ASSOCIATIONS BETWEEN POLYMORPHISMS AND ABNORMAL RPFNA CYTOMORPHOLOGY IN HIGH-RISK POSTMENOPAUSAL WOMEN TAKING HRT

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Abstract

Introduction: Hormone replacement therapy (HRT) is an effective treatment option for women experiencing symptoms of menopause but long-term use is associated with an increased risk of breast cancer. HRT-related breast cancer risk is dependent on many other factors including age at menopause, age at initiation of therapy, duration of use, dose and method of delivery. Differences in genetic factors, specifically single nucleotide polymorphisms (SNP) may identify those women whose breast tissue is likely or unlikely to be seriously affected by HRT. **Methods:** Post-menopausal women at increased risk for breast cancer underwent breast tissue sampling by random periareolar fine needle aspiration (RPFNA) and epithelial cells were characterized as to whether they exhibited cytologic evidence of hyperplasia with atypia. DNA was isolated from buccal cells for assessment of candidate genes involved in steroid metabolism, receptor function, cell cycle control, DNA repair and/or carcinogen metabolism. Associations between each SNP and RPFNA atypia were examined by unconditional logistic regression in three different genetic models: a log-additive, dominant, and co-dominant. Linear regression was used to assess the association between each SNP and worsening cytomorphology while taking systemic HRT for $\geq$ 6 months vs. off systemic HRT. The adjusted significance level, $p<0.001$ was used to account for multiple testing. **Results:** *RAD54* $Gln^{929}Glu$, *TFR* $Gly^{142}Ser$, *VEGF* 3'UTR and *ACE16* (I/D) were associated with RPFNA atypia in all women at $p<0.05$ and *TFR* $Gly^{142}Ser$ reached borderline significance ($p=0.0025$). Interestingly, the cohort of women with RPFNA cytomorphology both while taking HRT and off HRT showed the most significant results. *RAD23B* Ala249Val was significantly associated with worsening cytomorphology while on HRT vs. off HRT ($p=0.0009$) and *ERCC1* 3'UTR was borderline ($p=0.0015$). SNPs associated with worse cytomorphology showed similar effects when we defined the outcome variable as evidence of atypia while on systemic HRT with no atypia while off systemic HRT. **Conclusion:** Promising associations exist between polymorphisms involved in DNA repair and worse RPFNA cytomorphology as a consequence of HRT use. However, given the probability of chance finding due to multiple testing, these results will need to be validated in an independent cohort.

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Introduction

Hormone replacement therapy (HRT) is an effective treatment option for women experiencing symptoms of menopause. However, long-term use is associated with an increased risk of breast cancer in post-menopausal women [Beral 2003, Rossouw 2002, Chlebowski 2009]. It is well-established that breast cancer risk associated with HRT differs by the type of therapy, with combined estrogen-progestin therapy posing a greater risk than estrogen monotherapy [Rossouw 2002; Beral 2003; Li 2003; Anderson 2004]. However, HRT-related breast cancer risk is dependent on many other factors including age at menopause, age at initiation of therapy, duration of use, dose and method of delivery [Sood 2014, Manson 2013, Li 2003, Modena 2005] and it is likely that for the majority of women there is little to no increase in risk from taking low-dose HRT for 5 years or less during the menopause transition [Johansen 2008, North American Menopause Society 2012].

Women who are already at increased risk for breast cancer, due to family history or other risk factors, may be particularly anxious about initiating HRT despite the adverse effects of estrogen deprivation on their quality of life. While a 15-50% increase in relative risk would not appreciably elevate absolute risk in the average 50 year old whose baseline five year risk is 1%, a high risk woman with a baseline five year risk of 5% could have her absolute risk estimate increased to as much as 7.5% with more than five years of use of estrogen and progestin [Rossouw 2002]. However, high risk women who undergo oophorectomy for risk reduction before age 45, resulting in early or pre-mature menopause are at increased risk for osteoporosis, heart disease or cognitive impairment if they do not take HRT until the age of natural menopause [Shuster 2009]. Given that the quality of life symptoms during the menopause transition and the long-term consequences of estrogen deprivation can be severe, it would be advantageous to develop a simple way to identify those women whose breast tissue is unlikely to be seriously affected by HRT.

A research program was started at The University of Kansas Medical Center (KUMC) in the late 1980s aimed at predicting the short-term risk of developing breast cancer in women who were already at increased risk on the basis of family and personal history. Given the principle that histologic hyperplasia

with atypia on a diagnostic biopsy is known to increase 5 year risk of breast cancer by 4 fold [Hartmann 2005; Dupont 1985; Pearlman 2010] and reported evidence that proliferative changes in the breast are seen in a multi-focal and multi-centric pattern [Neilson 1987, Hoogerbrugge 2003], CJF developed the procedure of random per-areolar fine needle aspiration (RPFNA). This minimally invasive, non-lesion directed technique assesses multiple areas of benign breast tissue in order to detect a field effect in the entire breast. The rationale was that women with evidence of atypia upon random sampling of the entire breast are likely to have multiple areas of proliferative tissue and increased likelihood that one of these areas will progress to invasive cancer. The RPFNA technique was assessed in a cohort of 480 high-risk women. The results of this prospective study indicated that evidence of atypia by RPFNA strongly predicts the development of subsequent DCIS or invasive breast cancer and that cytomorphology along with the Gail model [Gail 1989] could be used to identify a cohort of women at a very high short-term risk (Fabian 2000). RPFNA is currently used in the Breast Cancer Prevention Center at KUMC for risk stratification and as a surrogate end point biomarker in chemoprevention trials (Fabian 2007).

