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ORIGINAL ARTICLE

Detection of serum p53 antibodies from Chinese patients with papillary thyroid carcinoma using phage-SP-ELISA: correlation with clinical parameters

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Abstract The goal of the present study was to investigate whether p53 antibodies (Abs) could be a relevant marker for papillary thyroid carcinoma (PTC). Three types of enzyme-linked immunosorbent assay (ELISA) methods were developed for the detection of p53 Abs, including p53-ELISA, phage-SS-ELISA, and phage-SP-ELISA. A total of 304 patients, including 117 cases with thyroid adenoma and 187 PTC patients, were enrolled in this study. Expression of p53 protein and mutation in *BRAF* gene were evaluated in paraffin-embedded tissue from 44 patients with PTC, in order to elucidate their correlations with the presence of p53 Abs. Compared with p53-ELISA and phage-SS-ELISA, phage-SP-ELISA presented the highest detection efficiency of p53 Abs in patients with PTC, and a combination of these three ELISA systems could make the detection of p53 Abs more sensitive than using each of the individual ELISA methods. Furthermore, p53 Abs was positively associated with clinical stage ($P = 0.044$), node metastasis ($P = 0.010$), and p53 protein accumulation ($P = 0.019$). These results indicate that serum p53 Abs could be a useful marker for PTC.

Keywords p53 Antibodies · Papillary thyroid carcinoma · Phage display · Tumor marker

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Introduction

Thyroid cancer is the most common malignant tumor of endocrine system and accounts for approximately 1 % of all newly diagnosed cancer cases. Its incidence has increased significantly in China. The most frequent type of thyroid malignancy is papillary thyroid carcinoma (PTC), which accounts for approximately 80 to 90 % of all thyroid malignancies [1–3]. Fine-needle aspiration (FNA) and ultrasound are widely used in clinical practice as diagnostic techniques for PTC. More recently, the oncogenic T1799A transversion mutation of *BRAF* has been described and represents the most common genetic alternation in PTC [4, 5], with a prevalence of approximately 45 % [6, 7]. However, there is no effective serum marker for PTC so far. Therefore, it is of high necessity to identify the additional diagnostic and prognostic markers that facilitate the improved and personalized management of patients with PTC.

Serum p53 antibodies (Abs) were first described by Crawford et al. in breast cancer patients [8]. Since then, it was gradually found in patients with various types of cancer, such as esophageal, lung, colorectal, breast, liver, stomach, ovarian, and so on [9]. Numerous studies demonstrated that p53 Abs was rarely detected in healthy donors and patients with benign diseases and that in cancer patients and their prevalence varied form 9 to 60 % according to the tumor studied [8, 10–13]. Furthermore, clinical studies have shown that p53 Abs is an independent prognostic factor of poor survival in many studies for various tumor sites [14–16].

To our knowledge, no data are available in the literature on the prevalence of p53 Abs and their usefulness as a tumor marker for PTC. To determine whether p53 Abs could be a relevant marker for PTC, we conducted a

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prospective study to explore whether p53 Abs was more frequent in PTC patients than in thyroid adenoma patients. In this study, we prepared recombinant p53 protein and two types of filamentous bacteriophages for the detection of serum p53 Abs. In order to confirm p53 Abs, which usually correlates with p53 accumulation [17], we checked for p53 protein stabilization in some samples of PTC by immunohistochemistry. Additionally, we also detected the *BRAF* mutation in PTC patients of China and analyzed the relationship between *BRAF* mutation and p53 Abs.

Materials and methods

Patients and controls

A total of 304 patients, including 117 cases with thyroid adenoma (36 men, 81 women; median age 50.9, range from 26 to 67) and 187 PTC patients (41 men, 146 women; median age 42.0, range from 18 to 63), were enrolled in this study. All of them were from China-Japan Union Hospital, Changchun, Jilin from June 2012 to November 2012. For each patient, age, clinical stage, and lymph node status were recorded. Serum samples were obtained before the patients received any treatment and stored at −40 °C until used. Resected specimens were fixed in 10 % formalin, paraffin embedded, and conserved for immunohistochemical and *BRAF* mutation analysis. Clinical staging was defined according to the international TNM classification proposed by American Joint Committee on Cancer (AJCC). Meanwhile, a population of 150 normal controls with no further clinical information who received routine physical examinations from the Northeast Normal University Attached Hospital constituted the normal control group.

