A Polymorphic Locus in the Promoter Region of the *IGFBP3* Gene Is Related to Mammographic Breast Density

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Abstract

Background: Mammographic breast density is a significant risk factor for breast cancer. Women with dense tissue accounting for more than 60–75% of the area of the breast have a 4- to 6-fold increase in their risk of breast cancer, compared to women with little or no breast density. A high circulating level of insulin-like growth factor-I (IGF-I) and low IGF binding protein 3 (IGFBP-3) level have been associated with increased breast density in premenopausal women. Genetic polymorphisms in the *IGF1* and *IGFBP3* genes may influence breast and serum levels of these growth factors. The aims of this study were to determine whether polymorphic variations in the *IGF1* and *IGFBP3* genes are associated with breast density, and serum IGF-I and IGFBP-3 levels, and whether serum IGF-I and IGFBP-3 levels are associated with mammographic density. **Methods:** A total of 441 white women, recruited from Women's College Hospital (Toronto, Ontario), enrolled in this study. Each woman completed a questionnaire, detailing information on age, menstrual history, hormone use, diet, and medical and mammography history. Blood samples were taken for DNA extraction to genotype the subjects for polymorphic variants in the two candidate genes, and for measurement of circulating levels of IGF-I and IGFBP-3. Mammographic films were retrieved from Women's College Hospital and digitized using a laser film scanner. The digitized images were assessed for breast density using a computer-assisted method. **Results:** There was a positive association between serum IGFBP-3 levels and the number of *A* alleles at a previously described polymorphic locus in the promoter region of the *IGFBP3* gene among premenopausal women ($P = 0.01$). There was also a positive trend in the mean percentage of breast density by the number of *A* alleles of the *IGFBP3* gene among premenopausal women ($P = 0.0005$). Women with two *A* alleles had a 5-fold increase in the odds of having a percentage of breast density greater or equal to 28%, compared with women with no *A* allele ($P = 0.002$). However, there was no association between serum IGF-I and IGFBP-3 levels and breast density among premenopausal women ($P > 0.05$). **Conclusions:** This is the first study to report a strong relationship between a polymorphic gene locus (*IGFBP3*) and mammographic breast density. However, we could not confirm an association between serum IGF-I levels and breast density among premenopausal women, as demonstrated in previous studies.

Introduction

The radiographic appearance of the breast varies among individuals according to the relative amounts of fat and connective and epithelial tissues. On mammograms, fat appears dark, whereas connective and epithelial tissues, which absorb X-rays more strongly, appear as lighter regions in the image. Breast density is an important determinant of breast cancer risk. In general, women for whom dense tissue accounts for more than 60–75% of the breast have a 4- to 6-fold increase in their risk of breast cancer, compared to women with little or no density (1). Estimates from two nested case-control studies (2, 3) show that 28–33% of breast cancers may be attributed to having density in >50% of the breast. Mammographic density has been associated with breast cancer risk in both pre- and postmenopausal women (1, 4).

Insulin-like growth factor-I (IGF-I) plays a key role in cell proliferation, growth, and embryonic development (5). IGF-I is bound in the circulation to one of six IGF binding proteins (IGFBPs) that modulate its activity. IGF binding protein 3 (IGFBP-3) binds more than 75% of IGF-I in the serum and regulates the interaction between IGF-I and its receptor (IGF-IR). Once released from IGFBP-3, IGF-I can leave the circulation and enter the target tissue to exert its effects. At the tissue level, IGFBP-3 can enhance the growth-promoting effects of IGF-I, but it can also inhibit growth in an IGF-independent manner (5).

Epidemiological studies (6–9) show that the high circulating levels of IGF-I and low levels of IGFBP-3 are associated with an increased risk of premenopausal breast cancer. Fewer positive associations were found among postmenopausal women (9–11). In a nested case-control study from Sweden, Kaaks et al. (10) found that serum IGF-I levels were predictive of breast cancer risk in women aged 55 and over, particularly among those women who used hormone replacement therapy (HRT). This association was not confirmed in a recent Dutch study (11), but the sample size was much smaller in the later study. Studies have also shown that high serum IGF-I levels (12–13) and low IGFBP-3 levels (12) are associated with increased premenopausal breast density. To date, one study (14) has investigated the association between tissue IGF-I concentrations in the breast and breast density. Guo et al. (14) showed that breast tissue sections from premenopausal subjects with extensive breast densities had larger stained areas of IGF-I, compared with those from subjects with little breast density.

Studies (15–17) have shown that there is a strong hereditary component to breast density, but to our knowledge, only one study (18) has investigated the relationship between specific genes and breast density. Haiman et al. (18) found no consistent relationships between polymorphisms in genes involved in steroid hormone metabolism (CYP17, COMT, 17HSDB1, and 3HSDB1) and breast density.

Genetic polymorphisms in the IGF1 and IGFBP3 genes that have the potential to influence the expression of these proteins may correlate with IGF-I and IGFBP-3 levels in the breast and in the circulation. The aim of this study is to assess the role of IGF1 and IGFBP3 genotypes as predictors of circulating growth factor concentrations and of breast density.

