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13. ABSTRACT (Maximum 200 Words)

Enhanced estrogen responsiveness of the breast, which may contribute to breast cancer development, could result from changes in estrogen receptor (ER)-α or ER-β expression, or mutation of ER which may lead to receptor hypersensitivity, e.g., the ER-α A908G mutation. We are investigating whether ER markers may serve independently or jointly with histopathology to improve predictive capability for breast cancer. The present study is based on a cohort of 6800 women biopsied for benign breast disease at the Mayo Clinic between 1967 and 1981, and were followed until 1987 for breast cancer. During this time period, 226 cases of breast cancer were identified, and age/race-matched women who did not develop cancer were selected as controls. We have measured expression of ER-α by immunohistochemistry in benign breast lesions and surrounding normal breast epithelium of postmenopausal cases and controls, and have been screening benign and malignant breast tissues for the ER-α A908G mutation. Thus far, we have identified the A908G mutation in benign breast biopsies exhibiting hyperplasia and/or apocrine metaplasia, and in malignant breast tumors, including lobular tumors. Immunoistochemical expression of ER-β has not been successful, therefore, we propose to genotype CYP19/aromatase for the C/T variant in exon 10 in cases and controls.

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Introduction

Although certain histopathologic subtypes of benign breast disease (BBD), such as atypical hyperplasia, are associated with an increased risk of breast cancer, histologic appearance fails to accurately distinguish among the majority of women with BBD those who will subsequently develop cancer. Analysis of molecular or immunohistochemical (IHC) alterations in benign breast biopsies may therefore serve independently or jointly with histopathology to improve predictive capability for breast cancer. Because of the importance of estrogen in breast cancer development and progression, in this study, we had selected for analysis several markers related to estrogen signaling and responsiveness, including IHC expression of estrogen receptor (ER)-α and ER-β, and a somatic point mutation in ER-α (A908G; Lys→Arg) that results in hypersensitivity of the receptor (Fuqua et al, 2000; Wang et al, 2001). These markers were to be evaluated in a breast cancer case-control study nested within a cohort of women who were underwent biopsy for BBD at the Mayo Clinic between 1967 and 1981 and who were followed until 1987 for the development of breast cancer. Formalin-fixed, paraffin-embedded benign and subsequent malignant breast tissues were obtained from cases in the study, while benign biopsies were obtained from control women who did not develop breast cancer during the same time interval, and all tissues have undergone a standardized histopathologic review. Once IHC and molecular laboratory assays are completed, using statistical analyses, we plan to compare the prevalence of each marker in benign tissues in cases and controls, and between the benign and subsequent tumors in the cases, and determine the relationship between each marker and histopathologic characteristics, including proliferation.

Body

The progress made on this study within the past year is described in relation to each specific task as outlined below.

Task 1: Immunohistochemical staining of breast tissues for ER-α and ER-β

a. Carry out ER-α staining using the DAKO ER1D5 antibody on benign and malignant breast tissues from postmenopausal women in the Mayo Clinic study.

ER-α IHC staining using the Dako ER1D5 antibody has been completed for the breast tissues of the postmenopausal women in this study.

b. Score ER-α-staining in the non-proliferative and proliferative benign breast lesions, their histologically-normal surrounding regions, and in malignant tumors.

Detailed scoring of ER-α-stained benign breast tissues has been completed by the study pathologist, Dr. Hasharan Singh, M.D., at UNC-Chapel Hill. ER-α staining was evaluated both in the surrounding histologically-normal epithelium and in the benign lesions. The staining in benign lesions was noted as being within proliferative or non-proliferative lesions. The normal epithelial staining categories were defined as follows: none; no staining or any % epithelial cell staining having a staining intensity of less than 2 out of a maximum of 4, which was considered background, low; 1-30% of epithelial cell nuclear staining with intensity of 2 or greater, moderate to high; 31-100% of epithelial cell nuclear staining with intensity of 2 or greater. Our results indicate that ER alpha expression in the normal epithelial component is significantly higher in control benign tissues and than in case benign tissues (P=0.02) (Figure 1). Higher ER-α expression in normal breast epithelium of controls is in contrast to the study of Khan et al (1998) which found more staining among cases. However, there are several important differences in study design between the two studies that could account for the

