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ORIGINAL RESEARCH

Further Study of Circulating Antibodies to P16, CD25 and FOXP3 in Hepatocellular Carcinoma

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Jiaxin Wang[1]
Yangchun Xu[2]
Yanjun Wang[1]
Xuan Zhang[2]
Guizhen Zhang[2]

[1]Department of Endocrinology, The Second Hospital of Jilin University, Changchun, People's Republic of China; [2]Jilin Provincial Key Laboratory on Molecular and Chemical Genetic, The Second Hospital of Jilin University, Changchun, People's Republic of China

Purpose: It has been reported that circulating levels of IgG antibodies against p16, CD25 and FOXP3 proteins were significantly changed in patients with lung cancer, breast cancer and esophageal cancer. However, different peptide fragments appear to trigger different immune responses. This work aimed to analyze the alteration of plasma IgG for p16-derived peptide antigen called p16a, CD25-derived peptide antigen called CD25a and a FOXP3-derived antigen in hepatocellular carcinoma (HCC).

Patients and methods: An enzyme-linked immunosorbent assay (ELISA) was developed in-house to detect plasma IgG to p16a, CD25a and FOXP3 in 119 patients with HCC and 132 control subjects.

Results: Circulating levels of IgG antibodies for all three peptide antigens were significantly higher in HCC patients than control subjects ($P<0.001$ for all 3 assays); male patients mainly contributed to increase ($P<0.01$ for all 3 assays). Further analysis showed that plasma anti-p16a, anti-CD25a and anti-FOXP3 IgG levels were increased mainly in patients with intermediate and late-stage HCC ($P<0.01$ for both assays). Receiver operating characteristic (ROC) curve analysis showed that with a specificity of >95%, the area under the ROC curve (AUC) was 0.62 with 11.4% sensitivity for anti-p16a assay, 0.68 with 14.3% sensitivity for anti-CD25a IgG assay and 0.64 with 10.1% sensitivity for anti-FOXP3 assay. Of the three groups of HCC patients, group 3 (BCLC stage C+D) showed the best sensitivity for the detection of plasma anti-p16a and anti-FOXP3 IgG levels with an AUC of 0.66 and 0.65.

Conclusion: Circulating IgG antibody to p16a, CD25a and FOXP3 proteins may be a useful biomarker for assessment of HCC prognosis of this malignancy, especially in male patients with HCC.

Keywords: autoantibody, p16, CD25, FOXP3, hepatocellular carcinoma

Introduction

Liver cancer was the fourth leading cause of cancer-related deaths in 2015 following lung, colorectal, and stomach cancer.[1] The most common type of liver cancer is hepatocellular carcinoma (HCC).[2] In our recent studies, we found that circulating IgG antibodies against linear peptide antigens derived from p16 protein, interleukin 2 (IL-2) receptor α-subunit (also called CD25) and forkhead/winged-helix transcription factor box P3 (FOXP3) were significantly changed in liver cancer,[3] non-small cell lung cancer (NSCLC),[4–8] breast cancer[9,10] and esophageal cancer.[11–13] Therefore, circulating IgG antibodies for these target molecules may be either diagnostic or prognostic values for solid tumors.

While the reports on circulating IgG antibodies against CD25-derived peptide antigens in NSCLC showed inconsistent results,[4–8] further investigation suggested that the

Correspondence: Xuan Zhang; Guizhen Zhang
Jilin Provincial Key Laboratory on Molecular and Chemical Genetic, The Second Hospital of Jilin University, 218 Ziqiang Street, Changchun, People's Republic of China
Tel +86 0431-81136436
Email zhangxuankj@163.com; zhangguizhenjl@163.com

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immune system had different responses to distinct CD25-derived peptides. For example, a significant low anti-CD25b IgG level was observed in patients with an early-stage NSCLC but anti-CD25a IgG levels were significantly increased[7] in this malignancy. Interestingly, our previous study revealed that anti-CD25b IgG levels were significantly increased in patients with HCC.[3] In this study, therefore, we attempted to confirm if anti-CD25a IgG levels were significantly changed in HCC. Because circulating IgG for both p16 and FOXP3-derived peptide antigens have been found to be associated with several types of solid cancer,[7,8] it is important to see if circulating IgG antibodies for these 2 self-antigens could serve as biomarkers for clinical assessment of HCC.

