

## Epstein–Barr Virus Serology as a Potential Screening Marker for Nasopharyngeal Carcinoma among High-Risk Individuals from Multiplex Families in Taiwan

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### Abstract

**Background:** Nasopharyngeal carcinoma (NPC) is an Epstein–Barr virus (EBV)–associated cancer that is highly treatable when diagnosed early, with 5-year disease-free survival of approximately 90%. However, NPC is typically diagnosed at advanced stages, in which disease-free survival is <50%. There is, therefore, a need for clinical tools to assist in early NPC detection, particularly among high-risk individuals.

**Methods:** We evaluated the ability of anti-EBV IgA antibodies to detect incident NPC among high-risk Taiwanese individuals. NPC cases ( $N = 21$ ) and age- and sex-matched controls ( $N = 84$ ) were selected. Serum collected before NPC diagnosis was tested for ELISA-based IgA antibodies against the following EBV peptides: EBNA1, VCAp18, EA p138, Ead\_p47, and VCAp18 + EBNA1 peptide mixture. The sensitivity, specificity, and screening program parameters were calculated.

**Results:** EBNA1 IgA had the best performance characteristics. At an optimized threshold value, EBNA1 IgA measured at baseline identified 80% of the high-risk individuals who developed NPC during follow-up (80% sensitivity). However, approximately 40% of high-risk individuals who did not develop NPC also tested positive (false positives). Application of EBNA1 IgA as a biomarker to detect incident NPC in a previously unscreened, high-risk population revealed that 164 individuals needed to be screened to detect 1 NPC and that 69 individuals tested positive per case detected.

**Conclusions:** EBNA1 IgA proved to be a sensitive biomarker for identifying incident NPC, but future work is warranted to develop more specific screening tools to decrease the number of false positives.

**Impact:** Results from this study could inform decisions about screening biomarkers and referral thresholds for future NPC early-detection program evaluations. *Cancer Epidemiol Biomarkers Prev*; 23(7); 1213–9. ©2014 AACR.

### Introduction

Epstein–Barr virus (EBV) proteins play a necessary role in the etiology and pathogenesis of nasopharyngeal carcinoma (NPC; refs. 1–10), and evidence from recent, prospective studies has demonstrated that higher antibody levels, particularly IgA antibodies directed against lytic and latent protein expression, precede the develop-

ment of NPC (11–13). Men from the general Taiwanese population who tested positive for IgA antibodies against the lytic viral capsid antigen (VCA) protein had an increased risk of developing NPC compared with VCA IgA–negative men, an association that persisted even  $\geq 5$  years after antibody measurement (HR, 13.9; 95% confidence interval, 3.1–61.7; ref. 11).

This association between altered EBV serology and NPC development has also been reported among Taiwanese individuals with an inherently elevated NPC risk. In individuals from multiplex NPC families, families with  $\geq 1$  first or second degree relatives affected by NPC, the incidence of disease is reported to be  $90 \times 10^5$ , 10-fold higher than the general Taiwanese population (14). Even among this group, elevated antibody titers of both VCA IgA and IgA antibodies against the latent EBV nuclear antigen 1 (EBNA1) protein before diagnosis were associated with higher rates of NPC, with EBNA1 IgA-positive individuals having >6-fold increased risk compared with EBNA1 IgA–negative individuals (RR = 6.6; 1.5–61; ref. 14).

Although the association of EBNA1 and VCA IgA with NPC risk has been established, the important question of

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whether antibody patterns can discriminate between individuals who will or will not develop NPC in the future (i.e., clinical utility) remains inadequately answered. In the prospective evaluation of anti-EBV antibodies and NPC risk among high-risk family members in Taiwan that used research-based assays for EBNA1 and VCA IgA, these two markers proved to be sensitive for detecting incident NPC but did not achieve specificity above 50% (14).