discrepancy in results. The study by Khan evaluated normal-appearing areas of breast epithelium surrounding malignant tumors in cases, while the Mayo study tissues were all benign and were obtained prior to the occurrence of any cancer. Additionally, the Khan study evaluated both pre- and postmenopausal women, while we evaluated only benign tissues from postmenopausal women because information on the timing of the breast biopsies relative to stage of the menstrual cycle was not available. Prior studies indicate that in premenopausal women, ER expression is 2 to 3-fold higher in the follicular compared with luteal phase of the menstrual cycle (Battersby et al, 1992). It is therefore possible that our results are specific to postmenopausal women.

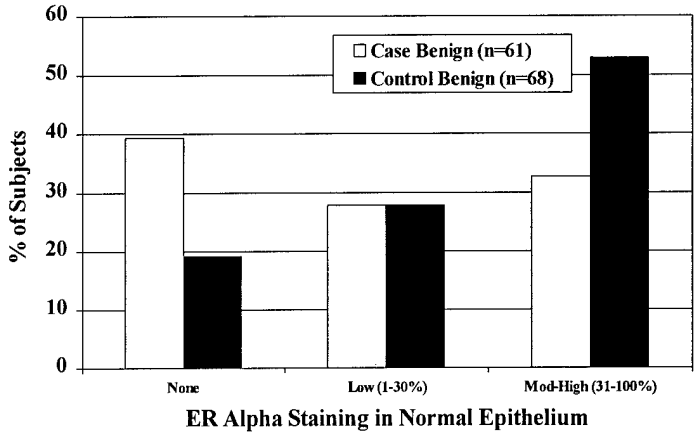

Figure 1: ER Alpha Immunohistochemical Staining in the Normal Epithelial Component of Benign Breast Tissues of Postmenopausal Cases and Controls.

c. Optimize and compare ER-β staining using the Biogenex, GlaxoSmithKline and Genetex antibodies, and identify appropriate control cell lines and formalin-fixed breast tissues.

d. Using the most appropriate ER-β antibody, carry out staining of postmenopausal benign and malignant breast tissues from the Mayo Clinic study.

As described previously, we have thoroughly tested several ER-β antibodies (and multiple lots of these) that have been described as being suitable for immunohistochemistry, including the Genetex 14C8 monoclonal antibody, made to the NH2 terminus of the ER-β protein (Skirlis et al, 2000), a polyclonal antibody from Biogenex, and an antibody described by Fuqua et al (2000) that was developed by GlaxoSmithKline. Testing has included several lots of each antibody, and slight modifications to the staining protocols. However, to our great disappointment, we have not been able to achieve what we consider to be satisactory ER-β immunohistochemical staining in the archival tissues from the Mayo Clinic cohort study, even though good quality staining has been obtained on control tissues with both the Genetex and Biogenex antibodies. Most likely, this result is due to the unique, old age of the tissue blocks, which were collected between 1967 and 1981. We plan to make several additional attempts, but in the event that the results are still unsatisfactory, we would like to propose the substitution of a different hormone-related marker, genetic variation in CYP19/aromatase (C/T in exon 10) in the breast cancer cases and control subjects for the ER-β beta marker.

Substitution of CYP19/Aromatase Genotyping (in lieu of original Task 1c and 1d):

Influence on Estrogen Levels of Genotypic Variation in Estrogen Biosynthetic Enzymes Such as Cytochrome P450 19 (CYP19)/Aromatase Although hormonal secretion and metabolism can be influenced by environmental factors, the control of hormonal patterns is largely genetically regulated. Thus, characterization of genes and their variants that control steroid hormone levels are crucial to understanding the

pathways of estrogen-mediated breast cancer. Increased activities of enzymes involved in the biosynthesis of estrogens may influence breast cancer risk by providing more estrogen for conversion to genotoxic metabolites, and/or stimulating breast epithelial cell division.

