Presence of Tumor DNA in Plasma of Breast Cancer Patients: Clinicopathological Correlations

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ABSTRACT

Using different molecular techniques, DNA has been detected in the plasma of cancer patients with various types of tumors. We undertook the present study to investigate the presence of plasma DNA, before mastectomy, in patients with breast cancer at diagnosis and to analyze the clinicopathological spectrum of this subgroup of patients with respect to patients without DNA with tumor characteristics. We studied 62 patients with breast cancer, who were selected sequentially after mastectomy and diagnosis of breast carcinomas. Genomic DNA extracted from tumor and normal tissues, normal blood cells, and plasma was used for molecular studies. Alterations in polymorphic markers selected because they had been found to show a high rate of alterations in breast cancer in previous studies (D17S85, D17S654, D16S421, TH2, D10S197, and D9S161), as well as mutations in the p53 gene and aberrant methylation at the first exon of p16INK4a, were used to identify and characterize tumor and plasma DNA. Thirteen clinicopathological parameters were analyzed in each patient. We identified 56 cases (90%) with at least one molecular event in tumor DNA, and 41 cases (66%) with a similar alteration in plasma DNA. Comparison of the clinicopathological parameters between patients with and without plasma DNA revealed significant differences in the axillary involvement, rate of invasive ductal carcinoma, high proliferative index, and the parameter comprised of lymph node metastases, histological grade III, and peritumoral vessel involvement. A high proportion of breast cancer patients exhibited plasma DNA at diagnosis similar to tumor DNA, and its presence correlated significantly with pathological parameters associated with a poor prognosis.

INTRODUCTION

Breast cancer is the most common malignancy in women in Western countries and accounts for 18.4% of all cancers in female patients (1). During recent decades, its prevalence rate has exhibited a clear increase, in part as a result of the aging population; however, the age-specific prevalence has risen by 25% in the last 20 years, affecting 1 in 10 women during their lifetime (2). We are also observing an increase in breast cancer in young women under 35 years of age (3). Qualitative changes in the lifestyle of women in developed countries that can influence risk factors for breast cancer, such as age at menarche, menopause, or first pregnancy, may partially explain this phenomenon (2). In contrast, the mortality rate for breast cancer has not increased to the same degree as its prevalence because patient survival has improved due to new treatments. In addition to specific treatments, the following strategies for reducing mortality should be considered: (a) a refinement of diagnostic techniques for early diagnosis; and (b) the investigation of new methods able to detect metastatic or recurrent disease in preclinical or symptomatic phases.

It is known that malignant cell transformation is accompanied by well-defined molecular genetic changes within the original cell. Today, it is possible to detect many of them not only in research studies but also in clinical diagnostics in tumor DNA of tissue samples obtained from diverse sources reached by the tumor cells, including sputum (4), urine (5), pancreatic juice (6), and stool (7). In breast cancer, a case has been reported with c-erbB-2 gene amplification found in nipple discharge (8). Previous studies have found that free DNA is circulating in both healthy and ill individuals. In control subjects, the mean concentration of soluble DNA in plasma was 14 ng/ml (9), and in patients with different types of neoplasias, the mean concentration rose to 180 ng/ml (10). A mean concentration of 118 ng/ml was also detected in benign gastrointestinal processes (9), and mean concentrations above those registered in controls have been reported in patients with autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, or other inflammatory conditions (11). An active process explaining the enrichment of plasma DNA in cancer patients has also been suggested (12). More recently, qualitative and quantitative studies have demonstrated the presence of fetal DNA in maternal serum, with implications for noninvasive prenatal diagnosis (13–15). Likewise, the examination of graft and host interactions has revealed the presence of donor-specific DNA in the plasma of kidney and liver transplant recipients (16).

In recent years, using molecular techniques such as PCR for amplification of small amounts of DNA, it has been possible to identify the same alterations observed in tumor DNA in the plasma DNA of patients bearing diverse types of tumors. The alterations found include K-ras, N-ras, and p53 gene mutations, aberrant promoter hypermethylation of tumor suppressor genes, and changes in microsatellites detected by polymorphic markers in the following cancers: pancreatic cancer (17, 18); colon cancer (19–21); myelodysplastic syndromes (22); small cell lung cancer (23, 24); non-small cell lung cancer (25, 26); head and neck carcinomas (27); clear cell renal carcinoma (28); and breast (24) and liver cancer (29).

