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Serum CYFRA21-1 as an effective tumor biomarker for patients with nasopharyngeal carcinoma

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We investigated if the serum cytokeratin 19 fragment 21.1 (CYFRA21-1) level was elevated in nasopharyngeal carcinoma (NPC) and can function as a biomarker for detection and monitoring of NPC. Three hundred and one study subjects were divided into two groups: the NPC group (n=126) and healthy control group (n=175). Serum CYFRA21-1 levels were measured before and after treatment using a chemiluminescent immunoassay, and its association with tumor stage and the clinical objective responses were analyzed. Receiver operating characteristic (ROC) curve analysis was performed to discriminate patients with NPC from the healthy controls. The pretreatment serum CYFRA21-1 level was significantly elevated in patients with NPC compared with the healthy controls (5.07±1.98 ng/ml vs 2.36±1.21 ng/ml, p<0.001), and it declined significantly after the entire treatment (2.14±0.72 ng/ml, p<0.001). The serum CYFRA21-1 level of patients with a classification of T3-4 was significantly higher than that of those with class T1-2 (5.64±2.23 ng/ml vs 4.62±1.64 ng/ml, p=0.006), and that of patients with clinical stage III-IV was higher than clinical stage I-II (5.31±2.02 vs 4.04±1.37 ng/ml, p=0.003). The AUC, sensitivity and specificity of elevated serum CYFRA21-1 in patients with NPC was 0.91, 0.83 and 0.89 respectively. In conclusion, the serum CYFRA21-1 level could be a reliable and effective biomarker for the detection and monitoring of NPC tumor progression.

Key words: nasopharyngeal carcinoma, CYFRA21-1, tumor biomarker, receiver operating characteristic curve

Nasopharyngeal carcinoma (NPC) is a common head and neck cancer that has a higher incidence rate in people living in the East and within the Asian population that has migrated to other parts of Asia or North America than in those in the Western world[1]. In southern China, the incidence is as high as 25 per 100 000. Each year, 80 000 new cases of NPC are diagnosed worldwide, and 50 000 individuals die of this disease[2]. The incidence in males is three-fold that of females, with a peak incidence age of 40-50 years old. Despite the many great achievements that have been made in treatment modalities, recurrence and distant metastasis are the main reasons for failure of treatment. This cancer is highly malignant with extensive capacity for infiltration, early lymphatic spread and high predilection for distant metastases. The majority of patients already have advanced disease at the time of diagnosis. The 5-year overall survival is as high as 90% and 84% for early-stage I and IIA, but it falls to 50-70% for advanced stage[3].

NPC has been pathologically classified by the World Health Organization (WHO) into three categories: type I, keratinizing squamous cell carcinoma; type II, non-keratinizing carcinoma; and type III, non-keratinizing undifferentiated carcinoma[4]. In the southern Chinese population, 2% of cases are Type I, 3% are Type II, and 95% are Type III[5]. Keratinizing undifferentiated carcinoma is associated with the Epstein-Barr virus (EBV)[6]. EBV DNA quantification and various anti-EBV antibodies had been studied as tumor biomarkers of NPC, and some had been widely applied in clinics. However, some studies suggested that anti-EBV antibody titers had limitations for post-treatment monitoring because they still remained at a high level in some patients who were obviously in remission; furthermore, no reliable threshold for a difference between relapse and remission existed[7]. Therefore, novel and reliable diagnostic biomarkers to complement EBV are urgently needed to facilitate early diagnosis.

CYFRA21-1 is known to react specifically with cytokeratin (CK) 19 fragments. CKs are the principal structural elements of the cytoskeleton of epithelial cells, including nasopharyngeal epithelial cells. Accelerated CK19 degradation occurs in neoplastically transformed epithelial cells as a result of increased protease activity of caspase 3, which is a regulator of the apoptosis cascade, and fragments are released into the blood. This leads to an increase in the serum CYFRA21-1 level, which can be recognized by two monoclonal antibodies[8]. CYFRA21-1 has been identified as a potential tumor marker for the diagnosis and prognosis of non-small-cell lung cancer (NSCLC). Measurement of the serum CYFRA21-1 level is a useful auxiliary test for NSCLC and shows particularly high specificity for the diagnosis of squamous cell carcinoma of the lung[9, 10]. Few reports have evaluated CYFRA21-1 as a highly sensitive and specific serological marker for NPC. The major issue addressed in this work is whether serum CYRFA21-1 can be used as an effective biomarker for diagnosis and monitoring of patients with NPC.

