Advances in Brief

Detecting Tumor-related Alterations in Plasma or Serum DNA of Patients Diagnosed with Breast Cancer[1]

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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 (T_1) 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 LOH[3] 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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[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

						DM1			D17S1325		
Patient no.	Age (yr)	Type[a]	Grade	Node	pTNM	T	P	A	T	P	A
1	55	IDC	1	0/20	$pT_{1c}N_oM_o$	–	–	–	–		
2	46	IDC	3	0/16	$pT_2N_oM_o$	–	–	–	–		
3	81	IDC	3	29/29	$pT_2N_2M_o$	L	–		L	–	
4	55	IDC	2	1/10	$pT_2N_{1bi}M_o$	–	–	–	–		
5	76	IDC	3	4/15	$pT_2N_{1biv}M_o$	–	–	–	–		
6	79	IDC	1	1/17	$pT_2N_{1bi}M_o$	L	L	L	S	–	S
7	68	DCIS	3		$pT_{is}N_xM_o$	L	–	–	–		
8	73	IDC	2	0/26	$pT_4N_oM_o$	S	S	–	–		
9	72	ILC	2	0/6	$pT_{1c}N_oM_o$	–	–	–	L	–	
10	66	ILC	2	0/14	$pT_2N_oM_o$	–	–	–	–		
11	81	IDC	2	1/14	$pT_2N_{1biii}M_o$	–	–	–	–		
12	54	ILC+DCIS	2		$pT_3N_{1bii}M_o$	–	–	–	–		
13	68	IDC	2	9/22	$pT_2N_{1biii}M_o$	–	–	–	–		
14	55	IDC	1	2/31	$pT_{1b}N_{1biii}M_o$	–	–	–	–		
15	77	DCIS	3			–	–	–	–		
16	63	IDC	2	1/11	$pT_2N_{1a}M_o$	–	–	–	–		
17	66	IDC	2	0/25	$pT_2N_oM_o$	L	–	–	–		
18	63	ILC	2	0/11	$pT_2N_oM_o$	–	–	–			
19	64	IDC	2	2/12	$pT_2N_{1biii}M_o$	–	–	–			
20	72	IDC	2	0/14	$pT_{1s}N_oM_o$	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.

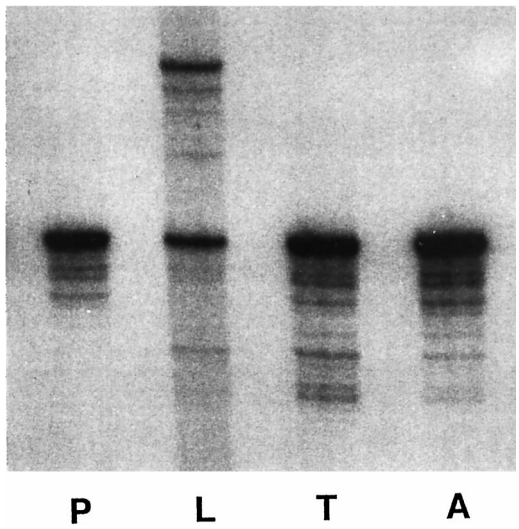

Fig. 1 Autoradiograph from microsatellite analysis. Representative microsatellite analysis of DNA of patient 6 with marker DM1. *P*, plasma; *L*, lymphocyte; *T*, tumor; *A*, normal tissue adjacent to the tumor. Plasma, tumor, and adjacent tissue reveal a loss of the upper allele.

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, histoprognostic 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 l 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/isoamylic alcohol (24:1 v/v) and ethanol precipitation, and finally dissolved in 10 mM Tris (pH 8)-1 mM EDTA diluted five times.

Table 2 Microsatellite alteration of plasma, tumor, and adjacent tissue DNA of breast cancer patients

