

# Pituitary Growth Hormone and Growth Hormone–Releasing Hormone Receptor Genes and Associations with Mammographic Measures and Serum Growth Hormone

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## Abstract

**Background:** Mammographic density is a strong risk factor for breast cancer that is heritable and associated with blood levels of growth hormone and insulin-like growth factor-I (IGF-I). We tested single nucleotide polymorphisms (SNP) in pituitary growth hormone (*GH1*) and growth hormone–releasing hormone receptor (*GHRHR*) genes for an association with mammographic density, hormones of the growth hormone/IGF-I axis, and anthropometric variables.

**Methods:** Mammograms from 348 women were measured using a computer-assisted method, blood collected, and DNA extracted. The SNPs genotyped were *GH1* –57G>T, *GH1* –75G>A, and *GHRHR* A57T. ANOVA and covariance were used to examine associations, adjusted for age, body mass index, ethnicity, and menopausal status, between each SNP and three measures of the mammogram: percent density, total dense area, and total nondense area. Similarly, the SNPs were

tested for associations with serum growth hormone, IGF-I, IGFBP3, prolactin, and anthropometric variables.

**Results:** *GH1* –57G>T and *GH1* –75G>A were both associated with percent density and total nondense area. *GH1* –57T homozygotes had 5.2 more mean adjusted percent density than other subjects combined ( $P = 0.03$ ) and 16.2 cm<sup>2</sup> (14.6%) less nondense area ( $P = 0.01$ ). *GH1* –75A homozygotes had 3.4 more percent density than subjects with at least one G allele ( $P = 0.04$ ) and also had 32% higher serum growth hormone levels ( $P = 0.02$ ).

**Conclusion:** We have found associations between mammographic density and two SNPs in the pituitary growth hormone gene, one of them also associated with serum growth hormone levels. These findings suggest that the *GH1* gene may also influence breast cancer risk. (Cancer Epidemiol Biomarkers Prev 2005;14(11):2648–54)

## Introduction

Variations in the mammographic appearance of the breast reflect variations in tissue composition that are strongly associated with risk of breast cancer in studies of percent mammographic density measured qualitatively and quantitatively (1–4). Menopausal status, weight, and number of live births all influence mammographic density but account for only 20% to 30% of the age-adjusted variance in mammographic density (5). Two recent twin studies in women ages 40 to 70 years estimated that the heritability of mammographic density, measured quantitatively using a computer-assisted method and adjusted for age and other covariates, was 67% (95% confidence interval, 59–75) in North American twins, 60% (95% confidence interval, 54–66) in Australian twins, and 63% (95% confidence interval, 59–67%) in the two groups of twins combined (6). Previous work has also shown associations between mammographic density and blood levels of growth hormone and insulin-like growth factor-I (IGF-I), which are both mitogens in the breast. IGF-I, which is produced in response to growth hormone, has also been associated with mammary carcinogenesis in animals and humans (7–10).

We selected three common polymorphism for this study: *GH1* –57G>T, *GH1* –75G>A, and *GHRHR* Ala57Thr. *GH1* –57G>T is located in the proximal promoter region of the gene encoding growth hormone, and a study found it to be associated with growth hormone levels (11). *GH1* –75G>A is located in a pituitary-specific transcription factor binding region, Pit-1, of the proximal promoter of *GH1* (12), and we hypothesized that due to its physical location, it may be functional. *GHRHR* encodes a 423-amino-acid seven transmembrane G-protein-linked receptor expressed primarily in the pituitary (13–15). The G-to-A base change at position 169 in exon 3 of the *GHRHR* results in an alanine-to-threonine substitution at codon 57, and threonine at this site confers increased sensitivity of the receptor to GHRH (13). In addition, a study of free fat mass in Quebec families found evidence of linkage to chromosome 7p15.3, and a strong candidate gene in this region is *GHRHR* (16). We hypothesized that the *GH1* –75G allele, *GH1* –57T allele, and *GHRHR* Thr57 would be associated with increased percent density.

It is thus plausible that these factors may influence both the tissue composition of the breast and risk of breast cancer in humans. The purpose of the present study was to examine the association of polymorphisms in genes related to growth hormone with mammographic density and with blood levels of growth hormone and IGF-I.

## Materials and Methods

**General Method.** DNA from subjects who participated in a cross-sectional study of hormone levels and mammographic density was used to assess the relationship between common

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polymorphisms of candidate genes and mammographic density. We studied 177 premenopausal and 171 postmenopausal women, who consented to allow their DNA to be used in studies investigating the genetic determinants of breast tissue density. The methods of this study are published in detail elsewhere (7, 17).

**Recruitment.** Between 1994 and 1997, potential subjects were identified from the mammographic units of Mount Sinai, Women's College, and St Michael's Hospital in Toronto. A radiologist visually estimated the percent mammographic density, and this was categorized into quintiles. Stratified sampling was used to recruit similar numbers of women into each quintile of percent density, with overrepresentation of the extreme categories. The number of subjects recruited into each quintile of mammographic density was as follows: <10%,  $n = 101$ ; 10% to <25%,  $n = 62$ ; 25% to <50%,  $n = 60$ ; 50% to <75%,  $n = 60$ ; and >75%,  $n = 99$ .

Potential subjects were sent a letter and subsequently telephoned about the study. Premenopausal subjects were eligible if they were menstruating regularly, not pregnant or breast-feeding, and had no hysterectomy or oophorectomy. Postmenopausal women were eligible if they had spontaneous amenorrhea for at least 12 months, or had a hysterectomy and were  $\geq 50$  years of age, or had a bilateral oophorectomy at any age. A subject was excluded if she was taking any type of exogenous hormones, had breast augmentation or reduction, a personal history of breast cancer, or was being investigated for breast cancer. In total, 382 women agreed to participate in the study, representing 88% of subjects who were contacted and found eligible.

