prevalent nasopharyngeal malignant tumor with remarkable differences in distribution that gravitates toward Southern Asia. Even though many improvements in NPC therapy have been achieved, the diagnosis at an advanced stage led to reducing the success rate of treatment as well as the survival of patients [1]. Thus, early screening and diagnosis represents beneficial opportunities to increase the survival of patients as well as the effects of nasopharyngeal cancer treatment. Because of the nonspecific symptoms related to the early stage of NPC as well as the deeply seated location of nasopharynx, it leads to major obstacles to early screening of NPC [2]. Therefore, effective biomarkers are truly needed [3]. According to the etiology of NPC, a strong association between the infection of Epstein-Barr virus (EBV), also known as human γ-herpesvirus 4 (HHV4), and NPC has been postulated [4-8]. In the stage of latency, the limited latent genes are expressed, have been reported to play the key role in nasopharyngeal tumorigenesis [9-11]. Among them, the latent membrane protein 1 (*LMP-1*) gene, its encoded protein, has profound effects on the proliferation as well as the metastasis of NPC cells, resulting in the highly invasive and malignant growth of nasopharyngeal tumors [10]. In addition to the expression of *LMP-1*, the establishment of an epigenetic change on the *E-cadherin* gene, located on chromosome 16q22.1, which is responsible for the main key mediator of cell-cell adhesion in epithelial tissue, by the forming of E-cadherin/catenin complex that is further linked to the actin cytoskeleton, is likely to be a major contributing factor to the development and metastasis of nasopharyngeal tumors [12,13]. Recent growing evidence indicated that the hypermethylation, a kind of epigenetic alteration of *E-cadherin* resulting in the silence of its function, has been reported to play a key role

in NPC development and metastasis [14-17]. Are there any associations between the expression of *LMP-1* and meth-ylation of *E-cadherin* in NPC tumorigenesis? And, emphatically, that is to determine whether a combination of *LMP-1* expression and methylation of *E-cadherin* could be a significant molecular characteristic of nasopharyngeal carcinomas, as well as it could be the potential biomarker for NPC. To answer the issue mentioned above, the present case-control study was designed to explore the relationship in the expressions of *LMP-1* and the hypermethylation of *E-cadherin* in NPC tumor and non cancerous specimens of Vietnamese population.

MATERIALS AND METHODS

Ethics Statement, Sample Collection. Institutional Ethics Board approval was obtained from the Medical Ethics Committee of the Cho Ray Hospital, Ho Chi Minh City, Vietnam [the decision number of the permission: 516/BVCR-HDDD]. All the samples used in this study were approved by Cho Ray Hospital and obtained from all participants from January 2017 to December 2018. The patients enrolled in this study were required to sign consent forms to approve the usage of the samples for laboratory studies and analyses.

A total of 93 archived NPC biopsy tissues that were confirmed by immunohistochemistry, were obtained from the Cho Ray Hospital, Ho Chi Minh City, Vietnam. Notable clinicopathological characteristics, including patients' gender, age, and histological type were recorded. One hundred non cancerous swab samples were collected from volunteers. In brief, a 15 cm-long cotton stick was inserted into the nasal cavity and moved toward the nasopharyngeal wall; it was then swept over the surface of the posterior and lateral nasopharyngeal wall. The cotton stick was withdrawn and immediately immersed in phosphate-buffered saline stored at –20 °C for further experiments.

Nucleic Acid Isolation and Bisulfite Modification. Total RNA was isolated using TRIzol™ Reagent (Cat: 15596026; Thermo Fisher Scientific Inc., Waltham, MA, USA). cDNA was reverse-transcribed from using ~1 µg isolated RNA using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific Inc.). The reverse-transcription assay was performed according to according to the manufacturer's guideline.

Total of genomic DNA was isolated from biopsy and swab samples by the phenol/chloroform method. Then, total genomic DNA was isolated and purified using standard phenol-chloroform and ethanol precipitation. The bisulfite conversion of 500 ng purified DNA was performed using EpiJET Bisulfite Conversion Kit (Thermo Fisher Scientific Inc.). The final precipitation was eluted in a volume of 20 µL and stored at –20 °C for further studies.

