

RESEARCH COMMUNICATION

Detection of Serum Anti-P53 Antibodies from Patients with Colorectal Cancer in China Using a Combination of P53- and phage-ELISA: Correlation to Clinical Parameters

Jing Wu^{1&}, Tian Qiu^{2&}, Pengtao Pan¹, Dehai Yu¹, Zhigang Ju¹, Xuewei Qu³, Xiang Gao¹, Chuanbin Mao^{3*}, Li Wang^{1*}

Abstract

Background: Colorectal cancer is one of the most common malignant tumors in China. The aims of this research were to increase the sensitivity of anti-p53 antibody detection in the sera of patients with colorectal cancer and to assist in their diagnosis. **Methods:** Sixty-seven non-selected Chinese with colorectal cancer were involved in this study. Anti-p53 antibodies in serum were detected by ELISA using recombinant human wild-type p53 protein and hybrid phage as the coating antigen. Correlations between the anti-p53 antibodies and clinicopathological parameters were also analyzed. **Results:** The detection efficiency of anti-p53 antibodies in the patients with colorectal cancer was increased (46.3%, 31/67) through the combination of the two ELISA methods compared with each method alone. The titer of serum anti-p53 antibodies was not associated with clinicopathological parameters, but there was a significant correlation between their presence, the CEA level, and the stage of the patient's colorectal cancer. **Conclusions:** These results demonstrate that combination of the two ELISA methods increased the detection rate of anti-p53 antibodies in patients with colorectal cancer. This research may provide a useful method to complement conventional clinical diagnosis.

Keywords: p53 - antibodies - colorectal cancer - ELISA - phage display technique

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Introduction

The p53 gene is located at chromosome 17p1 3.1, which encodes a 53-kilodalton nuclear phosphoprotein. The wild-type p53 gene functions as a tumor suppressor. The cell cycle is composed of a series of steps that can be negatively or positively regulated by various factors. Chief among the negative regulators is the p53 protein. Alteration or inactivation of p53 by mutation or through interactions with oncogene products of DNA tumor viruses, can lead to cancer (Levine et al., 1991). These mutations seem to be the most common genetic changes in human cancers (Levine et al., 1991). Levels of wild-type p53 are usually quite low due to its short half-life. However, p53 protein is stabilized in response to various DNA-damaging agents, and its level increases (Maki et al., 1997). The accumulation of p53 protein may lead to the production of anti-p53 antibody in serum.

Colorectal cancer is one of the most frequent malignant tumors in the world (Abdel-Aziz et al., 2009). In China, especially in some large cities, the growth of colorectal cancer morbidity is significant. Therefore, a more

favorable marker for the diagnosis of colorectal cancer is needed.

Anti-p53 antibodies have been detected in the sera of patients with various types of cancers (Soussi, 2000). It had been demonstrated that the immune response of patients with p53 antibodies is restricted to a small subset of peptides localized in the amino and carboxy termini of the p53 protein and few individuals have antibodies to the central DNA binding domain (Lubin et al., 1993). The phage display technique simulated the natural epitope effectively, and the displayed peptides were exposed well. ELISA using phage displaying the epitope of the p53 protein can detect anti-p53 antibodies in sera of patients with various types of cancers (Portefaix et al., 2002).

In the present study, the ELISA procedures were carried out using the recombinant human wild-type p53 protein and the phage-displayed peptide SQAMDDLMLS as coating antigen respectively. We applied this ELISA format to detect serum anti-p53 antibodies in 67 Chinese patients with colorectal cancer. Furthermore, we evaluated the presence of anti-p53 antibodies in correlation to CEA and to conventional clinicopathological data.

