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EPIDEMIOLOGY

Ages at menarche- and menopause-related genetic variants in relation to terminal duct lobular unit involution in normal breast tissue

Hannah Oh[1] · Clara Bodelon[1] · Maya Palakal[1] · Nilanjan Chatterjee[1] · Mark E. Sherman[1,2] · Laura Linville[1] · Berta M. Geller[3] · Pamela M. Vacek[3] · Donald L. Weaver[3] · Rachael E. Chicoine[3] · Daphne Papathomas[1] · Deesha A. Patel[1] · Jackie Xiang[1] · Susan E. Clare[4] · Daniel W. Visscher[5] · Carolyn Mies[6,7] · Stephen M. Hewitt[8] · Louise A. Brinton[1] · Anna Maria V. Storniolo[9] · Chunyan He[10,11] · Montserrat Garcia-Closas[1] · Stephen J. Chanock[1] · Gretchen L. Gierach[1] · Jonine D. Figueroa[1,12]

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Abstract Reduced levels of terminal duct lobular unit (TDLU) involution, as reflected by higher numbers of TDLUs and acini per TDLU, have been associated with higher breast cancer risk. Younger age at menarche and older age at menopause have been previously related to lower levels of TDLU involution. To determine a possible genetic link, we examined whether single-nucleotide polymorphisms (SNPs) previously established in genome-wide association studies (GWAS) for ages at menarche and menopause are associated with TDLU involution. We conducted a pooled analysis of 862 women from two studies. H&E tissue sections were assessed for numbers of TDLUs and acini/TDLU. Poisson regression models were used to estimate associations of 36 menarche- and 21 menopause-SNPs with TDLU counts, acini counts/TDLU, and the product of these two measures, adjusting for age and study site. Fourteen percent of evaluated SNPs (eight SNPs) were associated with TDLU counts at $p < 0.05$, suggesting an enrichment of associations with TDLU counts. However, only menopause-SNPs had >50 % that were either significantly or nonsignificantly associated with TDLU measures in the directions consistent with their relationships shown in GWAS. Among ten SNPs that were statistically significantly associated with at least one TDLU involution measure ($p < 0.05$), seven SNPs (rs466639: *RXRG*; rs2243803: *SLC14A2*; rs2292573: *GAB2*; rs6438424: *3q13.32*; rs7606918: *METAP1D*; rs11668344:

Gretchen L. Gierach and Jonine D. Figueroa have contributed equally.

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✉ Hannah Oh
hannah.oh@nih.gov

[1] Division of Cancer Epidemiology and Genetics, National Cancer Institute, 9609 Medical Center Dr., Bethesda, MD 20892, USA

[2] Division of Cancer Prevention, National Cancer Institute, Bethesda, MD, USA

[3] The University of Vermont, Burlington, VT, USA

[4] Department of Surgery, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA

[5] Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN, USA

[6] Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA, USA

[7] Genomic Health, Inc., Redwood City, CA, USA

[8] Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, Bethesda, MD, USA

[9] Susan G. Komen Tissue Bank at the Indiana University Simon Cancer Center, Indianapolis, IN, USA

[10] Department of Epidemiology, Richard M. Fairbanks School of Public Health, Indiana University, Indianapolis, IN, USA

[11] Indiana University Simon Cancer Center, Indianapolis, IN, USA

[12] Usher Institute of Population Health Sciences and Informatics, Institute of Genomics and Molecular Medicine, Edinburgh Cancer Research Centre, University of Edinburgh, Edinburgh, UK

TMEM150B; rs1635501: *EXO1)* were associated in the consistent directions. Our data suggest that the loci associated with ages at menarche and menopause may influence TDLU involution, suggesting some shared genetic mechanisms. However, larger studies are needed to confirm the results.

Keywords Menarche · Menopause · Lobular involution · TDLU · SNP · Breast histology

Introduction

Terminal duct lobular units (TDLUs) are the milk-producing structures of the breast and the predominant anatomical structures from which breast cancers originate [1]. As women age, the total number of TDLUs and acini (epithelial substructures) decrease, a process called age-related TDLU involution [2]. Studies have shown that less advanced TDLU involution, indicated by higher numbers of TDLUs and acini measured at a single time point, is a significant risk factor for breast cancer [3–6]. Recent longitudinal data also support that women whose TDLUs fail to involute are at higher subsequent breast cancer risk as compared with those who do not [7]. Further, measures of TDLU involution likely reflect a global process occurring throughout the breast as data show TDLU measures to be largely correlated within and between breasts in a woman [8–10]. Therefore, identification of factors that are associated with TDLU involution may reveal underlying biological pathways related to breast cancer risk.

Menarche indicates the onset of female sexual maturation and attainment of reproductive capacity that involves important physiologic changes of breast development [11, 12]. Before or around the time at menarche, ovaries start producing estrogens and progesterone, which stimulate the branching of the duct system, appearance of TDLUs, and accumulation of fat in the connective tissue of breasts. During puberty, breasts continue to mature, growing in size and number of glands and lobules, to support lactation at a future birth. In contrast, menopause is marked by significant atrophy in the breast compared with premenopausal women. In response to the reduction in estrogen levels at menopause, glandular tissue of the breast shrinks, which is then replaced by adipose tissue, and connective tissue becomes inelastic [11, 12].

Younger age at menarche and older age at menopause are well-established risk factors for breast cancer [13], although the exact biologic mechanisms by which they are associated with risk have not been identified. Younger age at menarche and older age at menopause are thought to indicate high cumulative exposure to sex hormones [14] that stimulate cell proliferation [15] and breast development [11, 12, 16]. Previously, we have observed that women with higher estrogens and prolactin levels have higher TDLU counts [17, 18]. We have also shown younger age at menarche and fewer years since menopause to be associated with higher TDLU counts [19], suggesting that increasing epithelial cell content, which indicates delayed involution, in part may mediate their associations with breast cancer risk. However, we have limited knowledge of the biological mechanisms that may influence TDLU involution.