Quantitative Polymerase Chain Reaction and Nested-Methylation-Specific Polymerase Chain Reaction. For evaluation of *LMP-1* expression, quantitative polymerase chain reactions (qPCRs) were done by means of a qSYBR-green and the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene was used as an endogenous control. The internal control candidate was used to normalize the cycle threshold (Ct) values of each *LMP-1*. The primers, which were used in the current study, were obtained by the previous studies and are shown in Table 1 [16,18,19].

The methylation status of the *E-cadherin* gene promoter in the clinical samples was examined by two-stage nested-methylation-specific PCR (nested-MSP). The primers of stage 1 and stage 2 PCR are shown in Table 1. The primers of stage 1 PCR were used for preceding amplification that recognize the bisulfite-modified template, and notably, do not discriminate between methylated and un-methylated

Table 1. The primer sequences used.

For evaluation of ***LMP-1*** expression	For evaluation of ***LMP-1*** expression
Primers [Refs.]	Sequences (5'>3')
LMP-1-F [18] LMP-1-R [18]	CAG TCA GGC AAG CCT ATG A CTG GTT CCG GTG GAG ATG A
GAPDH-F [19] GAPDH-R [19]	TGC CTC CTG CAC CAC CAA CT CGC CTG CTT CAC CAC CTT C

For evaluation of ***E-cadherin*** methylation				
	Primers [Refs.]	Sequences (5'>3')	Temperature	Product
Stage 1	Seq-F [16] Seq-R [16]	TTT TAG GTT AGA GGG TTA T CTA CAA CAA CAA CAA CAA C	50 °C	350 bp
Stage 2	E-cad-MF [16] E-cad-MR [16]	GGT GAA TTT TTA GTT AAT TAG ***CG***G TA***C*** CAT AAC TAAC ***CG***A AAA ***CG***C ***CG***	60 °C	205 bp
Stage 2	E-cad-UF [16] E-cad-UR [16]	GGT AGG ***TG***A ATT TTT AGT TAA TTA G***TG*** GTA ACC ***CA***T AAC TAA C***CA*** AAA A***CA*** A***CA***	60 °C	211 bp

Refs.: references; F: forward primer; R: reverse primer; M: methylated U: unmethylated; CpG islands in italics and bold characters.

sequences. In stage 2 PCR, two pairs of primer were used to amplify the regions of interest. One pair recognized a sequence in which CpG sites were methylated (unmodified by bisulfite treatment). Another pair recognized a sequence in which CpG sites were unmethylated (modified to UpG treatment). Each stage of PCR was performed in a total of 15 µL containing 3 µL bisulfite-modified template DNA (in case of stage 1 PCR) or 3 µL stage 1 PCR product (in case of stage 2 PCR), 0.75 unit iTaq DNA polymerase (Bio-Rad Laboratories, Hercules, CA, USA), 0.5 µM each primer, 7.5 µL MyTaqTM Mix (Bioline Reagents Ltd., London, UK). Thermal cycling was initiated at 95 °C for 5 min., followed by 40 cycles of denaturation at 95 °C for 30 seconds, annealing at X °C for 30 seconds, extension at 72 °C for 30 seconds, and a final extension at 72 °C for 10 min. (X °C was the specific annealing temperature for each primer, shown in Table 1). Finally, the methylated and unmethylated PCR products were separated on 2.0% agarose gel and visualized by ethidium bromide staining. Representative nested-MSP products were sequencing to confirm the specificity of primers, examine the efficiency of bisulfite modification and the hypermethylation status of target gene.

Statistical Analyses. Statistical analyses were performed by Medcalc® Version 12.7.0.0 (MedCalc Software bv, Ostend, Belgium). The average frequency of methylation and *LMP-1* expression was calculated. The χ^2 test was used to determine the association between the expression of *LMP-1*, *E-cadherin* methylation and NPC status. Moreover, the association between hypermethylation of *E-cadherin*, expression of *LMP-1* and risk of cervical cancer was estimated by computing the odds ratio (OR) and 95% confidence interval (CI).

