Plasma Macrophage Inhibitory Cytokine-1 as a Complement of Epstein-Barr Virus Related Markers in Identifying Nasopharyngeal Carcinoma

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Abstract

Background: We evaluated the diagnostic value of plasma Macrophage inhibitory cytokine-1 (MIC-1) in distinguishing patients with nasopharyngeal carcinoma (NPC) and explored its complementary role with widely used Epstein-Barr virus (EBV) related markers, EBV capsid antigen-specific IgA (VCA-IgA) and EBV copy number. Methods: ELISA was used to analyze the plasma MIC-1 levels in 190 NPC patients, 72 VCA-IgA-positive healthy donors (VP), and 219 normal subjects with negative VCA-IgA (VN). 10 pairs of plasma samples before and after radiotherapy were also included. Results: The plasma MIC-1 levels were significantly higher in NPC patients (Median: 678.39 ng/mL) than those in VN and VP (310.29 and 294.59, \(p < 0.001\)). Receiver operating characteristic (ROC) curves of the MIC-1 concentrations revealed that the area under the ROC curve (AUC) was 0.790 (95% confidence interval [CI]: 0.748-0.832), with a sensitivity of 63.7%, and a specificity of 85.9% respectively, for distinguishing NPC patients from the healthy donors. Similarly, between NPC and VP, ROC was 0.796 (0.738-0.853) with sensitivity of 63.7%, and specificity of 88.9%. In addition, between NPC and VN, ROC was 0.788 (0.744-0.832) with sensitivity of 63.7%, and specificity of 84.9%. Further, we found that MIC-1 could complement VCA-IgA and EBV DNA markers, with a negative rate of 88.9% in VCA-IgA-positive healthy controls, and a positive rate of 59.0% in EBV DNA-negative NPC patients, respectively. Also, the MIC-1 plasma concentration dropped significantly after radiotherapy (\(p = 0.027\)). Conclusions: MIC-1 can complement VCA-IgA titers and EBV DNA copy number tests in NPC detection, improve identification of EBV DNA-negative NPC patients, and distinguish NPC from VCA-IgA-positive healthy controls.

Keywords
MIC-1, nasopharyngeal carcinoma, VCA-IgA, EBV DNA, radiotherapy

List of abbreviations
AUC, area under the ROC curve; CI, confidence interval; EBV, Epstein-Barr virus; MIC-1, Macrophage inhibitory cytokine-1; NPC, Nasopharyngeal Carcinoma; NPV, negative predictive value; PPV, positive predictive value; ROC, Receiver operating characteristic; Sen, sensitivity; Spe, specificity; TGF-\(\beta\), transforming growth factor beta; VCA-IgA, EBV capsid antigen-specific IgA; VP, VCA-IgA-positive healthy donors; VN, subjects with negative VCA-IgA.

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Introduction

Nasopharyngeal carcinoma (NPC) is largely endemic in Southeast Asia, specifically Southern China. In these areas, the annual incidence rate of the disease peaks at 50 cases per 100,000 people, almost 25 times higher compared to the rest of the world.\(^1\) Though the etiology of NPC includes both environmental and genetic factors, Epstein-Barr virus (EBV) infection impacts strongly.\(^2\) Therefore, elevated levels of EBV-related antibodies, such as immunoglobulin against the EBV viral capsid antigen (VCA), have been widely used to screen NPC.\(^3\) Also, the circulating EBV DNA has been used for the diagnosis of NPC. However, in isolation, these serological tests are often insufficient in identifying NPC with confidence.\(^4\) Despite remarkable sensitivity, the serology test for VCA is highly unsatisfactory due to a high false-positive ratio.\(^5\) Greater than 20% of NPC patients turn out negative in early antigen (EA) tests, therefore it is challenging to preclude NPC with a negative EA serology result.\(^5\) Several groups observed the presence of EBV DNA in the plasma of NPC patients.\(^6\) They consistently found a low detection rate in healthy subjects, whereas observed high variability in the NPC patients.\(^6\) Moreover, EBV DNA tests used in diagnosing early-stage or recurrence of NPC have been reported of limited use.\(^7\) Thus, developing new NPC diagnostic markers and complementing EBV related markers are imperative.

