Advances in Brief

p53 Mutation in Plasma DNA and Its Prognostic Value in Breast Cancer Patients[1]

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Abstract

***Purpose*: Tumor-specific DNA has recently been detected in the plasma of lung, head and neck, breast, and colon cancer patients. Detection of tumor-specific genetic materials in cancer patients at sites distant from the tumor, such as in the blood, may provide a unique and valuable tumor marker for diagnosis and prognosis.**

***Experimental Design*: The present investigation was aimed at determining the presence of p53 mutations in the peripheral blood of breast cancer patients and its prognostic value in these patients.**

***Results*: In this study, we found that the mean concentration of plasma DNA in healthy women was 21 ng/ml whereas in patients with breast cancer the mean concentration was 211 ng/ml ($P < 0.01$). p53 mutations were detected in the primary tumors of 46 of 126 (36.5%) breast cancer patients. Of these 46 patients, 30 (65.1%) were found to have p53 mutations in their plasma DNA. p53 mutations in plasma DNA were strongly correlated with clinical stage, tumor size, lymph node (LN) metastasis, and estrogen receptor status ($P < 0.05$). After a median follow-up of 29 months, univariate and multivariate analysis revealed that both primary tumor and plasma DNA p53 mutations were significant prognostic factors for both relapse-free and overall survival. Furthermore, we demonstrated that patients with both primary tumor and plasma p53 mutations have the worst survival. This outcome occurs in both LN-positive and LN-negative groups. Thirteen of the 22 (59%) patients with recurrence and/or metastasis later had detectable p53 mutations in their plasma DNA.**

***Conclusions*: Detection of p53 mutations in plasma DNA may be used as a prognostic factor and an early marker to indicate recurrence or distant metastasis.**

Introduction

Recent advances in tumor genetics have revealed that malignant transformation follows an accumulation of multiple genetic alterations, including inactivation of tumor suppressor genes and/or activation of proto-oncogenes (1). Several genetic changes, such as the activation of the *ras* oncogene and the inactivation of the *p53* gene, are involved in the pathogenesis of breast cancer (2). Assays based on the molecular detection of these genetic changes have been shown as potential diagnostic and prognostic tools for breast cancer (3, 4). The identification of these genetic changes at sites away from the primary tumor may help to assess the extent of disease and overall tumor burden at initial diagnosis as well as during follow-up care.

There is evidence that naked DNA is released, enriched, and remains stable in the blood of cancer patients (5, 6). Recently, tumor-specific DNA has been detected in the plasma of lung, head and neck, breast, and colon cancer patients (7–10). This suggests that cell-free plasma is a source for detecting cancer-specific DNA markers. In the past, tumor-associated markers such as proteins or glycoproteins have been used for diagnosis of disease progression in patients. However, the specificity of these assays is limited because the majority of these markers are not tumor specific and are found in normal cells. To date, tumor-specific genetic markers have been assessed primarily in tumor biopsies. The detection of tumor-specific genetic alterations in cancer patients at sites distant from the tumor, such as in the blood, provides a unique and valuable tumor marker for diagnosis and prognosis.

Breast cancer is associated with different types of molecular genetic aberrations such as somatic mutations of oncogenes and tumor suppressor genes (11–15). The determination of these abnormalities can be used as a specific tool in histological diagnosis and can possibly be used as a tumor marker. As observed in other tumors, the specific molecular alterations shown by the primary breast carcinoma may also be found in the plasma DNA of breast cancer patients. p53 mutations are found in 50–75% of breast carcinoma patients (15). This high incidence of mutations suggests that the *p53* gene may constitute a useful tumor marker (16). The present investigation was aimed at determining the presence of p53 mutations in the peripheral blood of breast cancer patients and its prognostic value in these patients.