Materials and Methods

Subjects

The study cohort consisted of 251 participants, of whom 119 were diagnosed with HCC at the Second Hospital of Jilin University, Changchun, China, and 132 were used as control subjects. These 119 HCC patients aged 54.7±9.7 years consisted of 102 males and 17 females; their blood samples were taken during the first hospitalization and before any anticancer treatment was received. HCC staging was made based on the Barcelona Clinic Liver Cancer (BCLC) staging system,[14] and these 119 patients with HCC were classified into three subgroups: group 1 (stage 0+A), group 2 (stage B) and group 3 (stage C+D). These 132 healthy control subjects (106 males and 26 females), aged 54.9±8.6 years, were recruited from local communities, and they were included in this study based on the following criteria: (1) they had no any history of liver cancer and other malignancies; (2) they had no any severe autoimmune conditions, such as autoimmune thyroid disease, pernicious anemia, type-1 diabetes, celiac disease, ankylosing spondylitis, systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis and inflammatory bowel diseases. All the subjects were of Chinese Han origin and all provided informed written consent to take part in the study as approved by the Institutional Review Boards of the Second Hospital of Jilin University and conformed to the Declaration of Helsinki.

Antibody Testing

Linear peptide antigens were used to develop an in-house enzyme-linked immunosorbent assay (ELISA) for the detection of anti-p16a, anti-CD25a and anti-FOXP3 IgG antibodies in plasma as described in our previous studies.[7,8,15,16] The peptide sequences used in this study are given in Table 1. The in-house ELISA was then developed with these linear peptides as described in previous reports.[7,8] Briefly, the synthetic peptides were dissolved in 67% acetic acid to 5mg/mL, respectively, and diluted with the coating buffer (0.1 M phosphate buffer containing 0.15 M NaCl and 10 mM EDTA, pH 7.2) at 20 μg/mL to coat Maleimide-activated 96-well microplates (Thermo Scientific, Shanghai, China) according to the manufacturer's instructions. Just prior to use, the antigen-coated plates were washed twice with 200 μL Wash Buffer (phosphate-buffered saline (PBS; P4417, Sigma-Aldrich, Shanghai, China)) containing 0.05% Tween-20. A 50 μL plasma sample diluted 1:200 in Assay Buffer (PBS) containing 0.5% bovine serum albumin (BSA) was added to each well, while 50 μL Assay Buffer was added to the negative control (NC) wells, and 50 μL positive control (PC) sample was added as well. The binding reaction system was incubated at room temperature for 1.5 hrs, followed by washing and incubation with 50 μL peroxidase-conjugated goat anti-human IgG antibody (ab98567, Abcam, Guangzhou, China), diluted 1:50,000 in Assay Buffer at 4°C for 1 hr. The color development was made by adding 50 μL Stabilized Chromogen (SB02, Life Technologies, Beijing, China) and terminated after 20 mins by adding 25 μL Stop Solution (SS04, Life Technologies). The optical density (OD) at 450 nm was measured on a microplate reader with a reference wavelength of 620nm. All the samples were tested in duplicate and the specific binding ratio (SBR) was used to represent the relative levels of plasma IgG antibodies. The SBR was computed as follows:

$$SBR = (OD_{Sample} - OD_{NC})/(OD_{PC} - OD_{NC})$$

To minimize the intra-assay deviation, the ratio of the difference between the duplicated OD values of each sample to their sum was used to assess the precision of ELISA.

Table 1 Information for Peptide Antigens Derived from Three Antigens

Antigen	Sequence{N→C}	NCBIA Accession	Position{aa}
p16a	CGFLDTLVVLHRAGARLDVRDAWGRLPVD	NP_000068	89–102
CD25a	KPGHCREPPPWENEATERIYHFVVGQMVY	NP_000408	99–126
FOXP3	CDWFTRMFAFFRNHPATWKNAIRHNLSLHKD	NP_001107849	331–358

If the ratio was >10%, the test of the sample was treated as invalid and not used for data analysis.

Data Analysis

The mean ± SD in SBR was used to present the data. The Mann–Whitney *U*-test was used to examine the differences in plasma IgG levels between the patient and the control groups and Pearson's correlation analysis was used to examine the correlation between plasma IgG levels and clinical parameters (BCLC stages, serum bilirubin, albumin and prothrombin time). Receiver operating characteristic (ROC) curve analysis was used to calculate the area under the ROC curve (AUC) with 95% confidence interval (CI) and the sensitivity of the IgG assay against a specificity of >95% (Figure 1). A p-value of <0.017 was considered to be significant as three antigens were tested in this study.

