Short Communication

Loss of Heterozygosity at the *BRCA1* and *BRCA2* Loci Detected in Ductal Lavage Fluid from *BRCA* Gene Mutation Carriers and Controls

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Abstract

Female *BRCA* gene mutation carriers are at increased risk for developing breast cancer. Ductal lavage is a novel method for sampling breast ductal fluid, providing epithelial cells for cytologic assessment and a source of free DNA for molecular analyses. Loss of heterozygosity (LOH) at the *BRCA* loci in ductal lavage fluid is a potential biomarker of breast cancer risk. The LOH rate was measured at the *BRCA1/2* loci and compared with that at a control locus (*APC*) using free DNA from the ductal lavage fluid of *BRCA* carriers and predictive test negative controls. We evaluated the reproducibility of these analyses. Free DNA sufficient for PCR amplification was obtained from 33 ductal lavage samples of 17 healthy women of known *BRCA* status (14 *BRCA* carriers and 3 controls). LOH rates of 36.4% to 56.3% at the *BRCA1* locus and 45% to 61.5% at the *BRCA2* locus were found among *BRCA* carriers. The LOH rate at the *APC* locus was lower (18.5%). The interaliquot reproducibility for the D17S855 marker of the *BRCA1* locus was 66.7%. Intraaliquot reproducibility was 90%. Although we successfully isolated sufficient free DNA from ductal lavage fluid for PCR amplification, the degree of reproducibility of these LOH studies raises questions about the robustness of this technique as a risk assessment tool in the evaluation of high-risk women. Further studies are required to evaluate the specificity and predictive value of LOH in ductal lavage fluid for breast cancer development. (Cancer Epidemiol Biomarkers Prev 2006;15(7):1399–402)

Introduction

Women carrying pathogenic *BRCA* gene mutations are at increased risk for developing breast cancer under the age of 50 years (1). It is well recognized that mammograms are less sensitive in younger women who have more radiodense breast tissue, and although alternative imaging modalities, such as magnetic resonance imaging, have shown promise, there is still a clear need for better risk assessment and earlier breast cancer detection in this high risk group (2, 3).

Somatic loss of heterozygosity (LOH), the loss of a normal functioning allele at a heterozygous locus, is a frequent genetic event in the multistep process of breast cancer tumor development and progression (4). Carriers of germ-line mutations in tumor suppressor genes, such as *BRCA1* or *BRCA2*, are at risk of acquired loss of the wild-type allele, one of the common mechanisms of inactivation in hereditary breast cancer (5).

Ductal lavage allows repeated minimally invasive sampling of breast duct fluid, and sufficient exfoliated epithelial cells for cytologic diagnosis may be collected (6). More than 60 women from *BRCA* gene mutation carrying families are taking part in the ductal research program at The Royal Marsden NHS Foundation Trust evaluating the usefulness of nipple aspiration and ductal lavage as risk assessment tools. We are doing a variety of molecular analyses on the ductal fluid collected in the search for surrogate biomarkers of risk. The purpose of this study is to evaluate the frequency of LOH at the *BRCA1* and *BRCA2* loci detected in free DNA from the ductal lavage fluid of *BRCA* gene mutation carriers and controls, to assess the reproducibility of doing these microsatellite analyses, and to compare the LOH rate with that at a control locus of uncommon loss in breast cancer.

Materials and Methods

Subjects. Prospective ethics committee approval was gained for this study assessing epithelial cell atypia and potential biomarkers in the ductal lavage fluid of *BRCA* gene mutation carriers and predictive genetic test negative controls. Informed consent was gained from all subjects. Ductal lavage was attempted on all healthy unaffected breasts, and where possible, multiple ducts in the same breast were cannulated. Thirty-three ductal lavage specimens were available for LOH analysis from 17 women, of whom 14 were *BRCA* mutation carriers (5 *BRCA1* and 9 *BRCA2*) and 3 were predictive test negative controls. Five ductal lavage samples were repeat samples, collected 1 year apart, from 3 *BRCA* carriers. Five of the *BRCA2* carriers had previously contralateral breast cancer but were currently disease free.

Specimen Collection and Processing. Ductal lavage was done as described previously with the modification that cannulation of both nipple aspirate fluid–yielding and nipple aspirate non fluid–yielding ducts was attempted (6). Ductal

Received 12/26/05; revised 2/27/06; accepted 5/2/06.

