Detection of Circulating Antibodies to p16 Protein-Derived Peptides in Hepatocellular Carcinoma

Yangchun Xu, MM,[1] Litong Gu, MM,[2] Jiaxin Wang, MD,[1] Zhenqi Wang, MD,[3] Ping Zhang, MD, PhD,[4] Xuan Zhang, MD, PhD[1]**

Laboratory Medicine 2020;51:574-578

DOI: 10.1093/labmed/lmaa006

ABSTRACT

Objective: This study aimed at confirming the alteration of circulating anti-p16 immunoglobulin G (IgG) levels in hepatocellular carcinoma (HCC).

Methods: An in-house-developed enzyme-linked immunosorbent assay was used for determining plasma IgG antibodies against p16-derived antigens in 122 HCC patients and 134 healthy controls.

Results: Plasma anti-p16 IgG levels were significantly higher in HCC patients than in the controls ($Z = 3.51$, $P = 0.0004$), with no difference between males and females. A trend of increasing plasma anti-p16 IgG levels was associated with increasing HCC stage, with group 3 patients having the highest anti-p16 IgG levels ($Z = 3.38$, $P = 0.0008$). Group 3 exhibited the best sensitivity (19.6%) and specificity (95%) for plasma anti-p16 IgG detection, with an area under the receiver operating characteristic curve of 0.659 (95% confidence interval, 0.564–0.754).

Conclusion: Circulating IgG antibody to p16 protein might be a useful biomarker for HCC prognosis assessment rather than for early malignancy diagnosis.

Keywords: autoantibody, biomarker, hepatocellular carcinoma, p16 protein, tumor-associated antigens, receiver operating characteristic

Hepatocellular carcinoma (HCC) is a common malignant disorder associated with a very unfavorable prognosis and a high incidence and fatality rate.[1] Despite the significance of early HCC diagnosis in its treatment, robust biomarkers for early clinical HCC diagnosis remain unavailable. To date, alpha-fetoprotein (AFP) levels in circulation and abdominal ultrasound examination are recommended for routine HCC surveillance and diagnosis. However, due to their low specificity and sensitivity and limited prognostic value, their clinical usefulness is debatable.[2, 3] Therefore, the need for more sensitive and specific early HCC diagnosis methods remains urgent. Numerous studies have suggested using the overexpression of tumor-associated antigens (TAAs) in many cancers, which triggers the secretion of corresponding autoantibodies, potentially in cancer diagnosis.[4–6] Although several circulating anti-TAA antibodies have served as serological biomarkers for early HCC diagnosis and HCC prognosis prediction in the last 2 decades,[7–11] the identification of robust antibody markers with optimal specificity and sensitivity for early HCC diagnosis remains vital.

The malfunction of the cyclin-dependent kinase (CDK) inhibitor called cyclin-dependent kinase inhibitor 2A (CDKN2A), also called p16, considered to be an important tumor suppressor, may result in uncontrolled cell proliferation.[12,13] Promoter hypermethylation of p16 is reportedly a characteristic of HCC patients.[14] Several studies have demonstrated that hypermethylation-induced p16 expression inhibition plays crucial roles in HCC progression and is closely related to a poor prognosis in recurrent early-stage HCC.[14–16]

Abbreviations:

HCC, hepatocellular carcinoma; IgG, immunoglobulin G; AFP, alpha-fetoprotein; TAAs, tumor-associated antigens; CDK, cyclin-dependent kinase; CDKN2A, cyclin-dependent kinase inhibitor 2A; NSCL, non–small cell lung; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PC, positive control; NC, negative control; OD, optical density; SBR, specific binding ratio; QC, quality control; SD, standard deviation; ROC, receiver operating characteristic; AUC, area under the ROC curve; CI, confidence interval.