Macrophage inhibitory cytokine-1 (MIC-1) is a divergent member of the cytokine superfamily transforming growth factor-beta (TGF-\(\beta\)).\(^8\) In humans, MIC-1 is predominantly expressed in the placenta tissue. However, its expression levels can be rapidly elevated by p53, and are also strongly regulated by cytokines such as TGF-\(\beta\) and interleukin-1.\(^9\) This suggests that MIC-1 could act downstream of the signaling pathways, such as the ERK signaling pathway and Akt signaling pathway, involved in the regulation of apoptosis and cell cycle arrest.\(^10\)

Interestingly, in several cancers, such as prostate, breast, and colorectal, the levels of MIC-1 are dramatically upregulated.\(^11\)-\(^13\) Therefore MIC-1 could of great interest in stratification, diagnosis, and prognosis of such diseases.\(^14\) Nevertheless, the diagnostic significance of the blood MIC-1 level in NPC has not been explored yet. Here, we evaluate the diagnostic importance of plasma MIC-1 and assess if it may complement clinically used EBV related markers in NPC. Additionally, we explored if it can be used to monitor the patient’s progress after radiotherapy.

Materials and Methods

Patients and Ethical Statement

The plasma samples from 190 pathologically confirmed NPC patients were drawn during diagnosis at the Sun Yat-sen University Cancer Center before any treatment between January 2017 and March 2018. TNM stage was established based on the 2009 Union for International Cancer Control/American Joint Committee on Cancer staging system for NPC. Patients accompanying another malignancy or skin disease were excluded.

The patient parameters are collected from medical files and displayed in Table 1. Apart from that, we also recruited 72 cancer-free healthy controls with positive VCA-IgA (VP) and followed them up for 6-12 months to rule out inflammation-related diseases and cancer. 219 volunteers undergoing routine physical examinations with negative results comprised the

| Table 1. Levels of MIC-1 and Clinical Characteristics in 190 Untreated NPC Patients. |
|---|---|---|---|
| Characteristics | No. of patients | MIC-1(pg /ML) Median (IQR) | p value\(^a\) |
| Age (yr) | | | |
| < 48 | 94 | 525.70 (295.30-797.10) | < 0.001 |
| ≥48 | 96 | 867.30 (551.50-1448.00) | |
| Sex | | | 0.113 |
| Female | 139 | 699.30 (443.04-1135.28) | |
| Male | 51 | 549.15 (301.74-916.44) | |
| EBV DNA copy number | | | 0.2317 |
| \(\leq 10^3\) | 78 | 640.86 (443.04-1135.28) | |
| \(10^2-10^4\) | 57 | 748.19 (466.24-1070.67) | |
| \(10^4-10^5\) | 37 | 692.92 (352.11-1395.22) | |
| \(\geq 10^5\) | 16 | 648.60 (529.91-1595.15) | |
| VCA-IgA titers | | | 0.5842 |
| ≤1:40 | 72 | 623.35 (316.95-897.67) | |
| ≤1:80 | 27 | 583.64 (316.95-897.67) | |
| ≤1:160 | 73 | 717.52 (393.22-931.36) | |
| ≥1:320 | 18 | 789.26 (424.50-1188.21) | |
| EA-IgA titers | | | 0.9632 |
| ≤1:10 | 64 | 668.44 (404.28-1106.06) | |
| ≤1:20 | 18 | 594.98 (295.62-1202.12) | |
| ≤1:40 | 72 | 689.66 (320.15-966.96) | |
| ≥1:80 | 35 | 692.92 (443.04-966.11) | |
| EBV-DNase antibody | | | 0.1788 |
| Negative | 17 | 533.04 (280.26-1111.21) | |
| Positive | 148 | 700.84 (379.96-1104.14) | |
| Rta-IgG | | | < 0.001 |
| Negative | 26 | 317.52 (246.15-538.02) | |
| Positive | 125 | 748.45 (435.77-1185.98) | |
| Zta-IgG | | | 0.0616 |
| Negative | 36 | 536.86 (278.16-860.03) | |
| Positive | 113 | 673.27 (361.42-1147.92) | |
| pT status | | | 0.276 |
| pT1-3 | 117 | 603.70 (304.30-917.40) | |
| pT4 | 48 | 700.80 (397.40-1102.00) | |
| pTx | 25 | 928.60 (730.80-1318.00) | |
| pN status | | | 0.066 |
| pN 0-1 | 56 | 553.70 (272.60-855.90) | |
| pN 2-3 | 109 | 647.10 (390.50-1010.00) | |
| pNx | 25 | 928.60 (730.80-1318.00) | |
| pM status | | | 0.768 |
| pM 0 | 153 | 624.90 (321.40-931.36) | |
| pM 1 | 12 | 666.00 (314.30-1072.00) | |
| pMx | 25 | 928.60 (730.80-1318.00) | |
| Overall stage | | | 0.817 |
| Stage I-III | 89 | 647.10 (301.90-937.30) | |
| Stage IV | 76 | 599.60 (359.20-982.90) | |
| Stage X | 25 | 928.60 (730.80-1318.00) | |