The CYP19 gene encodes a steroid aromatase that mediates the rate-limiting step in the metabolism of C19 androgens to estrogens. In postmenopausal women, local aromatization of androgens to estrogens is the main source of estradiol in breast tissue (Pike et al, 1993). Both immunohistochemistry and in situ hybridization studies have identified CYP19/aromatase expression in the epithelial cells of the terminal ductal lobular units and surrounding stromal cells of the normal human breast (Brodie et al, 1998). Genotypic variation resulting from polymorphisms in CYP19 has been proposed to influence expression of this gene. Possession of the A1 allele of CYP19 (12 tetranucleotide (TTTA) repeats in intron 5) was nearly 2.5 times more common in breast cancer cases than in controls (Kristensen et al, 1998). However, this variant is rare, occurring in only 2-3% percent of white populations. A more recently described variant of CYP19, a C to T substitution in exon 10 that is in strong linkage disequilibrium with the (TTTA)n repeat polymorphism, was found to be associated with an increase in aromatase mRNA and an increased breast cancer risk (Kristensen et al, 2000). This allelic variant occurs at a frequency of 33% in Caucasian populations, making it an ideal marker for this study which is comprised of predominantly white subjects. Given its correlation with aromatase expression and breast cancer risk, it would be of interest to determine whether the CYP19 T variant is correlated with the presence of the ER-α A908G mutation in breast tissue or if it modifies the risk of breast cancer defined by ER alpha expression in postmenopausal subjects. Our hypothesis is that women carrying these variants together with the hypersensitive mutant ER-α in their breast tissue may be at especially high risk of breast cancer.

Genotyping of CYP19/Aromatase Genotyping of CYP19/aromatase for the C/T alleles will be performed on DNA obtained by microdissection from histologically-normal surrounding breast epithelium from the benign tissues of controls (n=160) and cases (n=160) in the Mayo Clinic study. We will use the ABI SNaPshot fluorescent method for genotyping CYP19, which has been extensively in our laboratory. We have access to a ABI 377 automated sequencer on which fluorescently-tagged allelic fragments will be separated and identified using GeneScan software. PCR will be performed using the following primers: CYP19 5'-TCAGACAGGTGTCTGGAAC-3' and CYP19-3' 5'-GGATGGATGATTGTATGTG-3'.

Although peripheral blood-derived DNA is preferable for such studies, this is not available from the Mayo Clinic cohort. Previous studies evaluating loss of heterozygosity have utilized a similar approach to obtain control DNA from benign tissue blocks. In order to ensure that the genotyping results obtained in these studies are not influenced by PCR artifacts or template-induced errors associated with the use of formalin-fixed tissues, genotyping assays will be repeated on all samples. The likelihood of PCR-related errors occurring twice within the restriction enzyme or primer recognition sites and producing erroneous genotyping results is extremely low. However, if we obtain different genotyping results in the second assay compared with the first, we will repeat the assay a third time. We will then determine the association of the T variant with breast cancer, and determine whether cases with the CYP19 T variant are more likely to also carry the ER-α A908G mutation or exhibit altered ER-α protein expression among postmenopausal subjects.

TASK 2. Screening of benign biopsies from cases and controls, and the paired malignant tumors from cases for the ER-α A908G mutation

Note: this report contains unpublished data.

a. Perform microdissection of benign and malignant tissues to enrich for abnormal cells.

Hematoxylin and eosin (H&E)-stained tissue sections were reviewed by the study pathologist, who encircled the areas of benign or malignant lesions. To obtain DNA for molecular analysis, slides of unstained sections are superimposed on the H&E slide and the lesion of interest is dissected away from surrounding normal tissue. Because we could not predict exactly what histologic entity within the benign breast biopsies would carry the ER mutation, we initially have not separated areas within the benign lesion into subcomponents, e.g., ductal hyperplasia, sclerosing adenosis, apocrine metaplasia, However, given our preliminary results, this is something we plan to do.

b. Screen benign and malignant tissues for the ER-α A908G mutation.