**Measurements.** Data and blood samples were collected after a 12-hour overnight fast and during the luteal phase of the menstrual cycle (days 20-24) for premenopausal subjects. The mammogram closest to the time of the blood draw was used (the mean difference between mammogram and blood draw was 32 weeks).

**Obtaining Consent for Genetic Study.** Subjects who participated in the hormone study were mailed a letter describing the goals of the genetic component of this study and asked to provide written consent to analyze DNA for genetic polymorphisms that may affect breast density levels. Of 382 eligible subjects (193 premenopausal and 189 postmenopausal women), 357 (93%) gave consent for use of their DNA. Eight subjects could not be contacted because they had moved and could not be traced. Sixteen subjects did not respond after repeated reminders, and one subject declined to provide consent. Four premenopausal subjects who consented to participate were excluded from this study because there was insufficient DNA. A total of 348 subjects (177 premenopausal and 171 postmenopausal) had DNA and provided consent.

**Ethnicity.** Each subject was asked their country of birth as well as the countries of birth for each of their parents and grandparents. Subjects were also asked the question "What is your ethnic or cultural background?" and given instructions to mark all appropriate categories. Subjects were classified as (a) Black; (b) White (e.g., British, French, European, Latin/South American of European background); (c) Native/aboriginal people of North America (North American Indian, Inuit, Metis); (d) East Asian (e.g., Chinese, Japanese, Korean, Vietnamese); (e) South Asian (e.g., Indian from India, Pakistani, Punjabi, Tamil); (f) other, with specification; and (g) Don't know. When adjusting for ethnicity in the analyses shown here, these groups were combined as follows: (a) Caucasian (White) and (b) other.

**Epidemiologic, Anthropometric, and Dietary Data.** Information about epidemiologic risk factors for breast density and breast cancer was collected by questionnaire. Each subject was

weighed and measured for height and waist and hip circumference.

**Measurement of Blood Samples.** Prolactin was measured by standard RIA methods at the Wellesley Hospital in Toronto. IGF-I, IGFBP3, and growth hormone were measured by Esoterix (Calabasas Hills, CA). The first two were measured by a competitive binding RIA. Growth hormone was measured by a two-site immunometric assay. Values for growth hormone were missing in 19 (premenopausal,  $n = 3$ ; postmenopausal,  $n = 16$ ) and undetectable in 120 (premenopausal,  $n = 53$ ; postmenopausal,  $n = 67$ ) of 357 (34%) subjects in the study. Nondeterminant values were assumed to be due to the episodic and pulsatile nature of growth hormone release. A value of 0.2 ng/L was assigned to the 120 undetectable measurements and represents the lower limit of sensitivity for the assay. Consequently, for statistical analyses that involved growth hormone, analyses were done on the subset of 218 women with detectable growth hormone values and repeated in the entire set including the 120 subjects with assigned values. In all instances, the results were similar, although statistical significance was sometimes lost because of the smaller sample size available for those with detected values. Results are presented here with all study subjects included.

**Mammographic Density.** Breast density measurements were made using a randomly selected, craniocaudal (viewing from above down) mammographic view of one breast from each subject. Mammograms were digitized using a Lumisys model 85, randomly ordered, and presented to the observer (N.F.B.) for analysis as an array of  $675 \times 925$  pixels (0.0676 mm<sup>2</sup>/pixel). The observer selected a threshold grey value to separate the image of the breast from the background, using a previously described computer assisted method (18).

**DNA Isolation.** DNA was purified from buffy coats of blood samples using a modified chaotropic method (19). DNA samples were stored at  $-80^{\circ}\text{C}$ . DNA was extracted from whole blood using Puregene DNA isolation kit (Gentra, Minneapolis, MN). Yields of DNA were determined by light spectroscopy.

**Genotyping.** Both single nucleotide polymorphisms (SNP) in *GHI* were measured using the same 311-bp PCR product that was amplified with the following primers: 5'-AGCCA-GGGGGCATGATCCC-3' and 5'-GCCCCCGTCCCATCTA-CAGGT-3' (Invitrogen/Life Technologies, Life Technologies Bethesda Research Laboratories Custom Primers, Burlington, Ontario, Canada; ref. 12). The PCR product was verified by 2% agarose gel electrophoresis and then incubated overnight at  $37^{\circ}\text{C}$  with *HincII* (*HindII*) restriction enzyme, which cut the PCR product containing a T nucleotide at position -57 into fragments of lengths 109 and 202 bp (MBI Fermentas, Burlington, Ontario, Canada). Digested PCR product was separated on 2% agarose gels by gel electrophoresis at 160 V for 30 minutes and viewed under UV transilluminator. Using the same 311-bp PCR product, *GHI* -75A>G was measured using Snapshot method. Four microliters of the PCR product were cleaned using 6  $\mu\text{L}$  of double-distilled H<sub>2</sub>O, 2  $\mu\text{L}$  of alkaline phosphatase from shrimp Roche (Laval, Quebec, Canada), and 0.1  $\mu\text{L}$  of exonuclease I (New England Biolabs, Beverly, MA); this mixture was incubated for 1 hour at  $37^{\circ}\text{C}$  followed immediately by 15 minutes at  $72^{\circ}\text{C}$ . The mixture was then stored at  $4^{\circ}\text{C}$  until the genotyping was completed. The snapshot primer was 5'-TCCAGGACTGAATCGTGCTC-3' (Invitrogen/Life Technologies, Life Technologies Bethesda Research Laboratories Custom Primers). Negative controls were run with each PCR to ensure that contamination did not occur.

*GHRHR* G-to-A base change at position 169, which results in an alanine-to-threonine change at codon 57, was measured by PCR based snapshot method (13). The PCR product was 265 bp in length and verified using 2% agarose gel electrophoresis.