RESULTS

Sample Collection. A total of 93 NPC biopsies and 100 non cancerous samples were collected in local hospital. The characteristics of all 93 NPC samples are summarized in Table 2. The mean age of NPC specimens ranged from 20 to 81 (mean: 53.51 ± 1.43). According to sex distribution, the number of males (accounting for 73.12%) was more than that of females (accounting for 26.88%). Histological type: the number of type 3 (undifferentiated carcinoma) was the highest, accounting for 67.74%. The stage 4 (advanced stage), accounting for 48.39%, was the highest among stages of NPC patients.

***LMP-1* Expression in Nasopharyngeal Carcinoma and Non Cancerous Samples.** The expression of *LMP-1* and *GAPDH* were evaluated by qPCR. The *GAPDH* gene was used as the internal control for evaluating the expression of *LMP-1*. As the expression of *GAPDH*, both NPC samples and control samples were positive. The Ct values of *GAPDH* were 27.14 ± 1.79 and 27.75 ± 2.14 in case and control groups, respectively. There are no significant differences between the expression of *GAPDH* in the case and control groups (p >0.05) (Figure 1). According to the expression of *LMP-1*, 71 of the 93 NPC specimens (accounting for 76.34%) were positive, while none of the controls were positive (Figure 1). A value of p <0.0001 indicated that *LMP-1* gene expression was significantly associated with

Table 2. Characteristics of enrolled clinical nasopharyngeal carcinoma NPC samples.

***n* = 93**	***n* (%)**
Gender:	
males	68 (73.12)
females	25 (26.88)
Age (years:	
<20	1 (1.08)
20 to 40	17 (18.28)
40 to 60	43 (46.23)
60 to 80	31 (33.33)
>80	1 (1.08)
Histological type[a]:	
type 1	4 (4.30)
type 2	26 (27.96)
type 3	63 (67.74)
Stage:	
I	0 (0.00)
II	33 (35.48)
III	15 (16.13)
IV	45 (48.39)

n: number.

[a] Type 1: keratinizing squamous cell carninoma; type 2: nonheratinizing squamous cell carcinoma; type 3: undifferentiated carcinoma.

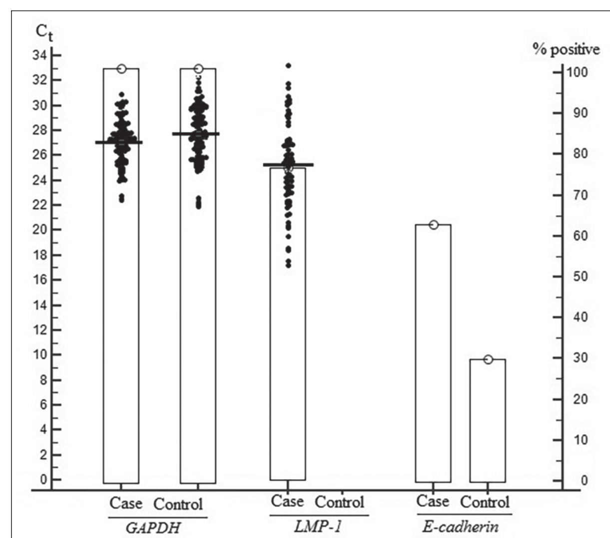

Figure 1. The mean Ct value and percentage of *GAPDH*, *LMP-1* and *E-cadherin* in the case and control groups. Each black dot indicated the Ct value of *GAPDH*, *LMP-1* in each sample. Each bar indicated the positive percentage of *GAPDH*, *LMP-1* and *E-cadherin* in case and control groups.

