

*Advances in Brief***Detecting Tumor-related Alterations in Plasma or Serum DNA of Patients Diagnosed with Breast Cancer¹**

Xu qi Chen, Hervé Bonnefoi,
Sophie Diebold-Berger, Jacqueline Lyautey,
Christine Lederrey, Ellen Faltin-Traub,
Maurice Stroun, and Philippe Anker²

Département de Biochimie et de Physiologie Végétale, Faculté des Sciences [X. q. C., J. L., C. L., M. S., P. A.] and Département de Pathologie, Faculté de Médecine [S. D-B.], Université de Genève, and Département de Gynécologie, Hôpital Cantonal Universitaire de Genève [H. B., E. F-T.], 1211 Geneva, Switzerland

Abstract

Chromosomal abnormalities are associated with the development of breast cancer, and widespread allelic loss or imbalance is frequently found in tumor tissues taken from patients with this disease. Using different markers, we studied a total of 61 patients (divided into three groups) for the presence of microsatellite instability and loss of heterozygosity (LOH) in plasma or serum DNA. Of the initial 27 patients, 35% of the tumor samples displayed LOH, whereas 15% had identical alterations in the corresponding plasma samples. In addition, the adjacent normal breast tissue of two patients also displayed LOH. In a second group of 11 patients, 45% of the tumors displayed LOH, and 27% displayed identical plasma DNA alterations; one case displayed an identical LOH in adjacent nontumor tissue. In a third series of 23 patients also studied with tetranucleotide repeats, 81% of the tumor samples displayed LOH, whereas 48% had LOH in the corresponding serum samples. The fact that small tumors (T₁) of histoprognostic grade 1 or *in situ* carcinomas could present DNA alterations in the plasma/serum at an early stage, allied to the widely increased range of available microsatellite markers, suggests that plasma or serum DNA may become a useful diagnostic tool for early and potentially curable breast cancer.

Introduction

Breast Cancer and Screening. Adenocarcinoma of the breast is the most common malignancy in the female Western

population, affecting up to one in eight women in the United States. Breast cancer is responsible for up to one in five cancer-related deaths among women, and long-term survival following breast cancer has improved little over the past 20 years (1).

An important strategy to reduce mortality from breast cancer is the introduction of mammography screening in an attempt to detect cancers at an asymptomatic and pathologically early stage. Although several studies indicate that mass screening might be a useful strategy for reducing breast cancer mortality, there are a number of disadvantages associated with this form of cancer screening (2–6). These include a high rate of false positive tests (7), frequent false negative tests, and the enormous public health costs involved (8, 9). Thus, when the benefits of mammography screening are weighed against its costs and other disadvantages, it is perhaps not surprising that this form of screening has engendered an enthusiastic and contentious debate over the past 20 years (10, 11).

Chromosomal Abnormalities in Tumor DNA. Our knowledge of the genetic changes associated with cancer has grown rapidly since the introduction of the PCR in the late 1980s. Chromosomal abnormalities, including mutations, insertions, deletions, allelic losses of oncogenes and tumor suppressor genes, and microsatellite alterations have been discovered in cancer cell DNA, and the application of molecular biological techniques now allows us to identify these alterations in tumors. In relation to breast cancer, several chromosomes appear to be frequently affected by somatic genetic abnormalities. Allelic losses or imbalances have also been reported in numerous chromosomal subregions, and knowledge of microsatellite alterations in breast cancer is constantly expanding (12).

Tumor-related Abnormalities in Plasma/Serum DNA. Increased quantities of DNA have been found in the plasma of patients suffering from different malignancies (13–17). This circulating extracellular DNA exhibits tumor-related alterations such as decreased strand stability (17), ras or p 53 mutations (18–25), microsatellite alterations (26–29), or aberrant promoter hypermethylation of tumor suppressor genes (30, 31). In relation to breast cancer, p53 mutations have been found in the plasma DNA of 2 patients in a series of 15 patients (25).

Thus, the aim of our study was to isolate DNA from the plasma or serum of patients with a suspected diagnosis of breast cancer to analyze this DNA for the presence of microsatellite instability and LOH³ and to compare it with corresponding tumor DNA.