Breast cancer is associated with different types of molecular genetic aberrations such as somatic mutations of oncogenes (30–32) and tumor suppressor genes (33–36) as well as allelic loss and MI in several chromosomal regions (37–42). The determination of these anomalies can be used as a specific tool in the histological diagnosis, even early diagnosis, and can possibly be used as a prognostic factor. As observed in other tumors, the specific molecular alterations shown by breast carcinomas may also be found in the plasma DNA of patients harboring a breast cancer tumor.

Based on these facts, we designed the present study with two goals: (a) to determine the presence at diagnosis of tumor DNA in the plasma of patients with breast cancer, characterized by alterations in microsatellites and in tumor suppressor genes; and (b) to analyze the distribution of 13 clinicopathological parameters in patients with and without specific plasma DNA.

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3 The abbreviations used are: MI, microsatellite instability; dNTP, deoxynucleotidetriphosphate; L0H, loss of heterozygosity; C1, confidence interval; SSCP, single-strand conformational polymorphism; PVI, peritumoral vessel involvement; LNM, lymph node metastasis; IDC, invasive ductal carcinoma.
Tissue Sampling and DNA Extraction. All participants were informed of the nature of the study and gave their informed consent. Between October 1, 1997 and October 30, 1998, samples from tumor tissue and corresponding normal tissue were obtained sequentially, immediately after mastectomy, from 62 patients with a diagnosis of breast cancer. They were then snap-frozen in liquid nitrogen until processing. All specimens underwent histological examination to confirm the diagnosis of breast carcinoma. Pathological diagnosis and clinical evaluation disclosed no evidence of metastatic dissemination in any patient. A blood sample was collected from each patient with a clinical diagnosis of breast cancer on the day of surgery (before the mastectomy) to avoid the possible clearance of plasma DNA after removal of the primary tumor, and the sample was later discarded if histological diagnosis did not conclusively indicate the presence of a malignant lesion. Blood samples of 17 healthy controls were also obtained. DNA was extracted from normal blood cells and plasma immediately thereafter. DNA extraction from tumor and normal tissue samples and from peripheral blood mononuclear cells (which were also used as normal DNA to avoid possible molecular alterations that might have occurred in normal breast tissues; Ref. 42) was performed by a nonionic method (Oncor, Gaithersburg, MD). Plasma DNA was purified on Qiagen columns (QIamp Blood Kit; Qiagen, Hilden, Germany) according to the protocol for blood and body fluids, introducing the following modifications. Between 7.5 and 12 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 (13). Proteinase K (20 mg/ml; Boehringer Mannheim, Mannheim, Germany) 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.

Analysis of Clinicopathological Parameters. The following parameters were obtained from the medical records of the 62 patients studied: (a) birth and diagnosis dates; (b) family history of the disease; (c) menopausal status; (d) tumor size; (e) LIMs; (f) presence of steroid receptors (estrogen and progesterone); (g) histological type; (h) peritumoral vessel invasion; and (i) histological grade. Pathological stage was assessed using the tumor-node-metastasis (TNM) classification. All tumors were graded with regard to two parameters: (a) nuclear polymorphism (uniform and regular size, 1; moderate pleomorphism, 2; highly pleomorphic with giant nucleus, 3); and (b) mitotic index (<1 mitosis, 1; 2; 2, 3; >3, 3). The final grade was determined by adding the two scores: grade 1, 1–2; grade 2, 3–4; grade 3, 5–6. Presence of peritumoral vessel invasion was analyzed, and the steroid receptor content (estrogen and progesterone) was determined by an immunohistochemical procedure, the results of which were considered to be positive when 25% or more of the cells stained positively. The proliferative index of the tumors was demonstrated by Ki-67 antigen (Immunotech, Westbrook, ME) in an immunohistochemical analysis; the Ki-67 labeling index was considered high when 15% or more of the cells stained positively.