We have tested and compared three methods for detecting the ER-α A908G mutation in formalin-fixed paraffin-embedded breast tissues, including ligase chain reaction (LCR), SNaPshot, and single strand conformational polymorphism (SSCP) analysis. The SNaPshot fluorescent dideoxy primer extension assay (ABI), like LCR, is designed to detect only the A→G base change at position 908, whereas single strand conformational polymorphism (SSCP) detects a broader range of sequence variations in the region of exon 4 surrounding and including the mutation site at base 908 in codon 303. SSCP has proven to be superior, more reliable, and more sensitive than the other two methods in detecting the A908G mutation, hence we have used SSCP as the screening method in this study. SSCP produces a distinctive bandshift pattern when the A908G mutation is present, although the intensity of the shifted bands is often faint, suggesting that only a minor population of cells is carrying the mutation. An example of SSCP is provided in Figure 2 (upper panel), with tumor 257 showing positivity for the band shift.

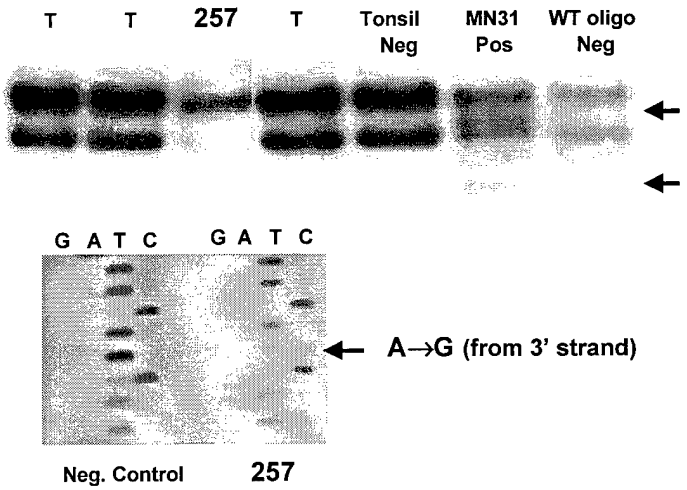

Figure 2: SSCP and sequencing analysis of ER-α A908G mutatio in breast tumors of the Mayo BBD Study. Tumor 257 shows a similar band shift in SSCP as the positive control MN31 (upper panel), and sequencing from the 3' strand confirms the presence of the A908 mutation (A:T to G:C) (lower panel).

c. Sequence tissues to confirm the presence of the mutation.

A total of 411 of 515 (80%) study tissues have been evaluated by SSCP for the ER-α mutation (Table 1). Many of the study tissues screened thus far (n=352) exhibited a band shift on SSCP and were designated as either positive or potentially positive for a mutation in ER exon 4. Only a minority of samples exhibiting a band shift on SSCP carried the diagnostic A908G band, however, we have sequenced all samples that showed even the slightest hint of an aberrant band so as not to miss any mutations. Thus far, 235 samples have been sequenced from the initial PCR product, and of these, 64 samples appeared to carry a mutation. However, based on our >10 years of experience in working with paraffin-embedded tissues and screening for various types of somatic mutations, we are well aware that artifactual mutations commonly occur,

particularly in tissues that are old or that yield poor quality DNA. Therefore, any sample that appears to carry a mutation based on the initial sequence result ('Tube A') is then subjected to re-PCR from the same DNA source and re-sequencing ('Tube B'). True mutations will persist in the subsequent Tube B reaction, while artifactual mutations will drop out. Using this algorithm, we have thus far identified 10 tissues that carry the ER-α A908G mutation in codon 303 and 12 tissues that carry other ER exon 4 mutations. The lower panel of Figure 2 shows the A908G mutation confirmed by sequencing in tumor 257. We are continuing to evaluate the remaining study tissues according to the SSCP/Tube A sequence/Tube B sequence algorithm.