Materials and Methods

Patients and Sample Collection. Tumor samples and, in some cases, corresponding adjacent tissue samples were collected during surgery from consenting and informed patients.

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² To whom requests for reprints should be addressed, at Laboratoire de Biochimie et de Physiologie Végétale, Université de Genève, Pavillon des Isotopes, 20 Boulevard d'Yvoy, 1211 Geneva, Switzerland. Phone: 41-22-702-63-38; Fax: 41-22-781-51-93; E-mail: anker@sc2a.unige.ch.

³ The abbreviation used is: LOH, loss of heterozygosity.

Table 1 Microsatellite alteration of plasma and tumor DNA and clinical data concerning breast cancer patients

Patient no.	Age (yr)	Type ^a	Grade	Node	pTNM	DM1			D17S1325		
						T	P	A	T	P	A
1	55	IDC	1	0/20	pT _{1c} N ₀ M ₀	-	-	-	-	-	-
2	46	IDC	3	0/16	pT ₂ N ₀ M ₀	-	-	-	-	-	-
3	81	IDC	3	29/29	pT ₂ N ₂ M ₀	L	-	-	L	-	-
4	55	IDC	2	1/10	pT ₂ N _{1bi} M ₀	-	-	-	-	-	-
5	76	IDC	3	4/15	pT ₂ N _{1biv} M ₀	-	-	-	-	-	-
6	79	IDC	1	1/17	pT ₂ N _{1bi} M ₀	L	L	L	S	-	S
7	68	DCIS	3		pT _{is} N ₀ M ₀	L	-	-	-	-	-
8	73	IDC	2	0/26	pT ₄ N ₀ M ₀	S	S	-	-	-	-
9	72	ILC	2	0/6	pT _{1c} N ₀ M ₀	-	-	-	L	-	-
10	66	ILC	2	0/14	pT ₂ N ₀ M ₀	-	-	-	-	-	-
11	81	IDC	2	1/14	pT ₂ N _{1biii} M ₀	-	-	-	-	-	-
12	54	ILC+DCIS	2		pT ₃ N _{1bii} M ₀	-	-	-	-	-	-
13	68	IDC	2	9/22	pT ₂ N _{1biii} M ₀	-	-	-	-	-	-
14	55	IDC	1	2/31	pT _{1b} N _{1biii} M ₀	-	-	-	-	-	-
15	77	DCIS	3			-	-	-	-	-	-
16	63	IDC	2	1/11	pT ₂ N _{1a} M ₀	-	-	-	-	-	-
17	66	IDC	2	0/25	pT ₂ N ₀ M ₀	L	-	-	-	-	-
18	63	ILC	2	0/11	pT ₂ N ₀ M ₀	-	-	-	-	-	-
19	64	IDC	2	2/12	pT ₂ N _{1biii} M ₀	-	-	-	-	-	-
20	72	IDC	2	0/14	pT _{1s} N ₀ M ₀	L	L	-	-	-	-
21	31	BD				-	-	-	-	-	-
22	78	BD				-	-	-	-	-	-
23	28	BD				-	-	-	-	-	-
24	43	BD				-	-	-	-	-	-
25	62	BD				-	-	-	-	-	-
26	61	BD				-	-	-	-	-	-
27	72	BD				-	-	-	-	-	-

^a IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; DCIS, ductal carcinoma *in situ*; BD, benign disease; T, tumor DNA; P, plasma DNA; A, adjacent tissue DNA; L, LOH; S, shift (appearance of new bands); -, absence of these findings.

