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Correlation of Epstein-Barr virus copy numbers, MICA expression and rs2596542 variant in nasopharyngeal carcinoma tumour

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Abstract:

Major histocompatibility complex class I chain-related A (MICA) is a tumour antigen that is greatly expressed on the surfaces of human malignancies and infection cells, which trigger attachment to immune cells. Although many studies have investigated the role of the MICA rs2596542 variant involved in high risk of hepatocellular carcinoma (HCC), the expression regulation of rs2596542 MICA in nasopharyngeal carcinoma (NPC) susceptibility with the Epstein-Barr virus (EBV) is still ambiguous. Therefore, this study was conducted to elucidate the association of rs2596542C/T and EBV load to MICA expression in NPC tissues. A total of 70 tumour tissues from NPC patients were enrolled in the current study. The genetic variant of rs2596542 was identified by Real-time PCR genotyping, and MICA protein expression was quantified by immunohistochemistry staining. A significant difference was observed in the expression of MICA regarding EBV copy numbers ($p<0.01$). High expression of MICA presented a considerably lower EBV status. In addition, higher expression of homologous MICA rs2596542 CC was significantly associated with a lower EBV load ($p<0.001$) thus suggesting a protective role of allele C against the infection of EBV. In summary, rs2596542 MICA plays an important role in the immune response preventing the EBV among NPC patients as well as developing a new predictive biomarker for virus susceptibility to cancers.

Keywords: **Epstein-Barr virus, MICA expression, rs2596542.**

Classification numbers: **3.2, 3.6**

1. Introduction

NPC is a common epithelial cancer that arises from the head and neck area and has a high prevalence in Southeast Asia and Southern China [1]. According to GLOBOCAN 2020, there were 133,354 new cases accounting for 0.69% of all diagnosed cancers, and the mortality rate was approximately 0.8%. Driven from the surface epithelium of the posterior nasopharynx, NPC is classified by World Health Organization (WHO) into three subtypes: squamous cell carcinoma (type I), non-keratinizing carcinoma (type II), and undifferentiated carcinoma (type III) [2]. Various etiological factors of NPC were investigated consisting of genetic susceptibility, environmental, dietary, and EBV infection [3-5]. Of these factors, EBV infection plays a predominant role when mostly associated with NPC type II and III [6]. The presence of EBV is detected in more than 90% of NPC type II, which is the most common type of NPC in the high-risk regions [7]. The relationship between NPC and EBV infection has been evaluated by the existence of EBV-DNA or transcripts determined from tumour cells and precancerous lesions [8]. Also, EBV-DNA load in plasma has been investigated as a potential biomarker of NPC, as it is highly associated with tumour burden and disease stage [9].

EBV, also known as human herpesvirus type 4 (HHV4), was the first human oncogenic virus to be discovered and belongs to the Gamma herpesvirus group. EBV infection affects 90% of people worldwide where primary infection typical occurs in early childhood and

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is mostly asymptomatic. However, in progressive cases, EBV can cause infectious mononucleosis (IM), an acute symptomatic, and associated with carcinogenesis as NPC [10]. B lymphocytes are the primary target of EBV infection into epithelial cells through CD21 expression on the cell surface where this virus can establish different latency stages in these lifelong carriers [11]. The EBV latency programs are categorised into four types, arranged from Latency 0 to Latency III, based on the specific genes expressed in normal B cells [12]. During primary infection, both CD8+ T cells and NK cells play a critical role in inhibiting the proliferation of EBV [13, 14]. Immune responses against virus-infected cells occur by downregulation of immunoreceptors including NKG2D (immunoreceptor natural killer group 2 member D) on the surface of effector cells [15]. NKG2D is the key stimulating receptor that remains on CD8+ T cell and NK cell surfaces. The interaction between NKG2D and its ligands and MICA (major histocompatibility complex (MHC) class I-related chain A polypeptides) can mediate signalling pathways to produce cytotoxicity and cytokine to kill EBV-infected cells.