Materials and Methods

Sample Collection and DNA Preparation. This study was performed in the Cancer Hospital of the Shanghai Medical University. Between March 1997 and December 1999, 126 patients with histologically confirmed local breast carcinoma

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Table 1 Biochemical and clinical characteristics of breast cancer patients

No. of patients	126
Age: range (median)	27–71 (47)
Menopausal status	
Premenopausal	82 (65.0%)
Postmenopausal	44 (35.0%)
Tumor size	
<2 cm	76 (60.3%)
>2 cm	50 (39.7%)
LN status	
LN −	70 (55.5%)
LN +	56 (44.5%)
ER status	
ER +	65 (51.5%)
ER −	48 (46.0%)
Unknown	3 (2.4%)

Table 2 Clinical characteristics and the concentration of plasma DNA in healthy women and breast cancer patients

	Breast cancer patients	Healthy women
No.	126	92
Age: range (median)	27–71 (47)	29–69 (51)
Menopausal status		
Premenopausal	82 (65%)	60 (66%)
Postmenopausal	44 (35%)	32 (34%)
Plasma DNA concentration (ng/ml)		
Range	21–329	0–52
Mean	211	21

Table 3 Relationship between the concentration of plasma DNA and clinical stage, LN status, and tumor size

	No.	Plasma DNA (ng/ml)	*P*
Stage			
I	7	26 ± 5	
II	50	181 ± 29	<0.05
III	69	254 ± 32	<0.05
LN status			
−	70	86 ± 19	
+	56	248 ± 28	<0.05
Tumor size			
<2 cm	76	76 ± 15	
>2 cm	50	232 ± 26	<0.05

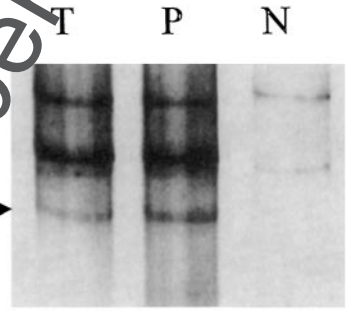

Fig. 1 SSCP photograph of exon 7 of the *p53* gene in one case of breast cancer. Normal tissue (*N*), tumor (*T*), and plasma (*P*) DNA were examined in same patient.

were included in the present study. The mean patient age was 58 years, and the clinical data are shown in Table 1. Peripheral venous blood samples (10 ml) were obtained by means of a standard venipuncture technique, using heparinized tubes. In patients with breast cancer, the blood was collected before and 1 week after surgery. During the course of this study, patients were retested when they developed recurrence or distant metastasis. The protocol of this study was approved by the human research committee of the Cancer Hospital, and informed consent was obtained from each patient. All patients were followed to determine their clinical outcome.

Tumor specimens were obtained at surgery and frozen immediately in liquid nitrogen for DNA extraction and assay of ERs.[3] Routine fixation in formalin and paraffin was done for histological assessment. Histological types were determined according to the WHO criteria (17). DNA extraction from tumor and tissue samples was performed by a nonorganic method (Oncor, Gaithersburg, MD). Plasma DNA was purified on Qiagen columns (QiampBlood Kit; Qiagen, Hilden, Germany) according to the manufacturer's protocol for blood and body fluids. Ten ml of plasma were heated at 99°C for 5 min on a heat block. The heated sample was then centrifuged at 14,000 rpm for 30 min, after which the clear supernatant (about 1 ml) was collected. Proteinase K (20 mg/ml) and buffer AL (Qiagen) were added in a 1:10 ratio with respect to the collected supernatant and incubated overnight at 55°C. One column was used repeatedly until the whole sample had been processed. The DNA extracted was quantified spectrophotometrically.

