Original Article

Rapid determination of serological cytokine biomarkers for hepatitis B virus-related hepatocellular carcinoma using antibody microarrays

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Hepatocellular carcinoma (HCC) is one of the most frequent tumors worldwide with an increasing incidence. The exploration of biomarkers for HCC is one of the main aims for improving the efficacy of diagnosis and treatment. The microarray technology provides a high-throughput platform for parallel exploration of biomarkers for clinics. In this study, we used antibody microarrays to screen the novel cytokine biomarkers of hepatitis B virus (HBV)-related HCC. Cytokine-secreting patterns in sera were determined from 109 cases including 43 HBV-related HCC patients, 33 chronic hepatitis B patients, and 33 normal controls by RayBio® Biotin label-based human antibody array. The correlation analysis was performed with conventional clinical diagnostic biomarkers, including serum alanine aminotransferase, alpha-fetoprotein (AFP) and hepatitis B surface antigen. Our results showed that in HBV-related HCC group, which had the highest percentage of AFP positive (>20 ng/ml) ratio, six cytokines were found differentially expressed in HCC patients (P < 0.05), compared with either normal controls or chronic hepatitis B group. Two macrophage-related cytokines, macrophage-derived chemokine (MDC) and macrophage-stimulating protein α (MSPα), displayed significant difference in the HCC group. Furthermore, an HCC diagnostic model for prediction was constructed, by which the combination of MDC and MSPα together with AFP had improved the diagnostic sensitivity from 60% (AFP alone) to 73.2% with similar specificity. Our results suggested that MDC and MSPα screened by antibody microarrays might serve as novel cytokines biomarkers for potential auxiliary diagnosis of HBV-related HCC.

Keywords antibody microarray; biomarkers; hepatocellular carcinoma; cytokines

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Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent tumors worldwide with an increasing incidence. Diagnosis, treatment, and prevention of HCC impose one of the most widely concerned public health problems in many countries [1,2]. So far, the most widely used serum biomarker for HCC screening is alpha-fetoprotein (AFP) that is increased (>20 ng/ml) in 30–60% patients with HCC. As a considerable number of patients with chronic liver disease display AFP levels in the range of 20–200 ng/ml [3], it is difficult to distinguish between HCC cases and those of chronic liver diseases with AFP only. Therefore, the specificity for diagnosis with AFP only reaches around 70–80%. If the cut-off value of AFP for HCC is increased, the sensitivity will be reduced to as low as 5–15% accordingly [4]. AFP only as a biomarker for prediction and diagnosis of HCC has its limitations for HCC screening.

Persistent infection by HBV/HCV virus, which results in continuous or recurrent chronic hepatitis (CHB), is recognized as an important risk factor for HCC [5]. The regional differences in the prevalence mostly reflect the different incidence of chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV). In most of the Asia-Pacific regions, as well as in Africa, endemic HBV is the most important etiological factor, while in Japan, by far HCV is the most common risk fact. In China, the observation of high infection rate of HBV with high incidence of HCC explosion provides strong evidence for it. Two major HBV-specific mechanisms contribute to the development of HCC. The first is the integration of the viral genome into the host chromosome, resulting in the activation of tumor-promoting genes or suppression of tumor suppression genes. The second is the expression of viral proteins in the host cells, which promotes liver cell necrosis, inflammation, and thus cytokine synthesis and fibrosis.
Novel biomarkers for HCC have been undergone exploration. Though some of them show promise, none is presently applied in the routine assay [6]. The discovery of useful biomarkers requires novel strategies and methodology.

In recent years, the association between inflammation and tumorigenesis has attracted more and more attentions. As a ‘two-edged sword’, inflammation either promotes the activation of immune system to prevent the progression of tumor, or alters the microenvironment around tumor which might lead to the progression of tumor. In patients bearing HBV-related HCC, not only tumor antigens, but also inflammatory effectors against HBV infection might constitute the pool of biomarkers in the periphery for future diagnosis. The panels of cytokines derived from inflammatory immune response are among the good candidates for investigation.