\(^a\)Kruskal-Wallis test or Mann-Whitney U test, \(p < 0.05\) was considered statistically significant. MIC-1: Macrophage inhibitory cytokine-1; IQR: interquartile range
EBV VCA-IgA negative (VN) group. Samples from the VP and VN groups were collected from the health examination department at our center. The preliminary screening phase included plasma from 20 pathologically confirmed cervical carcinoma, 20 prostate carcinoma, 20 leukemia, 20 NPC, 20 gastric cancer, 20 thyroid carcinoma, 20 colorectal carcinoma patients, and 20 healthy controls. To evaluate the relationship between MIC-1 plasma levels and radiotherapy, we enrolled 10 pairs of samples from NPC patients before and after radiotherapy.

Venous blood samples (3 mL) obtained from all the participants were drawn into EDTA-K2 anticoagulant tubes, centrifuged at 3600 rpm for 8 min, and then stored at -80°C till further use.

Ethics, Consent, and Permissions
This study was reviewed and approved by the Institutional Review Board and Ethics Committee of SYSUCC(GZR2018-147). At the time of patients’ admission, as a General standard procedure at our center, their written informed consent was obtained to use Clinical parameters and collected samples for further studies. The records were anonymous and de-identified before use.

ELISA Assay
Plasma MIC-1 concentrations were measured using a double-antibody sandwich ELISA at room temperature (RT) as per the manufacturer’s instructions (R&D Systems, Minneapolis, MN, USA). Briefly, 96-well microplates (Costar, USA) were coated with mouse anti-human MIC-1 antibody (100 µL/well, 2.0 µg/mL) overnight. After blocking, 100 µL of the standard or serum (5-fold dilution in 3% BSA) were added and incubated for longer than 1 h. Hereafter, 100 µL of the biotinylated goat anti-human MIC-1 antibody (12.5 ng/mL) was added into each well and incubated for 2 h. Then, Streptavidin-HRP (100 µL/well) diluted to 200 times was incubated for 20 min. Finally, the substrate solution (tetramethylbenzidine) was added and the reaction was stopped using 2 N H2SO4. Absorbance was recorded at a dual-wavelength of 450/630 nm. Each plate also contained a standard control (coefficient of variation < 12%).

Immunoenzymatic Assay of Plasma EBV VCA-IgA
Plasma EBV titers were assessed using the classic immunoenzymatic assay (IEA), obtained from the Shanghai Institute of Biological Products. Two independent observers determined the degree of staining.