Patients and methods

Patients. The sample collection for this study was conducted from Oct 2010 to Feb 2014 at the Eye, Ear, Nose & Throat Hospital of Fudan University, Shanghai. One hundred and twenty-six patients with confirmed nasopharyngeal carcinoma without metastasis were enrolled. None of the patients had other malignancies or significant disease, and only the newly diagnosed who had not received any treatment were used in this study. One hundred and seventy-five healthy, age and sex-matched volunteers were recruited as the healthy control group. The healthy controls had no malignancy or benign tumors after routine examination that included chest X-ray, liver function tests and complete blood tests (blood routine examination, blood biochemical analysis, tumor markers analysis, virus index analysis and blood coagulation system analysis). The study protocol was approved by the Research Ethics Committee of the Eye & ENT Hospital, Shanghai, and all patients and volunteers gave informed consent.

Treatment. All patients received definitive intensity modulation radiation therapy (IMRT) or 3D conformal radiotherapy (3D-CRT). Radiation was administered using 6-MV photon beams. The planned dose for the IMRT group was 66-69.75 Gy (2.2-2.25 Gy/F, QD, 5 times/week) to the gross tumor volume (GTV) and 54-62 Gy (1.8-2.0 Gy/F, QD, 5 times/week) to the clinical target volume (CTV) using varying treatment plans according to the tumor volume and cancer stage. The primary tumor and the upper neck were treated with IMRT, and the lower neck and supraclavicular fossae were treated with a CRT anterior field. The fields were matched using a split-beam technique. The treatment plan was validated after review by a physician. In the CRT group, the planned total dose was 68-73 Gy (1.9-2.0 Gy/F, QD, 5 times/week) to the GTV, and the planned dose to the initial large-fields was 36-40 Gy. The fields were then divided into small, matching, lateral neck fields and posterosuperior neck electron fields depending on the boost volumes and residual neck nodes. The final cone-down fields were boosted to 68-73 Gy with the preauricular and prenasal fields. The lower neck was initially treated with an anterior, 6-MV field and boosted with electrons. The treatment dose to the involved nodes in the lower neck was 56-60 Gy, and the prophylactic dose was 50-55 Gy. A boost dose of 4-6 Gy was given to the residual neck nodes at the end of the initial treatment with small local fields. The patient with Stage II-IV also received chemotherapy that included neoadjuvant, adjuvant or concurrent chemotherapy based on fluorouracil and platinum. In addition, some patients received at least 6 cycles of nimotuzumab (a humanized monoclonal antibody) therapy. The clinical examinations and CT/MRI scans were performed pre-treatment, mid-treatment and post-treatment to evaluate the tumor sizes.

Sample collection and assays. Venous blood samples were collected immediately before treatment and at the end of radiotherapy. Peripheral blood samples (5 ml) were collected from the subjects of the study and placed in procoagulant tubes containing separating gel. The samples were left standing for 30 minutes at room temperature and then centrifuged at 3000 g at 4°C for 15 minutes. The supernatants were separated manually into freezing tubes and, without any further treatment, were frozen at -80°C for a maximum of 3 years. Repeated freeze-thawing of the samples was forbidden

The serum level of CYFRA21-1 was measured using ELISIA. CYFRA21-1 measurement was automatically performed using a (Chemclin Biotech, CHINA) chemiluminescent immunoassay. This test was performed in the Department of Laboratory Medicine at our hospital. Before testing, the samples and the kit components were restored to room temperature (20~27°C), slightly inverted and mixed evenly. Twenty-five microliter aliquots of the samples and 100 µl of the enzyme conjugate were added to the appropriate wells and covered with a plate sealer. The plate was then incubated for 1 hour at 37°C. After 5 washes with buffer, 400 µl of each cleaning solution was added for 10 seconds and drained blotted. One hundred microliters of freshly prepared chemiluminescent substrate was added to each well and left for 5 minutes at room temperature in the dark. The luminescence values of each microplate were measured immediately using a luminometer.

Statistical analysis. For each group, the data were described as the mean±standard deviation for all continuous variables and as absolute numbers (percentage, %) for all categorical variables. The Mann-Whitney U test for continuous variables with the chi-square test or Fisher's exact test for categorical data was used to assess differences between two groups. ANOVA was used to compare the means of two or more samples. Differences were considered statistically significant if the p-value was lower than 0.05 (2-tailed test). All statistical procedures were performed using SigmaPlot version 12.3.