Concerning the comprehensive network of cytokines in immune responses, antibody-based microarrays are well suitable for biomarker screening in cancer research with advantage of high-throughput and rapid parallel detection of multiple proteins as well as low sample requirement [7,8]. This technique has already been applied in researches in different cancers including prostate cancer [9], breast cancer [10], and bladder cancer [11]. To date, the application of antibody microarrays searching for serum biomarkers of HCC has not been assessed.

In the present study, we intended to determine novel cytokine biomarkers for HBV-related HCC by using antibody microarray technique.

**Materials and Methods**

**Patients**

Serum samples were obtained from 43 Chinese patients with histologically confirmed HCC and HBV infection, who were hospitalized between January and September 2008 in the liver surgery department of Zhongshan Hospital, Fudan University (Shanghai, China). Blood samples were collected before surgical resection. Thirty-three Chinese patients who were diagnosed with CHB and receiving regular follow-ups in the special outpatient clinic of hepatitis in Zhongshan Hospital took part in this study during the same period. CHB was defined as persistent seropositivity for hepatitis B surface antigen (HBsAg) and recurrent elevated serum alanine aminotransferase (ALT) levels for more than 6 months. Thirty-three healthy cases who were receiving regular health examinations in the same hospital were recruited as normal controls. Informed consent forms were given to them before they took part in this study. Approval was obtained from the Ethics Committee of Zhongshan Hospital.

All subjects were negative for antibodies to HCV (anti-HCV) and HEV (anti-HEV) and had no serological markers for autoimmune disease. None had a history of alcohol abuse (>60 g/day), parenteral drug use, or hepatitis exposure. No specific antivirals or immune modulators were given during the same period. Sera samples were collected by centrifugation and stored at −80°C before microarray handling.

**Serum parameters**

HBsAg, anti-HCV, and anti-HEV antibodies were measured by commercially available enzyme-linked immunosorbent assay kits (Abbott Laboratories, North Chicago, USA). Other serum parameters applied in this study were extracted from the medical records of the patients at the time of prognosis including AFP, ALT and HBsAg.

**Antibody microarray processing**

Cytokine protein membranes coated with 507 cytokine antibodies were probed with serum samples from three normal cases, three CHB patients, and three HCC patients according to the manufacturer’s instructions using RayBio human antibody array I kit (RayBiotech, Inc., Norcross, USA). Briefly, the membranes were blocked by incubation with blocking buffer at room temperature for 60 min and incubated with serum sample (2.5 μl) at room temperature for 120 min. Membranes were washed three times with wash buffer I and three times with wash buffer II at room temperature for 5 min per wash and incubated with biotin-conjugated streptavidin at room temperature for 5 min. Finally, the membranes were washed and incubated with horseradish peroxidase-conjugated streptavidin at room temperature for 2 h and with detection buffers for 2 min. Cytokine-antibody complexes on membranes were then detected by chemiluminescence and exposed with X-ray films. The images were scanned with Artixscan 120 tf scanner (Microtek, Carson, USA) and then analyzed using ScanAlyze 2.51 software. The positive, negative, blank, and internal controls were used in all antibody microarray assays. By subtracting the background staining and normalizing to the positive controls on the same membrane, we obtained relative protein concentrations. The manufacturer claimed that the coefficient of variation of the array was <10%. The sensitivity of the antibodies present in the arrays ranged from 1–2000 pg/ml (www.raybiotech.com/human_array_sensitivity.pdf).

Additional serum samples of 30 normal controls, 30 hepatitis cases, and 40 HCC cases were processed on custom-ordered membranes coated with 30 cytokine antibodies (RayBiotech, Inc.) selected after the first step. Antibody array processing was the same as the first round screening.
**Statistical analysis**

The relative protein concentration of cytokine was used for comparison analysis. In the first step, the mean values of the 507 cytokines were compared among three groups. We selected 30 cytokines after comprehensive considerations of the difference among the three groups together with the coefficient of variations within groups for validation assay. Data were presented as means ± SD and compared by one-way analysis of variance (ANOVA). Diagonal linear discriminant analysis was applied using MATLAB 7.5 software (MathWorks Inc., Natick, USA.). Reported values were two-sided and were considered significant if $P < 0.05$.