Real-Time Quantitative Measurement of Plasma EBV DNA
EBV DNA, extracted from the pretreated plasma, was subjected to real-time quantitative polymerase chain reaction to obtain copy number as described previously. The clinically used copy number, 1000 copies/mL, was set as the cut-off level.

Statistical Analysis
Statistical analyses were performed using the GraphPad Prizm 8.0 or SPSS 23.0 (SPSS Inc.) programs. The relationships between the plasma MIC-1 levels and the clinicopathologic parameters, as well as the comparisons of MIC-1 concentration between different groups, were analyzed using the Mann-Whitney U test. The diagnostic ability of MIC-1 was assessed using the area under the receiver operating characteristic (ROC) curve (AUC). The maximal Yuden index value was considered as the cut-off value for MIC-1. Also, sensitivity (Sen), specificity (Spe), positive predictive value (PPV), and negative predictive value (NPV) were applied to evaluate the overall diagnostic performance of MIC-1. The plasma levels of MIC-1, before and after radiotherapy in NPC patients, were compared using the paired t-test. All statistical tests were 2-sided, and p < 0.05 were considered as statistically significant.

Results
MIC-1 Levels in the Preliminary Screening Phase
Blood plasma from 7 cancer subjects was subjected to MIC-1 ELISA assay along with healthy controls. The Mann-Whitney U test revealed that MIC-1 levels were significantly elevated in prostate cancer, colorectal cancer, and NPC but not cervical cancer, gastric cancer, thyroid carcinoma, and leukemia (Figure 1A). Interestingly, MIC-1 levels were particularly high in NPC, signifying its importance in NPC detection.

Plasma MIC-1 Levels in NPC and Its Association With Clinicopathological Characteristics
We further explored the diagnostic role of MIC-1 in NPC. The plasma concentrations of MIC-1 in 3 groups (VN: n = 219, VP: n = 72 and NPC: n = 190) are presented in Figure 1B. MIC-1 levels were upregulated in NPC patients compared to both VN group (p < 0.001) and VP group (p < 0.001). However, the plasma levels of MIC-1 in VN were similar to VP (p = 0.951). The median plasma levels of MIC-1 were 678.39 pg/mL (IQR, 368.52 to 1083.48 pg/mL) in NPC patients, 310.29 pg/mL (IQR, 215.62 to 404.51 pg/mL) in the VP group, and 294.6 pg/mL (IQR, 368.52 to 1083.48 pg/mL) in NPC patients, 310.29 pg/mL (IQR, 215.62 to 404.51 pg/mL) in the VP group, and 294.6 pg/mL (IQR, 202.56 to 457.86 pg/mL) in the VN group. The association between the plasma MIC-1 concentrations and the clinicopathological features are presented in Table 1. Though we found that MIC-1 levels were significantly associated with age and Rta-IgG (p < 0.001), the other parameters such as sex, EBV DNA copy number, VCA-IgA, EA-IgA, EBV-DNase antibody, Zta-IgG, pT, pN, pM, and overall stage (p > 0.05) were not related.

Diagnostic Ability of Plasma MIC-1 Levels in NPC Patients
To validate the discriminative competence of plasma MIC-1 levels, we utilized plasma from a total of 481 participants, including 190 NPC, 72 VP, and 219 VN samples. ROC curves analyses illustrated that the MIC-1 plasma levels strikingly distinguished the subjects with or without NPC, with an AUC value of 0.790.
When the cutoff value was set to the optimal point (528.27 pg/mL), the sensitivity, specificity, PPV, and NPV were 63.7%, 85.9%, 74.7%, and 78.4% (Table 2), respectively. For discriminating the NPC patients from the VP cohort, the AUC value of plasma MIC-1 was 0.796 (95% CI, 0.738 to 0.853, Figure 2B), with 63.7% sensitivity, 88.9% specificity, a striking 93.8% PPV and 48.1% NPV. Also, the AUC of MIC-1 for differentiating NPC patients from the VN cohort was 0.788 (95% CI, 0.744-0.832, Figure 2C), the sensitivity was the same whereas the specificity was 84.9% (Table 2).

