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Allele Imbalance or loss of heterozygosity, in normal-appearing breast epithelium as a novel biomarker to predict future breast cancer

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14. ABSTRACT The goal of this study is to determine whether the occurrence of AI/LOH in the DNA of histologically normal epithelium from noncancerous breasts predicts future breast cancer development. If so, then AI/LOH would be an excellent candidate molecular marker of increased sporadic breast cancer risk: its incidence increases during cancer development, it can be quantified and standardized, it is likely to reflect dysregulation of genetic mechanisms that could be potential targets for pharmacological modulation. In the past year, we have completed Task 2 and 3. Thirty-three subjects (16 control [no cancer] and 17 case subjects [developed cancer]) remained after subjects and blocks dropped out based on study criteria. They included 6 pairs and 21 unmatched subjects. Statistical analyses were performed but due to small sample size, no definitive predictions could be made. However, there were trends: a) AI, in aggregate, increases the likelihood of cancer (OR 1.78, p 0.47), b) AI at 17pVNTR (OR 0.167, p 0.194) might be associated with reduced cancer risk and c) increased subject age appears to be associated with reduced risk of AI. If more matched pairs could be identified, we would be able to increase the power of the study and the trends we observed might become statistically significant. Task 4 is in process.					
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I. INTRODUCTION

In July, 2010 Dr. Carol Rosenberg, PI for the funded project “Allelic imbalance, or loss of heterozygosity, in normal-appearing breast epithelium as a novel biomarker to predict breast cancer” left Boston University Medical Center. As I have been actively involved with the research proposed, I have been asked to compile and submit the final report for this funded project. Since the last Annual Project (submitted in February, 2010), all experiments have been completed, the study cohorts unblinded as to path status, and the findings tabulated as to rates and specific locations of AI/LOH [abbreviated here as AI] based on clinical outcome.

II. BODY.

Task 1. Identify 70 subjects from the Nurses’ Health Study Benign Breast Disease (NHS-BBD) nested case-control study who had benign breast biopsies with available tissue blocks and full clinical follow-up. Months 1-16.

This task is complete.

1) Dr Tamimi selected and assembled the benign breast disease tissue blocks. As planned, she selected breast cancer cases from the nested case-control study of benign breast disease and breast cancer who were between ages 35 and 55 at the time of the benign biopsy, had no atypia on the benign biopsy, and had no family history of breast cancer. For each breast cancer case, she selected one control who was matched as closely as possible to the case on age at benign biopsy, year of biopsy and who also lacked atypia on the biopsy and a family history of breast cancer. A total of 44 cases and 44 controls (with a total of 343 tissue blocks) met the criteria. Extra cases and controls are included because when the blocks are examined in the lab, some will be found to be unusable for technical reasons such as: no or too few normal lobules, or degraded DNA. The 343 blocks were sent to the Rosenberg lab blinded as to identity and outcome. The blocks were stored in a locked room accessible only to the PI and one laboratory associate. We developed a spreadsheet to track the blocks, and examined each block to determine which were physically able to be cut, sectioned each block that could be cut to confirm that the sections contained normal epithelium. After this was done, we returned all 343 blocks to Dr Tamimi. We have no NHS-BBD blocks in our possession.

2) The number of subjects was increased (with IRB approval) to 88 (44 cases, 44 controls).

Task 2: Microdissect multiple normal-appearing epithelial samples (TDLU) from each eligible NHS-BBD subject’s blocks, extract DNA, quantify AI using an optimized marker panel and a high-throughput system. Months 1-33.

This task is now complete.

Task 2A: Optimize panel of ~ 25 markers to be used. Months 1-12.

1) We completed the switch from a radioactive, gel and autoradiography-based detection system to a fluorescent, capillary electrophoresis-based detection system. This detection system is more automated, and therefore permits higher throughput for detection of AI/LOH.

2) We optimized our marker panel. After testing numerous samples of DNA extracted from the NHS-BBD blocks, we found that large-sized amplicons (larger than about 125 bp) had to be eliminated because they did not amplify reliably. This is almost certainly due to the blocks' old age (from the 1960s - 1990s) and heterogeneous fixation procedures the tissues underwent at time of biopsy. We refined our marker list to include primers that amplify products < 125 bp in length and that can be purchased already fluorescently labeled at ABI.

