# Microsatellite Changes in Nipple Aspirate Fluid and Breast Tissue from Women with Breast Carcinoma or Its Precursors

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## ABSTRACT

***Purpose:*** **Loss of heterozygosity (LOH) and microsatellite instability (MSI) have been identified in a variety of human cancers. The purpose of this prospective study was to determine whether (*a*) DNA can be isolated from nipple aspirate fluid (NAF) and PCR amplified to large fragments, (*b*) LOH and MSI are detectable in NAF, and (*c*) LOH and MSI in tissue and NAF increase with disease progression from precursor lesions to cancer.**

***Experimental Design:*** **Forty-six matched samples from breast lesions, normal breast, and NAF were microdissected, and DNA was extracted. Eleven microsatellite markers from seven chromosomes that have a high frequency of LOH/MSI in breast cancer were designed and respectively amplified.**

***Results:*** **LOH and/or MSI were identified in 22 of 46 (48%) breast lesions, including LOH in 8 of 36 (22%) proliferative/papilloma (P/Pap) and 7 of 10 (70%) cancer specimens, whereas MSI was found in 14 of 36 (39%) P/Pap and 6 of 10 (60%) cancer specimens. LOH/MSI loci in which alterations were detected in the 22 tissue specimens were PCR amplified using matched NAF DNA. LOH/MSI was detected in NAF from both P/Pap (5 of 15; 33%) and breast cancer (3 of 7; 43%) samples.**

***Conclusions:*** **Our findings suggest that (*a*) DNA from NAF, a physiological fluid collected noninvasively, can be PCR amplified and used to screen for LOH and MSI alterations that are known to be linked to breast cancer, suggesting that this methodology might prove useful for breast cancer screening, and (*b*) similar to findings in breast tissue, LOH and MSI alterations increase in frequency with disease progression in NAF, which suggests that NAF is a surrogate for breast tissue which has important prognostic implications.**

## INTRODUCTION

Over 39,000 women in the United States will die of breast cancer this year (1). Prevention and early detection are the best ways to decrease morbidity and mortality. Standard screening with mammography and physical examination miss 10–40% of early breast cancers. The development of DNA-based noninvasive techniques to detect breast cancer shows great promise in aiding in the early detection of disease. LOH,[3] MSI, oncogene amplification, mutation of tumor suppressor genes, and mitochondrial DNA mutations have been identified in a variety of human physiological fluids, including sputum (2), urine (3), pancreatic juice (4, 5), stool (6), blood (7, 8), and SND (9, 10).

NAF has been used for many years as a noninvasive method to identify markers of breast cancer risk. Our data analyzing NAF demonstrate that cytologic and protein markers (11–13) can be evaluated and are associated with the presence and stage of breast cancer. The purpose of this study was (*a*) to assess whether nuclear DNA can be isolated from NAF and PCR amplified, (*b*) to determine whether LOH and MSI are detectable in NAF, and (*c*) to assess whether LOH and MSI in tissue and NAF increase in frequency with disease progression from precursor lesions to cancer.

A comprehensive study identified LOH at 26 cytogenetic regions on 16 different chromosomes in breast cancer tissue specimens (14). Until recently, studies of molecular changes from diverse tumor types, including breast cancer, have primarily focused on specific genes, including K-*ras*, N-*ras*, and *p53* (15–18). Amplification of the c-*erb*B2 gene (9) was observed in one woman diagnosed with breast cancer who presented with SND, and LOH (10) was observed in 7 of 10 women diagnosed with breast cancer who presented with SND. We chose to analyze 11 microsatellite markers from seven chromosomes that have a high frequency of LOH/MSI in breast cancer tissue (19–23) to determine whether these changes, when present in tissue, are also present in NAF. Like SND, NAF is a physiologic fluid from the breast ducts, but unlike SND, the volume and cellularity of NAF are generally lower. On the other hand, the number of women from whom a NAF sample can be collected is far greater than those with SND. In this study, we collected NAF from 33 women and SND from 13 women and compared our ability to detect LOH/MSI in specimens from women with similar histology. If LOH/MSI alterations could be identified in even a subset of NAF samples from women in whom these alterations were detected in tissue, this DNA-based noninvasive method to detect breast cancer using NAF would provide an

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[3] The abbreviations used are: LOH, loss of heterozygosity; MSI, microsatellite instability; NAF, nipple aspirate fluid; SND, spontaneous nipple discharge; P/Pap, proliferative/papilloma; DH, ductal hyperplasia.

attractive adjunct to current screening tools in the early detection of breast cancer.

