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13. ABSTRACT (Maximum 200 Words)

The importance of DNA abnormalities to tumorigenesis is not in doubt but their clinical utility has not yet been fully elucidated. We performed a pilot study to evaluate detection of occult circulating tumor DNA using tumor-specific microsatellite alterations in subjects previously diagnosed with breast cancer. Using 22 microsatellite markers located at sites of frequent loss of heterozygosity (LOH) in breast cancer, we analyzed DNA from 16 primary tumors (Stage IIA or more advanced) and 30 longitudinally collected plasma specimens. Clinical data at time of plasma collection was obtained. Every tumor (16/16, 100%) was characterized by an individual pattern of LOH, its LOH "fingerprint." LOH in plasma DNA was detected in 12/30(40%) plasma DNA samples, from 8/14(57%) subjects. However, the number of LOH in plasma DNA was small (n=15), and the mean proportional LOH was much lower than in tumors (0.05vs0.52). Although they were infrequent, most LOHs in plasma DNA (12/15, 80%) were concordant with abnormalities in the paired tumors, suggesting that they were authentic tumor-derived abnormalities. Despite this, we found no association between plasma DNA LOH and original tumor stage or clinical status at time of blood collection (i.e., LOH was seen as often in subjects who had no evident disease as in those with evident disease). In addition, detection of LOH was not consistent between serial samples from half of subjects (5/11, 45%), despite stable clinical condition. No association with clinical outcome was evident, although the sample size may be too small to be conclusive. Microsatellite instability (MI) in plasma DNA was infrequent, non-concordant with paired tumor, and inconsistent in serial samples. The results of this study suggest that detecting microsatellite abnormalities in plasma DNA of breast cancer subjects may not be useful either for detecting occult metastases or for monitoring disease. Other techniques may be more promising but it is possible that circulating tumor DNA does not reflect tumor biology.

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Introduction

Cancer is a genetic disease, believed to develop as genetic alterations arise, each conferring some growth advantage to the cell in which it occurs. Numerous DNA alterations affecting oncogenes and tumor suppressor genes relevant to the pathogenesis of breast cancer have been identified. Although their importance to tumorigenesis is not in doubt, the clinical utility of identified abnormalities has not yet been fully elucidated. We proposed to investigate whether a tumor's individual pattern of genetic abnormalities could be used as a molecular marker to detect unrecognized neoplastic tissue. Recent studies had detected tumor-specific genetic abnormalities in the peripheral blood of patients with lung, head and neck and colorectal carcinoma. These findings lead us to hypothesize that a well-selected panel of molecular markers may be used to detect circulating DNA from breast cancer cells. These cells may be from sites of known disease, or from unrecognized occult sites. Detection of occult tumor cells could dramatically alter the staging, treatment and prognosis of breast cancer patients.

To investigate our hypothesis, we proposed, 1) to use a panel of 20 breast-tissue specific molecular markers, that we had already defined, to identify a unique genetic fingerprint for each of 40 primary breast tumors from women with locally advanced or metastatic disease. This fingerprint would consist of each tumor's individual pattern of allele imbalances, otherwise known as loss of heterozygosity (LOH) or novel microsatellite alleles, otherwise known as microsatellite instability (MI); 2) to investigate how often the tumor's unique genetic fingerprint can be detected in these patients' peripheral blood; 3) to examine whether detection of a circulating, tumor-specific genetic fingerprint can identify the presence of tumor cells patients with otherwise unrecognized, occult disease. If so, use of this straightforward genetic fingerprinting technique might eventually enhance existing diagnostic methodology, more accurately predict prognosis, and influence treatment decisions for women diagnosed with breast cancer.

Body

Research accomplishments associated with each *Task* in approved Statement of Work.

Task 1: Subject enrollment and collection of serial plasma samples (Months 1-24)

Work on this task is completed.

A total of 21 subjects with metastatic or locally advanced breast cancer were enrolled. Approximately half of the subjects had locally advanced tumors, the other half had metastatic disease. As noted in last year's annual report, we anticipated that we would not

be able to enroll our original target of 40 subjects because evaluating the increasing number of serial plasma samples and determining the clinical status of enrolled subjects at numerous time points has been more time consuming than we anticipated. Evaluating the serial plasma samples and obtaining clinical correlations are unique aspects of this study and merit the unexpected effort. Because 40 subjects was originally selected as an estimate of what might be possible, and not because of statistical considerations, we do not feel that this should substantially impact the validity of our final conclusions. From the enrolled subjects, we obtained analyzable DNA from 16 primary tumors (Stage IIA or more advanced) and 30 longitudinally collected plasma specimens.

Task 2: Genetic fingerprinting (Months 4-32)

Work on this task is completed.

a. Separation of mononuclear cells (MNC) from plasma and/or serum, extraction of DNA.
We try to obtain blood samples using a 19-gauge needle. However, because the samples are often drawn in the clinic from implanted phlebotomy access devices (portacaths), or from patients with relatively poor venous access because of chemotherapy, age, other medical problems, we sometimes have to obtain blood using a needle of the next smallest size, i.e., a 21-gauge. Although not optimal, this size needle approximates what has been reported in the literature and, importantly, this size needle is what would be most commonly used if peripheral blood fingerprinting is tested more widely. After the blood is obtained, we have had no difficulty separating MNCs from plasma and extracting DNA from either type of sample using the commercially available Qiagen kit.

b. Retrieval of archival tissue blocks; review of slides with pathologist; additional slide cutting and staining, and reexamination; microdissection of desired tumor sections; DNA extraction.
We have been able to locate archival tissue blocks for all subjects enrolled, identify areas enriched for tumor, microdissect these areas using the laser capture microdissection apparatus and extract the cells' DNA with little difficulty. We have found few samples whose DNA is too degraded to be useful. We microdissected and analyzed multiple tumor samples from each subject, in order to minimize the possibility of missing an abnormality in the tumor that might be apparent in the plasma. Each independently microdissected tumor specimen is identified as a separate specimen (i.e., T_1, T_2, etc).