To move the use of EBV serology toward a clinically applicable tool for screening or management of individuals at high-risk for NPC, we previously evaluated and reported the reproducibility of a panel of chemically defined, peptide-based anti-EBV antibodies (15). The IgA antibodies on this panel proved to have acceptable performance characteristics, providing a reproducible EBV serology tool for application in future studies.

We selected IgA antibodies from this panel with  $\geq 70\%$  intraclass correlation coefficients (ICC), a measure of the proportion of the total assay variability attributed to true variability in IgA antibody levels between individuals. Using these IgA antibodies, we evaluated whether EBV serology measured years before disease presentation could be used to identify high-risk individuals in this specific Taiwanese subpopulation who developed NPC over time. We also simulated the application of the best-performing IgA marker on this panel as a screening tool for NPC in this high-risk population and report the approximate number of individuals that would need to be screened per case of NPC detected under varying, realistic screening program parameters.

## Materials and Methods

High-risk individuals for this study were selected from an ongoing NPC multiplex family study in Taiwan. Details of this study population have been published previously (14, 16, 17). In brief, 2,557 unaffected family members recruited from 358 multiplex families had blood drawn and have been followed since 1996 for the development of incident NPC. Ascertainment of NPC is determined through both linkage to the Taiwan National Cancer Registry and active clinical evaluation of a substantive portion of the cohort at a follow-up visit.

All NPC cases ascertained through December 31, 2010, were selected for this study ( $N = 21$ ). For each of the 21 incident NPC cases, 4 individuals from this high-risk population who did not develop NPC were selected, matched on both gender and age (5-year intervals), resulting in 84 controls. Cases and controls were not matched on family so that findings could be applied broadly to multiplex families.

Blood was drawn at study enrollment for each of these 105 individuals. Serum from the blood collection was tested for IgA antibodies against the following EBV proteins according to previously published protocols (15): VCAp18, EBNA1, EA<sub>p</sub>138, EA<sub>d</sub>p47, and a combined mixture of VCAp18 + EBNA1 peptides (18). The ELISA assays used were chemically defined (peptides) rather

than recombinant or cell-based antigens, facilitating standardization that would be important for future clinical utility. In each ELISA test, four known EBV negative reference sera were tested at 1:100 in duplicate calibrators, and the cutoff value (COV) for each ELISA plate was defined by calculating the mean OD<sub>450</sub> reactivity + two times the SD of these four samples. Results were reported as the mean of the duplicate absorbance value observed for each IgA antibody, divided by the COV.

To ensure that the performance characteristics of the peptide-based anti-EBV antibody assays measured in this set of samples fell within the reported reproducibility values previously published (15), 29 individuals had duplicate samples included in the testing (12 across plate and 17 within plate). Coefficients of variation (CV) and ICCs were calculated for each anti-EBV IgA antibody in the panel using PROC GLM (SAS version 9.3).

The percentage of incident NPC cases and unaffected high-risk individuals who tested positive at a standardized threshold value ( $\geq 1.0$ ) was calculated. To ensure that these IgA antibodies measured before diagnosis were valid for predicting the development of future disease, blood drawn after NPC diagnosis for 142 prevalent NPC cases (67 early stage and 75 late stage) and 75 controls from the general Taiwanese population recruited in a previous case-control study (19) was also tested for the presence of these five anti-EBV IgA antibodies at the same threshold value ( $\geq 1.0$ ). If titers of these IgA antibodies measured before diagnosis were hypothesized to predict future disease, we expected to observe a high prevalence above the threshold in samples taken concurrent to the time of NPC diagnosis. Furthermore, we expected that the percentage above the threshold should be lower among population controls with low risk for NPC.

We calculated a delta statistic for each respective anti-EBV serologic marker, defined as the difference in the mean IgA antibody level between cases and controls, divided by the variance in this population. According to the method published by Wentzensen and Wacholder (20), this delta statistic is a measure of the potential ability of the screening tool to discriminate between cases and noncases and can be used to generate an ROC curve to illustrate the performance characteristics for the screening marker being evaluated at different threshold values.

