

Tumor DNA in Plasma at Diagnosis of Breast Cancer Patients Is a Valuable Predictor of Disease-free Survival¹

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ABSTRACT

Purpose: We examine prospectively whether the presence of plasma DNA with tumor characteristics before mastectomy is a predictive factor related to recurrence and disease-free survival (DFS).

Experimental Design: A series of 147 patients with breast carcinomas, selected sequentially, was analyzed. The characterization of plasma DNA, based on similar alterations in tumor and plasma DNA, was achieved with six polymorphic markers (D17S855, D17S654, D16S421, TH₂, D10S197, and D9S161) and mutations in the TP53 gene. Recurrence, DFS, overall survival, and 12 other clinicopathological parameters were obtained. Univariate and Cox's multivariate studies were performed.

Results: A total of 142 patients were eligible for study. A total of 104 tumors (73.2%) showed at least one molecular alteration. In 61 patients (42.9%), a similar molecular alteration was detected in plasma DNA and tumor DNA. No alterations were found in the plasma DNA of the remaining 81 patients (57%). During the follow-up period (median, 22 months; range, 1–46 months), we observed 23 recurrences (16%), the distribution of which was significantly different ($P = 0.005$) with regard to plasma DNA [17 patients (74%) with circulating tumor DNA and 6 patients (26%) without tumor plasma DNA]. Univariate statistical analysis confirmed the prognostic significance of the already known parameters (tumor size, lymph node metastases, and stage) and demonstrated that tumor plasma DNA was a predictor

of DFS. In multivariate analysis, an independent borderline significance was observed for tumor plasma DNA.

Conclusions: Tumor DNA in plasma at diagnosis in breast cancer patients can predict DFS, and its determination could be used as a prognostic factor in these patients.

INTRODUCTION

Breast cancer is the most common malignancy in women, mainly in Western countries, accounting for about 30% of all female cancers, and it is the second leading cause of death by cancer (1). The ratio of estimated incidence to mortality is close to 4:1 (1), and this decrease in the mortality rate can be attributed both to the success of early detection and to the improvement in treatment, particularly in systemic-specific therapies. As result of these strategies, patients with breast cancer can survive longer. These patients require an appropriate and optimal follow-up, mainly during the first 3 years, a period marked by a large number of both locoregional and distant recurrences (2). The surveillance methods used to detect asymptomatic recurrence are generally the same as those used at initial diagnosis, and they are based on physical examination, X-rays, ultrasounds, radionuclide scans, and hematological and biochemical profiles (3). However, the use of the serological markers available at present, such as CA 15.3, carcinoembryonic antigen, and so forth, has not improved the rate of recurrences detected by classical clinical methods (4). Therefore, a DNA marker that could be used to identify high-risk patients or to predict a relapse in a presymptomatic phase could have a great impact on the management of patients and probably on survival.

Malignant cell transformation is associated with numerous genetic changes that are hallmarks of cancer growth and progression. Regardless of whether these alterations play a role in malignant transformation or whether they are a secondary expression of tumor cells, their analysis and correlation studies can provide useful data in both research studies and clinical trials (5–8). Several molecular genetic alterations including LOH³ and MI (9–11) and somatic mutations in oncogenes (12) and tumor suppressor genes (13, 14) have been described in breast adenocarcinomas.

Some of these alterations, which are identical to those found in primary tumor DNA, have been observed in extracellular circulating DNA in blood samples of breast cancer patients. These include alterations in microsatellites, LOH or MI (15–18) detected by polymorphic markers, TP53 gene mutations (19), and aberrant methylation of the promoter gene of *INK4a* (20). In an attempt to evaluate whether circulating DNA with characteristics of tumor DNA in cancer

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³ The abbreviations used are: LOH, loss of heterozygosity; MI, microsatellite instability; CI, confidence interval; DFS, disease-free survival; NSCLC, non-small cell lung cancer; RR, relative risk.

patients has prognostic value, several studies have reported a direct correlation with poor survival in pancreatic carcinoma (21), esophageal cancer (22), NSCLC (23), melanoma (24), head and neck carcinomas (25), and B-cell neoplasias (26). As observed in these malignancies, breast cancer patients with circulating tumor DNA may have a different outcome than those patients who do not have this molecular change. To test this hypothesis, on the basis of our previous report (15) demonstrating tumor plasma DNA at diagnosis, we studied a series of breast cancer patients to analyze their early relapses and corresponding DFS in relation to extracellular circulating DNA with features of tumor DNA.