c. *Fingerprinting of normal (mononuclear cell), tumor, and plasma and/or serum DNA.*
Using 22 microsatellite markers located at sites of frequent loss of heterozygosity (LOH) in breast cancer, we fingerprinted tumor, MNC and plasma samples. No unexpected difficulties were encountered. Consistent with what has been reported in the literature, we found that all tumors demonstrate LOH, and that the tumors' proportional LOH (pLOH)

(pLOH= # arms with LOH / # informative arms) = 52%. Also consistent with previous reports, we found only a few instances of microsatellite instability (MI).
Of considerable interest is our observation that every tumor was characterized by its own unique genetic fingerprint. No 2 tumors were alike. In fact, in 2 subjects who each had 2 tumors, each tumor had an individual fingerprint. This observation has led to a separate, but related, project being developed by another member of the PI's laboratory, whose goal is to develop a quantifiable genetic test, based on assessment of allelic imbalances, to distinguish new primary breast cancers from disease recurrence (see **Reportable Outcomes**).

d. *Data analysis by visual inspection and densitometry*
Most examples of loss of heterozygosity (LOH) seen in tumor tissue are visually unambiguous. Many potential plasma abnormalities are more subtle, however, and we used densitometry to scan a large proportion of candidate LOHs. LOH in either tumor or plasma is defined as a reproducible 25% change in allele intensity compared with normal (MNC), using the equation: $n_2/n_1 \times t_1/t_2$, where n_1 = MNC's larger allele, n_2 = MNC's smaller allele, t_1 = tumor or plasma's larger allele, t_2 = tumor or plasma's smaller allele. Novel microsatellite alleles were scored by visual inspection.

e. Preliminary determination of proportion of patients with detectable tumor-specific fingerprints in circulation.
LOH in plasma DNAwas fairly common: it was detected in 12/30 (40%) plasma DNA samples, from 8/14 (57%) subjects. However, the number of LOH in plasma DNA was small (n = 15), and the mean proportional LOH was much lower than in tumors (0.05 *vs* 0.52). Although they were infrequent, most LOHs in plasma DNA (12/15, 80%) were concordant with abnormalities in the paired tumors, suggesting that they were authentic tumor-derived abnormalities.

As explained in a previous report, because the number of LOHs in plasma is much less than in tumor, we reevaluated the sensitivity of our assay. We performed a mixing study of normal and tumor DNA and confirmed that we can detect tumor DNA when it constitutes 25% of total DNA. It is possible that we can detect tumor DNA when it constitutes even less. These data suggest that when collected using standard, clinically applicable techniques, relatively little plasma DNA is derived from the tumor.

In contrast to LOH, microsatellite instability (MI) in plasma DNA was infrequent, non-concordant with paired tumor, and inconsistent in serial samples.

Task 3: *Determine efficacy of fingerprinting to detect occult disease (Months 6-36).*

Work on this task is completed.

a. Preparation of clinical data sheet.
A template was prepared in the first year of the award to record clinical and genetic data for each subject. A sample was included in the first annual report.

b. Recording of clinical information via examination of records.
Charts are reviewed each time a subject returns to clinic to determine whether any significant clinical events have occurred in the interval.

c. Evaluation of whether circulating tumor DNA can identify neoplasia: final determination of proportion of subjects with detectable tumor-specific genetic fingerprints in the peripheral circulation, and association with clinical status.
We found no association between plasma DNA LOH and original tumor stage or clinical status at time of blood collection (i.e., LOH was seen as often in subjects who had no evident disease as in those with evident disease). In addition, detection of LOH was not consistent between serial samples from half of subjects (5/11, 45%), despite stable clinical condition. No association with clinical outcome was evident, although the sample size may be too small to be conclusive. Microsatellite instability (MI) in plasma DNA was infrequent, non-concordant with paired tumor, and inconsistent in serial samples.

In conclusion: the results of this pilot study suggest that detecting microsatellite abnormalities in plasma DNA of breast cancer subjects may not be clinically useful either for detecting occult metastases or for monitoring disease. Other techniques may be more promising, but it is possible that circulating tumor DNA does not reflect tumor biology.

Key Research Accomplishments

1. Successful testing of hypothesis that detection of circulating tumor DNA might be clinically useful. The conclusion of this study is that evaluation of circulating tumor DNA using LOH analyses is **not** likely to be useful to evaluate the potential presence of clinically significant occult disease; a related conclusion is that there appear to be a considerable fraction of subjects with clinically obvious disease who have little or no plasma DNA detectable.

2. An observation made during these investigations, i.e., that each tumor has its own, individual LOH fingerprint, is provocative and has generated new testable hypotheses about the role of LOH fingerprinting in human breast cancer diagnosis and treatment.

Reportable Outcomes

1. Presentation of data at Department of Defense Breast Cancer Research Program's Era of Hope Conference, September 25-28, 2002 (Orlando, Florida). Abstract was included with last year's report.

2. Publication of a manuscript describing the study's results. A copy of this MS is included in the **Appendix**.

3. Funding of new grant application from the Susan G Komen Breast Cancer Foundation to examine DNA fingerprinting of breast tumors in another context, based on observations made during the course of this award.

Conclusions

The question posed (whether tumor-specific DNA products can be detected in the blood of women with locally advanced or metastatic cancer, and whether their detection can identify occult disease) appears to have been answered, i.e., in this pilot study, we found that although tumor DNA circulates, it constitutes a small proportion of total DNA in cancer patients and its detection is not necessarily associated with disease burden. Therefore, further refinements to this technique, or use of more sensitive techniques may be necessary for screening to be of clinical utility. An observation made during these investigations, i.e., that each tumor has its own, individual LOH fingerprint, is provocative and has generated new testable hypotheses about the role of LOH fingerprinting in human breast cancer diagnosis and treatment.