**Results**

**Patient characteristics**

The characteristics of the recruited cases of three groups were listed in Table 1. The distribution of age was similar among three groups. The average age of hepatitis group (40 years) was the lowest compared with the normal group (45 years) and the HCC group (50 years). There was no difference in gender among all the cases. The normal group presented with HBsAg negative and ALT within the normal range. The hepatitis and HCC groups were HBV-infected cases with HBsAg positive. The mean ALT level of the hepatitis group was higher than the normal. HCC group showed the highest AFP positive ratio (62.8%) than others (6.1% for hepatitis group and 0 for normal group).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal cases</td>
<td></td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>33/0</td>
</tr>
<tr>
<td>Age (≥49/≥49 years)</td>
<td>23/10</td>
</tr>
<tr>
<td>HBsAg (positive/negative)</td>
<td>0/33</td>
</tr>
<tr>
<td>ALT (≤75/&gt;75), U/L</td>
<td>33/0</td>
</tr>
<tr>
<td>Serum AFP (≤200/&gt;200), ng/ml</td>
<td>33/0</td>
</tr>
<tr>
<td>CHB cases</td>
<td></td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>33/0</td>
</tr>
<tr>
<td>Age (≥49/≥49 years)</td>
<td>26/7</td>
</tr>
<tr>
<td>HBsAg (positive/negative)</td>
<td>33/0</td>
</tr>
<tr>
<td>ALT (≤75/&gt;75), U/L</td>
<td>19/14</td>
</tr>
<tr>
<td>Serum AFP (≤200/&gt;200), ng/ml</td>
<td>31/2</td>
</tr>
<tr>
<td>HCC cases</td>
<td></td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>43/0</td>
</tr>
<tr>
<td>Age (≥49/≥49 years)</td>
<td>22/21</td>
</tr>
<tr>
<td>HBsAg (positive/negative)</td>
<td>43/0</td>
</tr>
<tr>
<td>ALT (≤75/&gt;75), U/L</td>
<td>32/11</td>
</tr>
<tr>
<td>Serum AFP (≤200/&gt;200), ng/ml</td>
<td>16/27</td>
</tr>
</tbody>
</table>

**Spectrum of cytokines from three groups**

Serum samples from three normal cases, three CHB patients, and three HCC patients were spotted on the membranes composed of 507 cytokines (for details see www.raybiotech.com/L_series_map.asp#1). Three different profiles were shown in Fig. 1. We chose the most differentially detected ones among these three groups and those with the lowest coefficient of variations for further validation assay with more samples. Validations assays in larger groups ($n = 100$) were lately performed. Spectrums of 30 cytokines from different groups were shown in Fig. 2. The validation assays for comparison of cytokine patterns among three groups were performed by using additional samples including 30 normal controls, 30 hepatitis cases, and 40 HCC cases with pre-selected 30 cytokines. Relative protein concentrations were calculated for further comparison among three groups.

**Differential cytokine patterns in the sera of HBV-related HCC patients**

After comparing the relative protein concentrations of 30 cytokines among three groups, six cytokines were selected as differentially expressed after one-way ANOVA analysis. They were Fas/TNFRSF6, Glut3, IGFBP-rp1/IGFBP-7, MDC, MSPα chain, and MSPβ chain. As listed in Table 2, the HCC group displayed the relative different concentrations of IGFBP-rp1/IGFBP-7, MDC, MSPα chain, and MSPβ chain significantly from the non-HCC group. We found that these four proteins were dramatically altered in the HCC group compared with those in the normal controls and CHB groups, whereas the difference of IGFBP and MSPβ were exhibited between normal controls and HBV-infected groups (either CHB patients or HCC patients).