Microsatellite Analysis and PCR Conditions. 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× PCR buffer, 200 μM dNTP, 0.6 μM of each primer, and different concentrations of MgCl₂, depending on the polymorphic marker. A 30-cycle amplification was done in a thermal cycler (Perkin-Elmer, Foster City, CA). Six microsatellite markers were used to determine LOH on the following chromosomes: (a) on chromosome 17, D17S855 (43) and D17S654 (44); (b) on chromosome 16, D16S421 (45); (c) on chromosome 11, TH2 (42); (d) on chromosome 10, D10S197 (37); and (e) on chromosome 9, D9S161 (46). These markers were chosen because they have been reported to show a high rate of alterations in breast carcinomas. The alleles were separated by mixing 25 μl of the PCR products with a 10×-μl volume of loading buffer (total volume, 35 μl), 0.02% xylene cyanol, and 0.02% bromophenol blue. Electrophoresis was run on nondenaturing 8%-12% polyacrylamide gels for 12–15 h at 300 V. After gel electrophoresis, the allelic band intensity was detected by a nonradioisotopic technique using a commercially available silver staining method (47). The amplified products were denatured by mixing with 15 μl of denaturing stop solution that contained 98% formamide, 10 mM edathamil (pH 8.0), 0.02% xylene cyanol and 0.02% bromophenol 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 nonradioisotopic method using a commercially available silver staining method (47). The specimens that showed a differential band at SSCP 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 Gene clean Kit (Bio-101, Inc., La Jolla, CA), and used for direct DNA sequencing by the dNTP method with the Sequenase Kit (United States Biochemical Corp. Cleveland, OH) according to the manufacturer’s instructions.

Methylation Study of the First Exon of p16INK4a. We also used the methylation status of the first exon of p16INK4a as a molecular alteration to identify patients with tumor DNA in plasma. The study was performed in the 43 cases in which plasma DNA was available after microsatellite and p53 gene mutational analysis. A PCR-based methylation assay was performed, based on the inability of some restriction enzymes to cut methylated sequences (49). The PvrI and SacII restriction enzyme sites were examined. Analysis of DNA digests was performed according to the manufacturer’s instructions (Promega, Madison, WI), and the digested DNA was amplified with primers flanking the restriction sites. Amplification of β-globin was used as internal control of the reaction in a multiplex PCR. There are no restriction sites in the β-globin sequence selected for PvrI and SacII. The primer set used for methylation analysis of exon 1 of p16INK4a was 5'-GGGACAGCCTAGGGCCGG and 5'-AGTCGCCCGCATCCTCCCC, and the primer set for β-globin was 5'-CAACCTCTACAGTGCACC and 5'-GAAGGCGAAAAAGGACGTAC. The conditions used were as follows: 2.5 μl of 10× buffer II (Perkin-Elmer, Roche Molecular Systems Inc.,) 200 μM dNTPs; 2.5 mM MgCl₂; 0.6 μM exon 1 primers; 0.6 μM β-globin primers; 100 ng of the digested DNA as template; and 25 μl of distilled H₂O. This reaction mix was amplified with Ampli Taq Gold (Perkin-Elmer, Roche Molecular Systems Inc.) at 94°C for 12 min with 35 cycles of 94°C for 30 s, 61°C for 30 s, and 72°C for 30 s, followed by incubation at 72°C for 11 min. PCR products were resolved in 6% acrylamide and stained with a nonisotopic silver nitrate method (47). PCR-based methylation analysis using restriction enzymes may be subject to variability if the DNA digestions are not complete. To rule out the possibility of incomplete restriction, all samples were digested overnight in two independent experi-
ments. PCR amplifications from each of the duplicate digests were repeated twice to ensure reproducibility of the results.

Statistical Analysis. A descriptive statistical study was performed in which the categorical variables were tabulated according to their absolute value proportion and 95% CI. The continuous variables are expressed in terms of the mean and 95% CI. The categorical variables were contrasted by means of the $\chi^2$ test with the Yates correction (50) or Fisher’s exact test when any of the expected frequencies was less than 5. We considered $P < 0.05$ to be significant

Statistical analyses were performed using the EPI-INFO package, version 6.04.

RESULTS

We studied 62 breast cancer patients for the presence of molecular genetic alterations in their plasma and tumor DNA. The objective was to detect molecular events in serum DNA that supported evidence of its tumor origin. Thus, as molecular markers, we used the allelic status of six different chromosomal regions (37, 42–46), point mutations of the $p53$ gene because they are detected in $16–81\%$ of breast carcinomas (51, 52), and the methylation status of the first exon of $p16^{INK4a}$, which has been reported in $31\%$ of these tumors (53).