References

An extensive list of references related to work related to this project are included in the MS attached in the appendix.

Appendix

Publication:

1. Wang Q, Larson PS, Schlechter BL, Zahid N, Finnemore E, de las Morenas A, Blanchard RA, **Rosenberg CL**. Microsatellite Alterations In Serial Plasma DNA Samples During Follow-up Of Women With Breast Cancer. Int J Cancer 2003: 106: 923-929.

Int. J. Cancer: **106**, 923–929 (2003)

Publication of the International Union Against Cancer

LOSS OF HETEROZYGOSITY IN SERIAL PLASMA DNA SAMPLES DURING FOLLOW-UP OF WOMEN WITH BREAST CANCER

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We evaluated the potential utility of occult circulating tumor DNA as a molecular marker of disease in subjects previously diagnosed with breast cancer. Using 24 microsatellite markers located at sites of frequent loss of heterozygosity (LOH) or allele imbalance in breast cancer, we analyzed DNA from 16 primary tumors (Stage IIA or more advanced) and 30 longitudinally collected plasma specimens. Clinical data at the time of plasma collection were obtained. All 16 tumors were characterized by an individual pattern of LOH. LOH was detected in 12 of 30 (40%) plasma samples, taken from 8 of 14 (57%) subjects. However, the number of LOH in plasma was small (n = 15), and the mean proportion of LOH was much lower than in the tumors (0.05 *vs.* 0.52). Although infrequent, 12 of 15 (80%) plasma LOH were concordant with abnormalities in the paired tumors, and the mean percent LOH was higher than in normal plasmas, suggesting that they were authentic tumor-derived abnormalities. We found, despite this, no association, between plasma LOH and tumor stage or clinical status at time of blood collection (i.e., LOH was as common in subjects with no evident disease as in those with evident disease). In addition, detection of LOH was not consistent between serial samples from 5 of 11 subjects (45%), despite stable clinical conditions. No association with clinical outcome was evident, although the sample size was small. Microsatellite instability in plasma was infrequent, nonconcordant with paired tumor and inconsistent in serial samples. This pilot study suggests that identifying tumor-specific LOH in the plasma of breast cancer subjects may not be useful for detecting occult metastases or for monitoring disease. Other detection techniques may be more promising, but circulating tumor DNA may not be a sufficiently accurate reflection of breast cancer clinical status or tumor activity.

Key words: *detection; microsatellite; prognosis; peripheral blood; staging; breast cancer; loss of heterozygosity*

Improved noninvasive testing to detect cancer or predict disease outcome would be very useful. One logical approach to achieve this goal has been to investigate tumor-specific DNA that circulates in the peripheral blood. Early studies showed that free soluble DNA that is released from cells under normal and pathologic conditions circulates in the plasma.[1,2] Recently, tumor-specific microsatellite DNA alterations were proposed as clonal markers for detecting malignant disease.[3] Numerous studies that investigate subjects with a variety of cancers have identified these abnormalities in accessible body fluids such as blood, urine, sputum and stool.[4–6] Use of microsatellite alterations as tumor markers is particularly attractive in breast cancer. First, the disease is genetically, pathologically and clinically heterogeneous, and existing prognostic markers are imperfect. Second, the disease can spread hematogenously. Third, breast cancers lack a signature genetic abnormality, but nearly all tumors are characterized by microsatellite alterations, specifically allele imbalance (AI) or loss of heterozygosity (LOH) at multiple loci.[7,8] Nearly every breast tumor has an individual pattern of AI/LOH,[7] which constitutes its "fingerprint." Taken together, it is not surprising that several reports identify microsatellite alterations in the peripheral blood of patients with breast cancer.[9–14]

In those reports, LOH is seen in plasma or serum DNA of breast cancer subjects, with prevalence ranging between 18–66% of cases.[9–14] Novel microsatellite alleles, representing micro-satellite instability (MI) are occasionally seen. In contrast, LOH or MI are rarely, if ever, detected in normal controls' plasma DNA.[11,13,15–17] Blood samples are usually collected at a single time point, either pre-[10,12–14] or postoperatively,[11] from subjects who usually have early stage disease, although some with advanced disease have been included. The proportion of LOH (pLOH) detected in primary tumors varies (12–90% of informative sites), as does the concordance of LOH (i.e., matched LOH) between tumor and blood DNA (estimated between 33–100%[9–11,14]).

Important questions remain about the utility of this approach in breast cancer. Associations between circulating microsatellite alterations and adverse pathologic parameters are noted,[11,12] but follow-up data linking circulating microsatellite alterations to clinical status is preliminary.[14] Evaluation of circulating DNA alterations in subjects whose blood is repeatedly sampled over time has not been performed. The proportion of subjects with known metastatic disease (mets) who have plasma DNA LOH is not known. Finally, more DNA often circulates in the plasma of subjects with cancer than normal controls,[1,15,18,19] but whether the amount fluctuates with disease stage or activity is unclear. The relative proportions of tumor *vs.* normal DNA have only recently been addressed.[20]

To investigate these issues, we evaluated microsatellite alterations in serial plasma DNA samples collected during follow-up of women previously diagnosed with breast cancer. Using 24 microsatellite markers on 11 chromosomal arms that are located at frequent sites of LOH in breast tumors, we generated a fingerprint

Abbreviations: AI, allele imbalance; CI, confidence interval; +DS, clinically evident disease; LOH, loss of heterozygosity; LOH+, plasma DNA with LOH; LOH-, plasma DNA without LOH; mets, metastatic disease; MI, microsatellite instability; MNC, mononuclear cell; NED, no evident disease; pLOH, proportion of LOH. No commercial sponsorship or affiliations, stock/equity interests and patent licenses exist that constitute real or apparent conflict of interest with the work presented in this study.