NPC. The relative sensitivity, specificity, negative likelihood ratio, positive predictive value, as well as negative predict value were 76.34% (95% CI = 66.40-84.54), 100.00% (95% CI = 96.37-100.00), 0.24 (95% CI = 0.17-0.34), 100.00%, 81.97% (95% CI = 75.93-86.75), respectively. We found no significant association was found between *LMP-1* expression and the clinicopathological characteristics, including patients' gender (p = 0.06), age (p = 0.19), tumor histological types (p = 0.29) and stage of NPC (p = *0.07*) (Table 3). Additionally, the OR value was 638.73 (95% CI = 38.12-10,703.57, p <0.0001), indicated that the positive association between the expression of *LMP-1* and risk of NPC.

Methylation of *E-Cadherin* Status in Nasopharyngeal Carcinoma Samples and Non-Cancerous Samples. In the current case-control study, nested-MSP was applied in evaluation of the methylation status of the *E-cadherin* gene promoter in Vietnamese samples. The methylation and unmethylation frequencies of the *E-cadherin* gene promoter in NPC biopsy samples were 73.12% (68 of 93 samples) and 26.88% (25 of 93 samples), respectively. In the case of non-cancerous samples, the methylation and unmethylation frequencies were 30.00% (30 of 100 samples) and 70.00% (70 of 100 samples), respectively. The relative sensitivity, specificity, positive likelihood ratio, negative likelihood ratio, positive predictive value, as well as negative predictive value were 73.12% (95% CI = 62.92-81.79), 70.00% (60.02-78.76), 2.44 (95% CI = 1.76-3.37), 0.38 (95% CI = 0.27-0.55), 69.39% (95% CI = 62.12-75.81) and 73.68% (95% CI = 66.17-80.04), respectively. Additionally, the methylation status of the *E-cadherin* gene promoter in NPC samples was found to be significant higher than in healthy samples, and strongly associated to the cancer of nasopharynx (p <0.0001). The nested-MSP products (stage 2) of methylation and/or un-methylation in the promoter of *E-cadherin* in samples were observed in electrophoresis with visualized by ethidium bromide staining and are shown in Figure 2.

The sequencing of samples methylated in the promoter region of representative sample revealed a conversion of unmethylated cytosine, but not methylated cytosine (Figure 2). By sequencing, comparison between the non-bisulfite-modified and bisulfite-modified, all methylated cytosines were unchanged, which were marked as square symbols. Otherwise, the unmethylated cytosines, which were marked as triangle symbols, were changed into thymine in the bisulfite sequence (Figure 3).

In this study, the OR values were computed between the methylation of *E-cadherin* and NPC. The results show that the OR value was 6.35 (95% CI = 3.39-11.88, p <0.0001). It indicated that there was significant association between the methylation of the *E-cadherin* gene promoter and risk of NPC. Additionally, only significant association between *E-cadherin* methylation and patients'

Table 3. Association between the expression of *LMP-1*, methylation of *E-cadherin* and clinicopathological parameters.

	LMP-1		**E-Cadherin**	
	Positive, *n* (%)	Negative, *n* (%)	Methylated, *n* (%)	Unmethylated, *n* (%)
Gender:				
males	51 (54.83)	17 (18.28)	46 (49.46)	22 (23.66)
females	20 (21.51)	5 (5.38)	22 (23.66)	3 (3.23)
p Value	0.06		0.05	
Age (years):				
<20	1 (1.08)	0 (0.00)	1 (1.08)	0 (0.00)
20 to 40	11 (11.83)	6 (6.45)	12 (12.90)	5 (5.38)
40 to 60	36 (38.71)	7 (7.53)	32 (34.41)	11 (11.83)
60 to 80	23 (24.73)	8 (8.60)	23 (24.73)	8 (8.60)
>80	0 (0.00)	1 (1.08)	0 (0.00)	1 (1.08)
p Value	0.19		0.52	
Histological type[a]:				
type 1	3 (3.23)	1 (0.00)	4 (4.30)	0 (0.00)
type 2	17 (18.28)	9 (9.68)	16 (17.20)	10 (10.75)
type 3	51 (54.83)	12 (12.90)	48 (51.61)	15 (16.13)
p Value	0.29		0.16	
Stage:				
I	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
II	26 (27.96)	7 (7.53)	22 (23.66)	11 (11.83)
III	8 (8.60)	7 (7.53)	9 (9.68)	6 (6.45)
IV	37 (39.78)	8 (8.60)	37 (39.78)	8 (8.60)
p Value	0.07		0.14	

n: number.

