ELSEVIER

European Journal of Cancer

Volume 50, Supplement 4, May 2014, Page e64

P0192 The concentration of soluble mica as prognostic factor in nasopharyngeal carcinoma

H. Ratnawati [a], S. Mubarika [b], I. Astuti [b], B. Hariwianto [b], D. Retnoningrum [c]

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https://doi.org/10.1016/j.ejca.2014.03.236

Background

In Indonesia, nasopharyngeal carcinoma (NPC), a malignancy of the head and neck, has a high mortality rate because patients are usually diagnosed at an advance stage and, in some, metastasis has even occured. When diagnosed at an early stage, 5-year survival rates can reach more than 80%, but at advance stages, 5-year survival rate is only 10-40%. Epstein-Barr virus is associated with the pathogenesis of NPC. These viral-induced carcinoma express the stress surface protein molecules MHC class I-related chain A (MICA), part of an important activation pathway to trigger the immune system to attack tumour cells. However, the virus and cancer cells might downregulate the expression of membrane-bound MICA (mMICA) molecules and increased the soluble MICA (sMICA) concentration, therefore reducing the immunogenicity of the tumour cell and decreasing the prognosis. We aimed to determine the concentration of sMICA as prognostic factor in NPC.

Methods

We immunohistochemical analysis to determine mMICA and ELISA to determine sMICA concentration from the sera. We used Kruskal-Wallis and Mann-Whitney test for analysis.

Findings

mMICA expression was present in all NPC tissue (100%) in various concentrations. Mean sMICA concentrations was 245.8 pg/mL in NPC stage II, 369.9 pg/mL in stage III, and 464,8 pg/mL in NPC stage IV. The higher the sMICA concentration, the higher the NPC stage. There was no significant difference in sMICA concentration between stage III and stage IV NPC (p=0.344).

Interpretation

Increased concentration of sMICA protein is a prognostic factor in NPC.

Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common head and neck carcinomas with a unique distribution pattern. It seems that some ethnic groups are prone to this carcinoma. According to the International Agency for Research on Carcinoma report in 2008, more than 80% of patients with nasopharyngeal carcinoma are in Asia, especially East and Southeast Asia. In Indonesia, the prevalence rate is 6.2/100,000 people with 12,000 new cases per year and ranked as the world's second-highest mortality rate of nasopharyngeal carcinoma. Nasopharyngeal carcinoma is more common in men with a ratio of 2.4 to 1 and is commonly associated with the productive age population; therefore, the incidence of nasopharyngeal carcinoma will affect the economic condition of the patient and the state health financing (1-2).

The pathogenesis of NPC is a multifactorial process in which chronic Epstein-Barr virus (EBV) infection, genetic susceptibility, and environmental risk factors, including certain dietary factors, play an essential role in the pathogenesis (3-5). EBV genome was detected in almost all NPC cases. It encoded viral proteins that could precede malignant transformation, especially in undifferentiated nasopharyngeal carcinoma (NPC WHO type II and type III) with linkages close to 100% (6-8).

NPC diagnosis is often delayed due to the hidden location of the tumor and the atypical symptoms in the early stages. As a result, most patients are diagnosed with an advanced stage, with a survival rate of less than 50%, which results in a high mortality rate (9)**.** Furthermore, population screening through serology of EBV antibody and endoscopy has not been satisfactory since almost all human adults carried the virus.

Cellular stress such as infected viral cells or tumor cells will express a protein known as MHC class I chain-related gene A (MICA) (10-13). In some malignancies, MICA is expressed on the cell surface known as membrane-bound MICA (mMICA), but through proteolysis, this molecule could also be found in the serum as soluble MICA (sMICA). The release of sMICA will be accompanied by the decrease of mMICA expression on the carcinoma cell surface; it means a reduction in the activity of the immune cells against carcinoma cells because the MICA molecule is a ligand for immune cells activation receptor against carcinoma cells. That is part of a carcinoma immune evasion mechanism (14-15). Since NPC is a complex disease caused by an EBV chronic infection (6-7) and MICA as the primary molecule produced by carcinoma cells (16), we thus sought to analyze the concentration profile of MICA protein in healthy people with and without EBV infection and nasopharyngeal carcinoma patients, to determine whether it can predict malignant transformation in a healthy individual with EBV infection and as a prognostic factor in NPC patients. In addition, this study also determines the correlation between sMICA concentration and mMICA expression in NPC patients at various stages.