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Qiu Wang, Pamela Larson and Benjamin Schlechter contributed equally to this work.

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for each of 16 primary breast tumors from 14 subjects and available nodal or distant metastatic sites. Using the same markers, we then examined 30 plasma DNA samples collected longitudinally during routine follow-up; the subjects' clinical status was known at the time of each collection. Our goals were to determine: *(i)* how frequently plasma DNA microsatellite abnormalities are present in subjects previously diagnosed with Stage II-IV breast cancer; *(ii)* whether plasma DNA and tumor DNA abnormalities were concordant; *(iii)* whether detection of abnormalities correlated with clinical status at the time of each blood collection (i.e., no evident disease [NED] or clinically evident disease [+DS]); *(iv)* whether detection of abnormalities remained constant during follow-up; and *(v)* if the presence of abnormalities at any collection might correlate with outcome.

MATERIAL AND METHODS

Subjects

Twenty-one subjects with breast cancer were enrolled after signing an informed consent approved by our Institutional Review Board. Only women with Stage I disease were ineligible because they were unlikely to relapse or have occult metastases. Because of tumor DNA degredation or small tumor size, we could fully evaluate 16 tumors from 14 subjects; 2 subjects had synchronous bilateral cancers. Five anonymous healthy female volunteers each provided 10 ml of blood for analysis as normal control samples.

Specimens

An amount of 10–15 ml of blood was collected in routine EDTA-containing tubes at the time of enrollment. After that, it was collected at 1–7 month intervals, except 2 samples were collected at 11 and 54 month intervals. To minimize plasma contamination by peripheral blood mononuclear cells (MNCs), specimens were collected using 19-, 20- or 21-gauge needles, stored on ice and processed within 2 hr. The subjects' clinical status at the time of each blood collection was designated as either NED or as +DS based on standard diagnostic evaluations performed by the treating oncologist. All but one +DS subject had documented distant metastases: #17 had an axillary recurrence (nodal). Cancer treatment was also recorded as on *vs.* off. Treatments included chemo-, hormonal or radiation therapy, and anti-Her2/Neu antibody. Formalin-fixed and paraffin-embedded tissue blocks from the original diagnostic procedure and available subsequent surgeries were retrieved from the Pathology Department archives.

DNA

MNCs were separated from plasma by diluting whole blood 1:1 with cold PBS and then centrifugation over Ficoll-Paque (Amersham, Piscataway, NJ). Using a glass pipette and leaving behind a visible interface, first plasma, then MNCs, were isolated. DNA was extracted from each via solid phase extraction with a commercially available column (QiAmp Blood Kit; Qiagen, Valencia, CA), with which we have previous experience.[21] Slides of the primary cancer plus any involved nodes or distant metastatic sites were reviewed by a single experienced breast pathologist to identify areas enriched in tumor cells. Serial sections were cut from the desired blocks and areas of interest were microdissected via laser capture (Arcturus Engineering, Mountain View, CA). DNA was extracted from the captured cells with techniques used previously.[22,23] When tumors were sufficiently large, multiple microdissected samples were examined independently in order to detect any distinct coexisting clones. MNC DNA was quantitated by spectrophotometry. DNA from a subset (~50%) of tumor and plasma samples (generally those collected during the latter half of the study) was quantitated fluorometrically (PicoGreen dsDNA Quantitation Kit; Molecular Probes, Eugene, OR).

Microsatellite selection

Twenty-four markers on 11 chromosome arms were selected for high percent heterozygosity, small size of amplified fragment and location at sites frequently undergoing LOH/AI in breast cancer. Markers at regions not believed relevant to breast tumorigenesis were also included. Markers used were 1p(468), 1q(549, 213), 3p(1283), 7q(486, 796), 9p(156), 11p(THO1, 2071), 11q(PYGM, 1818, 1819), 16q(265, 512, 402, 413), 17p(TP53, 796, 525, VNTR), 17q(1290, 855, 579) and Xq (AR). Primers were obtained from Research Genetics (Huntsville, AL) and Marshall University (Huntington, WV).

PCR/electrophoresis

Six multiplexed PCRs were performed using 30–35 cycles of amplification, incorporation of α^{32}P-dCTP and annealing temperatures between 55–60°C. When DNA quantitation was available, 0.5 ng of template was used per PCR. When DNA quantitation was not available (i.e., in a subset of tumor and plasma samples), the template used was a volume of plasma DNA solution that yielded approximately the same signal intensity as the MNCs on the autoradiograph. One-third of the amplified products was electrophoresed through 7% denaturing acrylamide gels that were then exposed to autoradiography film.

Determination of LOH or MI

Autoradiographs were scanned by a laser densitometer (Molecular Dynamics, Sunnyvale, CA) to calculate relative ratios of allele intensity in MNC, tumor and plasma samples. The normal pattern at each microsatellite in each individual was defined as the pattern in MNCs. LOH in either tumor or plasma DNA was defined at heterozygous loci as imbalance of allele intensities greater than 25%, i.e., when $(n1)(t2)/(n2)(t1) > 1.33$ or < 0.75, where n1 = normal samples' larger allele, n2 = normal samples' small allele, t1 = test samples' larger allele, t2 = test samples' smaller allele. The pLOH was calculated as: # LOH / # informative sites. MI was defined as reproducible presence of a novel-sized allele upon visual inspection of autoradiographs. All abnormal results were documented at least twice.

Plasma DNA control study

MNC and plasma samples were isolated from 5 anonymous healthy female volunteers with no history of cancer. DNA was extracted and quantitated as described above. Triplicate PCRs were performed on each of the 5 MNC and 5 plasma DNAs using 3 microsatellite markers (D1s549, D17s579 and D17s525) and 0.5 ng template. The normal allele ratio was derived from each controls' MNC sample.