Task 2B: Section NHS-BBD blocks, microdissect normal epithelium, isolate DNA, perform PCRs, and run reaction products through capillary electrophoresis. Months 6- 33.

1) By June 2009, we had examined all of the blocks, and divided the subject into 5 groups, based on block age (which ranged from the 1990s to the 1960s). We had analyzed blocks from the 3 groups dated in reverse chronological order, i.e., those from the 1990s, the late 1980s, and the early 1980s. We had sectioned all blocks with features permitting analysis – i.e., blocks that contain normal epithelial lesions (terminal ductal lobular units, or TDLUs) of adequate size to generate sufficient DNA, and of great enough number per subject for statistical analysis ($n \geq 3$), and that can fit into a modern microtome (since some blocks are fairly old and come from diverse hospitals, a few subjects' blocks were of eccentric shape that could not technically fit into any microtome configuration currently in use). Then all potential lesions were microdissected. Subsequently, we extracted their DNA, and then performed multiplex PCR using the optimized markers, capillary electrophoresis, quantification of the output, replicates on all samples with AI.

2) At the present time, we have examined and sectioned all NHS-BBD blocks ($n=343$). We have found that a larger-than-expected number of TDLUs contained DNA of insufficient quality to yield reliable results. These subjects also had to be removed from analysis. When we finished examining all potential subjects, a total of 33 of the original 88 (37%) subjects were analyzable. **Table 1** (attached) provides information about the criteria used to identify subjects with usable DNA.

3) As planned, we calculated AI using a reproducible 33% difference in allele intensity as our cut-off. This cut-off corresponds to an allele intensity ratio of 1.5 or 0.67. This ratio is a good choice to discern authentic AI: the old age of the NHS-BBD subject blocks

leads to a greater likelihood that there might be a higher background level of DNA damage manifesting as AI than there might be in “younger” or more recently fixed/embedded blocks. If we were to use a lower cut-off (such as 25%, corresponding to an allele intensity ratio of 1/33 or 0.75), the noisiness, or false positives, might mask real differences between groups.

Task 2C: Enter results onto Excel spreadsheet, send spreadsheet to statistician. Months 12-33.

Data has been entered into Excel spreadsheets and descriptive analyses are complete. Originally each case was matched with a control. After subjects and blocks dropped out based on study criteria, 33 subjects remained [16 control subjects (no cancer) and 17 case subjects (developed cancer)]. They included 6 matched pairs and 21 unmatched subjects. **Table 2A** summarizes characteristics of the 33 NHS-BBD subjects (such as age at diagnosis, number of blocks/case, number of TDLU/case). **Table 2B** summarizes the characteristics of the 6 matched pairs for the same variables. Group characteristics across all 33 subjects or across the 6 matched pairs are vertically identical.

Table 3 summarizes information about AI in the normal epithelium of the 33 subjects [A] and for the 6 matched pairs [B]. This table provides information on the total number of TDLU, of informative markers, of AI, etc. While the number of AIs are slightly lower in the case subjects [25 versus 20]. The number of subjects with AI/ total number of subjects is higher for the case subjects [65% versus 50%]. Multiple AI per subject occurred only once in the case subjects while it occurred 6 times in the control subjects. In the matched pairs, the control subjects had the same number of TDLU as the case subjects, but 1.6 times more informative markers and 2.4 more AIs than the case subjects. The fact that the total number of cases with AI/ total number subjects is the same in the both groups [67%] indicates that the case subjects tended to have a single AI/subject while the controls tended to have multiple AIs/ subject.

To show that multiple TDLUs isolated from the same tissue block were independent events, we examined the relationship of AI to number of blocks and number of TDLUs. In every case with multiple AI [whether control or case subject], the fingerprints were different indicating that each AI was in fact an independent event. There was only one subject where the AIs were the same and came from TDLUs from the same block. This was control subject N017 with AIs in N4 and N5 at 16q [loss of the upper allele]. In every other case loss of either the upper or lower allele had occurred, so the fingerprints were different.

