# Microsatellite Alterations in Plasma DNA of Primary Breast Cancer Patients[1]

**Jacqueline A. Shaw,[2] Brendan M. Smith, Tom Walsh, Suzanne Johnson, Lindsay Primrose, Martin J. Slade, Rosemary A. Walker, and R. Charles Coombes**

Breast Cancer Research Unit, Department of Pathology, University of Leicester, Clinical Sciences Wing, Glenfield Hospital, Leicester LE3 9QP, United Kingdom [J. A. S, S. J., L. P., R. A. W.]; Cancer Research Campaign Laboratories, Cancer Cell Biology Section, Imperial College School of Medicine-Hammersmith Hospital Campus, London W12 0NN, England [B. M. S., M. J. S., R. C. C.]; Health Sciences, Division of Medical Genetics, University of Washington, Seattle, Washington 98195-7720 [T. W.]

## ABSTRACT

**The aim of this study was to analyze plasma DNA from primary and metastatic breast cancer cases for tumor-specific alterations and to compare these findings with immunocytochemistry and estimation of cytokeratin 19 (CK19) mRNA for detection of micrometastases. DNA was extracted from plasma, lymphocytes, and microdissected tumor tissue sections obtained from 71 patients with breast cancer and 9 controls. DNA samples were analyzed for loss of heterozygosity (LOH) and/or microsatellite instability (MI) by PCR with two polymorphic markers (DM-1 and D16S400). Reverse transcription-quantitative PCR (QPCR) and immunocytochemistry were used for detection of CK19 mRNA and protein. Breast cancer plasma DNA displayed frequent LOH (31.3%) and MI (11.6%) supported by the same alteration in microdissected tumor DNA. Most notably, 10 of the 39 patients with primary breast cancer showed LOH ($n = 6$) or MI ($n = 4$). We compared plasma tumor DNA, plasma and bone marrow QPCR, and blood and bone marrow immunocytochemistry in 32 of the patients with primary cancer. Of these, only one patient had immunocytochemically detectable carcinoma cells in the blood, and three showed abnormally high levels of plasma CK19 mRNA. All four of these patients had plasma DNA alterations. We then compared bone marrow findings: of the 10 primary breast cancers that showed LOH or MI, 6 had elevated CK19 mRNA and 5 had immunocytochemically positive cells. Tumor DNA is readily detectable in plasma of primary and metastatic breast cancer patients, and plasma DNA alterations (LOH and MI) reflect those seen in the tumor. The application of microsatellite analyses to plasma DNA may be useful in assessing tumor burden in breast cancer patients, particularly when combined with QPCR, and is preferable for patients with breast cancer, for whom sequential bone marrow aspiration is undesirable.**

## INTRODUCTION

Recently it has been suggested that DNA derived from plasma or serum could be used for molecular diagnosis (1). Small amounts of free DNA (~1 ng of soluble DNA/ml) are found circulating in plasma from healthy individuals (2). Increased concentrations of free plasma or serum DNA are often found in patients with rheumatoid arthritis, systemic lupus erythematosus, pancreatitis, pulmonary embolism, ulcerative colitis, peptic ulcer, and other inflammatory conditions (2–5). Patients with cancer have been shown to have even higher levels of free plasma DNA, often in excess of 100 ng DNA/ml (2, 6–8), but quantitative estimates are not sufficiently sensitive or specific for cancer diagnosis (6).

Two publications were first to show that DNA exhibiting tumor specific microsatellite alterations (*e.g.*, LOH[3]) can be detected by PCR in the plasma and serum of patients with advanced small cell lung cancer (9) and patients with head and neck cancer (10), respectively. Two recent reports have shown the same phenomenon in breast cancer patients (11, 12). We have also investigated whether tumor-specific microsatellite alterations can be detected in the plasma DNA of primary breast cancer patients and compared these results with other tests to detect micrometastases. We have analyzed plasma (taken prior to and following surgery in some cases) for microsatellite alterations from primary breast cancer patients and also obtained samples from patients with metastatic breast cancer and compared our results with those obtained from age- and sex-matched controls.

We performed a limited study with two markers only that were selected for the simultaneous analysis of LOH and MI. These markers were one-trinucleotide (DM-1) and one-dinucleotide (D16S400) repeats that had shown a high frequency of LOH (D16S400) or MI (DM-1) in our previous studies of screen-detected early breast cancers and preinvasive cases of ductal carcinoma *in situ* (13–15).