The Complement Role of MIC-1 for EBV-Related Markers in the Diagnosis of NPC

We further explored the relationship between plasma MIC-1 concentrations and EBV-related markers, like VCA-IgA titer and EBV DNA content. To assess the association between MIC-1 concentrations and VCA-IgA titer, the NPC patients were divided into 4 groups, based on VCA-IgA titers: ≤ 1:40 (n = 72), 1:80 (n = 27), 1:160 (n = 73), and ≥ 1:320 (n = 18).
We found no significant differences in plasma MIC-1 concentrations among these 4 groups ($p > 0.05$) (Figure 3A). Moreover, similar results were observed when we separated the NPC patients into 4 groups according to the plasma EBV DNA copy number: $\geq 10^5$ (n = 16), $10^4 - 10^5$ (n = 37), $10^3 - 10^4$ (n = 57), and $\leq 10^3$ (n = 78). The results suggest that MIC-1 concentrations are unlikely to be directly related to VCA-IgA titer and EBV DNA copy numbers (Figure 3B).

We further explored whether plasma MIC-1 can supplement VCA-IgA in distinguishing healthy controls. Figure 3C shows the percentages of healthy controls stratified by $528.27$ pg/mL cutoff values of plasma MIC-1. In VP, the rate of negative results for plasma MIC-1 was 88.9%, which is slightly higher than VN (84.9%).

Dramatically, a larger proportion of NPC patients were positive for plasma MIC-1 (63.7%) than the EBV DNA (58.5%). We also explored the complementary effect of MIC-1 to EBV DNA. 78/188 (59.0%) of NPC patients were EBV DNA-negative. Among them, 46 out of 78 (59.0%) EBV DNA-negative NPC patients were positive for MIC-1.

**The Effect of Plasma MIC-1 in NPC Surveillance**

Plasma samples were collected from 10 patients after radiotherapy. The mean plasma MIC-1 concentration before radiotherapy was 778.75 pg/mL, which afterward dropped to $511.26$ pg/mL, $p = 0.027$ (Figure 4) except 1 patient.

**Discussion**

Our results suggest that irrespective of VCA-IgA and EBV DNA status, detecting plasma MIC-1 level is of robust diagnostic value for NPC. MIC-1, a growth factor, is known to play regulatory roles in apoptosis, cell cycle, tumor metastasis,
Similarly, Hsiao JR et al. also reported a positive rate of merely 14 patients were positive for EBV DNA in the circulation.\(^{24}\)

In conclusion, the current study reveals that plasma MIC-1 levels are significantly higher in NPC than other tumors. Therefore, we explored the diagnostic value of this widely described potential tumor marker in NPC.

A strong correlation between NPC and EBV infection has been already established. VCA, strongly immunogenic capsid antigen of EBV, is released shortly after EBV infection and has a long half-life. More than 90\% of patients with NPC are VCA-IgA positive. Hence, it is frequently used for the screening of the disease in symptomatic patients or living in high incidence areas. However, most positive results are not truly NPC. In several large population screens in the NPC-high incidence areas, such as Taiwan,\(^{21}\) Hong Kong,\(^{22}\) Wu Zhou, and the other southern China areas,\(^{23}\) the positive ratio of VCA-IgA was only 3-10\%. However, a long period follow-up of these revealed that only 1-5\% of them developed into pathologically-confirmed NPC, suggesting the positive predictive value (PPV) of VCA-IgA is rather low. Due to this, several subjects, even in the absence of NPC, suffered unnecessary psychological burden of nasopharyngoscopy, radiological examinations, or aggressive biopsy, along with loss of money and time. Subsequently, EBV DNA levels in the circulation found to be more reliable in the detection of NPC. Though the detection rates in healthy control were low, several studies reported a high variability of the test among the NPC patients. For instance, Mutirangura et al. reported that in a group of 42 NPC patients, only 14 patients were positive for EBV DNA in the circulation.\(^{24}\) Similarly, Hsiao JR et al. also reported a positive rate of merely 38.9\%.\(^{25}\) However, Lo et al. found that EBV DNA could be detected in the blood of 96\% of NPC patients. Overall, this suggests that EBV DNA detection does not have standardized methods and techniques as of yet, and lacks the sensitivity.\(^{6}\) In our study, 58.5\% of NPC patients were EBV DNA positive, and this is consistent with Shotelersuk K, et al.\(^{26}\) Interestingly, EBV DNA could also be detected in patients suffering from several other diseases,\(^{27,28}\) such as infectious mononucleosis, gastric cancer, and Hodgkin's disease. Therefore, a new diagnostic marker that can detect and monitor NPC, complementing the widely used EBV markers, is of great value.