Other variables, such as subject age, block age, number of TDLU or number of informative markers, could bias the findings. As expected as the number of TDLUs and informative markers increased, the number of AI increased. An increase in block age might have been expected to produce an increase in detection of artifactual AI, however this was not observed indicating that the DNA was so damaged or degraded, due to older aged, that even the small amplicons [<125 bp in length] could not be amplified. An unexpected finding was that as the subjects aged, the number of AI decreased. In fact in

women aged 50 or older, only 6/45 [13%] AI occurred compared with 39/45 [87%] AI in the women aged 35-49. In the 19 subjects with any AI, 3/19 [16%] were aged 50-55 yrs while 16/19 [84%] were 49 yrs or younger. While there appeared to be a trend between a women's increase age and a decrease of AIs, this finding was not statistically significant [see **Table 6C**]. One possible explanation might be the result of higher estrogen levels present in younger women. Recent reviews examined a) the role of estrogen in initiation and 2) the genotoxic metabolites of estradiol in the breast as a potential mechanism of estradiol induced carcinogenesis [1-3]. The introduction of DNA damage along with the proliferative effects of estrogen during menstrual cycling represent an example of the classical model of carcinogenesis: initiation and proliferation leading to cancer development.

We examined FAL [fractional allele loss, a measure of allele loss] at chromosomal sites by groups [paired and unpaired subjects]. For Example: N017 at 265 [2 AI/ 6 informative TDLU = 0.33]; or N015 at 579 [3 AI/ 6 informative TDLU=0.50]. Examination reveals that the control subjects have AI at 17p VNTR [located within the TP53 gene] while the case subjects have loss only at 17p TP53. Both 11q markers [1818 and 1819] located near the ATM locus have been lost in N024-N4 indicating loss of a large portion of that chromosome arm. Loss of the 17q marker located near the BRCA1 locus is also prevalent. Of the 5 subjects with AI in this region, 3/5 [60%] are 37 years or younger, two have gone on to develop cancer [N015 and N020] and one is a control with no cancer [N001]. The remaining two are also control subjects. Three of these 5 subjects [N001, N015 and N017] have multiple AI in at least 3 chromosome locations and involve multiple TDLUs suggesting a possible field defect. ATM, TP53 and BRCA1 have all been implicated in breast cancer tumorigenesis.

Table 4 compares the present results with the results of our original study [4]. We find that, in the present study, the patients are the same age as in previous study [mean age ~ 44 yrs]. The proportion of patients with AI falls within the range seen in the previous study [55%]. In the earlier study 28% of the control group [RM, reduction mammoplasty subjects] displayed AI while in the present study 50% of the control subjects [no cancer] displayed AI. In the present study 65% of our case subjects [developed cancer] displayed AI while in the previous study 81-83% of the test groups [sporadic breast cancer subjects or BRCA1 mutation carriers] displayed AI. The number of AI per informative site [5.4 versus 1.2], the number of AI/TDLU [0.285 versus 0.142] and the mean block age [23.5 versus 2.7 yrs] are all higher than in the original study. These results may reflect authentic differences between patient populations between studies, the age of the blocks, or the uses of the 33% allele ration cut off.

Task 3. Correlate prevalence and chromosome sites of AI with clinical outcome. Months 12-36.

This task is now complete.

Since February 2010 we have finished all the experimental work, the study population has been unblinded, and the data analyzed. In **Table 5** we have tabulated AI by chromosome site for a) all subjects and b) for the matched pairs.

While 55 [63%] of potential subjects were excluded from the study, modified statistical analyses were conducted on the remaining 33 subjects.

a) Cancer and AI

Primary analysis focused on the presence of AI, as a dichotomous outcome, and its impact on the likelihood of developing cancer. As a crude assessment, Chi-Square tests and Fisher Exact test were performed. In order to adjust for covariates, logistic regression was used with the development of cancer as the dependent variable, and AI and covariate as independent. AI was analyzed both in aggregate [across all markers], and distinctly for each marker. If an individual had presence of AI on any TDLUs for a specific marker, that person was considered AI positive for that marker. If an individual were AI positive for any marker, that person was considered to be AI positive in aggregate. Because the number of TDLUs per person would increase the probability of finding AI, the number of TDLUs analyzed for each person was an important covariate in the logistic regression. Secondary analysis examined the relationship between age and the likelihood of developing AI. Logistic regression was performed with AI as the dependent variable, with age and covariates as independent. Again, the analysis was performed both by aggregating AI across all markers and by distinctly analyzing AI