Mutational Study of the *p53* Gene. To establish the presence of point mutations in the conserved exons of TP53, PCR-SSCP analysis was performed according to a modification of the method reported previously (3, 18). We amplified exons 5, 6, 7, and 8 of the *p53* gene, and the primers used were as follows: (a) exon 5, 5′-TCCTTCCTCTTCCTACAG and 5′-ACCCTGGGCAACCAGCC CTGT; (*b*) exon 6, 5′-ACAGGGCTGGTTGCCCAGGGT and 5′-AGTTGCAAACCAGACCTCAGGCG; (*c*) exon 7, 5′-TCCTAGGTTGGCTCTGACTGT and 5′-AGTGGCCCTGACCTGGAGTCT; and (*d*) exon 8, 5′-GGGACAGGTAGGACCAGACCTGATTTCCTT and 5′-ATCTGAAGGCATAACT GCACCCTTGG. The annealing temperatures were 65°C, 67°C, 62°C, and 68°C, respectively. PCR was performed under standard conditions in 25 μl containing 100 ng of DNA template (tumor, normal, or plasma DNA), 2.5 μl of 10× PCR buffer, 0.75 unit of AmpliTaq Gold (Perkin-Elmer, Roche Molecular System Inc., Branchburg, NJ), 200 μM deoxynucleotide triphosphate mixture; 0.6 μM each primer, and different concentrations of magnesium chloride. For PCR amplification, the samples underwent 40 cycles at 94°C for 1 min (subjected to different annealing temperatures, depending on the primer) and

[3] The abbreviations used are: ER, estrogen receptor; LN, lymph node; SSCP, single-strand conformational polymorphism; CEA, carcinoembryonic antigen; CA-15-3, cancer-associated antigen 15-3; RFS, relapse-free survival.

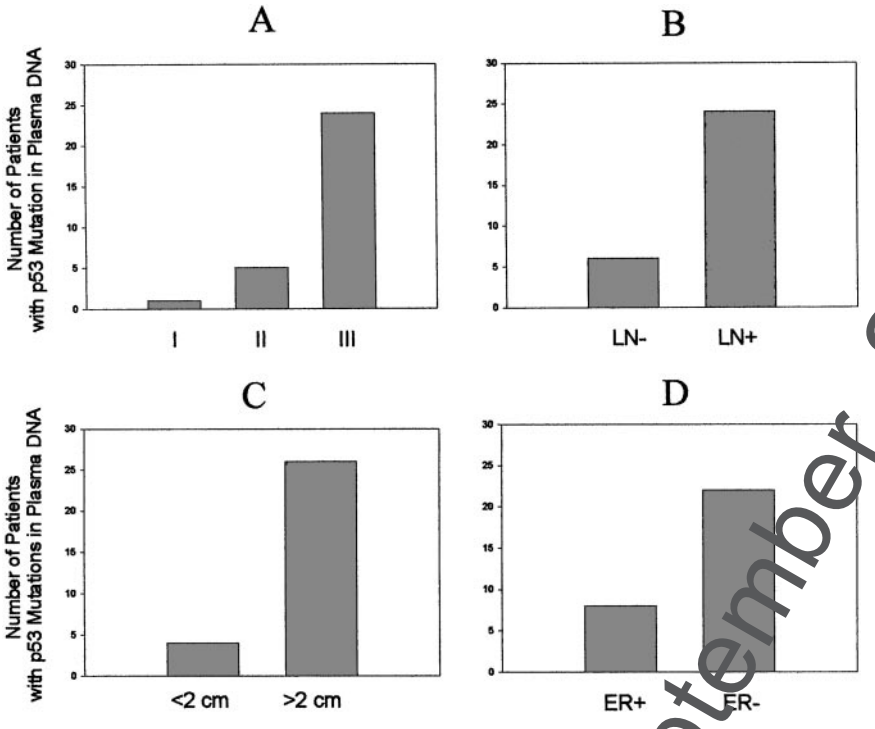

Fig. 2 Relationship between the p53 mutation of plasma DNA and clinical stage (*A*), LN status (*B*), tumor size (*C*), and ER status (*D*).

70°C for 1 min. The amplified products were denatured by mixing with 15 μl of denaturing stop solution containing 98% formamide, 10 μM edathamil (pH 8.0), 0.02% xylene cyanol, and 0.02% bromphenol blue; heated to 95°C for 5 min; and cooled rapidly on ice. Electrophoresis was run on nondenaturing 8–12% polyacrylamide gels for 12–15 h at 250 V. The allelic band intensity on the gels was detected by a nonradioisotopic method using a commercially available silver staining method (19). The specimens that showed a differential band at SSCP analysis were amplified to obtain templates for DNA sequencing. These amplifications were independent from those used for SSCP analysis. Amplified DNA fragments were purified from 0.9% agarose gels using the Geneclean Kit (Bio-101, Inc., La Jolla, CA) and used for direct DNA sequencing by the deoxynucleotide triphosphate method with the Sequenase Kit (United States Biochemical Corp., Cleveland, OH).