The delta statistics were calculated for each of the five anti-EBV IgA antibodies evaluated in this study, and the optimal threshold value for a given IgA marker was defined as the value on the ROC curve with the highest specificity that successfully identified at least 80% of incident NPC cases (80% sensitivity) in this high-risk population. We also identified an alternative threshold with the highest specificity that maintained at least 90% sensitivity.

For the IgA antibody on this panel with the highest delta statistic, or greatest ability to discriminate between high-risk individuals who would or would not go on to develop NPC, we further estimated two screening parameters:

(i) the number of high-risk individuals needed to be screened to detect 1 NPC case, and (ii) the number of high-risk individuals with IgA antibody titers above the threshold (test positives) for each NPC case detected. The number of high-risk individuals needed to be screened for each detected NPC case is equivalent to the inverse of the probability of detecting NPC using a given IgA marker in this high-risk population:  $[1/(\text{sensitivity} \times 5\text{-year risk})]$ . The number of high-risk individuals testing positive for each detected NPC case is equivalent to the inverse of the probability of detecting NPC among the test positives, or the inverse of the positive predictive value of a given IgA marker:

$$\left( \frac{1}{\frac{\text{Sensitivity} \times 5\text{-year risk}}{(\text{sensitivity} \times 5\text{-year risk}) + [(1 - \text{specificity}) \times (1 - 5\text{-year cumulative risk})]}} \right)$$

Finally, these two screening parameters were also estimated assuming application of less specific IgA markers on the panel to this high-risk population.

## Results

The average duration of follow-up between baseline blood draw and diagnosis among the 21 incident NPC cases was 5.4 years (SD = 3.0 years; median = 4.9 years). The CV and ICC for VCAp18 IgA, EBNA1 IgA, EAp138 IgA, and Ead\_p47 IgA measured in these 105 individuals were consistent with values previously reported (15). The mixture of VCAp18 + EBNA1IgA peptides that was introduced to the panel for the first time in this study had a CV of approximately 10% and an ICC >90%, consistent with the premise that the variability in this partic-

ular IgA marker between samples was due to true differences between individuals rather than assay variability.

Using a threshold of  $\geq 1.0$ , the percentage of individuals with blood drawn at the time of NPC diagnosis (i.e., prevalent NPC) with a positive result exceeded 90% for both EBNA1 IgA and the combined VCAp18 + EBNA1IgA markers (Table 1). In contrast, less than half of the prevalent NPC cases tested above the threshold for either early antigen IgA antibody, although both EAp138 IgA and Ead\_p47 IgA were highly specific markers, testing above the threshold in <20% of general population controls. Importantly, EBNA1 IgA, in addition to being very sensitive, had a specificity of 80% and proved to be the EBV serologic marker with the best performance characteristics for prevalent NPC.

The percentage of high-risk multiplex family members developing NPC during follow-up (i.e., incident NPC) whose baseline serum antibody titer tested above the  $\geq 1.0$  threshold was lower as compared with prevalent NPC cases for each anti-EBV IgA marker evaluated (Table 2). However, the trends observed were the same as those observed for prevalent disease; EBNA1 IgA still proved to be the best serologic marker for incident NPC.

The difference in the mean EBNA1 IgA antibody titer measured at baseline in high-risk multiplex family members who developed NPC (mean = 2.67) versus those who did not develop NPC (mean = 0.91) was used to calculate a delta statistic ( $\Delta = 1.04$ ) and generate an ROC curve (Fig. 1; ref. 20). The optimized threshold value chosen for EBNA1 IgA (cutoff = 0.72) in high-risk multiplex family members had a specificity of 58% while still identifying at least 80% of individuals who developed NPC (80% sensitivity). The alternative threshold (cutoff = 0.61) that identified 90% of