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[2] To whom requests for reprints should be addressed, at Breast Cancer Research Unit, Department of Pathology, University of Leicester, Glenfield Hospital, Groby Road, LE3 9QP, United Kingdom. Phone: 0116-2563023; Fax: 0116-2523274; E-mail: js39@le.ac.uk.

[3] The abbreviations used are: LOH, loss of heterozygosity; MI, microsatellite instability; QPCR, quantitative PCR; CK19, cytokeratin 19.

## PATIENTS AND METHODS

**Patients.** We recruited, with informed consent, 71 patients (age range, 32–82 years) with primary or metastatic breast cancer for the study, and 9 age-matched women attending for other nonneoplastic diseases. Blood samples were obtained preoperatively and 1 day postoperatively in 21 patients and preoperatively only in an additional 18 patients with primary breast cancer. These patients had no evidence of overt metastases as judged by negative bone and liver scans and chest radiology. Thirty of these patients had infiltrating ductal carcinomas, 8 had lobular carcinomas, and 1 had a mucinous carcinoma. Eleven patients had histological evidence of node involvement; 32 had estrogen receptor-positive carcinomas. Samples were obtained from 18 patients with progressive metastatic or locally advanced breast cancer, prior to commencing treatment, and 14 patients whose disease was either in remission (6) or had stabilized (8) following cytotoxic chemotherapy or endocrine therapy. All histology was confirmed by R. A. W.

**DNA Extraction.** Blood samples were processed within 1 h after venesection by Ficoll gradient extraction. Plasma was transferred into new polypropylene tubes and stored at −80°C. After removal of the plasma, the lymphocytes were stored at −80°C for later DNA extraction. Plasma was processed for PCR by boiling and centrifugation as described by Lo *et al.* (16); 10 μl were used directly for PCR. Lymphocyte DNA was extracted by digestion with proteinase K at 48°C, and 5 μl of crude digest were used directly for each PCR.

All primary tumor tissues had been fixed in 4% formaldehyde in saline for 18–24 h, and blocks were selected and processed through graded alcohols to paraffin wax. A microdissection procedure was used to analyze different components within the same tumor section and to separate tumor cells from adjacent normal stroma as described previously (14, 15). Three μl of the digest were used as template in PCR amplification.

**Microsatellite PCR.** The microsatellite markers studied were as follows: DM-1 $(CTG)_n$ located in the 3′ untranslated region of the *myotonic dystrophy protein kinase* gene (17), and the D16S400 $(CA)_n$ dinucleotide that maps to chromosome 16q (18). The PCR primer sequences, amplification, and PAGE conditions have been described previously (13, 14). In brief, PCR reaction conditions were as follows: 45 mM Tris-HCl (pH 8.8); 11 mM $(NH_4)_2SO_4$; 4.5 mM $MgCl_2$; 200 μM dTTP, dCTP, and dGTP; 25 μM dATP (Pharmacia, St. Albans, United Kingdom); 0.3 μl of α-$^{35}$S-labeled deoxyadenosine-5′-triphosphate (600 Ci/mmol; 10 mCi/ml; ICN Pharmaceuticals, Inc., Basingstoke, United Kingdom); 113 μg/ml BSA (Boehringer Mannheim), 6.7 mM β-mercaptoethanol, 4.4 μM EDTA (pH 8.0), 10 pmol of forward and reverse primers; 3–10 μl of DNA; and 1 unit of Taq DNA Polymerase (Life Technologies, Inc., Paisley, United Kingdom) in a total volume of 25 μl. PCR was carried out using the following cycles: 5 min of denaturation at 94°C, followed by 30 cycles of 1 min of denaturation at 94°C, 1 min of annealing, and 1 min of extension at 72°C, with a final extension of 7 min at 72°C on a DNA Thermal Cycler (Perkin-Elmer Cetus, Warrington, United Kingdom).

Where necessary, plasma samples were subjected to two rounds of amplification as follows: the first round of PCR was 30 cycles, and 0.1 μl of product was then used as template for a subsequent round of 25 cycles of PCR with the same primer pair.