Here, we showed that plasma MIC-1 concentrations in the NPC patients were significantly higher than those in VN and VP healthy controls. Also, levels of MIC-1 were markedly higher in NPC compared to gastric cancer and leukemia, suggesting high specificity in discriminating NPC from the other EBV DNA-positive cancers. Additionally, excluding age and Rta-IgG (\(p < 0.001\)), MIC-1 levels showed no association with patient clinical characteristics. Notably, we observed no significant difference in the plasma MIC-1 levels between the early-stage and advanced-stage NPC patients, suggesting its applicability in both the scenarios. Also, no significant difference was noticed among the NPC patients having different VCA-IgA titer and EBV copy number, suggesting that MIC-1 is a non-EBV related marker. Additionally, ROC curve analysis revealed the diagnostic accuracy of plasma MIC-1 in distinguishing NPC from healthy controls with VCA-IgA positive or negative with similar efficiency. Compared with VCA-IgA titer, plasma MIC-1 level exhibited higher PPV and specificity. Overall these results signify that Plasma MIC-1 level alone could be a better diagnostic marker of NPC. Moreover, it can aid the diagnosis with EBV-related markers in EBV DNA negative individuals and VCA-IgA positive healthy controls. Most VP were MIC-1 negative, therefore, healthy individuals with VCA-IgA positive can be easily distinguished from the NPC patients. On the contrary, many NPC patients were positive for MIC-1 while being negative for EBV DNA. Therefore, combined testing for plasma MIC-1 and EBV DNA copy numbers could improve the diagnostic results. Additionally, this test can be utilized for population studies in high-risk areas.

The decreased plasma MIC-1 levels after radiotherapy suggests that it can also be used in assessing the radiotherapeutic response in NPC patients. However, notably in 1 sample, the MIC-1 protein level was increased after the radiotherapy. Interestingly, upon following up, we found that the particular patient was not sensitive to radiotherapy. As the previous finding showed that MIC-1 was involved in radioresistance of NPC cell lines,\(^{20}\) perhaps evaluated MIC-1 level could be a reflection of radioresistance in the NPC patients. However, such a conclusion needs further verification due to the limitation of the sample size in our study.

Although our results are promising, there were few limitations. Larger and multicenter samples could rule out selection bias, control bias, and the incidence of NPC bias in the following research. Also, the oncological and biological significance of MIC-1 must be investigated further.

Conclusions

In conclusion, the current study reveals that plasma MIC-1 levels are significantly increased in NPC, which easily distinguished the NPC patients from the healthy controls. Also, a combination of MIC-1 and EBV-related markers could aid the
diagnosis. MIC-1 is an easy-to-access and non-invasive marker that could help clinicians. We will continue to verify the performance of MIC-1 in NPC screening and surveillance in samples from larger and multicenter patient cohorts.

