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 (D17S855, 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 auxiliary 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 hyper-methylation 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 MI3 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, deoxynucleotide triphosphate; LOH, loss of heterozygosity; CI, confidence interval; SSCP, single-strand conformational polymorphism; PFI, peritumoral vessel involvement; LNM, lymph node metastases; IDC, invasive ductal carcinoma.

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PATIENTS AND METHODS

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 nonisotopic 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) LNM; (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 (0 on chromosome 16, D16S421 (45); (b) on chromosome 11, TH2 (42); c-erbB-2 (43); on chromosome 10, D10S197 (37); and (c) family history of the disease; (d) birth and incubation overnight at 55°C. One column was used repeatedly until the whole sample had been processed. The DNA extracted was quantified spectrophotometrically.

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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 PvuII and SacII restriction enzyme sites were examined. Analysis of DNA digest 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 PvuII and SacII.) The primer set used for methylation analysis of exon 1 of p16INK4a was 5′-GGGACGAGCTTGGGACCG and 5′-AGTTGCGCCCTCTCAGTGCT; and the primer set for β-globin was 5′-CAACCTCATCAGGCTCG and 5′-CAAGAGCAGGAAGCACGAGTG. The conditions used were as follows: 2.5 μl of 10× buffer II (Promega-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-
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). In three cases (5%), 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.

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
following independent variables: (a) involvement of three or more lymph nodes ($P = 0.005$); (b) IDC ($P = 0.05$); and (c) a high proliferative index ($P = 0.02$). When we considered the subgroup of patients whose tumors concomitantly exhibited three characteristics classically associated with high-grade malignancy (LNMs, histological grade III, and PVI) and analyzed them with respect to the presence or absence of plasma DNA, a significant difference ($P = 0.009$) was also observed. The rest of the variables analyzed displayed no statistically significant differences (Table 2). During the 1-year study period reported here, there were no cases of relapse or death, regardless of the presence or absence of plasma DNA.

Plasma DNA of healthy controls was extracted in lower concentrations than in cancer patients, ranging between 0 and 45 ng/ml. The same six markers were tested in plasma and normal blood cell DNA. Plasma 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) 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).

### Table 2: Pathological characteristics of breast carcinomas showing a statistically significant difference between patients presenting plasma DNA with and without tumor features

<table>
<thead>
<tr>
<th>No. of eligible patients</th>
<th>Tumor plasma DNA</th>
<th>No tumor plasma DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>N % 95% CI</td>
<td>N % 95% CI</td>
<td>P</td>
</tr>
<tr>
<td>LNM&lt;3</td>
<td>26 63 (47–78)</td>
<td>15 100 (78–100)</td>
</tr>
<tr>
<td>³3</td>
<td>15 37 (22–53)</td>
<td>0 0 (0–22)</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IDC</td>
<td>33 80 (65–91)</td>
<td>8 53 (27–79)</td>
</tr>
<tr>
<td>Others</td>
<td>8 20 (9–35)</td>
<td>7 47 (21–73)</td>
</tr>
<tr>
<td>PVI, LNM, grade III</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>13 32 (18–48)</td>
<td>0 0 (0–22)</td>
</tr>
<tr>
<td>No</td>
<td>28 68 (52–82)</td>
<td>15 100 (78–100)</td>
</tr>
<tr>
<td>Ki-67 index</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>32 78 (62–89)</td>
<td>7 47 (21–73)</td>
</tr>
<tr>
<td>Low</td>
<td>9 22 (11–38)</td>
<td>8 53 (27–79)</td>
</tr>
</tbody>
</table>

DISCUSSION

The results of this study provide evidence of the presence of plasma DNA with features of tumor DNA in breast cancer patients. In 1977, Leon et al. (10) reported the presence of free DNA at concentrations ranging between 0 and 2 µg/ml in the serum of breast cancer patients 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.

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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...
current radiographic procedures. Moreover, the identification of pre-malignant disease with defined molecular alterations (54) would permit 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 undergo 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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