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# Extensive serum biomarker analysis in patients with nasopharyngeal carcinoma

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ABSTRACT

Nasopharyngeal carcinoma (NPC) is a fast-growing cancer characterized by high occurrences of nodal and distant metastases and poor prognosis. It is therefore important to identify new serum biomarkers for the early diagnosis and prognostic prediction of this disease. The present study identifies biomarkers in NPC patient serum using a solid-phase antibody array detecting the expression profiles of 174 cytokines in a single experiment. ELISA was performed to validate the array results. The levels of TIMP-2, SELL, CCL24, MMP-1, MMP-3, IGF-I and IL-8 were significantly higher in serum from NPC patients, while the levels of MSP-alpha and HCC-4 were lower. Furthermore, the validation results were identical to those obtained from the antibody array. These results indicate that these cytokines might serve as novel biomarkers for the diagnosis and prognostic prediction of NPC.

## 1. Introduction

Nasopharyngeal carcinoma (NPC) is a cancer that arises from the squamous epithelial cells covering the surface of nasopharynx in the nasopharyngeal region. NPC is characterized by a high frequency of nodal and distant metastases at the time of diagnosis [1,2]. NPC is a relatively rare malignancy in most parts of the world, but is common in Southern China and Southeast Asia where the incidence is as high as 20–50 cases per 100,000 person-years [3,4].

Environmental factors, genetic susceptibility, Epstein-Barr virus (EBV) infection, smoking and drinking have been thought to be risk factors for NPC [5–7]. Most NPC patients tend to present at a more advanced stage due to nonspecific clinical symptoms, and have unfavorable prognoses because of limited knowledge of the molecular pathogenesis, lack of reliable and robust biomarkers for early detection, and poor responses to available therapies [8]. At present, radiotherapy is the standard therapy for NPC, but chemotherapy has been increasingly applied in combination with radiotherapy to improve long-term survival probability. However, for different NPC patients with the same pathological stages and/or therapeutic regimens, the outcomes may vary due to individual differences in each case. Therefore, identifying novel biomarkers for sensitive diagnosis and prognostic prediction of NPC is of great importance.

Many cancers arise from sites of infection, chronic irritation and inflammation. Epstein-Barr virus (EBV) infection is known as a critical component of NPC. The tumor microenvironment, which is largely orchestrated by inflammatory cells, is an indispensable participant in the neoplastic process, fostering proliferation, survival and migration. In addition, tumor cells have co-opted some of the signaling molecules of the innate immune system, such as selectins, chemokines and their receptors for invasion, migration and metastasis [31]. Therefore, in the present study, human cytokines-array technology was utilized to investigate the difference of inflammation/immune factor between patient and health people in a high-throughout format.

## 2. Materials and methods

### 2.1. Patients

Serum samples were obtained from 12 NPC patients who were

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**Table 1**
Patient clinical data (samples from the First Affiliated Hospital of Foshan, Southern Medical University).

| | |
|---|---|
| *Patient* | |
| n | 12 |
| Age (mean ± SD), year | 49.4 ± 8.9 |
| Sex | male = 50%, female = 50% |
| Disease stage | II = 75%, III = 25% |
| Treatment | no |
| *Control* | |
| n | 12 |
| Age (mean ± SD), year | 44.1 ± 5.1 |
| Sex | male = 50%, female = 50% |
| P-value (Age, patient vs control) | 0.085 |

diagnosed clinically and histologically at the Department of Laboratory Medicine, the First Affiliated Hospital of Foshan, Southern Medical University (Foshan, China) and 53 NPC patients in I, II, III, IV stage at the Department of Laboratory Medicine, The Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University. In addition, 15 lung cancer cases and 15 liver cancer cases was collected for assay from The Sun Yat-Sen Memorial Hospital. All samples were collected before surgical resection from patients who had not yet received chemotherapy or radiotherapy. 22 normal healthy controls receiving regular health examinations in two hospitals were recruited as control subjects. Clinical data regarding all subjects is listed in Tables 1 and 3. All subjects were informed of the investigational nature of this study and signed informed consent forms prior to their inclusion. All procedures were approved by the Ethics Committee of the First Affiliated Hospital of Foshan, Southern Medical University (2015SMU_EC0035) and The Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University.

### 2.2. Antibody array

A Human Cytokine Antibody Array G series 2000 kit (Raybiotech, Inc, Norcross, GA, USA) was used to detect proteins of interest in the patient serum samples. This antibody array is capable of detecting 174 cytokines (Appendix 1) in a single experiment. This assay consisted of three arrays (array 6, 7 and 8) with 174 antibody dots, in duplicate, and utilizes a sandwich assay technique. The detection of cytokines was performed following the antibody-array manufacturer's instructions. Briefly, serum was diluted 2-fold, incubated in these assay pools for 2 h and washed. The arrays were incubated with a mix of 174 biotin-conjugated antibodies for an additional 2 h at room temperature. The arrays were again washed and developed for 2 h via incubation with Cy3-conjugated streptavidin. The slides were scanned with a GenePix 4000B scanner (GenePix version 5.0, Axon Instruments, Union City, CA, USA) and the signal values were extracted using the GenePix 4000B software. The signal data were analyzed with the Raybiotech analysis tool, a Microsoft Excel plugin specifically designed to analyze data in the Human Cytokine Antibody Array G series 2000 assay. Additionally, a human cytokine array G8 (Raybiotech AAH-CYT-G8-4) was used to detect MMP-1 level in serum from The Sun Yat-Sen Memorial Hospital (Appendix 3).