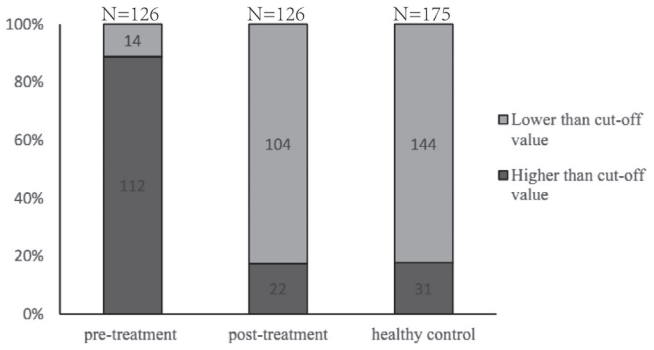

Figure 1. Serum CYFRA21-1 level in patients with NPC and the healthy controls
According to the cut-off value of 3.09 ng/ml, 88.9% (112/126) of patients had a high serum level before therapy, but only 17.7% (31/175) of the healthy controls had a high serum level. After the radiotherapy, 17.5% (22/126) of patients' serum CYFRA21-1 level was above 3.09 ng/ml.

The diagnostic sensitivity, diagnostic specificity, Receiver Operating Characteristic (ROC) curves and area under the ROC curve (AUC) were computed and compared to those of the controls, and the cut-off values were chosen. The positive predictive (PPV) and negative predictive values (NPV) were calculated as follows:

$$\text{Sensitivity} = \frac{\textit{number of true positive}}{\textit{number of true positive+ number of fale negative}} \qquad \text{Specificity} = \frac{\textit{number of ture negative}}{\textit{number of fale positive+ number of true negative}}$$

$$\text{PPV} = \frac{\textit{number of ture positive}}{\textit{number of ture positive+ number of fale positive}} \qquad \text{NPV} = \frac{\textit{number of ture negative}}{\textit{number of true negative+ number of fale negative}}$$

Table 1. Baseline characteristics of 301 participants

Characteristic	No. of Patients (%)	No. of controls (%)
Age		
>50	58(46.0)	90(51.4)
≦50	68(54.0)	85(48.6)
Gender		
Male	101(80.2)	96(54.9)
Female	25(19.8)	79(45.1)
T stage		
T1-2	69(54.8)	
T3-4	57(45.2)	
N stage		
N0	19(15.1)	
N1	26(20.6)	
N2	74(58.7)	
N3	7(5.6)	
Clinical stage		
I	8(6.3)	
II	16(12.7)	
III	67(53.2)	
IV	35(27.8)	

Results

Characteristics of the study groups. We recruited 126 patients with NPC (101 males, 25 females) and 175 healthy volunteers (96 males, 79 females) to serve as healthy controls. The age range was 17-78 years (mean 45.9±12.3) for the patients and 18-76 years (mean 44.6±9.8) for the healthy controls. According to the tumor-node-metastasis (TNM) staging system for nasopharyngeal carcinoma by the American Joint Committee on Cancer (AJCC) 2010, the baseline characteristics of the patients were determined and were summarized in *Table 1*. The populations were comparable with respect to age and sex.

The serum CYFRA21-1 level in the study group. The mean baseline serum level of CYFRA21-1 was 5.07±1.98 ng/ml (1.8-13.09 ng/ml) in patients with nasopharyngeal carcinoma, which was higher than that of the healthy controls 2.36±1.21 ng/ml (0.65-7.10 ng/ml; $p<0.001$). The serum CYFRA21-1 level declined significantly to 2.14±0.72 ng/ml (0.64-4.19 ng/ml) at the end of treatment ($p<0.001$). Because the cut-off value of the serum CYFRA21-1 level was set as 3.09 ng/ml, the proportion of positive patients in the nasopharyngeal carcinoma group was 88.9% (112/126) before treatment and 17.5% (22/126) after treatment. The proportion of positive patients in the healthy controls was 17.7% (31/175). All of the above-mentioned results are shown in *Fig* 1. No significant serum CYFRA21-1 level difference was observed in the NPC group according to age (>50y: 5.23±2.01 ng/ml vs ≦50y : 4.93±1.87 ng/ml; $p=0.092$) and sex (male: 5.16±2.14 ng/ml vs femal: 4.71±1.95 ng/ml; $p=0.437$).