Recently, genome-wide association studies (GWAS) have identified single-nucleotide polymorphism (SNP) markers that are associated with ages at menarche [20] and menopause [21], which should further enhance our understanding of the biology of menarche and menopause as well as normal breast development. Findings from these studies suggest that ages at menarche and menopause are complex traits that involve various markers and pathways, some of which are related to obesity, hormonal regulation, coenzyme A and fatty acid biosynthesis, and DNA repair [20, 21]. Given that menarche and menopause are critical periods when the breast undergoes large changes, many of the mechanisms related to menarche and menopause may also influence TDLU involution and thus breast cancer risk. In a pooled analysis of two studies, the Susan G. Komen Tissue Bank (KTB) at the Indiana University Simon Cancer Center [22] and the NCI Breast Radiology Evaluation and Study of Tissues (BREAST) Stamp Project [23], we examined whether SNPs related to age at menarche (36 SNPs) and age at menopause (21 SNPs) are associated with standardized measures of TDLU involution in order to provide some insights into the influence of potentially biologically relevant loci on the histology of the breast.

Materials and methods

Study population

The KTB is an annotated specimen bank which has recruited healthy volunteer women, aged 18–91 years, since 2007. The current analysis targeted a subset of 2321 KTB participants who were recruited from January 10, 2009 to September 14, 2012. Participants provided demographic, lifestyle, and cancer-related information via self-administered questionnaire. Details of the KTB have been described elsewhere (http://komentissuebank.iu.edu) [19, 22].

The NCI BREAST Stamp Project is a cross-sectional study of mammographic density conducted among 465 women, aged 40–65 years, who were referred for diagnostic image-guided breast biopsy from 2007 to 2010 at the University of Vermont College of Medicine and University of Vermont Medical Center. Women with a prior history of

breast cancer or receipt of nonsurgical treatment for any cancer, who had undergone breast surgery within one year, had breast implants, or were taking breast cancer chemoprevention were ineligible. A self-administered questionnaire and a supplementary telephone interview assessed health and breast cancer risk factor information. Details of the study have been described elsewhere [23].

Women were excluded if they had a diagnosis of in situ or invasive carcinoma at biopsy ($n = 177$ KTB, $n = 78$ BREAST) and if they were of nonwhite, non-European descent ($n = 555$ KTB, $n = 23$ BREAST), pregnant at biopsy ($n = 21$ KTB), currently using hormone therapy ($n = 95$ KTB, $n = 23$ BREAST), and missing genotype data ($n = 796$ KTB, $n = 17$ BREAST) or TDLU measurements ($n = 1$ KTB, $n = 14$ BREAST). Repeated data from the previous donors ($n = 124$ KTB) were also excluded. A total of 862 women ($n = 552$ KTB, $n = 310$ BREAST) were included in the final analytic population.

Participants provided written informed consent and the studies were approved by the Institutional Review Board at the Indiana University and the NIH Office of Human Subjects Research for the KTB and by the Institutional Review Boards at the University of Vermont and the National Cancer Institute for the BREAST Stamp Project.

KTB sample collection and DNA extraction

Whole blood samples were collected using Vacuette® EDTA tubes. DNA was extracted from blood cells at the Indiana CTSI Specimen Storage Facility (ICTSI-SSF) lab using an AutogenFlex Star (SN 401033) instrument and the Flexigene AGF3000 blood kit for DNA extractions from whole blood specimens following manufacturer's specifications. For this study, a 50 µL aliquot of sample was stored using Biomatrica® DNAstable® Handbook. Samples were reconstituted at the Cancer Genomics Research laboratory (CGR) (Frederick, MD, USA) for genotyping.

BREAST Stamp sample collection and DNA extraction

Whole blood samples were collected using standard techniques and allowed to clot for 30 min. Mouthwash samples were collected as previously described [24]. Blood and mouthwash samples were processed at the University of Vermont General Clinical Research Center. Frozen samples were shipped to SeraCare Life Sciences (Gaithersburg, MD), where they were stored in liquid nitrogen. At SeraCare, leukocyte DNA was isolated from blood clots using phenol chloroform extraction methods, and DNA was isolated from buccal cells using Puregene methods (Gentra Puregene Buccal Cell Kits, Qiagen). DNA was quantified at the CGR with the QuantiFluor® dsDNA System (Promega) according to the manufacturer's instructions.

Genotyping assay

Genotyping was performed at the CGR using Taqman nuclease assay (Taqman®), with reagents designed by Applied Biosystems (http://www.appliedbiosystems.com/) as Assays-by-Design™ and using the ABI PRISM 7900HT, 7700 or 7500 Sequence Detection Systems or Fluidigm format. Duplicate concordance was 100 % for all SNPs.

SNPs were selected a priori from previous GWAS meta-analyses that examined associations with ages at menarche [20] and menopause [21, 25] as continuous traits in years. We confirmed that the allele frequencies and the directions of associations between the selected SNPs and ages at menarche and menopause estimated in our data were largely consistent with those previously reported in the GWAS, although we generally had limited power to detect the modest associations (Supplementary Table 1, methods in Appendix).

TDLU measurements

In the KTB, tissue cores were obtained from the upper outer quadrant of the breast using a 10-gauge needle. The tissue samples were then fixed in formalin to construct formalin-fixed paraffin embedded (FFPE) blocks. In the BREAST Stamp Project, FFPE blocks of benign breast tissue were collected from diagnostic ultrasound-guided core needle (14-gauge) or stereotactic-guided vacuum-assisted (9-gauge) breast biopsies. For both studies, each prepared FFPE tissue block was sectioned at 5 µm and stained with hematoxylin and eosin (H&E). All TDLU measures in the H&E slides were centrally annotated at the NCI by a single study pathologist (MES) as previously described [9, 19]. The H&E slides were scanned as digital images suitable for web-based viewing, electronic marking of regions of interest, and image analysis on Digital Image Hub software (SlidePath/Leica, Dublin, Ireland). TDLU analyzer software [18, 26] was used to quantify the number of acini per TDLU ("acini count/TDLU") for up to ten TDLUs per woman and the median value was selected as a summary measure for each woman. A previous study [19] demonstrated high intra-observer agreement (Spearman $r > 0.90$) for the study pathologist for the TDLU measures. The TDLU measures were also inversely correlated with the subjective, qualitative evaluation of TDLU involution (none, partial, complete involution) that had been previously linked to breast cancer risk [3]. To estimate the cumulative epithelial content, a product of the TDLU counts and the median acini counts/TDLU was calculated for each woman.