**QPCR Measurement of CK19 and Immunocytochemistry.** We obtained the mononuclear fraction of 20 ml of blood after Ficoll separation for CK19 mRNA QPCR and to obtain cytospins for immunocytochemistry. QPCR for CK19 after reverse transcription-PCR was done by estimating the number of CK19 transcripts by normalizing to the number of ABL transcripts as an internal control for quality and quantity of RNA (19). Modifications of this method, described by Slade *et al.* (20), showed that a CK19:ABL ratio of >1:1000 is a significantly higher ratio than in normal individuals. Briefly, competitive PCR for CK19 was carried out with 2.5 μl of cDNA plus 2.5 μl of competitor dilution in a total volume of 25 μl for 30 cycles of 96°C for 1 min, 69°C for 25 s, and 72°C for 1 min, followed by a 10-min extension at 72°C. The wild-type product was 463 bp, and the competitor was 588 bp. Products were analyzed by agarose gel electrophoresis, and the equivalence point was estimated by inspection. Quantification of ABL transcripts was performed for 2.5 μl of cDNA plus 2.5 μl of competitor dilution in a total volume of 25 μl. The wild-type product was 385 bp, and the competitor was 486 bp. Results were expressed as the CK19:ABL ratio for samples that were positive for CK19.

Immunocytochemistry was done by staining cytospins of $1 \times 10^6$ mononuclear cells for CK8, 18, and 19, using A45-B/B3 (Micromet, Munich, Germany) as described previously (21). In brief, cells were centrifuged at a concentration of $5 \times 10^5$ per area and air dried. The primary antibody, A45-B/B3 (Micromet, Munich, Germany), was used at a final concentration of 2 mg/ml. Rabbit antimouse antiserum (Z259; Dako, Hamburg, Germany) and alkaline phosphatase anti-alkaline phosphatase complex (D651; Dako, Hamburg, Germany) were used as recommended by the manufacturer, and the reaction was developed with new fuschin. An isotype mouse myeloma antibody, MOPC-21 (Sigma), served as a negative control, and the MCF-7 cell line served as a positive control. The cytospins were screened without counter staining

## RESULTS

PCR analysis of microsatellite markers yielded amplified DNA from plasma for all except two breast cancers (69 of 71 cases). No alterations (LOH or MI) were detected in plasma DNA with either D16S400 or DM-1 in any of the nine control cases studied (*e.g.*, Fig. 1*a*). In contrast, specific alterations were detected in plasma DNA isolated from both primary and metastatic breast cancer cases, supported by the same alteration in microdissected tumor DNA (*e.g.*, Fig. 1*c*). For some cases, because of the paucity of available tumor tissue blocks, tumor DNA was prepared from a single whole tumor tissue section. For example, Fig. 1*b* shows analysis of D16S400 in a patient with metastatic breast cancer. LOH was clearly identified in the plasma sample, but it also appeared to be present in the DNA from the whole tumor tissue section, as indicated by reduced intensity of the upper allele when compared with the intensity in lymphocyte DNA.

We found one primary case (Fig. 1*d*) that showed MI in plasma DNA that was not supported by any alteration in either