[1]Second Hospital of Jilin University, [2]Department of Hepatobiliary & Pancreatic Surgery, Jilin Province People's Hospital, [3] School of Public Health, Jilin University, [4]Department of Hepatobiliary & Pancreatic Surgery, First Hospital of Jilin University, Changchun, China

*To whom correspondence should be addressed.
zhangxuankj@126.com; azhangpinga@126.com

Similarly, a meta-analysis revealed that hypermethylation-induced p16 gene loss is related to the risk of increased liver cirrhosis and HCC development.[17] Notably, p16 is reportedly overexpressed in many cancers, including cervical, breast, and non–small cell lung (NSCL) cancers,[18–20] and compared with controls, patients with such cancers highly express circulating antibodies against p16 protein, suggesting the clinical diagnostic value of anti-p16 immunoglobulin G (IgG) levels. Previous studies have proved the reliability and sensitivity of an in-house enzyme-linked immunoassay (ELISA) (using linear peptides as antigens) in detecting circulating autoantibodies against some TAAs, including circulating anti-p16 IgG.[21,22] Therefore, the in-house ELISA was applied for confirming the associations between plasma anti-p16 IgG levels and HCC in this study in order to further elucidate the clinical applicability of plasma anti-p16 IgG levels in HCC diagnosis.

Table 1. Demographics and Clinical Characteristics of HCC Patients

Patient Characteristics		Patients (n)	% of Patients
Age (y)	≥60	40	32.79
	<60	82	67.21
Sex	Male	103	84.73
	Female	19	15.57
AFP (ng/mL)	<20	51	41.80
	≥20	66	54.10
	Not available	5	4.10
HCC stage	0	6	4.92
	A	19	15.57
	B	45	36.89
	C	47	28.52
	D	5	4.10
Groups	1 (stages 0 + A)	25	20.49
	2 (stages B)	45	36.89
	3 (stages C + D)	52	42.62

HCC, hepatocellular carcinoma; AFP, alpha-fetoprotein.

Materials and Methods

Subjects

A total of 122 patients were recruited for this study by the First and Second Hospitals of Jilin University, Changchun, China, ranging from March 2010 to November 2015. Their HCC diagnosis was performed via CT scans and circulating AFP levels, and their median age was 54.8 years ± 9.7 years. HCC staging was confirmed as per the Barcelona Clinic Liver Cancer staging system.[23] **Table 1** contains the patient demographics and clinical characteristics. To explore the correlation between clinical stages and plasma anti-p16 IgG levels, all 122 patients were classified into 3 subgroups, as follows: group 1 (stages 0 + A), group 2 (stage B), and group 3 (stages C + D). Plasma specimens were collected prior to anticancer therapy. Also, 134 healthy control subjects (107 males and 27 females) aged 55.2 years ± 9.2 years were recruited from local communities for this study. The controls, with no malignancy or severe autoimmune disease history, were included based on a clinical interview and image examination.

The recruited patients and controls were all of Chinese Han origin, and all provided informed written consent. This work was approved by the ethics committee of the Second Hospital of Jilin University, Changchun, China, and conformed to the requirements of the Declaration of Helsinki.

Plasma Anti-p16 IgG Level Detection

According to B-cell and human leukocyte antigen-II restricted epitopes, we designed a human p16-derived peptide antigen (CGFLDTLVVLHRAGARLDVRDAWGRLPVD) via computational prediction (http://www.iedb.org) and synthesized this peptide antigen using solid-phase chemistry to ensure >95% purity. It was then applied to the development of an in-house ELISA assay based on the plasma anti-p16 IgG, as the method described in previous study.[24] Briefly, the peptide antigen was dissolved in 67% acetic acid to obtain a 5 mg/mL stock solution, and a 20 μg/mL working solution was then obtained by diluting with coating buffer (0.1 M phosphate buffer containing 0.15 M NaCl and 10 mM ethylenediaminetetraacetic acid, pH 7.2). Next, antigen coating of the maleimide-activated plates (Thermo Scientific) was completed as per the corresponding specifications and then washed twice using 200 μL wash buffer (phosphate-buffered saline [PBS] containing 0.05% Tween 20). Fifty microliters of specimens and 50 μL of assay buffer (PBS containing 0.5% bovine serum albumin) were added to each specimen well (positive control [PC]) and negative control (NC) well incubating for 1.5 h at room temperature, respectively, in which plasma specimens were diluted (1:200) in assay buffer in advance. After washing the plates thrice, 50 μL of peroxidase-conjugated goat anti-human IgG antibody (1:50,000,