						DM1			D17S1325			D7S522			D9S169			D10S197			Tp53			ER		
Patient no.	Age (yr)	Type[a]	Grade	Node	pTNM	T	P	A	T	P	A	T	P	A	T	P	A	T	P	A	T	P	A	T	P	A
28	74	IDC	2	1/18	$pT_3N_{1biii}M_o$	–	–	–				–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
29	58	IDC	3	3/21	$pT_2N_{1biv}M_o$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
30	68	IDC	2	0/21	$pT_2N_0M_o$							S	S	–	–	–	–	S	S	–	–	–	–	–	–	–
31	46	IDC	2	5/18	$pT_{4d}N_{biii}M_1$	L	L	L	L	L	–	–	–	–	S	S	S	–	–	–	–	–	–	–	–	–
32	62	IDC	1	0/15	$pT_{1c}N_oM_o$	–	–	–	–			–	–	–	–	–	–	–	–	–	–	–	–	L	L	–
33	50	ILC	2	0/20	$pT_3N_oM_o$	–	–	–	–			–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
34	50	IDC	3	0/17	$pT_{1c}N_oM_o$	L	–	–	–			–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
35	75	IDC	2		$pT_{1c}N_xM_o$	–	–	–	–			–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
36	65	ILC	2	0/21	$pT_2N_oM_o$	–	–	–	–			–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
37	55	IDC	1	1/12	$pT_{1c}N_{1bi}M_o$							–	–	–	–	–	–	–	–	–	L	–	–	–	–	–
38	72	IDC	1		$pT_2N_xM_o$	–	–	–	–			–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; T, tumor DNA; P, plasma DNA; A, adjacent tissue DNA; L, LOH; S, shift (appearance of new bands); –, absence of these findings.

The amount of plasma/serum DNA was estimated by minigel colored with either SYBR green (Molecular Probes, Eugene, OR) or Gelstar nucleic acid gel stain (FMC Bioproducts, Rockland ME).

PCR Amplification. After PCR amplification, plasma or serum DNA was compared to tumor DNA. Lymphocytic DNA of the peripheral blood served as a control of normality.

The following oligonucleotide primers (Amersham-Pharmacia, Freiburg, Germany) were selected according to the Genome Database: D6S311; D7S522; D8S137; D8S321; D9S169; D10S197; D11S488; D13S260; D16S398; D16S402; D16S421; D17S579; D17S1325; Tp 53; Tp53.5; Tp53.6; Tp53.8; ACTBP2; DM1; EABMD; ER; THRA; and UT5320.

For the first 38 patients, we used radioactive autoradiography. Ten and 20 ng of plasma DNA and 20 ng of lymphocyte, tumor, and adjacent normal tissue DNA were used as a template in a hot-start PCR in a 25-μl reaction mixture [1.5 mM $MgCl_2$, 20 μM deoxynucleotide triphosphate, 0.6 unit of Taq polymerase, and 10–20 pmol of each primer (depending on the primer)]. One primer was labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. Lymphocyte, tumor, and plasma DNA was subjected to 30–35 cycles of PCR at 94°C for 1 min, 56°C to 60°C for l min (depending on the primer), and 1 min at 72°C, with a final extension step of 72°C for 10 min, and products were separated by denaturing gel electrophoresis (5.6 M urea, 7% polyacrylamide, and 32% formamide gel) followed by autoradiography (26).

After having checked that the results were comparable, an additional series of 23 patients was studied by laser fluorescence. One primer of each set was labeled with a fluorescent dye (5′Cy 5) at the 5′ end, and all primers were purified by fast protein liquid chromatography. Ten and 20 ng of serum DNA and 20 ng of lymphocyte and tumor DNA were used as a template in a hot-start PCR in a 25-μl reaction mixture [1.5 mM $MgCl_2$, 20 μM deoxynucleotide triphosphate, 0.6 unit of Taq polymerase, and 2–10 pmol of each primer (depending on the primer)]. Each PCR reaction was carried out at 94°C for 5 min, 94°C for 1 min, 55°C to 64°C (depending on the primer) for 1 min, and 72°C for 1 min for 33 cycles, with a final extension of 10 min at 72°C. PCR products were separated electrophoretically on 8% polyacrylamide gels (Reprogel high resolution; Amersham-Pharmacia) and detected by laser fluorescence using an automated gene sequencer (Alfexpress; Amersham-Pharmacia). Fluorescent gel data were analyzed with the Allele-link 2 program. PCR products from lymphocytes and corresponding serum and tumor tissue were analyzed on the same gel. The size (in bp) of the microsatellite alleles was calculated automatically by using internal size markers. Automatic analysis of peak areas allowed relative quantification of PCR products and determination of allelic ratios. LOH was scored if the allelic ratios of tumor or serum PCR products and corresponding lymphocyte PCR products were below a cutoff value of 70% (29). Results indicating LOH were repeated at least twice.