Statistical analysis

All analyses were conducted using the pooled data of the two studies. For each outcome, Poisson regression models, with a robust variance estimator, were performed to examine the relationships with candidate SNPs. To confirm the robustness of results, linear regression models were additionally performed as secondary analyses. Because different needle sizes were used for the breast biopsies, we standardized TDLU counts by total tissue area on the H&E slides by using an offset in the Poisson models and by including total tissue area within the denominator of the outcome in the linear models. For linear models, TDLU measures were log-transformed to improve normality of the data. To avoid log-transformation of zeros, we added one to each TDLU measure before the transformation. Per-allele risk estimates were presented adjusted for age (10-year intervals) and study (KTB, BREAST), where coding of each variable with values of 0, 1, and 2 was based on the number of risk alleles (i.e., alleles associated with younger age at menarche or older age at menopause implicating increased breast cancer risk) that each woman carried. Additional adjustment for menopausal status, percent fat on the H&E slides, body mass index, parity, and ages at menopause and menarche did not materially change the results, and thus were not included in the final models. *P*-values for trend were estimated using the Wald test. In secondary analyses, we stratified by study, menopausal status, and parity to assess the variation in associations by these variables; we tested for heterogeneity by these variables using likelihood ratio tests for interaction terms in the pooled model. For SNPs showing heterogeneous associations by study, we further stratified by low- to high-risk benign lesion type ($n = 128$ nonproliferative, 152 proliferative without atypia, 28 atypical hyperplasia) within the BREAST Stamp Project to examine whether the associations varied by biopsy diagnosis. In separate secondary analyses, we also created a composite polygenic risk score to assess the polygenic contribution of loci associated with ages at menarche and menopause by summing the number of risk alleles multiplied by the corresponding beta estimates from the previously published GWAS. Associations with TDLU involution measures were estimated for quintile categories of the polygenic risk scores.

All statistical tests were two sided with 5 % type I error. Because we used established SNPs identified from previous GWAS [20, 21], we used the threshold of $p < 0.05$ to identify significant associations. We tested for enrichment of associations using binomial tests comparing the proportion of SNPs with significant associations to 5 % which was expected by chance alone at 5 % type I error. We also adjusted for multiple testing using Bonferroni correction (corrected alpha = 0.0003 for 171 tests). To account for directions of associations, we compared the proportion of evaluated SNPs associated (regardless of significance) in the directions consistent with our expectation (RR > 1.0) based on their relationships with ages of menarche or menopause as shown in GWAS to 50 % which would have been expected by chance.

Analyses were conducted with SAS software, version 9.3 (SAS Institute Inc., Cary, NC).

Results

Participant characteristics

Of the 862 women evaluated in the two studies, the majority was premenopausal (72 % in the KTB, 63 % in the BREAST) (Table 1). Forty-seven percent of KTB and 39 % of BREAST participants had menarche at age ≤12 years. The mean age of the women was 43.1 years and, among postmenopausal women, the mean age at natural menopause was 49.8 years (49.4 years in the KTB, 50.3 years in the BREAST). Women from the KTB and BREAST studies were enriched for family history of breast cancer in a first-degree relative compared with the general population at a similar frequency (23 % in the KTB and 25 % in the BREAST vs. 11 % in the general U.S. population [27, 28]). The median numbers of TDLUs per 100 mm^2 tissue area and acini per TDLU were 21.7 and 13.0 in the KTB and 18.8 and 11.3 in the BREAST, respectively.

TDLU counts

Out of 36 menarche SNPs and 21 menopause SNPs evaluated, eight SNPs (five menarche and three menopause SNPs) were significantly associated with TDLU counts ($p < 0.05$) (Table 2, results for all 57 SNPs in Supplementary Table 2). The number of significant associations was significantly higher than that would have been expected by chance alone (14 vs. 5 %; $p = 0.007$), suggesting an enrichment of association with TDLU counts. Among these, the risk alleles of rs466639 (*RXRG*), rs2243803 (*SLC14A2*), and rs2292573 (*GAB2*), which have been associated with younger age at menarche, were also associated with higher TDLU counts. Rs7606918 (*METAP1D*), rs11668344 (*TMEM150B*), and rs1635501 (*EXO1*), which have been associated with older age at menopause, were associated with higher TDLU counts. Regardless of statistical significance, 50 % of

Table 1 Characteristics of participants by study

	KTB ($n = 552$) N (%)	BREAST ($n = 310$)
Age (years)		
<30	180 (32.6)	0
30–39	105 (19.0)	0
40–49	127 (23.0)	162 (52.3)
50–59	85 (15.4)	112 (36.1)
≥60	55 (10.0)	36 (11.6)
Age at menarche (years)		
≤12	260 (47.1)	120 (39.2)
13	169 (30.6)	113 (36.9)
≥14	123 (22.3)	73 (23.9)
Postmenopausal	157 (28.4)	113 (36.5)
Surgical menopause (among postmenopausal women)	18 (15.0)	7 (8.3)
Age at natural menopause (years)		
<45	9 (11.8)	5 (8.1)
45–49	22 (29.0)	13 (21.0)
50–54	34 (44.7)	41 (66.1)
≥55	11 (14.5)	3 (4.8)
Body mass index (kg/m^2)		
<25	222 (40.6)	140 (45.2)
25–29.9	160 (29.3)	86 (27.7)
≥30	165 (30.2)	84 (27.1)
Parity		
Nulliparous	267 (48.4)	79 (25.5)
1	64 (11.6)	39 (12.6)
2	140 (25.4)	125 (40.3)
≥3	81 (14.7)	67 (21.6)
First-degree family history of breast cancer	127 (23.0)	76 (24.9)
TDLU involution measure		
TDLU observed	382 (69.2)	226 (72.9)

	KTB ($n = 552$) Median (IQR)	BREAST ($n = 310$)
Among women with at least one TDLU		
TDLU counts per 100 mm^2 area	21.7 (41.5)	18.8 (32.4)
Median number of acini per TDLU	13.0 (13.5)	11.3 (10.0)
Product of TDLU counts per 100 mm^2 area and median number of acini per TDLU	284 (728)	212 (440)

KTB Susan G. Komen Tissue Bank, *BREAST* NCI Breast Radiology Evaluation and Study of Tissues Stamp Project, *TDLU* terminal duct lobular unit, *IQR* interquartile range

menarche SNPs and 69 % of menopause SNPs were associated with TDLU counts in the consistent directions based on their associations with ages at menarche or menopause shown in GWAS.