Estrogen and its metabolic compounds are involved in breast carcinogenesis indirectly through their influence on cell growth and proliferation [Horwitz 1998, Zumoff 1998] and directly via oxidative reactions with DNA, forming adducts which generate mutations [Yue 2003, Yager 2006, Cavalieri 2006]. Progesterone can influence breast carcinogenesis through increased formation of estradiol by induction of 17β-hydroxysteroid dehydrogenase. [Poutanen 1995] Progesterone metabolites regulate estrogen receptor levels in breast epithelial cells [Pawlak 2007] as well as proliferation, apoptosis and cell adhesion in tumor and normal breast cells [Weibe 2006, Wiebe 2006]. The effects of exogenous hormones on benign breast tissue are likely to be modulated by genes important in steroidogenesis, catabolism and elimination of estrogens, transcriptional activation in response to hormones and growth factors, cell cycle control, differentiation, and genes involved in carcinogen metabolism and DNA repair. Common polymorphisms in these genes have been associated with increased breast cancer risk in prior studies [Haiman 2003; Tworoger 2004; Lisgarten 2004; Brandt 2004; Liede 2003; Haiman 2002; Zheng 2001; Aston 2004; Coughlin 1999] and some have been shown to influence hormone levels [Feigelson 1998, Dunning 2004,

Tworoger 2004]. However, associations with HRT-related breast cancer risk have been inconsistent [Campa 2011, Kawase 2009, Prentice 2009, Travis 2010, Hein 2012, MARIE-GENICA 2010 (1-4), Nickels 2013, Rebbeck 2007, Reding 2009, Conway 2007, Lee 2011, Andersen 2013, Diergaarde 2008] and few validated interactions between single nucleotide polymorphisms (SNP) and HRT use exist to date [Hein 2012, Justenhoven 2011, Obazee 2013].

To our knowledge, no study has associated candidate polymorphisms with risk biomarkers, such as benign breast tissue cytology. Since risk biomarkers may be subject to modulation, they are ideally suited to monitor change in breast cancer susceptibility with to the use of HRT. Additionally, breast tissue cytology has the advantage of being directly related to the underlying neoplastic process [Fabian 2005]. Thus, high risk women who develop abnormal cytomorphology as a consequence of HRT are likely more susceptible to the carcinogenic effects of exogenous hormones. In order to examine the relationship between relevant polymorphisms, HRT use and RPFNA cytomorphology, we have conducted a genetic association study in post-menopausal women at increased risk for breast cancer.

Materials and Methods

Study Subjects

High-risk post-menopausal women were selected from those being followed at the KUMC Breast Cancer Prevention Center as part of the High Risk Breast Clinic (HRBC) cohort. The HRBC database was queried to identify potential subjects with prior RPFNA (performed by CJF between January 2000 and October 2004) or those planning RPFNA for short-term risk assessment or for ongoing clinical trials between October 2004 and January 2006. The cohort was later expanded to include women (at high risk for breast cancer) with RPFNA performed between January 2000 and March 2009. In order to meet the criteria for the RPFNA procedure as previously described [Fabian 2007, Fabian 2013], subjects were required to be at increased risk for breast cancer by virtue of one or more of the following: (1) five-year Gail predicted probability of breast cancer $\geq$1.67% [Gail 1989]; (2) prior biopsy showing atypical hyperplasia or lobular carcinoma in situ; (3) prior treated contralateral DCIS or invasive cancer; (4)

known BRCA1/2 mutation carrier. Potential subjects were post-menopausal at the time of aspiration, defined as complete surgical (a prior hysterectomy and bilateral oophorectomy), partial surgical (a prior hysterectomy with ovaries intact and age >50 years) or natural menopause (no period for at least 12 months prior to RPFNA), and were either not using hormone replacement therapy or had been on a stable dose of estrogen alone or estrogen plus a progestin for at least 6 months prior to RPFNA.

Upon entry into the HRBC cohort and before each RPFNA procedure, women were asked to sign a consent form which stated that the RPFNA was still considered a research procedure and outlined any additional specimen collection procedures, including buccal cell collection, to be conducted for future studies in the HRBC cohort. Potential subjects for our study were contacted either in-person or by phone and asked to provide a buccal cell sample for SNP genotyping. Women who agreed to participate were either mailed or given a buccal cell collection kit and asked to sign a separate consent form. At the time of buccal cell sample collection, women were asked to verify information in the HRBC database corresponding to their baseline risk and reproductive history factors. A separate database was created to record baseline data, RPFNA history and HRT use at the time of each RPFNA for our study cohort. HRT use was divided into four categories: (1) on well-absorbed systemic HRT (oral, patch, troches, vaginal creams or gels, estrogen + progestin +/- testosterone, estrogen alone, estrogen + testosterone); (2) on poorly-absorbed vaginal estrogen (estring, vagifem tabs); (3) on phytoestrogens (flaxseed, soy, black cohash); (4) off HRT (none of these). Only those women with RPFNA while on well-absorbed systemic HRT for at least six months prior to aspiration or off HRT for at least six months prior to aspiration were considered for this study. RPFNA data and HRT information was updated prospectively for each subject from the time of entry into the study database until March 2009, when data was extracted for analysis.

RPFNA and cytomorphology

RPFNA was performed under local anesthesia from two sites (upper outer and upper inner quadrant) and cells were pooled from both breasts [Fabian 2000] in a single 15 cc tube containing 9 cc of CytoLyt™ and 1 cc of 10% neutral buffered formalin. Cells were spun, washed and re-suspended in PreservCyt™ after approximately 24 h in CytoLyt™. At least three aliquots were processed to slides using a

ThinPrep™ (Cytyc Corporation, Malborough, MA) non-Gyn protocol. Slides for cytomorphology were Papanicolaou-stained using an RNase-free technique [Fabian 2000, Fabian 2005]. All slides were assessed by a single cytopathologist (Carolla M. Zalles) who assigned a categorical assessment of non-proliferative, hyperplasia, borderline hyperplasia with atypia, or hyperplasia with atypia [Zalles 1995, Zalles 2006]; a Masood semi-quantitative index score [Masood 1990], and a Consensus Panel Designation [Uniform 1997].

Sample collection, DNA isolation and genotyping assays

Buccal cells collected via an oral rinse with Scope mouthwash were obtained either by mail or in person from each participant and shipped to Intergenetics, Inc. (Oklahoma City, OK). Genomic DNA was isolated using the Gentra PureGene DNA extraction kit (Gentra, Minneapolis, MN). Genotyping was done by high throughput microsphere-based, allele-specific primer extension (ASPE) assays followed by analysis on the Luminex 100 flow cytometer (Luminex, Austin, TX) as previously described [Ashton 2005, Diergaarde 2008]. DNA was pre-amplified by multiplex PCR using HotStar Taq DNA polymerse (Qiagen Inc., Valencia, CA).