In the case of aggregated AI, Chi-square analysis yielded an odds ratio [OR] of 1.83 and a corresponding p-value of 0.39. While the presence of AI in aggregate increases the odds of cancer by 1.83, the p-value is not significant at the $\alpha=0.05$ level. In logistic regression, adjusting for age of subject, block age, and number of TDLUs per person, the OR is 1.78 with a p-value of 0.47. AI, in aggregate, increases the likelihood of cancer but the effect is not statistically significant.

In the case of marker specific AI, p-values are reported unadjusted for multiple testing. Due to small sample size, Fisher Exact tests were used in place of Chi-Square tests to provide crude results. The lowest p-value obtained was 0.22 at marker 17pVNTR, with crude OR of 0 [see **Table 6A**]. In order to run logistic regression, in cases with any 0 cells in the AI cancer cross-tabulation, 1 individual was added to each cell in order to attain convergence. This technique was used for markers 17p [VNTR and 17pTP53]. While this **biases results toward the null** for these markers, it provides an interpretable result [see **Table 6B**]. In these logistic regressions, 3 out of 9 markers had an OR of greater than 1, indicating AI at only three markers seemed to increase cancer risk. More specifically, 17pVNTR had an OR of 0.167 with a p-value of 0.194, suggesting that AI at this marker might be associated as reduced risk of cancer. None of these results, however, are statistically significant at the $\alpha=0.05$ level. In marker specific AI, adjusting for block age, number of TDLUs per person and cancer, 3 out of 9 markers had an OR greater than 1. The remaining 6 had ORs < 1, implying age of subject decreases the chance of AI at these markers. None of these results, however, were significant at the $\alpha=0.05$ level.

b) **AI and Subject age**

Secondary analysis examined the relationship between age and the likelihood of developing AI. Logistic regression was performed with AI as the dependent variable, with age and covariates as independent. The analysis was performed both by aggregating AI across all markers and by distinctly analyzing AI at each marker.

In the situation of aggregate AI, adjusting for block age, number of TDLUs per person and cancer status, a single year in age was associated with OR of 0.93 with a corresponding p-value of 0.32. An increase in subject age appeared to decrease the chance of aggregate AI, but the effect was not statistically significant at the $\alpha=0.05$ level.

In marker specific AI, also adjusting for block age, number of TDLUs per person and cancer status, 3 out of 9 markers had an OR greater than one. The remaining six had $ORs < 1$ implying age of subject decreases the chance of AI at these markers. None of these results, however, are significant at the $\alpha = .05$ level [see **Table 6C**].

So we can conclude that aggregating over all markers, AI seems to increase risk for cancer, but the results is not statistically significant. Independently, AI on specific markers does not seem to have a consistent effect on cancer status, but the sample sizes are very small. The relationship between AI and age is not clear-cut either. In aggregate, age appears to reduce the risk of AI, but the result is not statistically significant. Across markers, the effect of age on the risk of AI varies, but again, no results are statistically significant.

Task 4. Manuscript write-up and presentation of results. Months 24-36.

This is not yet complete.

We have begun work on a manuscript. Since no association was found primarily due to study size, we are looking for the best journal to publish the “negative” results. One possibility might be PloS One. Whatever the publication, it will acknowledge the support of the US Army Medical Research and Material Command support.

III. KEY RESEARCH ACCOMPLISHMENTS.

July 2006 - July 2007

> NHS BBD substudy cases and controls identified and blocks pulled; blocks sent blinded as to identify and outcome to the Rosenberg lab; blocks received and stored as required by regulations.

> switch made from radioactive gel-based system to fluorescent capillary electrophoresis based system to detect AI/LOH.