ER Assays. The standard dextran-coated-charcoal assay was used as described previously (20). In all cases, the Scatchard plot analysis was done with eight points, and the protein content in the reaction was 1 mg/ml. Receptor levels of 10 fmol/mg protein or greater were considered positive. ER biochemical measurements were confirmed by ER immunohistochemistry.

CEA and CA-15-3 Assays. Both CEA and CA-15-3 were determined by a two-site ELISA (Oncogene Research, Cambridge, MA). The cutoff limits of 10 ng/ml (CEA) and 30 units/ml (CA-15-3) were taken as recommended by the manufacturers of the assay (21).

Statistical Analysis. Comparisons of the differences among the p53 mutations of plasma and primary tumor DNA *versus* wild-type p53 of plasma and primary tumor DNA were made using the one-tailed Student's *t* test. The Kaplan-Meier method was used to calculate RFS and overall survival curves. The log-rank test was used to assess the univariate effect of p53 mutations in plasma DNA and the effect of other variables on the RFS and overall survival. Cox's proportional hazards model was used to conduct a multivariate analysis of prognostic factors and assess differences in overall survival and RFS after adjustment for other covariates.

Patient Follow-up. All patients were followed after surgical treatment. Physical examination was performed every 3 months in all patients for the first 2 years and then performed twice per year. Mammography, radiographic studies (chest X-ray), and liver ultrasounds were performed every 12 months; bone scan and computed tomography scan were performed as clinically indicated. Blood tests, including electrolyte and liver function profiles and complete blood cell counts, were repeated at every follow-up visit. RFS was calculated as the period from surgery until the date of the first recurrence.

Results

The Quantitation of Plasma DNA in Breast Cancer Patients and Healthy Women. Plasma DNA has been detected both in healthy individuals and in individuals with cancer. We measured the concentration of plasma DNA in 126 breast cancer patients and 92 healthy women. As shown in Table 2, we found that the mean concentration of plasma DNA in healthy women was 21 ± 20 ng/ml, whereas in patients with breast cancer, the mean concentration was 211 ± 18 ng/ml ($P < 0.01$). The concentration of plasma DNA in breast cancer patients correlated with the following clinicopathological parameters: (*a*) clinical stage ($P = 0.0021$); (*b*) involvement of LN metastasis ($P = 0.005$); and (*c*) tumor size ($P = 0.04$; Table 3).

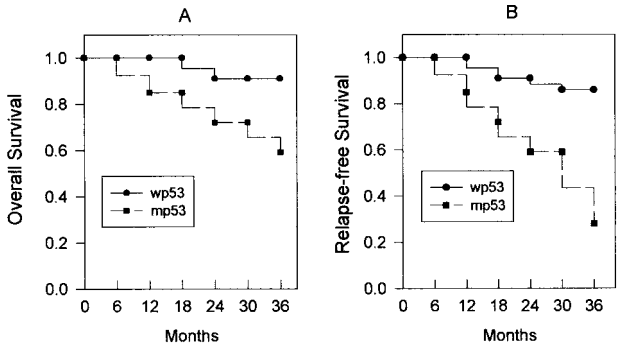

Fig. 3 Overall survival and disease-free survival curves in breast cancer patients with mutant p53 (*mp53*) and wild-type p53 (*wp53*) in plasma DNA.

Table 4 Univariate analysis for prognostic factors for breast cancer patients

Factors	*P*	
	RFS	Overall survival
Age	0.134	0.233
Menopausal status	0.343	0.486
Tumor size	0.079	0.112
LN status	0.003	0.002
ER status	0.049	0.046
p53 mutations in plasma DNA	0.012	0.021
p53 mutations in primary tumor	0.032	0.042

Mutation Status of the *p53* Gene. p53 mutations were detected in the primary tumors of 46 of 126 (36.5%) breast cancer patients (Fig. 1). Of these 46 patients, 30 (65.1%) were found to have p53 mutations in plasma DNA. We found that the status of p53 mutations in plasma DNA was strongly correlated with clinical stage, LN metastasis, tumor size, and ER status ($P < 0.05$; Fig. 2).