One-hundred and seventeen common single nucleotide polymorphisms (SNPs) were selected in collaboration with Intergenetics, Inc. [Jupe 2007, Ralph 2007, Diergaarde 2008, Jupe 2008]; a summary of these SNPs can be found in the supplementary materials (**Supp. Table 1**). The SNPs were located in breast cancer candidate genes involved in steroid hormone metabolism (CYP17, CYP19, CYP1A1, CYP1B1, COMT, SULT1A1, UGT1A1) and receptor function (AR, A1B1, ESR1, PGR), cell cycle control (PHB, CCDN1, EGFR), DNA repair (ERCC2, RAD23B) and/or carcinogen metabolism (SULT1A1, NQ01, GSTP1) [Haiman 2003; Tworoger 2004; Lisgarten 2004; Brandt 2004; Liede 2003; Haiman 2002, 2003; Zheng 2001; Aston 2004; Coughlin 1999].

We identified 361 potential subjects from the HRBC database; of these potential subjects, 258 were genotyped and had at least one RPFNA performed by March 2009. Following quality-control, 218 women & 79 SNPs passed with >95% call rate. A flow-chart of the exclusion of subjects and SNPs can

be found in the supplementary materials (**Supp. Table 2**). To note, no SNP deviated from Hardy-Weinberg equilibrium (HWE) with a p-value <0.05.

Statistical Analysis

Descriptive statistics were generated using mean and standard deviation for continuous variables and frequency and percent for categorical variables. Distribution of risk, reproductive, RPFNA history and hormone use factors among women with RPFNA atypia and women without RPFNA atypia were compared using t-tests for continuous variables and X^2 test for categorical variables. Genotype frequencies were tested for Hard-Weinberg equilibrium (HWE) using X^2 goodness-of-fit test. All analyses were performed using R 3.0.2 [R Core Team 2013].

To examine associations between each SNP and RPFNA atypia we used three different genetic models: a log-additive (genotype coded in terms of the number of minor alleles), dominant (carrier vs non-carrier of minor allele), and co-dominant (genotype) genetic models. These analyses were completed in all women regardless of HRT use and then stratified by HRT use. Analysis of each SNP with evidence of RPFNA atypia was computed using unconditional logistic regression models with odds ratio (OR) and 95% confidence interval (CI) estimated using Wald statistics. In addition to the SNP association analysis stratified by HRT use, we also completed tests for gene-environment interaction with logistic regression models including each SNP, the HRT use variable (stable dose for ≥6 months at time of RPFNA) and a gene-environment (GE) interaction term. P-values for the interaction effects were obtained using likelihood-ratio tests (i.e. Ho: interaction effect = 0).

Women with RPFNA performed both on and off HRT were excluded from the stratified analysis (described above) and made up the group of women for which we assessed the change in RPFNA cytomorphology while on HRT versus off HRT. Linear regression was used to evaluate the correlation between each SNP and change in cytomorphology (worst on HRT minus worst off HRT) based on the numeric cytomorphology categorization index (1=no cells, 2=normal, 3=normal borderline EH, 4=apocrine metaplasia, 5=EH, 6=EH borderline atypia, 7=atypia, 8=suspicious for cancer). Most changes in RPFNA cytomorphology categorization index occurred between category 5 and category 7 (EH to AH)

although a few women developed atypia (category 7) on HRT with normal cytology (category 2) off HRT. Logistic regression was used to evaluate the association between each SNP and the development of RPFNA atypia only while on HRT (with no atypia while off HRT). All significance tests were two-sided and p-value <0.001 was considered significant when we adjusted for multiple testing; however, $p<0.05$ was reported due to the exploratory nature of this analysis.

Results

Risk and reproductive history

The study population consisted of 218 post-menopausal women at increased risk for breast cancer. Of these women, 95 (43.6 %) had evidence of atypia by RPFNA and 123 (56.4 %) did not have evidence of atypia by RPFNA. Baseline characteristics for the study population are presented in **Table 1**. In general, the cohort consisted of young post-menopausal women, mean age at menopause = 45.3 years and age at entry < 60 years (data not shown), who were at increased risk for breast cancer compared to the general population, mean 5-yr GAIL risk = 3.80% (data not shown). Women with evidence of atypia by RPFNA do not differ from women without evidence of atypia by most risk or reproductive history factors. However, those with evidence of RPFNA atypia were slightly younger at baseline (54.5 ± 6.53 vs. 56.48 ± 7.38, p=0.042) and more likely to have a negative family history, although this did not reach statistical significance (p=0.052). These findings are consistent with other studies using the high risk breast clinic (HRBC) post-menopausal cohort.

Women with evidence of atypia had an average of 4.66 ± 2.15 aspirations throughout the duration of this study while women without evidence of atypia had an average of 2.68 ± 1.78 aspirations ($p<0.001$). Two factors contribute to this finding: (1) the RPFNA procedure involves random, non-lesion directed sampling of the breast tissue and thus cytologic atypia is more likely to be recognized in those women with increased sampling and (2) those with evidence of atypia by RPFNA are more likely to enroll in chemoprevention trials and continue to receive aspirations as part of the HRBC cohort. Women with evidence of atypia as part of this study were also more likely to have had evidence of atypia prior to 2000.

Evidence of atypia in random sampling of benign breast tissue is indicative of widespread proliferative changes in the breast [Fabian 2005] and the high-risk density is not removed as part of the RPFNA procedure.

Table 1: Description of risk and reproductive history for 218 women by evidence of atypia on RPFNA