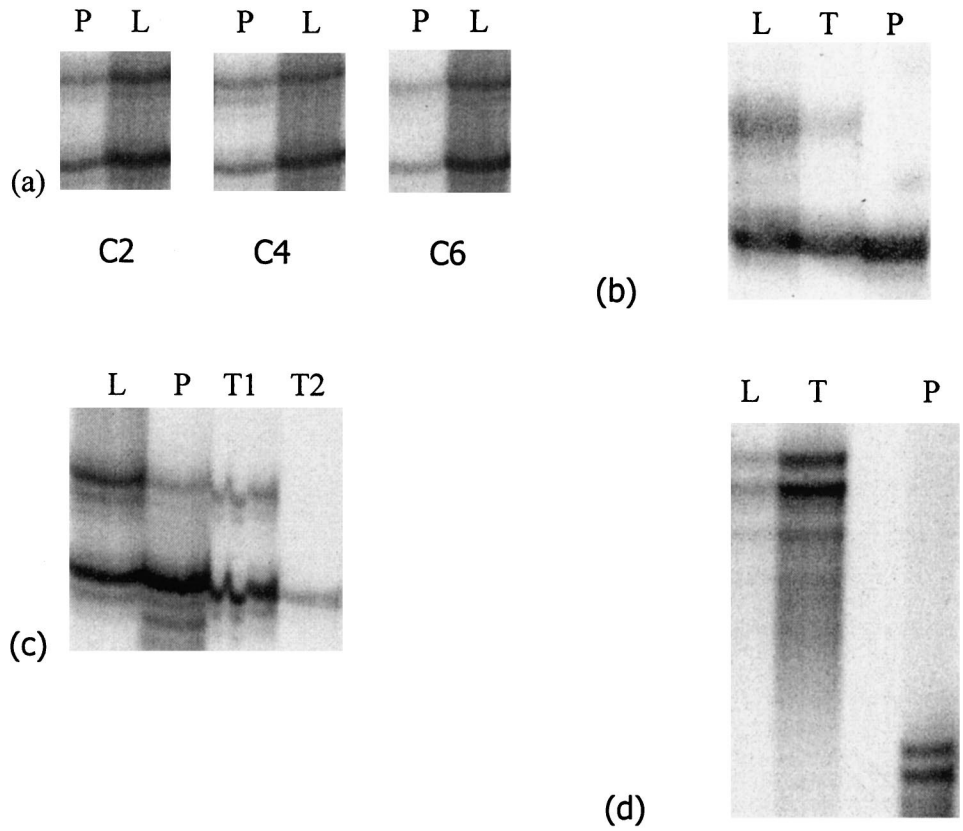


*Fig. 1* Detection of LOH and MI in plasma and tumor DNA from breast cancer patients. *a*, analysis of control cases 2, 4, and 6 (*C2*, *C4*, and *C6*) at D16S400; *b*, LOH at D16S400 in tumor and plasma from metastatic case 5; *c*, LOH at D16S400 in microdissected tumor and plasma from metastatic case 13; *d*, MI at DM-1 in plasma from primary case 3. *P*, plasma DNA; *L*, lymphocyte DNA; *T*, whole tumor tissue section DNA; *T1* and *T2*, foci of tumor cells prepared by microdissection.

whole tumor or any of four separate areas of tumor prepared by microdissection. This result was reproducible, and there was no apparent sample error. This may indicate that the DNA in the plasma was not derived from the primary tumor in this case.

Twenty-one cases (2 of the metastatic and 19 of the primary patients) were uninformative for LOH. In total, 15 of 48 (31.3%) informative cancer cases showed LOH, and 8 of 69 (11.6%) cases showed MI. Where subsequent blood samples were available ($n = 21$) for patients with primary breast cancer, the second plasma and lymphocyte DNA samples showed the same genotypes as the preoperative sample.

Ten of 39 patients with primary breast cancer showed LOH (6) or MI (4); 16 patients with metastatic breast cancer showed either LOH (10) or MI (6). In the case of metastatic patients, there was no correlation between the incidence of finding LOH/MI in the plasma and whether the patients had progressive disease (10 of 16 positive) or stable/regressing disease (6 of 14 positive). Nor was there any correlation with site(s) of metastatic disease.

Because the results of the first cohort of patients with metastatic breast cancer seemed promising, we carried out a prospective comparison of plasma DNA, QPCR for CK19 mRNA in plasma, and immunocytochemistry to detect circulating carcinoma cells in the blood in a group of 32 patients with primary breast cancer. Immunocytochemistry was positive only in 1 patient and QPCR in 3 patients despite the fact that 10 patients with primary breast cancer showed positive plasma DNA displaying LOH or MI (see Table 1). There were no significant differences observed in terms of node involvement or tumor size between the 10 patients who displayed LOH or MI in plasma DNA and the 20 patients who had no alterations detected.

We then compared bone marrow findings for CK19 and immunocytochemistry with blood tests for each of the three methods in the 32 patients with primary breast cancer. Eighteen patients had evidence of CK19 transcripts in the bone, and 9 had evidence of bone marrow micrometastases by immunocytochemistry. Twelve and 6 patients who had positive bone marrow CK19 mRNA or immunocytochemistry, respectively, showed no alterations to plasma DNA. Eight of 10 primary patients who displayed LOH or MI had positive tests on either immunocytochemistry or CK19 mRNA measurements in the bone marrow (Table 1).