Acini counts per TDLU

One menarche SNP and none of menopause SNPs were significantly associated with acini counts/TDLU ($p < 0.05$) (Table 3, results for all 57 SNPs in Supplementary Table 3), which does not demonstrate a significant enrichment of associations (1.8 vs. 5 %; $p = 0.37$). Rs6438424 (3q13.32) was associated with both younger age at menarche and higher acini counts/TDLU. Regardless of statistical significance, 37 % of menarche SNPs and 67 % of menopause SNPs were associated with acini counts/TDLU in the consistent directions based on their associations with ages at menarche or menopause shown in GWAS.

The product of TDLU counts and acini counts per TDLU

Five menarche SNPs and one menopause SNP were significantly associated with the product of TDLU counts and acini counts/TDLU ($p < 0.05$) (Table 4, results for all 57 SNPs in Supplementary Table 4); however, significant enrichment of associations was not found (11 vs. 5 %; $p = 0.16$). The risk alleles of rs2243803 (*SLC14A2*) and rs6438424 (3q13.32) have been associated with younger at age menarche and were related to higher product of TDLU counts and acini counts/TDLU. The risk allele of rs7606918 (*METAP1D*) has been associated with older age at menopause and was related to higher product of TDLU counts and acini counts/TDLU in this study. Regardless of statistical significance, 45 % of menarche SNPs and 71 % of menopause SNPs were associated with the product in the consistent directions.

Secondary analyses

Similar results were observed with linear models (Supplementary Tables 2–4). However, none of the associations remained statistically significant after Bonferroni adjustment.

Although the directions of associations did not vary by study, menopausal status, and parity, we observed statistically significant heterogeneity in the strength of associations by study and menopausal status (p-heterogeneity < 0.05) (data not shown). Specifically, a positive association of rs2243803 with acini counts/TDLU and the inverse associations of rs1398217 with product of TDLU counts and acini counts/TDLU were stronger in premenopausal women. Positive associations of rs6438424 with TDLU counts, acini counts/TDLU, and product of the two measures were stronger in the KTB. Associations of rs466639 and rs1635501 with higher TDLU counts were stronger in the BREAST. Within the BREAST, positive associations of rs466639 and rs1635501 with TDLU counts were suggestively stronger among women with proliferative versus

Table 2 Top associations ($p < 0.05$) of ages at menarche- and menopause-SNPs with TDLU counts in the pooled analysis

	Genomic region	Nearest gene(s)	Risk/nonrisk allele[a]	AF[b]	N	Poisson model[c]	
						RR (95 % CI) per risk allele	*p*-trend
Menarche SNPs							
rs466639[d]	1q23.3	*RXRG*	A/G	0.14	843	1.26 (1.05, 1.51)	0.01
rs1079866	7p14.1	*INHBA*	C/G	0.85	814	0.82 (0.69, 0.97)	0.02
rs2243803[d]	18q12.3	*SLC14A2*	T/A	0.59	814	1.16 (1.02, 1.32)	0.02
rs9389666	6q16.2	*PRDM13, MCHR2*	C/G	0.40	803	0.87 (0.77, 0.98)	0.02
rs2292573[d]	11q14.1	*GAB2*	T/C	0.84	804	1.23 (1.02, 1.48)	0.03
Menopause SNPs							
rs7606918[d]		*METAP1D*	A/G	0.85	806	1.31 (1.07, 1.60)	0.01
rs11668344[d]	19q13.42	*TMEM150B*	T/C	0.65	821	1.17 (1.01, 1.36)	0.04
rs1635501[d]	1q43	*EXO1*	T/C	0.53	798	1.14 (1.00, 1.30)	0.04

TDLU terminal duct lobular unit, *AF* allele frequency, *RR* relative risk, *CI* confidence interval, *SNP* single-nucleotide polymorphism

[a] Risk allele indicates an allele associated with younger age at menarche or older age at menopause. Based on their associations with ages at menarche and menopause in GWAS, we expect to observe RR >1 for their associations with TDLU counts

[b] Allele frequency of the risk allele

[c] Adjusted for study (KTB, BREAST) and age (<30, 30–39, 40–49, 50–59, ≥60 years)

[d] Alleles with expected directions of associations based on their associations with ages at menarche or menopause as shown in GWAS

Table 3 Top associations ($p < 0.05$) of ages at menarche- and menopause-SNPs with acini counts per TDLU in the pooled analysis

	Genomic region	Nearest gene(s)	Risk/nonrisk allele[a]	AF[b]	N	Poisson model[c]	
						RR (95 % CI) per risk allele	*p*-trend
Menarche SNPs							
rs6438424[d]	3q13.32	*3q13.32*	T/G	0.52	840	1.15 (1.05, 1.28)	0.005

None of the 21 menopause-SNPs was significantly associated with acini counts per TDLU ($p \geq 0.05$)

TDLU terminal duct lobular unit, *AF* allele frequency, *RR* relative risk, *CI* confidence interval, *SNP* single-nucleotide polymorphism

[a] Risk allele indicates an allele associated with younger age at menarche. Based on their associations with age at menarche in GWAS, we expect to observe RR > 1 for their associations with acini counts per TDLU

[b] Allele frequency of the risk allele

[c] Adjusted for study (KTB, BREAST) and age (<30, 30–39, 40–49, 50–59, ≥60 years)

[d] Alleles with expected directions of associations based on their associations with age at menarche as shown in GWAS

nonproliferative lesions (rs466639: RR = 1.38 nonproliferative vs. 1.76 proliferative without atypia vs. 2.17 atypical hyperplasia; rs1635501: RR = 1.25 vs. 1.41 vs. 1.31), although the differences were not statistically significant (p-heterogeneity = 0.80 for rs466639, 0.86 for rs1635501).