July 2007 - July 2008

> all blocks sectioned and returned to NHS

> optimization of primer multiplexes for small amplicons

> experiments begun, starting with most recent samples and working backwards chronologically. These experiments are: laser capture microdissection of each lesion separately, DNA extraction, amplification, and analysis using fluorescently labeled microsatellite markers (n=9); AI/LOH determination [33% cut-off] and confirmation by replicate PCR at each marker; data entry onto spreadsheet. Results from the initial, most contemporary, batch of subjects (n=11) indicated that the rate of AI/LOH approximated what we saw in our initial paper. Results from these subjects were submitted to the DoD EoH conference.

July 2008 – June 2009

> experiments (as described above) continue, data accumulates and initial analyses begin.

June 2009 – February 2010

> all usable subjects [n=33], blocks [142] and lesions [n=158] have now been identified; all lesions have been microdissected; DNA has been extracted, amplified, and analyzed by fluorescent capillary electrophoresis; AI/LOH [33% cut-off] calculated and confirmed for each lesion at each microsatellite marker; data entered into spreadsheets. Descriptive analyses being done, unblinding to occur in the near future.

February 2010 – February 2011

> all experiments have been completed, the study cohorts unblinded as to cancer status, all findings tabulated as to rates and specific locations of AI/LOH, statistical analyses completed and tabulated. Manuscript in preparation.

IV. REPORTABLE OUTCOMES.

> Results from the initial batch of samples were submitted in Jan 2008 as an abstract to the DoD Era of Hope meeting (Abstract No P25-12, which was attached to July 2008's Annual Report.)

V. CONCLUSIONS.

The goal of this study was to determine whether the occurrence of AI/LOH (abbreviated here as AI) in histologically normal epithelium from non-cancerous breasts predicts future breast cancer development. By July 2008, we had completed Task 1 and Task 2A, and had made substantial progress on Tasks 2B and 2C. Data from the first 11 subjects were submitted as an abstract to the DoD Era of Hope meeting in January 2008. By July 2009 we had made further progress on Tasks 2A and 2B and started Task 3 and 4. By February 2010, we had completed all of Tasks 2 and had made further progress on Tasks 3 and 4. At the present time, we have completed Tasks 2 and 3 and work on Task 4 is being completed.

Specifically, all subjects have been identified and their tissue blocks sent to Dr Rosenberg's laboratory. All blocks that could be sectioned were cut, and all blocks were returned to Dr Tamimi at the NHS. The techniques to study the AI/LOH in a more automated fashion have been finalized, and now all lesions have been microdissected, each lesions' DNA has been extracted, quantified, and tested for quality, and assessment of AI (or not) has been determined for each lesion at each marker. Descriptive statistics are completed. As mentioned earlier several recent publications [4-8], as well as our own data [9] continue to provide evidence that supports the hypothesis that genomic changes in histologically normal-appearing tissues have cancer relevance. These recent references are consistent with our hypothesis that AI/LOH in histologically normal breast epithelium may be a clinically meaningful predictor of outcome.

Unblinding of the NHS-BBD subjects with regard to cancer outcome yielded 16 controls [no cancer] and 17 case subjects [developed cancer]. They included 6 matched pairs and 21 unmatched subjects. As the number of matched pairs was so few, we analyzed the data across unmatched groups [control versus case subjects] and between the 6 matched pairs whenever possible. We found it impossible to make a definitive prediction due to the sample size. However, there were a few trends suggested. AI seemed to increase risk for cancer, but the results were not statistically significant. Independently, AI on specific markers did not seem to have a consistent effect on cancer status, but again the sample sizes are very small. The relationship between AI and age was not clear-cut either. In aggregate, age appeared to reduce the risk of AI, but the result was not statistically significant. Across markers, the effect of age on the risk of AI varied, but again, no results were statistically significant. Future studies exploring the differences seen between the two groups at 17p [TP53 locus] might elucidate how AI in the TP53 gene [VNTR marker] may be a protective factor in women presenting with benign breast disease.

In summary, the study was hampered by two technical problems. The first problem concerned the block appearance where 22/88 [25 %] subjects had tissue blocks that were either in cassettes too large to fit a standard microtome [7/22] or were in large wax blocks [15/22]. We requested that the tissue be re-embedded but Dr. Tamimi denied the request. The second problem concerned the remaining blocks. Before receiving the tissues, many of them were used in a TMA [tissue microarray] study and arrived with many large punched holes in the tissues greatly limiting the number of TDLUs available for the study. We had to reject 13/88 (14.7%) subjects based on TDLU issues. These two technical problems greatly compromised our proposed study design. If more matched pairs could be identified, we might increase the power of the study so that the trends observed would become statistically significant.