**Table 1.** Sensitivity and specificity for anti-EBV IgA antibodies, applied to prevalent NPC and healthy controls from Taiwan

| IgA Marker                           | Sensitivity for prevalent NPC <sup>a</sup> | Specificity in healthy, community controls |
|--------------------------------------|--|--|
| Viral capsid antigen (VCAp18)        | 78.9                                       | 49.3                                       |
| Early prevalent disease              | 76.1                                       |  |
| Late prevalent disease               | 81.3                                       |  |
| Epstein-Barr nuclear antigen (EBNA1) | 92.3                                       | 80.0                                       |
| Early prevalent disease              | 95.5                                       |  |
| Late prevalent disease               | 89.3                                       |  |
| Early antigen (Ead_p47)              | 43.0                                       | 81.3                                       |
| Early prevalent disease              | 38.8                                       |  |
| Late prevalent disease               | 46.7                                       |  |
| Early antigen (EAp138)               | 47.9                                       | 82.7                                       |
| Early prevalent disease              | 38.8                                       |  |
| Late prevalent disease               | 56.0                                       |  |
| Combined VCAp18 + EBNA1 ELISA        | 99.3                                       | 32.0                                       |
| Early prevalent disease              | 100  |  |
| Late prevalent disease               | 98.7                                       |  |

NOTE: Early disease, WHO stage I/II; late disease, WHO stage III/IV.

<sup>a</sup>The percentages are based on a threshold value for determining a positive result of  $\geq 1.0$ .

**Table 2.** Sensitivity and specificity for anti-EBV IgA antibodies, applied to high-risk multiplex family members from Taiwan

| IgA marker                           | Sensitivity for incident NPC <sup>a</sup> | Specificity in high-risk controls |
|--------------------------------------|---|-----------------------------------|
| Viral capsid antigen (VCAp18)        | 52.4                                      | 46.4                              |
| Epstein-Barr nuclear antigen (EBNA1) | 66.7                                      | 85.7                              |
| Early antigen (Ead_p47)              | 0.0                                       | 92.9                              |
| Early antigen (EAp138)               | 0.0                                       | 95.2                              |
| Combined VCAp18 + EBNA1 ELISA        | 85.7                                      | 35.7                              |

<sup>a</sup>The percentages are based on a threshold value for determining a positive result of  $\geq 1.0$ .

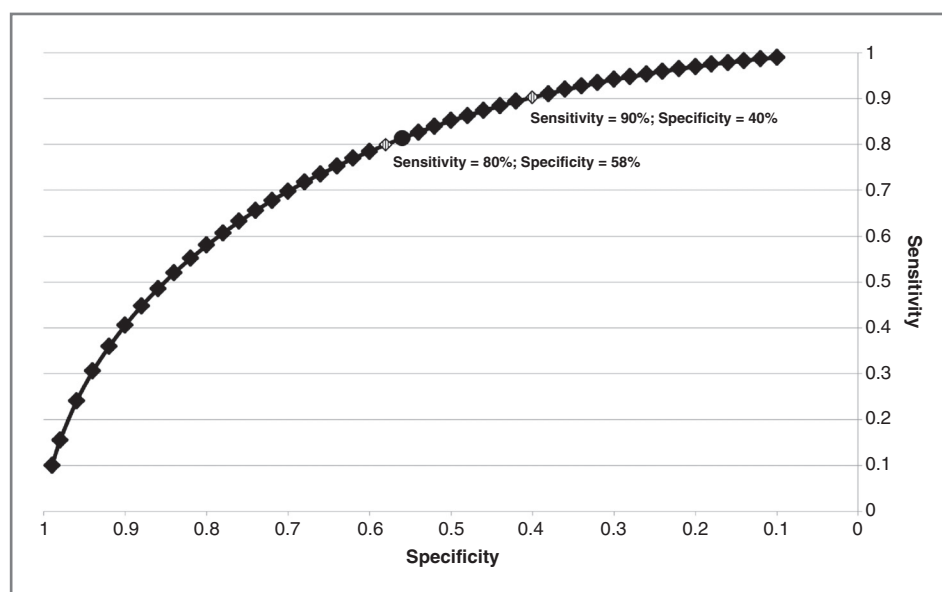
individuals who developed NPC (90% sensitivity) had lower specificity (40%).