MICA is a gene of MHC class I and is related to human tumorigenesis. It is approximately ~15.5 kb in length and located on the short arm of chromosome 6 (6p21.33). Of all MHC class I genes, MICA is the most polymorphic gene with 108 alleles and 87 identified protein variants [16]. Single nucleotide polymorphism (SNP) rs2596542C/T is located at the MICA promoter region and has been considered a valuable marker to screen HCC. Allele T of rs2596542 could have a higher risk to develop HCC when it can affect the bindings of stress-inducible transcription factors and thus can alter the expression of MICA [17]. Although MICA polymorphisms have been investigated to be highly associated with several types of cancer, only a few studies have been performed in NPC susceptibility EBV [18]. To identify the effect of EBV in tumour cells related to MICA expression and MICA polymorphism, specifically rs2596542, we performed this study to investigate the association between MICA expression and EBV copy numbers in NPC.

2. Materials and methods

2.1. Patients and specimens

This study was accepted by the ethics committee at Hanoi Medical University, Vietnam under number 26/HMUIRB. The case group included 70 patients who were diagnosed with NPC at Hanoi Medical University Hospital from October 2019 to November 2021. Patients who had NPC and did not receive any treatment were confirmed by histology at the Department of Pathology, Hanoi Medical University. All individuals signed an informed consent before the study.

2.2. DNA extraction and SNP genotyping

DNA samples were extracted from formalin-fixed paraffin-embedded (FFPE) tissue sections by QIAamp DNA FFPE Tissue Kit (Qiagen, 56404). The genotypes of the MICA rs2596542C/T polymorphism were determined by Real-time PCR on a Quantstudio3™ Real-Time PCR Systems (Applied Biosystems Inc., Foster City, USA) according to the manufacturer's instructions. In total, the 10-µl volume of the PCR reaction mixes contained 5 µl 2X TaqMan™ Genotyping Master Mix (Applied Biosystems), 0.5 µl 20X Taqman probe (TaqMan®: C___27301153_10, Applied Biosystems), and 4.5 µl of isolated DNA sample. The PCR conditions are as follows: 60ºC for 30 s; denaturation at 95ºC for 10 min; and 40 cycles of (95ºC for 15 s and 60ºC for 1 min) and final step at 60ºC for 30 s.

2.3. EBV copy number measurement

EBV in tumour tissues were detected and measured by Realtime PCR using the GeneProof EBV PCR kit (Cat. No. EBV/ISEX/100, GeneProof) by targeting the EBNA1 gene, according to the manufacturer's protocol. In total 40 µl PCR reactions mix volume contained 30 µl MasterMix and 10 µl DNA isolated sample using the conditions: 37ºC for 2 min, denaturation at 95ºC for 10 min, and 45 cycles of (95ºC for 5 s, 60ºC for 40 s and 72ºC for 20 s). A positive standard curve of EBV copy number was analysed and calculated with a QuantStudio 3 Real-time PCR system (Applied Biosystems, CA, USA). Quantification of EBV in FFPE tumour tissues were calculated as EBV copy numbers per microgram of DNA (copies/µg DNA).

2.4. Immunohistochemical staining (IHC)

The expression of MICA in NPC tissue samples were assessed by IHC examination using an ultraView Universal DAB detection kit (Ventana, USA). Briefly, paraffin-embedded NPC specimens were sliced, deparaffinized in xylene, rehydrated through a graded ethanol series (100-90-80%), and then blocked with endogenous peroxidase. Subsequently, sections were dipped into rabbit anti-human MICA polyclonal antibody (diluted 1:100, Abcam) for 32 min at 37ºC. Positive and negative controls were also stained following the manufacturer's recommendation. After washing by PBS,

sections were incubated with ultraView Universal HRP Multimerto primary detection (Ventana, USA) and then visualized by ultraView Universal hydrogen peroxide (Ventana, USA) and ultraView Universal DAB (Ventana, USA) for 8 min at 37ºC. The DAB was toned by the addition of ultraView Universal Copper (Ventana, USA) for 4 min at 37ºC before specimens were counter-stained with hematoxylin II (Ventana, USA) and then incubated with Bluing Reagent (Ventana, USA) under the same condition. The sections were rinsed by a detergent-water mixture, dehydrated through a graded ethanol series, cleared with xylene, covered by Richard-Allan Scientific Mounting Medium (ThermoFisher Scientific, USA), manually cover-slipped, and finally observed under the microscope.