VI. REFERENCES.

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VII. APPENDIX

1. Table 1. Criteria leading to identification of 33 NHS-BBD subjects for analysis
2. Table 2. Characteristics of NHS-BBD subjects and tissues
3. Table 3. Descriptive statistics of AI in NHD-BBD tissue
4. Table 4. AI in NHS-BBD vs. original study
5. Table 5. AI per chromosome arms in NHS-BBD tissue
6. Table 6. Statistical Observations from the NHS-BBD study
7. Abstract No. P25-12 submitted to the 2008 DoD Era of Hope meeting

Table 1. NHS-BBD cases -reasons for exclusion from present genomic study

Summary

24 cases were rejected after tissue blocks were cut and the H&E stains were examined

(i.e., too few TDLUs, aberrant tissue histology)

9 cases were rejected because of DNA insufficiency, i.e., inadequate quality or quantity

22 cases were rejected because of the manner in which the tissue was preserved

(i.e., cassettes couldn't fit into microtome)

Case	DNA analyzed but reject			Tissue cut and rejected after H&E review					Initially unuseable		
	Not enough DNA	Poor quality (acid)	Poor quality (other)	TDLUs too small	TDLUs lost	<3 TDLUs	Stroma only	Too many lymphs	Histo logically abnl	Large wax blocks	Cassettes too large
N004			1								
N006		1									
N007							1				
N009			1								
N012					1						
N016										1	
N018								1			
N019										1	
N022								1			
N023			1								
N025											1
N026				1							
N027								1			
N029						1					
N032				1							
N033						1					
N034						1					
N035				1							
N036										1	
N037	1										
N040	1										
N043				1							
N044								1			
N046											1
N047								1			
N048						1					
N050				1						1	
N052											
N053							1				
N054				1							
N055										1	
N056						1					
N057								1			
N058											1
N059									1		
N060						1					
N061	1										
N063										1	
N064											1
N065										1	
N067										1	
N068											1
N070								1			
N072										1	
N074				1							
N075											1
N076										1	
N079	1										
N080										1	
N081										1	
N082										1	
N083										1	
N084										1	
N085											1
N087											1
Total	4	2	3	7	1	6	2	7	1	15	7

Table 2A. Characteristics of NHS-BBD subjects+tissue: all subjects

	Control Group [no cancer]	Case Group [developed cancer]
Subjects	16	17
Age at diagnosis, years		
Mean	43.6	43.1
Median	42	43
Range	37-54	35-55
No. of TDLU	76	82
No. of TDLU/subject		
Mean	4.8	4.8
Median	4.5	4.0
Range	3-7	3-8
Tissue block age, years		
Mean	24.0	23.2
Median	25.0	21.0
Range	13-39	14-34
Blocks/case		
Mean	4.5	4.1
Range	2-8	1-15
TDLU/case		
Mean	4.8	4.8
Range	3-7	3-8

Table 2B. Characteristics of NHS-BBD subjects+tissue: matched subjects

	Control Group [no cancer]	Case Group [developed cancer]
Subjects	6	6
Age at diagnosis, years		
Mean	39.3	38.3
Median	39.5	37.0
Range	37-42	35-43
No. of TDLU	32	34
No. of TDLU/subject		
Mean	5.3	5.7
Median	5.5	5.5
Range	3-7	4-8
Tissue block age, years		
Mean	23.0	23.2
Median	21.5	21.5
Range	13-34	14-33
Blocks/case		
Mean	5	5
Range	3-8	1-13
TDLU/case		
Mean	5.3	5.7
Range	3-7	4-8