¹Institute of Genetics and Cytology, School of Life Sciences, Northeast Normal University, Changchun, ²Department of Pathology, Cancer Institute and Cancer Hospital, Chinese Academy of Medical Sciences, Beijing, China ³Department of Chemistry and Biochemistry, University of Oklahoma, Norman, USA ⁴Equal contributions *For correspondence: wanglee57@163.com and cbmao@ou.edu

Materials and Methods

Patients and controls

A total of 67 patients (40 men and 27 women) who had been diagnosed with colorectal cancer and treated at China-Japan Union Hospital of Jilin University, Changchun, Jilin, from Sept. 2004 to Nov. 2007 were enrolled in this study. The mean age of the patients was 53.9 years (range 24–82). Serum samples were obtained before the patients received any treatment and stored at -80°C until used. The clinicopathological characteristics of the patients were summarized in Table 1. The stages of the tumors were determined according to the UICC TNM classification. Serum samples from a panel of 400 healthy volunteers of a mean age of 53.4 years (range 24–82), were used to determine the cut-off value and reliability of the ELISA methods for detection of anti-p53 antibodies. Ten serum samples with colon polyp, at a mean age of 42.7 years (range 26–58), were used as a control group. Patient recruitment and sample collection were performed within the guidelines of protocols approved by the institutional review boards. All cases have been analyzed by clinicians.

Preparation of the Coating antigen

In the previous study, we prepared the recombinant p53 protein and detected serum anti-p53 antibodies from patients with breast tumor (Gao et al., 2005). The preparation of the phage-displayed peptide, 37-46 (SQAMDDLMLS) was based on previous reports (Qiu et al., 2007). The samples were analyzed by 20% SDS-PAGE and visualized by silver staining.

Western blot analysis of phage-displayed peptide

The protein and phage-displayed peptide were electrophoretically separated and subsequently transferred to a nitrocellulose membrane. The filters were cut into strips which were then blocked overnight at 4°C in blocking buffer (5% powered nonfat milk in TBS) to block nonspecific binding sites. After washing with TBST, each strip was incubated for 1h at 37°C with a p53 antibody positive or control serum, followed after three times washings by the addition of the peroxidase-conjugated goat anti-human Ig G (Sigma) and incubation for 1 h at 37°C . Thereafter the strips were stained with 3-amino-9-ethylcarbazole (AEC, AMRESCO, American) used as a chromogen.

Detection of serum p53 antibodies

The ELISA procedures for detecting anti-p53 antibodies in serum were based on our previous study (Qiu et al., 2007). The optimal conditions of both ELISA methods were determined according to checkerboard titration. Anti-p53 antibodies in sera from 400 healthy volunteers were detected using optimal conditions of the two ELISA methods to determine the cut-off values.

A serum has been chosen as the control serum. All the results were expressed as p53 index: $\text{p53 index} = \text{OD}_{450 \text{ nm}} \text{ absorbance of a sample} / \text{OD}_{450 \text{ nm}} \text{ absorbance of the serum control}$. Serum samples with a p53 index >1.7 were considered positive, when the wild-type p53 protein was used as the coating antigen. Serum samples with a p53

index > 1.1 were considered positive, when the hybrid phage-displayed peptide was used as the coating antigen.

Detection of CEA

The levels of CEA in sera were assayed with the electrochemiluminescent immunoassay (Elecsys 2010, Roche, Switzerland). A sample was regarded as CEA positive when the level of CEA in serum was higher than 3.40 ng/ml.

Statistical analysis

A comparison was made between the detection results of the two ELISA systems. The χ^2 -test was used to determine the associations between anti-p53 antibodies and clinicopathological characteristics. A value of $p < 0.05$ was considered significant.

Results

SDS-PAGE and Western Blot results of phage-displayed peptide

As shown in Figure 1A, the peptide was successfully displayed on the surface of filamentous bacteriophage virions. Figure 1B revealed that the phage-displayed peptide reacted with a p53 antibody positive serum from cancer patients. These results also demonstrated that the phage-displayed peptide could be used as antigen for the detection of anti-p53 antibodies in cancer patients.

Clinicopathological characteristics of colorectal cancer patients

Clinicopathologic backgrounds of the 67 colorectal cancer patients are shown in Table 1. Among the patients, 36 had colon cancer and 31 had rectal cancer. Before operation, 20 (29.9%) of the patients were at clinical stage I or II, and 47 (70.1%) were at stage III or IV. A total of 8 patients (11.9%) were well differentiated, 35 (52.2%) were moderately differentiated, and 24 (35.8%) were poor differentiated.