2.5. Quantitation of MICA protein level on IHC

MICA protein was expressed on the cell membrane of the epithelial cells. Levels of MICA expression were determined by assessing the staining intensity and the percentage of positively stained epithelial cells. The staining intensity and the percentage of positively stained cells were scored following the Allred scoring system, as shown in Table 1. The overall MICA expression score was calculated by adding intensity and percentage score results, which ranged from 0 to 8P. In total, 70 slides were assessed and evaluated by two independent pathologists at Hanoi Medical University after using a blind number code. Conflicting scores were not displayed. Scores of 0 to 5 are considered low expression and scores of 6 to 8 are considered high expression.

Table 1. Allred scoring system.

Positive cells (%)	Proportion score (A)	Intensity	Intensity score (B)
0	0P	None	0P
<1	1P	Weak	1P
1 to 10	2P	Intermediate	2P
11 to 33	3P	Strong	3P
34 to 66	4P	Final score: A+B Range: 0-8P	

2.6. Statistical analysis

Differences in EBV copy numbers based on MICA expression levels associated with the rs2596542 genotype in NPC tissues were calculated using the Student's T-test. In all statistical analyses, $p<0.05$ was considered to indicate a statistically significant difference. All tests were performed with the GraphPad Prism 8 software package (San Diego, USA).

3. Results

3.1. Main characteristics of the study

Among the 70 enrolled patients, 68.57% were male and 31.43% were female. Histopathology of all subjects showed two subtypes of NPC accounting for 82.86% in undifferentiated carcinoma type and 17.14% in squamous cell carcinoma type. Among participants, the average age of participants was 51.73±13.04. All patient characteristics are listed in Table 2.

Table 2. Study population characteristics.

Sex	Proportion/Amount
Male	48 (68.57%)
Female	22 (31.43%)
Histopathological	
Undifferentiated carcinoma	58 (82.86%)
Squamous cell carcinoma	12 (17.14%)
Age (Mean±SD)	51.73±13.04
Total	70

3.2. Frequencies of MICA rs2596542 variant in NPC

The genotype frequencies for the MICA rs2596542 variant in our study were analysed in 70 NPC patients and are described in Table 3. The haplotype frequencies of CC, CT, and TT were 40, 41.43 and 18.57%, respectively. These frequencies were obtained following the Hardy-Weinberg equilibrium (p=0.7).

Table 3. Allele and genotype frequencies of MICA rs2596542 in NPC patients.

Total (n=70)	Genotype			Allele	
	CC	*CT*	*TT*	*C*	*T*
	28 (40%)	29 (41.43%)	13 (18.57%)	85 (60.71%)	55 (39.29%)

3.3. Association between MICA expression and EBV measurements according to rs2596542 genotype

MICA expression in immunohistochemistry was indicated in low and high expression groups by the Allred-scored system. The expression of MICA showed a significant difference with regard to EBV copy numbers ($p<0.01$) (Fig. 1A). High expression of MICA presented a lower significance of EBV status. In addition, in comparison with the different rs2596542 genotypes, patients who carried homozygous CC displayed a high correlation with EBV copy numbers ($p<0.001$) while no significant difference was recorded in the other (Fig. 1B).