Characteristic	Never Atypia (n=123)[a]	Ever Atypia (n=95)[a]	p-value[b]
Breast Cancer Risk Information[c]			
Age (yrs), mean (SD)	56.48 (7.38)	54.5 (6.53)	0.042
BMI (kg/m^2), mean (SD)	27.02 (6.65)	27.48 (6.68)	0.62
Mammographic Breast Density, n (%)			0.27
<5%	14 (11.48%)	8 (8.42%)	
5-25%	40 (32.79%)	25 (26.32%)	
26-50%	35 (28.69%)	25 (26.32%)	
51-75%	19 (15.57%)	16 (16.84%)	
>75%	10 (8.2%)	11 (11.58%)	
Number of affected 1st Degree Relatives			0.064
None	30 (24.4%)	36 (37.9%)	
1	61 (49.6%)	43 (45.3%)	
At least 2	32 (26.0%)	16 (16.8%)	
5-yr Gail Risk (%), mean (SD)	3.84 (2.3)	3.74 (2.64)	0.76
Prior AH or LCIS biopsy, n (%)	22 (17.89%)	21 (22.11%)	0.55
Prior contralateral breast cancer, n (%)	14 (11.38%)	13 (13.68%)	0.76
Reproductive History Information[c]			
Parity (0 vs. ≥1), n (%)	15 (12.2%)	16 (16.84%)	0.44
Age at Menopause (yrs), mean (SD)	45.42 (7.24)	45.05 (7.11)	0.71
Interval from Menopause (yrs), mean (SD)	11.11 (8.39)	9.45 (7.57)	0.13
Type of Menopause			0.17
Natural	50 (41.0%)	30 (31.9%)	
Surgical	72 (59.0%)	64 (68.1%)	
RPFNA History in High Risk Breast Clinic (HRBC)[d]			
Number of RPFNAs since 2000, mean (SD)	2.68 (1.78	4.66 (2.15)	<0.001

RPFNA atypia prior to 2000, n (%)	17 (14.05%)	26 (27.66%)	0.021

a. Numbers do not add up to the total for never atypia (n=123) and ever atypia (n=95) due to missing data for some participants
b. Two-sided t-test p-value for continuous variable; chi-square p-value for categorical variables
c. Risk information and reproductive history were collected prospectively upon entry and at each follow-up visit with CJF as part of the High Risk Breast Clinic (HRBC); information stored in the HRBC database was verified at the time of buccal sample collection
d. RPFNA data was collected prospectively at each aspiration visit and entered into the HRBC database; relevant information was collected for each subject throughout the duration of this study and entered in a separate database

Hormone Replacement Therapy (HRT) use history

As shown in **Table 2**, most women in this post-menopausal cohort had used hormone replacement at some point in their personal history to control symptoms of menopause (n=172/218, 78.9%). The proportion women with a history of HRT use appears to be slightly greater among those women with evidence RPFNA atypia (85.3% vs. 74.0%, p=0.064). In order to examine this further, we considered three important factors which contribute to HRT-related breast cancer risk: duration of use, type of therapy and whether dose was expected to increase risk.

Most women in our study cohort, with or without evidence of RPFNA atypia, were likely advised to initiate low-dose and/or estrogen alone hormone replacement therapy; especially since this cohort consisted of young post-menopausal women (mean age at menopause = 45.3, data not shown) and more than 50% had undergone partial or complete surgical removal of their uterus and/or both ovaries. Consistent with this, combined estrogen + progesterone therapy use was low in our cohort (37.5%). However, many women were taking doses of estrogen that have been associated with increased risk of breast cancer ($\geq$ 0.625 mg oral estrogen, 0.05 mg transdermal estradiol or 1 mg oral estradiol) and there appears that more women with evidence of RPFNA atypia were on these higher doses compared to women without evidence of RPFNA atypia (74.5% vs 60.7%, p=0.046).

Duration of HRT use appears to differ among those with and without evidence of RPFNA atypia (p=0.0014); the greatest proportion of women with evidence of atypia were on HRT for $\leq$ 5 years while the greatest proportion of women without evidence of atypia were on HRT for > 5 years. This difference indicates that certain women in our cohort may have continue on hormone replacement long-term, either

for symptom relief or until the age of natural menopause (if <40 years), without affecting their breast cancer risk as predicted by benign breast tissue cytomorphology.

Table 2: Description of Hormone Replacement Therapy (HRT) for 218 women by evidence of atypia on RPFNA

Post-menopausal Hormone Use[e]	Entire Cohort (n=218)[a]	Never Atypia (n=123)[a]	Ever Atypia (n=95)[a]	p-value[b]
On Systemic HRT, n (%)				0.064
Never	46 (21.1%)	32 (26.02%)	14 (14.7%)	
Ever	172 (78.9%)	91 (74.0%)	81 (85.3%)	
Duration of systemic HRT (years)				0.0014
Never	46 (21.1%)	32 (26.02%)	14 (14.7%)	
≤ 5 years	78 (35.8%)	31 (25.2%)	47 (49.5%)	
>5 years	94 (43.1%)	60 (48.8%)	34 (35.8%)	
Estrogen Dose Increased Risk[c], n (%)				0.046
No	72 (33.3%)	48 (39.3%)	24 (25.5%)	
Yes	144 (66.7%)	74 (60.7%)	70 (74.5%)	
On Combined (E + P) HRT[d], n (%)				0.99
Never	141 (65.3%)	80 (65.6%)	61 (64.9%)	
Ever	75 (34.7%)	42 (34.4%)	33 (35.1%)	

a. Numbers do not add up to the total for never atypia (n=123) and ever atypia (n=95) due to missing data for some participants
b. Two-sided t-test p-value for continuous variable; chi-square p-value for categorical variables
c. Estrogen dosages expected to increase risk include: ≥ 0.625 mg oral estrogen, 0.05 mg transdermal estradiol or 1 mg oral estradiol
d. Use of systemic Estrogen + Progesterone (or Progestin) hormone replacement therapy
e. Post-menopausal hormone use was collected prospectively at each BCPC clinic visit and recorded in the HRBC database. The drug name, dose, type, dose-class, method of delivery, start and end date of HRT use was collected at each RPFNA throughout the duration of this study and entered in a separate database

SNPs associated with RPFNA atypia

Polymorphisms in four genes were associated with evidence of RPFNA atypia with a p-value < 0.05. However, these polymorphisms did not remain significant when we adjust for multiple testing, p<0.001. Results for select polymorphisms (p<0.05) are shown in **Table 3** and for all polymorphisms in the supplementary materials (Supp. Table 3). Carriers of the variant allele for *RAD54* $Gln^{929}Glu$, *TFR* $Gly^{142}Ser$ or *VEGF* 3'UTR polymorphisms showed the highest frequency of RPFNA atypia (OR_{dom}= 1.74, 1.98 and 1.86, p_{dom}=0.046, 0.026, 0.022). Log-additive genetic models for both *RAD54* $Gln^{929}Glu$ and *TFR* $Gly^{142}Ser$ showed an increase in the frequency of RPFNA atypia in relationship to the number of minor alleles a woman carried (OR_{ord}=1.71, 1.81 and p_{ord}=0.013, 0.0025). The

VEGF 3'UTR polymorphism was not associated with RPFNA atypia in this model and this was most likely due to a very small number of women with two minor alleles (T/T=2). The D/D genotype for the ACE16 I/D polymorphism is considered to be rare in the general population and associated with high expression of the ACE16 gene. However, in our cohort the deletion genotype was most common, with genotype frequencies D/D=33.2%, D/I=45.8%, I/I=21.03%, and DD was significantly associated with RPFNA atypia (OR_{dom}=0.55, p_{dom}=0.036).