PATIENTS AND METHODS

Patients, Tissue Sampling, DNA Extraction, and Clinicopathological Parameters

A prospective study started in October 1997 allowed us to identify and recruit patients with breast cancer. By July 2001, we had recruited 147 individuals. Participants were informed of the nature of the study, which was approved by the Research Ethics Board of our hospital, and they gave their informed consent. Healthy and tumor tissues were obtained sequentially, immediately after mastectomy. These samples were then snap-frozen in liquid nitrogen and stored at -80°C until processing. All specimens were examined pathologically to confirm the diagnosis of breast carcinoma. No evidence of disseminated disease was observed at diagnosis in any patient; however, in five cases, a direct extension to chest wall or skin only was observed. A peripheral venous blood sample (20 ml) was collected from each patient by venipuncture on the day of surgery (before mastectomy). Blood samples of 35 healthy controls were also obtained. DNA was extracted from healthy and tumor samples and from peripheral blood mononuclear cells (which were also used as normal DNA) by a nonorganic method (S-4520 kit; Oncor Inc., Gaithersburg, MD). Plasma DNA was purified as described previously (15). Briefly, between 7.5 and 12 ml of plasma were heated at 99°C for 5 min on a heat block. Plasma was then centrifuged at 14,000 rpm for 30 min, and the supernatant was incubated with proteinase K and buffer AL (Qiamp Blood Kit; Qiagen, Hilden, Germany) overnight at 55°C . Following the protocol for blood and body fluids (Qiamp Blood Kit; Qiagen), one column was used repeatedly until the whole sample had been processed. The DNA extracted was quantified spectrophotometrically.

The following variables were obtained from the medical records of the 142 patients: date of birth; tumor size; lymph node metastases; presence of steroid receptors (estrogen and progesterone); menstrual status; pathological stage; histological grade; proliferative index; c-erbB2 and bcl2 status; histological type; vascular invasion; systemic treatment administered; and dates of recurrence or death (when applicable) and follow-up. Pathological stage was assessed using the tumor-node-metastasis (TNM) classification. All tumors were graded according to the Bloom-Richardson system, and the steroid receptor content, the proliferative index, and c-erbB2 and bcl2 expression were determined by immunohistochemical procedures.

Clinical Follow-Up and Treatment

Prospective follow-up, starting after surgery and diagnosis, was based on a regular (every 3 months during the first year, every 6 months during the second year, and then yearly until relapse) clinical, biochemical, and radiological examination (chest X-ray, mammography, and other areas as clinically indicated), including bone scan and liver ultrasound when liver function was impaired. DFS was used as end point and was defined as the period from enrollment to first relapse, death without relapse, or last follow-up. After surgery, 44 patients (30%) underwent radiotherapy by conservative surgical treatment or locally advanced disease. All but six patients received systemic treatment (27). Seventy-five patients (53%) received hormone therapy (tamoxifen, 20 mg daily), and 61 patients (43%) received chemotherapy (100 mg/m² cyclophosphamide p.o. on days 1–14; 40 mg/m² methotrexate i.v. on days 1 and 8 and 600 mg/m² 5-fluorouracil i.v. on days 1 and 8; or 60 mg/m² Adriamycin i.v. on day 1 and 600 mg/m² cyclophosphamide i.v. on day 1).