Table 3A. Descriptive statistics of AI in NHS-BBD tissue: all subjects

Characteristic	Control Group	Case Group
	[no cancer]	[developed cancer]
No. of subjects	16	17
No. of TDLUs	76	82
TDLUs/subject	76/16 [=4.8]	82/17 [4.8]
No. of informative markers	429	401
Informative markers/TDLU	429/76 [=5.6]	401/82 [=4.9]
Informative markers/subject	429/16 [=26.8]	401/17 [=23.5]
No. of AI	25	20
AI/TDLU	25/76 [=0.329]	20/82 [0.244]
AI/subject	25/16 [=1.56]	20/17 [=1/18]
AI/informative marker	25/429 [0.058]	20/401 [=0.050]
TDLUs with AI/total TDLUs (%)	19/76 [25]	18/82 [22]
Subjects with AI/total subjects (%)	8/16 [50]	11/17 [65]

Table 3B. Descriptive statistics of AI in NHS-BBD tissue: matched subjects

Characteristic	Control Group	Case Group
	[no cancer]	[developed cancer]
No. of subjects	6	6
No. of TDLUs	32	34
TDLUs/subject	32/6 [=5.3]	34/6 [5.7]
No. of informative markers	206	124
Informative markers/TDLU	206/32 [=6.4]	124/34 [=3.6]
Informative markers/subject	206/6 [=34.3]	124/6 [=20.7]
No. of AI	12	5
AI/TDLU	12/32 [=0.375]	5/34 [0.147]
AI/subject	12/6 [=2.00]	5/6 [=0.83]
AI/informative marker	12/206 [0.058]	5/124 [=0.040]
TDLUs with AI/total TDLUs (%)	9/32 [28]	5/34 [14.7]
Subjects with AI/total subjects (%)	4/6 [67]	4/6 [67]

AI, allele imbalance; TDLU, terminal ducto-lobular unit

Table 4: Comparison of original RM-SP-BRCA1 study compared to current NHS-BBD study

	RM	SP	BRCA1	Control Subj [#] [no cancer]	Case Subj [#] [developed cancer]
No. of AI/informative sites	14/2012	25/1793	26/1598	25/429	20/401
%	0.6	1.4	1.6	5.8	5.0
No. of AI/TDLU	0.087	0.150	0.191	0.329	0.244
Subj with AI/total subj	5/18 [28%]	15/18 [83%]	13/16 [81%]	8/16 [50%]	11/17 [65%]
Mean subj age [range] yrs	41 [31-50]	42 [30-49]	42 [34-54]	43.6 [37-54]	43.1 [35-55]
Mean block age [range] yrs	1.4 [0-6]	3.7 [0-11]	3.1 [0-11]	24.0 [13-39]	23.2 [14-34]

[#]: to help reduce artifacts introduced by block age, a 33% allele imbalance ratio cut off was employed

Table 5A. AI per Chromosome Arm in NHS-BBD tissue: all subjects

Chromosome Arm	Control Subjects [no cancer]			Case Subjects [developed cancer]		
	No of Subjects with AI	Informative Subjects	%	No of Subjects with AI	Informative Subjects	%
	1q	4	14	29	3	14
7q	2	6	33	2	5	40
8p	1	12	8	1	8	13
11q	1	14	7	4	16	25
16q	2	13	15	2	11	18
17p	3 ^a	14	21	2 ^b	17	12
17q	2	14	14	2	10	20

Table 5B. AI per Chromosome Arm in NHS-BBD tissue: matched subjects

Arm	Control Subjects [no cancer]			Case Subjects [developed cancer]		
	No of Subjects with AI	Informative Subjects	%	No of Subjects with AI	Informative Subjects	%
	1q	1	6	17	0	4
7q	2	4	50	1	1	100
8p	1	5	20	1	2	50
11q	0	5	ns	1	5	20
16q	0	5	ns	1	4	25
17p	3 ^a	5	60	1 ^b	6	17
17q	1	6	17	1	3	33