Materials and Methods

Recruitment of Study Subjects. Between October 1999 and December 2001, healthy study subjects were recruited from the HealthWatch Clinic of the Sunnybrook and Women's College Hospital (Toronto, Ontario). This clinic provides primary preventive care for women throughout Ontario with a focus on prevention of disease and promotion of healthy aging. Routine screening mammography is also provided. Women who were not currently pregnant, who were 30 years of age and above, who were willing to complete a questionnaire, and who were willing to provide a blood sample were eligible. All participants provided written informed consent. Subjects completed a self-given questionnaire, detailing information on age, ethnic group, menstrual history, current and past hormone use, alcohol and coffee consumption, smoking, anthropometric measures, medical history, and mammography history. Blood samples were taken for DNA extraction and hormone level measurements.

Subject Eligibility and Exclusion Criteria. Of the 1083 women who were approached to participate in our study between 1999 and 2001, 866 women (80%) were willing to participate. Forty-three women were ineligible because they did not complete the questionnaire or did not provide a blood sample. Eight hundred and twenty-three women were eligible. Subjects were excluded from the study if they were non-white ($n = 12$), had breast cancer ($n = 29$) or other cancers (except for basal cell carcinoma) ($n = 14$) or diabetes ($n = 18$), or had unknown menopausal status ($n = 23$). This left a total of 727 eligible subjects.

Additional exclusion criteria were used for each of the three principal sub-analyses: (a) Subjects were excluded from the serum-gene analysis if they were currently or previously on HRT ($n = 340$), or currently on oral contraceptives (OCs) ($n = 22$) or on raloxifene ($n = 4$), or had no serum available ($n = 4$). Three hundred and fifty-seven subjects were included in this analysis; (b) Subjects were excluded from the mammogram-gene analysis if they had no mammogram available to study before HRT use ($n = 249$), had never had a mammogram ($n = 37$), if their mammograms were missing ($n = 27$), or had breast implants ($n = 2$). Four hundred and twelve subjects were included in this analysis; (c) Subjects included in the mammogram-gene analysis were excluded from the mammogram-serum analysis if they were currently or previously on HRT ($n = 70$), currently on OCs ($n = 3$) or on raloxifene ($n = 3$), had no mammogram in the 24 months before the blood draw ($n = 11$), had no blood serum ($n = 4$), or were premenopausal at the time of mammography and postmenopausal at the time of blood draw ($n = 9$). Fifty-two percent, 26% and 22% of the women included in the mammogram-serum analysis had a mammogram done on the day of blood draw, 1–12 months before the date of blood draw, and 13–24 months before the date of blood draw, respectively. Three hundred and twelve subjects were included in this analysis.

A total of 441 subjects was included in one or more components of this study.

Determination of Menopausal Status. Women were classified as premenopausal at the time of study entry (i.e., time of blood draw) if they stated that they had a recent menstrual period. Women were classified as postmenopausal at the date of study entry if they reported that they had not menstruated for a year.

Women who had a hysterectomy before the date of study entry were considered to be postmenopausal at the date of study entry if they also had a bilateral oophorectomy. If a woman reported a hysterectomy without removal of the ovaries, menopausal status was assigned according to the following rule. Women who were, at the time of study entry, at or below the 10th percentile of menopausal age among women (out of the 823 eligible subjects) who had natural menopause were assigned premenopausal status (the age cut-point was 44). Women who were, at the time of study entry, at or above the 90th percentile of menopausal age among women who had natural menopause were assigned postmenopausal status (the age cut-point was 54). Women between the 10th and 90th percentile were considered to have "unknown" menopausal status and were excluded.

Women would be classified as postmenopausal at the date of mammography if they reported that they had not menstruated for a year before the mammogram. If a mammogram was taken before the date of study entry, the age at menopause as provided in the self-given questionnaire was used to determine a subject's menopausal status on the date of mammography.

Women who reported having had a hysterectomy were assigned menopausal status at the date of mammography according to the criteria described above. The menopausal status was determined according to the rule for 28 women (6 pre, 22 post) included in the serum-gene analysis, for 36 (11 pre, 25 post) women included in the mammogram-gene analysis, and for 26 (6 pre, 20 post) women included in the mammogram-serum analysis.

Retrieval of Mammograms. Subjects' mammograms were retrieved from the radiology department of Women's College Hospital (Toronto, Ontario). Mammograms that were done on the date closest to the date of the subjects' entry into the study (date of blood draw) were requested. For subjects who were currently on HRT, a mammogram done before the initiation of HRT was requested. The cranio-caudal image of one breast, chosen at random by coin toss, was evaluated for each subject. A high degree of symmetry in area of density exists between the left and right breasts, and between the cranio-caudal and medio-lateral views of the same breast (19).

Digitization of Mammograms and Assessment of Breast Densities. The mammograms were digitized at 260 μm/pixel with a Lumysis 85 laser film scanner, which covers a range of 0–4.0 absorbance. Digitization was done at the Sunnybrook & Women's Health Sciences Centre Imaging Research Unit (Toronto, Ontario), where bitmap files generated by the digitization of films were prepared for presentation to the assessor. All mammograms were assessed by one of us (M.Y.). Breast density was assessed using a software algorithm for computer-assisted thresholding developed at the University of Toronto (Toronto, Canada) (19, 20). The mammograms were assessed without any information on the subjects. The images were presented on a 17-in. screen with color overlay. For each image, the observer selected a gray value as a threshold to separate the image of the breast itself from the darker background (the "edge threshold"). An algorithm automatically surveyed the image counting pixels, the brightness of which was above that threshold, that is, those lying within the projection of the breast (number of pixels in breast projection = T). A second threshold was then selected by the observer to identify regions of the breast that are representative of radiographically dense tissue (light appearance). Pixels that correspond to radiographically dense breast were then identified (number of pixels in areas of dense tissue = D). The area on the mammogram showing the pectoralis muscle and prominent veins was excluded from both calculations. The percentage of the breast area that appeared dense was then calculated (% breast density = $D/T \times 100$). This measure of mammographic breast density was highly reproducible as measured within this study (within-person intraclass correlation coefficient = 0.94; $P < 0.001$; $n = 90$).