### 2.3. ELISA

Samples were further analyzed using ELISA kits (Raybiotech, Inc Norcross GA, USA), according to the manufacturer's instructions. Serum dilution factors were specific to the individual serum biomarkers. After dilution, samples were incubated in the plates coated with capture antibodies for 2.5 h at room temperature. The plates were washed and biotin-conjugated antibody was added and allowed to bind for 2 h. The plates were again washed and HRP-conjugated streptavidin was added to catalyze the TMB reagent for 30 min. The catalytic reaction was stopped by the addition of 100 µl sulfuric acid and the optical density ($OD_{450}$) was determined via an ELx800NB microplate reader (BioTek Instruments, Winooski, VT, USA).

### 2.4. EA-IgA assay and EBV quantative PCR assay

15 samples in each group were further analyzed using Epstein - Barr virus IgA ELISA (IBL, German), according to the manufacturer's instructions. After dilution, samples were incubated in the precoated plate for 60 min at 37 °C. The plates were washed and 100 µl of labeled antibody solution was added and allowed to bind for 30 min at 4 °C. The plates were again washed and HRP-conjugated streptavidin was added to catalyze the TMB reagent for 30 min. The optical density ($OD_{450}$) was determined via an ELx800NB micro-plate reader (BioTek Instruments, Winooski, VT, USA). EBV copy number was also measured in the same batch of samples by quantitative Taqman PCR assay via an ABI7500 real-time PCR (Applied Biosystems, Thermo Fisher SCIENTIFIC). Quantification was performed using standard curve method. Standard curves were constructed in each PCR run and the copy numbers of the genes in each sample were interpolated using these standard curves. Serial dilutions of EBV standard DNA was used as template for the standard curve.

### 2.5. Statistical analysis

Data were presented as means ± standard deviations. All statistical analyses were performed using SPSS version 20 (IBM Corp., Armonk, NY, USA). Differences between groups were determined by Mann-Whitney *U* test and were considered significant when $P < 0.05$. Fold change (FC) was calculated to indicate the relative expression levels of cytokines in NPC patient serum.

## 3. Results

### 3.1. Cytokine data analysis

After analysis with the Raybiotech analysis tool, normalized (background-subtracted) data was statistically analyzed by Mann-Whitney *U* test using SPSS v13.0 software. Eight cytokines were found to be differentially expressed between NPC patients and the disease-free control subjects (detailed data in Appendix 2). The levels of TIMP-2, SELL, CCL24, MMP-3, IGF-I and IL-8 were increased in NPC serum samples, while the levels of MSP-alpha and HCC-4 were decreased, compared against the control values. The mean values of these eight biomarkers (Table 2) and the fold changes in the NPC and control serum samples showed that the expressions of these factors in patient serum were clearly different from those in the controls (Fig. 1). Furthermore, to prove the differences of these markers between NPC and controls, the signal values of the markers from each sample were subjected to unsupervised-hierarchical cluster analysis. The results showed

**Table 2**
Antibody array data of differentially expressed cytokines from patient and control serum (samples from the First Affiliated Hospital of Foshan, Southern Medical University).

| Cytokine | P value (Mann-Whitney *U* test) | Fold change (Cancer/Control) | Mean (NPC, n = 12) | Mean (Control, n = 12) |
|---|---|---|---|---|
| CCL24 | 0.00003 | 2.734 | 3976.8 ± 363.2 | 1454.5 ± 179.0 |
| IGF-I | 0.00003 | 2.865 | 1258.5 ± 193.3 | 439.3 ± 51.3 |
| IL-8 | 0.00003 | 3.570 | 1507.7 ± 328.7 | 422.3 ± 105.7 |
| TIMP-2 | 0.00003 | 3.448 | 8281 ± 568.6 | 2401.9 ± 470.4 |
| MMP-3 | 0.00003 | 5.109 | 8160.7 ± 1192.3 | 1597.3 ± 311.6 |
| SELL | 0.00003 | 2.359 | 10109.9 ± 1036.4 | 4285.8 ± 560.9 |
| MSP-alpha | 0.00003 | 0.528 | 3927.2 ± 362.1 | 7442.0 ± 629.2 |
| HCC-4 | 0.00003 | 0.375 | 780.3 ± 354.4 | 2080.8 ± 264.2 |