Next, we analyzed the correlations between the serum CYFRA21-1 level and the clinical characteristics of the patients. The mean level of serum CYFRA21-1 in patients with stage T3-4 (5.64±2.23 ng/ml) was significantly higher than that in patients with stage T1-2 (4.62±1.64 ng/ml; $p=0.006$). The mean level of serum CYFRA21-1 in patients with clinical stage III-IV (5.31±2.02 ng/ml) was significantly higher than that in patients with I-II (4.04±1.37 ng/ml; $p=0.003$). However, no significant differences in the mean serum CYFRA21-1 levels between N-stage NPC patients were observed (N0: 4.80±2.02; N1: 4.58±1.73, N2: 5.32±2.07, N3:4.92±1.50 ng/ml; $p=0.420$). The relationship between the serum CYFRA21-1 level and tumor stage was shown in *Table 2*.

Table 2. The relationship between clinical characteristics and serum level of CYFRA21-1

Stage	CYFRA21-1 (ng/ml) Mean±SD	p-value
T1-2	4.62±1.64	
T3-4	5.64±2.23	0.006
N0	4.80±2.02	
N1	4.58±1.73	
N2	5.32±2.07	
N3	4.92±1.50	0.420
I-II	4.04±1.37	
III-IV	5.31±2.02	0.003

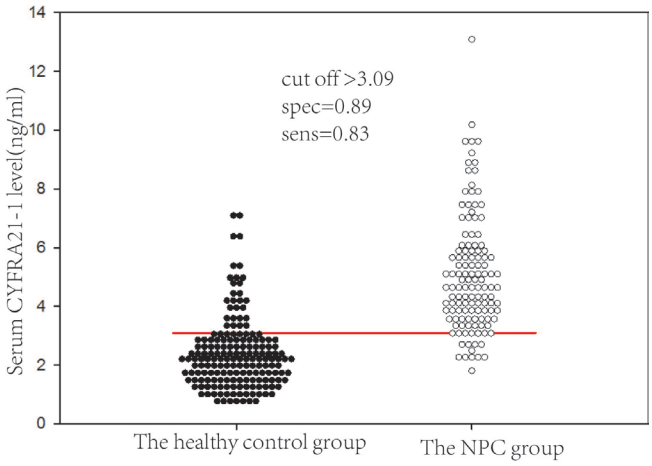

Figure 2. The cut-off, sensitivity and specificity of serum CYFRA21-1 levels
The NPC group was set as the high group in the positive direction, and the healthy control group was set as the low group in the positive direction. According to the cut-off value of 3.09 ng/ml, the sensitivity and specificity of diagnosis of NPC was 0.83 and 0.89, respectively.

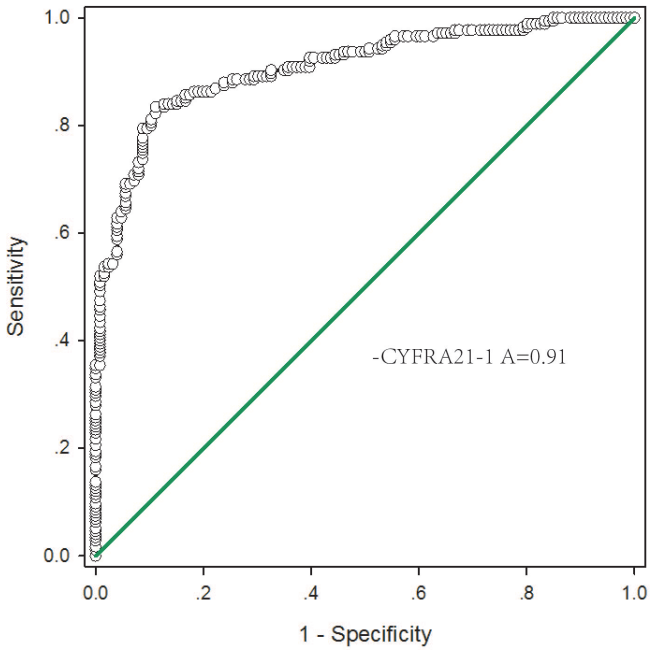

Figure 3. ROC curve analyses of the use of CYFRA21-1 to differentiate NPC cases and the healthy controls
Receiver operator characteristic curves for serum CYFRA21-1 level as diagnostic test in NPC, levels of serum CYFRA21-1 level was measured in 126 patients with NPC and 175 healthy controls. With cut-off value of 3.09 ng/ml, the area under ROC curve was 0.91 (95% confidence interval, 0.876-0.941).