Detection of serum anti-p53 antibodies by two ELISA Methods

Anti-p53 antibodies in sera were detected by two ELISA methods using recombinant human wild-type p53 protein and hybrid phage protein as the coating antigen. Using checkerboard titration ELISA, the optimal coating concentration of antigen was determined to be $5\mu\text{g/ml}$

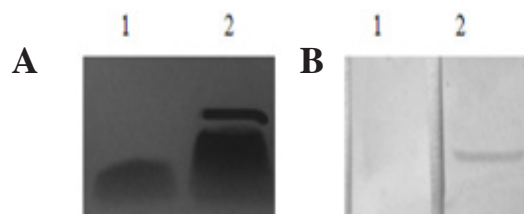


Figure 1. SDS-PAGE and Western Blot Analysis of the Phage-displayed Peptide. (A) Samples were analyzed by SDS-PAGE and visualized by silver staining. Line 1, wild-type phage protein; line 2, hybrid phage protein. (B) Western blot analysis of phage-displayed peptide. Line 1, probing with a negative serum from healthy persons; line 2, probing with a p53 antibody positive serum from cancer patients

Table 1. Patient Characteristics (n=67)

Characteristics	n (%)	
Age(year)	≤60	44 (65.7)
	>60	23 (34.3)
Sex	Male	40 (59.7)
	Female	27 (40.3)
Tumor location	Right colon	13 (19.4)
	Left colon	23 (34.3)
	Rectum	31 (46.3)
Differentiation degree	Well	8 (11.9)
	Moderate	35 (52.2)
	Poor	24 (35.8)
Stage	I	2 (3.0)
	II	18 (26.9)
	III	7 (10.4)
	IV	40 (59.7)
CEA	+	24 (35.8)
	-	43 (64.2)

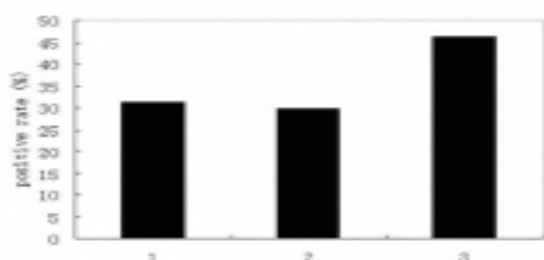


Figure 2. Comparison of Positive Rate for Serum Anti-p53 Antibodies Between Two ELISA Methods. 1. p53-ELISA(31.3%); 2. phage-ELISA(29.9%); 3. p53-ELISA or phage-ELISA(46.3%)

recombinant p53 protein and 60µg/ml hybrid phage, and the optimal degree of dilution of serum was 1:200. Based on the reference values that were calculated using parametric determination of the lower 0.95 fraction of the reference distribution in the 400 healthy control donors, the cut-off values of p53 index were determined to be 1.7 when wild-type p53 protein is the coating antigen and 1.1 when hybrid phage displaying protein is the coating antigen. Of the 400 healthy control patients, 20 tested positive for anti-p53 antibodies in their sera according to the p53- ELISA and 19 tested positive according to the phage-ELISA (specificity 95.0% and 95.3%).

Furthermore, we assayed the preoperative sera from 67 patients with colorectal cancer and 10 patients with colon polyp for the presence of p53 antibodies. Among the 67 patients, 21 (31.3%) patients were tested positive for anti-p53 antibodies in their sera in p53-ELISA and 20 (29.9%) patients in phage-ELISA. None of the 10 patients with colon polyp had serum anti-p53 antibodies detectable by p53-ELISA or phage-ELISA. All the sera samples were assayed again one month after the first detection, using the same sera as controls. Both assays gave the same results, confirming the repeatability and stability of this ELISA procedure. To unite the two ELISA systems, the detection rate was 46.3% (31/67). The combination of these two ELISA systems could increase the detection rate of anti-p53 antibodies compared with that of either p53-ELISA or phage-ELISA (Figure 2).