No significant associations were observed with polygenic risk scores for ages at menarche and menopause (data not shown).

Discussion

In our pooled analysis of a priori selected SNPs and standardized TDLU involution measures, we observed that a subset of SNPs previously found to be related to ages at menarche or menopause was also associated with standardized measures of TDLU involution. Specifically, out of 36 menarche SNPs and 21 menopause SNPs tested, 14 % of evaluated SNPs were found to be significantly associated with TDLU counts, whereas only 5 % would have been expected to be found by chance alone; these findings suggest an enrichment of associations with TDLU counts and are consistent with our previous data showing that self-reported early age at menarche is associated with higher TDLU counts [19]. Further, a total of ten SNPs were associated with at least one TDLU involution measure; seven (four menarche SNPs and three menopause SNPs) of these SNPs were associated in the directions consistent with our expectation based on their associations with ages at menarche or menopause as shown in GWAS. Our data suggest that some menarche- and menopause-SNPs may be related to variation in normal breast histology, specifically

Table 4 Top associations ($p < 0.05$) of ages at menarche- and menopause-SNPs with the product of TDLU counts and acini counts per TDLU in the pooled analysis

	Genomic region	Nearest gene(s)	Risk/nonrisk allele[a]	AF[b]	*N*	Poisson model[c]	
						RR (95 % CI) per risk allele	*p*-trend
Menarche SNPs							
rs1079866	7p14.1	*INHBA*	C/G	0.85	802	0.73 (0.59, 0.90)	0.003
rs9389666	6q16.2	*PRDM13/MCHR2*	C/G	0.40	791	0.82 (0.70, 0.95)	0.01
rs1398217	18q21.1	*FUSSEL18*	C/G	0.41	835	0.81 (0.68, 0.96)	0.01
rs2243803[d]	18q12.3	*SLC14A2*	T/A	0.59	802	1.19 (1.02, 1.39)	0.03
rs6438424[d]	3q13.32	*3q13.32*	T/G	0.52	833	1.22 (1.02, 1.45)	0.03
Menopause SNPs							
rs7606918[d]		*METAP1D*	A/G	0.85	793	1.51 (1.16, 1.95)	0.002

TDLU terminal duct lobular unit, *AF* allele frequency, *RR* relative risk, *CI* confidence interval, *SNP* single-nucleotide polymorphism

[a] Risk allele indicates an allele associated with younger age at menarche or older age at menopause. Based on their associations with ages at menarche and menopause in GWAS, we expect to observe RR >1 for their associations with the product of TDLU counts and acini counts per TDLU

[b] Allele frequency of the risk allele

[c] Adjusted for study (KTB, BREAST) and age (<30, 30–39, 40–49, 50–59, ≥60 years)

[d] Alleles with expected directions of associations based on their associations with ages at menarche or menopause as shown in GWAS

with regard to the number of TDLUs. In two cohorts of women with benign breast disease, women with lower levels of TDLU involution were at higher risk of developing breast cancer [3, 29]. Menarche and menopause are critical periods of marked changes within the breast and both are related to hormonal fluctuations and cellular proliferation [11, 12, 16], which are likely to be reflected in the process of TDLU involution. However, when taking into account the direction of associations regardless of statistical significance, only menopause SNPs, but not menarche SNPs, had more than the expected number of SNPs (>50 %) that were associated in the consistent directions based on their relationships with ages at menarche or menopause, suggesting that the loci associated with delayed menopause may be more closely related to the biological mechanisms underlying delayed lobular involution. Further, our data suggest that the biological mechanisms related to these loci may also influence TDLU involution, independent of ages at menarche and menopause, as their associations with TDLU and acini/TDLU counts persisted after adjustment for these variables in the models.

As expected based on their associations with ages at menarche or menopause reported in GWAS, the risk alleles for four menarche SNPs (rs466639: *RXRG*; rs2243803: *SLC14A2*; rs2292573: *GAB2*; rs6438424: 3q13.32) and three menopause SNPs (rs7606918: *METAP1D*; rs11668344: *TMEM150B*; rs1635501: *EXO1*) were significantly associated with higher TDLU counts, higher acini counts/TDLU, and/or higher product of these two measures. Although there is limited knowledge about the causal variants in each locus or the biological functions of the involved genes, some of these SNPs were located near or in genes that are implicated in hormonal regulation (rs466639: *RXRG*), cell proliferation (rs2292573: *GAB2*), and DNA repair (rs1635501: *EXO1*), suggesting some plausible biologic mechanisms that may be involved in TDLU involution and breast tissue aging.

In contrast, two alleles related to younger age at menarche showed significant associations with lower TDLU counts. The findings could be due to chance or suggest that the alleles related to age at menarche are not related to normal breast microanatomy.

There was no overlap between the SNPs that were significantly associated with TDLU counts and the SNP that was associated with acini counts/TDLU. Although little is known about how TDLU involution occurs in the breast, TDLU counts and acini counts/TDLU may indicate different biologic processes of involution, as suggested by a modest correlation between these two measures [9, 19]. TDLU involution may occur in women by either reducing the number of TDLUs or reducing the number of acini within the TDLUs, both resulting in decreased overall epithelial content in the breast. Our data suggest that genetic variants may differentially influence each of these processes.