**Table 3**
Patient clinical data (Samples from The Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University).

| Disease stage | NPC I | NPC II | NPC III | NPC IV | Liver cancer | Lung cancer | Healthy control |
|---|---|---|---|---|---|---|---|
| n | 16 | 17 | 16 | 8 | 10 | 15 | 10 |
| Age (mean ± SD) | 58.9 ± 6.8 | 54.8 ± 5.2 | 58.3 ± 6.3 | 60.0 ± 6.2 | 50.0 ± 8.1 | 58.9 ± 9.9 | 52.2 ± 10.7 |
| Sex | Male = 50%<br>Female = 50% | Male = 47%<br>Female = 53% | Male = 50%<br>Female = 50% | Male = 62%<br>Female = 38% | Male = 70%<br>Female = 30% | Male = 60%<br>Female = 40% | Male = 50%<br>Female = 50% |
| p-value (Age, patient vs control) | 0.101 | 0.483 | 0.081 | 0.087 | 0.611 | 0.124 | |

that the NPC and control groups were accurately distinguished based on the samples (Fig. 2).

### 3.2. The differentially expressed cytokines

The expression levels of the cytokines in the antibody array are proportional to their fluorescent intensities and eight markers were expressed at different levels in the NPC and control samples. Fig. 3 shows images from each array deemed to be the most representative of each group (samples from the First Affiliated Hospital of Foshan, Southern Medical University). The level of MMP1 increases gradually as the progression of PNC (Fig. 5 and Appendix 3).

### 3.3. ELISA results

The validation of 8 differentially expressed markers identified by antibody array was performed using additional samples including 20 NPC cases and 20 cancer-free control samples in an ELISA analysis. The concentrations of the biomarkers in these samples were analyzed using Mann-Whitney *U* test. The levels of the eight cytokines were confirmed to be significantly different in patients when compared with controls (Fig. 4), confirming the reliability of the protein microarray results. Validation of MMP-1 identified by antibody array was also performed using additional samples including 53 NPC cases, 15 cancer-free control samples, 15 lung cancer cases and 15 liver cancer cases in an ELISA analysis. The ELISA results were consistent with array data (Figs. 5 and 6).

### 3.4. EA-IgA assay and EBV quantative PCR assay

Except screening of serum marker with antibody array and ELISA kit, we also compared the role of Epstein-Barr virus (EBV) DNA load and EBV IgA serology as tools for screening patients with NPC. 15 samples in each group were further analyzed using EA-IgA assay kits, according to the manufacturer's instructions. As the malignancy of cancer increases, the EA-IgA level in serum increased as shown in Fig. 7. The EBV copy numbers were not related with the progression of NPC patients when compared to that of normal health controls (Fig. 7).