Laboratory Assays. Blood samples were processed and sera were frozen immediately after venipuncture. Genomic DNA was extracted from 20 ml of peripheral blood using Puregene DNA extraction kits.

The polymorphism in the *IGF1* gene is a variable number tandem repeat (VNTR) type polymorphism (21),

consisting of eight alleles, ranging in size from 16 to 23 repeat units. The polymorphism was evaluated by a PCR-based assay. The procedures have been previously described (22).

The polymorphism in the *IGFBP3* gene is a single bp polymorphism at position −202 relative to the CAP site. A RFLP assay was carried out for this polymorphism. The procedures have been previously described (23).

Levels of total IGF-I and IGFBP-3 in serum were assayed using ELISA methodology with reagents from Diagnostic Systems Laboratory (Webster, TX). Two IGF-I and two IGFBP-3 measurements were taken for each subject, and the average of the pair of values was used in the statistical analysis. The correlation coefficient for the pair of IGF-I values was 0.99 ($P < 0.001$). The correlation coefficient for the pair of IGFBP-3 values was 0.98 ($P < 0.001$). All assays were carried out in a blinded manner.

Statistical Methods. The distributions of serum IGF-I and IGFBP-3 levels, and the percentage of breast density, were consistent with a normal distribution in all three groups of women (all women; premenopausal; postmenopausal), except for the percentage of breast density among all women, which was slightly off-normal. Square-root transformation of percentage of breast density among all women generated a normal distribution. However, the results were similar with or without transformation and therefore the results using the nontransformed variable were presented.

First, the relationships between the *IGF1* genotype and serum IGF-I levels, and between the *IGFBP3* genotype and serum IGFBP-3 levels were assessed using linear regression methods with serum IGF-1 as the dependent variable. Second, significant determinants of breast density in this data set were identified. The relationships between the *IGF1* and *IGFBP3* genotypes and mammographic density and between serum IGF-I and IGFBP-3 levels and mammographic density were then assessed.

Potential predictors of breast density which were evaluated in this analysis included: age, BMI (kg/m^2), menopausal status (pre- or post-), age at menopause (years), age at menarche (years), age at first birth (years), parity, family history of breast cancer (yes/no), previous OC use (yes/no), smoking history (never, past, current), alcohol consumption (number of drinks per week), and coffee consumption (number of cups per day). These variables were evaluated because of previous reported associations with either IGF-I or IGFBP-3 levels (24–27), with breast density (1, 28), or with breast cancer (29). All alcoholic drinks (*i.e.*, beer, wine, liquor) were considered to be equivalent. Menopausal status, family history of breast cancer, and previous OC use were evaluated as dichotomous variables. Parity and coffee consumption were evaluated as continuous and as dichotomous (*e.g.*, parity ≥4 *versus* <4) variables. Smoking history and alcohol consumption were evaluated as ordinal and dichotomous variables. The effects of these variables on mammographic density were evaluated using linear regression.

The associations between genotypes and the percentage of mammographic density and between serum protein levels and the percentage of mammographic density were measured using linear regression models (*i.e.*, proc glm in SAS) adjusting for age, BMI, and for other potential confounders. The adjusted means

presented are those calculated according to the fitted linear model. In these analysis, the final model included covariates if they generated *P* values of less than or equal to 0.2 in the linear regression model described above.

Logistic regression was also used to study the relationships between the *IGFBP3* genotype and mammographic density among premenopausal women, and between the *IGF1* genotype and mammographic density among postmenopausal women. In the first analysis, the percentage of breast density was dichotomized using a cut-point based on the mean percentage breast density observed among premenopausal women with no *IGFBP3 A* allele (*i.e.*, 28% breast density). In the second analysis, the percentage of breast density was dichotomized using a cut-point based on the mean percentage of breast density observed among postmenopausal women with no *IGF1 19* repeat allele (*i.e.*, 18%). Covariates were determined in the same manner as that used for the linear models described above. Continuous variables were transformed to categorical variables in the logistic regression models. A *P* value <0.05 (two-sided test) was considered to be statistically significant. Data were analyzed using SAS (30).

Results

Subject Characteristics. The characteristics of the study subjects are described in Table 1. Subject characteristics are similar in all three analysis subgroups.

Serum-Gene Analysis

Variation in Serum IGF-I Level by Number of Alleles of the IGF1 Genotype. The number of CA repeats of the *IGF1* gene varies among individuals, ranging from 17 to 23. There was no significant trend in the mean serum IGF-I level by number of *19* CA repeat alleles among premenopausal (*P* = 0.7); there was a borderline effect among postmenopausal women (*P* = 0.07) (Table 2). No significant associations were found between serum IGF-I level and *IGF1* CA repeat genotypes using other categorizations (data not shown).

Variation in Serum IGFBP-3 Level by Number of Alleles of the IGFBP3 Genotype. There was a significant trend in the mean IGFBP-3 level by number of *A* alleles at a previously described polymorphic locus in the *IGFBP3* promoter region, located at position −202 relative to the CAP site (31), among premenopausal women (*P* = 0.01), but only a borderline effect was observed among postmenopausal women (*P* = 0.07). There was a significant negative trend in the mean IGF-1:IGFBP-3 ratio by the number of *IGFBP3 A* alleles among both premenopausal women (*P* = 0.001) and postmenopausal women (*P* = 0.0002).