[a] Type 1: keratinizing squamous cell carcinoma; type 2: nonkeratinizing squamous cell carcinoma; type 3: undifferentiated carcinoma.

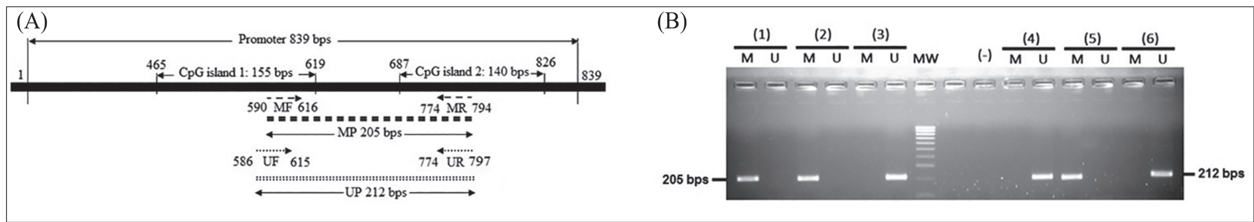

Figure 2. (A) The schematic diagram represented the *E-cadherin* gene promoter and its CpG island. MF: methylated forward primer; MR: methylated reverse primer; UF: unmethylated forward primer; UR: unmethylated reverse primer; MP: amplified methylated product by methylated primers; UP: amplified unmethylated product by unmethylated primers. Numbers indicated the position of nucleotide. (B) Methylated promoter of the *E-cadherin* gene was analyzed on some clinical samples by MSP. (The MSP product was 205/211 bps in length. Lanes 1, 2 and 3: NPC biopsy samples; lanes 4, 5 and 6: healthy samples; lane (–): negative control; MW: 100 bps ladder; M: methylated; U: unmethylated.

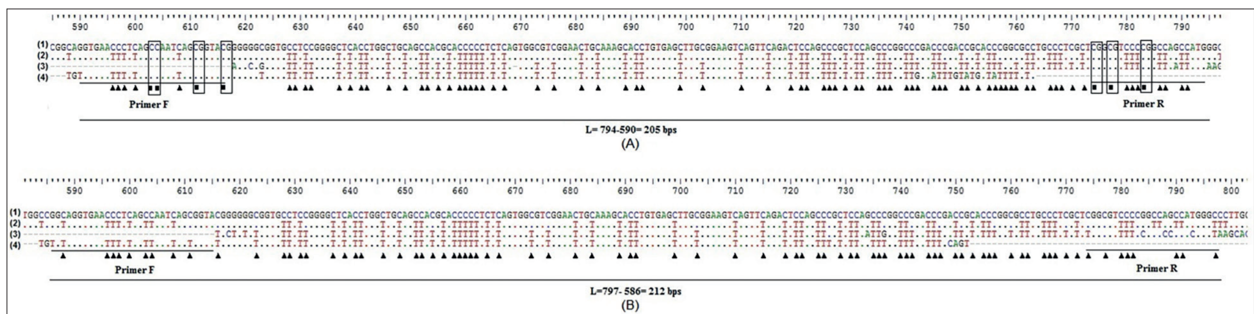

Figure 3. Sequencing profile of (A) methylated and (B) unmethylated of the *E-cadherin* gene promoter. (1) DNA sequence was without bisulfite modification; (2) DNA sequence was bisulfite-modified; (3) amplified sequencing DNA by forward primer; (4) amplified sequencing DNA by reversed primer; square symbols: methylated cytosine; triangle symbols: unmethylated cytosine.

gender was observed (p = 0.05). No significant association was found between *E-cadherin* methylation and the clinicopathological characteristics, including patients' age (p = 0.52), tumor histological types (p = 0.16) and stage of NPC (p = 0.14) (Table 3).