Results

A total of 27 initial cases were studied with two microsatellite markers (DM-1 and D17S1325). Of these 27 patients, 7 had benign tumors, and 20 had malignant tumors (2 had *in situ* tumors and 18 had invasive tumors). Neither tumor nor plasma DNA extracted from patients with benign disease displayed microsatellite alterations (Table 1). In contrast, DNA extracted from 7 of 20 tumors (35%) displayed microsatellite alterations (either LOH or a bandshift; Table 1). Three of these cases also displayed an identical alteration in corresponding plasma DNA. In addition, two cases exhibited identical changes in normal tissue adjacent to the tumor.

Our second series of 11 patients (all of whom had invasive cancer) was studied with 15 microsatellite markers (D6S311, D7S522, D8S137, D9S169, D10S197, D13S260, D16S402, D16S421, D17S1325, D17S579, THRA, ER, EABMD, DM1, and TP53). No LOH was observed in any case with markers D6S311, D8S137, D13S260, D16S402, D16S421, D17S579, THRA, and EABMD. Tumor tissue taken from 5 of the 11 cases (45%) demonstrated an abnormality of at least one of the remaining seven markers (Fig. 1; Table 2). Three of 11 cases (27%) had identical alterations within plasma DNA, and tissue adjacent to one tumor displayed LOH with markers D9S169 and DM1.

The serum of the third group of 23 patients was studied using laser fluorescence on an automatic sequencer. Samples from these cases (19 cases of invasive carcinoma, 2 cases of *in*

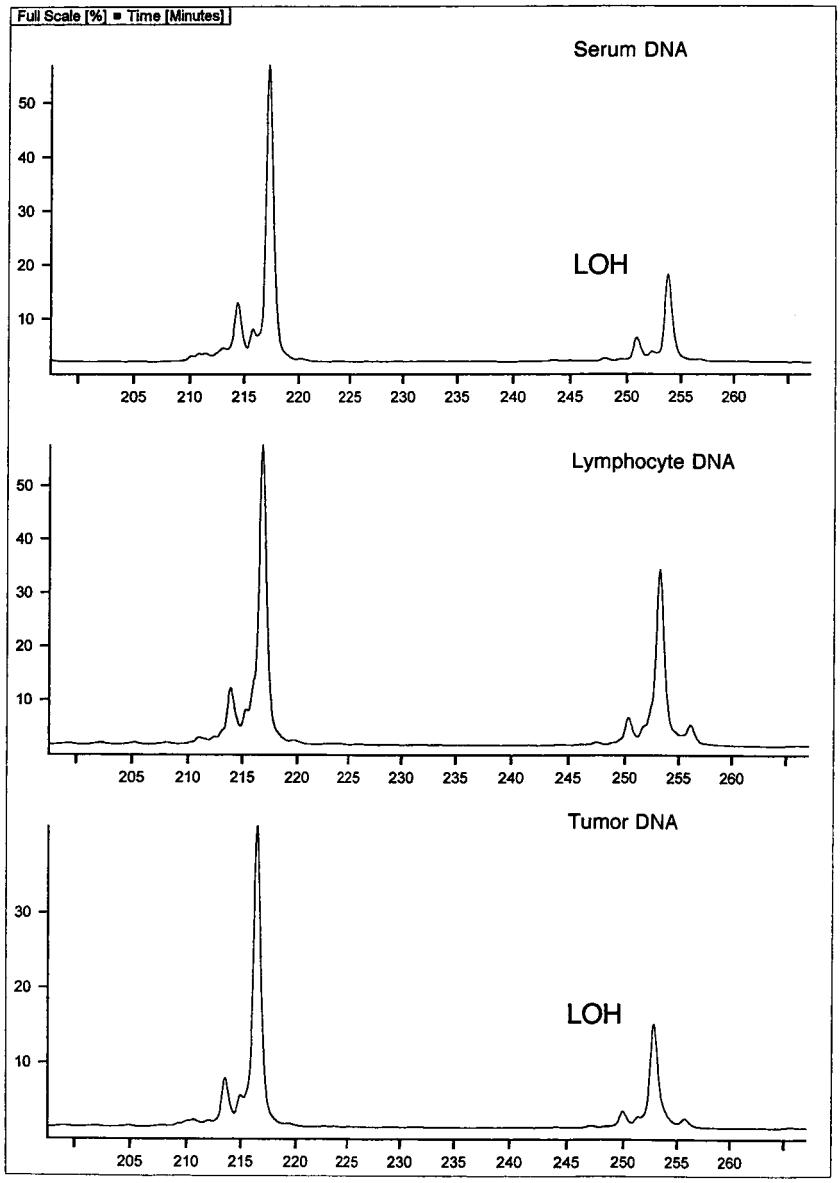

Fig. 2 Original tracings from microsatellite analysis of serum, lymphocyte, and tumor DNA of patient 42 with marker ACTBP2. Plasma and tumor DNA reveal LOH.