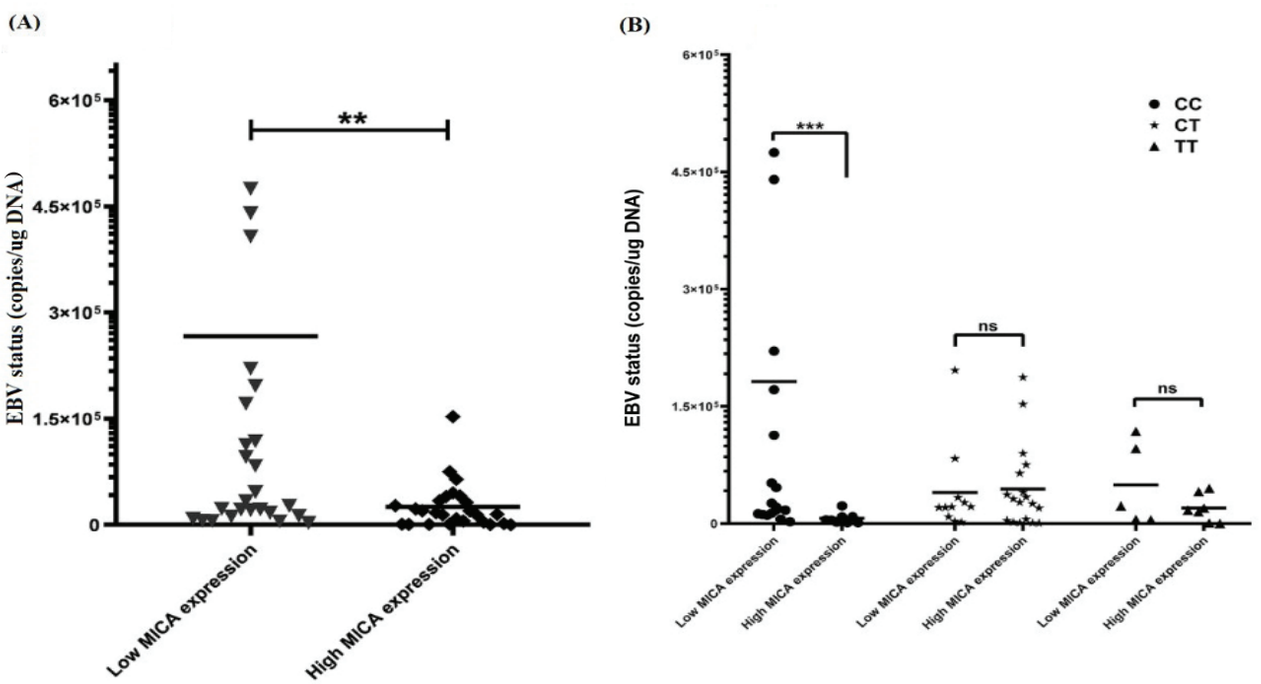

Fig. 1. EBV copy numbers in NPC tissues associated with MICA expression and association with rs2596542 variant. Each dot represents the EBV copy numbers (copies/μg of DNA) of each tumour sample by quantification Realtime-PCR. **(A)** Association between the expression of MICA and EBV copy numbers. **(B)** EBV copy numbers in NPC tissues according to MICA expression related to rs2596542. ns: not significant. **p<0.01 and ***p<0.001.

4. Discussion

Here, we evaluated the correlation between the expression level of MICA and EBV copy numbers in NPC tissue. We found that low MICA expression was associated with higher EBV copies, which suggest that EBV in tumour cells could contribute to regulating MICA expression. MICA is a member of the NKG2D ligand family and expresses broadly in human malignancies and virally-infected tissues. High expression of MICA can increase the cytotoxic activities of effector cells and the subsequent lysis of tumour cells. Numerous regulators have been elucidated to modulate MICA expression including viral infection, oxidative stress, heat sock, and the NF-κB pathway [19-21].