Authors’ Note
SX and LL carried out the main work, draft the manuscript and contributed equally. YP carried out the Immunoenzymatic assays. GO and NX designed and guided the study. TZ and QH helped collect the sample and the clinical parameters. All authors read and approved the final manuscript. Shan Xing and Huilian Li contributed equally to this work. The study was in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was reviewed and approved by the Institutional Review Board and Ethics Committee of Sun Yat-sen University Cancer Center (Ref: GZR 2018-147). The consent has been obtained from the participants, for the use of clinical parameters and collected samples for further studies at the time of patients’ admission. The records were anonymous and de-identified before use. The consent we obtained from study participants was written.

Declaration of Conflicting Interests
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References
1. Wee JT, Ha TC, Loong SL, Qian CN. Is nasopharyngeal cancer really a “Cantonese cancer”? Chin J Cancer. 2010;29(5):517-526.
2. Chua MLK, Wee JTS, Hui EP, Chan ATC. Nasopharyngeal carcinoma. Lancet. 2016;387(10022):1012-1024. doi:10.1016/S0140-6736(15)00055-0
3. Lo KW, To KF, Huang DP. Focus on nasopharyngeal carcinoma. Cancer Cell. 2004;5(5):423-428. doi:10.1016/S1535-6108(04)00119-9
4. Levine PH, Connelly RR, Milman G, Easton J. Epstein-Barr virus serology in the control of nasopharyngeal carcinoma. Cancer Detect Prev. 1988;12(1-6):357-362.
5. Low WK, Leong JL, Goh YH, Fong KW. Diagnostic value of Epstein-Barr viral serology in nasopharyngeal carcinoma. Otolaryng Head Neck. 2000;123(4):505-507. doi:10.1067/mhn.2000.108201
6. Chan KCA, Lo YMD. Circulating EBV DNA as a tumor marker for nasopharyngeal carcinoma. Semin Cancer Biol. 2002;12(6):489-496. doi:10.1016/S1044-557X(02)00091-3
7. Tay JK, Chan SH, Lim CM, Siow CH, Goh HL, Loh KS. The role of Epstein-Barr virus DNA load and serology as screening tools for nasopharyngeal carcinoma. Otolaryng Head Neck. 2016;155(2):274-280. doi:10.1177/0194599816641038
8. Paralkar VM, Vail AL, Grasser WA, et al. Cloning and characterization of a novel member of the transforming growth factor-beta/bone morphogenetic protein family. J Biol Chem. 1998;273(22):13760-13767. doi:10.1074/jbc.273.22.13760
9. Lawton LN, Bonaldo MF, Jelicc PC, et al. Identification of a novel member of the TGF-beta superfamily highly expressed in human placenta. Gene. 1997;203(1):17-26.
10. Li PX, Wong J, Ayed A, et al. Placental transforming growth factor-beta is a downstream mediator of the growth arrest and apoptotic response of tumor cells to DNA damage and p53 overexpression. J Biol Chem. 2000;275(26):20127-20135. doi:10.1074/jbc.M090580199
11. Welsh JB, Sapinosa LM, Kern SG, et al. Large-scale delineation of secreted protein biomarkers overexpressed in cancer tissue and serum. P Natl Acad Sci USA. 2003;100(6):3410-3415. doi:10.1073/pnas.0530278100
12. Buckhaults P, Rago C, St Croix B, et al. Secreted and cell surface genes expressed in benign and malignant colorectal tumors. Cancer Res. 2001;61(19):6996-7001.
13. Cheung PK, Woolcock B, Adomat H, et al. Protein profiling of microdissected prostate tissue links growth differentiation factor 15 to prostate carcinogenesis. Cancer Res. 2004;64(17):5929-5933. doi:10.1158/0008-5472.Can-04-1216
14. Weide B, Schafer T, Martens A, et al. High GDF-15 serum levels independently correlate with poorer overall survival of patients with tumor-free stage III and unresectable stage IV melanoma. J Invest Dermatol. 2016;136(12):2444-2452. doi:10.1016/j.jid.2016.07.016
15. Yi Z, Yuxi L, Chunren L, et al. Application of an immunoenzymatic method and an immunoaautoradiographic method for a mass survey of nasopharyngeal carcinoma. Intervirology. 1980;13(3):162-168. doi:10.1159/000149121
16. An X, Wang FH, Ding PR, et al. Plasma Epstein-Barr virus DNA level strongly predicts survival in metastatic/recurrent nasopharyngeal carcinoma treated with palliative chemotherapy. Cancer-Am Cancer Soc. 2011;117(16):3750-3757. doi:10.1002/cncr.25932
17. Subramanian S, Strelau J, Unskicer K. Growth differentiation factor-15 prevents low potassium-induced cell death of cerebellar granule neurons by differential regulation of Akt and ERK pathways. J Biol Chem. 2003;278(11):8904-8912. doi:10.1074/jbc.M210037200
18. Lee DH, Yang Y, Lee SJ, et al. Macrophage inhibitory cytokine-1 induces the invasiveness of gastric cancer cells by up-regulating the urokinase-type plasminogen activator system. Cancer Res. 2003;63(15):4648-4655.
19. Bootcov MR, Bauskin AR, Valenzuela SM, et al. MIC-1, a novel macrophage inhibitory cytokine, is a divergent member of the TGF-beta superfamily. Proc Natl Acad Sci U S A. 1997;94(21):11514-11519. doi:10.1073/pnas.94.21.11514
20. Chang JT, Chan SH, Lin CY, et al. Differentially expressed genes in radioresistant nasopharyngeal cancer cells: gp96 and GDF15. Mol Cancer Ther. 2007;6(8):2271-2279. doi:10.1186/1535-7163.MCT-06-0801
21. Pickard A, Chen C, Diehl SR, et al. Epstein-Barr virus seroreactivity among unaffected individuals within high-risk nasopharyngeal carcinoma families in Taiwan. Int J Cancer. 2004;11(1):117-123. doi:10.1002/ijc.20222
22. Luo JH, Chia KS, Chia SE, Reilly M, Tan CS, Ye WM. Secular trends of nasopharyngeal carcinoma incidence in Singapore, Hong Kong and Los Angeles Chinese populations, 1973-1997. *Eur J Epidemiol*. 2007;22(8):513-521. doi:10.1007/s10654-007-9148-8