## 4. Discussion

Although NPC is characterized by atypical clinical features, the

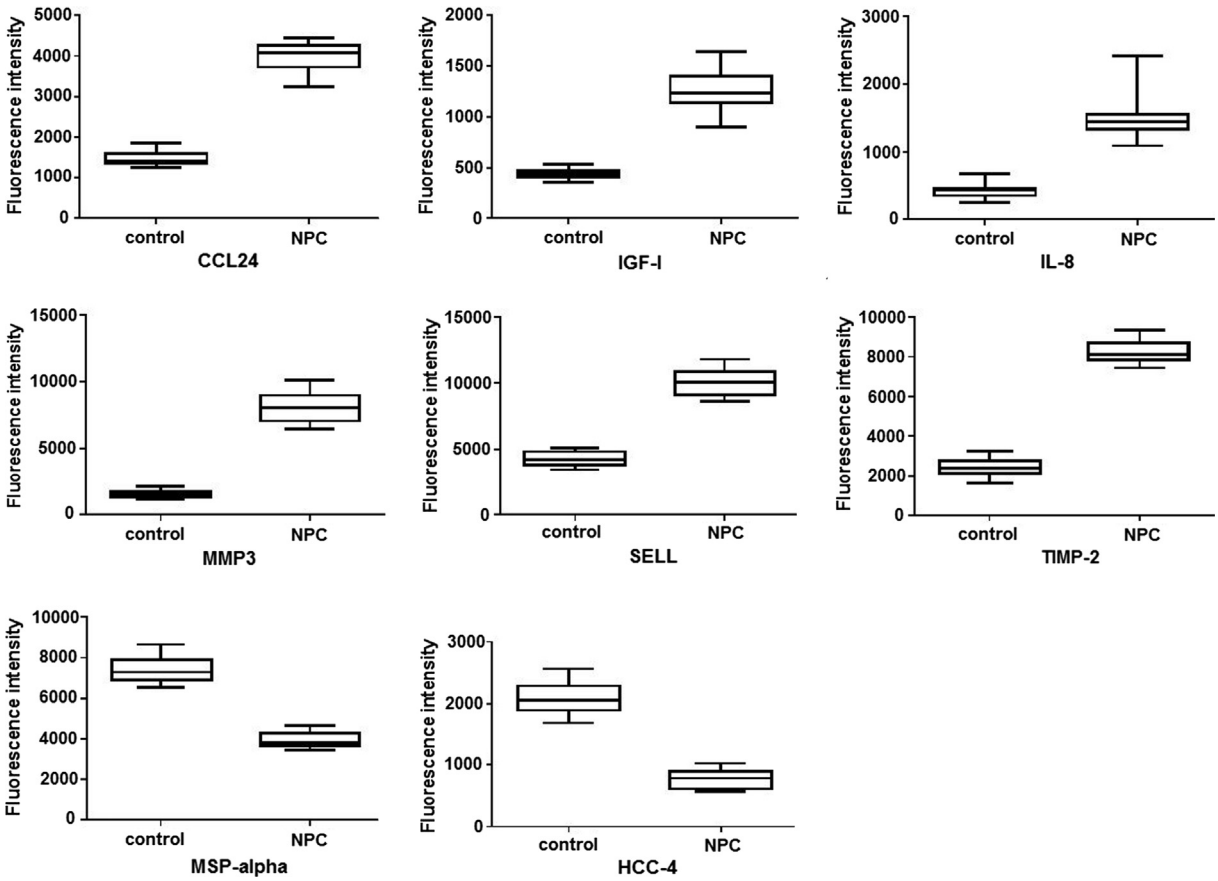


**Fig. 1.** Boxplot of the antibody array data. After statistical analysis with Mann-Whitney *U* test, the data of serum biomarkers expressed differentially between the NPC samples and the controls are shown by boxplot with $P < 0.05$. The centerline in the boxplot indicates the median data point in each group.

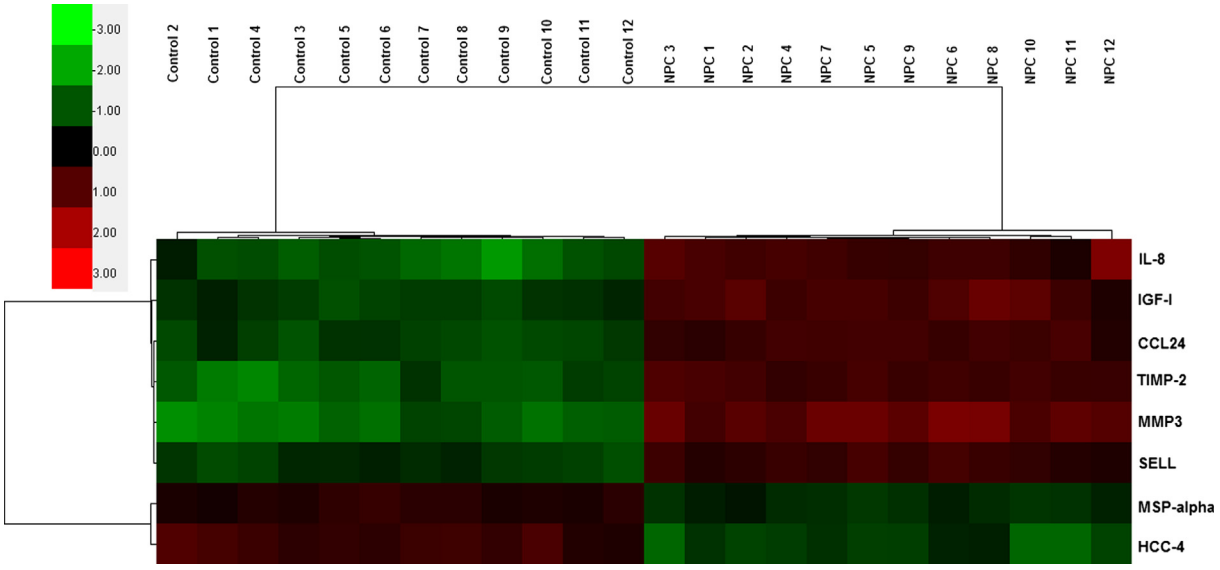


**Fig. 2.** Unsupervised-hierarchical cluster analysis of differentially expressed cytokines. The array data of the eight differentially expressed biomarkers was used for unsupervised-hierarchical cluster analysis by Cluster 3.0 software. The results show that the NPC and control groups were discriminated accurately. In the figure, low levels of protein are shown in green, median levels in black, and high levels in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