Materials and Methods

Clinical data and sample collection

Serum samples were obtained from Molecular Biology Laboratory, Gajah Mada University, and the paraffin blocks from the biopsy tissue of the same patients were obtained from Pathology Department, Sardjito Hospital. From all samples that met the inclusion criteria (untreated NPC patients, NPC patients before surgery, chemotherapy, or radiotherapy and with positive EBV infection), 150 µL of serum was taken and then stored minus 20°C until the examination of sMICA concentration. As the control group for the sMICA assessment, we collected the serum from the healthy adult donor. The number of samples required for each NPC patients group and control group was 21 subjects (Calculated using *software version* designed by KC Lun and Peter

Chiam, *National University of Singapore)*. Peripheral blood was collected after obtaining informed consent and got the approval for using the patients and controls material from the Research Ethics Committee of the Gadjah Mada University.

We had difficulties obtaining stage I NPC patients. There was only one patient with stage II NPC; therefore, statistical analysis of sMICA and mMICA only from NPC stage III and stage IV. Clinical information (age, gender, tumor size, tumor grade of differentiation, tumor location, disease stage, and regional lymph node metastasis) was derived from the patients' medical records. Primary tumor size, lymph node status, and disease stage grouping were classified according to the 1997 UICC criteria.

ELISA

Before the MICA concentration test, the control individuals' blood serum was evaluated to determine the EBV infection by sandwich ELISA (IgA VCA p18 + EBNA1 test) at Molecular Biology Laboratory Gadjah Mada University (Yogyakarta, Indonesia). The sera from the healthy control group were divided into two groups, as healthy control with EBV positive (HC EBV+) and healthy control with EBV neg (HC EBV-), each group consisting of 21 sera.

The soluble MICA (sMICA) examination from 42 sera NPC patients and 42 sera healthy individuals was determined by sandwich ELISA technique with ELISA kit from BAMOMAB (Munich, Germany). Plates were coated with the capture anti-MICA monoclonal antibody (mAB) AMO1 at 2 µg/ml in phosphate-buffered saline solution (PBS), then blocked by the addition of 100 µl of 15% bovine serum albumin (BSA) for 2 hours at 37°C and washed. Next, the serial dilutions standard (recombinant soluble MICA_04 in 7.5% BSA-PBS) and 100 ul diluted samples (1:10 in 5% BSA) for each plate, incubated for 2 hours at 37°C, washed. Next, for monoclonal antibody detection, incubated with anti-mouse IgG2a– horseradish peroxidase (1:8000 in 7.5% BSA-PBS) at 37°C for 1 hour (R&D Systems, Minneapolis, MN, USA). Finally, washed the plates, and development was done using tetramethylbenzidine peroxidase substrate system (R&D Systems, MN, USA), then added 100 ul 1 M phosphoric acid, and absorbance was measured at 450 nm wavelength.

MICA *Immunohistochemistry*

To determine the correlation between the level of sMICA and the intensity expression of mMICA, we did the MICA immunohistochemistry towards the NPC tissue from the same patients. The anti-MICA SR99 antibody was kindly provided by S.Caillat-Zucman, Laboratoire d'Immunologie, Hospital Saint-Vincent de Paul, Paris, France.

Immunohistochemistry was performed on 4 µm-thick paraffin sections of NPC tissue to evaluate MICA expression. Sections were deparaffinized and endogenous peroxidase was blocked with 1.5% H2O2 in phosphate-citrate buffer, pH 5.8. Antigen retrieval was performed at 100°C in citrate buffer, pH 6.0, for 20 minutes. Sections were subsequently incubated with the anti-MICA SR99 mouse monoclonal antibody with the concentration of 1:100 for 1 hour at room temperature (17). After washing with phosphate-buffered saline, sections were incubated with Envision System/HRP for 30 minutes (DAKO Cytomation, Glostrup, Denmark). Visualization was performed with the highly sensitive substrate-chromogen system DAB (DAKO) and counterstained with Mayer's hematoxylin. Intestine tissue material was used as a positive control for MICA expression. The tissue used as a negative control was incubated with an isotype-matched control Immunoglobulin.