Mixing study

One subject's tumor and uninvolved lymph node DNA, which served as normal, were quantified by fluorometry. A series of solutions was made, each containing a total of 0.5ng of DNA, but comprised of different proportions of normal and tumor DNA (0.5ng + 0.0ng, 0.45ng + 0.05ng, 0.375ng + 0.125ng, 0.25ng + 0.25ng, 0.0ng + 0.05ng). These solutions were used as PCR templates for amplification by 2 markers, D17s1290 (on 17q) and VNTR (on 17p).

Statistical analyses

The chi-square test was used to determine whether clinical status at the time of blood collection was associated with LOH in that plasma. The confidence interval (CI) for the mean percent LOH in the plasma samples was calculated and compared to the estimated false positive percent LOH in the normal controls. The mean percent LOH in the 30 plasma samples equaled the mean value of each sample's (# LOH/ # informative loci) × 100.

RESULTS

Subjects and samples

From 14 subjects with 16 primary tumors (Stage IIA or more advanced), 30 blood samples were collected longitudinally during routine follow-up for disease. Subjects were heterogeneous, reflecting the spectrum of disease (see Table I). Using 50 years as a surrogate for menopause, 5 subjects were premenopausal, 3 were

TABLE I – CLINICAL CHARACTERISTICS AND LOH IN PLASMA AND TUMOR DNA

Subject number	Stage	Samples		Clinical status at plasma collection	# LOH/ # informative sites		Ongoing therapy	Sites of LOH	Concordance of plasma LOH with tumor LOH
		Tumor	Plasma		Tumor	Plasma			
17	IIIA	T1			5/12				
			P1	Nodal		0/12	On		
			P2	NED		1/10	On	17pVNTR	N
34	IIA	T1-2 (L)[1]			7.5/13[2]				
	IIA	T1-3 (R)			10/13				
			P1	NED		1/13	Off	11p2071	Y (R)
50	IV	T1-3			5/6				
			P1	Mets		0/6	On		
51	IIIB	T1-2			2/5				
			P1	NED		0/3	On		
			P2	NED		0/5	On		
52	IIIA	T1-3			5/16				
			P1	Mets		0/16	On		
			P2	Mets		0/16	On		
			P3	Mets		0/12	On		
53	IIB	T1			7/13				
			P1	NED		0/13[3]	On		
			P2	Mets		0/12	Off		
54	IIB	T1-3			6/13				
			P1	NED		0/13	On		
			P2	NED		0/11	On		
			P3	NED		1/10	On	17p796	Y
			P4	NED		0/13	On		
55	IIB	T1			4/9				
			P1	Mets		0/12	On		
			P2	Mets		0/6	On		
56	IIA	T1-2			8/11				
			P1	Mets		0/11	On		
			P2	Mets		1/11	On	16q402	Y
59	IIA	T1-3			9/12				
			P1	Mets		1/12	On	17q579	Y
			P2	Mets		0/12	On		
			P3	Mets		1/12	On	17q579	Y
60	IV	T1-3			5/8				
			P1	Mets		1/8	On	17p796	Y
			P2	Mets		1/8	On	17p796	Y
			P3	Mets		1/7	On	17p796	Y
65	IIB	T1-3 (R)			1/14				
	IIIA	T1-6 (L)			1/14				
			P1	Mets		1/13	On	11pTHO1	N
			P2	Mets		2/11	On	7q796	N
								17pVNTR	Y (R)
67	IIA	T1-5			12/16[3]				
			P1	NED		0/13	On		
68	IIIB	T1-2			4/9[2]				
			P1	Mets		3/6	Off	1q549	Y
								9p156	Y
								17pTP53	Y
			P2	Mets		0/5[3]	On		
Total: 14		16	30	10 NED 20 + DS	91.5/175 pLOH = 0.52 (1 MI)	15/312 pLOH = 0.05 (3 MI)			12/15 (80%) concordant (0/3 MI)

[1]L, left; R, right.–[2]Average LOH in multiple samples from a single primary tumor.–[3]MI at 2 markers in # 53 P1, but not tumor; at 1 marker in #68 P2, but not tumor; and at 1 marker in #67 tumor but not plasma. Thus, no concordant abnormalities were present.

perimenopausal (48–52 years) and 6 were postmenopausal. There were 9 Stage II tumors, 5 Stage III tumors and 2 Stage IV tumors based on the American Joint Committee on Cancer criteria.

Each subject's MNC DNA constituted the control tissue defining her normal pattern at each microsatellite marker. Between 1 and 6 microdissected samples (mean: 2.7) from each subject's primary tumor and available nodal and metastatic deposits defined her cancer's unique genetic fingerprint(s). The serial plasma samples were examined for evidence of tumor-specific circulating DNA abnormalities. Eleven of 14 subjects had multiple plasma samples collected, with a mean number of 2.5 samples, and a mean interval between collections of 7 months (range: 1–54 months). Three of the 14 subjects had a single collection, due either to death or loss of follow-up.

Sensitivity of method

Detection of LOH in normal plasma DNA. We obtained MNC and plasma DNA from 5 anonymous healthy female volunteers. Using 0.5 ng of each plasma DNA, we tested for LOH at 3 markers (D1s549, D17s525 and D17s579) that were selected to be on chromosome arms demonstrating relatively frequent LOH (1q, 17p and 17q). Nine of 15 sites were informative, and each was amplified in 3 independent reactions resulting in a total of 27 independent PCRs. No reproducible abnormalities were seen.