23. Liu ZW, Ji MF, Huang QH, et al. Two Epstein-Barr Virus-Related serologic antibody tests in nasopharyngeal carcinoma screening: results from the initial phase of a cluster randomized controlled trial in Southern China. *Am J Epidemiol*. 2013;177(3):242-250. doi:10.1093/aje/kws404

24. Mutirangura A, Pornthanakasem W, Theamboonlers A, et al. Epstein-Barr viral DNA in serum of patients with nasopharyngeal carcinoma. *Clin Cancer Res*. 1998;4(3):665-669.

25. Hsiao JR, Jin YT, Tsai ST. Detection of cell free Epstein-Barr virus DNA in sera from patients with nasopharyngeal carcinoma. *Cancer*. 2002;94(3):723-729. doi:10.1002/cncr.10251

26. Shotelersuk K, Khorprasert C, Sakdikul S, Pornthanakasem W, Voravud N, Mutirangura A. Epstein-Barr virus DNA in serum/plasma as a tumor marker for nasopharyngeal cancer. *Clin Cancer Res*. 2000;6(3):1046-1051.

27. Gan YJ, Sullivan JL, Sixbey JW. Detection of cell-free Epstein-Barr virus DNA in serum during acute infectious mononucleosis. *J Infect Dis*. 1994;170(2):436-439. doi:10.1093/infdis/170.2.436

28. Lo YM, Chan WY, Ng EK, et al. Circulating Epstein-Barr virus DNA in the serum of patients with gastric carcinoma. *Clin Cancer Res*. 2001;7(7):1856-1859.