Two investigators examined histological evaluation and scores in a random blinded manner to ensure accuracy of quantification of immunohistochemical slides using the

same scale subsequently counted the positive cells. A total of 300 cells were examined in at least three areas, and determined the percentage of positive cells was confined to one of the following categories: (a) --: none; (+): less than 25% positive cells; (++): 25-75% positive cells; (+++): more than 75% positive cells (18). After the independent evaluation, differences between the two persons were re-evaluated in the presence of a third party. The sMICA and mMICA analysis were examined in Medisch Centrum Vrije University, Amsterdam, Netherland.

Statistical Analysis

The difference in the distribution of soluble MICA levels between the NPC patients and healthy control subjects with EBV positive and EBV negative were analyzed with nonparametric analysis, Kruskal-Wallis dan Mann-Whitney Test with 95% confidence interval ($p \leq 0.05$) was considered to be statistically significant. Spearman's rho nonparametric correlations were used to analyze the correlation between mMICA expression and sMICA concentration.

Results

The result showed that the characteristics of the NPC patients aligned with the previous research, in which men have 2.8 times NPC incidence higher than women. In NPC stage III, 6 patients (28.6%) range between 41 – 50 years old, with an average age is 47.3 years. In stage IV, the highest range is between 51 – 60 years old (52%), with an average age is 50.6 years. During this study, there was no stage I patient and only had one patient with stage II NPC; therefore, stage I and stage II were excluded from the statistical analysis.

The clinical result showed that 50% of the tumor had invaded the paranasal sinuses or the bone structure (T3); even 19.1% were T4 which means that cancer has metastasized to the intracranial or the central nervous, infratemporal fossa, hypopharynx, or eyeball. Nodal status showed that 88% had metastasized to the lymph nodes, either unilateral (21.4%) or bilateral (66.6%). All histopathologic features in this study were undifferentiated type, and 71.4% of the tumor was located bilaterally.

Table 1. Clinical features of NPC patients

Parameter	Frequency (%)
Range of Age (yrs)	
Stage III	47.3 (25-76)
Stage IV	50.6 (18-70)
Gender (Men/Women)	31/11 (74/26)
Tumor Size	
T1	8 (19.0)
T2	5 (11.9)
T3	21 (50.0)
T4	8 (19.1)
Lymph Nodes	
N0	5 (11.9)
N1	9 (21.4)
N2	16 (38.0)
N3	12 (28.6)
Stage	
III	21 (50.0)
IV	21 (50.0)
Tumor Differentiation	
Keratinizing squamous cell ca	0 (0.0)
Non-keratinizingsquamouscell ca	9 (0.0)
Undifferentiated carcinoma	42 (100.0)
Tumor Location	
Right	8 (19.0)
Left	4 (9.6)
Bilateral	30 (71.4)

The level concentration of sMICA

The average concentration of sMICA in HC EBV- was 96 pg/ml compared to sMICA in HC EBV+, which was 136.8 pg/ml and statistical analysis showed a significantly different ($p = 0.027$). It means that EBV infection causes the increase of sMICA concentration. The average concentration of sMICA in NPC stage II (only one patient), stage III, and stage IV NPC are 245.8 pg/ml, 369.9 pg/ml, and 464.8 pg/ml, respectively. The result clearly showed that NPC stages and the level of sMICA concentration are

linear; as the NPC stages increases, sMICA concentration increases too.

In NPC IV, seven samples (33.3%) have higher sMICA concentration than the average concentration (464.8 pg/ml), and it turned out that 6 of these samples were NPC stage IVB. The higher the NPC stage (from stage IVA to IVB) also caused the higher the sMICA concentration. The highest concentration of sMICA (1314.6 pg/ml) in NPC stage IV was sample number 6, in which the patient was a 68 years old man, T3N3aM0 (NPC stadium IVB), undifferentiated and bilateral location.