The test characteristics of the optimized threshold value for EBNA1 IgA were used, in addition to the 5-year cumulative NPC risk among individuals  $\geq 40$  years of age in this target population (14), to calculate the number of high-risk multiplex family members needed to be screened per NPC case detected ( $N = 164$ ) as well as the number of who tested above the threshold for EBNA1 IgA per NPC case detected ( $N = 69$ ; Table 3). Application of the more sensitive alternative threshold for classifying individuals as positive for EBNA1 IgA yielded slightly different results: The number of high-risk multiplex family members needed to be screened per NPC case detected was lower ( $N = 146$ ), but the number who tested above the threshold and would require further diagnostic evaluation per NPC case detected was higher ( $N = 88$ ).

The VCAp18 IgA and combined VCAp18 + EBNA1 IgA markers were not highly specific when applied to this high-risk multiplex family population, with lower delta statistics than EBNA1 IgA alone (VCAp18,  $\Delta = 0.08$ ; VCAp18 + EBNA1,  $\Delta = 0.98$ ) illustrating a diminished

capacity to distinguish between cases and noncases. For VCAp18 IgA, the choice of a threshold value with  $\geq 80\%$  sensitivity resulted in a specificity of only 20% (Supplementary Fig. S1), less than half the specificity of EBNA1 IgA. For the combined VCAp18 + EBNA1 IgA marker, the specificity for the threshold value with  $\geq 80\%$  sensitivity was 54% (Supplementary Fig. S2), which although superior to VCAp18 alone was still slightly inferior to EBNA1 IgA alone. Application of these less specific screening tools to the same unscreened, high-risk population resulted in greater numbers of multiplex family members who tested above the threshold per NPC case detected (VCAp18, 128; VCAp18 + EBNA1, 75), particularly for VCAp18, in which a halving in specificity resulted in nearly double the number of individuals requiring additional diagnostic workup despite not developing NPC for each true NPC case detected (Table 4).

Using the data from both the single VCAp18 IgA marker and single EBNA1 IgA marker rather than considering the combined VCAp18 + EBNA1 IgA peptide mixture did not offer superior discriminatory capacity. We created a composite score for each individual by multiplying the log



**Figure 1.** The ROC curve for EBNA1 IgA marker ( $\Delta = 1.04$ ). The optimized threshold value was defined as the value on the ROC curve with the highest specificity that successfully identified at least 80% of incident NPC cases (80% sensitivity) in this high-risk population. The alternative threshold had the highest specificity while identifying at least 90% of incident NPC.



**Table 3.** Application of EBNA1 IgA as screening tool for incident NPC in high-risk multiplex Taiwanese family members (40+ years of age)

| Screened population                      | 5-year NPC risk          | Sensitivity      | Specificity | No. of screened/<br>NPC case <sup>c</sup> | No. of screen-positive/<br>NPC case <sup>d</sup> |
|--|--------------------------|------------------|-------------|---|--|
| Taiwanese multiplex family members (40+) | 762/100,000 <sup>a</sup> | 80% <sup>b</sup> | 58%         | 164                                       | 69   |
| Taiwanese multiplex family members (40+) | 762/100,000              | 90% <sup>e</sup> | 40%         | 146                                       | 88   |

<sup>a</sup>Eleven NPC cases were ascertained among 1,444 high-risk Taiwan multiplex family study participants at least 40 years of age at the time of the baseline blood draw (14).

<sup>b</sup>Sensitivity and specificity generated from EBNA1 IgA COV = 0.72.

<sup>c</sup>Number of screened per NPC case detected =  $1/(\text{sensitivity} \times \text{5-year NPC risk})$ .