Estimation of false positive rate of LOH in plasma DNA. Because low quantity DNA may result in artifactual abnormalities, we estimated our rate of detecting false positive LOH. Using the 5 MNC and 5 plasma DNA samples obtained from healthy donors,

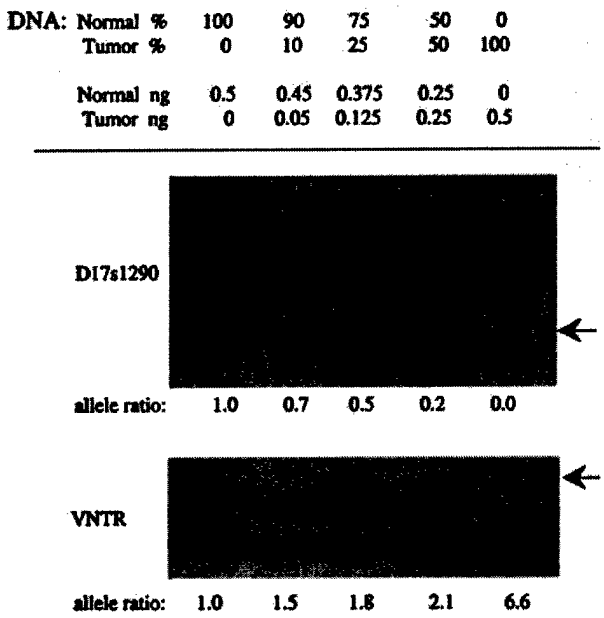

FIGURE 1 – Detection of LOH in templates with varying proportions of normal and tumor DNA. LOH can be detected when ≥10% of template DNA arises from the tumor. Known quantities (0.05–0.5 ng) and proportions (0–100%) of tumor and normal DNA, from uninvolved lymph nodes, were mixed and then PCRs were performed. Results from 2 markers are shown. LOH is defined as a normalized allele ratio >1.33 or <0.75, although in most cases the values are greater. Upper panel: LOH of the lower allele of D17s1290 (on 17q) is seen in samples containing ≥10% tumor DNA. Lower panel: LOH of the upper allele of the marker VNTR (on 17p) is seen in samples containing ≥10% tumor DNA.

we amplified 0.5 ng DNA with 3 markers (D1s549, D17s525 and D17s579). Each informative marker was amplified in 3 independent reactions resulting in a total of 54 independent PCRs (half using MNC, half using plasma DNA). We found limited variation in allele ratios, with the mean normalized allele ratio = 1.03, standard deviation = 0.17. Four of 54 (7.4%) reactions (one from MNC, 3 from plasma) had allele ratios outside our predetermined cutoff values (>1.33, <0.75), although no sample's LOH was ever reproducible (see above). Because our criteria for LOH require at least 2 independent demonstrations of abnormal allele ratios, this indicates that the maximum estimated rate of false positive LOH is $0.074 \times 0.074 = 0.006$ or 0.6%. This rate falls outside the 95% confidence interval for the mean percent LOH detected in study plasma samples, which was 5.6% (CI 2, 9.2). Thus, the abnormalities in study plasma samples are unlikely to be due to chance.

Detection of LOH using templates with varying proportions of tumor and normal DNA. Because the proportions of circulating tumor and normal DNA are unknown and may not be constant, we determined the lowest proportion of tumor DNA that we could detect. We performed a mixing study using known quantities and proportions of tumor and normal DNA. As shown in Figure 1, we found that we could reliably detect LOH when 10% or more of total DNA came from the tumor. MI could presumably be detected at even lower proportions of tumor DNA, since PCR should be more sensitive for detecting the presence of a novel-sized allele than the relative absence of a normal-sized one.

Tumor DNA abnormalities

The mean number of informative sites per subject was 11.5. All 16 tumors demonstrated one or more LOH, and the mean pLOH per tumor was 0.52 (range: 0.07–0.83), which is consistent with previous reports.[7,8] MI was detected at one marker in one subject's tumor (#67) (see Table I). Most abnormalities were present in all microdissected samples from a subject's tumor, but intratumoral heterogeneity was seen occasionally. In those instances, the tumor's fingerprint included all detected abnormalities.

TABLE II – TUMOR STAGE (AT DIAGNOSIS) IS NOT ASSOCIATED WITH LOH + PLASMA DNA

Stage	Plasma DNA		Total no. of tumors
	LOH+	LOH−	
IIa	3	2	5
IIb	2	2	4
IIIa	1	2	3
IIIb	1	1	2
IV	1	1	2
Total	8	8	16

Plasma DNA abnormalities

Table I provides details of these results. Altogether, 15 LOHs were detected among 312 informative sites, leading to a mean pLOH in plasma DNA of 0.05. The 15 LOHs were distributed among 12 of 30 (40%) plasma DNA samples (designated as LOH+ samples) from 8 of 14 (57%) subjects. The mean percent LOH in the 30 plasmas was 5.6%. The 15 plasma DNA LOHs involved 10 markers on 7 chromosome arms. LOHs were not concentrated at any particular marker, but LOH on arm 17p was overrepresented because 7 of the 15 LOHs were located on 17p. These 7 LOH were at 3 markers in 5 subjects. The overrepresentation of 17p LOH in plasma DNA did not simply reflect more tumor LOH on 17p than on other arms, since tumor pLOH on 17p was 65%, on 17q was 73% and on 1q was 89%. However, only 2 plasma LOHs from one subject were at 17q sites, and only one plasma LOH from one subject was at a 1q site. In addition to the 15 LOHs, 3 novel alleles were detected in plasma DNA in 2 of 30 (7%) samples from 2 of 14 (14%) subjects.