In stage III NPC, the highest concentration was 767.3 pg/ml, a man 65 years old, T3N0M0, undifferentiated and bilateral. On the contrary, the lowest sMICA concentration was 181.9 pg/ml, a woman 28 years old, undifferentiated and location only at extra. Statistical results showed that p = 0.171, which means no significant difference in sMICA concentration between NPC III and NPC IV. To see whether the increased concentration of sMICA could predict a malignant transformation, did statistical analysis towards sMICA concentration in healthy control and NPC patients. The lowest sMICA concentration was showed for HC EBV- (96 pg/ml), followed by HC EBV+ (136.8 pg/ml), NPC stage II (245.8 pg/ml), NPC stage III (369.9 pg/ml) and the highest is NPC stage IV (464.8 pg/ml). Statistical analysis showed that sMICA concentration in healthy control (both HC EBV- and HC EBV+) was a highly significant difference compared to NPC patients stage III and stage IV with p = 0.000.

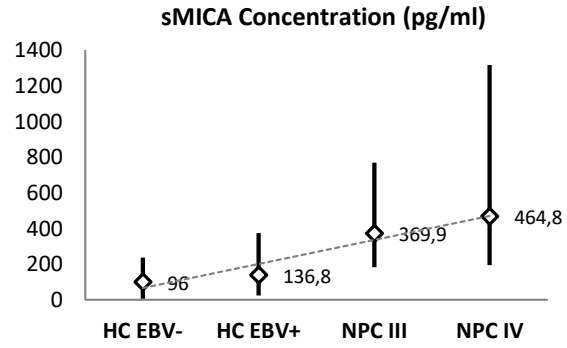

Fig 1. Soluble MICA concentration in healthy control (HC EBV-) and HC with EBV infection (HC EBV+) and NPC patients (stage III and IV).

It clearly showed that the concentration of sMICA in healthy control either without EBV infection or with EBV infection is significantly different from the sMICA concentration of NPC patients.

The graph showed that the lowest concentration of sMICA in NPC III was 181.9 pg/ml and in NPC IV was 192.2 pg/ml. However, compared with the average sMICA concentration in HC EBV+ (136.8 pg/ml), it remains higher in patients with NPC. From the data obtained, the increasing value of sMICA concentration in people with EBV infection can be a marker of the development toward malignancy.

***Expression of* mMICA *on NPC patients and the correlation with* sMICA.**

Analysis of mMICA expression was performed on NPC stage III and NPC stage IV tissue from the paraffin blocked. The result showed that all NPC tissue expressed mMICA but with different percentages. Statistical analysis towards the expression of mMICA in NPC stage III showed that p = 0.806, which means that there was no significant difference in mMICA expression between NPC III and NPC IV. It is consistent with the result from sMICA concentration, where there was no significant difference between NPC III and NPC IV (p=0.473).

Spearman's rho nonparametric correlations between the level of sMICA concentration and expression of mMICA showed a negative correlation between sMICA concentration and mMICA expression with the coefficient correlation in NPC stage III and IV are -0.842 and -0.860. It means the increased concentration of sMICA will be followed by the decreased expression of mMICA.

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Discussion

The results showed that the epidemiological characteristic of NPC patients was aligned with the previous studies. Men are 2.8 times higher than women, and the average age of the patients was 47.3 years for NPC stage III and 50.6 years for NPC stage IV. This result is consistent with previous results, which stated that the number of NPC patients in men is 2 – 3 times higher than women with the highest frequency between 40 – 60 years old (3-5). In Indonesia, the average age of NPC patients was 45.5 years (1).

Clinical features showed that as many as 50% of tumors invaded the paranasal sinuses or invaded the bone structure (T3), and 19.1% of intracranial tumors had spread to the intracranial or the central nervous, infratemporal fossa, hypopharynx, or eyeball (T4). It is mainly due to the difficulties in diagnosing NPCs that cause patients to come to the hospital with an advanced stage because NPC has no specific symptoms, and the location of the tumor, which is generally in the fossa Rosenmuller and Eustachian tubes are difficult to detect. Lymph node status in this study showed that 88.1% had metastasized to the lymph nodes either unilateral (23.8%) or bilateral (64.3%). Swollen lymph nodes are often the primary symptoms that cause the patient to come for treatment. This is consistent with the meta-analysis research by Ho (2012) that 85% of NPC patients had metastasized to the lymph nodes of the neck (19).

