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Serum p53 antibodies in correlation to other biological parameters of breast cancer

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Abstract

Breast cancer is the second most frequent cancer of Thai women. Mutation of *p53* is a common event in breast cancer. This alteration can result in cellular accumulation of p53 and may also found in serum p53 antibodies (p53-Abs). To clarify prognostic significance of these antibodies, we evaluated p53-Abs in 158 sera of patients with breast cancer. Thirty (19%) patients were found to have p53-Abs. The incidence of p53-Abs tended to be higher in patients with advanced disease group (stages III and IV) than patients with early disease group (stages I and II) ($P = 0.055$). Strong correlations were found between the presence of p53-Abs and p53 protein expression ($P < 0.001$) and lymph node status ($P = 0.021$). The presence of p53-Abs was associated with lack of estrogen (ER) receptor expression ($P = 0.035$) but was not related to progesterone receptor (PR) ($P = 0.567$). In addition, there was a statistically significant correlation between p53-Abs and proliferation associated antigen Ki-67 ($P = 0.006$), but no relation between c-erbB2 oncoprotein and p53-Abs was observed ($P = 0.112$). Additionally, no correlation was noted between the presence of p53-Abs and serum carcinoembryonic antigen (CEA) or carbohydrate antigen (CA15-3). Our findings indicate that p53-Abs appears to be a promising new parameter to evaluate the cellular biology and prognosis of breast cancer.
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Keywords: p53 antibodies; Breast cancer; ELISA

1. Introduction

Activation of p53 in response to cellular or genotoxic stress induces several responses, including DNA repair, senescence, differentiation, cell cycle arrest and apoptosis [1,2]. These functions are achieved, in part, by the transactivational properties of p53, which activate a series of genes involved in cell cycle regulation. Mutation in the *p53* gene are found in 50% of all human malignancies. Most of the known *p53* gene alterations are missense mutations clustered in the evolutionarily highly conserved exons 4–8 [3,4]. These mutations result in a biologically inactive p53 protein that stably accumulates in the cell nucleus and can be detected by immunohistochemistry. In the absence of wild-type p53 protein, genetic aberrations are more likely to accumulate leading to genetic instability and cell transformation.

It has been demonstrated that *p53* mutations can lead to the production of p53-Ab which can be detected in the sera of patients with various types of cancers [5]. These antibodies recognize immunodominant epitopes localized in the amino-terminus and, to a lesser extent, in the carboxy-terminus of human p53 [6–8]. Antibodies specific for the central region is always very low or absent [6]. The mechanism by which p53 is presented in the immune system is unknown. Isotyping of p53-Abs has shown that they correspond mainly to IgG1 and IgG2 subclasses [9], corresponding to a secondary immune response. The usefulness of anti-p53 serology for detection of *p53* gene alteration has been studied in several malignancies including breast cancer [10–15]. The present study was performed to evaluate the prevalence of p53-Abs in correlation to p53 accumulation, ER, PR, c-erbB2, Ki-67 protein's expression, and circulating tumor markers CEA, CA15-3, and to the conventional clinicopathological parameters.

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2. Materials and methods

2.1. Serum collection

Patients had donated a blood sample for routine clinical examination prior to any treatment and excess sera were kept frozen at −80 °C and were used for the present analysis. For each patient, age, histopathological type and staging were recorded. Staging was defined according to the international TNM classification proposed by American Joint Committee on Cancer (AJCC) [16].