ab98567; Abcam) was added and incubated for an hour at room temperature, followed by color development using 50 μL of stabilized chromogen (SB02; Life Technologies, Beijing, China). Twenty minutes later, 25 μL of stop solution (SS04; Life Technologies) was added to terminate the reaction. A microplate reader was used to determine the optical density (OD) within 10 min at 450 nm, and 620 nm was selected as the reference wavelength.

Two repeats were set up for all specimens. The relative levels of plasma IgG antibodies were displayed with specific binding ratio (SBR), calculated as (OD specimen – OD NC)/(OD PC – OD NC). Additionally, to minimize intra-assay deviation, the accuracy of the in-house ELISA antibody detection method was evaluated using the ratio of the repeated OD value of each specimen to its sum. Specimens with ratios of >10% were excluded from the analysis. Unrelated healthy subjects (>100) were randomly selected as quality control (QC) specimens for calculating interassay deviation, and ELISA repeatability was represented by the coefficient of variation.

Data Analyses

Data are expressed as the mean ± standard deviation (SD) in SBR. The Mann-Whitney U test (2-tailed; IBM SPSS Statistics 21.0) was used to compare the difference in plasma anti-p16 IgG levels between patients and controls. Receiver operating characteristic (ROC) curve analysis was performed to obtain the area under the ROC curve (AUC) with the 95% confidence interval (CI), as well as the sensitivity of the anti-p16 IgG assay against a specificity of 95%.

Results

The in-house ELISA used in this study had a better repeatability (the comparison among the repeats), with a coefficient of variation of 15.2%, calculated based on the SBR (1.84 ± 0.28) from QC specimens tested independently in 13 plates. Anti-p16 IgG levels of HCC patients were obviously elevated compared with those of the controls ($Z = 3.51$, $P = 0.0004$). Anti-p16 IgG levels were significantly higher in both male ($Z = 2.86$, $P = 0.004$) and female ($Z = 2.36$, $P = 0.018$) patients than in the healthy controls, implying that plasma anti-p16 IgG levels were elevated in all HCC patients, regardless of sex (Table 2).

Table 2. Comparison of the Circulating Antibodies to p16-Derived Peptide Antigen of HCC Patients and Control Subjects

Patient Group	Antibody Level SBR (mean ± SD [n])		Z	P[a]
	Patients	Controls		
Male	1.92 ± 0.93 (103)	1.60 ± 0.67 (107)	2.86	0.004
Female	1.86 ± 0.46 (18)	1.53 ± 0.49 (27)	2.36	0.018
Combined	1.91 ± 0.87 (121)	1.59 ± 0.64 (134)	3.51	0.0004

HCC, hepatocellular carcinoma; SBR, specific binding ratio; SD, standard deviation.
[a]Mann-Whitney test (2-tailed).

Table 3. Relationship between HCC Stages and Circulating IgG Antibodies to p16-Derived Peptide Antigen Levels

Stage(s)	Antibody Level SBR (mean ± SD [n])		Z	P[a]
	Patients	Controls		
0 + A	1.66 ± 0.58 (25)	1.59 ± 0.64 (134)	0.97	0.331
B	1.86 ± 0.79 (45)	1.59 ± 0.64 (134)	2.51	0.012
C + D	2.08 ± 1.03 (51)	1.59 ± 0.64 (134)	3.36	0.0008

HCC, hepatocellular carcinoma; IgG, immunoglobulin G; SBR, specific binding ratio; SD, standard deviation.
[a]Mann-Whitney test (2-tailed).