The histopathologic features of all NPC samples in this study are the undifferentiated type because almost 95% of NPC cases in endemic areas are non-keratinizing undifferentiated carcinoma. The tumor location is 71.4% bilateral.

This study examined IgA VCA p18, and EBNA1 EBV as the parameters of a person who has been infected with EBV. Viral capsid antigen (VCA) is an antigen protein expressed by the infected EBV cells when virus particles are released during lytic infection (20). The results showed that 97.6% (41 samples) of NPC patients showed positive results. Then we change the EBV negative sample (1 sample) with the EBV positive to fulfill the inclusion criteria. Fachiroh (2009) found that 90.7% out of 151 NPC patients indicated positive results in IgA/EBNA1+VCA-p18, whereas Cao found that the sensitivity is 95.29% and specificity 94.07% (21-22). It is evidence that EBV plays a vital role in the pathogenesis of NPC. Furthermore, EBV infection is one of the highest etiologic factors in the pathogenesis of NPC in people with immunosuppressive conditions, especially on undifferentiated nasopharyngeal carcinoma, with a correlation close to 100% (1,6).

According to Houali, the viral proteins neither play a role in epithelium oncogenesis nor immunosuppression. EBV can express several types of protein that can protect itself from cytotoxicity by the immune effector cells. EBNA1 protein can decrease the

expression of MHC-I, which causes the virus to remain in the cell and avoid cytotoxicity by T CD8+ cells. EBNA1 protein is also an antiapoptosis which causes the virus to stay in the cell and long life persistence (23).

Other EBV protein that influences the progression of NPC is LMP1 and LMP2. LMP1 protein may increase the secretion of IL-8, and it will increase the secretion of matrix metalloproteinase (MMP), especially MMP2 and MMP9, which can cause the degradation of the extracellular matrix. MMP is an enzyme that causes proteolytic shedding in membrane-bound MICA, specifically in the ectodomain of mMICA in which mMICA will be released as soluble MICA. MMP2 protein increases cell movement in the extracellular matrix and reduces adhesion between cells. It can also cause degradation of NKG2D receptor as well as mMICA release become sMICA (24-26).

Soluble MICA caused the degradation and internalization of the NKG2D receptor, resulting in decreased expression of NKG2D on NK cells, CD8+ T cells, and γδ T cells as the immune effector cells. Carcinoma cells also release TGF-β, leading to down-regulating NKG2D receptor and MICA ligand. The virus can also bind to MICA molecules and hold these molecules inside the cell; with consequences, the MICA expression on the cell surface decreases. LMP1 may also increase the production of IL-10 by T regular cells, which inhibit T cell proliferation and IFN-g secretion (27-28).

This study showed that all NPC tissues, with a total of 42 specimens, express mMICA but with different frequencies and intensities. However, there was no significant difference in mMICA expression between stage III and stage IV NPC. This study differs from Vetter's study about the expression of mMICA on melanoma tissue, which found that out of the 40 melanoma tissues examined, only 31 (77.5%) expressed mMICA and exhibited variability in frequency and intensity. This mMICA expression is related to tumor invasion since the negative results were found in benign tumors. Vetter concluded that mMICA expression is associated with the beginning of the malignancy transformation (18). The tissues that express mMICA are also infiltrated by immune cells with NKG2D receptors, so it can say that the interaction of NKG2D/MIC has a role in carcinoma immuno-surveillance in the uveal melanoma primary tumor tissue (29). This study found mMICA expression throughout all the NPC tissue (100%), both for NPC stage III and stage IV. It might be because NPC is a malignancy of epithelial tissue, and the virus (EBV) plays a role in its pathogenesis, causing mMICA to be expressed with high frequency on NPC. As it has been said by Mistry (2007) MICA ligand is often found in epithelial tumors, and one of the factors affecting the mMICA expression is viral infections (30).