Table 1: Summary of ER Alpha A908G Mutation Screening Studies

Subject - Tissues/ Menopausal Status	No. Blocks Screened by SSCP/ Total	No. SSCP + or ?	No. Sequenced (Tube A)	No. Potentially Mutation-Positive in Tube A Seq.	No. Re-sequenced (Tube B)	No. Positive for ER A908G Mutation	No. Positive for Other ER Exon 4 Mutation	No. Positive for Any ER Exon 4 Mutation
Control - Benign								
Premenopausal	50/67 (75%)	44	27	5	4	0	2[A]	2[A]
Postmenopausal	65/93 (70%)	59	37	12	7	1	3	4
Case - Benign								
Premenopausal	67/78 (86%)	59	46	18	13	4	1	5
Postmenopausal	66/81 (81%)	58	42	14	7	1	2	3
Case - Malignant								
Premenopausal	68/86 (79%) (from 70 cases)	55	49 (from 34 cases)	9	4	0	4	4
Postmenopausal	95/110 (86%) (from 48 cases)	77	34 (from 26 cases)	6	5	4	0	4

Table 2 provides a list of the ER-α exon 4 mutations identified to date. We have detected the G>A codon 311 polymorphism in one tissue, which has been shown to be a germline polymorphism. Three tissues exhibited a A>G base change in codon 299; we have found that this change is also a germline variant. One benign tissue from control subjects and 4 benign tissues from case subjects were found to carry the A908G mutation in codon 303. Several additional mutations in ER-α exon 4 were also detected. Interestingly, as noted previously, ER-α alterations resided in benign tissues exhibiting apocrine metaplasia or some degree of epithelial proliferation (hyperplasia, atypical hyperplasia or sclerosing adenosis). Six malignant tumors from cases also carried the A908G mutation, with two of the 6 tumors having lobular or mixed lobular histology.

Note: this report contains unpublished data.

Table 2: ER Alpha Mutations Identified and Histolopathologic Characteristics

Subject -Tissue ID No.	ER Alpha Mutation Confirmed	Primary Histologic Diagnoses	Presence of Apocrine Metaplasia in Benign	Proliferation in Benign Tissue
Control - Benign				
Premenopausal				
M056	A>G codon 299 (germline variant)	normal tissue only	no	no
M223	A>G codon 299 (germline variant)	mild hyperplasia, apocrine hyperplasia	yes	yes
Postmenopausal				
M198	C>T codon 312	duct ectasia, apocrine metaplasia	yes	no
M316	A>G codon 299 (germline variant)	apocrine metaplasia	yes	no
M425	C>T codon 307, C>T codon 310	duct ectasia, apocrine metaplasia	yes	no
M440	**A>G codon 303 (A908G)**	fibrosis, apocrine metaplasia	yes	no
Case - Benign				
Premenopausal				
M140	**A>G codon 303 (A908G)**	sclerosing adenosis	no	yes
M168	**A>G codon 303 (A908G)**	atypical hyperplasia, apocrine metaplasia	yes	yes
M370	C>T codon 301, C>T codon 307	sclerosing adenosis	no	yes
M504	**A>G codon 303 (A908G)**	mild hyperplasia, apocrine metaplasia	yes	yes
M509	**A>G codon 303 (A908G)**	atypical hyperplasia	no	yes
Postmenopausal				
M094	G>A codon 311 (germline polymorphism)	normal tissue only	no	no
M176	G>A codon 310	fibrosis	no	no
M249	C>T codon 300	apocrine metaplasia	yes	no
Case - Malignant				
Premenopausal				
M164	A>T codon 315	Invasive ductal carcinoma	n/a	n/a
M237	G>A codon 297	Invasive ductal carcinoma	n/a	n/a
M292	G>A codon 307	Invasive ductal carcinoma	n/a	n/a
M313	C>T codon 309	Invasive ductal carcinoma	n/a	n/a
Postmenopausal				
M129	**A>G codon 303 (A908G)**	Invasive ductal carcinoma	n/a	n/a
M170	**A>G codon 303 (A908G)**	Mixed lobular/ductal carcinoma	n/a	n/a
M257	**A>G codon 303 (A908G)**	Invasive ductal carcinoma	n/a	n/a
M330	**A>G codon 303 (A908G)**	Invasive lobular carcinoma	n/a	n/a
M442	**A>G codon 303 (A908G)**	Invasive ductal carcinoma	n/a	n/a
M497	**A>G codon 303 (A908G)**	Ductal carcinoma in situ	n/a	n/a