Table 4. ROC Curve Analysis of Circulating Anti-IgG Levels in HCC Patients

Stage(s)	AUC	95% CI	SE	Sensitivity[a]
0 + A	0.562	0.442–0.680	0.061	0
B	0.623	0.531–0.717	0.048	8.9
C + D	0.659	0.564–0.754	0.049	19.6
Overall	0.622	0.554–0.690	0.035	11.4

ROC, receiver operating characteristic; IgG, immunoglobulin G; HCC, hepatocellular carcinoma; AUC, area under the ROC curve; CI, confidence interval; SE, standard error.
[a]Against 95% specificity.

Further analysis of the association between plasma anti-p16 IgG levels and clinical stages revealed that plasma anti-p16 IgG levels displayed an increasing trend with HCC stages (Table 3), with group 3 (stages C + D) patients having the highest anti-p16 IgG levels ($Z = 3.38$, $P = 0.0008$). However, there was no obvious correlation ($Z = 0.97$, $P = 0.331$) between anti-p16 IgG levels and clinical stages in group 1 (stages 0 + A) patients.

An AUC of 0.622 (95% CI, 0.554–0.690) was obtained for all HCC patients, based on the ROC curve analysis, with a sensitivity of 11.4% and a specificity of 95% (Table 3). As shown in Table 4, of the 3 groups of HCC patients, group 3 (stages C + D) showed the best sensitivity for plasma anti-p16 IgG

level detection, with an AUC of 0.659 (95% CI, 0.564–0.754), followed by group 2 (stage B), with an AUC of 0.623 (95% CI, 0.531–0.717). The AUC for group 1 (stages 0 + A) patients was 0.562 (95% CI, 0.442–0.680), with a sensitivity of 0, suggesting the potential use of plasma anti-p16 IgG as a more biomarker for HCC prognosis, compared to early HCC diagnosis.

Discussion

The CDK inhibitor p16 protein plays a crucial role in cell proliferation regulation via G_1-phase cell arrest; thus, its malfunction may result in uncontrolled cell proliferation.[25,26] It is reportedly overexpressed in several cancers.[20,22] In the present study, HCC patients were found to have significantly higher circulating anti-p16 IgG levels than did control subjects. This finding was consistent with those from previous studies where higher circulating anti-p16 IgG levels were observed in cervical,[18] breast,[19] NSCLC,[20,27] and esophageal[21] cancer patients than that of control subjects. Additionally, 2 other studies which applied an ELISA method utilizing recombinant p16 protein for measuring circulating anti-p16 IgG levels found that HCC patients displayed markedly higher anti-p16 IgG levels than did controls.[22,28] The specific mechanism responsible for higher anti-p16 IgG levels in cancer remains unknown; however, this might be owed to higher p16 protein expression in cancer tissues.[29,30] However, hypermethylation-induced p16 expression inhibition is suggested to be a characteristic of HCC patients and is associated with an increased risk of HCC.

Further, plasma anti-p16a IgG levels showed an increasing trend with increasing HCC stage, and advanced- and terminal-stage HCC patients had the highest anti-p16a IgG levels, suggesting that plasma anti-p16 IgG might be a more promising biomarker for HCC prognosis evaluation than early HCC diagnosis. A similar phenomenon was observed in non–small cell lung cancer in a study by Zhang et al,[20] contrary to those in esophageal cancer reported by Jin et al,[21] where circulating anti-p16 IgG levels showed a decreasing trend with increasing tumor stage. Anti-p16 antibody levels are suggestively altered in diverse patterns among cancer types. Nevertheless, there remain several limitations to our work. First, the antibody testing sample size was small, and a large sample size was needed to permit a firm conclusion. Additionally, the control was a poor representative, as the subjects were only recruited from local communities, and reportedly, healthy relatives of HCC patients might be more reliable for identifying a serological biomarker for familial HCC risk.