Results

The levels of plasma IgG antibodies to p16a, CD25a and FOXP3 were compared between HCC patients and control subjects (Table 2). When compared with control subjects, patients with HCC had a significant higher level of anti-p16a IgG (Z= 3.51, P=0.0004), anti-CD25a IgG (Z=−3.834, P<0.001) and anti-FOXP3 IgG (Z=−4.959, P<0.001); the male patients primarily contributed to the increased IgG levels (Z=−2.86, P=0.004 for anti-p16a IgG, Z=−5.210, P<0.001 for anti-CD25a IgG and Z=−4.060, P<0.001 for anti-FOXP3 IgG, respectively).

Further analysis showed that increased levels of plasma anti-p16a, anti-CD25a and anti-FOXP3 IgG levels were mainly shown in groups 2 and 3 (Table 3), in which patients with advanced and terminal stage HCC had the highest anti-p16a, and anti-FOXP3 IgG levels (Z=3.38, P=0.0008 in group 2 and Z=−3.038, P=0.002 in group 3, respectively). Plasma anti-CD25a IgG levels were slightly higher in group 1 patients (stage 0+A) than controls (Z=−2.317, P=0.020),

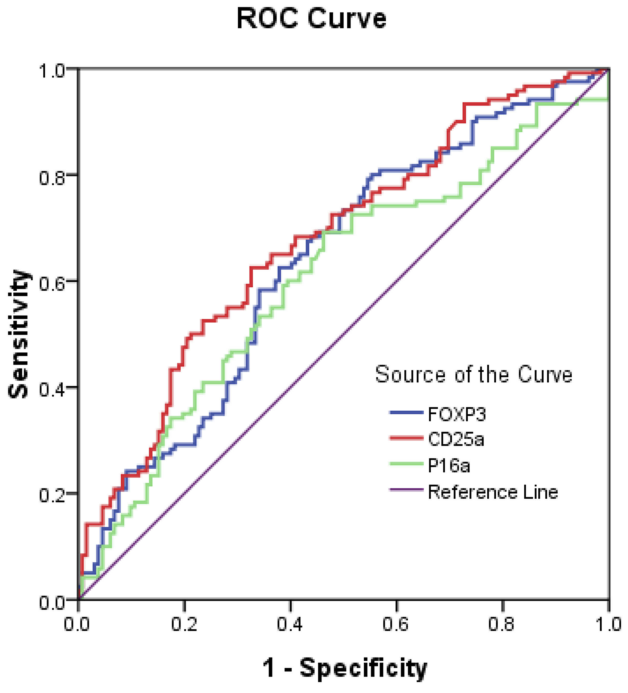

Figure 1 ROC curve analysis of circulating levels of anti-p16a, anti-CD25a and anti-FOPX3 IgG antibodies in hepatocellular carcinoma.

Table 2 Comparison of Circulating IgG Levels for 3 Target Peptide Antigens Between HCC Patients and Control Subjects

Group	Patients (n)	Control (n)	Z	P*
p16a				
Male	1.92±0.93 (102)	1.60±0.67 (106)	−2.86	0.004
Female	1.86±0.46 (17)	1.53±0.49 (26)	−2.36	0.018
Both	1.91±0.87 (119)	1.59±0.64 (132)	−3.51	0.0004
CD25a				
Male	1.04±0.14 (102)	0.93±0.12 (106)	−5.210	<0.001
Female	1.01±0.13 (17)	0.99±0.13 (26)	−0.621	0.535
Both	1.04±0.14 (119)	0.95±0.13 (132)	−3.834	<0.001
FOXP3				
Male	1.09±0.22 (102)	0.97±0.20 (106)	−4.060	<0.001
Female	0.98±0.17 (17)	1.00±0.23 (26)	−0.248	0.804
Both	1.08±0.22 (119)	0.97±0.21 (132)	−4.959	<0.001

Note: *P-value of <0.017 is considered statistically significant as 3 peptide antigens were tested.

Table 3 Analysis of Circulating IgG Levels for 3 Target Peptide Antigens in HCC Patients at Different Stages

Stage	Patients (n)	Control (n)	Z	P
p16a				
Group 1	1.66±0.58 (25)	1.59±0.64 (132)	0.97	0.331
Group 2	1.86±0.79 (43)	1.59±0.64 (132)	2.51	0.012
Group 3	2.08±1.03 (51)	1.59±0.64 (132)	3.36	0.0008
CD25a				
Group 1	1.00±0.13 (25)	0.95±0.13 (132)	−2.317	0.020
Group 2	1.05±0.12 (43)	0.95±0.13 (132)	−4.603	<0.001
Group 3	1.04±0.15 (51)	0.95±0.13 (132)	−3.228	<0.001
FOXP3				
Group 1	1.05±0.20 (25)	0.97±0.21 (132)	−2.044	0.044
Group 2	1.06±0.16 (43)	0.97±0.21 (132)	−2.797	0.005
Group 3	1.11±0.26 (51)	0.97±0.21 (132)	−3.038	0.002

Notes: The antibody levels are expressed as mean ± SD in SBR. Group 1= BCLC stage 0+A, Group 2 = BCLC stage B, Group 3 = BCLC stage C+D.