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

						D8S321		D11S488		D17S579		D17S1325		ACTBP2		DM1		UT5320	
Patient no.	Age (yr)	Type[a]	Grade	Node	pTNM	T	S	T	S	T	S	T	S	T	S	T	S	T	S
39	57	IDC	2	1/19	$pT_2N_{1bi}M_o$	L	−	−		NI		NI		L	−	−		L	−
40	74	DCIS				−		−		−		NI		−		−		L	L
41	57	IDC	2	0/10	$pT_2N_oM_o$	NI		L	−	−		−		L	−	−		−	
42	54	IDC	3	11/13	$pT_3N_{1biv}M_1$	L	−	NI		NI		NI		L	L	−		L	L
43	57	IDC+LCIS	1	0/11	$pT_{1c}N_oM_o$	−		−		NI		NI		−		−		−	
44	38	IDC	2	5/17	$pT_2N_{1biii}M_o$	−		L	−	NI		NI		L	−	−		−	
45	52	IDC	2	29/29	$pT_2N_{1biv}M_1$	L	−	NI		NI		NI		−		NI		L	L
46	63	DCIS				−		NI		−		−		−		NI		−	
47	68	IDC	2	0/10	$pT_{1c}N_oM_o$	L	−	−		−		NI		−		L	−	L	L
48	44	IDC	2	2/11	$pT_2N_{1biv}M_o$	−		L	−	−		−		−	−	−		−	
49	50	IDC	2	1/15	$pT_2N_{1biii}M_o$	−		NI		L	−	L	−	L	−	NI		−	
50	60	ILC	2	0/15	$pT_{1c}N_oM_o$	−		L	−	NI		−		L	−	NI		−	
51	70	IDC	2		$pT_{1c}N_xM_o$	−		L	−	−		−		L	−	L	L	−	
52	72	IDC	1	0/26	$pT_{1c}N_oM_o$	−		−		−		−		−	−	−	−	−	
53	50	ILC	2	0/13	$pT_{1c}N_oM_o$	−		−		NI		−		−	−	L	L	−	
54	83	IDC	3	0/13	$pT_2N_oM_o$	L	−	−		NI		L	L	NI	NI	L	−	−	
55	57	IDC	2	0/19	$pT_2N_oM_o$	−		−		−		NI		−	−	−		−	
56	71	IDC	3	0/8	$pT_2N_oM_o$	L	−	L	−	L	−	L	−	L	−	NI		L	−
57	82	IDC	3		$pT_2N_xM_o$	NI		L	L	NI		−		−	−	−	−	−	
58	49	IDC	2	7/14	$pT_2N_{1bii}M_o$	L	−	NI	NI			−	−	L	L	NI	NI	L	−
59	64	IDC	2	1/17	$pT_{1c}N_{1a}M_o$	−	−	−	−			−	−	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 preference 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.

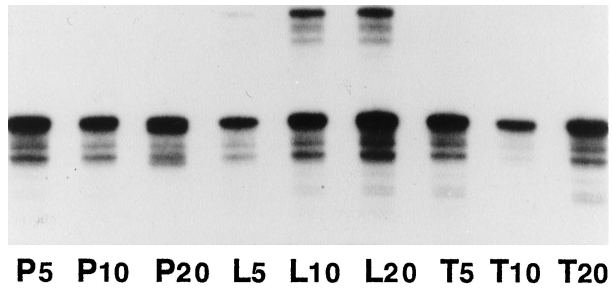

Fig. 3 Autoradiograph from microsatellite analysis. Representative microsatellite analysis of DNA of patient 20 with marker DM1. *P*, plasma; *L*, lymphocyte; *T*, tumor. The amount of DNA (in ng) used in the PCR reaction is designated below each lane (*5*, *10*, and *20*). A false LOH can be seen when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

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 histoprognostic 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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References

1. Wingo, P. A., Ries, L. A., Parker, S. L., and Heath, C. W. Long-term cancer patient survival in the United States. Cancer Epidemiol. Biomark. Prev., *7:* 271–282, 1998.