Mammogram-Gene Analysis

Mammographic Density versus Predictor Variables. Mammographic density was correlated with age, menopausal status, BMI, parity, and coffee consumption (Tables 3 and 4). Premenopausal women had a significantly higher mean percentage of breast density (33.7%) than postmenopausal women (19.1%) (*P* < 0.001). Mammographic density was negatively correlated with age among postmenopausal women (*r* = −0.32; *P* < 0.001), but not before menopause (*P* = 0.7). Mammographic density was negatively associated with BMI among both pre- and postmenopausal subjects (*P* < 0.001). Percentage of breast density was negatively associated with parity

Table 1. Characteristics of subjects[a] at study entry

Characteristic	All (*n* = 312)	Premenopausal (*n* = 142)	Postmenopausal (*n* = 170)
Age [yrs; mean (range)]	55.4 (30−85)	46.5 (30−54)	62.8 (46−85)
Height [inches; mean (range)]	64.4 (55−71)	64.7 (59−71)	64.1 (55−71)
Weight [pounds; mean (range)]	156.2 (105−280)	154.0 (108−280)	158.0 (105−250)
BMI [kg/m^2; mean (range)]	26.3 (18.2−47.7)	25.6 (18.2−47.7)	26.9 (19.0−41.3)
IGF-1 [ng/ml; mean (range)]	160.7 (74.5−414.0)	175.4 (96.5−414.0)	148.4 (74.5−256.5)
IGFBP-3 [ng/ml; mean (range)]	2734.7 (511.4−4329.9)	2775.8 (1791.1−3845.1)	2700.3 (511.4−4329.9)
Percentage of breast density [mean (range)]	25.8 (0.3−76.4)	33.7 (1.0−76.4)	19.1 (0.3−69.2)
Age at menopause[b] [yrs; mean (range)]			50.1 (35−58)
Age at menarche [yrs; mean (range)]	12.8 (9−18)	12.8 (9−18)	12.8 (9−17)
Age at first birth[c] [yrs; mean (range)]	25.3 (16−41)	25.6 (16−41)	25.1 (16−40)
Parity [mean (range)]	2.1 (0−7)	2.0 (0−6)	2.2 (0−7)
Family history of breast cancer (%)[d]	16.3	16.9	15.9
Previous OC use (%)	49.0	63.4	37.1
Smoking history (%)			
Never	53.8	57.0	51.2
Past	38.1	38.7	37.6
Current	8.0	4.2	11.2
Alcohol consumption (# drinks/wk; %)			
Do not drink	24.0	12.0	34.1
0−3 drinks per week	45.2	52.5	38.2
4−9 drinks per week	24.4	26.8	22.4
10 or more drinks per week	6.4	7.7	5.3
Coffee consumption [# cups/day; mean (range)]	1.8 (0−12)	1.7 (0−10)	1.8 (0−12)

[a]Subjects included in the mammogram-serum analysis.
[b]Age at menopause only includes women who had natural menopause or bilateral oophorectomy.
[c]Age at first birth of parous women.
[d]Percentage of women with at least one first degree relative (mother/sister/daughter) who had breast cancer.

Table 2. Mean serum IGF-I and IGFBP-3 levels by number of alleles of the *IGF1 19* CA repeat genotype and *IGFBP3* genotype

IGF1 genotype	Mean IGF-I[a] (SD)		Adjusted mean IGF-I[b]		P value for trend[c]	
	Pre[d] (n = 174)	Post[e] (n = 183)	Pre[d] (n = 174)	Post[e] (n = 183)	Pre[d] (n = 174)	Post[e] (n = 183)
No *19* repeat allele (n = 27/21)[f]	176.5 (47.6)	166.9 (43.9)	176.1	163.1	0.7[g]	0.07[h]
One *19* repeat allele (n = 80/89)	178.2 (47.6)	148.8 (37.3)	177.1	149.2		
Two *19* repeat allele (n = 67/73)	172.0 (41.9)	143.3 (41.5)	173.5	143.9		

IGFBP3 genotype	Mean IGFBP-3[i] (SD)		Adjusted mean IGFBP-3[j]		P value for trend[k]	
	Pre[d] (n = 174)	Post[e] (n = 183)	Pre[d] (n = 174)	Post[e] (n = 183)	Pre[d] (n = 174)	Post[e] (n = 183)
No *A* allele (n = 49/54)	2645 (313)	2589 (505)	2642	2611	0.01[l]	0.07[m]
One *A* allele (n = 91/93)	2774 (439)	2709 (603)	2782	2684		
Two *A* alleles (n = 34/36)	2884 (533)	2803 (529)	2867	2834		