Prognostic Significance of p53 Mutations in Plasma DNA. After a median follow-up of 29 months (range, 4–39 months), clinical data revealed recurrence or distant metastasis in 16 of 126 (13.3%) patients. An additional six patients (4.7%) developed distant metastasis and died. Patients with preoperative plasma p53 mutations exhibited a shorter survival than did those with the wild-type gene (Fig. 3). This outcome was observed in both the LN-positive and LN-negative groups. Univariate analysis of tumor characteristics and patient outcome revealed that primary and plasma DNA p53 mutations, LN status, and ER were significant prognostic factors for both RFS and overall survival (Table 4). Multivariate analysis revealed that LN and ER status were independent predictors of overall survival time. Both primary and plasma DNA p53 mutations independently predicted RFS and overall survival (Table 5).

p53 Mutations of Plasma DNA, CEA, and CA-15-3 in Breast Cancer Patients with Recurrence or Distant Metastasis. All breast cancer patients again underwent analysis of p53 mutations in their plasma DNA at 1 week after surgery. We did not detect p53 mutations in plasma DNA in 29 of 30 patients who previously had p53 mutations in plasma DNA. The one case who had p53 mutations in plasma DNA after surgery had early liver metastasis at 5 months after surgery. Thirteen of the 22 (59.0%) patients with recurrence and/or metastasis later had detectable p53 mutations in their plasma DNA (Table 6). In these 22 patients, we also measured the levels of CEA and CA-15-3. As shown in Table 6, only five patients (22.7%) had high levels of CEA, and six patients (27.2%) had elevated levels of CA 15-3.

Table 5 Multivariate analysis with Cox's proportional hazards model for prognostic factors for breast cancer

Factors	*P*	
	RFS	Overall survival
Age	0.218	0.435
Menopausal status	0.430	0.413
Tumor size	0.329	0.560
LN status	0.001	0.013
ER status	0.051	0.045
p53 mutation in plasma DNA	0.003	0.004
p53 mutation in primary tumor	0.048	0.031

Discussion

The development of molecular markers is needed to improve the diagnosis and assessment of tumor progression in breast cancer patients. The results of this study present evidence for the presence of plasma DNA with features of malignant genetic transformation. In the early 1970s, Leon *et al.* (10) reported that free DNA was present at concentrations ranging between 0 and 2 μg/ml in the serum of breast cancer patients and that it was possible to analyze the variation in the amount depending on the stage of disease and the response to treatment received by the patients. Our results also demonstrate that the concentration of plasma DNA is much higher in breast cancer patients in comparison with healthy women. In breast cancer patients, the level of plasma DNA also correlates with stage, LN metastasis, and tumor size.

Recently, tumor-specific DNA has been detected in the plasma of a number of cancer patients, including breast cancer patients (7–10), which suggests that cell-free plasma is a useful source for detecting cancer-specific DNA markers. In the past, tumor-associated markers such as proteins/glycoproteins have been used for diagnosis or prognosis of progression in patients. However, the specificity of these assays is limited because the majority of these markers are not tumor specific and are found in normal cells. To date, tumor-specific genetic markers have been assessed primarily in pathological specimens. However, surgery is not always performed on patients with advanced-stage disease, which limits the availability of tumor tissue for genetic assessment. The detection of tumor-specific genetic alterations in the blood provides a unique and valuable tumor marker for diagnosis and prognosis.

Previous studies have identified several tumor-specific gene alterations in the plasma DNA of cancer patients. Mutant K-ras and p53 DNA have been detected in the plasma of patients with colorectal, pancreatic, and hematological neoplasms (22). Several studies have shown that the presence of tumor DNA in the plasma correlates with disease stage (14–15, 18, 22).