Grant support: Cancer Research UK. Z. Kote-Jarai was funded by the legacy of the late Marion Silcock and is now funded by Breast Cancer Campaign. Prostate Cancer Research Foundation (S. Bullock).

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doi:10.1158/1055-9965.EPI-05-0971

Table 1. LOH rates at the *BRCA1*, *BRCA2*, and *APC* loci

Subject	Status	Duct	Cytology	BRCA1			BRCA2				APC
				D17S855	D17S1322	D17S1325	D13S260	D13S171	D13S1493	D13S267	D5S346
1	BRCA1	Right a	Benign	LOH (2)	No LOH	LOH (2)	No LOH	NI	No LOH	No LOH	No LOH
		Right b	Benign	No LOH	No LOH	No LOH	LOH (2)	NI	No LOH	No LOH	No LOH
2	BRCA1	Right a1	Benign	LOH (1)	LOH (2)	F	LOH (2)	LOH (1)	LOH (1)	LOH (2)	No LOH
		Right b1	Mild atypia	No LOH	LOH (1)	No LOH	LOH (2)	LOH (1)	No LOH	No LOH	No LOH
		Left a1	ICMD	F	LOH (2)	LOH (2)	LOH (2)	LOH (2)	F	LOH (1)	LOH (2)
		Left b1	ICMD	F	F	F	LOH (1)	F	F	F	F
		Right a2	Benign	No LOH	NR	NR	NR	NR	NR	NR	No LOH
		Right b2	Benign	No LOH	NR	NR	NR	NR	NR	NR	No LOH
		Left a2	Benign	No LOH	NR	NR	NR	NR	NR	NR	LOH (2)
3	BRCA1	Right a1	Benign	No LOH	No LOH	No LOH	NI	No LOH	No LOH	No LOH	No LOH
		Right a2	Benign	No LOH	No LOH	No LOH	NI	F	F	No LOH	No LOH
		Left a2	Benign	No LOH	No LOH	No LOH	NI	No LOH	No LOH	No LOH	No LOH
4	BRCA1	Right a	ICMD	No LOH	No LOH	No LOH	NI	No LOH	No LOH	No LOH	No LOH
5	BRCA1	Left a	Benign	NI	No LOH	No LOH	LOH (2)	LOH (1)	No LOH	NI	No LOH
6*	BRCA2	Right a	Benign	LOH (1)	LOH (1)	LOH (2)	NI	LOH (1)	NI	LOH (1)	LOH (2)
		Right b	Benign	LOH (1)	LOH (1)	No LOH	NI	F	NI	LOH (2)	No LOH
7	BRCA2	Right a	Benign	LOH (2)	LOH (2)	LOH (2)	LOH (1)	No LOH	NI	LOH (1)	No LOH
8*	BRCA2	Right a	Benign	No LOH	LOH (1)	No LOH	No LOH	No LOH	No LOH	No LOH	No LOH
9†	BRCA2	Right a	ICMD	F	LOH (1)	LOH (2)	No LOH	NI	NI	No LOH	No LOH
		Left a	Benign	LOH (1)	LOH (1)	LOH (2)	No LOH	NI	NI	LOH (1)	F
10	BRCA2	Right a1	Benign	LOH (1)	NI	LOH (2)	NI	NI	LOH (1)	LOH (2)	No LOH
		Right b1	Benign	No LOH	NI	No LOH	NI	NI	LOH (1)	No LOH	No LOH
		Left a1	Benign	No LOH	NI	No LOH	NI	NI	LOH (1)	LOH (2)	No LOH
		Right a2	ICMD	LOH (1)	NI	LOH (1)	NI	NI	LOH (1)	LOH (1)	No LOH
		Right c2	ICMD	F	NI	F	NI	NI	LOH (1)	F	No LOH
11*	BRCA2	Right a	ICMD	NI	NI	No LOH	No LOH	No LOH	LOH (1)	No LOH	No LOH
12	BRCA2	Left a	ICMD	No LOH	NI	No LOH	LOH (1)	LOH (1)	No LOH	NI	LOH (2)
13*	BRCA2	Left a	ICMD	F	F	F	F	F	NI	F	LOH (1)
14*	BRCA2	Left a	Benign	LOH (1)	NI	No LOH	NI	NI	LOH (1)	F	No LOH
			LOH rate ‡	9/22	9/16	8/22	8/13	6/12	8/17	9/20	5/27
15	CONTROL	Left a	Benign	F	NI	LOH (1)	F	LOH (1)	F	NI	No LOH
16	CONTROL	Right a	Benign	No LOH	No LOH	NI	LOH (2)	LOH (1)	NI	NI	NI
17	CONTROL	Right a	Benign	No LOH	No LOH	No LOH	NI	LOH (2)	NI	LOH (1)	No LOH
		Left a	Benign	No LOH	No LOH	No LOH	NI	No LOH	NI	No LOH	No LOH
			LOH rate ‡	0/3	0/3	1/3	1/1	3/4	NI	1/2	0/3