We determined MICA expression was associated with EBV levels among those with CC haplotype but found no significant difference between allele C and T of rs2596542. High expression of rs2596542 CC on the cell surface can inhibit virus infection, which causes the low copy number of EBV in NPC tumours. MICA can be shed on the infected cell surface and thus can lead to downregulating expression and reducing the recognition of NK cells and T-CD8$^+$ cells. H.V. Tong, et al. (2013) [22] postulated the hypothesis that the SNP rs2596542 in the promoter region of MICA has a significant association with MICA expression. Various studies have demonstrated the genetic variant of rs2596542, but their model was mainly focused on patients having HBV or HCV infection and liver cancer. By GWAS analysis, rs2596542 was considered as the molecular marker for HCC. Allele T of rs2596542 showed a higher risk factor than allele C in the occurrence of HCC-induced HCV. A.A. Mohamed, et al. (2017) [23] indicated that patients who carried allele T had a significantly higher risk for HCC (p=0.013, OR=1.93) while allele C was observed to reduce HCC risk. In our data, rs2596542 C (57/70) has a higher frequency among NPC patients suggesting a protective role of allele C against the infection of EBV. These results were in agreement with X.J. Kuang, et al. (2019) [24]. Meta-analysis of the relationship between rs2596542 genotype and the risk of HCC indicated that a higher homozygous rs2596542 CC proportion among healthy controls could indicate a protective effect of the CC genotype in progressive HCC. By contrast, several studies indicated that minor allele T of rs2596542 also presents a protecting effect on the HCC progression in Caucasians [25, 26]. However, we did not observe any significant difference between allele C and T of rs2596542 in our study, which may be related to ethnic characteristics.

Our data suggest that rs2596542 may be a possible genetic target for MICA shedding. Moreover, low expression of rs2596542 CC may be associated with MICA shedding, thus leading to EBV immune evasion by increasing EBV copy number in the tumour cells. To date, the genetic mechanisms for MICA shedding are still ambiguous. MICA shedding contains multiple proteases and has a crucial role in the immunotherapeutic target of cancer. These processes are related to several factors including MICA alpha-3 domain, ERp5 and protease of which the alpha-3 domain is a key proteolytic cleavage initiator. After ERp5 removes a disulfide bond between the amino acid residues 202 and 259, the alpha-3 domain is unfolded and thus exposed to the proteolytic cleave site [27]. Next, under metalloproteases, MICA is cut in somewhere or flank of the stalk in the alpha-3 domain, thus releasing the entire extracellular portion of MICA including the alpha-1 and alpha-2 domains that are the NKG2D binding sites. Using a monoclonal antibody, MICA shedding can be inhibited by blocking the alpha-3 domain that will activate NK cells to secrete IFN-γ and TNF-α thus killing infected cells [28]. Our data suggest that rs2596542 may be a possible genetic target for MICA shedding. Further study should be performed to clarify the contribution of rs2596542 to the shedding of MICA. This application will be a promising strategy for cancer immunotherapy.

On the other hand, MICA expression can be prevented by soluble MICA (sMICA). Overexpression of sMICA in circulation induces a decrease in NKG2DL expression on the cell surface, which results in suppressing NKG2D-mediated anti-tumour immunity [29]. Higher sMICA can have a higher rate of vascular invasion and poorer survival. Additionally, the high TMN (III/IV) stage of HCC patients shows higher levels of sMICA than patients with low TMN stage (I/II) of HCC [30]. Allele rs2596542 T has been demonstrated to be strongly associated with the low-level production of sMICA indicating an important role as a tumour suppressor [17, 31]. P.H.Y. Lo, et al. (2013) [32] explored that the carrying allele T of rs2596542 had a higher significant sMICA level in serum than those of allele C (p=0.00016). V. Kumar, et al. (2012) [31] found a significant level of sMICA in HBV-induced HCC patients. Thus, rs2596542 T was significantly associated with increased sMICA levels (p=0.009). Therefore, the genetic variation of MICA rs2596542 can be applied as a predictive risk biomarker for virus susceptibility to cancers.