a, AI in VNTR; b, AI in TP53

Table 6. NHS-BBD: Statistical Results

a) Crude ORs: AI and cancer			
Marker	OR	Fisher p-value	N
m11q1818	2.45	0.615	24
m11q1819	1.10	1.00	23
m16q265	1.22	1.00	24
m17pTP53	infinity	0.499	27
m17pVNTR	0.00	0.228	19
m17q579	0.92	1.00	24
m1q213	0.68	1.00	28
m7q486	1.33	1.00	11
m8p1121	1.57	1.00	20
b) Logistic regression adjusted* ORs: AI and cancer			
Marker	OR	p-value	N
m11q1818	2.572	0.482	24
m11q1819	0.674	0.8058	23
m16q265	0.995	0.9967	24
m17ptp53	2.412	0.4759	31
m17pvnr	0.167	0.1936	23
m17p579	0.496	0.5747	24
m1q213	0.998	0.9982	28
m7q486	<0.001	0.2634	11
m8p1121	1.595	0.7819	20
*Adjusted for age of subject, block age and TDLUs per person			
c) Logistic regression adjusted* ORs: Age of subject and AI			
Marker	OR	p-value	N
m11q1818	1.078	0.5348	24
m11q1819	0.841	0.4374	23
m16q265	1.036	0.770	24
m17ptp53	0.964	0.991	27
m17pvnr	0.778	0.2416	19
m17p579	0.871	0.1877	24
m1q213	0.987	0.8744	28
m7q486	0.391	0.8602	11
m8p1121	1.051	0.9039	20
*Adjusted for block age, TDLUs per person and cancer status			

Title: Allele imbalance (AI) or loss of heterozygosity (LOH) in normal-appearing breast epithelium as a novel marker to predict future breast cancer.

Authors: Pamela S Larson, Stuart Schnitt, Rulla Tamimi, Carol L Rosenberg

Abstract: Background: Predicting breast cancer development remains challenging. AI, or LOH, is a DNA abnormality that is common to almost all breast tumors. We had shown that AI in histologically normal breast epithelium [terminal ducto-lobular units (TDLU)] is strongly associated with cancer risk (Larsen et al, J Clin Oncol 2005). This led to the hypothesis that women who have AI in their TDLUs are at an increased risk for breast cancer. This study tests that hypothesis, by examining TDLUs from benign breast biopsies of women in the Nurses' Health Studies - Benign Breast Disease (NHS-BBD) nested case-control study. The NHS-BBD study consists of a subset of women enrolled in the NHS. Cases are women with breast cancer diagnosed by June 1, 1995, with a previous benign breast biopsy and available pathology specimens. Controls are women matched for age and year of benign biopsy, who had not been diagnosed with breast cancer at the time the case was diagnosed, and available pathology specimens. Baseline information and clinical follow-up are available for all subjects.

Methods: Cases (n=44) and controls (n=44) were women 35-55 years of age at the time of benign biopsy whose biopsies lacked atypia, and who had no family history of breast cancer. 10- μ sections were cut from paraffin blocks and TDLUs were removed by laser microdissection. DNA was extracted, and 10-20 ng were used per PCR. AI was determined by a fluorescent, capillary electrophoresis-based detection system (Applied Biosystems 3100) with a standard cut-off (reproducible 33% change in peak height). The microsatellite panel is being optimized to add markers with high heterozygosity and small amplified fragments. AI analyses were performed blinded to case-control status.

Results: To date, 11 subjects have been studied. Seven of 11 could be analyzed, generating 38 TDLUs (range 3-8/subject). (Two subjects had degraded DNA, and 2 had insufficient TDLUs). The 38 TDLUs were examined with the current marker panel consisting of 9 markers on 7 chromosome arms (1q, 7q, 8p, 11q, 16q, 17p, 17q); a total of 211 heterozygous sites could be scored. Six of the 211 (2.8%) sites, from 4/7 (57%) subjects, demonstrated AI. The mean number of AI per TDLU was 0.158. These rates resemble those found in our previous study, in which women with no increased cancer risk vs those with cancer [or high cancer risk] had a rate of AI = 0.6% vs 1.5%; the proportion of women with any AI = 28% vs ~ 82%; and the number of AI per TDLU = 0.087 vs ~ 0.170.

Conclusions: Despite the age of the paraffin blocks used in the current study, AI could be analyzed in histologically normal breast epithelium from benign breast biopsies of women in the NHS-BBD study. Initial results demonstrate that the rate of AI, the proportion of subjects with AI, and the number of AI per TDLU are consistent with those found in our earlier study. This suggests that analysis of the remaining subjects, and subsequent unblinding of cases and controls, will help to elucidate whether AI in normal epithelium can predict future development of breast cancer.