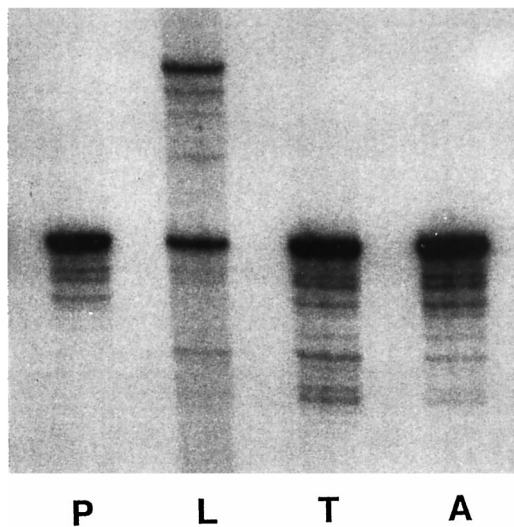


Fig. 1 Autoradiograph from microsatellite analysis. Representative microsatellite analysis of DNA of patient 6 with marker DM1. P, plasma; L, lymphocyte; T, tumor; A, normal tissue adjacent to the tumor. Plasma, tumor, and adjacent tissue reveal a loss of the upper allele.

Part of each tumor and adjacent tissue specimen was histopathologically characterized to confirm its diagnosis, and the other part was used for molecular analysis. Blood was collected before surgery. For the first 38 patients, 10 ml of blood with

EDTA for plasma collection were taken. For the last 23 patients, a sample of 10 ml of blood was collected without additive for serum. Altogether, we analyzed 61 patients. The following clinical and biological parameters were studied: tumor size, lymph node status (pTNM), histological type, histoprognoctic grade, and hormonal receptors. All molecular analyses were performed by personnel who were blinded to clinical and pathological details.

Blood samples were also taken from healthy donors as controls. The EDTA fraction yielded the lymphocytes and the plasma that were separated by Ficoll gradient. Serum was collected by centrifugation (1000 × g; 10 min) after clotting.

Breast tissue, lymphocyte, plasma, and serum specimens were stored at -20°C until further use.

DNA Isolation. Fresh frozen tissue and lymphocytes were treated with SDS and proteinase K, followed by phenol and chloroform extraction. Paraffin-embedded tissue scraped from the slides was washed in xylol to remove paraffin. After the addition of 1 volume of ethanol, the mixture was centrifuged, and the pellet was digested with proteinase K and SDS, followed by phenol and chloroform extraction.

Plasma or serum (200 μl) was purified on Boehringer columns (High Pure Viral Nucleic Acid Kit; Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's protocol, followed by treatment with phenol/chloroform (1:1 v/v) and chloroform/isoamyl alcohol (24:1 v/v) and ethanol precipitation, and finally dissolved in 10 mM Tris (pH 8)-1 mM EDTA diluted five times.