Note: Data presented for women included in the serum-gene analysis.
[a]Mean serum IGF-I level (ng/ml).
[b]Multivariate adjusted mean serum IGF-I level.
[c]Test for trend in mean serum IGF-I level by number of alleles of the *IGF1* 19 CA repeat genotype.
[d]Premenopausal women.
[e]Postmenopausal women.
[f](n = pre/post).
[g]Adjusted for age (yrs, continuous), BMI (kg/m^2, continuous), family history of breast cancer (yes/no), alcohol (10 or more drinks a week *versus* <10), and coffee (1 a day *versus* rest of group) for premenopausal women.
[h]Adjusted for BMI (kg/m^2, continuous) and parity (4 and above *versus* <4) for postmenopausal women.
[i]Mean serum IGFBP-3 level (ng/ml).
[j]Multivariate adjusted mean serum IGFBP-3 level.
[k]Test for trend in mean serum IGFBP-3 level by number of alleles of the *IGFBP3* genotype.
[l]Adjusted for age (yrs, continuous), alcohol (4–9 drinks a week *versus* rest of group), and coffee (1 a day *versus* rest of group) for premenopausal women.
[m]Adjusted for age (yrs, continuous) and parity (4 and above *versus* <4) for postmenopausal women.

among postmenopausal women ($P = 0.003$), but not among premenopausal women ($P = 0.1$). Mammographic density was negatively associated with coffee consumption among premenopausal women ($P = 0.04$), but not among postmenopausal women ($P = 0.7$).

Variation in Percentage of Breast Density by Number of Alleles of the IGF1 Genotype. There was no significant trend in the mean percentage of breast density by the number of *19* CA repeat alleles of the *IGF1* gene among premenopausal women ($P = 1.0$) (Table 5). Among postmenopausal women, after adjusting for age, BMI, and parity, there was a marginally significant trend in mean percentage of breast density by number of repeat alleles ($P = 0.03$).

Distribution of Postmenopausal Women with Breast Density Less Than 18% and Breast Density Greater Than or Equal to 18% According to the Number of 19 Repeat Alleles of the IGF1 Gene. There was no statistically significant difference in the proportion of women with ≥18% of breast density and of women with <18% of breast density in the two *19* repeat alleles group compared to the no *19* repeat allele group (OR = 1.12, 95% CI: 0.43–2.89, $P = 0.8$) (Table 6).

Variation in Percentage of Breast Density by Number of Alleles of the IGFBP3 Genotype. There was a significant trend in the mean percentage of breast density by number of *A* alleles of the *IGFBP3* gene at the −202 locus for

Table 3. Associations between predictor variables and percentage of breast density

Predictor variables	All (n = 412)		Premenopausal (n = 206)		Postmenopausal (n = 206)	
	r[a]	P[b]	r[a]	P[b]	r[a]	P[b]
Age (yrs)	−0.42	2×10^{-19}	0.03	0.7	−0.32	2×10^{-6}
Height (inches)	0.09	0.3	0.02	0.7	0.09	0.4
Weight (pounds)	−0.35	2×10^{-14}	−0.41	1×10^{-9}	−0.32	2×10^{-6}
BMI (kg/m^2)	−0.40	2×10^{-17}	−0.43	2×10^{-10}	−0.38	2×10^{-8}
Age at menopause[c] (yrs)					0.07	0.08
Age at menarche (yrs)	0.05	0.8	0.10	0.3	0.02	0.6
Age at first birth[d] (yrs)	0.07	0.3	0.14	0.2	−0.04	0.9

Note: Data presented for women included in the mammogram-gene analysis.
[a]Pearson correlation coefficient (unadjusted).
[b]P value for trend (generalized linear model) adjusted for age (yrs, continuous) and BMI (kg/m^2, continuous). Height, weight, and BMI *versus* breast density were adjusted for age only. Age *versus* breast density was unadjusted.
[c]Age at menopause only includes women who had natural menopause or bilateral oophorectomy.
[d]Age at first birth of parous women.

Table 4. Trend in mean percentage of breast density across categories of predictor variables

Predictor variables	All (n = 412)			Premenopausal (n = 206)			Postmenopausal (n = 206)		
	Mean[a] (SD)	r[b]	P[c]	Mean[a] (SD)	r[b]	P[c]	Mean[a] (SD)	r[b]	P[c]
Parity		−0.22	0.003		−0.14	0.1		−0.25	0.003
None	31.5 (18.9)			38.8 (21.2)			24.7 (13.7)		
One	27.7 (19.6)			35.6 (17.7)			19.8 (18.4)		
Two	29.5 (17.7)			34.2 (17.2)			22.7 (16.3)		
Three	24.5 (17.2)			34.2 (16.1)			14.5 (11.7)		
Four and above	15.5 (11.9)			21.8 (14.2)			14.1 (11.1)		
Family history of BC[d]		0.02	0.7		0.11	0.3		−0.01	0.9
No	26.6 (17.9)			33.6 (17.6)			19.2 (15.1)		
Yes	27.6 (18.2)			39.1 (17.2)			18.8 (13.4)		
Previous OC use		0.2	0.2		0.03	0.8		0.2	0.2
No	23.4 (17.3)			33.7 (18.6)			17.3 (13.2)		
Yes	29.9 (18.0)			34.8 (17.0)			22.0 (16.7)		
Smoking history		−0.03	0.3		−0.09	0.08		0.09	0.4
Never	27.4 (18.0)			36.4 (17.1)			17.9 (13.4)		
Past	25.8 (17.8)			31.3 (17.1)			20.1 (16.7)		
Current	27.0 (18.6)			36.5 (22.8)			21.6 (13.6)		
Alcohol (# drinks/wk)		0.16	0.4		0.08	0.6		0.13	0.8
Do not drink	20.0 (15.9)			32.1 (18.0)			14.7 (11.5)		
0–3 drinks	29.2 (17.7)			34.8 (17.1)			22.1 (15.7)		
4–9 drinks	26.3 (17.7)			32.1 (17.2)			20.3 (16.3)		
10 or more drinks	34.2 (20.7)			41.4 (19.7)			18.0 (12.5)		
Coffee (# cups/day)		−0.05	0.2		−0.16	0.04		0.06	0.7
None	26.6 (18.4)			36.9 (17.2)			17.0 (13.7)		
One a day	28.8 (17.4)			36.5 (16.1)			20.1 (14.7)		
Two a day	28.9 (18.9)			36.3 (17.5)			21.6 (17.5)		
Three or more a day	24.2 (16.7)			29.4 (18.1)			18.9 (13.4)		