The primers for the PCR reaction were 5'-GTGGTGGCTTCTCTGATTCCTTTAT and 3'-TTCCAGATGAAAGCACCTCCCTTTC (in 5' to 3'; Invitrogen/Life Technologies, Life Technologies Bethesda Research Laboratories Custom Primers). The Snapshot primer was GGCCCCATGCATAAATGT (in 5' to 3'; Invitrogen/Life Technologies, Life Technologies Bethesda Research Laboratories Custom Primers). All snapshot procedures were carried out by the Sequencing Facility of the Robarts Research Institute in London, Ontario, Canada.

All genotypes were read twice by two reviewers (C.M. and H.C.) when necessary to verify the genotype. PCR and digestion were repeated to resolve any genotyping problems. For each polymorphism, genotyping was repeated in a random sample of 50 subjects to check the accuracy of genotyping. The error rate for each of the SNPs measured was <2%. To verify that the genotyping was correct for the SNPs in the *GH1* gene, the PCR product of 20 subjects was sequenced by the Sequencing Facility of the Robarts Research Institute. The PCR product was isolated and prepared for sequencing using the QIAquick Gel Extraction kit (Qiagen, Inc., Mississauga, Ontario, Canada). Data were double entered into a computer database to minimize errors.

**Statistical Analysis.** All analyses were conducted using SAS version 8.1. Initially, the allele frequencies for the genotypes were checked for Hardy-Weinberg equilibrium using a  $\chi^2$  test. This was done for all subjects, premenopausal and postmenopausal subjects separately, and then by ethnic group. Allele frequencies were compared between ethnic groups using a  $\chi^2$  test. Descriptive statistics were carried out by menopausal status and ethnicity.

Four measures of density were examined for associations with the three polymorphisms: total area, total area of dense tissue, total area of nondense tissue, and percent density. Data were transformed if necessary to approximate a normal distribution. The log transformation was applied to the total area and the total nondense area. A square root transformation was applied to the percent density and the total area of dense tissue. The relationships between the transformed measures of breast density and genotypes were explored using ANOVA and an analysis of covariance to adjust for potential confounders. All statistical test values are reported for the transformed outcome variables. Unless otherwise specified, all means of density measures reported are the untransformed least square means and SEs adjusted for age, body mass index (BMI), ethnicity for each of the menopausal groups, and menopausal status for analyses of all women. Because waist circumference was often more strongly correlated with some mammographic measures than BMI, the analyses were repeated, replacing BMI with waist in the model. The purpose of these analyses was to verify that the associations between SNPs and mammographic measures persisted with adjustment for another measure of body size than BMI. The results of analyses adjusted for waist were similar to those adjusted for BMI. Because associations with mammographic density are more commonly adjusted for BMI as an indicator of adiposity, we reported the results adjusted for BMI.

Initially, analyses were conducted using a codominant model of inheritance; meaning, the genotypes were treated as a categorical variable with three levels. After examining the distribution of subjects and the adjusted means of the outcome variables, we combined some of the groups of genotypes as follows: for *GH1* -57G>T, the G homozygotes and heterozygotes were grouped together and compared with the homozygous T individuals; for *GH1* -75G>A, the G homozygotes and heterozygotes were grouped together and compared with the A homozygotes; for the SNP in *GHRHR*, G homozygotes and heterozygotes were grouped together for the analyses. The Duncan *t* test was used to compare the means for each genotype before grouping GG/GT for *GH1*

-57G>T and GG/GA for *GH1* -75G>A. However, for the *GHRHR* SNP, the grouping of genotypes was dictated by the small-number G homozygotes.

All analyses were conducted in three ways to account for ethnicity: first, without including a variable for ethnicity in the models, then with ethnicity in the model, and finally on the subgroup of Caucasian women only. The results shown in this article are all adjusted for ethnicity, and we comment on the results for Caucasian women alone.

## Results

**Study Subjects.** Selected characteristics of the 177 premenopausal and 171 postmenopausal women in this study are shown in Table 1. The two groups were similar in height, weight, BMI, and waist-to-hip ratio. Postmenopausal women had a slightly later mean age of menarche and earlier mean age of first birth. As expected, the mean percent density was greater in premenopausal women (29.1%) than in postmenopausal women (23.8%).

**Allele Frequencies and Hardy-Weinberg Equilibrium.** The allele frequencies for all SNPs in this study were in Hardy-Weinberg equilibrium, overall and when calculated by menopausal status and ethnic group. For *GH1* -57G>T, the G allele frequency for Caucasians, East Asians, Jewish, and "other" ethnic groups were 0.64 ( $n = 294$ ), 0.36 ( $n = 14$ ), 0.69 ( $n = 16$ ), and 0.46 ( $n = 24$ ), respectively. The allele frequencies were similar among Caucasian and Jewish women ( $P = 0.53$ ), but Caucasians differed from East Asians ( $\chi^2 = 7.82$ ,  $P = 0.005$ ), and from "other" ethnic groups ( $\chi^2 = 5.35$ ,  $P = 0.02$ ). For *GH1* -75A>G, the frequency of the A allele among all women was 89%; this was similar across all ethnic groups ( $\chi^2 = 5.15$ ,  $P = 0.16$ ). This polymorphism was in weak linkage disequilibrium with the *GH1* -57G>T SNP ( $r = -0.10$ ,  $P < 0.0001$ ). The allele frequencies of the G allele of *GHRHR* Ala>Thr at codon 57