Table 3: SNPs associated with RPFNA atypia (n=218) (p < 0.05)

SNP ID	Gene (var)	Genotype	RPFNA atypia / total[a] (%)	P_{2df}	OR (95% CI)	P_{dom}	OR (95% CI)	P_{ord}
rs4646994	ACE16 (I/D)	D/D D/I I/I	38/71 (53.5%) 38/98 (38.8%) 17/45 (37.8%)	0.11	0.55 (0.31, 0.96)	0.036	0.7 (0.48, 1.02)	0.067
rs3088074	RAD54 (Q929E)	C/C C/G G/G	37/103 (35.9%) 44/94 (46.8%) 13/20 (65.0%)	0.050	1.74 (1.01, 2.99)	0.046	1.71 (1.12, 2.62)	0.013
rs3817672	TFR (S142G)	A/A A/G G/G	21/67 (31.3%) 43/100 (43.0%) 30/50 (60.0%)	0.013	1.98 (1.09, 3.61)	0.026	1.81 (1.23, 2.65)	**0.0025***
rs3025039	VEGF (3'UTR)	C/C C/T T/T	62/162 (38.3%) 30/50 (60.0%) 0/2 (0%)	0.037	2.12 (1.13, 3.99)	0.022	1.86 (1.02, 3.39)	0.042

***borderline statistically significant when we adjust for multiple testing using Bonferroni type-1 error correction, p<0.001**
a. total may not add to 218 due to incomplete genotype data for certain polymorphisms

SNPs associated with RPFNA atypia and HRT use

In order to determine whether the associations between candidate polymorphisms and RPFNA atypia would be modified by HRT, we stratified our cohort by use of systemic hormone replacement therapy at the time of RPFNA. This analysis excluded women with RPFNA results both on and off HRT (n=55) so that gene-environment interaction could be assessed using a single RPFNA for each subject's worst benign breast tissue cytomorphology. Results for select polymorphisms (p<0.05)

are shown in **Table 4** and for all polymorphisms in the supplementary materials (**Supp. Table 4**). We found 13 polymorphisms associated with RPNFA atypia in only one of the two strata ($p<0.05$), indicating a gene-environment effect or a modifying effect of HRT use on genetic risk.

Table 4: SNPs associated with RPFNA atypia in cohort (n=163[a]) stratified by systemic HRT use at time of aspiration ($p < 0.05$)

SNP ID	Gene (var)	Genotype	OFF Systemic HRT (n=77)					ON Systemic HRT (n=86)				
			RPFNA atypia / total[b] (%)	OR (95% CI)	P_{dom}	OR (95% CI)	P_{ord}	RPFNA atypia / total[b] (%)	OR (95% CI)	P_{dom}	OR (95% CI)	P_{ord}
rs16260	ECAD (nt-160)	C/C C/A A/A	14/32 (43.8%) 12/36 (33.3%) 3/9 (33.3%)	0.64 (0.25, 1.64)	0.35	0.74 (0.37, 1.51)	0.41	12/43 (27.9%) 17/32 (53.1%) 4/10 (40.0%)	2.67 (1.09, 6.54)	0.032	1.65 (0.87, 3.13)	0.12
rs3088074	RAD54 (Q929E)	C/C C/G G/G	26/70 (37.1%) 3/7 (42.9%) NONE	0.81 (0.32, 2.05)	0.66	0.93 (0.43, 2.03)	0.86	31/79 (39.2%) 2/6 (33.3%) NONE	2.28 (0.93, 5.58)	0.07	1.89 (1.01, 3.53)	0.046
rs3817672	TFR (S142G)	A/A A/G G/G	11/30 (36.7%) 10/32 (31.3%) 8/15 (53.3%)	1.07 (0.42, 2.76)	0.89	1.31 (0.7, 2.44)	0.4	5/23 (21.7%) 14/38 (36.8%) 14/25 (56.0%)	0.36 (0.14, 0.93)	0.034	0.47 (0.25, 0.87)	0.017
rs1139793	TXNRD2 (I340T)	C/C C/T T/T	11/30 (36.7%) 14/38 (36.8%) 4/9 (44.4%)	1.07 (0.42, 2.76)	0.89	1.12 (0.56, 2.26)	0.75	24/29 (49.0%) 7/29 (24.1%) 1/7 (14.3%)	0.29 (0.11, 0.75)	0.011	0.37 (0.16, 0.83)	0.016
rs3025039	VEGF (3'UTR)	C/C C/T T/T	18/53 (34.0%) 11/23 (47.8%) 0/1 (0.0%)	1.65 (0.62, 4.4)	0.32	1.42 (0.57, 3.57)	0.45	22/68 (32.4%) 10/15 (66.7%) 0/1 (0.0%)	3.41 (1.1, 10.53)	0.033	2.57 (0.91, 7.29)	0.076
rs25487	XRCC1 (Q399R)	G/G G/A A/A	13/37 (35.1%) 13/34 (38.2%) 3/6 (50.0%)	1.23 (0.49, 3.1)	0.66	1.26 (0.61, 2.62)	0.53	14/27 (51.9%) 17/45 (37.8%) 2/14 (14.3%)	0.44 (0.17, 1.12)	0.085	0.45 (0.22, 0.9)	0.024
rs700519	CYP19 (R264C)	C/C C/T T/T	23/66 (34.8%) 6/9 (66.7% NONE	3.91 (0.9, 17.09)	0.07	3.74 (0.86, 16.35)	0.08	32/80 (40.0%) 1/6 (16.7%) NONE	0.3 (0.03, 2.69)	0.28	0.3 (0.03, 2.69)	0.28
rs10012	CYP1B1 (R48G)	C/C C/G G/G	13/44 (29.5%) 12/25 (48.0%) 4/7 (57.1%)	2.46 (0.96, 6.34)	0.062	1.92 (0.94, 3.93)	0.072	13/38 (34.2%) 13/37 (35.1%) 5/9 (55.6%)	1.07 (0.45, 2.56)	0.88	1.37 (0.7, 2.66)	0.36
rs5918	ITGB3 (L33P)	T/T T/C C/C	19/60 (31.7%) 10/17 (58.8%) NONE	3.08 (1.02, 9.34)	0.047	3.08 (1.02, 9.34)	0.047	23/81 (37.7%) 10/22 (45.5%) 0/2 (0.0%)	1.21 (0.46, 3.17)	0.7	0.98 (0.42, 2.32)	0.97