Correlation between serum anti- p53 antibodies and

Table 2. Correlation Between p53 and Clinical Characteristics of Colorectal Cancer Patients (n=67)

Characteristics	p53-antibodies		phage-antibodies	
	+	-	+	-
Total No.	21 (31.3%)	46	20 (29.9%)	47
Sex	Male	13	27	11
	Female	8	19	9
Stage	p-value	0.80	0.61	
	I/II	2	18	2
CEA	III/IV	19	28	18
	p-value	0.01	0.02	
p-value	+	11	15	12
	-	10	31	8
	p-value	0.02	0.03	

p53-antibodies, serum anti-p53 antibodies while using p53 protein as the coating antigen; phage-antibodies, serum anti-p53 antibodies while using the hybrid phage as the coating antigen

clinical Characteristics

Table 2 shows the relationships between presence of anti-p53 antibodies and clinical features. Among the 20 patients with stage I or II cancer, 2 (10%) had anti-p53 antibodies detected in their sera by both p53-ELISA and phage-ELISA, while in the advanced stages III and IV were 19 of 47 (40.4%) and 18 of 47 (38.3%), respectively. Twenty-six of 67 patients with colorectal cancer were positive for CEA (38.8%). The value of CEA became higher as stage advanced. The presence of anti-p53 antibodies above cut-off values in serum was associated with the patient’s stage of cancer and level of CEA.

Discussion

Crawford et al. (1982) first detected anti-p53 antibodies in the serum of breast cancer patients. Thereafter the anti-p53 antibodies were detected in the sera of patients with various malignancies (Angelopoulou et al., 1994; Soussi, 2000; Shaarawy and Sheiba, 2004; Yamazawa et al., 2007). In this study, we applied two ELISA methods to detect serum anti-p53 antibodies of 400 healthy controls and 67 Chinese colorectal cancer patients. Among the 67 patients, 21 (31.3%) patients were positive for anti-p53 antibodies according to the p53-ELISA and 20 (29.9%) patients tested positive according to the phage-ELISA. This result is comparable to other studies of the presence of anti-p53 antibodies in sera of patients with colorectal cancer (Shiota et al., 2000).

The studies between serum anti-p53 antibodies and other clinical parameters remain debatable (Kressner et al., 1998; Bielicki et al., 1999; Hammel and Soussi, 2000; Lu et al., 2007; Atta et al., 2008; Suppiah et al., 2008). In this study, 2 (10%) of 20 patients with stage I or II cancer tested positive for anti-p53 antibodies by both p53-ELISA and phage-ELISA, while in the advanced stages III and IV were 19 of 47 (40.4%) and 18 of 47 (38.3%), respectively. The presence of anti-p53 antibodies in serum was associated with the patient’s stage of cancer. For the 10 patients with colon polyp, no anti-p53 antibodies were detectable by p53-ELISA or phage-ELISA. These data, which are consistent with the study of Hammel et

al. (1997) show that none of those with non-malignant disease displayed anti-p53 antibodies. However, Shiota et al. (2000) has reported that 1 of 18 (6%) sera from patients with colon polyp were positive for anti-p53 antibodies. Fujita et al. (2003) found that p53 mutations were frequently observed in serous carcinomas but not found in adenomas and rarely found in borderline tumors, suggesting that p53 mutations might be directly involved in malignant transformation.

Matina and colleagues (2006) found that testing for anti-p53 antibodies could increase diagnostic sensitivity when used in combination with measurement of other conventional tumor markers. In our study, there was a relationship between serum anti-p53 antibodies and CEA. Owing to its high specificity for malignancy, the anti-p53 antibodies detection could make up for the limitations of CEA detection and improve the diagnosis for colorectal cancer.

In this study, the hybrid phage displayed the immunodominant epitope of the p53 N-terminal region. This displayed peptide was more exposed than the epitope in the recombinant p53 protein. Therefore, the hybrid phage when used as a coating antigen might be more sensitive to anti-p53 antibodies than the recombinant p53 protein sometimes, and the combination of phage-ELISA and p53-ELISA could make up for the limitation of the previous only using the p53-ELISA. Moreover, the phage-displayed epitope could be prepared simply and fusion protein could be produced in large quantities at a lower cost. The phage-ELISA as an auxiliary diagnosis method still has its striking attraction and potential clinical application value. We suppose this result may be similar in other types of cancers, so future research is necessary.

In conclusion, the sera of 67 Chinese patients with colorectal cancer were tested for the anti-p53 antibodies using the recombinant p53 protein and the hybrid phage as the coating protein. These results demonstrated that the combination of the two ELISA methods increased the detection rate of anti-p53 antibodies in patients with colorectal cancer. This research may provide a useful method to complement conventional clinical diagnosis for patients with colorectal cancer.

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