2.2. ELISA

p53 protein was prepared from recombinant baculoviruses by infecting Sf9 insect cell. The harvested cells were lysed, and protein was extracted. Ninety-six-well microtiter plates were coated with 100 μl of recombinant wild-type human p53 protein or control protein for 24 h at 37 °C. Immunoplates were blocked with PBS containing 2% caseine and 0.2% Tween 20 to detect non-specific interactions. Duplicate immunoplates were then incubated with patient serum (100 μl per well) diluted 1:100 in PBS containing 5% non-fat milk at room temperature for 1 h and with anti-human IgG peroxidase conjugate human diluted 1:5000. The anti-human peroxidase activity was then visualized with 100 μl of tetramethylbenzidine solution. The reaction was stopped by adding 100 μl of 1 M sulforic acid. Plates were then read at 450 nm using a MR5000 (Dynatech Laboratories). The result was then validated by comparison of the optical density plot of this series compared to the negative control, and the cut-off point was defined as 1.6 times the negative control [9].

2.3. Immunohistochemistry (IHC)

Immunohistochemical analysis was performed on tissues using conventional peroxidase method. After undergoing dewaxing, inactivation of endogeneous peroxidase, the sections were incubated with monoclonal antibodies in case of p53, Ki-67, ER, PR, (Dako, Denmark) and polyclonal antibody for c-erbB2 (Dako, Denmark) overnight at 4 °C. Subsequently, detected with biotinylated horse anti-mouse or anti-rabbit IgG antibody and streptavidine-biotin-conjugated horse radish peroxidase (Dako, Denmark). Peroxidase activity was detected using diaminobenzidine tetrachloride. For p53, ER, PR and Ki-67 nuclear staining of invasive tumor cells was scored as positive. For c-erbB2 membranous staining of invasive tumor cells was scored as positive. The threshold for p53 was 5%, for ER and PR was 10% and Ki-67 was 13%. ELISA and IHC were performed independently by two of the authors (SS and AK, respectively).

2.4. Assay for CEA and CA15-3

For the analysis of CEA and CA15-3, we used commercially available kits (Roche, Mannheim, Germany). The assay was performed according to the manufacturer's recommendations. The cut-off values were taken from the manufacturer's data, 5 ng/ml for CEA and 30.8 U/ml for CA15-3.

2.5. Statistical analysis

Data are presented as percentages or mean as appropriate. Statistical analysis of the data was performed using Microstat software. A χ^2-test was performed to determine the association between the presence of p53-Abs and clinicopathological features of the patients. Statistical significance was assessed at 5% level.

3. Results

3.1. Relationship between the presence of p53-Abs and clinicopathologic features

Of the 158 sera assayed from breast cancer patients (116 for preoperative evaluation and 42 for pre-chemotherapy investigation), 30 (19%) were positive for circulating p53-Abs including 3 early breast cancer (Table 1). Table 2 shows the relationship between the presence of p53-Abs and various clinical/pathologic characteristics. The presence of p53-Abs was independent of patient age. There was a difference in the incidence of p53-Abs between the early disease group (stages I and II) (14.3%) and the advanced disease group (stage IV) (26.7%), although this difference did not reach statistical significance ($P = 0.055$). A statistically significant relationship was noted between the presence of p53-Abs and local-regional lymph node involvement ($P = 0.021$). All tumors were invasive ductal carcinoma histological subtype. All patients were sero negative for HIV and Hepatitis B (HBs).

3.2. Relationship between the presence of p53-Abs and nuclear accumulation of p53 protein

A total of 28 (43.8%) of the 64 tumor assayed for nuclear accumulation of p53 were IHC positive (Table 3). Twenty tumors (71.4%) of the p53-Abs positive patients with available tissue had been immunohistochemically stained for cellular p53 accumulation (overexpression). Six tumors (16.7%) of p53-Abs positive patients with available tumor tissue had IHC negative. A highly significant association was found between p53 protein accumulation in tumors and the presence of p53-Abs ($P < 0.001$).