Conclusion

The results of this study, which was designed to explore the clinical applicability of circulating anti-p16 IgG levels in HCC diagnosis, suggest that circulating IgG antibodies to p16 protein might be a useful biomarker for HCC prognosis assessment compared to early HCC diagnosis. **LM**

Acknowledgments

We thank all the patients and control subjects for their participation in this study.

This work was supported by Hailanshen Biomedical & Technology Ltd, Shenzhen, China.

References

1. Ghouri YA, Mian I, Rowe JH. Review of hepatocellular carcinoma: epidemiology, etiology, and carcinogenesis. *J Carcinog*. 2017;16(1):1.
2. Farinati F, Marino D, De Giorgio M, et al. Diagnostic and prognostic role of alpha-fetoprotein in hepatocellular carcinoma: both or neither? *Am J Gastroenterol*. 2006;101(3):524–532.
3. Lee JM, Yoon JH, Kim KW. Diagnosis of hepatocellular carcinoma: newer radiological tools. *Semin Oncol*. 2012;39(4):399–409.
4. Liu W, Peng B, Lu Y, Xu W, Qian W, Zhang JY. Autoantibodies to tumor-associated antigens as biomarkers in cancer immunodiagnosis. *Autoimmun Rev*. 2011;10(6):331–335.
5. Poletaev A, Pukhalenko A, Kukushkin A, Sviridov P. Detection of early cancer: genetics or immunology? Serum autoantibody profiles as markers of malignancy. *Anticancer Agents Med Chem*. 2015;15(10):1260–1263.
6. Dai L, Tsay JCJ, Li J, et al. Autoantibodies against tumor-associated antigens in the early detection of lung cancer. *Lung Cancer*. 2016;99:172–179.
7. Hong Y, Huang J. Autoantibodies against tumor-associated antigens for detection of hepatocellular carcinoma. *World J Hepatol*. 2015;7(11):1581–1585.
8. Himoto T, Kuriyama S, Zhang JY, Chan EK, Nishioka M, Tan EM. Significance of autoantibodies against insulin-like growth factor II mRNA-binding proteins in patients with hepatocellular carcinoma. *Int J Oncol*. 2005;26(2):311–317.
9. Ying X, Zhao Y, Wang JL, et al. Serum anti-osteopontin autoantibody as a novel diagnostic and prognostic biomarker in patients with hepatocellular carcinoma. *Oncol Rep*. 2014;32(4):1550–1556.