NOTE: Ducts are identified as from the left or right breast. Individual ducts are identified sequentially as a, b, or c, and the suffix 1 or 2 indicates the first or second lavage visit, respectively. For example, Left a2 indicates sample that has been taken from the left breast, duct a at the second ductal lavage visit.

Abbreviations: LOH (1), loss of smaller size allele; LOH (2), loss of larger size allele; NI, noninformative marker result; NR, no result available; F, PCR failure; ICMD, insufficient cellular material for diagnosis.

**BRCA2* carriers affected by contralateral breast cancer. Ductal lavage samples were collected only from their healthy unaffected breasts.

† Subject 9 had previous atypia in both breasts on nipple aspirate fluid cytology and subsequently had mild atypia found in a ductal lavage sample taken from the right breast (not included in this study).

‡ LOH rate is the number of samples with LOH per total number of informative DL sample results for each marker.

lavage samples were centrifuged at 1,500 rpm for 10 minutes at 4°C, and the Shandon cytospin technique was used to produce two slides for cytologic assessment. Slides with <10 epithelial cells were classed as inadequate cellular material for diagnosis. Slides with sufficient epithelial cells were further categorized as benign ductal epithelial cells, mildly atypical epithelial cells, markedly atypical cells, or malignant epithelial cells. Free DNA was extracted from 200 μL ductal lavage supernatant, using the DNeasy Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and eluted in 50 μL buffer. Two aliquots of free DNA were extracted separately for reproducibility studies assessing intraaliquot and interaliquot reproducibility. DNA concentration was measured using a NanoDrop ND-1000 spectrophotometer.

LOH Analysis. Fluorescence-based PCR amplification of three *BRCA1* polymorphic microsatellite marker, two intragenic (D17S855 and D17S1322) and one telomeric marker (D17S1325), and four markers flanking the *BRCA2* locus, one centromeric (D13S260) and three telomeric (D13S171, D13S1493, and D13S267), was done. Intraaliquot and interaliquot reproducibility was evaluated using the *BRCA1* intragenic marker D17S855 in a subset of 21 ductal lavage samples, where at least two informative marker results were available. The chromosomal region 5q21, using marker D5S346 close to the region of the *APC* gene, was chosen as a representative control locus, as it has been found not to be a site of preferential allelic loss in breast cancer (4). Markers were assessed for informativeness using genomic DNA extracted from the subject's peripheral blood lymphocytes. PCR for the *BRCA* microsatellite markers was conducted in 15 μL reactions containing 1.5 to 3.0 mmol/L magnesium chloride (optimized for each primer set), deoxynucleotide triphosphate mix (250 μmol/L each of dATP, dGTP, dTTP, and dCTP), 1 unit Taq polymerase (AmpliTaq Gold, Applied Biosystems, Foster City, CA), 1.5 μL of each primer (final optical density 0.1), and 5 μL extracted DNA from the ductal lavage samples (equating to ~10 ng DNA). PCR for the *APC* locus was done as described by Zauber et al. (7) Genotyping and analysis were done using an ABI 3100 Genetic Analyser and Genotyper version 3.7 (Applied Biosystems).

LOH was defined as a 50% decrease in the allelic height ratio in DNA from an individual duct compared with the height ratio of the alleles in blood lymphocyte DNA. Allelic loss was determined using the normalized allelic ratio equation [LOH ratio = (D1)(N2) / (D2)(N1)], where D1 and D2 are the heights of the smaller and larger allelic peaks, respectively, from the duct and N1 and N2 are the heights of the allelic peaks from blood lymphocyte DNA (8). Ducts for which the calculated LOH ratio was ≤0.5 or ≥2.0 were scored as LOH. The LOH rate was defined as the number of samples

in which LOH was shown divided by the total number of informative sample results available for each microsatellite marker. We deemed the LOH result to be reproducible if the D17S855 marker results were concordant (both heterozygous or both showing loss of the same allele). We defined inconsistency of the LOH result as a discrepancy between replicate experiments as to whether LOH was present or absent or as a discrepancy in which of the two alleles was lost.