The results of the peripheral blood of 18 of the patients with metastatic breast cancer were also studied. Whereas 7 patients showed LOH or MI in plasma DNA, 12 showed evidence of CK19 transcripts; however, only 3 showed the presence of circulating immunocytochemically detectable cancer cells. Only 3 of 18 patients were negative on all tests (Table 2).

## DISCUSSION

The data presented in this study show that DNA is present in the plasma of patients with primary and metastatic breast cancer and is amplifiable and detectable by PCR. In addition, specific alterations were found in plasma DNA (LOH and MI)

*Table 1* Details of patients with primary breast cancer showing tumor-specific alterations in plasma DNA

| | Patient no. | Age (years) | ICC[a] BM blood | | CK19 BM blood | | Histology | Pathology, tumor size (mm) | Lymph node status (no. of +ve nodes) | ER | Grade |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Loss of heterozygosity | | | | | | | | | | | |
| | 1 | 49 | −[b] | − | + | | IDC | 5 | 0/19 | − | 3 |
| | 2 | 80 | − | − | + | − | ILC | 15 | ND | +++ | 2 |
| | 3 | 42 | + | − | − | − | IDC | 60 | 8/18 | +++ | 2 |
| | 4 | 78 | + | − | + | + | IDC | 65 | 4/8 | − | 2 |
| | 5 | 56 | − | − | + | − | ILC | 15 | 0/27 | ++ | 2 |
| | 6 | 74 | − | − | + | + | ILC | 37 | ND | +++ | 2 |
| Microsatellite instability | | | | | | | | | | | |
| | 7 | 51 | + | − | − | + | ILC | 20 | 0/12 | +++ | 2 |
| | 8 | 52 | Isotype pos. | | − | − | IDC | 13 | 0/21 | +++ | 2 |
| | 9 | 56 | + | + | + | − | IDC | 16 | 0/25 | +++ | 1 |
| | 10 | 77 | − | − | − | − | ILC | 22 | ND | +++ | 3 |

[a] ICC, immunocytochemistry for CK19; BM, bone marrow; ER, estrogen receptor; IDC, infiltrating ductal carcinoma; ILC, infiltrating lobular carcinoma; ND, not done.
[b] −, negative; +, positive; ++, strongly positive; +++, very strongly positive.

*Table 2* Comparative results of plasma tumor DNA, CK19 mRNA, and immunocytochemistry in patients with locally advanced or metastatic breast cancer

| Patient no. | Sites of metastases | Plasma DNA result | CK19 QPCR[a] | Immunocyto-chemistry | Treatment |
|---|---|---|---|---|---|
| 1 | Bo/Li/LN[b] | MI | 1:200 (+) | Neg | Nil |
| 2 | Li/LN | NAD | 1:40 (+) | Neg | Nil |
| 3 | Bo/Li | MI | 1:1600 (−) | Neg | Nil |
| 4 | Bo/Li | LOH | 1:800 (+) | Neg | Nil |
| 5 | Sk/LN | LOH | 1:200 (+) | Pos | Nil |
| 6 | Bo/Li/LN | NAD | 1:120 (+) | Pos | Nil |
| 7 | Br | NAD | 1:2500 (−) | Neg | Nil |
| 8 | Bo/Lu | LOH | 1:1000 (−) | Neg | Nil |
| 9 | Bo/Li | MI | 1:100 (+) | Neg | Nil |
| 10 | Li/Lu/LN | NAD | 1:400 (+) | Neg | Megace |
| 11 | Bo/Li/Br | NAD | 1:400 (+) | Neg | Mitozantrone, methotrexate |
| 12 | Bo/Lu/Br | NAD | 1:600 (+) | Pos | Docetaxel |
| 13 | Bo | NAD | 1:1300 (−) | Neg | Tamoxifen |
| 14 | Bo | MI | 1:1300 (−) | Neg | Arimidex |
| 15 | LN | NAD | 1:400 (+) | Neg | Tamoxifen |
| 16 | LN | NAD | 1:800 (+) | Neg | Formestane |
| 17 | Bo | NAD | 1:800 (+) | Neg | 5-fluorouracil, epirubicin, cyclophosphamide |
| 18 | Bo | NAD | 1:1700 (−) | Neg | Arimidex |

[a] QPCR for CK19: +, ABL mRNA ratio greater than normal; −, ABL mRNA ratio less than normal.
[b] Bo, bone; Li, liver; LN, lymph node; Neg, negative; NAD, no alteration detected; Sk, skin; Pos, positive; Br, breast; Lu, lung.

that were usually identical to those present in the corresponding tumor DNA, indicating that plasma DNA is derived predominantly from the primary tumor. Indeed, identification of clear LOH in plasma DNA strongly supports the hypothesis that tumor DNA forms a significant proportion of DNA in the plasma (22). One primary case (Fig. 1*d*) showed MI in plasma DNA but not tumor, and may suggest that the plasma DNA was not derived from the primary tumor in this case.