2. Ransohoff, D. F., and Harris, R. P. Lessons from the mammography screening controversy: can we improve the debate? Ann. Intern. Med., *127:* 1029–2034, 1997.

3. Shapiro, S., Venet, W., Strax, P., Venet, L., and Rooeser, R. Ten to 14 year effects of breast cancer on mortality. J. Natl. Cancer Inst., *69:* 349–455, 1982.

4. Larsson, L. G., Nystrom, L., Wall, S., Rutqvist, L., Andersson, I., Bjurstam, N., Fagerberg, G., Frisell, J., and Tabar, L. The Swedish randomised mammography screening trials: analysis of their effect on the breast cancer related excess mortality. J. Med. Screen., *3:* 129–132, 1996.

5. Kerlikowske, K., Grad, D., and Rubin, S. M. Efficacy of screening mammography a meta-analysis. J. Am. Med. Assoc., *273:* 149–154, 1995.

6. Smart, C. R., Hendrick, R. E., and Rudledge, J. H. Benefit of mammography screening in women aged 40 to 49 years: current evidence from randomized controlled trials. Cancer (Phila.), *75:* 1619–1626, 1995.

7. Wright, C. J., and Mueller, C. B. Screening mammography and public health policy: the need for perspective. Lancet, *346:* 29–32, 1995.

8. Marteau, T. Psychological cost of screening. Br. Med. J., *299:* 527–528, 1989.

9. Kattlove, H., Liberati, A., Keeler, E., and Brook, R. Benefits and costs of screening and treatment for early breast cancer. J. Am. Med. Assoc.**,** *273:* 142–148, 1995.

10. Margolese, R. Screening mammography in young women: a different perspective. Lancet, *347:* 881–882, 1996.

11. Dilhuydy, M. H., and Barreau, B. The debate over mass mammography: is it beneficial for women? Eur. J. Radiol., *24:* 86–93, 1997.

12. Kerangueven, F., Noguchi, T., Coulier, F., Allione, F., Wargniez, V., Simony-Lafontaine, J., Longy, M., Jacquemier, J., Sobol, H., Eisinger, F., and Birnbaum, D. Genome-wide search for loss of heterozygosity shows extensive genetic diversity of human breast carcinomas. Cancer Res., *57:* 5469–5474, 1997.

13. Leon, S. A., Shapiro, B., Sklaroff, D. M., and Yaros, M. J. Free DNA in the serum of cancer patients and the effect of therapy. Cancer Res., *37:* 646–650, 1977.

14. Shapiro, B., Chakrabarty, M., Cohn, E. M., and Leon, S. A. Determination of circulating DNA levels in patients with benign or malignant gastrointestinal disease. Cancer (Phila.), *51:* 2116–2120, 1983.

15. Fournié, G. J., Courtin, J. P., and Laval, F. Plasma DNA as a marker of cancerous cell death. Investigation in patients suffering from lung cancer and in nude mice bearing human tumour. Cancer Lett., *2:* 221–227, 1995.

16. Stroun, M., Anker, P., Lyautey, J., Lederrey, C., and Maurice, P. Isolation and characterization of DNA from the plasma of cancer patients. Eur. J. Cancer Clin. Oncol., *28:* 707–712, 1987.

17. Stroun, M., Anker, P., Maurice, P., Lyautey, J., Lederrey, C., and Beljanski, M. Neoplastic characteristics of the DNA found in the plasma of cancer patients. Oncology (Basel), *46:* 318–322, 1989.

18. Vasyukhin, V., Anker, P., Maurice, P., Lyautey, J., Lederrey, C., and Stroun, M. Point mutations of the N-*ras* in the blood plasma DNA of patients with myelodysplastic syndrome or acute myelogenous leukaemia. Br. J. Haematol., *86:* 774–779, 1994.

19. Sorenson, G. D., Pribish, D. M., Valone, F. H., Memoli, V. A., Bzik, D. J., and Yao, S. L. Soluble normal and mutated DNA sequences from single-copy genes in human blood. Cancer Epidemiol. Biomark. Prev., *3:* 67–71, 1994.