5. Conclusions

In this study of 70 patients with EBV-positive NPC, our study indicated that EBV in tumour cells might contribute to regular MICA expression. The patients harbouring the CC genotype of rs2596542 MICA showed a greater immune response against tumours with low EBV concentration than tumours with high EBV concentration. This suggests a protective role of rs2596542C with the infection of EBV among NPC patients. Moreover, SNP rs2596542 may be a possible genetic target for immunotherapy as it is related to the shedding of MICA, which can cause EBV immune evasion. Further studies should be conducted to clarify the affection of rs2596542 to MICA shedding. Our results can be used to investigate the role of rs2596542 as a new predictive biomarker for EBV susceptibility to NPC.

CRediT author statement

Thanh Dat Ta: Data curation, Writing - Original draft preparation; Khanh Tran Van: Visualization, Investigation; Thanh Binh Nguyen: Software, Validation; Manh Thuong Le: Conceptualization, Methodology, Software; Long Ha Le Hai: Writing - Reviewing and Editing; Hoang Viet Nguyen: Supervision.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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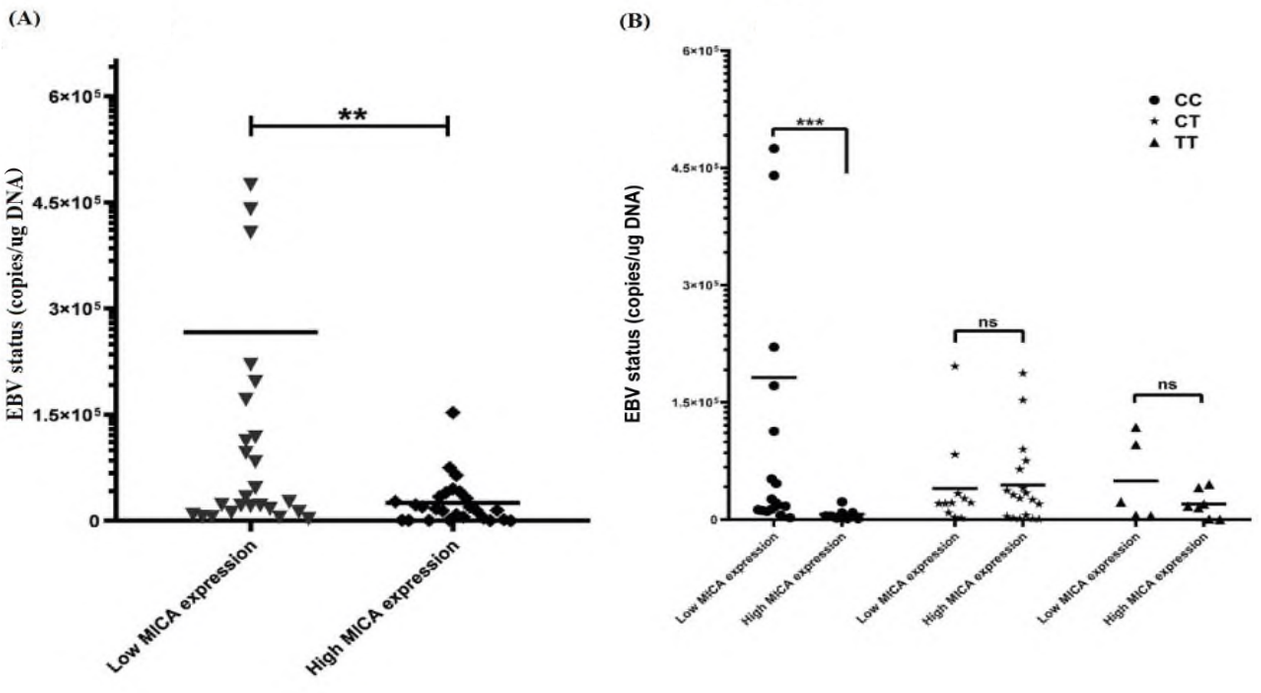

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