Table 4 Correlation Between IgG Levels and Biochemical Parameters in the Circulation

Parameters	P16a		CD25a		FOXP3	
	r	*P*	*r*	*P*	*r*	*P*
BCLC stages	0.119	0.197	0.060	0.514	0.098	0.287
Total bilirubin	0.048	0.632	0.027	0.790	0.006	0.951
ALb	−0.194	0.051	−0.416	<0.001	−0.475	<0.001
PT	0.048	0.637	0.227	0.024	0.357	<0.001

although there were no significant correlations between BCLC stages and plasma IgG levels (P>0.05 for all 3 assays), but anti-CD25a IgG levels were positively correlated with anti-FOXP3 IgG levels (r=0.766, P<0.001) (Table 4). Moreover, plasma anti-CD25a and anti-FOXP3 IgG levels were negatively correlated with albumin and positively correlated with prothrombin time in HCC patients.

ROC curve analysis showed that with a specificity of >95%, the anti-p16a IgG assay had an AUC of 0.62 with 11.4% sensitivity, the anti-CD25a IgG assay had an AUC of 0.68 with 14.3% sensitivity and the anti-FOXP3 IgG assay had an AUC of 0.64 with 10.1% sensitivity (Tables 5–7). Of these 3 groups of HCC patients, group 3 (BCLC stage C+D) showed the best sensitivity for detection of plasma anti-p16a IgG with an AUC of 0.66 (Table 5) and anti-FOXP3 IgG with an AUC of 0.65 (Table 7); group 2 (BCLC stage B) showed the best sensitivity for plasma anti-CD25a detection with an AUC of 0.73 (Table 6). The combination of plasma IgG antibodies for all 3 peptide antigens showed an AUC of 0.71 with 61.5% sensitivity against specificity of 71.5%, PPV of 66.1% and NPV of 67.4%. The combination of anti-CD25a IgG and anti-FOXP3 IgG showed an AUC of 0.681 with 63.0% sensitivity against specificity of 67.4%, PPV of 63.6% and NPV of 66.9% (Table 8).

Table 5 ROC Curve Analysis of Circulating Anti-P16a IgG Levels in HCC

Group	AUC	SE[a]	95% CI	Sensitivity (%)[b]
1	0.56	0.061	0.44–0.68	0.0
2	0.62	0.048	0.53–0.72	8.9
3	0.66	0.049	0.56–0.75	19.6
Overall	0.62	0.035	0.55–0.69	11.4

Notes: [a]Standard error; [b]against a specificity of 96.2%. Group 1 = BCLC stage 0+A, Group 2 = BCLC stage B and Group 3 = BCLC stage C+D.

Table 6 ROC Curve Analysis of Circulating Anti-CD25a IgG Levels in HCC

Group	AUC	SE[a]	95% CI	Sensitivity (%)[b]
1	0.65	0.061	0.53–0.77	8.0
2	0.73	0.041	0.65–0.82	14.0
3	0.65	0.045	0.57–0.74	17.6
Overall	0.68	0.033	0.62–0.75	14.3

Notes: [a]Standard error; [b]against a specificity of 96.2%. Group 1 = BCLC stage 0+A, Group 2 = BCLC stage B and Group 3 = BCLC stage C+D.

Table 7 ROC Curve Analysis of Circulating Anti-FOXP3 IgG Levels in HCC

Group	AUC	SE[a]	95% CI	Sensitivity (%)[b]
1	0.63	0.059	0.51–0.74	12.0
2	0.64	0.044	0.56–0.73	4.7
3	0.65	0.002	0.55–0.74	13.7
Overall	0.64	0.035	0.57–0.71	10.1

Notes: [a]Standard error; [b]against a specificity of 96.2%. Group 1 = BCLC stage 0+A, Group 2 = BCLC stage B and Group 3 = BCLC stage C+D.