<sup>d</sup>Number of screen-positive per NPC case detected =  $1/(\text{positive predictive value})$ .

<sup>e</sup>Sensitivity and specificity generated from EBNA1 IgA COV = 0.61.

odds for each IgA marker generated from a logistic regression model with the outcome of case status (VCAp18 IgA log odds, 0.0212; EBNA1 IgA log odds, 0.4714) by the VCAp18 IgA and EBNA1 IgA antibody titer values for each individual. However, the delta statistic comparing the average value of this composite score between cases and noncases was 1.04, identical to the EBNA1 IgA marker alone.

## Discussion

Our evaluation of a well-characterized scalable panel of five anti-EBV IgA antibodies suggests that EBV serology measured before diagnosis can sensitively identify individuals from high-risk multiplex families with an elevated risk of developing NPC. In particular, the EBNA1 IgA marker applied in this population sensitively detected those diagnosed with incident NPC. In contrast, the two early antigen markers investigated (EAp138 IgA, Ead\_p47 IgA) were present at very low levels before NPC diagnosis and were, therefore, not effective screening tools for incident disease. In a population at very high risk of developing NPC, such as the multiplex family members from Taiwan studied here, a screening program designed to detect disease and lower mortality needs highly sensitive screening tools that will target individuals at high disease risk for early detection and effective treatment, recognizing that increasing the sensitivity results in lower

specificity. EBNA1 IgA, despite being a sensitive screening tool, was not highly specific when applied to this population. At an 80% sensitivity threshold value, approximately 40% of high-risk individuals who did not develop NPC also tested positive. Although this test might be useful to reassure close to 60% of high-risk family members that they are not at an elevated risk of NPC within the next few years, screening efforts targeted toward the remaining 40% would benefit from marker combinations that increased test specificity, such as the incorporation of measures of nasopharyngeal EBV DNA levels (21).

Importantly, application of this EBNA1 IgA antibody marker resulted in 164 high-risk individuals needing to be screened, using a simple blood draw, for every incident case of NPC detected. This value is well within the range of the number of high-risk patients that need to be screened for currently recommended programs in the United States, such as low-dose CT screening of 302 high-risk patients for lung cancer to prevent one lung cancer-related death (22).

These findings are in agreement with the earlier data from Taiwan that used research-based EBV serology tests, suggesting that EBNA1 IgA is the most suitable serologic marker in this high-risk population for identifying NPC (14). Of note is that, despite similar conclusions, the values for sensitivity and specificity for the two respective EBNA1 IgA ELISA tests were not exactly the same, with

**Table 4.** Application of less specific screening tools for incident NPC in high-risk multiplex Taiwanese family members (40+ years of age)

| IgA marker     | 5-year NPC risk | Sensitivity | Specificity | No. of screened/<br>NPC case | No. of screen-positive/<br>NPC case |
|----------------|-----------------|-------------|-------------|------------------------------|-------------------------------------|
| VCAp18         | 762/100,000     | 82%         | 20%         | 160                          | 128                                 |
| VCAp18 + EBNA1 | 762/100,000     | 81%         | 54%         | 162                          | 75                                  |

the current peptide-based assay having slightly higher specificity. Our results that point to the utility of EBNA1 IgA as a sensitive screening marker are further supported by large, ongoing NPC screening trials being conducted in China in areas with high endemic rates of NPC (23–27). The sensitivity of the EBNA1 IgA assay used in that setting to diagnose NPC within a year of screening approached 90% and illustrated the potential application of this screening tool in a general population setting for identifying those most likely to be harboring prevalent NPC (28). However, it should be noted that the performance characteristics of any EBNA1 IgA test differ if applied to detect prevalent NPC that will present clinically within the following year versus incident NPC that will develop and present over the course of 5 years.