Molecular Characterization and Microsatellite Analysis

Six microsatellite markers were used to determine LOH on the following chromosomes: (a) chromosome 17, D17S855 (28) and D17S654 (29); (b) chromosome 16, D16S421 (30); (c) chromosome 11, TH₂ (11); (d) chromosome 10, D10S197 (31); and (e) chromosome 9, D9S161 (32). These markers were chosen because they show a high rate of alterations in breast carcinomas (15). PCR was performed in 25- μl volumes using 100 ng of template DNA, 0.75 unit of Ampli Taq Gold DNA polymerase (Perkin-Elmer, Roche Molecular Systems, Inc., Branchburg, NJ), 2.5 μl of $10\times$ PCR buffer, 200 μM deoxynucleotide triphosphate, 0.6 μM of each primer, and a range of concentrations of MgCl_2 , depending on the polymorphic marker. A 40-cycle amplification was done in a thermal cycler (Perkin-Elmer, Foster City, CA) for plasma DNA, and 30 cycles were used for the rest of the samples (normal and tumor breast tissue and mononuclear blood cell DNA). Alleles were separated by mixing 25 μl of the PCR product with 10 μl of loading buffer, 0.02% xylene cyanol, and 0.02% bromphenol blue. Electrophoresis was run on nondenaturing 8–12% polyacrylamide gels for 12–15 h at 500 V. After electrophoresis, the allelic band intensity was detected by silver staining (15), measured by a GS-690 Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA), and analyzed using the Multi-Analyst/PC (Bio-Rad Laboratories). An allele was considered to be lost when its signal was reduced by $>75\%$ with respect to that observed in its normal counterpart. In this study, only LOH was considered because MI was not detected in tumor DNA.

Analysis of TP53 Mutations

Using PCR single-strand conformational polymorphism analysis, we amplified the conserved exons of the TP53 gene, exons 5, 6, 7, and 8. The primers used and their corresponding annealing temperatures were reported previously (15). PCR was performed under standard conditions in a volume of 25 μl that contained 2 μl (100 ng) of DNA template (tumor, normal, or plasma DNA); 2.5 μl of $10\times$ PCR buffer and 0.75 unit of Ampli Taq Gold (Perkin-Elmer, Roche Molecular Systems, Inc.); 200

Table 1 Spectrum of the calculated rate of DFS probability

Time (months)	DFS probability	95% CI
12	92%	87–97
24	85%	78–92
36	76%	65–87
45	39%	7.8–71

μM deoxynucleotide triphosphate mix; 0.6 μM of each primer; magnesium chloride at a range of concentrations, depending on the primer; and distilled H_2O needed to reach the total volume. For PCR amplification, the samples underwent 40 cycles of 94°C for 1 min for plasma DNA and 30 cycles for the remaining biological samples. They were then subjected to a range of annealing temperatures, depending on the primer, and finally to 70°C for 1 min. The amplified products were denatured by mixing them with 15 μl of denaturing stop solution that contained 98% formamide, 10 mM edathamil (pH 8.0), 0.02% xylene cyanol, and 0.02% bromphenol blue; heated to 95°C for 5 min; and rapidly cooled 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 silver staining method. The specimens that showed a differential band at single-strand conformational polymorphism were rechecked, and PCR products were purified using the High Pure PCR Product Purification Kit (Boehringer Mannheim). The purified DNA fragments were sequenced using an ABI Prism 377 DNA Sequencer apparatus (PE Applied Biosystem).

Statistical Analysis

The clinicopathological parameters recruited for each patient were analyzed in a descriptive statistical study. The categorical variables were contrasted by the χ^2 test with the Yates correction (33) or Fisher's exact test when any of the expected frequencies was less than 5. We considered $P \leq 0.05$ to be significant. For the survival study, DFS was the end point.

Univariate Analysis. All variables included in this study were the used for this analysis. The relationship between the cumulative probability of DFS and the predictors analyzed was calculated following the Kaplan-Meier method (34), and significant differences between curves were evaluated with Mantel's log-rank test (35).

Multivariate Analysis. To identify the factors that might be of independent significance in influencing the DFS, a Cox stepwise proportional risk regression model was fitted (36). The variables included were those used in univariate study. Adjusted hazard ratios are presented with their corresponding 95% CIs. The model's basic assumptions (proportional hazards) were evaluated. In each hypothesis contrast a type I (α) error less than 0.05 of the null hypothesis was rejected. The SPSS version 10.0 software package was used for statistical analysis.