## MATERIALS AND METHODS

**Patient Samples.** We prospectively designed and submitted a protocol to our institutional review board[4] to determine whether LOH and MSI could be collected in NAF and SND and whether these alterations increased with breast cancer progression. The approved protocol was followed, and informed consent was obtained from 46 subjects. All subjects had a radiographically suspicious and/or palpable breast lesion requiring diagnostic biopsy. All had NAF/SND collected before surgery. Thirteen subjects provided SND, and 33 provided NAF. Forty-six matched lesional tissue specimens, adjacent histologically normal tissue, and NAF/SND specimens were collected. All 13 subjects with SND were found to have histologic papillomas. For the remaining 33 subjects, breast fluid was collected using breast massage and a modified breast pump as described previously (13). Frozen tissue provided by the pathologist immediately after removal from the subject was snap frozen until use. Frozen samples were batched, and each was formalin-fixed and paraffin-embedded just before cutting. Serial 10-μm histological sections were prepared. The first and sixth levels were stained with H&E, and unstained levels 2–5 were microdissected. Histologic diagnoses were verified by light microscopy for each block using levels 1 and 6, and the areas to be microdissected were marked. A 2 × 2-mm area was microdissected from matched normal and lesion slides to obtain a cell population sufficient to obtain a detectable band in each PCR reaction. Areas of dissection were chosen to obtain an epithelial cell purity of ≥80%. Dissection of unstained slides was performed in a laminar flow tissue culture hood. NAF was obtained using a modified breast pump before or concurrent with surgical resection and kept at −80°C until use (13).

**DNA Isolation.** DNA was extracted from normal and abnormal tissue samples using xylene/ethanol for deparaffinization followed by proteinase K digestion with the addition of Chelex-100 and heat inactivation at 95°C for 10 min, followed by phenol/chloroform plus ethanol precipitation. The DNA was resolved in tris ethylenediamine tetra-acetic acid buffer. A Qiagen Mini-Blood Kit was used to isolate DNA from NAF. Briefly, the NAF/SND was first diluted with 200 μl of PBS, and DNA was extracted following the kit protocol. Isolated DNA was kept at 4°C in a dedicated area that was used only for PCR assembly. No PCR products or equipment used in post-PCR analysis ever entered this area.

**LOH and MSI Analysis.** LOH and MSI analysis were first performed with the DNA obtained from normal and lesional tissue. If one or more informative change(s) were found in the lesion that were not present in matched normal tissue, DNA from corresponding NAF/SND was amplified and analyzed for the altered DNA marker. PCR primer pairs were prepared for 11 microsatellite markers that mapped to chromosomes 1, 3, 9, 11, 13, 16, or 17 (*D1S2878, D3S1277, D9S1690, D11S988, D11S4175, PGYM, D13S137, D16S503, D16S265, D17S921,* and *NF1*) and were previously found to possess high rates of LOH/MSI (19–23). Nine loci were synthesized, and the forward primers were fluorescence-labeled. Because D16S265 and PYGM are too similar in size to primers that had already been labeled, they were analyzed using silver staining (discussed below). The 50-μl PCR reaction contained 1× PCR buffer with 1.5 mM $MgCl_2$, 200 μM deoxynucleotide triphosphates, 1× Q-Solution, 1.25 units of HotStar Taq DNA polymerase, and primer sets with final concentrations ranging from 0.1 to 1.0 μM, depending on the primer sets used. Eight μl of normal and tumor DNA template were used. A water control was used to exclude results due to contamination. PCR was performed under the following cycling conditions: initial denaturation at 95°C for 15 min; followed by 30 cycles of 94°C for 45 s, 58°C for 45 s, and 72°C for 1 min; with extension for a final 45 min at 60°C to reduce PCR artifacts. Fluorescence-labeled PCR products were then detected and analyzed using an ABI 310 Genetic Analyzer and GeneScan Collection software.

*D16S265* and *PYGM* were analyzed using a non-fluorescence-labeled PCR/PAGE assay. Amplicon detection was performed as described previously with electrophoresis of a denatured 7% polyacrylamide gel followed by silver staining to detect bands in a DNA sequencing gel (24).

**Criteria Used to Determine Whether MSI or LOH Was Present.** MSI was defined as any change in DNA length produced by the insertion or deletion of >2 bp in a tumor/precursor lesion tissue microsatellite compared with matched normal tissue. Cases in which normal tissue showed two alleles and tumor tissue showed only one allele were not counted as MSI because we considered it difficult to clearly differentiate between LOH and MSI. LOH was defined as a >75% diminution in the intensity ratio of the "lost" allele relative to the retained allele when compared with a normal control. All results showing informative changes were repeated, and only those showing changes in two or three assays were considered positive for microsatellite alteration (LOH/MSI).