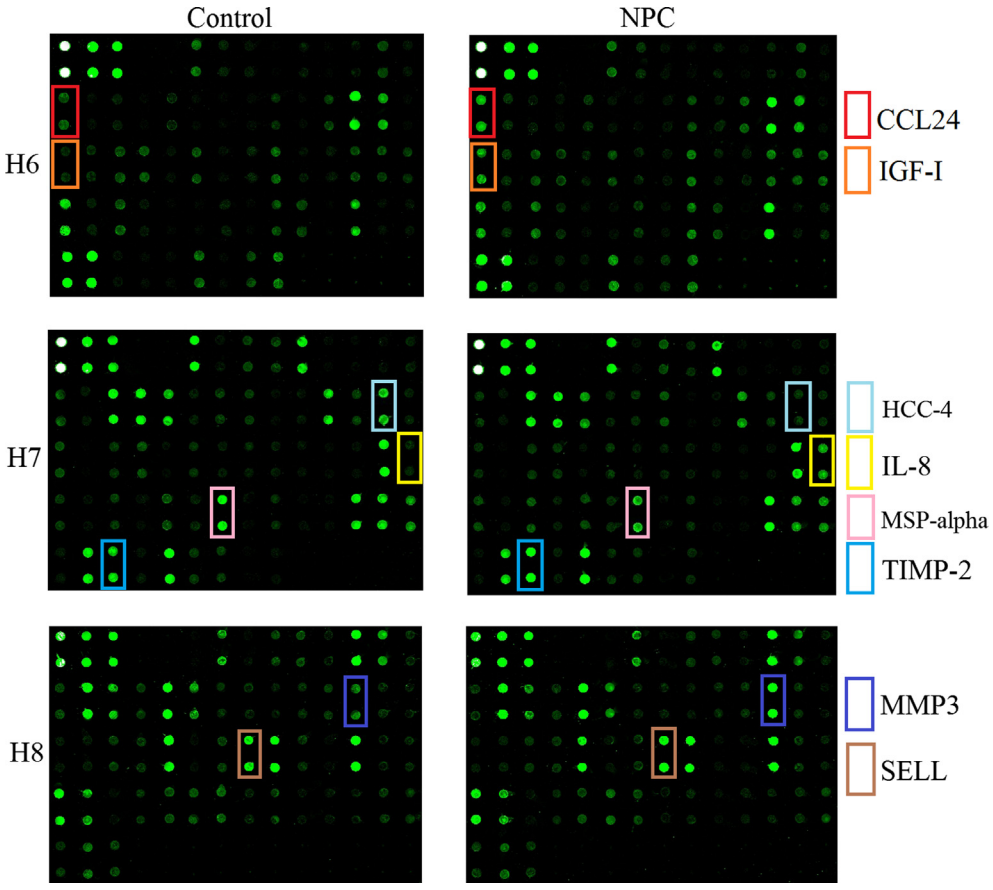


**Fig. 3.** The patterns of cytokines in the antibody arrays. The fluorescent signals of the cytokines were photographed and their levels are proportional to their signal intensity. In these arrays, each antibody was printed in duplicate. The locations of the eight significantly different proteins are noted in colored boxes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

prognoses of patients with NPC have improved significantly due to recent advances in diagnostic technologies and therapeutic interventions. However, patients within the same clinical stage of NPC still achieve different clinical outcomes when treated by identical therapeutic interventions. Therefore, identification of novel biomarkers for more effective prognoses is vital.

In the present study, an advanced technique simultaneously assaying 174 cytokines was utilized to identify novel serum biomarkers, with the results yielding nine differentially expressed cytokines in NPC serum: MPP-1, TIMP-2, SELL, CCL24, MMP-3, IGF-I, IL-8, MSP-alpha and HCC-4 from Two batches of samples. Unsupervised-hierarchical cluster analysis obtained 100% accuracy in sample classification using the microarray data, which confirmed the array results. Additionally, the results of ELISA, a secondary and independent analytical method,