As AFP is still considered as the ‘golden’ biomarker for prediction and diagnosis of HCC, we want to explore whether the above-mentioned four proteins would be significant for the auxiliary diagnosis in the HCC when combined with AFP. By using diagonal linear discriminant analysis method, a diagnostic model for HCC was constructed by applying the significant cytokines together with AFP. The model using MDC, MSPα chain, and AFP gave the best diagnostic value. The formula $F = -7.0989 + 6.4611 \times X + 4.1457 \times Y - 1.4157 \times Z$, where $X$ means the relative concentration of MDC, $Y$ means the relative concentration of MSPα chain, and $Z$ means the value of log10(AFP + 1), was used to discriminate HCC patients from others. $F$ value that was less than 0 had a diagnostic value for HCC, whereas the threshold $F$ value above 0 had a diagnostic value for non-HCC. The model discriminated the patients with HCC and without HCC into two parts (Fig. 3). The area under curve (AUC) of the receiver
operating curve (ROC) of this model was 0.89, its sensitivity was 73.2%, and its specificity was 95%. The AUC of the ROC of AFP alone was 0.85. Sensitivity of AFP was 60% and its specificity was 96%.

Prognostic significance of MDC and MSP\(\alpha\) in HBV-related HCC

According to the statistical analysis, MDC and MSP\(\alpha\) contributed significantly to the potential diagnosis of HCC. We further investigated the association of these two molecules with AFP. First, we studied the association between MDC and MSP\(\alpha\). As shown in Fig. 3, the association of MDC and MSP\(\alpha\) was very close in all groups. More interestingly, MDC and MSP\(\alpha\) levels had decreased simultaneously in the HCC group, which was consistent with the average low levels of these two molecules. But neither MDC nor MSP\(\alpha\) showed any significant association with AFP either in AFP positive or negative HCC patients.

Discussion

In the present study, we identified two novel cytokines in the serum, which exhibited potential for the diagnosis of HBV-related HCC by using antibody-based microarray. The low requirement for sample volumes results in the small amounts of both precious clinical samples and expensive antibodies. The assay can be performed efficiently and parallelly, enabling the possibility of biomarker discovery and validation in a high-throughput manner. In addition, the assay shows good reproducibility, high sensitivity, and quantitative accuracy over large concentration ranges [12]. In our study, we applied antibody microarray to explore serum cytokine profiling of HBV-related HCC patients and CHB.
patients. Using this sensitive method, as less as 2.5 μl serum was adapted for assay. To make the results more reliable and the assay less expensive, cytokine panels were pre-selected with small amount of samples and the validation assays were performed with large population. As the identities of the proteins measured in antibodies microarrays were definitely known compared with other proteomic methods used for biomarker identification such as 2-D electrophoresis plus mass spectrometry, no further experiment is needed for protein identification in this assay.

In China, male patients of HBV-related HCC or chronic hepatitis are more prevalent than female ones. That’s why we chose male cases to study in our research. However, caution should be drawn while our result being applied in female cases. In the disease progression of HBV infection, it takes years for patients to develop HCC from chronic hepatitis. In our study, most of the hepatitis cases are younger than HCC patients, which is consistent with natural process of HCC development.

More and more evidence supports the idea that inflammation contributes to cancer development and progression. The risk of developing cancers of the esophagus, colon, pancreas, lung, and gallbladder is increased by the presence of chronic inflammatory diseases. The inflammatory environment is likely enriched with macrophages that generate high levels of cytokines and chemokines, which endow tumor with an environment-enhancing survival, migration, and proliferation. In our study, by antibody

Table 2 The relative concentrations of six cytokines selected through one-way ANOVA

<table>
<thead>
<tr>
<th></th>
<th>Fas/TNFRSF6</th>
<th>Glut3</th>
<th>IGFBP-rp1/IGFBP-7</th>
<th>MDC</th>
<th>MSPα chain</th>
<th>MSPβ chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nomal group (m ± SD)</td>
<td>0.327 ± 0.032</td>
<td>0.300 ± 0.039</td>
<td>0.455 ± 0.032</td>
<td>0.925 ± 0.014</td>
<td>0.838 ± 0.020</td>
<td>0.568 ± 0.035</td>
</tr>
<tr>
<td>CHB group(m ± SD)</td>
<td>0.442 ± 0.030</td>
<td>0.460 ± 0.039</td>
<td>0.595 ± 0.029</td>
<td>0.923 ± 0.015</td>
<td>0.876 ± 0.020</td>
<td>0.655 ± 0.036</td>
</tr>
<tr>
<td>HCC group(m ± SD)</td>
<td>0.421 ± 0.028</td>
<td>0.399 ± 0.034</td>
<td>0.600 ± 0.028</td>
<td>0.884 ± 0.013</td>
<td>0.808 ± 0.017</td>
<td>0.535 ± 0.030</td>
</tr>
<tr>
<td>P value*</td>
<td>0.027</td>
<td>0.017</td>
<td>0.002</td>
<td>0.049</td>
<td>0.039</td>
<td>0.035</td>
</tr>
</tbody>
</table>