3.3. Relationship between the presence of p53-Abs and other biomarkers

Association analysis between hormone receptor status revealed a negative association between p53-Abs and estrogen receptor (ER) ($P = 0.035$) (Table 4), however, p53-Abs

Table 1
p53 antibodies in 30 breast cancer patients

No.	Age (years)	Stage	p53 protein	ER	RR	c-erbB2	Ki-67	CEA (ng/ml)	CA15-3 (U/ml)
1	40	IIA	+	−	−	−	ND	1.5	8
2	39	IIIA	+	−	−	+	ND	2	9
3	49	IIIA	+	−	−	−	+	4.3	18.3
4	38	I	−	+	+	+	−	1.8	15
5	62	IIA	+	−	+	+	+	6.3	20.3
6	57	IIA	ND	ND	ND	+	ND	2	10
7	43	IIA	−	−	−	−	−	2.7	18
8	29	I	−	+	+	+	−	2.4	18.6
9	71	IIIA	+	+	−	+	+	3.4	13
10	51	IIIA	+	−	−	+	+	2	17
11	45	IIIA	+	−	−	−	+	1	23
12	52	IIIB	+	−	+	+	+	1.8	21
13	51	IIA	+	−	+	ND	ND	2	10
14	39	IIA	−	+	+	+	+	2.9	15
15	55	I	+	−	−	−	−	3.1	18
16	50	IIIA	+	−	−	−	−	1.7	122
17	38	IIB	+	−	−	−	+	3.4	15
18	51	IIIA	ND	−	+	ND	ND	2.3	26
19	45	IV	+	−	−	−	−	1.5	10
20	43	IIIA	+	−	−	−	+	2	7.8
21	49	IIIA	ND	ND	ND	ND	ND	2.5	14
22	43	IIB	+	−	−	+	+	1.6	18
23	72	IIIB	−	+	+	−	+	3.7	10
24	32	IIIA	+	+	+	+	+	1.4	128
25	51	IIIA	+	−	−	+	+	3.9	27
26	37	IIB	+	−	−	−	+	1.8	19
27	41	IIA	−	−	−	+	−	1	23
28	46	IV	+	−	−	+	−	2	21
29	73	IIA	+	−	−	−	+	2.6	13
30	39	IIIA	ND	ND	ND	ND	ND	3.2	31

ND: not determined.

Table 2
Relationship between prevalence of p53-Abs with various clinicopathological features

Feature	No. of cases examined	p53-Abs (%)	*P*-value
Age (years)			
≤50	96	19 (19.8)	0.749
>50	62	11 (17.7)	
Stage			
I and II	98	14 (14.3)	0.055
III and IV	60	16 (26.7)	
Lymph node			
N+	86	22 (25.6)	0.021
N−	72	8 (11.1)	
Total	158	30 (19)	

Table 3
Relationship between the presence of p53-Abs and nuclear accumulation of p53 protein

Nuclear p53 staining	p53-Abs		*P*-value
	−	+	
p53+	8	20 (71.4)	<0.001
p53−	30	6 (16.7)	<0.001

Values in parentheses are percentages.

Table 4
Relationship between the presence of p53-Abs and other biomarkers

Various markers	p53-Abs		*P*-value
	−	+	
ER+	26	6 (18.8)	0.035
ER−	30	21 (41.2)	
PR+	11	9 (45)	0.567
PR−	30	18 (37.5)	
Ki-67+	12	15 (55.5)	0.006
Ki-67−	28	8 (22.2)	
c-erbB2+	27	14 (34.1)	0.0112
c-erbB2−	10	12 (54.5)	
CEA (mean ± S.D.; ng/ml)	2.3 ± 0.9 ($n = 45$)	2.5 ± 1.1 ($n = 30$)	0.668
CA15-3 (mean ± S.D.; U/ml)	21.4 ± 5.3 ($n = 30$)	20.6 ± 21.1 ($n = 30$)	0.470

Values in parentheses are percentages.

was not related to progesterone receptor (PR) expression ($P = 0.567$). The presence of p53-Abs was positively correlated with proliferation marker, Ki-67 ($P = 0.006$). No relationship was observed between p53-Abs and c-erbB2 oncoprotein expression ($P = 0.112$). Concerning the circulating tumor markers, there was no correlation between serum p53-Abs and CEA ($P = 0.668$) or CA15-3 ($P = 0.470$) statuses.