Construction and preparation of phage-SS and phage-SP

The f388-55 vector (kept in our lab) derived from filamentous bacteriophages was first double digested by *Bgl* I (Takara, Japan) and then the adaptor molecule created by annealing two oligonucleotides encoding the peptide SP (SDLWKLLP) of p53 protein N-terminus amino acids was inserted at an engineered restriction site in the bacteriophage gene III, and phage-SP was prepared and purified as described by Gandra et al. [18]. The phagemid pfd88 vector (kept in our lab) was double digested by *BstB* I and *Sac* II (Takara, Japan), then an appropriate DNA fragment encoding the peptide SS(SQAMDDLMLS) belonging to the N-terminal of p53 protein was directionally cloned into the modified gene VIII, and phage-SS was produced by infecting NM522 cells transformed with pfd88-SS with helper phage according to our previous study [19].

Western blot analysis of phage-SS and phage-SP

Both phage-SS and phage-SP were separated by electrophoresis and subsequently transferred to a nitrocellulose membrane (Amersham, American). 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 1 h at 37 °C with p53 polyclonal Abs (prepared in our laboratory) or anti-pIII monoclonal antibody recognizing amino acids 292 to 302 of the mature pIII coat protein (Mo Bi Tec, Germany). After the strip was washed for three times, the peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Sigma) was added to the strip, followed by incubation for 1 h at 37 °C. Thereafter, the strips were stained with 3-amino-9-ethylcarbozole (AEC, AMRESCO, American) used as a chromogen. Negative controls (wild-type phage) were carried out in parallel.

Enzyme immunoassays for the detection of serum p53 Abs

phage-SP-ELISA

Serum samples were analyzed for p53 Abs by enzyme-linked immunosorbent assay (ELISA) as described previously [19]. All samples were measured in duplicate and the mean of the duplicate values was taken as the final read out.

phage-SS-ELISA

The ELISA procedure was the same as the phage-SP-ELISA procedure, except that the coating antigen was phage-SS.

p53-ELISA

The ELISA procedure was the same as the phage-SP-ELISA procedure. But, the coating concentration of recombinant p53 protein (prepared in our lab) was 5 μg/ml according to our previous studies [17, 19].

Determination of cut-off value for each ELISA method

150 normal volunteers' sera were investigated under the optimal conditions of the three ELISA format to determine the cut-off values. For each ELISA method, the cut-off value designating positive reactions was conventionally defined as an absorbance value greater than the mean +2 standard deviations (SDs) of the normal cohort [20–22]. Because several hundred samples were analyzed at

different time periods, each run of ELISA included 2 samples as control sera [23].The two sera represented a range above and below the mean of 150 normal people, and the average of the two samples was used in each run to normalize all absorbance values.

Immunohistochemical analysis of p53 protein

Available tumor samples were analyzed for p53 protein accumulation by immunohistochemistry as our previous method [17]. Staining was considered positive when over 10 % of the cell nuclei had been stained [24, 25]. The percentages of tumor cells with positive p53 staining were determined independently by two researchers.

DNA isolation, PCR amplification of exon 15 segment of the *BRAF* and sequencing

Paraffin-embedded PTC samples from patients were microdissected and DNA was isolated. Two primers (forward, 5′-AATGCTTGCTCTGATAGGAAAA-3′; reverse, 5′-AGCATCTCAGGGCCAAAAAT-3′), were used to amplify a 230 bp fragment of the exon 15 of *BRAF* containing the site in which T1799A mutation occurs. All the sequencing data were obtained by sequencing with both the forward and reverse primers.

Statistical analysis

Significant difference for p53 Abs between PTC cases and benign controls was generated using Chi square test to compare the two groups. Correlations of serum p53 Abs with clinicopathologic parameters and p53 protein accumulation were examined by Chi square test or Fisher's exact test as appropriate. These results were generated using SPSS version 13.0 for Windows (SPSS Inc., Chicago, IL). Tests with $P < 0.05$ are considered statistically significant.

Ethics committee approval

This study was also approved by the Ethics Committee of China-Japan Union Hospital and conducted according to the Declaration of Helsinki for studies on human subjects. Informed consent was obtained from all participants.