Concordance of plasma and tumor DNA abnormalities

Twelve of 15 (80%) LOHs seen in plasma DNA were concordant with LOH seen in the paired tumor DNA. Three of 15 (20%) LOH and 3 of 3 (100%) instances of MI were not concordant with paired tumor abnormalities (see Table I). Thus, most LOHs in the subjects' plasmas likely represented authentic detection of tumor-derived DNA. The subset of plasma alterations not concordant with tumor abnormalities may represent areas of the tumor not sampled, aberrations that developed during clonal progression of the primary tumor or PCR artifact.[24]

Despite the relatively high level of concordance of plasma LOH with tumor LOH, the majority of tumor LOH was not detected in plasma. Overall, only 12 of 91 (13%) tumor LOH were seen in plasma, leaving 87% of tumor LOH not detected. Most LOH in plasma DNA was at single microsatellite markers, even though the paired tumor DNA usually contained multiple markers with LOH. LOH at multiple markers in plasma DNA was seen only in subjects #65 and #68, who both had mets. Since the proportion of tumor DNA in plasma was high enough to detect LOH, it is unclear why only a small subset of tumor LOH was seen. It may reflect different markers' variable efficiencies or sensitivities of LOH detection, irregular metabolism or stability of circulating DNA.

Association of plasma DNA LOH with stage at diagnosis or clinical status at time of blood collection

No association was seen between stage at original diagnosis and subsequent detection of LOH in any plasma sample (see Table II). Nor was an association seen between clinical status at the time of blood collection and LOH in that plasma DNA sample. Ten of 30 (33%) plasma samples were obtained when subjects were NED, and 20 of 30 (67%) samples when subjects had +DS. LOH was seen in 3 of 10 (30%) NED plasmas *vs.* 9 of 20 (45%) +DS plasmas. Although the numbers are small, these proportions are equivalent ($p = 0.43$, chi-square test) (see Table III). Because

some subjects may be more likely than others to have LOH detected in their plasma DNA, we also evaluated plasma DNA LOH in relation to the clinical status of subjects, as opposed to the status of individual plasmas, but again no association was noted. Thus, detection of plasma DNA LOH appeared independent of the presence of clinically evident disease.

Finally, we assessed the association of LOH+ plasma DNA with ongoing cancer treatment (see Table I). Conceivably, treatment could lead to tumor cell death and release DNA into the circulation, or could suppress tumor growth and diminish circulating DNA. Most plasma specimens (27 of 30) were obtained while subjects were on treatment. LOH+ and LOH– were seen in both treatment groups: 10 of 27 on therapy samples were LOH+ and 17 of 27 were LOH–; 2 of 3 off-therapy samples were LOH+ and 1 of 3 were LOH–. Thus, treatment did not appear to lead to clear-cut differences in the detection of LOH in plasma DNA.

Consistency of microsatellite abnormalities during follow-up

Six of 11 (55%) subjects with multiple plasma collections demonstrated consistent results (i.e., all plasma DNA samples were either LOH+ or LOH–), and 5 of 11 (45%) demonstrated inconsistent results (i.e., some plasma DNA samples were LOH + and others were LOH–). Inconsistent results were not attributable to changes in clinical status, which occurred in only one subject (#17). Even in this case, inconsistency did not seem due to changed clinical status because the plasma DNA was LOH+ when the subject was NED, and LOH– when she had evident disease (see Table I). There was no evidence that inconsistent results were related to whether or not cancer treatment was ongoing, since 4 of the 5 subjects with inconsistent plasma DNA results were treated continuously. However, we cannot rule out the possibility that some treatments influenced plasma DNA more than others. Inconsistent results were seen even when tumor burden was substantial, as illustrated by subject #68 (see Fig. 2). This subject had 2 plasma samples (P1 and P2) collected 1 month apart; at both times she had a large tumor burden and was receiving only palliative care. P1 revealed LOH at several markers concordant with tumor LOH; P2 appeared completely normal. Inconsistent results were also seen in serial plasma DNA of both subjects (#53, #68) with novel alleles. Because we could detect LOH (and presumably novel alleles) when 10% or more of the circulating DNA came from the tumor (see Fig. 1), the inconsistent results in serial plasma DNA samples suggest that the proportions of tumor and normal DNA, even in carefully collected specimens, may be highly variable.

Association of plasma DNA abnormalities with outcome

As expected given the heterogeneous subject population, there was variability in duration of follow-up and clinical outcome as illustrated in Table IV. Mean follow-up from first plasma collection was 27 months (range: 2–71 months). Among subjects who had any LOH+ plasma, mean follow-up was 21 months, and 3 of 8 (38%) remain alive. Mean follow-up was 35 months among subjects who had no LOH+ plasma, and 2 of 6 (33%) remain alive. Among subjects who are NED (the group for whom a marker would be most beneficial), LOH+ plasma may be even less strongly associated with outcome. Although follow-up time was short, 3 NED subjects were LOH + and 3 were LOH–, but the only one who has relapsed or died was LOH– (#53), developing metastases 9 months after an LOH– sample. Among DS^+ subjects, LOH+ may possibly reflect tumor burden or activity since all 5 LOH+ subjects have died, whereas 1 of 4 LOH– subjects was a long-term survivor (#55). Thus, although the sample size is too small to permit statistical analysis, the data do not suggest that LOH+ plasma during follow-up has a strong association with outcome.

TABLE III – CLINICAL STATUS AT TIME OF PLASMA COLLECTION IS NOT ASSOCIATED WITH LOH + PLASMA DNA

Clinical status at plasma collection	Plasma DNA			
	LOH+		LOH–	
	(*n*)	(%)	(*n*)	(%)
NED (n = 10)	3	(30)	7	(70)
+ DS (n = 20)	9	(45)	11	(55)

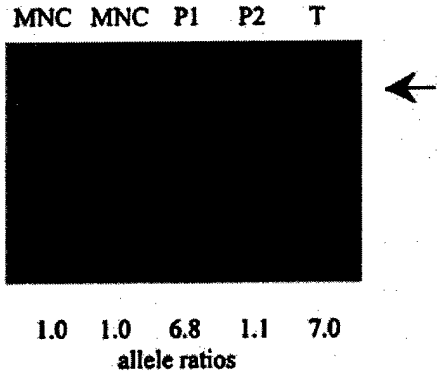

FIGURE 2 – LOH in plasma is not consistently detected despite stable clinical condition. LOH of microsatellite TP53's upper allele (arrow) in subject #68's tumor (T) and first plasma (P1), but not in her second plasma (P2). The plasmas were collected 1 month apart. At both time points the subject had progressive disease and she died 3 months later.