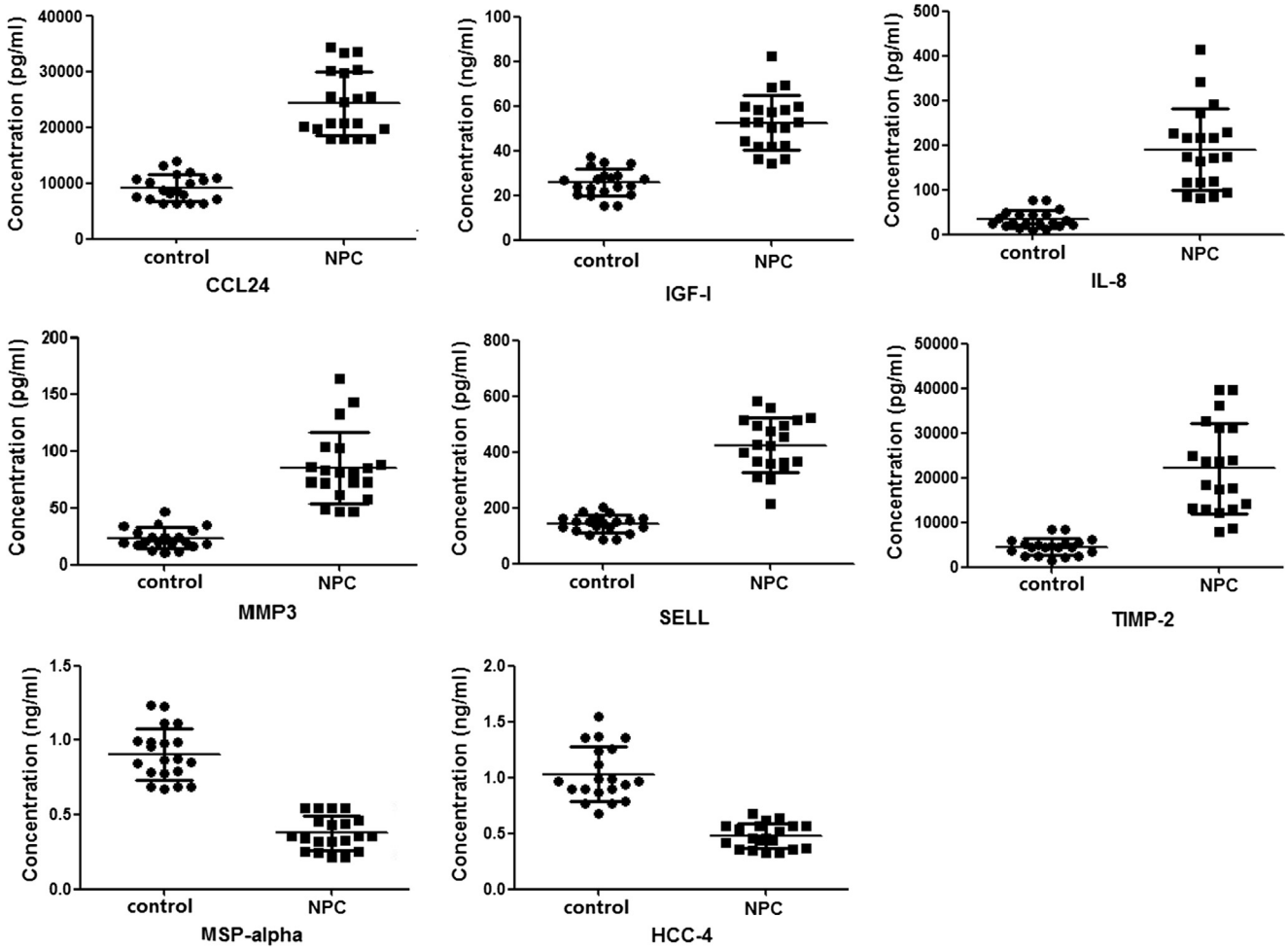


**Fig. 4.** ELISA validation of the eight differentially expressed cytokines. Mann-Whitney *U* test analysis showed the validation results were consistent with those of the protein array. The ELISA data is shown in the scatter diagram with median values. The P value of each factor obtained from the Mann-Whitney *U* test analysis was less than 0.05 between patients and controls.

analysis of additional serum samples showed that six cytokines (TIMP-2, SELL, CCL24, MMP-3, IGF-I and IL-8) were up-regulated in NPC patient serum, while MSP-alpha and HCC-4 were down-regulated, when compared against control samples. These results indicate that these markers have roles in the specific pathophysiological processes of NPC and are potential serum biomarkers. MMP-1 were analyzed by cytokines chip and ELISA from another group of samples collected from The Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University. It showed that the level of MMP-1 increases gradually in patient serum as the progression of the disease.

Matrix metalloproteinases (MMPs) are a family of zinc dependent endopeptidases that can degrade all extracellular matrix components, mediate the invasion, metastasis, proliferation, survival, and angiogenesis of tumors, and generally are over expressed in human tumors [18–20]. MMP-3 is expressed by fibroblastic cells and by normal and transformed squamous epithelial cells [21]. NPC is a cancer originating from the squamous epithelial cells in the nasopharyngeal region, suggesting that MMP-3 is interrelated with the progression of NPC. In a previous study, MMP-3 was highly expressed in nasopharyngeal carcinoma cells [22]. While there had not been previous reports of the high MMP-3 expression levels in NPC patient serum, the present study found MMP-3 to be up-regulated in NPC patient serum, compared with that of control serum, indicating that MMP-3 may be a serum biomarker for the prognosis of NPC.

Many reports suggest that MMP-1 is the most important to NPC development. MMP-1 expression is increased 124-fold in NPC versus 9-fold in other head and neck cancers [28]. LMP1 upregulates MMP-1 by increasing transcription with a new Ets binding site in the MMP-1 promoter and by increasing expression of MMP-3, an activator of latent MMP1 [29]. MMP-1 promotor polymorphism that prevents binding of the promoter repressor protein is in some NPC tumors. Patients with tumors that are homozygous for this polymorphism tended to have more aggressive tumors and late-stage disease at presentation [30]. The present study demonstrated that MMP-1 increased gradually from Stage I to stage IV compared with that of control serum. It is consistent with previous report that MMP-1 is the most important to NPC development.

TIMP-2, a member of the protease family inhibiting the proteolytic activity of matrix metalloproteinase 2, regulates cell growth and apoptosis. Previous studies suggest that the level of TIMP-2 expression is mainly associated with different types of cancer and metastasis [9–11]. The present study discovered that the level of TIMP-2 in NPC serum was higher than in healthy controls, although a previous study showed that the positive rates of TIMP-2 are significantly higher in NPC than in inflamed tissues [12]. These results suggest that TIMP-2 may promote NPC cell growth and inhibit apoptosis and may be a novel plasma biomarker of NPC.