Discussion

The present study demonstrated that circulating levels of IgG antibodies for p16a, CD25a and FOPX3 were significantly higher in HCC patients than control subjects, and the male patients mainly contributed to this increase (Table 2). Further analysis showed that increased levels of plasma IgG for these 3 peptide antigens were mainly shown in patients with the intermediate and late-stage HCC (Table 3). These observations suggest that anti-p16, anti-CD25a and anti-FOXP3 IgG antibodies have a prognostic value for HCC, consistent with our previous report on the alteration of anti-CD25b IgG levels in HCC.[3] Because men are three times more likely to develop HCC than women, circulating IgG antibodies to these 3 protein-derived peptide antigens may contribute to the gender disparity of HCC.

The profile of circulating anti-p16 IgG levels in HCC patients was quite similar to a number of previous studies that revealed an increase in anti-p16 IgG levels in several other types of cancer.[10,13,17,18] Meanwhile, another two studies applied an ELISA made from recombinant p16 protein to measure circulating anti-p16 IgG levels and found that HCC patients had significantly higher levels of anti-p16 IgG than control subjects[19,20] What mechanism is involved in raising anti-p16 IgG levels in cancer remains unknown, but high expression of p16 protein in cancer tissues may be one of the most possible reasons,[21,22] although a study reported lack of p16 expression in patients with HCC.[23]

Based on the present study, CD25+FOXP3+Treg cells could regulate the function of tumor-associated factors and liver biochemical parameters (Table 4). CD25 is a transmembrane protein present on the

Table 8 Analysis of Combined IgG Antibodies for 3 Target Peptide Antigens in HCC

Combination	AUC	Cut-Off value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
CD25a+FOXP3	0.681 (0.615,0.747)	0.493	63.0	67.4	63.6	66.9
CD25a+FOXP3+P16a	0.707 (0.642,0.772)	0.514	61.5	71.5	66.1	67.4

activated lymphocytes, especially Treg cells.[24] FOXP3 is a member of the forkhead/winged-helix family and a transcription factor that is specifically expressed in Treg cells.[25,26] Treg cells are critical immunomodulators in the immune system and may play a major role in the development of HCC;[27–29] the increased number of Treg cells has been found to be related to HCC stages,[30–32] and FOXP3 has also been found to contribute to HCC progression. For example, overexpression and low methylation of FOXP3 are involved in the oncogenic and progression of HCC.[33] Accordingly, the alteration of anti-CD25a and anti-FOXP3 IgG levels in HCC patients may directly affect the function of the Treg cells, and increased release of CD25 and FOXP3 molecules may stimulate autoreactive B cells to secrete antibodies against these two molecules.[34]

The combination of these 3 target molecules could increase the in-house ELISA sensitivity although the ELISA specificity was not ideal (Table 8). Further study is needed to improve the specificity for diagnostic purpose. Nevertheless, this work has provided an interesting clue that increased levels of circulating IgG antibodies for p16, CD25 and anti-FOXP3 could serve as a useful biomarker for assessment of HCC prognosis instead of early diagnosis of this malignancy.

There are a few limitations to this study. First, clinical information regarding biochemical examinations was incomplete, especially the alpha-fetoprotein (AFP) levels that were not collected. Second, clinical follow-up was not performed, so that the correlation between survival time and plasma IgG levels could not be estimated. Third, the sample size used in this study was rather small; the female sample number was much smaller than the male sample number, which may lead to an underpowered test. Accordingly, this work cannot draw a firm conclusion. Further replication of this initial finding with large sample size and detailed clinical information remains needed.

Conclusions

This study has confirmed that circulating IgG antibodies to p16, CD25 and FOXP3 are significantly increased in HCC patients, especially in late-stage HCC. These autoantibodies may be useful biomarkers for assessment of HCC prognosis, which would provide an in-depth insight into the prevention and treatment of HCC in cancer patients.

Abbreviations

AUC, area under the ROC curve; CI, confidence interval; FOXP3, forkhead box P3; HCC, hepatocellular carcinoma; IL2RA, interleukin 2 receptor; NC, negative control; OD, optical density; PC, positive control; ROC, receiver operating characteristic; SBR, specific binding ratio; Tregs, regulatory T-lymphocytes.

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Author Contributions

According to the IMCJE guidelines, all authors have met the following conditions:

1. Substantial contributions to conception and design, data acquisition, or data analysis and interpretation;
2. Final approval of the version to be published;
3. Drafting the article or critically revising it for important intellectual content;
4. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved.

Jiaxin Wang designed and performed laboratory work, and drafted the manuscript. Yanjun Wang and Yangchun Xu carried out recruitment of HCC patients and control subjects as well as collected clinical information. Guizhen Zhang and Xuan Zhang conceived of this study and carried out the data analysis, and edited the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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Disclosure

The authors declare that they have no conflict of interest.

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