Note: Data presented for women included in the mammogram-gene analysis.
[a]Mean = mean percentage of breast density.
[b]Pearson correlation coefficient (unadjusted).
[c]P value for trend (generalized linear model) adjusted for age (yrs, continuous) and BMI (kg/m^2, continuous).
[d]Family history of breast cancer.

premenopausal women ($P = 0.0005$) (Table 5). However, among postmenopausal women, there was no significant trend in the mean percentage of breast density by the number of *IGFBP3 A* alleles ($P = 0.5$).

Distribution of Premenopausal Women with Breast Density Less Than 28% and Breast Density Greater Than or Equal to 28% According to the Number of A Alleles at the −202 Locus of the IGFBP3 Gene. Women with two *A* alleles had a 5-fold increase in the odds of having a percentage of breast density greater or equal to 28%, compared with women with no A allele ($P = 0.002$) (Table 6).

Mammogram-Serum Analysis

Variation in Percentage of Breast Density by Serum IGF-I and IGFBP-3 Levels and IGF-1:IGFBP3 ratio. There was no association between percentage of breast density and serum IGF-I level among all three groups of women ($P > 0.5$) (Table 5). Similarly, there was no significant correlation between percentage of breast density and serum IGFBP-3 level or IGF-I:IGFBP-3 ratio in all three groups of women.

Discussion

The association between mammographic density and breast cancer risk is well established. Wolfe (32–2, 33) first described the association using a qualitative method of classification. Subsequently, several studies (1, 34, 35)

reported similar results using quantitative methods of classification, including the computer-assisted technique used in this study. In general, women for whom dense tissue accounts for more than 60–75% of the breast have a 4- to 6-fold increase in their risk of cancer compared to women with little or no density. This association is found in both pre- and postmenopausal women.

The epithelial cells and stromal tissue of the breast underlie the area of dense mammographic appearance. It is thought that breast density correlates with the volume of susceptible cells. It has also been suggested that breast density represents stromal and epithelial proliferation, which is influenced by local growth factors, including IGF-I and its binding proteins (36). IGF-I is a mitogen for the breast epithelium during breast development and in the adult breast (37–38). IGFBP-3 has been shown to enhance the growth-promoting effects of IGF-I, but can also inhibit growth in an IGF-independent manner (5).

This is the first study to demonstrate a clear relationship between a genetic polymorphism and mammographic breast density. We found a positive association between the number of *A* alleles at a polymorphic locus located in the *IGFBP3* gene promoter at a position −202 bp from the CAP site, and the percentage of breast density among premenopausal women. We also confirmed a positive relationship between the number of *A* alleles at this locus and serum IGFBP-3 levels among premenopausal women

(23, 31). Deal *et al.* (31) reported a significantly higher promoter activity of the *A* allele compared with the *C* allele *in vitro*, suggesting that the *IGFBP3* polymorphism influences gene expression.

IGFBP-3 functions as a growth inhibitor and antagonizes IGF-I action in many physiological contexts. The *A* allele has previously been shown to be associated with higher IGFBP-3 levels in the circulation (31); therefore, our hypothesis was that the A variant would be associated with reduced breast cell proliferation and lower density. This hypothesis was consistent with the results of Byrne *et al.* (12), in which plasma IGFBP-3 levels were negatively correlated with percentage of breast density. We propose two competing hypotheses to account for the unexpected association of the A allele with high breast density.

First, it is possible that within the breast, IGFBP-3 functions to enhance rather than inhibit IGF-I action. IGFBP-3, in common with other regulatory peptides such as TGFβ, can act to stimulate or inhibit growth based on the physiological context. While IGFBP-3 functions in many systems as an inhibitor, in vitro systems where it enhances IGF-I action have been described (39). If local expression of IGFBP-3 in the breast is higher in subjects with the A allele, and the peptide acts to stimulate IGF-I-stimulated proliferation, then it would be expected that the A allele would be associated with increased density, as observed.

A second model postulates that IGFBP-3 functions as a growth inhibitor in the breast, but that the effect of the U202 promoter polymorphism is more complex than previously suggested. While available evidence (31)

Table 5. Mean percentage of breast density by number of alleles the *IGF1* and *IGFBP3* genotypes, and by serum IGF-I and IGFBP-3 quartiles