SELL is a leukocyte adhesion molecule that mediates both lymphocyte homing to peripheral lymph nodes and leukocyte accumulation at sites of inflammation [13]. Reportedly, blocking SELL results in apoptosis of chronic lymphocytic leukemia cells and is therefore a potential therapeutic [14]. However, reports about the relationship between SELL and cancer are rare, especially concerning NPC. NPC is a

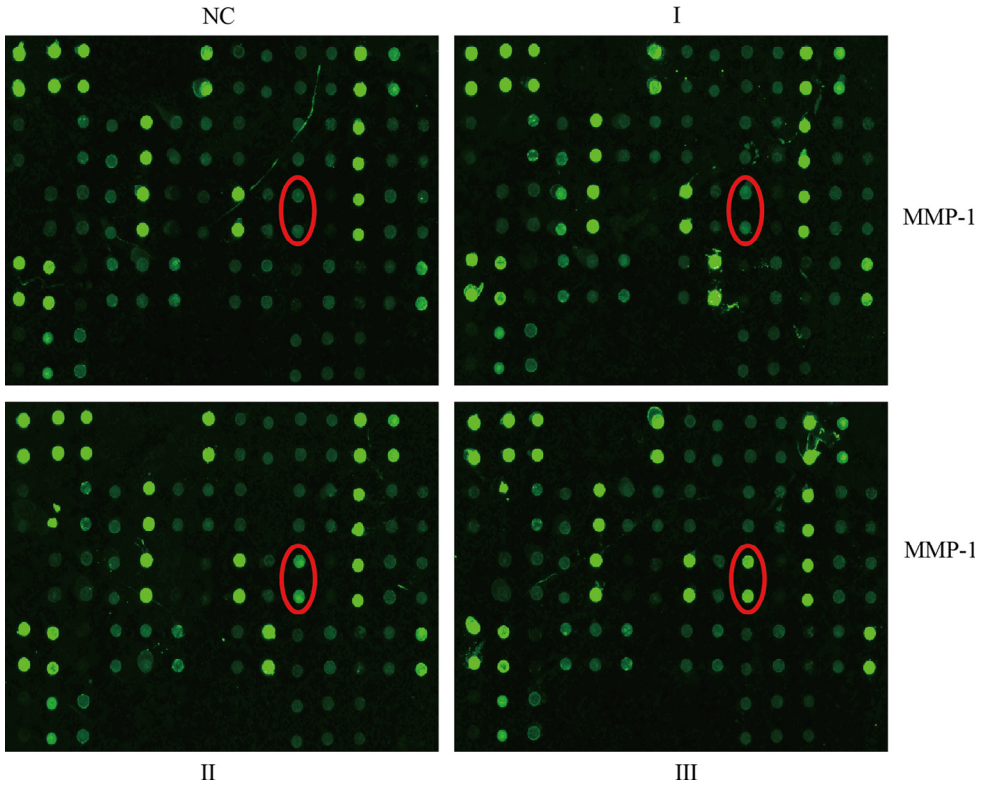


**Fig. 5.** Assay of MMP1 by human cytokine array G8. The fluorescent signals of the MMP-1 were photographed and their levels are proportional to their signal intensity. Each antibody was printed in duplicate. The locations of the MMP-1 are noted in colored boxes.

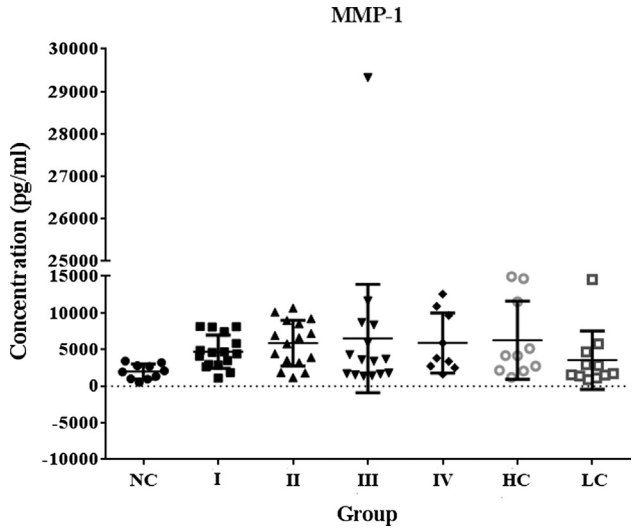


**Fig. 6.** ELISA validation of the MMP-1. The Mann-Whitney *U* test analysis showed the validation results were consistent with those of the protein array. The ELISA data is shown in the scatter diagram with median values. The P value obtained from Mann-Whitney *U* test analysis was less than 0.05 between each patient group and control group, and between different NPC stages. NC, Control group; I, NPC I stage; II, NPC II stage; III, NPC III stage; IV, NPC IV stage; HC, Liver cancer group; LC, Lung cancer group.