rs198977	KLK2 (G226W)	C/C C/T T/T	21/45 (46.7%) 6/28 (21.4%) 2/4 (50.0%)	0.38 (0.14, 1.03)	0.056	0.55 (0.24, 1.27)	0.16	14/43 (32.6%) 3/10 (30.0%) 15/36 (41.7%)	1.64 (0.68, 3.94)	0.27	1.71 (0.84, 3.47)	0.14
rs6917	PHB (3'UTR)	C/C C/G G/G	16/24 (33.3%) 12/35 (34.3%) 6/12 (50.0%)	0.29 (0.1, 0.9)	0.031	0.29 (0.1, 0.88)	0.029	13/35 (37.1%) 12/33 (36.4%) 7/10 (70.0%)	0.47 (0.16, 1.35)	0.16	0.68 (0.33, 1.37)	0.28
rs1799977	MLH1 (I219V)	A/A A/G G/G	19/40 (47.5%) 9/34 (26.5%) 1/3 (33.3%)	0.41 (0.16, 1.06)	0.067	0.48 (0.2, 1.15)	0.099	14/44 (31.8%) 18/41 (43.9%) 1/1 (100%)	1.77 (0.74, 4.26)	0.2	1.88 (0.81, 4.38)	0.14
rs4072037	MUC1 (E2)	A/A A/G G/G	7/24 (29.2%) 13/37 (35.1%) 9/16 (56.3%)	1.72 (0.61, 4.86)	0.30	2.64[c] (0.86, 8.1)	0.091[c]	7/26 (26.9%) 18/36 (50.0%) 6/21 (28.6%)	1.62 (0.63, 4.16)	0.32	0.56[c] (0.19, 1.64)	0.29[c]

a. Certain women (n=55) were excluded due to cross-over (multiple RPFNAs were performed both on and off systemic HRT); these women were analyzed separately (table 6, 7)
b. Total may not add to total women in each strata due to incomplete genotype data for some polymorphisms
c. Interaction most significant using the recessive model (those homozygous for the variant allele used as the reference group)

Gene-environment (GE) interaction was formally assessed by incorporating an interaction term into the logistic model. For this analysis, we used a stable dose of systemic HRT for at least 6 months prior to aspiration as our hormone environment variable. Again, we excluded women with RPFNA results both on and off HRT (n=55). The GE interaction results can be found in the supplementary materials (**Supp. Table 5**). GE interactions for ECAD (nt-160), TXNRD2 (I340T), CYP19 (R264C), KLK2 (G226W), MLH1 (I219V) were significant at $p<0.05$ but did not remain significant when we adjusted for multiple testing, $p<0.001$ (**Table 5**).

Table 5: Gene-Environment[b] interaction in cohort (n=163[a]) stratified by systemic HRT use at the time of aspiration ($p < 0.05$)

SNP ID	**Gene (var)**	**Dominance model**			**Log-additive model**		
		$OR_{OFF\ HRT}$ (95% CI)	**$OR_{SYS\ HRT}$ (95% CI)**	**P_{int}**	**$OR_{OFF\ HRT}$ (95% CI)**	**$OR_{SYS\ HRT}$ (95% CI)**	**P_{int}**
rs16260	ECAD (nt-160)	0.64 (0.25, 1.64)	2.67 (1.09, 6.54)	0.03	0.74 (0.37, 1.51)	1.65 (0.87, 3.13)	0.1
rs1139793	TXNRD2 (I340T)	1.07 (0.42, 2.76)	0.29 (0.11, 0.75)	0.05	1.12 (0.56, 2.26)	0.37 (0.16, 0.83)	0.04

rs25487	XRCC1 (Q399R)	1.23 (0.49, 3.1)	0.44 (0.17, 1.12)	0.12	1.26 (0.61, 2.62)	0.45 (0.22, 0.9)	0.04
rs700519	CYP19 (R264C)	3.91 (0.9, 17.09)	0.3 (0.03, 2.69)	0.04	3.74 (0.86, 16.4)	0.3 (0.03, 2.69)	0.04
rs198977	KLK2 (G226W)	0.38 (0.14, 1.03)	1.64 (0.68, 3.94)	0.03	0.55 (0.24, 1.27)	1.71 (0.84, 3.47)	0.04
rs1799977	MLH1 (I219V)	0.41 (0.16, 1.06)	1.77 (0.74, 4.26)	0.03	0.48 (0.2, 1.15)	1.88 (0.81, 4.38)	0.02
rs4072037	MUC1 (E2)	1.72 (0.61, 4.86)	1.62 (0.63, 4.16)	0.29	2.64[C] (0.86, 8.1)	0.56[C] (0.19, 1.64)	0.05[C]

a. Certain women (n=55) were excluded due to cross-over (multiple RPFNAs were performed both on and off systemic HRT); these women were analyzed separately (table 6, 7)
b. The environment variable considered for this analysis was on or off a stable dose of systemic HRT for ≥ 6 months prior to aspiration
c. Interaction most significant using the recessive model (those homozygous for the variant allele used as the reference group)