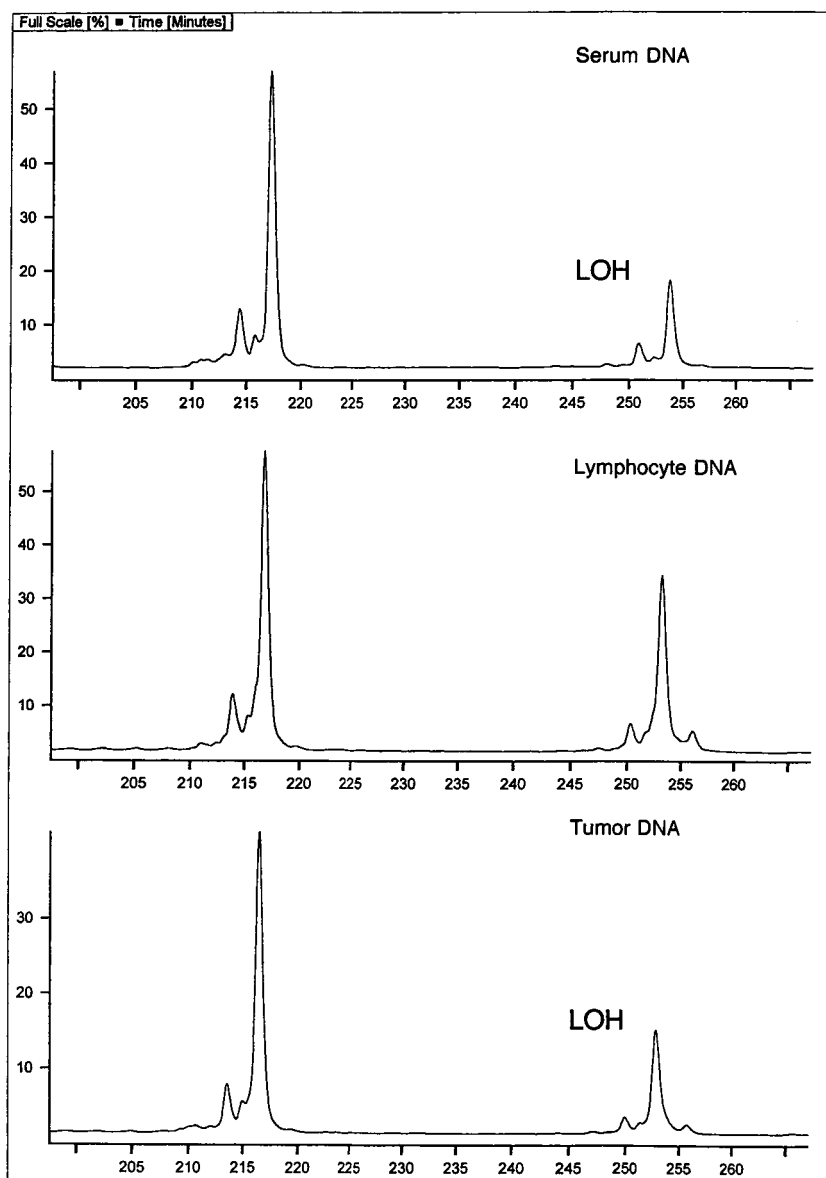


Fig. 2 Original tracings from microsatellite analysis of serum, lymphocyte, and tumor DNA of patient 42 with marker ACTBP2. Plasma and tumor DNA reveal LOH.

situ carcinoma, 2 cases of benign disease) were studied with 11 markers (D8S321, D11S488, D16S398, D17S1325, D17S579, ACTBP2, DM1, Tp53.5, Tp53.6, Tp53.8, and UT5320). No alterations were observed in any samples with markers D16S398, Tp53.5, Tp53.6, and Tp53.8. However, using the seven other markers, LOH was found in the tumor DNA of 17 of 21 patients with cancer (81%) in at least one locus, and 13 cases were positive for multiple loci (Fig. 2; Table 3). Ten of 21 cases (48%) had LOH in the corresponding serum samples, and 1 case was positive for multiple loci. DNA extracted from the tissue of one patient with benign fibroadenoma displayed LOH at two loci, but these alterations were absent in the corresponding serum sample. The plasma and serum of all healthy controls (at least 10 controls/microsatellite marker) did not demonstrate microsatellite alterations.

No significant associations were found between plasma/serum DNA alterations and the clinical and pathological features of cancer patients.

Discussion

Breast cancer is a common malignancy among the female population, and current screening methods fail to detect many cancers that present at a later date as a result of symptoms. The present study was performed to determine whether the plasma or serum of patients with breast cancer displays microsatellite alterations that are similar to those found within the primary tumor.

This study, which commenced in 1997, initially used two microsatellite markers that we found to be altered in only 35%

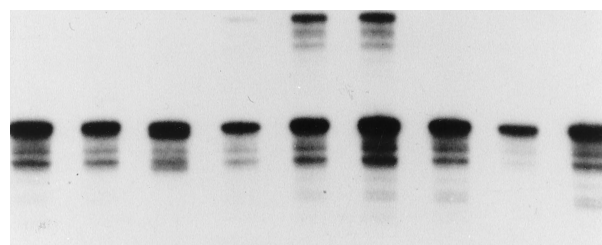
Table 3 Microsatellite alteration of serum and tumor DNA and clinical data concerning breast cancer patients