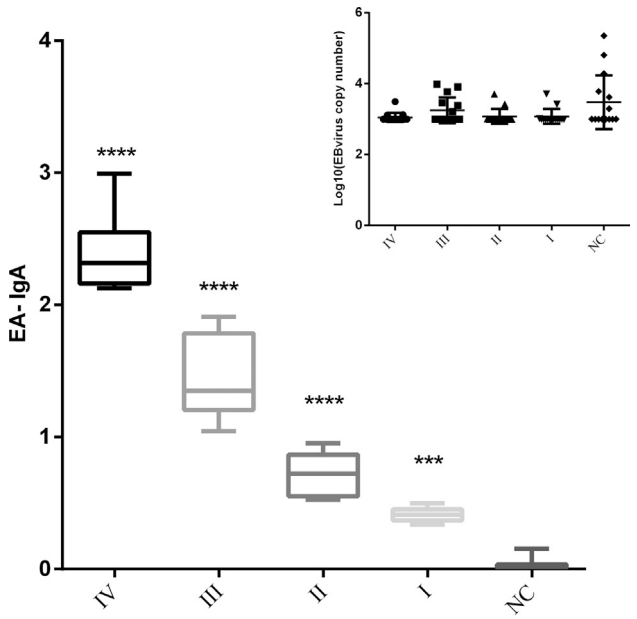


**Fig. 7.** Classification of nasopharyngeal carcinoma based on EA-IgA and EBV-Copy Numbers. 15 samples of each group from The Sun Yat-Sen Memorial Hospital have been recruited for the quantitative-PCR and EA-IgA assay. The Mann-Whitney *U* test analysis showed that The EA-IgA level increased with the progression of PNC. There is no significant difference of EBV copy numbers between normal health control and different stages of NPC patients (in put).

type of fast-growing cancer with a high frequency of nodal and distant metastases [2], and SELL was found to be highly expressed in NPC serum in this study, suggesting SELL may mediate the metastasis of NPC and be a novel biomarker for predicting prognosis.

CCL24 belongs to the CC class of chemokines and is one of three family members characterized as being the major ligands of the chemokine receptor CCR3 [15]. A previous study indicated that CCL24 is specifically expressed within colorectal cancer primary tumors and hepatic metastatic tumors and, to a far lower degree, within surrounding non-neoplastic tissue [16]. To our knowledge, the observation of CCL24 in NPC has not previously been reported. However, it has been noted that mean CCL24 levels measured in persistent allergic

rhinitis, non-allergic rhinitis with eosinophilia syndrome and chronic rhinosinusitis with polyps were significantly increased compared to controls [17], which suggests CCL24 may be involved in the pathogenesis of NPC. We found the level of CCL24 in NPC serum was significantly higher than that of the controls in this study, which further shows the role of CCL24 in the progression of NPC and its potential as a novel serum biomarker.

Insulin-like growth factors (IGFs) are involved in many normal physiological processes and pathological states, including cancer. Recently, it has been reported that higher concentrations of IGF-I were associated with prostate, colorectal, and premenopausal breast cancers [23]. Taylor et al. proposed that NPC is strongly associated with Epstein-Barr virus (EBV), a human herpesvirus [24], and Iwakiri et al. found that EBV infection induced IGF-I in NPC cell lines, suggesting that EBV directly affects the pathogenesis of NPC through the secretion of IGF-I [25]. In the present study, IGF-I was also found to be highly expressed in NPC patient serum, hinting that IGF-I can be used as a serum biomarker for NPC. But the further mechanism will be studied in future.

IL-8 is an essential inflammatory mediator and angiogenic chemokine that plays a role in both the development and the progression of many human malignancies, including nasopharyngeal carcinoma (NPC). Recent studies indicate that IL-8 over expression in NPC serum is associated with unfavorable prognoses and could be an independent unfavorable prognostic factor [26,27]. Thus, our results are consistent with those of previous studies, showing that IL-8 levels are promising markers for the presence of NPC and progression of the disease.

Additionally, to compare the role of Epstein-Barr virus (EBV) DNA load and EBV IgA serology as tools for screening patients with NPC. EA-IgA and EBV copy numbers had been measured in 75 samples from The Sun Yat-Sen Memorial Hospital. Notably, there was no correlation between EBV DNA load and EBV serology titers (Fig. 7). Although, this result was inconsistent with other literature reports [32], EBV IgA serology may reveal a good sensitivity and specificity as screening tools than EBV DNA load in samples from South China. A big cohort study are needed to confirm this bias.

In summary, this study employed a small number of patients and healthy controls to search for NPC serum biomarkers using protein arrays. Additional samples were obtained and analyzed by ELISA to confirm the protein array results and to establish the reliability of using the eight cytokines with significantly differential expression between NPC patients and controls as biomarkers. Notably, many of the cytokine markers were found, for the first time in the present study, to be differentially expressed in NPC serum, compared against normal controls. These results provide valuable leads deserving of further clinical validation with a greater number of cases to develop these novel serum biomarkers for the diagnosis and prognosis of NPC.

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## Conflict of interest

The authors declare no conflict of interest.

## Funding

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.cyto.2018.04.031.

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