RESULTS

We studied prospectively a consecutive series of 147 breast cancer patients to determine whether the presence of circulating plasma DNA with characteristics of tumor DNA is a factor related to disease recurrence and DFS. This study also included 35 healthy controls. The median age of the global series was 55

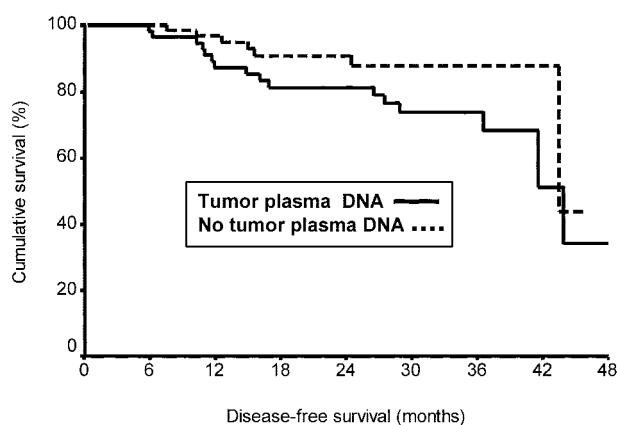


Fig. 1 Kaplan-Meier DFS curves for the 142 breast cancer patients evaluated according to the presence or absence of tumor plasma DNA at diagnosis ($P = 0.05$).

Table 2 DFS in relation to the clinicopathological characteristics analyzed

Variable	N ^a	45 months DFS	P (log-rank)
Stage			
I	29	90%	0.0001
II	88	60%	
IIIa	21	57%	
IIIb	4	0	
No. of lymph node metastases			
0	68	91%	0.05
1–3	41	32%	
>3	33	35%	
Tumor size (mm)			
0–20	44	89%	0.03
21–50	78	32%	
>50	20	61%	
Tumor plasma DNA			
Not detected	81	44%	0.05
Detected	61	34%	

^a N, number of patients.

years (range, 29–94 years), and the median age was 60 years for patients with recurrence.

No detectable levels of plasma DNA were observed in five patients, and these cases were excluded from the statistical analysis. The remaining 142 patients (97%) showed plasma DNA at concentrations ranging from 21 to 195 ng/ml (mean, 135 ng/ml). In healthy controls, plasma DNA was detected in lower concentrations (mean concentration, 15 ng/ml; range, 0–49 ng/ml) than in patients.

After screening the allelic status of six chromosomal regions and point mutations of the *TP53* gene with the molecular marker used to characterized plasma DNA as tumor DNA, we identified 104 primary breast carcinomas (73.2%) with at least one molecular alteration at the markers used, and of these, we identified 61 patients (42.9%) with at least one of these molecular alterations in plasma DNA. Fifty-nine patients (41.5%) showed LOH in at least one polymorphic marker, and 10 (7%) displayed *TP53* mutations; 2 did not show any concomitant

Table 3 Proportional hazard model for DFS (multivariate analysis)

Variable	Reference	β^a	p	RR	95% CI
Tumor plasma DNA	No tumor plasma DNA	0.97	0.08	2.6	0.84–8.2
Tumor size \geq 50 mm	Tumor size < 50 mm	1.1	0.03	3.0	1.1–8.2
Lymph node metastases	Lymph node without metastases	0.98	0.09	2.6	0.85–8.3
Stages IIIa + IIIb	Stages I + II	0.65	0.4	1.9	0.4–8.9

^a β , hazards model coefficient; RR, exponential β and RR.

LOH at any marker tested. In the remaining 81 patients with a detectable level of plasma DNA, no molecular changes were detected. These cases were considered as negative cases for the presence of tumor plasma DNA (57%). No alterations with the molecular markers used were detected in plasma DNA of healthy controls.

Patient Follow-Up, Recurrence Rate, and Distribution.

The follow-up comprised the interval between surgery and the time of the last visit or death. Median follow-up was 22 months (range, 1–46 months). During this period, 23 recurrences (16%) were detected; the most frequent were local relapse and bone metastases in 10 and 6 patients, respectively. Four patients died because of disease progression, two patients showed tumor plasma DNA, and two patients had no evidence of this alteration. On the basis of this recurrence rate, the calculated probability of DFS was 92% for the first year (95% CI, 87–97%), 85% for second year (95% CI, 78–92), 76% for the third year (95% CI, 65–87), and 39% for 45 months (95% CI, 7.8–71; Table 1; Fig. 1). Of the 23 patients with disease recurrence, 17 (74%) were positive for plasma DNA with tumor characteristics, and 6 were negative (26%). When this distribution of patients with relapse was analyzed in relation to the rest of the patients, a statistically significant difference was observed ($P = 0.005$).