Plasma DNA was found in all 62 patients at concentrations ranging from $24–170$ ng/ml (mean concentration, $115$ ng/ml).

Microsatellite Analysis. Although the microsatellites used were selected on the basis of their high rate of LOH in breast carcinomas, about $40\%$ of our cases were found to be uninformative. Fifty-one breast carcinomas ($82\%$) showed allelic loss at at least one locus. The same analysis of plasma DNA disclosed 38 patients ($61\%$) with the same microsatellite alterations (Fig. 1). The highest individual rate of LOH in tumor DNA corresponded to markers D17S654 and D16S421, with a $53\%$ and $50\%$ LOH, respectively (Table 1). All markers studied showed allelic loss in plasma DNA, but with variable percentages with respect to tumor DNA; TH2 displayed the highest coincidence rate, with $100\%$ of cases (Table 1). No MI was detected in tumor DNA. However, we observed that in five cases, plasma DNA demonstrated other additional molecular alterations not present in tumor DNA; two MI and one LOH for marker D17S654; and two LOH for D9S161.

Mutational Status of the $p53$ Gene. Tumor point mutations in $TP53$ were detected in seven tumors of our 62 patients (11%), an incidence that is lower than that reported elsewhere for breast cancer (51, 52). An extra band in plasma DNA corresponding to MI at the D17S654 marker in case 12. Plasma, P; tumor, T; normal breast tissue, N; lymphocyte, L.

In each molecular determination, microsatellite analysis, $TP53$ SSCP study, and methylation assay, the DNA pattern of normal blood cells and normal tissue was used as a control for comparison with tumor and plasma DNA. No differences were observed between normal blood cells and normal tissue DNA samples in every case.

In all, we identified 56 cases (90%) in which there was at least one molecular event in tumor DNA and 41 cases (66%) with a similar alteration in plasma DNA. Of the 15 patients that showed molecular change in tumor DNA but not in plasma DNA, 9 patients displayed the same molecular pattern in normal tissue, normal blood cells, and plasma DNA; in 6 cases, no amplification of the plasma DNA samples was obtained. The six cases in which there were no molecular alterations in the primary tumor had a mean plasma DNA concentration of $70$ ng/ml, but the molecular study revealed no differences among normal, normal blood cells, tumor, and plasma DNA.

Correlations between Clinicopathological Parameters and Molecular Changes. To establish a clinical meaning for the presence of plasma DNA with the characteristics of tumor DNA, we studied the correlation between the presence or absence of plasma DNA of tumor origin and 13 clinicopathological parameters at diagnosis (Table 2). Statistical analysis revealed statistically significant differences in the

| Table 1 Analysis of microsatellites, $TP53$ gene mutations, and aberrant methylation of the first exon of $p16^{INK4a}$ in tumor and plasma DNA of breast cancer patients |
|-----------------|-----|-----|-----|-----|
| A. Marker       | $n$ | MI  | Plasma LOH (%) | Plasma LOH (%) |
| TH2            | 62  | 35  | 6 (10)         | 6 (10)         |
| D10S197        | 62  | 30  | 9 (14)         | 6 (10)         |
| D16S421        | 62  | 22  | 11 (18)        | 8 (13)         |
| D17S5855       | 62  | 36  | 13 (21)        | 8 (13)         |
| D17S654        | 62  | 38  | 20 (32)        | 11 (18)        |
| D9S161         | 62  | 38  | 10 (16)        | 6 (10)         |
| B. $TP53$      |     |     |                |                |
| Tumor mutations (%) | Plasma mutations (%) |
| Methylation of $p16^{INK4a}$ | 43  | 10 (23) | 6 (14) | 0 |

*1, number of informative cases for each marker; $P$, number of cases with new alterations only in plasma DNA.