10. Yau WY, Shih HC, Tsai MH, Sheu JC, Chen CH, Chow LP. Autoantibody recognition of an N-terminal epitope of hnRNP L marks the risk for developing HBV-related hepatocellular carcinoma. *J Proteomics.* 2013;94:346–358.
11. Zhou JW, Li Y, Yue LX, Luo CL, Chen Y, Zhang JY. Autoantibody response to Sui1 and its tissue-specific expression in hepatocellular carcinoma. *Tumour Biol.* 2016;37(2):2547–2553.
12. Serrano M, Hannon GJ, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature.* 1993;366(6456):704–707.
13. Eleutério J Jr, Lima TS, Cunha MD, Cavalcante DI, Silva AM. Immunohistochemical expression of the tumor suppressor protein p16INK4a in cervical adenocarcinoma. *Rev Bras Ginecol Obstet.* 2017;39(1):21–25.
14. Csepregi A, Ebert MP, Röcken C, et al. Promoter methylation of CDKN2A and lack of p16 expression characterize patients with hepatocellular carcinoma. *BMC Cancer.* 2010;10:317.
15. Matsuda Y, Ichida T, Genda T, Yamagiwa S, Aoyagi Y, Asakura H. Loss of p16 contributes to p27 sequestration by cyclin D(1)-cyclin-dependent kinase 4 complexes and poor prognosis in hepatocellular carcinoma. *Clin Cancer Res*. 2003;9(9):3389–3396.
16. Ko E, Kim Y, Kim SJ, et al. Promoter hypermethylation of the p16 gene is associated with poor prognosis in recurrent early-stage hepatocellular carcinoma. *Cancer Epidemiol Biomarkers Prev.* 2008;17(9):2260–2267.
17. Zang JJ, Xie F, Xu JF, et al. P16 gene hypermethylation and hepatocellular carcinoma: a systematic review and meta-analysis. *World J Gastroenterol.* 2011;17(25):3043–3048.
18. Huangfu M, Liu L, Xu S, et al. Detecting of p16 autoantibody as a potential early diagnostic serum biomarker in patients with cervical cancer. *Clin Lab.* 2016;62(6):1117–1120.
19. Chen C, Huang Y, Zhang C, et al. Circulating antibodies to p16 protein-derived peptides in breast cancer. *Mol Clin Oncol.* 2015;3(3):591–594.
20. Zhang C, Ye L, Guan S, et al. Autoantibodies against p16 protein-derived peptides may be a potential biomarker for non-small cell lung cancer. *Tumour Biol.* 2014;35(3):2047–2051.
21. Jin Y, Guan S, Liu L, Sun S, Lee KH, Wei J. Anti-p16 autoantibodies may be a useful biomarker for early diagnosis of esophageal cancer. *Asia Pac J Clin Oncol*. 2015;11(4):e37–e41.
22. Looi K, Megliorino R, Shi F-D, Peng X-X, Chen Y, Zhang J-Y. Humoral immune response to p16, a cyclin-dependent kinase inhibitor in human malignancies. *Oncol Rep*. 2006;16(5):1105–1110.
23. Llovet JM, Fuster J, Bruix J; Barcelona-Clínic Liver Cancer Group. The Barcelona approach: diagnosis, staging, and treatment of hepatocellular carcinoma. *Liver Transpl.* 2004;10(2 Suppl 1):S115–S120.
24. Hallford P, Clair DS, Halley L, Mustard C, Wei J. A study of type-1 diabetes associated autoantibodies in schizophrenia. *Schizophr Res.* 2016;176(2-3):186–190.
25. Scully KM, Lahmy R, Signaevskaia L, et al. E47 governs the MYC-CDKN1B/p27^{KIP1}-RB network to growth arrest PDA cells independent of CDKN2A/p16^{INK4A} and wild-type p53. *Cell Mol Gastroenterol Hepatol*. 2018;6(2):181–198.
26. Leontieva OV, Blagosklonny MV. CDK4/6-inhibiting drug substitutes for p21 and p16 in senescence: duration of cell cycle arrest and MTOR activity determine geroconversion. *Cell Cycle.* 2013;12(18):3063–3069.
27. Zhao H, Zhang X, Han Z, Wang Y. Circulating anti-p16a IgG autoantibodies as a potential prognostic biomarker for non-small cell lung cancer. *FEBS Open Bio*. 2018;8(11):1875–1881.
28. Zhang JY, Megliorino R, Peng XX, Tan EM, Chen Y, Chan EK. Antibody detection using tumor-associated antigen mini-array in immunodiagnosing human hepatocellular carcinoma. *J Hepatol.* 2007;46(1):107–114.
29. Tong J, Sun X, Cheng H, et al. Expression of p16 in non-small cell lung cancer and its prognostic significance: a meta-analysis of published literatures. *Lung Cancer.* 2011;74(2):155–163.
30. Henshall SM, Quinn DI, Lee CS, et al. Overexpression of the cell cycle inhibitor p16INK4A in high-grade prostatic intraepithelial neoplasia predicts early relapse in prostate cancer patients. *Clin Cancer Res.* 2001;7(3):544–550.

HOLOGIC®

First and Only FDA Cleared
Digital Cytology System

Genius™ Cervical AI

Genius™ Review Station

Genius™ Digital Imager

Empower Your Genius With Ours

Make a Greater Impact on Cervical Cancer
with the Advanced Technology of the
Genius™ Digital Diagnostics System

Click or Scan
to discover more

genius™
DIGITAL DIAGNOSTICS