DISCUSSION

We evaluated the hypothesis that detecting occult circulating tumor DNA using a tumor-specific LOH fingerprint might have clinical utility in breast cancer. Our study is small, but the results are unique because of the serial collection of plasma specimens, collection of clinical data and fingerprinting of paired tumor and plasma DNA with a relatively large number of microsatellite markers.

From 14 subjects with 16 primary breast tumors (Stage IIA or more advanced), we evaluated a total of 30 plasma specimens. We found that all 16 tumors were characterized by an individual LOH fingerprint, and we observed LOH in 12 of 30 (40%) plasma DNA samples from 8 of 14 (57%) subjects. The number of LOH in plasma DNA, however, was small (n = 15), and the mean pLOH was much lower than in tumors (0.05 *vs.* 0.52). Although they were infrequent, 12 of 15 (80%) LOH in plasma DNA were concordant with abnormalities in the subjects' paired tumors, suggesting that they were authentic tumor-derived abnormalities. We found, despite this, no association between plasma DNA LOH+ and original tumor stage, or clinical status at time of blood collection (i.e., LOH+ was seen as often in subjects who were NED as in those with evident disease). In addition, detection of LOH was not consistent between serial samples from 5 of 11 (45%) subjects despite stable clinical condition and generally unchanging treatment status. No association with clinical outcome was evident, although the sample size is too small to be conclusive. MI in plasma DNA was infrequent, nonconcordant with paired tumor and inconsistent in serial samples. These results, taken together, suggest that monitoring microsatellite abnormalities in plasma DNA of breast cancer subjects may not be a clinically useful approach either for detection of occult metastases or for following disease.

What could explain these findings? One possibility is technical considerations. First, the technique may be insufficiently sensitive. Although tumor DNA was detected when it comprised as little as 10% of total DNA, this may be too large a proportion. Recent data, however, suggests that tumor DNA comprises anywhere from 3–93% of total circulating DNA.[20] Second, blood collection or plasma separation may result in contamination of plasma DNA with normal DNA despite collection techniques aimed at minimiz-

TABLE IV – LOH IN PLASMA DNA AND SURVIVAL

LOH+ in any plasma DNA				LOH − in all plasma DNAs			
Subject number	Clinical status at collections	F/U[1] (months)	Outcome	Subject number	Clinical status at collections	F/U[1] (months)	Outcome
34	NED	4	A[2]	50	+DS	2	D
68	+DS	4	D[3]	53	NED → +DS	12	D
17	+DS → NED	16	A	52	+DS	24	D
59	+DS	8	D	67	NED	50	A
56	+DS	24	D	55	+DS	51	D
65	+DS	26	D	51	NED	71	A
60	+DS	34	D				
54	NED	54	A				
	Mean no. of plasmas = 2.4	21 months	38% alive		Mean no. of plasmas = 1.8	35 months	33% alive

[1]F/U since first plasma collection.–[2]A, alive.–[3]D, dead.

ing MNC lysis (and yet being practical in clinical settings). Third, pertinent tumor clones may be missed despite sampling multiple areas of tumor and metastatic sites. However, if this were the explanation, we might have seen more plasma DNA LOH that was not concordant with tumor DNA LOH. Finally, plasma DNA LOH may reflect artifact. A value of 0.5 ng DNA, reflecting the genome of roughly 75 cells, should be adequate, but low quantity of DNA template has been reported as a cause of artifactual abnormalities[9,10,25–27] potentially leading either to spurious detection or omission of LOH. However, after testing multiple control samples using 0.5ng DNA template, we estimate the rate of artifactual LOH to equal ~0.6%, which falls well below the mean percent LOH in the study's plasma samples (5.6% [CI 2, 9.2]). In addition, the high degree of concordance with tumor DNA argues against artifact.

A more compelling explanation, however, is that the presence of circulating tumor DNA, as judged by concordant plasma DNA abnormalities, does not reflect tumor biology or the likelihood of mets. This is suggested by our observation that equal proportions (~50%) of NED and +DS subjects had LOH+ plasma. The fact that tumor DNA was found in the blood of only half the subjects with known mets suggests that access of tumor DNA (or of tumor cells, presumably) to the circulation is not the critical measure or determinant of the development of distant disease. Rather, other factors, for instance angiogenic capacity, susceptibility to apoptosis, immune mechanisms, proliferative potential or epithelial-mesenchymal interactions, may be more important. This idea is consistent with recent data suggesting that much circulating tumor DNA may originate from apoptotic or necrotic cells whose biologic significance is unknown.[20,28,29] It is also in accord with recent findings that occult bone marrow metastases are present at diagnosis in a large proportion of breast cancer patients, yet they have limited metastatic potential[30] and variable proliferative capacity.[31]

Devising a noninvasive test for detecting occult disease and monitoring breast cancer activity is a desirable clinical goal. In our pilot study, we found little evidence that monitoring circulating tumor-specific microsatellite alterations would be clinically useful. Evaluating more sensitive and specific approaches for detecting circulating genetic abnormalities,[32,33] or examination of different biomarkers, such as proteomic patterns in body fluids,[34,35] may yield more promising results. However, the present results also raise the possibility that in breast cancer circulating tumor DNA does not reflect tumor biology or correlate sufficiently well with clinical outcome to be a useful staging or diagnostic test.

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