Although the directions of associations were consistent, the magnitude of associations for three SNPs (rs6438424, rs466639, rs1635501) varied by study, possibly due to differences in population characteristics and biopsy methods. As women in the BREAST underwent diagnostic breast biopsies, they tended to be older and at higher risk of

breast cancer. TDLU measures in the BREAST were also estimated using tissues obtained by targeted image-guided biopsies, which inherently captured more epithelial-rich tissue area than in the KTB. Our findings of two SNPs (rs466639: *RXRG*; rs1635501: *EXO1*) that were more strongly associated with TDLU counts in the BREAST suggest that these SNPs may be related to mechanisms that are more strongly associated with TDLU involution among the higher risk group by acting later in the natural history of breast cancer (i.e., closer to the disease state in the risk spectrum). This hypothesis was further supported in our secondary analysis that showed these SNPs were also suggestively more strongly associated with TDLU counts among women with proliferative (vs. nonproliferative) lesions, as women with proliferative lesions (with and without atypia) have higher breast cancer risk as compared to women with nonproliferative lesions [30].

Strengths of this study are the use of standardized, reproducible measures of TDLU involution, the use of validated genetic markers associated with ages at menarche and menopause, and the use of two study populations including a unique resource of normal breast tissues (the KTB). Limitations of the study are the relatively small sample size, despite the pooling of two study populations; hence, we had limited statistical power to detect modest associations and none of the associations remained significant after Bonferroni adjustment for multiple testing. Additional studies with larger samples sizes are needed to replicate these findings. Furthermore, the results may not be generalizable to other populations since our study populations were enriched for family history of breast cancer. However, it is also important to understand and identify biomarkers among women at elevated breast cancer risk.

In conclusion, our data suggest that the processes of TDLU involution may be influenced by the genetic loci associated with ages at menarche and menopause may influence TDLU involution, suggesting some shared genetic mechanisms underlying these factors. However, given the limited sample size of this study, larger studies are needed to confirm the genetic correlations between TDLU involution and reproductive risk factors for breast cancer. Our findings also provide the basis to pursue a large-scale GWAS to identify susceptibility loci related to TDLU involution and provide further support for the evaluation of normal breast tissues, specifically TDLUs, in potentially clarifying etiologic pathways to breast cancer.

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Compliance with ethical standard Participants provided written informed consent, and the studies were approved by the Institutional Review Board at the Indiana University and the NIH Office of Human Subjects Research for the KTB and by the Institutional Review Boards at the University of Vermont and the NCI for the BREAST Stamp Project.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Russo J, Russo IH (2004) Development of the human breast. Maturitas 49(1):2–15. doi:10.1016/j.maturitas.2004.04.011
2. Henson DE, Tarone RE (1994) Involution and the etiology of breast cancer. Cancer 74(1 Suppl):424–429
3. Milanese TR, Hartmann LC, Sellers TA, Frost MH, Vierkant RA, Maloney SD, Pankratz VS, Degnim AC, Vachon CM, Reynolds CA, Thompson RA, Melton LJ 3rd, Goode EL, Visscher DW (2006) Age-related lobular involution and risk of breast cancer. J Natl Cancer Inst 98(22):1600–1607. doi:10.1093/jnci/djj439
4. Ghosh K, Hartmann LC, Reynolds C, Visscher DW, Brandt KR, Vierkant RA, Scott CG, Radisky DC, Sellers TA, Pankratz VS, Vachon CM (2010) Association between mammographic density and age-related lobular involution of the breast. J Clin Oncol 28(13):2207–2212. doi:10.1200/JCO.2009.23.4120
5. Ginsburg OM, Martin LJ, Boyd NF (2008) Mammographic density, lobular involution, and risk of breast cancer. Br J Cancer 99(9):1369–1374. doi:10.1038/sj.bjc.6604635
6. Figueroa J, Pfeiffer RM, Brinton LA, Palakal MM, Degnim AC, Radisky DC, Hartmann LC, Frost MH, Stallings Mann ML, Papathomas D, Hewitt S, Visscher D, Sherman M (2015) Standardized measures of lobular involution and subsequent breast cancer risk among women with benign breast disease. Cancer Res 75:4682. doi:10.1158/1538-7445.AM2015-4682
7. Radisky DC, Visscher DW, Frank RD, Vierkant RA, Winham S, Stallings-Mann M, Hoskin TL, Nassar A, Vachon CM, Denison LA, Hartmann LC, Frost MH, Degnim AC (2016) Natural history of age-related lobular involution and impact on breast cancer risk. Breast Cancer Res Treat 155(3):423–430. doi:10.1007/s10549-016-3691-5
8. Hutson SW, Cowen PN, Bird CC (1985) Morphometric studies of age related changes in normal human breast and their significance for evolution of mammary cancer. J Clin Pathol 38(3):281–287
9. Gierach GL, Patel DA, Pfeiffer RM, Figueroa JD, Linville L, Papathomas D, Johnson JM, Chicoine RE, Herschorn SD, Shepherd JA, Wang J, Malkov S, Vacek PM, Weaver DL, Fan B,

Mahmoudzadeh AP, Palakal M, Xiang J, Oh H, Horne HN, Sprague BL, Hewitt SM, Brinton LA, Sherman ME (2016) Relationship of terminal duct lobular unit involution of the breast with area and volume mammographic densities. Cancer Prev Res 9(2):149–158. doi:10.1158/1940-6207.CAPR-15-0282