Association Between *E-Cadherin* Methylation and *LMP-1* Expression. As shown in Table 4, the χ^2 test was applied to determine the relationship between the *LMP-1* expression and *E-cadherin* methylation. Based on the positive index (PI = 0: both *LMP-1* and methylation of *E-cadherin* were negative; PI = 0.5: either *LMP-1* or *E-cadherin* was positive; PI = 1.0: both *LMP-1* and methylation of *E-cadherin* were positive) among 93 NPC biopsy samples, 61 cases (accounting for 65.59%) were positive for both expression of *LMP-1* and *E-cadherin* methylation (PI = 1.0). This also meant that among 71 samples that were positive for *LMP-1* expression, 61 cases (85.92%) were positive for *E-cadherin* methylation. The p value (p <0.0001) indicated that the expression of *LMP-1* was positively significantly associated with the methylation of *E-cadherin*.

Analysis with clinicopathological characteristics, no significant association was between PI value and the clinicopathological characteristics, including patients' gender (p = 0.14), age (p = 0.42), and histological type (p = 0.42). Only significant association between PI value and patients' NPC stage was observed (p = 0.04) (Table 5). The result indicated that there was a significant tendency of *LMP-1* expression and methylation of *E-cadherin* in NPC biopsy samples of the advance stage (Table 5, Figure 4). As shown in Figure 4, among 37 samples that were positive for *LMP-1* expression, 35 cases of 37 samples (accounting for 94.60%) were positive for methylation of *E-cadherin*. Therefore, it could be inferred that the expression of *LMP-1* may have an ability to methylate the gene of *E-cadherin*, and those genes were associated with NPC in advanced stages.

Table 4. Statistical analysis of association between *LMP-1* expression and methylation of *E-cadherin* in nasopharyngeal carcinoma samples.

	LMP-1 Expression		
	Positive, *n* (%)	Negative, *n* (%)	*p* Value
E-cadherin methylation:			
positive, *n* (%)	61 (65.59)	7 (7.53)	<0.0001
negative, *n* (%)	10 (10.75)	15 (16.13)	

	NPC1	NPC2	NPC3	NPC4	NPC5	NPC6	NPC7	NPC8	NPC9	NPC10	NPC11	NPC12	NPC13	NPC14	NPC15	NPC16	NPC17	NPC18	NPC19	NPC20	NPC21	NPC22	NPC23
LMP-1	□	□	□	□	□	□	□	□	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
E-cadherin	□	□	□	□	■	□	□	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■

	NPC24	NPC25	NPC26	NPC27	NPC28	NPC29	NPC30	NPC31	NPC32	NPC33	NPC34	NPC35	NPC36	NPC37	NPC38	NPC39	NPC40	NPC41	NPC42	NPC43	NPC44	NPC45
LMP-1	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
E-cadherin	■	■	■	■	■	■	■	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■

Figure 4. Summary of *LMP-1* expression and methylation of the *E-cadherin* gene in 45 stage IV NCP samples. Filled box: positive, open box: negative.

Table 5. Association between the positive index and clinicopathological parameters.

	LMP-1, E-Cadherin		
	PI = 0, *n* (%)	PI = 0.5, *n* (%)	PI = 1.0, *n* (%)
Gender:			
males	14 (15.06)	11 (11.83)	43 (46.24)
females	1 (1.08)	6 (6.45)	18 (19.35)
p Value	0.14		
Age (years):			
<20	0 (0.00)	0 (0.00)	1 (1.08)
20 to 40	3 (3.23)	5 (5.38)	9 (9.68)
40 to 60	6 (6.45)	6 (6.45)	31 (33.33)
60 to 80	5 (5.38)	6 (6.45)	20 (21.51)
>80	1 (1.08)	0 (0.00)	0 (0.00)
p Value	0.42		
Histological type[a]:			
type 1	0 (0.00)	1 (1.08)	3 (3.23)
type 2	7 (7.53)	5 (5.38)	14 (15.05)
type 3	8 (8.60)	11 (11.83)	44 (47.31)
p Value	0.42		
Stage:			
I	0 (0.00)	0 (0.00)	0 (0.00)
II	4 (4.30)	10 (10.75)	19 (20.43)
III	5 (5.38)	3 (3.23)	7 (7.53)
IV	6 (6.45)	4 (4.30)	35 (37.63)
p Value	0.04		

PI: positive index; PI = 0: both *LMP-1* and methylation of *E-cadherin* were negative; PI = 0.5: either *LMP-1* or *E-cadherin* was positive; PI = 1.0: both *LMP-1* and methylation of *E-cadherin* were positive.