Patient no.	Age (yr)	Type ^a	Grade	Node	pTNM	D8S321		D11S488		D17S579		D17S1325		ACTBP2		DM1		UT5320	
						T	S	T	S	T	S	T	S	T	S	T	S	T	S
39	57	IDC	2	1/19	pT ₂ N _{1bi} M ₀	L	-	-	-	NI	-	NI	-	L	-	-	-	L	-
40	74	DCIS				-	-	-	-	-	-	-	-	-	-	-	-	L	L
41	57	IDC	2	0/10	pT ₂ N ₀ M ₀	NI	-	L	-	-	-	-	L	-	-	-	-	-	-
42	54	IDC	3	11/13	pT ₃ N _{1biv} M ₁	L	-	NI	-	NI	-	NI	L	L	-	-	-	L	L
43	57	IDC+LCIS	1	0/11	pT _{1c} N ₀ M ₀	-	-	-	-	NI	-	NI	-	-	-	-	-	-	-
44	38	IDC	2	5/17	pT ₂ N _{1biii} M ₀	-	-	L	-	NI	-	NI	L	-	-	-	-	-	-
45	52	IDC	2	29/29	pT ₂ N _{1biv} M ₁	L	-	NI	-	NI	-	NI	-	-	-	NI	-	L	L
46	63	DCIS				-	-	NI	-	-	-	-	-	-	-	NI	-	-	-
47	68	IDC	2	0/10	pT _{1c} N ₀ M ₀	L	-	-	-	-	-	NI	-	-	-	L	-	L	L
48	44	IDC	2	2/11	pT ₂ N _{1biv} M ₀	-	-	L	-	-	-	-	-	-	-	-	-	-	-
49	50	IDC	2	1/15	pT ₂ N _{1biii} M ₀	-	-	-	NI	L	-	L	-	L	-	NI	-	-	-
50	60	ILC	2	0/15	pT _{1c} N ₀ M ₀	-	-	L	-	NI	-	-	L	-	NI	-	-	-	-
51	70	IDC	2		pT _{1c} N ₀ M ₀	-	-	L	-	-	-	-	L	-	L	L	-	-	-
52	72	IDC	1	0/26	pT _{1c} N ₀ M ₀	-	-	-	-	-	-	-	-	-	-	-	-	-	-
53	50	ILC	2	0/13	pT _{1c} N ₀ M ₀	-	-	-	-	NI	-	-	-	-	-	L	L	-	-
54	83	IDC	3	0/13	pT ₂ N ₀ M ₀	L	-	-	-	NI	-	L	L	NI	NI	L	-	-	-
55	57	IDC	2	0/19	pT ₂ N ₀ M ₀	-	-	-	-	-	-	NI	-	-	-	-	-	-	-
56	71	IDC	3	0/8	pT ₂ N ₀ M ₀	L	-	L	-	L	-	L	-	L	-	NI	-	L	-
57	82	IDC	3		pT ₂ N ₀ M ₀	NI	-	L	L	NI	-	-	-	-	-	-	-	-	-
58	49	IDC	2	7/14	pT ₂ N _{1biii} M ₀	L	-	NI	NI	-	-	-	L	L	NI	NI	L	-	-
59	64	IDC	2	1/17	pT _{1c} N _{1a} M ₀	-	-	-	-	-	-	-	-	L	-	L	L	-	-
60	28	BD				-	-	-	-	-	-	-	-	-	-	NI	NI	-	-
61	43	BD				-	-	-	-	-	-	-	-	-	-	L	-	L	-

^a IDC, invasive ductal carcinoma; DCIS, ductal carcinoma *in situ*; ILC, invasive lobular carcinoma; LCIS, lobular carcinoma *in situ*; BD, benign disease; T, tumor DNA; S, serum DNA; L, LOH; S, shift (appearance of new bands); -, absence of these findings; NI, not informative.

of tumors and 15% of plasma samples. Later, 15 markers with reportedly higher alteration rates (50–80%) in breast cancer (12, 32–44) were used in an attempt to increase the sensitivity of the assay for malignant disease. We found a LOH rate of between 0 and 18% for these markers, which is lower than the rate given in previous reports. Let us stress that similar examples of discrepancies between laboratories have been found (32, 33). For instance, LOH at 7q31 has been claimed to occur in over 80% of all breast cancers and to be of prognostic significance (34), whereas a joint European study made by 17 institutes found a LOH rate ranging from 0–40%, with a mean of 19% (35). The reason for this is unclear but may be related to a number of methodological differences between laboratories. In preliminary experiments, we found that the microsatellite pattern of lymphocyte DNA and plasma DNA of healthy controls did not correspond in some cases. This led us to conduct an additional series of preliminary experiments assessing PCR quality as a function of the amount of DNA template contained in the primary reaction. Using between 5 and 80 ng of tumor, lymphocyte, or plasma DNA as a PCR template, it became clear that small quantities of template resulted in a poor-quality PCR product (see Fig. 3), probably as a result of incorrect reading of bases by Taq polymerase. Thus, it is important that all studies reporting results of microsatellite alterations in cancer should contain a large enough number of repeated experiments on healthy controls to identify potential problems with individual microsatellite markers. The quality of the DNA is also a determinant. When the DNA is not pure enough, Taq polymerase might also make mistakes. Neglecting these facts may result in an overestimation of LOH.