20. Anker, P., Lefort, F., Vasioukhin, V., Lyautey, J., Lederrey, C., Chen, X. q., Stroun, M., Mulcahy, H. E., and Farthing, M. J. G. K-ras gene mutations in the plasma of colorectal cancer patients. Gastroenterology, *112:* 1114–1120, 1997.

21. Mulcahy, H., Anker, P., Lyautey, J., Chen, X. q., Lederrey, C., Farthing, M. J. G., and Stroun, M. K-ras gene mutations in the plasma of pancreatic patients. Clin. Cancer Res., *4:* 271–275, 1998.

22. Kopreski, M. S., Benko, F. A., Kwee, C., Leitzel, K., Eskander, E., Lipton, A., and Gocke, C. D. Detection of mutant K-ras DNA in plasma or serum of patients with colorectal cancer. Br. J. Cancer, *76:* 1293–1299, 1997.

23. de Kok, J. B., van Solinge, W. W., Ruers, T. J., Roelofs, R. W., van Muijen, G. N., Willems, J. L., and Swinkels, D. W. Detection of tumour

DNA in serum of colorectal cancer patients. Scand. J. Clin. Lab. Investig., *57:* 601–604, 1997.

24. Hibi, K., Robinson, C. R., Booker, S., Wu, L., Hamilton, S. R., Sidransky, D., and Jen, J. Molecular detection of genetic alterations in the serum of colorectal cancer patients. Cancer Res., *58:* 1405–1407, 1998.

25. Silva, J. M., Gonzalez, R., Dominguez, G., Garcia, J. M., Espana, P., and Bonilla, F. TP53 gene mutations in plasma DNA of cancer patients. Genes Chromosomes Cancer, *24:* 160–161, 1999.

26. Chen, X. q., Stroun, M., Magnenat, J. L., Nicod, L. P., Kurt, A. M., Lyautey, J., Lederrey, C., and Anker, P. Microsatellite alterations in plasma DNA of small cell lung cancer patients. Nat. Med., *2:* 1033–1035, 1996.

27. Sanchez-Cespedes, M., Monzo, M., Rosell, R., Pifarré, A., Calvo, R., Lopez-Cabrerizo, M. P., and Astudillo, J. Detection of chromosome 3p alterations in serum DNA of non-small-cell lung cancer patients. Ann. Oncol., *9:* 113–116, 1998.

28. Nawroz, H., Koch, W., Anker, P., Stroun, M., and Sidransky, D. Microsatellite alterations in plasma DNA of head and neck cancer patients. Nat. Med., *2:* 1035–1037, 1996.

29. Goessl, C., Heicapell, R., Münker, R., Anker, P., Stroun, M., Krause, H., Müller, M., and Miller, K. Microsatellite analysis of plasma DNA from patients with clear cell renal carcinoma. Cancer Res., *58:* 4728–4732, 1998.

30. Sanchez-Cespedes, E. M., Rosell, M., Sidransky, D., Baylin, S. B., and Herman, J. G. Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from non-small cell lung cancer patients. Cancer Res., *59:* 67–70, 1999.

31. Wong, I. H., Lo, Y. M., Zhang, J., Liew, C. T., Ng, M. H., Wong, N., Lai, P. B., Lau, W. Y., Hjelm, N. M., and Johnson, P. J. Detection of aberrant p16 methylation in the plasma and serum of liver cancer patients. Cancer Res., *59:* 71–73, 1999.

32. Futreal, P. A., Söderksvist, P., Marks, J. R., Iglehart, J. D., Cochran, C., Barret, J. C., and Wiseman, R. W. Detection of frequent allelic loss on proximal chromosome 17q in sporadic breast carcinoma using microsatellite length polymorphisms. Cancer Res., *32:* 2624–2627, 1992.

33. Radford, D. M., Fair, K. L., Phillips, N. J., Ritter, J. H., Steinbrueck, T., Holt, M. S., and Donis-Keller, H. Allelotyping and ductal carcinoma in situ of the breast: deletion of loci on 8p, 13q, 16q, 17p and 17q. Cancer Res., *55:* 3399–3405, 1995.

34. Zenklusen, J., Bièche, I., Lidereau, R., and Conti, C. J. (C-A)n microsatellite repeat D7S522 is the most commonly deleted region in human primary breast cancer. Proc. Natl. Acad. Sci. USA, *91:* 12155–12158, 1994.