IGF1 genotype	Mean[a] (SD)			*P* value for trend[b]		
	All (n = 412)	Pre[c] (n = 206)	Post[d] (n = 206)	All (n = 412)	Pre[c] (n = 206)	Post[d] (n = 206)
No *19* repeat allele	27.1 (17.2)	34.7 (17.4)	17.9 (11.6)	0.2[e]	1.0[f]	0.03[g]
One *19* repeat allele	26.3 (17.6)	34.8 (16.8)	18.1 (14.2)			
Two *19* repeat allele	27.2 (18.6)	33.8 (18.7)	20.7 (16.2)			
IGFBP3 genotype						
No *A* alleles	23.5 (17.3)	28.2 (17.4)	18.8 (16.0)	0.003[h]	0.0005[i]	0.5[j]
One *A* allele	27.6 (17.8)	36.2 (17.3)	18.6 (13.4)			
Two *A* alleles	29.0 (18.7)	38.2 (16.9)	20.5 (16.2)			
IGF-I (ng/ml) quartiles[k]						
Q1	21.2 (18.4)	31.5 (18.8)	18.0 (17.5)	−0.04 (0.5)[m]	−0.01 (0.9)[n]	−0.06 (0.5)[o]
Q2	26.4 (17.7)	40.6 (15.2)	20.0 (15.0)			
Q3	29.1 (17.6)	28.2 (16.6)	20.0 (14.5)			
Q4	26.3 (15.8)	33.4 (16.1)	18.5 (13.1)			
IGFBP-3 (ng/ml) quartiles[p]						
Q1	24.7 (19.2)	36.0 (16.9)	19.3 (18.6)	−0.06 (0.3)[m]	−0.07 (0.4)[n]	−0.02 (0.8)[o]
Q2	27.5 (17.5)	32.6 (17.9)	19.2 (13.8)			
Q3	27.1 (17.8)	36.4 (16.7)	19.0 (14.5)			
Q4	23.7 (15.6)	29.8 (16.8)	19.0 (12.9)			
IGF-I:IGFBP-3 (molar ratio) quartiles[q]						
Q1	20.9 (16.8)	33.2 (16.7)	17.8 (14.9)	−0.05 (0.4)[r]	−0.02 (0.8)[s]	−0.06 (0.5)[t]
Q2	27.3 (17.5)	33.4 (20.0)	18.7 (14.0)			
Q3	27.7 (17.3)	35.2 (16.2)	22.2 (15.8)			
Q4	27.5 (17.9)	32.8 (16.6)	18.0 (15.5)			

Note: *Upper bound excluded.

[a]Mean percentage of breast density.

[b]Test for trend in mean percentage of breast density by number of alleles of the *IGF1* 19 CA repeat genotype or *IGFBP3* genotype.

[c]Premenopausal women.

[d]Postmenopausal women.

[e]Adjusted for age (continuous), BMI (continuous), menopausal status (pre/post), parity (4 and above *versus* <4), and coffee (3 or more *versus* <3).

[f]Adjusted for BMI (continuous), parity (4 and above *versus* <4), alcohol (10 or more *versus* <10), and coffee (3 or more *versus* <3).

[g]Adjusted for age (continuous), BMI (continuous), and parity (continuous).

[h]Adjusted for age (continuous), BMI (continuous), menopausal status (pre/post), and parity (4 and above *versus* <4).

[i]Adjusted for BMI (continuous), parity (4 and above *versus* <4), alcohol (10 or more *versus* <10), and coffee (3 or more *versus* <3).

[j]Adjusted for age (continuous), BMI (continuous), and parity (continuous).

[k]All—Q1 (74–128*), Q2 (128–156*), Q3 (156–188*), Q4 (≥188); Pre—Q1 (96–139*), Q2 (139–166*), Q3 (166–196*), Q4 (>= 196); Post-Q1 (74–118*), Q2 (118–139*), Q3 (139–173*), Q4 (≥173).

[l]Spearman's partial correlation between the continuous measure of percentage of breast density and the continuous level of IGF-I, IGFBP-3, or IGF-I:IGFBP-3 ratio adjusting for covariates.

[m]Adjusted for serum IGF-I or IGFBP-3 (continuous).

[n]Adjusted for serum IGF-I or IGFBP-3 (continuous).

[o]Adjusted for serum IGF-I or IGFBP-3 (continuous).

[p]All—Q1 (511–2381*), Q2 (2381–2719), Q3 (2719–3063), Q4 (≥3063); Pre—Q1 (1791–2472*), Q2 (2472–2722*), Q3 (2722–3044*), Q4 (≥3044); Post—Q1 (511–2316*), Q2 (2316–2719*), Q3 (2719–3065*), Q4 (≥3065).

[q]All—Q1 (0.030–0.051*), Q2 (0.051–0.057*), Q3 (0.057–0.066*), Q4 (≥0.066); Pre—Q1 (0.030–0.055*), Q2 (0.055–0.061*), Q3 (0.061–0.071*), Q4 (≥0.071); Post—Q1 (0.030–0.047*), Q2 (0.047–0.055*), Q3 (0.055–0.062*), Q4 (≥0.062).

[r]Adjusted for age (continuous), BMI (continuous), menopausal status (pre/post), parity (4 and above *versus* <4), coffee (3 or more *versus* <3).

[s]Adjusted for BMI (continuous), parity (4 and above *versus* <4), and coffee (3 or more *versus* <3).

[t]Adjusted for age (continuous), BMI (continuous), and parity (continuous).