Association between the therapeutic response and decreases in CYFRA21-1. The objective clinical response was assessed using the response evaluation criteria in solid tumor (RECIST). The tumor response was evaluated using physical examination, nasopharyngoscopy and CT/MRI of the head and neck after the completion of radiotherapy; and physical examinations were determined by at least two experienced oncologists. The complete response (CR) was defined as the complete disappearance of all measureable and assessable disease, and the partial response (PR) was defined as a subjective decrease with >50% tumor regression. Among the 126 patients with NPC, 117 (92.9%) attained CR at the end of radiotherapy, and 9 (7.1%) achieved PR. The mean values of the serum CYFRA21-1 levels prior to and after therapy were 5.07±1.98 ng/ml and 2.14±0.72 ng/ml, respectively, which clearly showed us that serum CYFRA21-1 was significantly decreased when compared with baseline levels ($p<0.001$).

Analysis of ROC curves. The efficacy of CYFRA21-1 as a serum biomarker for distinguishing nasopharyngeal carcinoma was assessed using ROC curve analysis. The pre-test probability and cost ratio was set as 0.05. The CYFRA21-1 levels in patients were defined as the high group, and those of the controls were defined as the low group. According to the dot histogram, the cut-off level of CYFRA21-1 was calculated as 3.09 ng/ml (*Fig 2*). The AUC was 0.91 (95% CI, 0.876-0.941) and accurately discriminated patients with nasopharyngeal carcinoma from the healthy controls (*Fig 3*). The sensitivity of detection was 0.83, and the specificity was 0.89. The cut-off levels of the serum CYFRA21-1 PPV and NPV were calculated as 0.783 and 0.911, respectively.

Discussion

Early diagnosis of NPC is essential to increase survival, but due to its silent, deep-seated location and presentation of nonspecific symptoms, the majority of patients had advanced disease at the time of diagnosis. Evaluated serum EBV-related protein has been used as a warning factor for people susceptible to NPC. However, due to limitations, EBV-related protein was not a useful biomarker for NPC. Ideal biomarkers must be specific, sensitive and easy to assay and interpret, and they must have high reproducibility and comparability between different laboratories[11].

CYFRA21-1 was an acidic protein of 40 kDa that has caspase-3 proteolytic sites between amino acids 311 and 367 of its protein sequence. It was released into the blood as soluble fragments from intracellular CK19 during cell lysis and tumor necrosis. The cytokeratin level was\s thought to sensitively reflect cell differentiation or tumorigenesis. Therefore, elevated serum levels of CYFRA21-1 might be a result of a greater degree of degradation and release of intracellular CK19 into the serum, which suggested an increasing tendency to differentiate into squamous cell carcinoma. Serum CYFRA21-1 had been reported as a prognostic factor in patients with a variety of cancer types, including head and neck cancer[12], NSCLC[13], esophageal cancer[14], pancreatic

cancer[15], cervical cancer[16] and biliary tract cancer[17]. In addition, it appeared more sensitive and more specific than other tumor biomarkers, such as Carcino Embryonie Antigen, Neuron-Specific Enolase and Squamous Cell Carcinoma antigen in squamous cell carcinoma. However, sufficient data to support CYFRA21-1 as a tumor biomarker in NPC is lacking.