In addition to recognizing the importance of the screening tool used, it is important to also define the prescribed clinical management of screen-positive individuals, as a good screening test in the absence of a sensitive diagnostic work-up does not have high utility. For example, sending 69 high-risk individuals who tested above the given threshold for EBNA1 IgA antibody titer for a routine, noninvasive clinical exam to detect 1 NPC case would not be as costly as sending those same 69 individuals for an invasive, expensive diagnostic procedure. Endoscopy, the current diagnostic procedure for NPC, is minimally invasive, so in a very high-risk population such as these multiplex family members, screening with a simple blood test that has modest specificity might be warranted, despite requiring a substantive number of individuals to be sent for endoscopy. However, endoscopy may not be sensitive enough to detect very early-stage NPC lesions. MRI or another more expensive, potentially more invasive, test might be required. In this case, the number of screen positives sent to receive this test for each real NPC case detected would become of greater concern.

Another important aspect to consider in the evaluation of potential screening tools is that selection of the target population alters the number of individuals needed to be screened per cancer case detected. Sensitivity of the EBNA1 IgA marker was lower when measured years before NPC diagnosis (incident NPC) in the high-risk multiplex family members as compared with measurement at the time of NPC diagnosis (prevalent NPC) in a general population setting. This result was not surprising; it is expected that EBNA1 IgA antibody titers measured years before NPC diagnosis may be more variable and less accurate at diagnosing future, incident disease when compared with serology measurements from blood drawn at the time of NPC presentation. Available data suggest that elevated antibody titer may be important for NPC risk for over a decade, although a more informative window may exist in the years immediately before NPC diagnosis (12). This is supported by our data, which demonstrate that at the same antibody titer threshold (EBNA1 IgA = 0.72), the sensitivity of the EBNA1 IgA marker among multiplex family members was marginally higher (sensitivity = 82%) when NPC cases diagnosed

<5 years following blood draw were considered as compared with the entire set of NPC cases diagnosed over approximately 10 years (sensitivity = 80%).

The underlying incidence of cancer also has a large impact on screening parameters. In high-risk multiplex family members from Taiwan with an underlying NPC incidence on the order of  $100 \times 10^5$ , it was estimated that in an unscreened population, only 164 high-risk individuals would need to have blood drawn and screened at baseline for each case of NPC successfully detected over the following 5 years. In contrast, approximately 1,250 individuals would need to be screened per patient with NPC detected if the same EBNA1 IgA marker were applied as a screening tool in the average-risk (NPC incidence =  $10 \times 10^5$ ) Taiwanese population (14). However, general population screening with EBNA1 IgA might be more feasible in high-risk areas such as the Guangdong province of China (NPC incidence =  $50 \times 10^5$ ; Supplementary Table S1; ref. 23).

The interpretation of our results is limited by the modest sample size of this study; due to the fact that we are targeting a small but very high-risk subset of the population, only 21 incident NPC cases were detected. Replication in other existing high-risk or family-based NPC studies would be of interest. Furthermore, there is a circular nature to defining and subsequently applying our "optimal" threshold value in the same population. Again, this could be addressed through independent replication or application of this threshold in another high-risk NPC population.

Our evaluation of the application of EBNA1 IgA as a screening tool for incident NPC suggests that the number of individuals that would need to be screened per case detected in a high-risk population in Taiwan is comparable with currently recommended cancer screening programs in the United States. High-risk target populations are an ideal setting for further evaluation of the utility of EBV serologic marker-based NPC screening. The currently available anti-EBV IgA antibodies tested here did prove to be sensitive tools for identifying individuals at elevated likelihood of developing future NPC, but further research is needed to identify more specific biomarkers that could triage EBNA1 IgA-positive individuals and move toward more efficient NPC screening implementation.

#### Disclosure of Potential Conflicts of Interest

J.M. Middeldorp is the owner and CEO and has ownership interest (including patents) in Cyto-Barr BV and is a consultant/advisory board member of Argen-X. No potential conflicts of interest were disclosed by the other authors.

#### Authors' Contributions

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