As with mMICA results, sMICA concentrations also increase along with higher diseases stage. Statistical analysis showed a highly significant difference ($p = 0.000$) between healthy individuals and NPC patients stage III and IV. The lowest sMICA concentration was in healthy control with EBV negative (96 pg/ml), followed by healthy control with EBV positive (136.8 pg/ml), NPC II (only one sample at a concentration of 245.8 pg/ml), NPC III (369.9 pg/ml) and NPC IV (464.8 pg/ml). The increase of sMICA concentrations indicates the increasing stage of NPC or worsening disease progression.

Previous studies on various types of malignancies also showed similar results. A study on neuroblastoma patients showed that the sMICA concentration was significantly different compared to healthy individuals ($p<0.0001$), with a median value of sMICA in neuroblastoma, was 120 pg/ml, while in healthy people 10 pg/ml (31).

The sMICA study at various stages of cervical carcinoma patients found that higher concentrations of sMICA in cervical carcinoma were highly significant difference compared to healthy individuals ($p<0.001$), and the research also found a significant

positive correlation between sMICA concentration and disease severity (32). A study on Oral Squamous Cell Carcinoma found a correlation between the sMICA concentration with stage and disease progression (33). Research on prostate carcinoma patients showed a correlation between the sMICA concentrations with disease progression (34).

Holdenrieder, who examined 512 patients with various carcinomas, found that there was a correlation between high concentrations of sMICA and sMICB with the stages of the disease. In contrast, Reinders *et al*., who studied various malignancies of the head and neck (head and neck squamous cell carcinoma but without NPC patients), said that sMICA concentration on the patients was not significantly different compared to the concentration on healthy people (15,35).

The high concentration of sMICA in these NPC patients can be due to excessive LMP1-induced matrix metalloprotease (MMP2) activity. LMP1 stimulates this enzyme to break down the surrounding matrix and allow the invasion of tumor cells into surrounding tissues and to the circulatory system. LMP-1 is the most oncogenic part of the EBV virus and acts as an initiator. Other viral proteins, LMP-2A and EBNA, regulate viral genomic transactivation with host genes and lead to downregulation of various host genes that affect oncogenesis (4,25,36).

Tumor cells and viruses develop a mechanism to protect them from NKG2D signaling to continue tumorigenesis. The tumor cells that express membrane-bound MICA (mMICA) will be down-regulated by proteolytic shedding mediated by metalloproteases secreted by tumor cells. This proteolytic leads to the release of the soluble form of the MICA ectodomain as an immune escape mechanism of tumor cells. The release of sMICA will be accompanied by decreased mMICA expression on the surface of tumor cells, leading to a decrease in stimulatory signals in cytotoxic T cells. Membrane-bound MICA stimulates antitumor immunity, whereas sMICA suppresses antitumor immunity. Soluble MICA also causes decreased NKG2D expression on the surface of CD8+ T cells and γδ T cells, thus inhibiting the antitumor activity of the immune cells (14,15). This result indicates that sMICA plays a vital role in an immune invasion against tumors. The virus also developed a mechanism to avoid signaling NKG2D by binding and holding the ligand inside the cell, thus reducing its expression on the cell surface (37-38).

Conclusion

The sMICA concentration in healthy individuals was significantly different from NPC patients. Therefore, it can be concluded that the increase of sMICA concentrations in individuals with EBV infection may function as a prognostic marker to predict the transformation towards NPC.

The limitation of this study is that we could not provide the sera samples from stage I and stage II NPC patients. Therefore, further detailed studies are still needed with more samples to investigate the cut-off point level of sMICA.

Conflict of Interest

All the authors hereby disclose that there is no conflict of interest.

Acknowledgments

The authors thank the staff of Molecular Biology Gadjah Mada University for providing sera samples; S.Caillat-Zucman, Laboratoire d'Immunologie, Hospital Saint-Vincent de Paul, Paris, France for providing anti-MICA SR99 antibody; Dr. Mary von Blomberg and Prof. dr. E. Bloemena and all the staff of Immunology and Pathology Department Vrije Universiteit Medisch Centrum, Amsterdam, Netherland, have allowed and assisted me in doing research.

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