Fig. 1. Representative photograph of two gels taken under normal light after staining with a (NO$_3$)$_2$Ag method showing (A) microsatellite LOH at the D9S161 marker in patient 25. The arrow indicates the loss of the same allele in tumor and plasma and its presence in the normal tissue and lymphocyte DNA of the same patient. B, appearance of a new band in plasma DNA corresponding to MI at the D17S654 marker in case 12. Plasma, P; tumor, T; normal breast tissue, N; lymphocyte, L.
and reported that it was possible to analyze variations in the amount depending on the stage of the disease and the response to the treatment received by the patients; however, the specific characterization of this DNA was not feasible. To date, several studies have reported that cancer patients exhibit DNA in plasma, and molecular studies have provided evidence that this DNA is similar to tumor DNA (17–29). Overall, in our series, 66% of patients were found to present molecular alterations in plasma DNA. To optimize the results, it will be essential to obtain a panel of positive markers capable of detecting the molecular changes in plasma DNA for each tumor.

Another aim of this study was to determine whether the presence of plasma DNA at diagnosis was significantly associated with some of the clinicopathological characteristics of the patients. Among the 13 parameters evaluated, we found 4 that showed statistically significant differences between patients with and without plasma DNA with tumor characteristics: (a) three (high proliferative index, three or more affected lymph nodes, and IDC) as independent variables; and (b) one comprised of PVI, axillary involvement, and high histological grade, which are three classical pathological parameters of poor prognosis (Table 2). Our patients showed no significant differences in other independent variables such as tumor size, stage, histological grade, steroid receptors, or PVI that are also indicative of tumor extension and aggressiveness and, secondarily, of tumor cell turnover and capacity for metastatic spread. To date, six studies have analyzed the presence of tumor DNA in plasma and disease stage, and the results have not been uniform. Two studies in head and neck carcinoma (27) and small cell lung cancer (23) reported a large number of patients with advanced disease and the presence of tumor DNA in plasma, and four studies also associated this molecular event with the early stages of non-small cell lung cancer (25), colon cancer (19, 20), and clear cell renal carcinoma (28). Taken together, the data available concerning the extension of tumor disease and the appearance of plasma DNA with tumor characteristics do not offer a conclusive explanation, suggesting that other unexplored biological characteristics of the tumor cells could be related to this phenomenon. Thus, the results of our study may indicate that the presence of plasma DNA of tumor origin in breast cancer patients is significantly associated with some histological features of highly malignant lesions.

In addition to the possible role of free plasma DNA as a prognostic factor in breast cancer, other applications can be also considered. The early diagnosis of breast cancer is currently one of the best strategies for improving the survival of these patients. If the results of this procedure were found to be positive in disease stage I or with tumors measuring less than 1 cm, it would be a good implementation of the

| Table 2: Pathological characteristics of breast carcinomas showing a statistically significant difference between patients presenting plasma DNA with and without tumor features |
|-------------------|-----------------|---------------|--------|
|                   | Tumor plasma DNA | No tumor plasma DNA | P     |
| No. of eligible patients | N | % | 95% CI | N | % | 95% CI |  |
| LNM | 41 | | | 15 | | |  |
| >3 | 26 | 63 | (47–78) | 15 | 100 | (78–100) | 0.005 |
| ≥3 | 15 | 37 | (22–53) | 0 | 0 | (0–22) |  |
| Histology | IDC | Others | | | | |  |
| 55 | 33 | 80 | (65–91) | 8 | 53 | (27–79) | 0.05 |
| PVI, LNM, grade III | Yes | No | | | | |  |
| Others | 8 | 20 | (9–35) | 7 | 47 | (21–73) | 0.009 |
| Ki-67 index | High | Low | | | | | 0.02 |
| 32 | 78 | (62–89) | 7 | 47 | (21–73) |  |
| 9 | 22 | (11–38) | 8 | 53 | (27–79) |  |
current radiographic procedures. Moreover, the identification of pre-malignant disease with defined molecular alterations (54) would per-mit the investigation of these molecular events in patients with pre-neoplastic lesions, hypothetically changing the management of these processes.

Previous observations have suggested that plasma DNA can un-dergo quantitative changes in cancer patients after radiation therapy (10); however, its utility in monitoring chemotherapy and radiotherapy has yet to be fully defined.

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 (55). Considering the prevalence and incidence of this disease, the discovery of a valid marker could have a strong impact on its management and probably on survival. We consider that the utilization of a simpler methodology that, as in our case, does not involve radioisotopic techniques may help to introduce plasma DNA analysis into the study and management of breast cancer. In the coming years, prospective studies, such as that recently reported in pancreatic carcinomas (56), will provide us with definitive information on the value of this research tool as a prognostic factor in cancer patients.

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