35. Devilee, P., Hermans, J., Eyfjörd, A. L., Borresen, R., Lidereau, R., Sobol, H., Borg, A., Cleton-Jansen, A. M., Olàh, E., Cohen, B. B., Scherneck, S., Hamann, U., Peterlin, B., Caligo, M., Bignon, Y. J., and Maugard, C. Loss of heterozygosity at 7q31 in breast cancer: results from an international collaborative study group. Genes Chromosomes Cancer, *18:* 193–199, 1997.

36. Patel, U., Grundfest-Broniatowski, S., Gupta, M., and Banerjee, S. Microsatellite instabilities at five chromosomes in primary breast tumors. Oncogene, *12:* 3695–3700, 1994.

37. Chuaqui, R. F., Sanz-Ortega, J., Vocke, C., Linehan, W. M., Sanz-Esponera, J., Zhuang, Z., Emmert-Buck, M. R., and Merino, M. J. Loss of heterozygosity on the short arm of chromosome 8 in male breast carcinomas. Cancer Res., *55:* 4995–4998, 1995.

38. Radford, D. M., Fair, K. L., Phillips, N. J., Ritter, J. H., Steinbrueck, T., Holt, M. S., and Donis-Keller, H. Allelotyping of ductal carcinoma *in situ* of the breast: deletion of loci on 8p, 13q, 17p, and 17q. Cancer Res., *55:* 3399–3405, 1995.

39. Radford, D., Philippe, N. J., Fair, K. L., Ritter, J. H., Holt, M., and Donis-Keller, H. Allelic loss and the progression of breast cancer. Cancer Res., *55:* 5180–5183, 1995.

40. Dorion-Bonnet, F., Mautalen, S., Hostein, I., and Longy, M. Allelic imbalance study of 16q in human primary breast carcinomas using microsatellite markers. Genes Chromosomes Cancer, *14:* 171–181, 1995.

41. Skirnisdottir, S., Eiriksdottir, G., Baldursson, T., Barkardottir, R. B., Egilsson, V., and Ingvarrson, S. High frequency of allelic imbalance at chromosome region 16q22–23 in human breast cancer: correlation with high PgR and low S phase. Int. J. Cancer, *64:* 112–116, 1995.

42. Fujii, H., Szumel, R., Marsh, C., Zhou, W., and Gabrielson, E. Genetic progression, histological grade, and allelic loss in ductal carcinoma *in situ* of the breast. Cancer Res., *56:* 5260–5265, 1996.

43. Shaw, J. A., Walsh, T., Chappell, S. A., Carey, N., Johnson, K., and Walker, R. A. Microsatellite instability in early sporadic breast cancer. Br. J. Cancer, *73:* 1393–1397, 1996.

44. Ahrendt, S. A., Chow, J. T., Xu, L. H., Yang, S. C., Eisenberger, C. F., Esteller, M., Herman, J. G., Wu, L., Decker, P. A., Jen, J., and Sidransky, D. Molecular detection of tumor cells in bronchoalveolar lavage fluid from patients with early stage lung cancer. J. Natl. Cancer Inst., *91:* 332–339, 1999.

45. Boland, C. R., Thibodeau, S. N., Hamilton, S. R., Sidranski, D., Eshleman, J. R., Burt, R. W., Meltzer, S. J., Rodriguez-Bigas, M. A., Fodde, R., Ranzani, G. N., and Srivastava, S. A National Cancer Institute workshop on microsatellite instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res., *58:* 5248–5257, 1998.

46. Deng, G., Lu, Y., Zlotnikov, G., Thor, A. D., and Smith, H. S. Loss of heterozygosity in normal tissue adjacent to breast carcinomas. Science (Washington DC), *274:* 2057–2059, 1996.

47. McCulloch, R. K., Sellner, L. N., Papadimitriou, J. M., and Turbett, G. R. The incidence of microsatellite instability and loss of heterozygosity in fibroadenoma of the breast. Breast Cancer Res. Treat., *49:* 165–169, 1998.

48. James, V., Kearsley, J., Irving, T, Amemiya, Y., and Cookson, D. Using hair to screen for breast cancer. Nature (Lond.), *398:* 33–34, 1999.

Clinical Cancer Research

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