**Table 1. Selected characteristics of study subjects by menopausal status**

|                                    | Mean (SD)                      |                                 |
|------------------------------------|--------------------------------|---------------------------------|
|                                    | Premenopausal<br>( $n = 177$ ) | Postmenopausal<br>( $n = 171$ ) |
| Risk factor                        |                                |                                 |
| Age (y)                            | 44.89 (4.62)                   | 56.04 (4.50)                    |
| Height (cm)                        | 163.27 (6.52)                  | 164.23 (6.69)                   |
| Weight (kg)                        | 67.24 (15.92)                  | 70.27 (16.62)                   |
| BMI (kg/m <sup>2</sup> )           | 25.23 (5.80)                   | 26.04 (5.99)                    |
| Waist/hip ratio                    | 0.75 (0.06)                    | 0.77 (0.08)                     |
| Age at menarche (y)                | 12.72 (1.47)                   | 13.03 (1.70)                    |
| Age at first birth (y)             | 28.19 (5.85)                   | 26.50 (5.75)                    |
| No. live births                    | 1.47 (1.19)                    | 1.67 (1.45)                     |
| Mammographic density (%)           | 29.07 (22.70)                  | 23.84 (20.47)                   |
| Growth factors ( $\mu\text{g/L}$ ) |                                |                                 |
| GH                                 | 1.71 (2.83) <sup>†</sup>       | 1.25 (2.46) <sup>†</sup>        |
| IGF-I                              | 154.57 (35.41) <sup>‡</sup>    | 130.18 (34.21) <sup>§</sup>     |
| IGFBP-3                            | 2.69 (0.46) <sup>†</sup>       | 2.83 (0.51) <sup>‡</sup>        |
| Allele frequencies                 |                                |                                 |
| <i>GH1</i> -57 G>T: G allele       | 0.64 (0.47) <sup>  </sup>      | 0.59 (0.29) <sup>  </sup>       |
| <i>GH1</i> -75 G>A: A allele       | 0.87 (0.15)                    | 0.91 (0.51)                     |
| <i>GHRHR</i> : G allele            | 0.93 (0.89)                    | 0.94 (0.44)                     |
| Ethnic origin, $n$ (%)             |                                |                                 |
| Caucasian                          | 153 (86.4)                     | 141 (82.5)                      |
| East Asian                         | 5 (2.8)                        | 9 (5.3)                         |
| Jewish                             | 10 (5.6)                       | 6 (3.5)                         |
| Other                              | 9 (5.1)                        | 15 (8.8)                        |

Abbreviation: GH, growth hormone.

\* $n = 174$ .

† $n = 155$ .

‡ $n = 176$ .

§ $n = 170$ .

|| $P$  for  $\chi^2$  testing, Hardy-Weinberg equilibrium.



were for Caucasians, East Asians, Jewish, and "other" ethnic groups 0.95, 0.93, 0.88, and 0.83 respectively, and differed significantly only between Caucasians and the "other" ethnic group ( $P = 0.005$ ). The allele frequencies differed between Caucasians and all other ethnicities combined; for *GH1* -57, *GH1* -75, and *GHRHR* A57T, the  $P = 0.01$ , 0.04, and 0.004, respectively, for the  $\chi^2$  statistic.

The allele frequencies measured for SNPs located in *GH1* are similar to those reported in other studies, but the frequency of the A allele in *GHRHR* has been reported to be 0.40 in a group of five subjects with somatotrophinomas (13) but was here found to be 0.06.

### Associations of Polymorphisms with Mammographic Density

*GH1* -57G>T: *Codominant Analysis*. As shown in Table 2, for all subjects, after adjustment for age, ethnicity, and BMI, the *GH1* -57T homozygotes had on average 3.2 (a relative difference of 11%) greater percent density than G homozygotes and 6.8 (a relative difference of 26%) greater percent density than heterozygotes ( $P = 0.04$ ). *GH1* -57T homozygotes had a nonsignificant 1.13 cm<sup>2</sup> (a relative difference of 3.5%) larger dense area than G homozygotes, and T homozygotes had a statistically significant 13.6 cm<sup>2</sup> (12.6%) smaller area of nondense tissue than G homozygotes. Adjusting for waist circumference, rather than BMI (data not shown), strengthened the significance of the result for percent density ( $F = 3.78$ ,  $P = 0.02$ ) and for nondense area ( $F = 4.77$ ,  $P = 0.009$ ) but not for dense area ( $F = 1.41$ ,  $P = 0.22$ ). Both premenopausal and postmenopausal *GH1* -57T homozygotes had nonsignificantly greater percent density and area of dense tissue and a smaller area of nondense tissue than G homozygotes.

When the analysis was confined to Caucasian women (data not shown), and adjusted for age, BMI, and menopausal status, *GH1* -57 was again significantly associated with percent density ( $F = 4.68$ ,  $P = 0.01$ ) and nondense area ( $F = 4.00$ ,  $P = 0.02$ ) in all women and showed a borderline association with dense area ( $F = 2.59$ ,  $P = 0.08$ ).

*GH1* -57G>T: *Dominant Analysis*. Results of the analyses with the T allele dominant to the G allele, given in Table 2, show that in all women, *GH1* -57T homozygotes had a 5.2 greater mean adjusted percent density than other subjects ( $P = 0.03$ ), a relative difference of 18.6%. *GH1* -57T homozygotes also had a 2.9 cm<sup>2</sup> (9.2%) smaller mean adjusted dense area ( $P = 0.20$ ) and a 16.2 cm<sup>2</sup> (14.6%) smaller nondense area ( $P = 0.01$ ).

In both premenopausal and postmenopausal women, *GH1* -57T homozygotes had a greater percent density and area of dense tissue and a smaller area of nondense tissue than other

subjects. In premenopausal women, this difference was of borderline significance for percent density ( $P = 0.08$ ) and nondense area ( $P = 0.10$ ). Both of these differences were significant if adjusted for waist circumference (data not shown) rather than BMI ( $P = 0.03$  and 0.05 for percent density and nondense area, respectively). Postmenopausal *GH1* -57T homozygotes had nonsignificant 14.6% greater percent density ( $P = 0.19$ ) and 14.4% less nondense tissue ( $P = 0.07$ ) than GT and GG subjects, when adjusted for age, BMI, and ethnicity.

When the analysis was confined to Caucasian women (data not shown), and adjusted for age, BMI, and menopausal status, *GH1* -57 was again significantly associated with percent density ( $F = 6.09$ ,  $P = 0.01$ ) and nondense area ( $F = 5.21$ ,  $P = 0.02$ ) and not with dense area ( $F = 3.32$ ,  $P = 0.07$ ) in all women. In the analysis of postmenopausal Caucasian women, adjusted for age and BMI, *GH1* -57 was significantly associated with nondense area ( $P = 0.03$ ) but not percent density ( $P = 0.06$ ) or dense area ( $P = 0.16$ ).