**Statistical Analysis.** Contingency tables of the categorical data were analyzed using $\chi^2$ or Fisher's exact test ($\alpha = 0.05$). All analyses were conducted using SigmaStat for Windows, Version 2.03S (SPSS, Inc., Chicago, IL).

## RESULTS

**DNA in NAF Can Be Isolated and PCR Amplified.** We were able to amplify NAF DNA from all (22 of 22) of the tested samples. The volumes required to amplify DNA in NAF ranged from 3 to 8 μl, with a median of 4.5 μl.

**LOH and MSI Are Detectable in Breast Tissue and Matched NAF.** Twenty-two of 46 (48%) subject samples demonstrated MSI and/or LOH in lesional tissue (Fig. 1; Table 1). MSI was found in 19 samples (41%), and LOH was found in 15 samples (33%). *D11S988* was the locus most frequently altered in breast lesional tissue, with 16 of 46 subject samples containing LOH/MSI [16 of 22 samples (73%) for which LOH/MSI was identified at any locus].

LOH and MSI were evaluated in NAF/SND DNA. Because our primary goal was to determine whether LOH/MSI present in lesional tissue could also be detected in NAF/SND, PCR am-

[4] This study began at Thomas Jefferson University and concluded at the University of Missouri-Columbia.

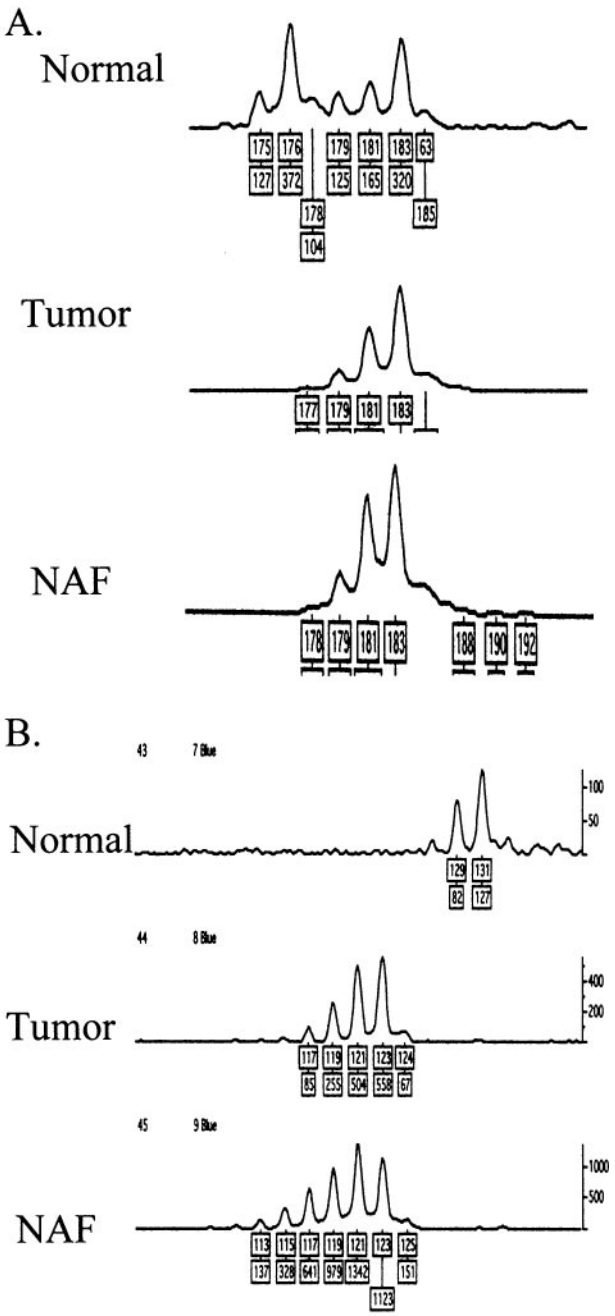


*Fig. 1* Representative (*A*) LOH and (*B*) MSI of locus *D17S921* in breast tumor tissue and NAF.

plification of NAF/SND DNA was carried out only at the loci for which alterations were detected in tissue. Of samples that could be PCR amplified, we were able to perform LOH and MSI determinations in all cases. LOH/MSI alterations were detected in NAF/SND DNA most often at two loci, *D11S988* and *D9S1690* (Table 1).