In our third series of 23 patients, we used serum in pref-



P5 P10 P20 L5 L10 L20 T5 T10 T20

Fig. 3 Autoradiograph from microsatellite analysis. Representative microsatellite analysis of DNA of patient 20 with marker DM1. P, plasma; L, lymphocyte; T, tumor. The amount of DNA (in ng) used in the PCR reaction is designated below each lane (5, 10, and 20). A false LOH can be seen when only 5 ng of lymphocyte DNA were used. The LOH observed in plasma and tumor DNA is true because it is maintained with increasing amounts of DNA.

erence to plasma, because greater quantities of higher-quality DNA were extractable from serum. In this series, a higher proportion of patients demonstrated alterations in both tumor and serum DNA. The primers D16S398, D17S579, D17S1325, Tp53.5, Tp53.6, and Tp53.8 were chosen because of their high incidence of LOH in breast cancer, as reported previously (12, 32, 33, 36, 38, 40, 41). In contrast, we also chose ACTBP2, D8S321, D11S488, and UT5320 because they are tetranucleotide repeats that have demonstrated a high rate of abnormality in other cancers (44). Although rarely studied in breast cancer (43), we also continued to use DM1 because this marker showed a relatively high and constant rate of abnormality in our series.

As in our previous two groups, the markers that we expected to result in high rates of LOH in breast cancer tended to perform poorly, whereas other markers yielded a relatively high rate of LOH in tumor DNA. It might be worthwhile to extend this work to look at other tetranucleotide repeats that have previously resulted a high incidence of alteration in lung cancer (44), bladder cancer, or head and neck cancer (45).

The three patients with distant metastases at the time of operation (one of whom later died of disease) displayed LOH in plasma or serum DNA at more than one locus. In addition two large (T_4) tumors displayed LOH in plasma DNA. Otherwise, no clear relationship between LOH and clinical or pathological characteristics was evident. Ductal and lobular cancers of all grades (grades 1–3) and all sizes demonstrated LOH in plasma DNA, indicating that other factors may be important in determining the presence of mutant DNA in plasma.

The alterations found in the samples of tissue adjacent to the tumor were detected in conjunction with those found in the plasma. One of the patients had a grade 1 tumor with size T_2 , and another patient had a grade 2 tumor with size T_1 and no metastases (Table 1), which confirms that the molecular heterogeneity that characterizes invasive cancers may occur at an early detectable stage (46).

One of nine patients with a benign tumor (a patient with a fibroadenoma accompanied by a fibrocystic mastopathy) showed LOH with two markers in the tumor DNA only. An increased risk of breast cancer among women previously diagnosed with fibroadenoma as well as the presence of LOH and microsatellite instability has been reported previously (47).

We found that two small (T_1) tumors of histoprogenic grade 1 or *in situ* carcinomas also displayed DNA alterations in serum/plasma DNA. This result is encouraging because it indicates that the presence of mutant DNA in plasma may occur at an early pathological stage. It is possible that further advances in detection techniques and the addition of reliable markers with a high incidence of mutation in breast cancer, such as p53 mutations (25) or hypermethylation of tumor suppressor genes (30, 31), might eventually lead to a noninvasive test for breast cancer. Such a test could also be valuable in the follow-up of patients after surgical or medical therapies. It will be interesting to compare it with the breast cancer screening technique using X-ray diffraction of hair that has just appeared (48).

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