[a] Type 1: keratinizing squamous cell carcinoma; type 2: nonkeratinizing squamous cell carcinoma; type 3: undifferentiated carcinoma.

DISCUSSION

In this study, we pointed out the significantly high expression of *LMP-1* in NPC biopsy samples, accounting for 76.34%, compared to no expression in non-cancerous samples. The increased expression of *LMP-1* was strongly associated with NPC risk, with statistical significance, based on the calculated OR value of 638.73 (p <0.05). Thus, all these results imply that the oncogenic role of *LMP-1* in NPC was well evaluated in this case/control. According to the etiological factor, as previously reported, *LMP-1* is an integral membrane protein that plays a key role in EBV-mediated oncogenesis involved in many signal pathways, including nuclear factor-κ-light-chain-enhancer of activated B cells (NF-κB), c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein (MAP) pathway [20-22]. Moreover, *LMP-1* is capable of inducing a range of phenotypic changes in epithelial cells [23]. In the latent stage of NPC, the expression of *LMP-1* are expressed in EBV-associated malignant type II, exemplified by NPC, contribute to cell survival, and NPC metastasis [11,24]. Thus, the *LMP-1* gene, which encoded the *LMP-1* protein, performed the oncogenic function in the case of EBV-associated malignancies, including NPC, has been identified as the meaningful diagnosis biomarker as well as potential therapeutic molecule target for NPC. Additionally, the finding of *LMP-1* characteristics was again confirmed in the current case-control study, and it may open up the treatment approach for NPC therapy based on the inhibitor of *LMP-1* expression in EBV-associated NPC. The inactivation of the *E-cadherin* gene through the methylation of its promoter has been postulated as the metastasis-associated factor of NPC development and progression [14-17,25]. In this study, the methylation of the *E-cadherin* gene was

examined by the nested-MSP method, which shows an advantage in the hypermethylation analysis by increased MSP sensitivity of approximately 50-fold [26]. Here, we found that the hypermeth-ylation of the *E-cadherin* gene promoter in 73.12% NPC biopsy samples, which was significantly higher than in control samples, accounting for 30.00%. This frequency of methylated *E-cadherin* gene in the current study was similar to that previously reported by Niemhom *et al.* [15], Ayadi *et al.* [16], and higher than the study by Ran *et al.* [17]. Even though the differences in E-cadherin methylation frequencies exist, they met the common point where the methylation of the *E-cadherin* gene was significantly associated with the risk of NPC based on the calculated OR (OR >1.0, p <0.05). Therefore, we believe that the aberrant methylation of the *E-cadherin* gene is significant in NPC and may serve as the promising biomarker that could be potentially used for diagnosis of NPC.