Our ability to detect LOH/MSI alterations in NAF/SND is expressed based on the number of samples analyzed (Tables 1 and 2). Although the ability to detect LOH and MSI at a given locus was low (Table 1), our success in detecting at least one alteration using this battery of markers was substantial (Table 2). Of the 22 subjects with LOH/MSI, we were able to detect LOH/MSI in NAF/SND from 8 (36%) subjects.

**NAF *versus* SND.** Among breast lesions, papilloma is unique in its frequent presentation of SND. Whereas the incidence of LOH and MSI was similar in tissue samples from DH and papilloma, the ability to detect LOH/MSI was greater in papilloma specimens (60% *versus* 17%; Table 2). Four of 5 of papilloma specimens were cellular, as compared with 3 of 6 DH and 7 of 17 nonpapilloma (includes DH) specimens. Obtaining a cellular specimen was not significantly related to whether or not the lesion was a papilloma. Cellular specimens generally had clusters of 10 to hundreds of epithelial cells, whereas specimens classified as noncellular had only scattered or no epithelial cells.

**LOH and MSI in Tissue and NAF Increase with Disease Progression from Precursor Lesions to Cancer.** LOH/MSI was found in 15 of 36 (42%) P/Pap and 7 of 10 (70%) cancer tissue specimens. Looking at the alterations separately, LOH was detected in 8 of 36 (22%) P/Pap and 7 of 10 (70%) cancer tissue specimens, whereas MSI was found in 13 of 36 (36%) P/Pap and 6 of 10 (60%) cancer specimens. The LOH/MSI alteration in a given tissue lesion was PCR amplified using matched NAF/SND DNA. LOH/MSI was detected in 5 of 15 (33%) subjects with P/Pap lesions and 3 of 7 (43%) subjects with breast cancer. The difference in LOH/MSI in NAF from P/Pap *versus* breast cancer specimens was not significantly different.

## DISCUSSION

Herein we demonstrate that DNA isolated in NAF/SND samples from subjects with papilloma, proliferative (hyperplasia and precancer), and cancerous lesions can be PCR amplified and analyzed for markers associated with breast cancer. Preliminary reports (9, 10) in sample sizes of 1 and 10 subjects, respectively, demonstrated that LOH could be detected in SND. SND is similar to NAF in that it comes from the breast ducts but dissimilar in that it is more voluminous, tends to have a pathological cause and therefore generally contains more cells than NAF, is an uncommon phenomenon and therefore is not appropriate for screening. NAF, on the other hand, can be collected in essentially all adult women who have an intact breast and is noninvasive and repeatable, making it appropriate to consider for breast cancer screening. In the two reports cited regarding LOH in SND, all samples were from women with breast cancer. In the current report, we prospectively collected NAF/SND samples from subjects who presented for diagnostic biopsy and were found to have a variety of pathological diagnoses.

In the current study, we found that DNA could be isolated and amplified, that LOH and MSI were detectable in NAF and SND, that the alterations in tissue in many cases were also detectable in NAF/SND, and that these alterations in NAF/SND DNA increased with disease progression to cancer. Now that we have demonstrated the feasibility of detecting LOH/MSI in NAF and SND, future studies should assess in NAF whether MSI and LOH are present in a given set of loci, regardless of whether an alteration is found in tissue.

Specific alterations (LOH and MSI) found in NAF/SND

*Table 1* Frequency of MSI/LOH at specific loci

| | MSI (%) | | LOH (%) | |
|---|---|---|---|---|
| Marker[a] | Tissue | NAF | Tissue | NAF |
| *D11S988* | 13/46 (28) | 2/13 | 3/46 | 1/3 |
| *D1S2878* | 9/46 (19) | 1/9 | 3/46 | 0/3 |
| *PYGM* | 6/46 (13) | 0/6 | 3/46 | 0/3 |
| *D9S1690* | 5/46 (11) | 1/5 | 3/46 | 2/3 |
| *D12S137* | 3/46 (7) | 0/3 | 3/46 | 0/3 |
| *D17S921* | 4/46 (9) | 0/4 | 2/46 | 1/2 |
| *D11S4175* | 4/46 (9) | 0/4 | 2/46 | 0/2 |
| *D16S503* | 5/46 (11) | 0/5 | 0 | NC |
| *D16S265* | 4/46 (9) | 0/4 | 1/46 | 0/1 |
| *NF1, D3S1277* | 0 | Not checked | 0 | Not checked |
| Totals[b] | 19/46 (41) | 4 /19 (21) | 15 /46 (33) | 4 /15 (20) |

[a] Marker is listed based on frequency of MSI/LOH alterations in tissue.
[b] Subjects positive at any locus/total subjects analyzed.