Analysis of DFS Intervals. The following parameters [c-erbB2 expression, proliferation index (Ki67), bcl2 expression, tumor DNA in plasma, pathological stage, menstrual status, histology, axillary lymph node metastases, tumor size, steroid receptor content, vascular invasion, and systemic therapy] were analyzed to calculate their effect on and possible relation with the failure of DFS. After univariate study of all variables analyzed, only tumor size, stage, lymph node metastases, and tumor plasma DNA demonstrated a statistically supported relation and were able to predict DFS (Table 2). The distribution of the 136 patients who received systemic therapy displayed a statistically significant difference between hormone therapy and chemotherapy ($P = 0.03$) with regard to tumor plasma DNA, suggesting a possible association of this molecular alteration with more aggressive tumor characteristics.

Multivariate Analysis for DFS. The variables that resulted in the univariate analysis with a significant relation in DFS were considered in a multivariate Cox's regression model for DFS: the model showed an acceptable fit ($\chi^2 = 13.03$; $P = 0.005$). Patients with tumor plasma DNA had a risk ratio in DFS of 2.6 (95% CI, 0.84–8.2; $P = 0.08$) when compared with patients without this molecular factor. Patients with a tumor larger than 5 cm had a RR in DFS of 3.0 (95% CI, 1.1–8.2; $P = 0.03$) in comparison with patients with smaller tumors. Axillary lymph node affection had a RR in DFS of 2.6 (95% CI, 0.85–8.3; $P = 0.09$) in compar-

ison with patients without positive lymph node metastases. Lower stages compared with higher ones had a RR of 1.9 (95% CI, 0.4–8.9; $P = 0.4$; Table 3).

DISCUSSION

The first description about the use of microsatellite polymorphic markers to identify and characterize extracellular circulating DNA in patients with cancer was made by Chen *et al.* (37) and Nawroz *et al.* (25) in September 1996. Since then, several follow-up studies, such as the present one, have been done to establish the possible value of free plasma DNA with features of tumor DNA as a prognostic factor in patients diagnosed with a malignancy (21–24).

Isolation and quantification of total plasma DNA in cancer patients are routinely feasible. However, identification of the corresponding amount of plasma tumor DNA requires more specific methods. We think that the use of nonradioisotopic techniques may facilitate the incorporation of tumor plasma DNA analysis into the study and management of cancer patients.

In breast cancer, an earlier detection of recurrent disease can contribute to a positive outcome of the process. In this way, the routine use of current serum tumor markers deserves a caution. For example, carcinoembryonic antigen and CA 15.3 increase at presentation in approximately 20% of patients with stage I or II breast cancer, but only when abnormal values progressively increase over time are metastases probable (38). It is obvious that the identification of valid prognostic markers in a preclinical stage could have a repercussion on disease behavior and patient survival.

The significant association found between the presence of plasma DNA with features of tumor DNA and disease recurrence, as well as its capability to predict DFS in univariate study, indicates that this molecular alteration could be used as a prognostic factor. It is possible that the prognostic value of this plasma tumor DNA could depend on its origin, but our results indicate that tumors with circulating tumor DNA are more aggressive, independent of its origin. The findings of this study are consistent with other studies on pancreatic carcinomas (21), esophageal adenocarcinomas (22), NSCLC (23), and melanoma (24).

Few studies have reported the correlation of patient outcome with characterized tumor DNA in the bloodstream, probably because of follow-up requirements; some of the tumors analyzed have those of shorter survival, such as small cell lung cancer (39) and pancreatic carcinomas (21). Other studies in esophageal adenocarcinomas (22) and NSCLC (23) and the present study have used longer follow-up periods and have also demonstrated a positive correlation between poor survival and the presence of plasma

DNA, indicating that independent of the production mechanisms and the molecular markers used, the presence of plasma DNA can be considered a negative prognostic factor.

The clinical behavior of breast cancer can usually be predicted by the well-established prognostic factors tumor size, axillary lymph node involvement, and pathological stage. Histological grade and steroid receptor content are also valuable factors (40). Our study confirms the prognostic value of these factors, although histological grade and steroid receptors did not demonstrate a predictable value for DFS in our series ($P = 0.8$ and $P = 0.5$, respectively; data not shown). We think that the study of tumor plasma DNA could mean a new molecular factor with prognostic implications in breast cancer patients.

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