Results and Discussion

In this study, we used free DNA extracted from the ductal lavage supernatant to allow the relatively limited cellular fraction of the ductal lavage sample to be retained for cytologic assessment and other molecular studies. Free tumor DNA has been isolated from various body fluids, including serum, plasma, urine, and peritoneal fluid (9, 10). The observation that levels of cell-free DNA are higher in the body fluids of cancer patients compared with healthy controls has led to interest in its use in the screening and early diagnosis of cancer (11).

We investigated whether LOH at the *BRCA* loci identified in free DNA from ductal lavage fluid could be used as a reproducible marker of breast cancer risk in healthy *BRCA* carriers. The mean ages of the *BRCA* carriers and controls at their first ductal lavage visit were 46.1 years (range, 35.1-62.8 years) and 49.7 years (range, 41.4-52.2 years), respectively. Thirty-three ductal lavage samples were obtained from 17 women (5 *BRCA1* carriers, 9 *BRCA2* carriers, and 3 controls). Twenty-three samples (69.7%) showed benign cytology. One ductal lavage sample from a *BRCA1* carrier showed mild atypia (subject 2). A further *BRCA* carrier had previously had atypia identified in both breasts by nipple aspiration cytology and has subsequently had mild atypia identified in a ductal lavage sample taken from the right breast (subject 9). The remaining nine ductal lavage samples contained insufficient ductal epithelial cells for cytologic diagnosis.

We were able to isolate sufficient free DNA from all samples, including apparently acellular samples, to do PCR amplification for at least one marker. LOH was found in 9 of 22 (40.9%) and 9 of 16 (56.3%) ductal lavage samples from *BRCA* carriers using the D17S855 and D17S1322 *BRCA1* markers, respectively, but in none of the 3 informative control samples. For the marker, D17S1325, 8 of 22 (36.4%) informative ductal samples from *BRCA* carriers showed LOH and 1 of the 3 informative control samples. Eight of 13 (61.5%) informative ductal lavage samples from *BRCA* carriers showed LOH for the centromeric *BRCA2* microsatellite marker D13S260. Only 1 control sample was informative for this marker, and LOH was found. LOH was found in 6 of 12 (50%), 8 of 17 (47.1%), and 9 of 20 (45%) of informative ductal lavage samples assessed using the D13S171, D13S1493, and D13S267 *BRCA2* markers, respectively. LOH was found in 3 of 4 informative samples from control women using the D13S171 marker and 1 of 2 using the D13S267 marker. No informative results were available for the control samples using the D13S1493 marker. We found LOH at the *APC* locus in 5 of 27 (18.5%) ductal lavage samples from *BRCA* carriers but none of the control samples (see Table 1).

One previous study has reported a LOH rate of 58% at the *BRCA1* locus using free DNA from the ductal lavage supernatant of known *BRCA1* carriers (12). Although Isaacs et al. reported that the LOH results were consistent using the same DNA preparations, reproducibility between different aliquots was not commented upon. We found that LOH results were consistent between separately extracted aliquots of DNA for 14 of the 21 (66.7%) ductal lavage samples analysed. For 6 of the 7 samples where there was inconsistency, there was interaliquot variation between LOH and heterozygous status. The remaining sample from subject 9, a *BRCA2* carrier, showed complete loss of the smaller allele in the first aliquot and loss of the larger allele in the second (see Fig. 1). Replicate PCRs were done to assess intraaliquot reproducibility in 10 ductal lavage samples, and results were consistent in 90% of the repeat experiments.

We examined whether LOH status varied over a 1-year period by examining repeat ductal lavage samples taken from three *BRCA* carriers. The LOH results were entirely consistent between the two time points for the *APC* locus. For the *BRCA1* marker, D17S855, one ductal lavage sample showed loss of the smaller allele at the first ductal lavage visit and heterozygosity in the sample from the second visit, one duct showed persistent loss of the smaller allele over time, and two further ducts remained heterozygous over time. We found no increase in the LOH rate in the ductal lavage fluid obtained from the contralateral breasts of those *BRCA2* carriers who had previously had breast cancer in their other breast. Two *BRCA* carriers had current or previous atypia but did not show a consistent pattern of LOH at the *BRCA* loci in ductal lavage samples from the ducts in which atypia was found.