*GH1* -75A>G: *Dominant Analysis*. Only seven subjects were *GH1* -75G homozygotes; thus, GA and GG subjects were combined for all analyses. The results are shown in Table 3. In all subjects, *GH1* -57A homozygotes had a 3.4 (a 13.1% relative difference) greater percent density ( $P = 0.04$ ) and a 10.1 cm<sup>2</sup> (8.6%) smaller nondense area ( $P = 0.07$ ) after adjustment for age, menopausal status, ethnicity, and BMI. Dense area was 1.6 cm<sup>2</sup> (5.4%) larger in A homozygotes, but this difference was not significant. When adjusted for waist circumference rather than BMI (data not shown), *GH1* -75 genotype was more strongly associated with percent density ( $P = 0.01$ ) and nondense area ( $P = 0.02$ ) and showed an association of borderline significance with dense area ( $P = 0.09$ ).

In premenopausal women, *GH1* -75A homozygotes had a 5.3 greater percent density (a relative difference of 20.8%;  $P = 0.02$ ), a 3.9 cm<sup>2</sup> (13.4%) larger dense area ( $P = 0.09$ ), and a 12.9 cm<sup>2</sup> (11.9%) smaller area of nondense tissue ( $P = 0.06$ ) after adjustment for age, BMI, and ethnicity. In postmenopausal women, *GH1* -75A homozygotes had nonsignificantly greater percent density and dense area and less nondense tissue.

When the analysis was restricted to Caucasian women (data not shown) and adjusted for age and BMI, *GH1* -75 was not associated with percent density ( $P = 0.10$ ), dense area ( $P = 0.29$ ), or nondense area ( $P = 0.15$ ). However, among premenopausal Caucasian women, *GH1* -75 was associated with percent density ( $P = 0.02$ ) but not dense area ( $P = 0.09$ ) or nondense area ( $P = 0.07$ ). There were no significant associations among Caucasian postmenopausal women.

*GHRHR*. Only one subject was homozygous A for the polymorphism measured in the *GHRHR* gene, and subjects who were homozygous A and heterozygous were combined

**Table 2. Association between genotypes and mammographic measures**

| Measurement                      | <i>GH1</i> -57G>T Genotype, mean (SE) |               |               | F (P)       | <i>GH1</i> -57G>T Genotype, mean (SE) |               | F (P)       |
|----------------------------------|---------------------------------------|---------------|---------------|-------------|---------------------------------------|---------------|-------------|
|                                  | GG                                    | GT            | TT            |             | GG/GT                                 | TT            |             |
| All women                        | n = 138                               | n = 153       | n = 57        |             | n = 291                               | n = 57        |             |
| % Density                        | 29.90 (1.79)                          | 26.29 (1.62)  | 33.13 (2.38)  | 3.25 (0.04) | 27.84 (1.36)                          | 33.02 (2.39)  | 4.82 (0.03) |
| Dense area (cm <sup>2</sup> )    | 32.67 (2.32)                          | 29.48 (2.09)  | 33.80 (3.08)  | 1.33 (0.26) | 30.85 (1.76)                          | 33.70 (3.08)  | 1.68 (0.20) |
| Nondense area (cm <sup>2</sup> ) | 108.58 (4.88)                         | 113.40 (4.40) | 94.95 (6.49)  | 4.16 (0.02) | 111.34 (3.70)                         | 95.10 (6.49)  | 6.25 (0.01) |
| Premenopausal women              | n = 75                                | n = 77        | n = 25        |             | n = 152                               | n = 25        |             |
| % Density                        | 31.41 (2.57)                          | 27.38 (2.37)  | 35.98 (3.80)  | 2.11 (0.12) | 29.16 (2.00)                          | 35.71 (3.81)  | 3.19 (0.08) |
| Dense area (cm <sup>2</sup> )    | 33.76 (3.38)                          | 29.58 (3.12)  | 35.21 (5.01)  | 0.89 (0.41) | 31.43 (2.63)                          | 34.94 (5.00)  | 1.05 (0.31) |
| Nondense area (cm <sup>2</sup> ) | 95.52 (6.06)                          | 103.44 (5.60) | 84.69 (8.98)  | 2.22 (0.11) | 99.93 (4.71)                          | 85.22 (8.97)  | 2.82 (0.10) |
| Postmenopausal women             | n = 63                                | n = 76        | n = 32        |             | n = 139                               | n = 32        |             |
| % Density                        | 27.74 (2.53)                          | 25.31 (2.22)  | 30.16 (3.03)  | 1.07 (0.34) | 26.31 (1.86)                          | 30.15 (3.03)  | 1.71 (0.19) |
| Dense area (cm <sup>2</sup> )    | 30.44 (3.18)                          | 29.75 (2.78)  | 32.11 (3.80)  | 0.36 (0.70) | 30.03 (2.33)                          | 32.11 (3.79)  | 0.62 (0.43) |
| Nondense area (cm <sup>2</sup> ) | 120.62 (7.82)                         | 124.85 (6.84) | 105.40 (9.35) | 1.94 (0.15) | 123.11 (5.74)                         | 105.42 (9.33) | 3.38 (0.07) |

NOTE: The least-squares means presented here are adjusted for age, ethnicity, and BMI. For all women, the means are also adjusted for menopausal status. The analyses were conducted using the transformed outcome variable, and the means presented are the least-squares-adjusted untransformed means.