We found amplifiable plasma DNA in all but two of the cancer samples, both primary and metastatic, as well as in non-cancer controls. We did not attempt to quantify the amount of DNA recovered per ml of plasma, but merely screened for amplifiable DNA recovered from plasma.

We reported previously the occurrence of immunocytochemically detectable micrometastases in the blood and bone marrow of patients with primary breast cancer (23). The chances of finding these cells in the peripheral blood of primary breast cancer patients is low, however, because only 1–2% of patients are positive. We have developed a QPCR technique in an attempt to improve the detection rate in blood (20), but this is far from optimal as exemplified in this study, where only three of the cases with primary breast cancer were found to be positive. The comparative study of circulating markers provided a useful indication of which tests may prove more reliable.

In this study, both detection of LOH and/or MI in plasma DNA and CK19 mRNA were more sensitive than immunocytochemistry in both primary and metastatic patients. Both plasma QPCR and plasma DNA testing together could detect 15

of 18 (83.3%) patients with metastatic disease and 10 of 32 patients with primary breast cancer, suggesting that the two tests could possibly be combined as a marker for minimal residual disease in the follow-up of patients after primary surgery. Importantly, our plasma DNA data were derived from the analysis of only two microsatellite markers. The frequency of detected alterations could be improved by analyzing several different polymorphic markers. The more laborious immunocytochemical method, often taking 3–4 h to screen slides and only detecting circulating positive cells in 3 of 18 patients (16.7%), may soon, therefore, be superseded by the combination of tests for tumor-specific alterations in plasma DNA and tumor-associated RNA.

It is of interest that of the 10 patients with primary breast cancer (Table 1) with evidence of LOH or MI in plasma DNA, 5 had negative lymph nodes and 8 showed no evidence of lymphovascular invasion. This contrasts with a recent study that found a significant correlation between microsatellite alterations and involvement of three or more lymph nodes (11). More markers and patients need to be studied to resolve these differences.

Importantly, our findings suggest that tumor cell access to the vasculature exists even in breast cancers where none can be seen by conventional histology. Presumably, apoptotic and/or necrotic tumor cells release DNA into the microvasculature from the primary tumor: alternatively, the source of tumor DNA could be from circulating or occult secondary deposits. Further studies will be required to determine the exact source of tumor DNA in these patients.

The frequency of LOH and MI detected with the markers analyzed were similar to those that have been reported previously by us (13–15) and others (24, 25). These frequencies are too low for any clinically useful diagnostic test. However, these data relate to a limited screen, carried out with two markers. It should be possible to broaden the panel of markers used to increase the frequency of detectable LOH (and/or MI) as well as to screen for specific mutations, such as *p53* or *BRCA1*. Other related studies support the feasibility of this approach. K-*ras* and *p53* gene mutations have been detected in the feces of patients with colorectal cancer (26, 27), and recently, mutant K-*ras* DNA was found in the plasma or serum of patients with colorectal cancer (23, 28). These investigators have suggested that such an approach may eventually form the basis of colorectal cancer screening tests. For breast cancer, our finding of tumor DNA in plasma from primary patients, with potentially curable disease, suggests that a plasma-based assay may have potential diagnostic and/or prognostic application. A further advantage of plasma DNA over the use of QPCR *per se* is the qualitative nature of the plasma DNA, compared with the QPCR methodology, which is subject to problems of contamination by minute quantities of extraneous mRNA.

Our data suggest that it should be possible to apply simple DNA, and possibly mRNA, screening methods to blood samples from all breast cancer patients. These findings are provocative and clearly require further investigation for assessment of tumor burden, metastatic status, and overall prognosis in breast cancer. The application of more DNA markers is being explored at present in a larger group of patients.

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