Results

Western blot analysis of phage-SS and phage-SP

In order to confirm the foreign peptide SS or SP is displayed on the surface of phage, we performed western blot analysis (Fig. 1). Compared with negative control, specific

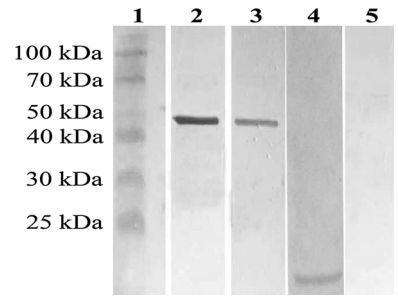

Fig. 1 Western blot analysis of phage-SS and phage-SP. Lane 1: protein Markers; Lane 2: wild-type phage probed with anti-pIII monoclonal antibody recognizing amino acids from 292 to 302 of the mature pIII coat protein; Lane 3–5: phage-SP, phage-SS, and wild-type phage, respectively, probed with p53 polyclonal Abs

pIII protein displaying the peptide SP was observed when phage-SP probed with p53 polyclonal Abs, and the same band was also found in the positive control. Recombinant pVIII protein displaying the peptide SS was also found when phage-SS reacted with p53 polyclonal Abs. These results indicate that the peptide SS or SP was successfully displayed on the surface of filamentous bacteriophage virions and the expressed peptide SS or SP also could identify p53 Abs, so phage-SS and phage-SP can be used as antigens for the detection of p53 Abs.

Detection of serum p53 Abs in benign and PTC groups by three ELISA methods

The specificity of three ELISA methods was 94.7 % (p53-ELISA), 95.3 % (phage-SS-ELISA), and 95.3 % (phage-SP-ELISA), respectively. Specificity was calculated as the number of true negatives expressed as a percentage of the number of true negative plus false positive in the normal control population.

A total of 117 thyroid adenoma patients (benign control group) and 187 PTC patients (PTC group) were evaluated by three ELISA methods. All the three ELISA methods showed that the detection rates of p53 Abs in the PTC group were higher than the benign control group (Fig. 2). Compared with p53-ELISA ($P = 0.258$) and phage-SS-ELISA ($P = 0.064$), the results of Chi square test showed that there was a statistically significant difference between the two groups for p53 Abs detection rates by phage-SP-ELISA ($P = 0.019$). This fact shows that the phage-SP-ELISA is superior to both p53-ELISA and phage-SS-ELISA for the detection of p53 Abs in patients with PTC.

Among the 187 PTC patients, a total of 33 (17.6 %) patients were tested positive for serum p53 Abs by p53-SS-SP-ELISA when the positive cases from all the three

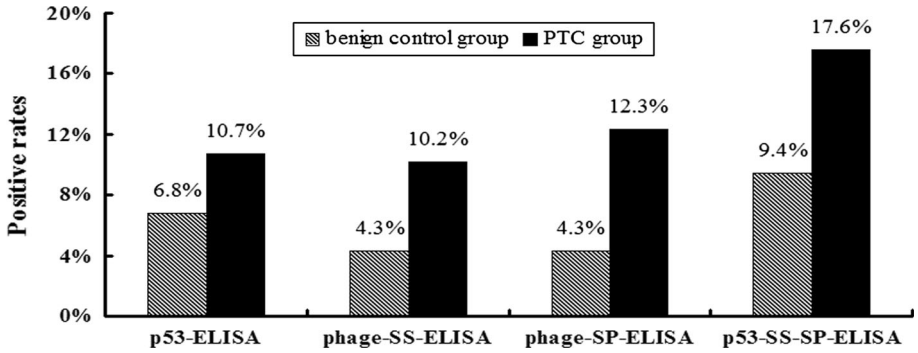

Fig. 2 Comparison of p53 Abs positive rates between benign control group and PTC group by three ELISA formats. *significant difference ($P < 0.05$) between 2 groups generated by Chi square test

Table 1 Correlation between serum p53 Abs and clinicopathologic parameters

Characteristics	Benign group	p53 Abs + [*n* (%)][b]	*P* value[a]	PTC group	p53 Abs + [*n* (%)][b]	*P* value[a]
Gender						
Male	36	3 (8.3)	1.0	41	7 (17.1)	0.913
Female	81	8 (9.9)		146	26 (17.8)	
Age (years)						
≥45	43	8 (18.6)	0.018*	77	18 (23.4)	0.086
<45	74	3 (4.1)		110	15 (13.6)	
Clinical stage						
I/II				172	27 (15.7)	0.044*
III/IV				15	6 (40.0)	
Lymph node						
Positive				56	16 (28.6)	0.010*
Negative				131	17 (13.0)	