4. Discussion

p53-Abs were originally described in 1982, by Crawford et al. [10] in the serum of 9% of breast cancer patients using a Western blotting method. Using ELISA, more than 15 studies have been performed recently in breast cancer [5]. The frequency of p53-Abs in breast cancer range from 15 to 20% but the majority of these studies were performed either in Europe or in US. No study have been performed in Thailand where the frequency of breast cancer is lower than in other countries. In the present study, we detected p53-Abs in 30 (19%) of 158 sera patients with breast cancer. The presence of p53-Abs is strongly associated to the group of tumors with p53 protein accumulation ($P < 0.001$) indicating that this immune response is triggered by the accumulation of p53 in the tumor as previously described in lung cancer [17]. The relationship between *p53* mutation, p53 accumulation in the tumor and p53 antibodies in the sera have been analyzed in several multifactorial analysis. It is now clear that all *p53* mutations will not lead to p53 accumulation as about 15% of *p53* mutation are frameshift or nonsense mutations that will not lead to the synthesis of a stable protein [4]. On the other hand, the presence of p53 antibodies is almost invariably associated with *p53* mutation in the tumor [18,19]. Patients with more advanced disease (stages III and IV) (26.7%) had a higher incidence of p53-Abs than in early disease (stages I and II) (14.3%), although the difference was not statistically significant ($P = 0.055$). We found that p53-Abs was associated with a lack of ER expression but was not related to PR suggesting that tumors eliciting antibody responses defined a subgroup with poor prognosis as previously described [20].

Few studies have analyzed the prognostic significance of coexpression of biomarkers. The observed association may further contribute to understanding of the biology of breast tumors. In breast cancer, the overexpression of p53 protein have been shown to be associated with rapid tumor cell proliferation [21] and overexpression of c-erbB2 [22]. Our result shows a good correlation between the presence of p53-Abs and Ki-67 expression, however, we have not found the relationship between c-erbB2 expression and p53-Abs. It should be noted that there was a difference between the frequency of c-erbB2 expression observed in this study (65%) and in the reports of Western authors (14–34%) [23,24]. The high prevalence of c-erbB2 expression (66%) was also reported in breast cancer in young Kuwaiti women [25]. Several research groups including our own found that there was no relationship between p53-Abs and CEA or CA15-3 statuses [26]. In addition, Takeda et al. [27] reported that the presence of p53-Abs was more significantly associated with stages 0, I and II colorectal cancer than was CEA.

Numerous studies have attempted to evaluate the clinical value of p53-Abs. Lubin et al. [28] showed that p53-Abs have been detected in two heavy smokers several years before clinical diagnosis of lung cancer. Similarly, Triver et al. [29] reported the presence of p53-Abs prior to a diagnosis for breast, lung, and prostate cancer. This finding suggested that p53-Abs may facilitate the early diagnosis of cancer. In addition, p53-Abs can be used to monitor patients during treatment. Zalcman et al. [30] showed that there was a good correlation between the specific evolution of the p53-Abs titer and the response to chemotherapy in patients with lung cancer. This had raised the possibility that p53-Abs could be a good candidate biomarker for several cancers.

p53-Abs are found predominantly in human cancer patients with specificity of 96%, but the sensitivity of such detection is only 30%. Because p53 was not detected in sera from patients with non-malignant diseases (0.5–1%) [5], the authors nevertheless suggested that serological testing for p53-Abs, despite its low positive results, can be regarded as a specific method to identify subgroups of patients with cancer. The development of circulating serum antibodies against oncogene and tumor suppressor gene product represents an interesting model system for studying immune response in cancer patients. The simple and rapid ELISA procedure suggested the potential usefulness of p53-Abs in clinical implications. However, further investigations in larger prospective homogeneous series of patients are necessary before definitive conclusion.

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