10. Vierkant RA, Hartmann LC, Pankratz VS, Anderson SS, Radisky D, Frost MH, Vachon CM, Ghosh K, Distad TJ, Degnim AC, Reynolds CA (2009) Lobular involution: localized phenomenon or field effect? Breast Cancer Res Treat 117(1):193–196. doi:10.1007/s10549-008-0082-6
11. Beccuti G, Ghizzoni L (2000) Normal and abnormal puberty. In: De Groot LJ, Beck-Peccoz P, Chrousos G et al (eds) Endotext. MDText.com Inc, South Dartmouth
12. Styne DM (1994) Physiology of puberty. Horm Res 41(Suppl 2): 3–6
13. Collaborative Group on Hormonal Factors in Breast Cancer (2012) Menarche, menopause, and breast cancer risk: individual participant meta-analysis, including 118 964 women with breast cancer from 117 epidemiological studies. Lancet Oncol 13(11): 1141–1151. doi:10.1016/S1470-2045(12)70425-4
14. Pike MC, Krailo MD, Henderson BE, Casagrande JT, Hoel DG (1983) 'Hormonal' risk factors, 'breast tissue age' and the age-incidence of breast cancer. Nature 303(5920):767–770
15. Yager JD, Davidson NE (2006) Estrogen carcinogenesis in breast cancer. N Engl J Med 354(3):270–282. doi:10.1056/NEJMra050776
16. Klein KO, Mericq V, Brown-Dawson JM, Larmore KA, Cabezas P, Cortinez A (1999) Estrogen levels in girls with premature thelarche compared with normal prepubertal girls as determined by an ultrasensitive recombinant cell bioassay. J Pediatr 134(2):190–192
17. Oh H, Khodr ZG, Sherman ME, Palakal M, Pfeiffer RM, Linville L, Geller BM, Vacek PM, Weaver DL, Chicoine RE, Falk RT, Horne HN, Papathomas D, Patel DA, Xiang J, Xu X, Veenstra T, Hewitt SM, Shepherd JA, Brinton LA, Figueroa JD, Gierach GL (2016) Relation of serum estrogen metabolites with terminal duct lobular unit involution among women undergoing diagnostic image-guided breast biopsy. Horm Cancer. doi:10.1007/s12672-016-0265-2
18. Khodr ZG, Sherman ME, Pfeiffer RM, Gierach GL, Brinton LA, Falk RT, Patel DA, Linville LM, Papathomas D, Clare SE, Visscher DW, Mies C, Hewitt SM, Storniolo AM, Rosebrock A, Caban JJ, Figueroa JD (2014) Circulating sex hormones and terminal duct lobular unit involution of the normal breast. Cancer Epidemiol Biomark Prev 23(12):2765–2773. doi:10.1158/1055-9965.EPI-14-0667
19. Figueroa JD, Pfeiffer RM, Patel DA, Linville L, Brinton LA, Gierach GL, Yang XR, Papathomas D, Visscher D, Mies C, Degnim AC, Anderson WF, Hewitt S, Khodr ZG, Clare SE, Storniolo AM, Sherman ME (2014) Terminal duct lobular unit involution of the normal breast: implications for breast cancer etiology. J Natl Cancer Inst 106(10):dju286. doi:10.1093/jnci/dju286
20. Elks CE, Perry JR, Sulem P, Chasman DI, Franceschini N, He C, Lunetta KL, Visser JA, Byrne EM, Cousminer DL, Gudbjartsson DF, Esko T, Feenstra B, Hottenga JJ, Koller DL, Kutalik Z, Lin P, Mangino M, Marongiu M, McArdle PF, Smith AV, Stolk L, van Wingerden SH, Zhao JH, Albrecht E, Corre T, Ingelsson E, Hayward C, Magnusson PK, Smith EN, Ulivi S, Warrington NM, Zgaga L, Alavere H, Amin N, Aspelund T, Bandinelli S, Barroso I, Berenson GS, Bergmann S, Blackburn H, Boerwinkle E, Buring JE, Busonero F, Campbell H, Chanock SJ, Chen W, Cornelis MC, Couper D, Coviello AD, d'Adamo P, de Faire U, de Geus EJ, Deloukas P, Doring A, Smith GD, Easton DF, Eiriksdottir G, Emilsson V, Eriksson J, Ferrucci L, Folsom AR, Foroud T, Garcia M, Gasparini P, Geller F, Gieger C, Consortium G, Gudnason V, Hall P, Hankinson SE, Ferreli L, Heath AC, Hernandez DG, Hofman A, Hu FB, Illig T, Jarvelin MR, Johnson AD, Karasik D, Khaw KT, Kiel DP, Kilpelainen TO, Kolcic I, Kraft P, Launer LJ, Laven JS, Li S, Liu J, Levy D, Martin NG, McArdle WL, Melbye M, Mooser V, Murray JC, Murray SS, Nalls MA, Navarro P, Nelis M, Ness AR, Northstone K, Oostra BA, Peacock M, Palmer LJ, Palotie A, Pare G, Parker AN, Pedersen NL, Peltonen L, Pennell CE, Pharoah P, Polasek O, Plump AS, Pouta A, Porcu E, Rafnar T, Rice JP, Ring SM, Rivadeneira F, Rudan I, Sala C, Salomaa V, Sanna S, Schlessinger D, Schork NJ, Scuteri A, Segre AV, Shuldiner AR, Soranzo N, Sovio U, Srinivasan SR, Strachan DP, Tammesoo ML, Tikkanen E, Toniolo D, Tsui K, Tryggvadottir L, Tyrer J, Uda M, van Dam RM, van Meurs JB, Vollenweider P, Waeber G, Wareham NJ, Waterworth DM, Weedon MN, Wichmann HE, Willemsen G, Wilson JF, Wright AF, Young L, Zhai G, Zhuang WV, Bierut LJ, Boomsma DI, Boyd HA, Crisponi L, Demerath EW, van Duijn CM, Econs MJ, Harris TB, Hunter DJ, Loos RJ, Metspalu A, Montgomery GW, Ridker PM, Spector TD, Streeten EA, Stefansson K, Thorsteinsdottir U, Uitterlinden AG, Widen E, Murabito JM, Ong KK, Murray A (2010) Thirty new loci for age at menarche identified by a meta-analysis of genome-wide association studies. Nat Genet 42(12):1077–1085. doi:10.1038/ng.714
21. Stolk L, Perry JR, Chasman DI, He C, Mangino M, Sulem P, Barbalic M, Broer L, Byrne EM, Ernst F, Esko T, Franceschini N, Gudbjartsson DF, Hottenga JJ, Kraft P, McArdle PF, Porcu E, Shin SY, Smith AV, van Wingerden S, Zhai G, Zhuang WV, Albrecht E, Alizadeh BZ, Aspelund T, Bandinelli S, Lauc LB, Beckmann JS, Boban M, Boerwinkle E, Broekmans FJ, Burri A, Campbell H, Chanock SJ, Chen C, Cornelis MC, Corre T, Coviello AD, d'Adamo P, Davies G, de Faire U, de Geus EJ, Deary IJ, Dedoussis GV, Deloukas P, Ebrahim S, Eiriksdottir G, Emilsson V, Eriksson JG, Fauser BC, Ferreli L, Ferrucci L, Fischer K, Folsom AR, Garcia ME, Gasparini P, Gieger C, Glazer N, Grobbee DE, Hall P, Haller T, Hankinson SE, Hass M, Hayward C, Heath AC, Hofman A, Ingelsson E, Janssens AC, Johnson AD, Karasik D, Kardia SL, Keyzer J, Kiel DP, Kolcic I, Kutalik Z, Lahti J, Lai S, Laisk T, Laven JS, Lawlor DA, Liu J, Lopez LM, Louwers YV, Magnusson PK, Marongiu M, Martin NG, Klaric IM, Masciullo C, McKnight B, Medland SE, Melzer D, Mooser V, Navarro P, Newman AB, Nyholt DR, Onland-Moret NC, Palotie A, Pare G, Parker AN, Pedersen NL, Peeters PH, Pistis G, Plump AS, Polasek O, Pop VJ, Psaty BM, Raikkonen K, Rehnberg E, Rotter JI, Rudan I, Sala C, Salumets A, Scuteri A, Singleton A, Smith JA, Snieder H, Soranzo N, Stacey SN, Starr JM, Stathopoulou MG, Stirrups K, Stolk RP, Styrkarsdottir U, Sun YV, Tenesa A, Thorand B, Toniolo D, Tryggvadottir L, Tsui K, Ulivi S, van Dam RM, van der Schouw YT, van Gils CH, van Nierop P, Vink JM, Visscher PM, Voorhuis M, Waeber G, Wallaschofski H, Wichmann HE, Widen E, van Wijnands-van Gent CJ, Willemsen G, Wilson JF, Wolffenbuttel BH, Wright AF, Yerges-Armstrong LM, Zemunik T, Zgaga L, Zillikens MC, Zygmunt M, Study TL, Arnold AM, Boomsma DI, Buring JE, Crisponi L, Demerath EW, Gudnason V, Harris TB, Hu FB, Hunter DJ, Launer LJ, Metspalu A, Montgomery GW, Oostra BA, Ridker PM, Sanna S, Schlessinger D, Spector TD, Stefansson K, Streeten EA, Thorsteinsdottir U, Uda M, Uitterlinden AG, van Duijn CM, Volzke H, Murray A, Murabito JM, Visser JA, Lunetta KL (2012) Meta-analyses identify 13 loci associated with age at menopause and highlight DNA repair and immune pathways. Nat Genet 44(3):260–268. doi:10.1038/ng.1051
22. Sherman ME, Figueroa JD, Henry JE, Clare SE, Rufenbarger C, Storniolo AM (2012) The Susan G. Komen for the Cure Tissue Bank at the IU Simon Cancer Center: a unique resource for