*Table 2* Frequency (%) of LOH/MSI based on lesion stage

| | Pathology | | | | | |
|---|---|---|---|---|---|---|
| Genetic changes | DH | Papilloma | ADH/LCIS[a] | DCIS | Invasive breast cancer | Total |
| LOH | | | | | | |
| Tissue | 3/14 (21) | 3/13 (23) | 2/9 (14) | 2/2 (100) | 5/8 (57) | 15/46 (33) |
| NAF[b] | 1/3 (33) | 1/3 (33) | 0/2 | 1/2 (50) | 1/5 (20) | 4/15 (27) |
| MSI | | | | | | |
| Tissue | 5/14 (36) | 5/13 (38) | 3/9 (33) | 1/2 (50) | 5/8 (57) | 19/46 (41) |
| NAF[b] | 0/5 | 2/5 (40) | 1/4 (25) | 0/2 (0) | 1/5 (20) | 4/19 (21) |
| LOH/MSI | | | | | | |
| Tissue | 6/14 (43) | 5/13 (38) | 4/9 (44) | 2/2 (100) | 5/8 (63) | 22/46 (48) |
| NAF[b] | 1/6 (17) | 3/5 (60) | 1/4 (25) | 1/2 (50) | 2/5 (40) | 8/22 (36) |

[a] ADH, atypical DH; DCIS, ductal carcinoma *in situ*; LCIS, lobular carcinoma *in situ.*
[b] Because NAF was only analyzed if tissue demonstrated MSI/LOH, the frequency listed is based on the samples analyzed.

DNA were identical to those present in the corresponding lesional breast tissue DNA. Our ability to detect LOH/MSI in NAF/SND from breast cancer patients was 3 of 10 (30%) overall and 3 of 7 (43%) specimens for which tissue demonstrated an alteration. Notably, in subjects with proliferative lesions, we were able to identify LOH/MSI in 5 of 36 (14%) NAF/SND specimens overall and in 5 of 15 (33%) specimens for which tissue demonstrated an alteration. This frequency is lower than the corresponding tissue samples. We believe that the frequency of detectable genetic alterations in NAF/SND samples could be improved by analyzing more markers or specific genes with more sensitive molecular techniques. Our data, along with results obtained from other investigators, indicate that NAF DNA may eventually be used for the development of noninvasive diagnostic, prognostic, and follow-up tests for breast cancer. Combining DNA-based techniques such as LOH/MSI and methylation-specific PCR (25) may improve our ability to detect breast cancer over cytomorphology alone.

Our ability to detect LOH/MSI was greater in SND specimens than in NAF (60% *versus* 17%) from subjects with similar histologic findings. This is not unexpected, and it is consistent with an earlier report (9, 10) of LOH in 70% of SND specimens.

The frequency of MSI and LOH in breast cancer tissue has been reported by a variety of investigators. The frequency of MSI varies widely and may be related to the author's definition of MSI as well as the loci chosen for analysis. Two recent reports (26, 27) document that MSI is a relatively frequent event, with the first observing MSI in 15 of 32 (47%) sporadic breast cancers, and the second detecting MSI in 37% of 52 primary invasive breast cancers. We observed an incidence of LOH that was lower than has been reported for some loci and similar to published reports at other loci. We are not sure why this might be, but possibilities include the technique used to determine LOH [fluorescence *versus* gel DNA intensity, as in the case of O'Connell *et al.* (22)] or the patient population [Caucasians and Africans *versus* Taiwanese (21)]. Normal tissue contamination is a possibility, although we observed a high rate of MSI. Using our 11 markers, LOH was detected in 70% (7 of 10) of breast cancers, and MSI was detected in 60% (6 of 10) of breast cancers.

LOH/MSI was present in premalignant breast disease at a lower rate compared with cancer tissue. This finding supports the concept that breast cancer results from progressive alteration of benign breast tissue. This finding is consistent with the cumulative molecular and epidemiologic studies suggesting that atypical DH/ductal carcinoma *in situ* may be a direct precursor of invasive breast cancer (22).

The results from this and other similar studies suggest that proliferative breast lesions share the same genotyping abnor-

malities as malignant breast disease, which may have important prognostic implications. Identifying low- and high-risk genotypes may enable us to match treatment to risk more appropriately in patients who have premalignant disease. A better understanding of the genetics of premalignant lesions, coupled with a noninvasive approach using NAF to collect samples for analysis, may also lead to safe and effective strategies to prevent disease development and progression.

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