*One-way ANOVA.
microarray techniques we have also determined two macrophage-related cytokines, MDC and MSPα, as differentially present in the sera of HBV-related HCC patients.

MDC is a CC chemokine originally identified in mature macrophages. It is also expressed in dendritic cells, B cells, T lymphocytes, as well as macrophages. MDC provides a paradigm for several aspects of chemokine immunobiology, ranging from allergic reactions to neoplasia [13]. It is responsible for controlling the homing of CCR4⁺ immune cells to infectious sites. Serum MDC has already been suggested as a marker of certain infectious diseases with immunological abnormality [14]. It has also been found that higher gene expression of MDC was significantly correlated with fine prognosis of lung cancer [15]. These results were consistent with the finding that the extra expression of MDC by gene transfer strategy could elicit better therapeutic potentials in animal models of lung cancer [16]. In our study, decreased serum level of MDC was detected in HCC patients compared with CHB patients. This might be due to the fact that the activation of immune cells have been suppressed by the existence of both chronic virus infection and tumor bearing, which in turn impeded the homing of immune cells to tumor sites. This might provide not only a novel indicator to monitor the immune status of patients, but also a new therapeutic target for HBV-related HCC patients.

MSP, also known as hepatocyte growth factor-like, is a heterodimeric protein composed of disulfide-linked α chain and β chain. It is synthesized mainly in liver cells [17–19]. Binding of MSP to its receptor, RON activation was initiated. The contribution of MSP/RON activation in tumor progression and metastasis lies in two aspects: both tumor cell autonomous manner (tumor cell growth, migration, survival and differentiation) and non-tumor cell autonomous (macrophage activation and polarization) manner. MSP-RON pathway was demonstrated to be involved in tumorigenesis [20] of several tumors (both in vivo and in vitro) such as pancreatic cancer [21], colorectal carcinoma [22], and renal cancer [23]. It was once reported that MSP mRNA was overexpressed in hepatoblastoma samples [24]. In our study, we found that the MSPα level in serum was significantly decreased in HBV-related HCC patients compared with normal controls and CHB patients. This was unexpectedly paradoxical to its biological function. Notably, the liver is the primary site of MSP production. The decreased level of MSPα in the sera of HBV-related patients might reflect the fact that undergoing the chronic infection of HBV for decades, these patients displayed impaired liver function. However, the mechanism that leads to the decreased level of MSPα in HBV-related patients needs further investigation in hepatoma cell lines or HCC tissues.

To further investigate the relationship between existing biomarkers such as AFP for HCC and our newly defined two molecules, we have constructed the diagnostic model for HCC with MDC, MSPα, and AFP. According to the deduced formula, it is suggested that with the combination of MDC and MSPα the diagnostic sensitivity has improved from 60% (AFP alone) to 73.2% with similar specificity. More interestingly, when compared the correlation between MDC/MSPα, they displayed good correlation. But there was no good correlation between AFP/MDC and AFP/MSP. This might lie to the possibility that AFP, MDC, or MSPα are expressed in different cell types.

In summary, this is the first report to define the serological alteration of macrophage-related MDC and MSPα in HBV-related patients compared with normal controls and chronic HBV patients. With the addition of these two molecules, it might expand the spectrum of biomarkers for clinical diagnosis either from the immune status of patients or from the status of tumor. More significantly, our study provides the evidence that the combination of biomarkers from tumor itself with that from immune status of patients might provide more precise diagnostic panels for future.

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