Given the lack of significant GE effect when we examined SNPs in two separate hormone environment strata, we were interested in examining the association between candidate polymorphisms and individual change in cytomorphology as a consequence of HRT use. For approximately ¼ of our cohort (n=55), RPFNA cytomorphology was assessed under multiple hormone environments. In this subset of women, we were able to identify SNPs associated with worse cytomorphology while on a stable dose of systemic HRT for ≥6 months compared to cytomorphology while off HRT for ≥6 months. **Table 6** shows select associations between polymorphisms ERCC1 (3'UTR), RAD23B (A249V), RAD51D (E233G), XRCC3 (T241M), INS-IGF2 (nt1107), MMP2 (nt-1306), NBS1 (E185Q) and worse RPFNA cytomorphology while on HRT vs. off HRT at the significance level $p < 0.05$. For this outcome variable, RPFNA cytomorphology is treated as a numeric index with more abnormal cytomorphology (ex. Hyperplasia with Atypia) receiving as higher score than normal or less abnormal cytomorphology (ex. Epithelial Hyperplasia). When we adjusted for multiple testing, RAD23B (A249V) remained significant with $p=0.0009$, while ERCC1 (3'UTR) was considered borderline significant with $p=0.0015$. Many SNPs associated with worse cytomorphology in Table 5 showed similar associations when we defined the

outcome variable as evidence of atypia while on systemic HRT with no atypia while off systemic HRT. **Table 7** shows select associations between polymorphisms, MMP2 (nt-1306), RAD23B (A249V), XRCC3 (T241M) and CYP19A1 (3'UTR), and evidence of RPFNA atypia which were significant at $p<0.05$. None of the associations presented in Table 6 remained significant when we adjust for multiple testing, $p<0.001$. However, the two outcomes are highly correlated and it is likely that we were under-powered to detect a significant association between the binary outcome variable (evidence of RPFNA atypia on vs. off HRT) in this smaller subset of women.

Table 6: SNPs associated with worse cytomorphology categorization on HRT vs. off HRT (n=55[a]) ($p < 0.05$)

SNP ID	Gene (var)	r	Beta (SE)	P_{ord}	Beta (SE)	P_{dom}	Beta (SE)	P_{rec}[b]
rs3212986	ERCC1 (3'UTR)	-0.43	-0.95 (0.28)	**0.0015***	-0.85 (0.37)	0.024	-1.67 (0.70)	0.022
rs1805329	RAD23B (A249V)	-0.45	-1.20 (0.34)	**0.0009****	-1.11 (0.36)	0.0035	-2.59 (1.39)	0.068
rs28363284	RAD51D (E233G)	0.34	1.75 (0.68)	0.013	3.52 (1.35)	0.012	3.52 (1.35)	0.012
rs861539	XRCC3 (T241M)	0.32	0.76 (0.31)	0.019	0.62 (0.39)	0.12	1.84 (0.70)	0.011
rs243865	MMP2 (nt-1306)	-0.25	-0.55 (0.30)	0.076	-0.45 (0.39)	0.25	-1.40 (0.71)	0.056

*** Borderline significant when we adjust for multiple testing using Bonferroni type-1 error correction, p<0.001**
**** Statistically significant association when we adjust for multiple testing**
a. Select cohort (n=55) with multiple RPFNAs performed both on and off systemic HRT
b. Recessive model indicates association with worse (more abnormal) cytomorphology categorization on sHRT in those homozygous for the variant allele vs. those with at least one common allele

Table 7: SNPs associated with RPFNA atypia on sHRT vs. no RPFNA atypia off HRT (n=55[a]) ($p < 0.05$)

SNP ID	Gene (Var)	Geno-type	RPFNA atypia / total[b] (%)	OR (95% CI)	P_{ord}	OR (95% CI)	P_{dom}	OR (95% CI)	P_{rec}[c]

rs1805329	RAD23B (A249V)	C/C C/T T/T	18/31 (58.1%) 6/20 (30.0%) 0/1 (0.0%)	0.29 (0.09, 0.91)	0.035*	0.36 (0.11, 1.14)	0.08 1	NA	0.99
rs861539	XRCC3 (T241M)	C/C C/T T/T	6/19 (31.6%) 14/31 (45.2%) 4/4 (100%)	2.94 (1.06, 8.14)	0.038*	2.47 (0.77, 7.91)	0.13	NA	0.99
rs10046	CYP19A1 (3' UTR)	C/C C/T T/T	5/12 (41.7%) 11/34 (32.4%) 6/7 (85.7%)	1.32 (0.52, 3.32)	0.56	0.71 (0.21, 2.39)	0.58	10 (1.1, 90)	0.04
rs243865	MMP2 (nt-1306)	C/C C/T T/T	17/31 (54.8%) 7/18 (38.9%) 0/4 (0%)	0.36 (0.14, 0.98)	0.045*	0.44 (0.14, 1.36)	0.15	NA	0.99

***similar association for worse cytomorphology (table 6) & for evidence of atypia (table 7) on HRT vs. off HRT**

a. Select cohort (n=55) with multiple RPFNAs performed both on and off systemic HRT

b. Total may not add to total women in each strata due to incomplete genotype data for some polymorphisms

c. Recessive model indicates association with worse (more abnormal) cytomorphology categorization on sHRT in those homozygous for the variant allele vs. those with at least one common allele

Discussion

This study evaluated associations between 79 polymorphisms in candidate genes important for breast carcinogenesis and the risk biomarker of cytomorphology of breast epithelial cells obtained by RPFNA in postmenopausal women either taking a stable dose of hormone replacement for ≥6 months at the time of cytomorphology assessment or not taking HRT. We were able to identify many promising associations between genetic variation and RPFNA atypia (1) in all women regardless of hormone use and (2) stratified by HRT use at the time of RPFNA which reached significance at $p<0.05$; however these did not remain significant when we adjust for multiple testing. Our most significant findings were for the cohort of women with RPFNA results both on and off systemic HRT. Given the probability of chance finding due to multiple testing, these results will need to be validated in an independent cohort.

None of the polymorphisms we examined were significantly associated with RPFNA atypia using the adjusted p-value, <0.001. However, the top association in this analysis, TFR ($Gly^{142}Ser$),

reached a borderline significant level (p=0.0025). This polymorphism is a missense variation located in codon 142 of the transferrin receptor gene (TFR) leading to a Glycine (GGC) to Serine (AGC) amino acid change [Evans and Kemp 1997]. The NCBI dbSNP database indicates that the G allele (Glycine) is more common in the general population with genotype frequencies: A/A=16.1%, G/A=54.8%, G/G=29.2%. However, the A allele (Serine) was more common in our cohort with genotype frequencies: A/A=30.7%, G/A=45.9% and G/G=22.9%. We found a higher frequency of RPFNA atypia with increasing numbers of the minor allele or G (Gly) in our cohort. A recent study in type 2 diabetes patients found that increased prevalence of the G allele ran in parallel to increase serum ferritin and increased soluble transferrin receptor levels (Fernández-Real 2010). Transferrin is the chief glycoprotein responsible for iron transport in mammalian blood. It binds to transferrin receptors leading to iron uptake by the cell [Elliot 1993]. Increased levels of transferrin receptors has been reported in proliferating and malignant cells, including breast cancer cells, [Shindelman 1981, Cavanaugh et al. 1999, Hogemann-Savellano et al 2003] with more malignant and aggressive tumors showing higher levels of transferrin receptors (Elliot 1993, Hogeman-Savellano 2003). Most published studies to date have found no associations of TFR genotypes with breast cancer risk when considered separately; however, in combination with the *HFE* $C^{282}Y$ polymorphism, *TFR* Ser^{142} was associated with increased risk for breast cancer (Beckman et al. 1999).