* $P < 0.05$ was statically significant

[a] Calculated by Chi square test or Fisher's exact test

[b] The positive cases from all of the three ELISA methods were added together

antigens were added together, and 13 (7.0 %) cases were tested positive in all the three ELISA methods. Among the 117 thyroid adenoma patients, 10 (8.5 %) patients were tested positive for serum p53 Abs by p53-SS-SP-ELISA, and only 1 case was tested positive in all the three ELISA methods. All these results demonstrated that p53-SS-SP-ELISA could detect more positive cases than just with any one of the individual ELISA, and there was a marked difference between the two groups for serum p53 Abs by p53-SS-SP-ELISA ($P = 0.047$).

Correlation between serum p53 Abs and clinicopathologic parameters

Table 1 shows the correlation between serum p53 Abs and clinicopathologic parameters of either the benign or PTC group. There was a significant correlation of serum p53 Abs with patients' age for the benign control group ($P = 0.018$), and the correlation was also observed in the PTC group ($P = 0.086$), although not statistically significance. We further considered the correlation of serum p53 Abs with clinical stage and lymph node in patients with PTC. The presence of serum p53 Abs was positively associated with clinical stage ($P = 0.044$) and node metastasis ($P = 0.010$).

Correlation of serum p53 Abs with p53 protein accumulation and *BRAF* mutation in PTC patients

Due to the loss of tissue samples, only 16 positive and 28 negative PTC patients of serum p53 Abs were further assessed for the presence of p53 protein accumulation and *BRAF* T1799A mutation analysis. 6 individuals of the 16 patients who were stained immunohistochemically positive for nuclear p53 accumulation were also positive for serum p53 Abs. In the 28 patients negative for p53 Abs, only 2 were detected positive for p53 protein accumulation. A very high significant correction was found between serum p53 Abs and p53 protein accumulation ($P = 0.019$), as shown in Table 2. Figure 3 shows examples of p53 immunohistochemical positivity and negativity in PTC tissue sample from a p53 antibody-positive patient and a p53 antibody-negative patient. Further, *BRAF* mutation was analyzed by DNA sequencing to evaluate whether there was a correlation between p53 Abs and *BRAF* mutation in PTC patients. However, no relationship was found as reported in Table 2.

Table 2 Correlation of serum p53 Abs with p53 protein accumulation and *BRAF* mutation

	p53 protein		*P* value[a]	*BRAF* 1799A mutation		*P* value[a]
	+	–		+	–	
P53 Abs (+)	6 (37.5)	10	0.019	14 (87.5)	2	1.0
P53 Abs (–)	2 (7.1)	26		23 (82.1)	5	

Values in parentheses are percentages. Due to the loss of tissue samples, we only analyzed 44 PTC patients, including 16 positive cases and 28 negative cases of serum p53 Abs

[a] The test way was Fisher's exact test and $P < 0.05$ was statically significant

Discussion

p53 antigenic sites recognized by serum Abs were systematically researched by Lubin et al. [26] and Schlichtholz et al. [27] using peptide scanning analysis. Their studies demonstrated that the immune response of patients with p53 Abs was restricted to a small subset peptides localized in the amino terminal of p53 protein such as the peptide SS and SP studied in this work, whatever the type of cancer. Furthermore, it has been shown that a peptide displayed on the surface of phage could simulate the natural epitope effectively and phage display system allowed the displayed peptides better exposed [28]. Therefore, we displayed the peptide SS and SP, respectively, which belong to the immunodominant regions of the amino-terminal part of p53 protein, in order to enable it to efficiently recognize and detect serum p53 Abs.

With the purpose of increasing the sensitivity of detection and studying the usefulness of p53 Abs as a tumor marker for PTC, we designed three types of ELISA detection systems to detect serum p53 Abs in patients with thyroid adenoma or PTC. Among the three tests used, phage-SP-ELISA presented the highest and detection efficiency of p53 Abs in patients with PTC (Fig. 1). This result indicated that serum p53 Abs in PTC patients mainly recognized the peptide SP, compared with the peptide SS and p53 protein. This finding was consistent with previously published studies [26, 27]. As is known, the phage-displayed peptide represents the amino-terminal region of the p53 protein, and the peptide is exposed better at the surface of phage than the recombinant p53 protein, so it may recognize p53 Abs against the displayed peptide more sensitively than recombinant p53 protein. This is probably the reason why phage-SP-ELISA had a higher detection efficiency of p53 Abs in comparison with p53-ELISA.