There was a trend toward a lower DNA concentration in ductal lavage samples showing LOH [mean, 2.8 ng/μL; (range, 1.2-3.8 ng/μL)] compared with samples in which heterozygosity was retained (mean, 4.1 ng/μL; range, 1.3-11.8 ng/μL),

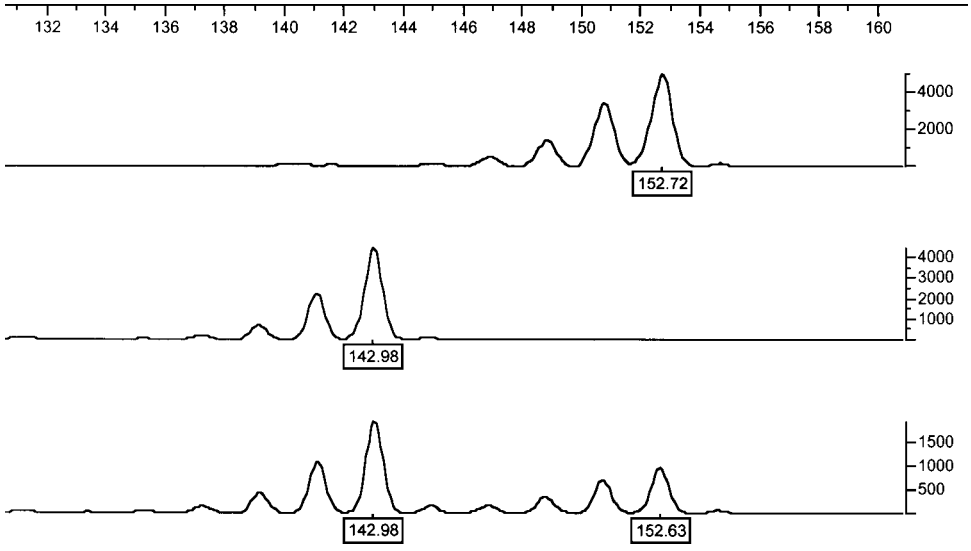

Figure 1. Discrepant D17S855 marker results between two different aliquots of DNA from duct left a of subject 9, a *BRCA2* carrier. *Top* and *middle traces,* complete loss of the smaller and larger size alleles, respectively; *bottom trace,* heterozygosity in the same subject's peripheral blood lymphocyte DNA for comparison.

suggesting that some of the LOH found may relate to the relatively low DNA yield from this material and the degree of fragmentation of the DNA close to marker sites. The interaliquot discrepancy in LOH result seen may in part be attributable to sampling variation of a mixed cell population, confounding the detection of free DNA from a relatively small atypical or malignant tumor cell population within a background of normal ductal cells. Furthermore, our finding of LOH at the *BRCA* loci in predictive test negative controls, at assumed population risk for developing breast cancer, raises the possibility that the result may not reflect true molecular events but rather a methodologic artifact.

We conclude that doing microsatellite marker analyses on free DNA from ductal lavage supernatant is possible, but we found unexpectedly high levels of LOH at the *BRCA* loci considering these samples were collected from apparently healthy women, although they are at increased risk for developing breast cancer. The rate of LOH at the *APC* locus was lower but was not negligible (18.5%). If loss of the wild-type allele at the relevant *BRCA* locus in germ-line *BRCA* mutation carriers represents an early event in breast tumorigenesis, analysis of free DNA from ductal lavage fluid may offer a useful alternative surrogate marker of risk particularly where samples are insufficient for cytologic diagnosis. However, the degree of reproducibility of these assays found in this study and that of Antill et al. (13) raises questions about the potential of this technique as a robust risk assessment tool in the evaluation of women with a genetic predisposition to breast cancer. Further studies are required to optimize the reproducibility of the methods and to evaluate the specificity and predictive value of LOH in ductal lavage fluid for subsequent breast cancer development.

Acknowledgments

We thank Dr. Gillian Mitchell who was instrumental in setting up the ductal program at The Royal Marsden NHS Foundation Trust, Audrey Ardern-Jones and Elly Lynch who identified suitable women to take part in the study, Dr Timothy Blackburn for his helpful comments on the article, and all the women who kindly took part in this study.

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