We observed that the genetic associations with RPFNA atypia differed when we stratified by systemic HRT use, with most SNP showing association in only one of the two strata. However, none of these associations reached statistical significance at $p<0.001$. Given that HRT use differed among those with and without evidence of RPFNA atypia, we would expect significantly different SNP effects (estimated by odds-ratios) for groups stratified by use of systemic HRT at the time of RPFNA. This difference was statistically assessed using gene-environment interaction models. In this study, gene-environment interactions for ECAD (nt-160), TXNRD2 (I340T), CYP19 (R264C), KLK2 (G226W), MLH1 (I219V) were significant at $p<0.05$ but did not remain significant when we adjusted for multiple testing, $p<0.001$.

Interestingly, the most significant genes associated with worse cytomophology and many genes associated with RPFNA atypia as a consequence of systemic HRT use were genes involved in DNA repair pathways. Exogenous estrogens are metabolized by phase 1 enzymes to form catechol estrogens and estrogen quinones [Spink 1992, Zhang 2007] and these metabolites have been shown to form base adducts in DNA [Ding 2003]. These adducts can either be removed by the nucleotide excision repair pathway or can spontaneously depurinate to produce apurinic sites that are then repaired by base excision repair [Cavalier 2006]. Thus, estrogens metabolites directly produce many of the base lesions that ERCC1, XRCC3, RAD51D and RAD23B proteins help repair. It is possible that polymorphisms in DNA repair genes may contribute to individual variation in the ability to recover from estrogen-mediated DNA damage.

The RAD23B $Ala^{249}Val$ polymorphism was significantly associated with worse cytomorphology ($p=0.0009$) and non-significantly associated with evidence of RPFNA atypia as a consequence of HRT use ($p=0.035$). The RAD23B protein forms a complex with XPC and this complex acts as the earliest DNA damage detector to initiate nucleotide excision repair (NER); it is also specifically involved in global genome NER [Sugasawa 1998]. This pathway is the main mechanism for the repair of bulky DNA adducts and non-bulky DNA lesions that result from oxidative damage [Hutsell 2005]. Several studies have associated genetic variants in RAD23B gene, including the Ala249Val polymorphism, with breast cancer [Mechanic 2006, Perez-Mayoral 2013]. In our cohort, we found that frequency of atypia by RPFNA with the use of systemic HRT was decreased for carriers of the variant T allele and further reduced in women homozygous for the T allele (OR = 0.29, $p=0.035$). We also identified an inverse correlation between number of homozygous alleles and worse cytomorphology by RPFNA in this cohort (OR = 0.30, $p=0.0009$). These findings are unexpected, considering a recent study by Perez-Mayoral et al. which showed that women carrying the T allele (Valine) at codon position 249 had decreased DNA repair capacity compared to women with the wild-type C allele (Alanine). If these findings are validated, it would be interesting to

assess the effects of this variation on RAD23B protein function and DNA repair capacity in our cohort.

This study revealed promising associations between certain polymorphisms and worse cytomorphology while on a stable dose of systemic HRT compared to off HRT. However, due to the increased probability of chance findings due to multiple testing, all associations need to be validated in an independent cohort. We have identified 48 potential subjects by querying the HRBC database for post-menopausal women with multiple RPFNA both while currently using systemic HRT and while not currently using systemic HRT. These women have been contacted by phone and asked to submit a saliva sample for genotyping with the original 107 SNPs [excluding 10 SNPs never genotyped] and 57 new SNPs which have been associated with HRT-related breast cancer risk in recent literature [Campa 2011, MARIE 2010, Reding 2009, Hein 2012, Justenhoven 2012, Conway 2007, Prentice 2010, Andersen 2013, Obazee 2013, Cerne 2012, Rebbeck 2009, Lee 2011]. These new candidate polymorphisms include genes related to hormone metabolism (CYP19A1, CYP2C19, CYP3A4, HSD17B1, SRD5A1, AKR1C3), transport (ABCB1, SLCO1B1) and receptor function (FGFR2, KRAS, MAP3K1, ESR1). The original cohort of women (n=55) and the independent cohort (n=48) will be genotyped for the new SNPs. This will serve to update our study to account for relevant findings from recent breast cancer GWAS and candidate gene studies.

The strengths of our study include the use of prospectively collected data regarding hormone therapy use and the use of an outcome variable, evidence of atypia by RPFNA, which divides our cohort into roughly two equal groups (43.58% had evidence of atypia by RPFNA and 56.42% did not have evidence of atypia by RPFNA). The RPFNA procedure allows for both continued monitoring of short-term breast cancer risk with changes in hormonal milieu and is ideally suited to monitor the biological effects of exogenous hormones on post-menopausal breast tissue. We tested a large number of polymorphisms in multiple candidate genes thought to be involved in breast cancer development; and many of these SNPs were suspected to affect protein function [Feigelson 1998, Dunning 2004, Tworoger 2004].Thus, we were well positioned to identify biologically relevant associations between SNPs and increased breast cancer risk

due to HRT use, predicted by the development of cytomorphologic atypia. One limitations of our study was the relatively small sample size and it is possible that some associations and/or interactions were not detected due to insufficient power. It must also be noted that multiple comparisons may have led to chance findings; with 80 SNPs tested in each analysis we would expect approximately one chance finding at $p<0.05$ for each analysis conducted. It is important to consider adjustment for multiple testing ($p< 0.001$) in the interpretation of these study results and to consider that certain associations will be confirmed in an independent cohort.

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