**Table 3. Association between genotypes and mammographic measures**

| Measurement                      | <i>GHI</i> -75G>A Genotype, mean (SE) |                | <i>F</i> ( <i>P</i> ) | <i>GHRHR</i> Genotype, mean (SE) |                | <i>F</i> ( <i>P</i> ) |
|----------------------------------|---------------------------------------|----------------|-----------------------|----------------------------------|----------------|-----------------------|
|                                  | GG/AA                                 | AA             |                       | AA/GA                            | GG             |                       |
| All women                        | <i>n</i> = 68                         | <i>n</i> = 280 |                       | <i>n</i> = 43                    | <i>n</i> = 305 |                       |
| % Density                        | 25.96 (2.37)                          | 29.36 (1.33)   | 4.25 (0.04)           | 28.72 (2.69)                     | 28.86 (1.37)   | 0.00 (0.95)           |
| Dense area (cm <sup>2</sup> )    | 30.03 (3.05)                          | 31.64 (1.71)   | 1.95 (0.16)           | 31.91 (3.45)                     | 31.29 (1.76)   | 0.04 (0.84)           |
| Nondense area (cm <sup>2</sup> ) | 116.74 (6.44)                         | 106.66 (3.61)  | 3.28 (0.07)           | 112.97 (7.32)                    | 107.22 (3.73)  | 0.43 (0.51)           |
| Premenopausal women              | <i>n</i> = 40                         | <i>n</i> = 137 |                       | <i>n</i> = 24                    | <i>n</i> = 153 |                       |
| % Density                        | 25.69 (3.25)                          | 31.03 (2.02)   | 5.36 (0.02)           | 28.69 (3.76)                     | 30.35 (2.08)   | 0.05 (0.82)           |
| Dense area (cm <sup>2</sup> )    | 28.75 (4.26)                          | 32.61 (2.65)   | 2.89 (0.09)           | 32.37 (4.90)                     | 31.80 (2.71)   | 0.07 (0.79)           |
| Nondense area (cm <sup>2</sup> ) | 108.46 (7.64)                         | 95.57 (4.76)   | 3.62 (0.06)           | 101.73 (8.84)                    | 97.10 (4.89)   | 0.56 (0.45)           |
| Postmenopausal women             | <i>n</i> = 28                         | <i>n</i> = 143 |                       | <i>n</i> = 19                    | <i>n</i> = 152 |                       |
| % Density                        | 26.13 (3.47)                          | 27.40 (1.74)   | 0.36 (0.55)           | 28.54 (3.89)                     | 27.00 (1.81)   | 0.00 (0.95)           |
| Dense area (cm <sup>2</sup> )    | 31.00 (4.34)                          | 30.47 (2.17)   | 0.10 (0.75)           | 30.90 (4.86)                     | 30.46 (2.25)   | 0.00 (0.95)           |
| Nondense area (cm <sup>2</sup> ) | 123.05 (10.76)                        | 118.24 (5.39)  | 0.29 (0.59)           | 124.79 (12.05)                   | 117.74 (5.59)  | 0.02 (0.89)           |

NOTE: The least-squares means presented here are adjusted for age, ethnicity, and BMI. For all women, the means are also adjusted for menopausal status. The analyses were conducted using the transformed outcome variable, and the means presented are the least square-adjusted untransformed means.

in the analysis shown in Table 3. There were no significant associations between this polymorphism and any of the mammographic features.

**Associations of *GHI* Polymorphisms with Anthropometric Variables and the Growth Hormone/IGF-I Axis.** In a codominant model, adjusted for age, ethnicity, height, and menopausal status, we found that *GHI* -57G homozygotes weighed on average 4.5 kg more (*P* = 0.09) and had a 1.45 kg/m<sup>2</sup> greater BMI (5.6%) than *GHI* -57T homozygotes (see Table 4). These differences were more marked in postmenopausal women, in whom *GHI* -57G homozygotes weighed on average 8.1 kg (12%) more (*F* = 4.37; *P* = 0.01) and had a 2.7 kg/m<sup>2</sup> (10.8%) greater BMI (*F* = 3.87; *P* = 0.02) than T homozygotes (data not shown). None of these associations was statistically significant among premenopausal women. *GHI* -75G>A was not significantly associated with any measure of body size.

When the analysis was restricted to Caucasian women (data not shown) and adjusted for age, menopausal status, and height, weight was not significantly associated with *GHI* -57 (*P* = 0.08, *n* = 294); however, postmenopausal TT subjects weighed on average 7.6 kg less than GG subjects (*P* = 0.02), a relative difference of 10%, and were 2.5 kg/m<sup>2</sup> (9%) lower BMI (*P* = 0.03). *GHI* -75 was not associated with any other anthropometric measures among Caucasian women.

*GHI* -57G>T was not significantly associated with levels of growth hormone, IGF-I, or IGFBP-3 in any group. The *GHI* -75 SNP was associated with growth hormone levels in all women, which were 31.8% higher, after adjustment for age,

BMI, and ethnicity, in homozygotes for the A allele than in others (*P* = 0.02). If the waist measure was substituted for BMI (data not shown), the *GHI* -75 SNP was significantly related to growth hormone levels in all women (*F* = 6.01 *P* = 0.01), and at borderline significance, in premenopausal (*F* = 2.94, *P* = 0.09) and postmenopausal women (*F* = 3.18, *P* = 0.08). This SNP was not significantly associated with IGF-I or IGFBP-3 levels.

When the analysis was restricted to Caucasian women, *GHI* -75 was again significantly associated with levels of growth hormone (*F* = 4.20, *P* = 0.04, *n* = 278), in the same direction as shown in Table 4.

When adjusted for age, ethnicity, and BMI, *GHRHR* A57T polymorphism was significantly associated with IGF-1 levels (*F* = 3.98, *P* = 0.05) in premenopausal women and when adjusted for waist rather than BMI (*P* = 0.03). Subjects who had at least one copy of the A allele had 13.8 μL/L (9.3%) significantly higher adjusted mean levels of serum IGF-I. Among postmenopausal women, none of these hormones was associated with the SNP in *GHRHR*.