C. Significance

Abnormalities of estrogen signaling pathways may be important in breast cancer development and progression. The ER-α A908G point mutation, described by Fuqua and coworkers (2000) in one-third of hyperplastic breast tissues, confers heightened ER-α biologic activity and may drive cellular proliferation in the early stages of neoplastic development. This extraordinarily important finding in certain benign breast lesions requires independent confirmation and further study to determine whether it also occurs in malignant breast tumors. In this study, we are determining whether the presence of the ER-α A908G mutation or abnormal ER expression in benign breast tissues is associated with histopathologic features of the tissues, and with an increased risk of subsequently developing breast cancer. The Mayo BBD resource provides malignant tumor tissue for analysis, but more importantly, the nested case-control study design will allow us to evaluate prior benign and subsequent malignancies within the same women, often occurring many years apart. This study should give us substantial

insight into the occurrence and heterogeneity of this molecular lesion in breast cancer development as well as providing confirmation of Fuqua's important observation.

Key Research Accomplishments

- ER-α expression by IHC staining in the normal epithelial component of benign breast tissue blocks from postmenopausal women is significantly greater in controls than in cases (P=0.02). This finding is in contrast to the previous study of Khan et al (1998) which found more staining in case tissues, although there were several differences in study design.

- The ER-α A908G mutation has been identified in some benign breast biopsies and malignant breast tumors of the Mayo benign breast cohort study. Based on the current data, it appears that the ER-α A908G mutation may occur more frequently in the case benign tissues than in the control tissues. Assessment of the A908G mutation as a predictive marker for breast cancer development will be conducted when all mutation screening work is complete.

- The presence of ER-α exon 4 mutations in benign tissues may be associated with histologic features of apocrine metaplasia and/or epithelial proliferation.

- We have identified a previously unreported germline variant in ER-α, a A>G base change in codon 299. This base change results in an amino acid change of Lys to Arg, which is the same change that occurs with the A908G mutation in codon 303. It is unknown whether the codon 299 Arg variant may be functionally abnormal, similar to the hypersensitive codon 303 somatic variant.

- Although lobular breast cancer is relatively uncommon (about 10-15% of all breast cancers), 2 of 6 invasive tumors carrying the A908G mutation were lobular or mixed lobular tumors. This finding is of interest because of the reports during 2003 (Chen, 2002; Newcomer, 2003) of an increased incidence of lobular breast cancer being associated with exogenous hormone use. A higher incidence of the A908G mutation in lobular breast tumors and in malignant breast tissue rather than benign tissue may also partially explain why several other groups (Tebbit et al, 2004; Tokunaga et al, 2004; Zhang et al, 2003) have previously failed to detect the mutation in breast tissues.

Reportable Outcomes

None yet, however several publications are anticipated over the next year.

Conclusions

The most important conclusion to be drawn thus far is that the ER-α A908G mutation can be detected in both benign and malignant breast tissues. In benign tissues, the mutation appears to be associated with histologic features of apocrine metaplasia and/or hyperplasia, while the mutation occurs in both lobular and ductal breast tumors. The detection of this mutation in tumors provides support for its role in breast cancer development.

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