In this study, we showed that the serum CYFRA21-1 level was significantly elevated in 126 patients with NPC compared with 175 healthy volunteers. This result was in accordance with the previous studies[18, 19]. According to the cut-off value calculated by software, elevated serum CYFRA21-1 levels were found in 17.7% of the healthy controls and in 88.9% of patients with NPC. In addition, at the end of the radiotherapy, only 17.5% of patients' serum CYFRA21-1 level was higher than the cut-off value. The serum CYFRA21-1 levels in the healthy controls (2.36±1.21 ng/ml) was higher than that (2.14±0.72 ng/ml) in patients with NPC who underwent therapy because irradiation might have damaged the formation of the cytoskeleton in normal epithelial cells[20, 21]. Radiotherapy-induced a large number of normal nasopharynx and oropharynx epithelial cell damage, resulted in decline of CYFRA21-1 level released into blood by normal respiratory epithelial cell. Because cytokeratin 19 was expressed in simple epithelia, including nasopharynx and oropharynx epithelial cells. However, the baseline serum CYFRA21-1 levels in patients was significantly higher than those in the healthy controls. Those data showed us that elevated serum CYFRA21-1 might be associated with NPC, and the degree of serum CYFRA21-1 could be associated with disease progression. To investigate our hypothesis, we analyzed the clinical variables in 126 patients with NPC, we demonstrated that pretreatment serum CYFRA21-1 levels were significantly related to tumor T (p=0.006) and clinical stages (p=0.003). This observation was consistent with a previous study[18]. However, no correlation between the serum CYFRA21-1 level and tumor N stage was observed (p=0.42). We deduced that soluble CYFRA21-1 was not easily dissolved into blood because of lymph nodes with complete and solid tumor capsules. A study by Lin et al.[19] showed a significant difference between the serum CYFRA21-1 levels in patients with T1-2 and T3-4, as well as between those with N0 to N1 and N2 to N3. Because of the significant positive association between T classification and clinical stage of NPC, the serum level of CYFRA21-1 might greatly reflect disease progression. According to the stage, CYFRA21-1 could be the most important prognostic factor in predicting the outcomes of patients with malignant tumors, and the serum level of CYFRA21-1 could be used as a powerful prognostic marker in NPC. Wei et al.[22] demonstrated that the pretreatment serum level of CYFRA21-1 could be a reliable biomarker for evaluating the long-term prognosis of patients with undifferentiated NPC. In our study, the relationship between the clinical objective tumor response and serum CYFRA21-1 change in patients submitted to systemic clinical treatment was synchronized. A promising result showed that the serum CYFRA21-1 level significantly declined from pre-treatment 5.07±1.98 ng/ml to post-treatment 2.14±0.72 ng/ml. In accordance with the serum CYFRA21-1 level decline, all 126 patients receiving systemic and standard treatments achieved an effective clinical objective tumor response. From another perspective, serum CYFRA21-1 level alteration could timely and accurately reflect the tumor state progress, and possibly be used to monitor treatment. The degree of serum CYFRA21-1 decline might be a prognostic factor for NPC.

To investigate the diagnostic efficacy of the serum CYFRA21-1 level for NPC, we established ROC curves and a dot histogram for the biomarker and determined the appropriate balance between sensitivity and specificity for choosing a cut-off point. The Area under the ROC curves (AUC) was 0.91 (95% CI, 0.876-0.941) and could be interpreted accurately for diagnosis. With the cut-off value of 3.09 ng/ml, 0.83 of sensitivity and 0.89 of specificity was obtained. Sensitivity was moderately shown in other studies, for example, 58.75% sensitivity in a study by Tai et al.[19] and 60% sensitivity in a study by Lin et al.[23]. The sensitivity and true-positive rate decreased at a higher cut-off level, while the sensitivity and the false-positive rate increased at a lower cut-off level. Therefore, the optimal cut-off was important for diagnostic sensitivity.

The PPV and NPV are the proportions of positive and negative results in statistics and determine what are true positive and negative results. A high result can be interpreted as indicating the accuracy of such a statistic. The concept of PPV has not been popular; however, it is clinically important when a tumor marker is used as a screening tool. Especially, the PPV of a tumor marker is a useful index for a high prevalence rate of a certain tumor. A study by Okamura and his colleagues[24] demonstrated that the PPV of a combination of positive tumor markers was greater than that of one alone in the diagnosis of lung cancer. In our study, the PPV and NPV were calculated as 0.783 and 0.911, respectively, which was useful for diagnosis. Combination of the serum CYFRA21-1 level with the EBV index might be more valuable for early and accurate diagnosis of NPC.

In conclusion, this study has several limitations. The number of studied subjects was small, all subjects from a single-center institute, and data from other benign diseases in the same anatomical sites were limited. However, our data clearly suggested that the serum CYFRA21-1 level could be a reliable and effective biomarker for the detection and monitoring of NPC tumor progression, with a sensitivity and specificity of 0.83 and 0.89, respectively. The combination of the serum CYFRA21-1 level with the EBV index might be more valuable for diagnosing NPC early and accurately. Further studies that include more cases with different treatment modes and their clinical results are undergoing.

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