**Variance Explained.** In a model adjusted for age, ethnicity, menopausal status, and BMI, *GHI* -75G>A explained 1% (*P* = 0.04) of the variance in percent density in all women and 1% (*P* = 0.10) among Caucasian women. *GHI* -57G>T explained 1% (*P* = 0.04) of the variance in percent density in all women and 2% (*p* = 0.01) among Caucasian women. Together, *GHI* -57G>T and -75G>A explained 2% of the variance in percent density among all women ( $F_{340}^3 = 3.26$ , *P* = 0.02), 3% among premenopausal ( $F_{170}^3 = 2.76$ , *P* = 0.04), and 1% among

**Table 4. Association between genotypes and anthropometric measures and hormones of the growth hormone/IGF-I axis**

| Variable                  | <i>GHI</i> -57G>T Genotype, mean (SE) |                      |                     | <i>F</i> ( <i>P</i> ) | <i>GHI</i> -75G>A Genotype, mean (SE) |                      | <i>F</i> ( <i>P</i> ) |
|---------------------------|---------------------------------------|----------------------|---------------------|-----------------------|---------------------------------------|----------------------|-----------------------|
|                           | GG ( <i>n</i> = 138)                  | GT ( <i>n</i> = 153) | TT ( <i>n</i> = 57) |                       | GG/GA ( <i>n</i> = 68)                | AA ( <i>n</i> = 280) |                       |
| All women                 |                                       |                      |                     |                       |                                       |                      |                       |
| Height* (cm)              | 163.18 (0.68)                         | 162.31 (0.61)        | 161.73 (0.90)       | 1.17 (0.31)           | 161.58 (0.89)                         | 162.65 (0.50)        | 1.41 (0.24)           |
| Weight† (kg)              | 69.45 (1.63)                          | 65.77 (1.48)         | 64.94 (2.18)        | 2.44 (0.09)           | 65.49 (2.17)                          | 67.11 (1.21)         | 0.35 (0.56)           |
| BMI* (kg/m <sup>2</sup> ) | 25.92 (0.61)                          | 24.61 (0.55)         | 24.47 (0.81)        | 2.07 (0.13)           | 24.59 (0.81)                          | 25.10 (0.45)         | 0.22 (0.64)           |
| Waist/hip ratio           | 0.76 (0.01)                           | 0.77 (0.01)          | 0.77 (0.01)         | 1.14 (0.32)           | 0.76 (0.01)                           | 0.76 (0.004)         | 0.00 (0.99)           |
| Waist‡ (cm)               | 73.11 (0.58)                          | 73.83 (0.53)         | 74.10 (0.77)        | 0.69 (0.50)           | 72.93 (0.76)                          | 73.77 (0.43)         | 0.77 (0.38)           |
| All women                 |                                       |                      |                     |                       |                                       |                      |                       |
| Growth hormone (μg/L)     | 1.48 (0.28)                           | 1.74 (0.26)          | 1.86 (0.37)         | 0.68 (0.51)           | 1.32 (0.37)                           | 1.74 (0.21)          | 5.42 (0.02)           |
| IGF-I (μg/L)              | 140.46 (3.46)                         | 141.10 (3.11)        | 146.72 (4.59)       | 0.51 (0.60)           | 139.35 (4.53)                         | 142.45 (2.54)        | 0.56 (0.45)           |
| IGFBP-3 (mg/L)            | 2.68 (0.05)                           | 2.77 (0.05)          | 2.82 (0.07)         | 2.03 (0.13)           | 2.68 (0.07)                           | 2.76 (0.04)          | 1.29 (0.26)           |
| IGF-I/IGFBP-3             | 0.20 (0.004)                          | 0.19 (0.004)         | 0.20 (0.005)        | 0.87 (0.42)           | 0.19 (0.005)                          | 0.19 (0.003)         | 0.00 (0.98)           |
| Prolactin (μg/L)          | 13.30 (0.99)                          | 12.94 (0.89)         | 14.22 (1.31)        | 0.37 (0.69)           | 13.50 (1.29)                          | 13.27 (0.73)         | 0.09 (0.76)           |

\*Adjusted for age, ethnicity, and menopausal status in all women.

†Adjusted for age, ethnicity, menopausal status, and height.

‡Adjusted for age, ethnicity, menopausal status, and BMI.

postmenopausal ( $P = 0.50$ ). When restricted to Caucasians, the results were similar in magnitude and statistically significant for all women and premenopausal women but not for postmenopausal women.

**Polymorphisms and Mammographic Measures: Effect of adjustment for the Growth Hormone/IGF-I Axis.** Adjustment for the hormones of the growth hormone/IGF-I axis, growth hormone, IGF-I, IGFBP3, and prolactin had little effect on the associations between the *GH1* -57G>T and measures of density. In premenopausal women, the relationship between *GH1* -75G>A and measures of percent density and nondense area became less significant when adjusted for hormones in the growth hormone/IGF-I axis ( $P = 0.05$  and  $P = 0.10$ , respectively).

## Discussion

We have found associations between mammographic density and two SNPs located in the proximal promoter region of the *GH1* gene, which have recently been shown to be functional (20). These SNPs were in weak linkage disequilibrium, and although both were significantly associated with percent density and nondense area in all women, there were some differences in associations with other variables. *GH1* -57G>T and *GH1* -75G>A were significantly associated with mammographic density in all women, and *GH1* -75G>A was significantly associated with mammographic density in premenopausal women. *GH1* -75G>A was also associated with serum growth hormone levels, whereas *GH1* -57G>T had a borderline association with weight, which is associated with the nondense area of the mammogram. However, both SNPs remained associated with percent density and the nondense area of the mammogram after adjustment for BMI or waist; thus, the associations of genotype with breast tissue characteristics are not simply the result of associations with body size or body fat. The results suggest that the *GH1* genotype influences breast tissue composition independently of an effect on body size. Similar results were seen when the analyses were restricted to Caucasian women, which suggests that ethnicity may not be a significant confounder here.