Table 6. Estimated odds of having a given percentage of breast density according to the number of *19* repeat alleles of the *IGF1* gene and the number of alleles of the *IGFBP3* genotype

Percentage of breast density	No. of *19* repeat alleles of the *IGF1* gene of postmenopausal women			
	No *19* repeat allele	One *19* repeat allele	Two *19* repeat alleles	Total
<18%	12	61	41	114
≥18%	12	38	42	92
Total	24	99	83	206
OR (95% CI)[a]	1.0 (reference)	0.68 (0.27–1.74)	1.12 (0.43–2.89)	
P value		0.4	0.8	

Percentage of breast density	No. of *A* alleles of the *IGFBP3* gene of premenopausal women			
	No *A* allele	One *A* allele	Two *A* alleles	Total
<28%	30	34	9	73
≥28%	28	71	34	133
Total	58	105	43	206
OR (95% CI)[b]	1.0 (reference)	2.53 (1.21–5.31)	5.05 (1.82–13.97)	
P value		0.01	0.002	

Note: *Upper bound excluded.
[a]Adjusted for age (yrs; <55, 55–62, 63–70, ≥71), BMI (kg/m^2; 18–26*, 26–30*, ≥30), parity (none *versus* 1 or more), and alcohol (none *versus* 1 or more a week).
[b]Adjusted for BMI (kg/m^2; 18–26*, 26–30*, ≥30), coffee (3 or more cups a day *versus* <3), smoking (never smoker *versus* current or past smoker), and family history of breast cancer (yes/no).

suggests that the *A* allele is associated with higher expression levels than the *C* allele under baseline conditions or in the presence of positive regulators such as growth hormone, it does not exclude the possibility that the *A* allele is associated with a higher degree of responsivity to various regulators, whether they are positive or negative. It is known that in estrogen-regulated tissues and cell lines, IGFBP-3 expression is down-regulated by estrogens (40–42). If we assume that locally produced IGFBP-3 (36) is the most important source of this peptide for the interstitial fluid of the breast (as circulating IGFBP-3 is mostly present in large molecular weight complexes with limited capillary permeability), and that the *A* allele is associated with the enhanced suppressibility by estradiol, then particularly in premenopausal women, we would expect to see enhanced suppression of IGFBP-3 expression in subjects with more *A* alleles, and this would be associated with lower IGFBP-3 concentrations, enhanced IGF-I action, and denser breasts. Clearly, detailed studies of the influence of this and related polymorphic loci on promoter function are a high priority.

We found a borderline significant association between postmenopausal serum IGFBP-3 levels and the *A* allele (*P* = 0.07). However, we found a highly significant inverse association between the IGF-I:IGFBP-3 ratio and the *A* allele (*P* = 0.0002). This observation requires further follow-up. While IGFBP-3 clearly is the major source of binding capacity of serum for IGFs, it is unclear why the −202 locus should influence the ratio to a larger extent than IGFBP-3 concentration.

The *IGF1* polymorphism does not appear to be strongly related to breast density among postmenopausal women. We found a marginally positive association between the *IGF1 19* repeat allele and breast density among postmenopausal women (*P* = 0.03, adjusted), using the linear model, but we failed to find a significant association using logistic regression with breast density

treated as a categorical variable. Studies of other genes suggest that the polymorphic CA repeats in the promoter region of a gene affects transcription activity of the gene (43) and that the length of CA repeats is inversely correlated with transactivation (44). Rosen *et al.* (21) first reported an association between this polymorphic locus and serum IGF-I levels. However, subsequent studies (22, 45–47) were not able to replicate their results. We found a negative trend between serum IGF-I and the number of *19* repeat alleles among postmenopausal women; however the trend did not reach statistical significance after adjusting for multiple covariates. Further studies are needed to define the role of this polymorphism in the expression of serum and tissue IGF-I levels.

We did not find a relationship between serum IGF-I and IGFBP-3 levels and mammographic density among premenopausal women. Byrne *et al.* (12) did not find a statistically significant correlation between plasma IGFBP-3 levels and breast density (Spearman correlation coefficient = −0.24, *P* = 0.07) among premenopausal women. Similarly, Boyd *et al.* (13) did not find an association between serum IGFBP-3 and breast density (regression coefficient = −0.02, *P* = 0.95). However, both studies found a significant positive correlation between blood IGF-I levels and breast density among premenopausal women. Future studies are needed to resolve this inconsistency. It is possible that IGF-I levels in the breast are a better predictor of premenopausal breast density. Guo *et al.* (14) showed that breast tissue sections from premenopausal subjects with extensive densities had larger stained areas of IGF-I when compared with subjects with little breast density (*P* = 0.02). However, we confirmed the results of Byrne *et al.* and Boyd *et al.* showing that blood IGF-I and IGFBP-3 levels did not correlate with breast density among postmenopausal women. Future studies will determine whether IGF-I and IGFBP-3 levels in the breast predict breast

density among postmenopausal women. Guo *et al.* (14), however, found no association between IGF-I concentrations in the breast and breast density among postmenopausal women.

Our study excluded women who were currently or previously using HRT or who currently used OCs. This was done to evaluate the relationship between endogenous levels of IGF-I and IGFBP-3 and mammographic density and to correlate genotypes with phenotypes. The inclusion of women who use exogenous hormones might mask these relationships. For example, the use of postmenopausal hormones has been reported to increase the percentage of breast density (48, 49). Breast density changes associated with HRT are dynamic, increasing with hormone initiation, and decreasing after discontinuation. Administration of oral estrogen decreases serum levels of IGF-I and IGFBP-3, and administration of transdermal estrogen increases serum levels of IGF-I (50, 51). We also excluded women who were current OC users because women who use OCs have reduced levels of IGF-I and increased levels of IGFBP-3 (23).

Despite longstanding evidence that mammographic density is a trait that is in part genetically determined (15–17), prior evaluations of various candidate genes (18) did not identify significant associations. Our results require confirmation, but suggest that polymorphic variation of *IGFBP3*, and perhaps other genes involved in the IGF system, influence breast density.

Acknowledgments

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