We observed that 17.6 % of patients with PTC had serum p53 Abs. This frequency is higher than the published report of thyroid cancer [26]. That may have something to do with the patients and methods used in this study. Furthermore, many p53 positive sera samples that could not be detected by p53-ELISA were detected by phage-ELISA; therefore, the combination of three ELISA systems could make the detection efficiency more sensitive compared with each of the individual ELISA methods. This conclusion is in line with our previous results of detecting p53 Abs in other types of cancer patients [19, 29, 30]. The results between p53 Abs and other clinical parameters revealed that serum p53 Abs positively correlated with higher clinical stage, positive lymph nodes metastasis, which indicated the presence of serum p53 Abs could be a prognostic factor of poor clinical outcome in patients with PTC. Such a finding is in accordance with the work performed in patients with other types of cancer [14–16].

The exact mechanism leading to the presence of p53 Abs is unknown but is thought to be associated with the presence of p53 protein accumulation [9, 17]. In this study,

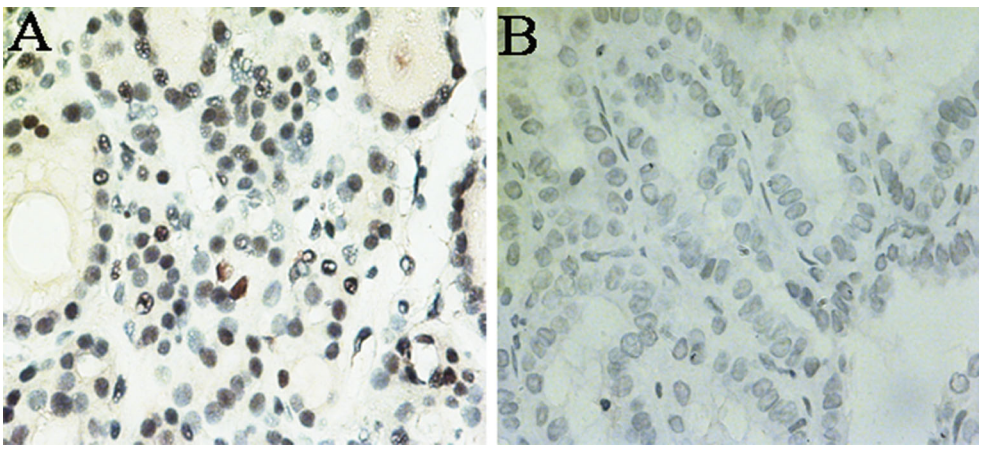

Fig. 3 Examples of p53 immunohistochemical positivity (**a**) and negativity (**b**) in PTC tissue from a p53 antibody-positive patient and a p53 antibody-negative case (400×)

the frequency of p53 protein overexpression in the p53 antibody-positive group and antibody-negative group of patients with PTC was 37.5 % (6/16) versus 7.1 % (2/28). There was a close correlation between the presence of p53 Abs and tumor p53 protein accumulation, indicating that this immune response is connected with the accumulation p53 in PTC.

We found that 84.1 % (37/44) of our PTC samples had the *BRAF* mutation, a higher proportion than that in previous reports [5, 31]. In fact, we analyzed about 100 FNA samples of PTC, and the mutation frequency of *BRAF* was also around 85 % (data not shown). Noteworthy, like p53 Abs, *BRAF* mutation predicts a poorer clinical prognosis for PTC [32, 33]. However, further analysis revealed that no correlation was found between p53 Abs and *BRAF* mutation.

In summary, this study showed that phage-SP-ELISA had a high detection efficiency in patients with PTC, and the combination of three ELISA methods could detect the most positive cases among patients with PTC. Statistical analysis revealed that serum p53 Abs is positively correlated to higher clinical stage and positive lymph nodes metastasis. Therefore, serum p53 Abs may be useful as a potential prognostic factor for PTC.

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Conflict of interest The authors declare no conflicts of interest.

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