The association of the *GH1* -75G>A with percent density also persisted after adjustment for hormones of the growth hormone/IGF-I axis. However, 30% of the subjects in this study had undetectable levels of growth hormone. Due to the difficulty in measuring growth hormone levels, we cannot be certain whether or not the association between mammographic density and *GH1* -75 is the result of the effects of this SNP on serum levels of growth hormone. However, subsequent analysis of urinary growth hormone levels in a sample of study subjects found that those subjects with undetectable serum growth hormone levels actually had low levels of urinary growth hormone. In addition, the polymorphisms in *GH1* were associated with peak growth hormone levels in provocative tests and with height in children and adults (11). Furthermore, polymorphisms in *GHRHR* were associated with differences in the sensitivity of *GHRHR* to stimulation by *GHRH* (13). There have been no previous studies of the association of polymorphisms in these genes and mammographic density.

Recently, *GH1* -57G>T and *GH1* -75G>A were determined to be functional. *GH1* -75 is located in a binding region of the proximal pituitary-specific transcription factor, Pit-1, and *GH1* -57 is located in a binding region for the vitamin D receptor (12, 20). Electrophoretic mobility shift assays showed that these SNPs, and three other SNPs in the proximal promoter region of *GH1*, showed allele-specific protein binding (20). Furthermore, functional tests have identified haplotypes in the *GH1* promoter region that were associated with either significantly reduced or elevated level of reporter gene expression.

Haplotype partitioning identified six SNPs, including *GH1* -57G>T, as major determinants of *GH1* gene expression, and *GH1* -75G>A was marginally important in determining *GH1* gene expression (20).

A study conducted among Japanese adults and children found that *GH1* -57G subjects had lower peak growth hormone levels in provocative tests, lower IGF-I levels, and were shorter (11). We found *GH1* -57G subjects to have significantly increased nondense area, which would be expected if these subjects had lower growth hormone levels. However, other studies found no association with growth hormone levels and these SNPs (12, 21). A recent population-based case-control study found that *GH1* -75AA was significantly associated with greater height in Chinese women (22). We found a similar, although nonsignificant, association with height in our study, as well as significantly higher growth hormone levels in the same subjects. However, Ren et al. did not find *GH1* -57G>T or *GH1* -75G>A to be associated with risk of breast cancer (22). This study did not include the six SNPs that have been identified as major determinants of *GH1* gene expression (20). In our study, *GH1* -57G and -75A allele frequencies in Caucasians were similar to other Caucasian populations (12) and were 0.65 and 0.86, whereas the same allele frequencies were 0.40 and 0.96 in Chinese women (22). Because these SNPs are important for *GH1* gene expression, variation in allele frequencies between populations may account for some interpopulation variation in heritable traits that are influenced by growth hormone, such as mammographic density and height, both of which are risk factors for breast cancer. The genetic basis of interindividual differences in *GH1* expression is extremely complex depending on variation in the proximal promoter region and an upstream locus control region (20). The evidence would suggest that the associations we observed in our study may be due to the functional nature of the SNPs measured; however, it is still possible that these results are due to linkage disequilibrium between the SNPs we measured and other causal SNPs.

Growth hormone secreted by the pituitary has both direct and indirect effects on breast cell proliferation (23, 24). Direct actions are mediated through growth hormone receptors present in the breast epithelium and stroma, as well as through prolactin receptors (25). The administration of growth hormone to aging female monkeys has been shown to induce hyperplasia of the mammary epithelium, with a striking increase in the number and cellularity of lobules, and an increase in the mitotic labeling index (26). This effect was seen whether growth hormone was administered alone or in combination with IGF-I and was unrelated to changes in the blood levels of estradiol and progesterone (26).

Indirect actions of growth hormone on the breast may be mediated through effects on the production of IGF-I in the liver and other organs, including the breast (27). IGF-I is a potent mitogen to breast epithelial cells that can function as an autocrine and paracrine growth factor, as well as a hormone. IGF-I is thought to play a key role in the growth and development of terminal end buds in the breast and to mediate in part the actions of growth hormone on normal mammary gland development at the time of puberty (28, 29).

In addition, the growth hormone gene, identical to the pituitary growth hormone gene studied here, is expressed in normal and neoplastic human breast tissue, in both epithelium and stroma (23, 30), and the overexpression of growth hormone in transgenic mice is associated with an increased incidence of breast cancer (31).

The association of growth hormone with risk of breast cancer has not been examined directly in any prospective study to date. However, IGF-I, which is produced in response to growth hormone, has been associated with risk of breast cancer and thus provides indirect evidence for an effect of



growth hormone on risk. Four case-control studies and three cohort studies have reported associations between higher levels of IGF-I and risk of breast cancer (32-37), whereas other studies found no associations (38, 39).

In the same subjects, as are studied in the present article, we have found an association between the growth hormone/IGF-I axis and mammographic density. Before adjustment for other risk factors, IGF-I and growth hormone were both directly associated with percent mammographic density in premenopausal women. In postmenopausal women, IGF-I, growth hormone, and prolactin, were associated directly with percent density. After adjustment for the waist measurement, only IGF-I in the premenopausal and prolactin in the postmenopausal remained significantly associated with percent density (7).

These results in part confirm the findings of a cross-sectional study in the Nurses Health Study by Byrne et al. that showed that blood levels of IGF-I were correlated positively with percent mammographic density in premenopausal but not in postmenopausal women (40). We have also found in previous work, using immunohistochemistry in breast biopsies, that the area of tissue stained for IGF-I is associated with mammographic density in women under the age of 50 but not in older women (41).

Twin studies have shown that both mammographic density and circulating levels of IGF-I are strongly heritable (6, 42). The present results suggest that inherited polymorphisms in the genes that influence growth hormone production may, at least in part, explain these effects and may be associated with the differences in risk of breast cancer that are associated with both of these factors. These findings should be replicated with improved measurement of growth hormone levels and an extended examination of the genetic variation in *GH1* gene.

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## Pituitary Growth Hormone and Growth Hormone–Releasing Hormone Receptor Genes and Associations with Mammographic Measures and Serum Growth Hormone

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