Table 6 The status of *p53* mutations in the plasma DNA of 22 patients with recurrence or metastasis

Patient no.	Stage	Preoperative *p53* mutations in plasma DNA	Follow-up			
			p53 mutations	High CEA	High CA15-3	Follow-up status
1	II	Yes	Yes	Yes	Yes	Metastasis, then death
2	II	Yes	Yes	No	Yes	Metastasis, then death
3	III	Yes	Yes	No	No	Metastasis, then death
4	III	Yes	Yes	Yes	No	Metastasis, then death
5	II	Yes	No	No	No	Metastasis, then death
6	III	No	No	No	No	Metastasis, then death
7	I	Yes	Yes	No	No	Metastasis
8	III	Yes	Yes	No	No	Metastasis
9	II	Yes	Yes	Yes	Yes	Metastasis
10	II	Yes	No	No	No	Metastasis
11	III	No	No	No	Yes	Metastasis
12	II	Yes	Yes	No	No	Metastasis
13	II	No	No	Yes	No	Recurrence
14	III	Yes	Yes	No	Yes	Recurrence
15	III	Yes	Yes	No	No	Recurrence
16	II	No	No	No	No	Recurrence
17	III	Yes	Yes	No	No	Recurrence
18	II	No	No	Yes	No	Recurrence
19	III	Yes	No	No	No	Recurrence
20	III	Yes	Yes	No	No	Recurrence
21	II	No	No	No	No	Recurrence
22	III	Yes	Yes	No	Yes	Recurrence

Nawroz *et al.* (8) have reported a large number of patients with advanced head and neck cancer who have detectable p53 mutations in plasma DNA. Recently, Silva *et al.* (2, 3) have reported that there is a significant difference between breast cancer patients with or without p53 mutations in plasma DNA with regard to LN involvement, proliferating index, and tumor cell growth. The results of the present study confirm that it is possible to identify p53 mutations in DNA extracted from the plasma of patients with breast cancer. Furthermore, we demonstrate that the status of p53 mutations in plasma DNA correlates strongly with clinical stage, tumor size, LN metastasis, and ER status ($P < 0.05$). Our study also shows that patients with plasma p53 mutations have a smaller probability of survival than those with the wild-type *p53* gene. Furthermore, the results of our study indicate that the presence of p53 mutations in both the plasma DNA and the primary tumor in breast cancer patients is significantly associated with highly malignant lesions and shorter survival.

The identification of recurrent breast cancer at a preclinical stage is an essential strategy in the fight against this disease, but no reliable serological predictive markers are currently available. Considering the prevalence of this disease, the discovery of a valid marker could have a strong impact on its management and probably also on survival. The status of p53 mutations in plasma DNA could be used as a marker of cancer recurrence or metastasis. The present study clearly shows that >97% of patients have undetectable p53 mutations in plasma DNA after complete excision of their primary tumors. However, of 22 patients with recurrence or metastasis, 13 who previously had p53 mutations in plasma DNA before surgery again develop detectable p53 mutations in plasma DNA. This would strongly suggest that the detection of p53 mutation in plasma DNA after primary treatment could be used as a marker of recurrence or distant metastasis.

CEA and CA-15-3 are common tumor markers used in breast cancer. CEA is a normal cell glycoprotein overexpressed by several adenocarcinomas, and CA-15-3 is a mucin-like membrane glycoprotein released by the tumor into the bloodstream (21). CEA and CA-15-3 are often used in the detection of recurrence and metastasis of breast cancer (23–25). In the present study, we find that only 5 of the 22 (22.7%) cases of recurrence and/or metastasis show high levels of CEA, and 6 (27.2%) of them show high levels of CA-15-3. On the other hand, p53 mutations in plasma were found in 13 of 22 (59%) of these patients.

In conclusion, our results indicate that in breast cancer, p53 mutations in plasma DNA are correlated strongly with clinical stage and are predictive of patient survival. Most importantly, detection of p53 mutations in plasma DNA after primary treatment may be used as an early marker to indicate recurrence or distant metastasis.

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