To answer whether or not there was the association between the expression of *LMP-1* and methylation of *E-cadherin* in NPC tumorigenesis, the statistical analysis was performed between the expression of *LMP-1* and *E-cadherin* methylation in 93 clinical NPC biopsy samples. Our results reported that there was strongly correlation between the *LMP-1* expression and *E-cadherin* methylation in NPC samples (*p <0.0001*). Especially, the status of both gene positive was significantly associated with the stage of NPC, in which the highest frequency of 37.63% (35 of 93) NPC samples were positive for both *LMP-1* expression and methylation of *E-cadherin* was observed. Additionally, in the stage IV, we found that in case of *LMP-1*-positive samples, 35 cases of 37 samples (counting for 94.60%) were positive for methylation of *E-cadherin*. Whereas, we found that 7 of 15 cases (counting for 46.67%), 19 of 33 cases (counting for 57.58%) in the stage II and stage 4, respectively, were positive for both genes. Therefore, it could be concluded that the expression of *LMP-1* and methylation of *E-cadherin* are more likely to be positive in the stage IV than the early stage of NPC. Based on these finding, it could be induced that the expression of *LMP-1*, which profound effects on the proliferation of NPC cells as well as the highly invasive, malignant growth of NPC tumors, and addition of epigenetics change, methylation of *E-cadherin*, a main key mediator of cell-cell adhesion, are likely to be a major contributing factor to the advance stage of nasopharyngeal tumor. These finding was similar to pervious study, the *LMP-1* have an ability to down regulation of expression of *E-cadherin* through the pathway of *E-cadherin*/β-catenin, in our study the inactivation of *E-cadherin* caused by the epigenetics event, resulting in enhancement of invasive capacity of metastasis of NPC cells [27]. *E-cadherin*, which is the important factor responsible for the development and metastasis of nasopharyngeal tumor, may combine to act in a complex sequential process of nasopharyngeal tumorigenesis that culminates in metastasis of EBV-infected tumor cells. Therefore, these combination testing will be used as a parameter in the diagnosis of NPC in further studies.

In this study, the *E-cadherin* gene has been reported to be a candidate therapeutic target in human cancers, such as hepatocellular carcinoma [28], nasopharyngeal cancer [17], breast cancer [29]. Especially, growing evidence supported epigenetics drug (epi-drug) has been generated and developed to explore the epigenetics reversion to treat human cancers. Giving the example, the inhibitors of DNA meth-ylation drugs, such as 5-azacytidine, 5-aza-2'-deoxycytidine, *etc.* acts as a potent inhibitor of the DNA demeth-ylase, enzymes that catalyze the process of DNA methylation [30]. In other study, they reported that 5-aza-2'-deoxycytidine could restore the *E-cadherin*-silenced lung cancer and metastasis of cancer cell [31]. Concerning to viral therapy, as reported, *LMP-1* gene, and its encoded protein play an oncogenic role in NPC, including the proliferation as well as the metastasis of NPC cells, whereby evidence of its expression can only be inferred by its inhibition being detrimental to the growth of the cell, therefore, *LMP1* may be a value therapeutic molecule target in the treatment of EBV associated disease, including NPC [32]. Therefore, the re-activation of *E-cadherin*, especially combined with viral therapy, will be a candidate therapeutic target in NPC. This study is believed to show the relationship between *LMP-1* expression, *E-cadherin* gene methylation reinforced the basis for pursuing *LMP1* and *E-cadherin* as a therapeutic molecule target in NPC. As reported, the striking different expression of *LMP-1* and aberrant methylation of *E-cadherin*, therefore, towards to activation of tumor suppressive E-cadherin and/or inhibition of oncogenic *LMP-1* expression may open up the molecule-biomarker for the development of therapeutics for NPC. This suggested a clinical trial could be performed on the group of NPC patients with advance stage to improve their survival and recovery. Therefore, it is necessary to design such a study to consider the effect treatment on two criteria described above by the treatment which focused on each molecular target or both targets.

Conclusions. Our data indicated that the *LMP-1* expression and *E-cadherin* methylation were significantly correlated with the risk of NPC. The frequency of *LMP-1* expression and *E-cadherin* methylation were higher in NPC biopsy samples, counting for 76.34% and 73.12%, respectively, compared to non-cancerous samples. Additionally, the strong association between the *LMP-1* expression and *E-cadherin* methylation was observed in NPC samples. The status of both gene positive was sig-

nificantly associated with the stage of NPC, in which the highest frequency of 37.63% (35 of 93) NPC samples were positive for both *LMP-1* expression and methylation of *E-cadherin*, was reported. In summary, the authors of this paper demonstrated that the relationship between *LMP-1* expression, *E-cadherin* gene methylation reinforced the basis for pursuing *LMP1* and *E-cadherin* as a molecule biomarker in NPC.

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Declaration of Interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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