defining the "molecular histology" of the breast. Cancer Prev Res (Phila) 5(4):528–535. doi:10.1158/1940-6207.CAPR-11-0234
23. Gierach GL, Geller BM, Shepherd JA, Patel DA, Vacek PM, Weaver DL, Chicoine RE, Pfeiffer RM, Fan B, Mahmoudzadeh AP, Wang J, Johnson JM, Herschorn SD, Brinton LA, Sherman ME (2014) Comparison of mammographic density assessed as volumes and areas among women undergoing diagnostic image-guided breast biopsy. Cancer Epidemiol Biomark Prev 23(11):2338–2348. doi:10.1158/1055-9965.EPI-14-0257
24. Garcia-Closas M, Egan KM, Abruzzo J, Newcomb PA, Titus-Ernstoff L, Franklin T, Bender PK, Beck JC, Le Marchand L, Lum A, Alavanja M, Hayes RB, Rutter J, Buetow K, Brinton LA, Rothman N (2001) Collection of genomic DNA from adults in epidemiological studies by buccal cytobrush and mouthwash. Cancer Epidemiol Biomark Prev 10(6):687–696
25. Stolk L, Zhai G, van Meurs JB, Verbiest MM, Visser JA, Estrada K, Rivadeneira F, Williams FM, Cherkas L, Deloukas P, Soranzo N, de Keyzer JJ, Pop VJ, Lips P, Lebrun CE, van der Schouw YT, Grobbee DE, Witteman J, Hofman A, Pols HA, Laven JS, Spector TD, Uitterlinden AG (2009) Loci at chromosomes 13, 19 and 20 influence age at natural menopause. Nat Genet 41(6):645–647. doi:10.1038/ng.387
26. Rosebrock A, Caban JJ, Figueroa J, Gierach G, Linville L, Hewitt S, Sherman M (2013) Quantitative Analysis of TDLUs using Adaptive Morphological Shape Techniques. Proceedings of SPIE–the International Society for Optical Engineering 8676. doi:10.1117/12.2006619
27. Mai PL, Wideroff L, Greene MH, Graubard BI (2010) Prevalence of family history of breast, colorectal, prostate, and lung cancer in a population-based study. Public Health Genom 13(7–8):495–503. doi:10.1159/000294469
28. Pinsky PF, Kramer BS, Reding D, Buys S, Team PP (2003) Reported family history of cancer in the prostate, lung, colorectal, and ovarian cancer screening trial. Am J Epidemiol 157(9):792–799
29. Baer HJ, Collins LC, Connolly JL, Colditz GA, Schnitt SJ, Tamimi RM (2009) Lobule type and subsequent breast cancer risk: results from the Nurses' Health Studies. Cancer 115(7):1404–1411. doi:10.1002/cncr.24167
30. Hartmann LC, Sellers TA, Frost MH, Lingle WL, Degnim AC, Ghosh K, Vierkant RA, Maloney SD, Pankratz VS, Hillman DW, Suman VJ, Johnson J, Blake C, Tlsty T, Vachon CM, Melton LJ 3rd, Visscher DW (2005) Benign breast disease and the risk of breast cancer. N Engl J Med 353(3):229–237. doi:10.1056/NEJMoa044383