Genetic variation and circulating levels of IGF-I and IGFBP-3 in relation to risk of proliferative benign breast disease

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Insulin-like growth factor-I (IGF-I) and its major binding protein IGFBP-3 have been implicated in breast carcinogenesis. We examined the associations between genetic variants and circulating levels of IGF-I and IGFBP-3 with proliferative benign breast disease (BBD), a marker of increased breast cancer risk, in the Nurses' Health Study II (NHSII). Participants were 359 pathology-confirmed proliferative BBD cases and 359 matched controls. Circulating IGF-I and IGFBP-3 levels were measured in blood samples collected between 1996 and 1999. Thirty single nucleotide polymorphisms (SNPs) in IGF-I, IGFBP-1, and IGFBP-3 genes were selected using a haplotype tagging approach and genotyped in cases and controls. Circulating IGF-I levels were not associated with proliferative BBD risk. Higher circulating IGFBP-3 levels were significantly associated with increased risk of proliferative BBD (highest *vs.* lowest quartile odds ratio (OR) [95% confidence interval (CI)], 1.70 (1.06–2.72); p-trend = 0.03). The minor alleles of 2 IGFBP-3 SNPs were associated with lower proliferative BBD risk (homozygous variant *vs.* homozygous wild-type OR (95% CI): rs3110697: 0.6 (0.4–0.9), p-trend = 0.02; rs2132570: 0.2 (0.1–0.6), p-trend = 0.02). Three other IGFBP-3 SNPs (rs2854744, rs2960436 and rs2854746) were significantly associated with circulating IGFBP-3 levels ($p < 0.01$). Although these SNPs were not significantly associated with proliferative BBD risk, there was suggestive evidence that the alleles associated with higher circulating IGFBP-3 levels were also associated with higher risk of proliferative BBD. These results suggest that genetic variants and circulating levels of IGFBP-3 may play a role in the early stage of breast carcinogenesis.

Key words: IGF-I, IGFBP-3, circulating levels, genetic variation, proliferative BBD

Abbreviations: AH: atypical hyperplasia; BBD: benign breast disease; BMI: body mass index; BPC3: Breast and Prostate Cancer Cohort Consortium; CI: confidence interval; CV: coefficient of variation; htSNP: haplotype tagging SNP; IGF-1: insulin-like growth factor-1; IGFBP-3: insulin-like growth factor binding protein 3; LD: linkage disequilibrium; MEC: Multiethnic Cohort; NHSII: Nurses' Health Study II; OCs: oral contraceptives; OR: odds ratio; SNPs: single nucleotide polymorphisms

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Breast cancer is the most commonly diagnosed cancer and the second leading cause of cancer deaths among women in the United States.[1] Benign breast disease (BBD) comprises a multiplicity of component histologic subtypes among which proliferative BBD is a marker of increased breast cancer risk and may even be in the pathway for a subset of breast cancers.[2] Women whose biopsies show proliferative changes without atypia have a 1.3–1.9-fold greater risk of subsequent breast cancer than women with nonproliferative lesions, and women with atypical hyperplasia (AH) have a 3.9–13-fold greater risk.[2]

Insulin-like growth factor I (IGF-I) is a polypeptide growth hormone that promotes proliferation of normal breast epithelial cells.[3–5] The vast majority of circulating IGF-I is bound to IGF binding proteins, in particular IGFBP-3, in conjunction with an acid-labile subunit.[6] IGF-I modulates cellular transformation and mammary carcinogenesis in animal studies.[7,8] By sequestering IGF-I, higher circulating IGFBP-3 levels were originally hypothesized to be protective against breast cancer.[9] However, epidemiological evidence of higher IGFBP-3 levels associated with an increased breast

cancer risk suggests that it may exert dual regulatory effects on IGF-I action and other IGF-I independent effects.[9]

The relations between circulating levels of IGF-I and IGFBP-3 and breast cancer risk have been inconsistent across studies, withpositive associations observed for IGF-I among premenopausal but not postmenopausal women in earlier studies.[10] Although several IGF-I and IGFBP-3 single nucleotide polymorphisms (SNPs) were associated with the corresponding biomarker levels,[11–19] results between IGF-I and IGFBP-3 genetic variants and breast cancer risk have largely been inconsistent.[11,12,15,19–23]

To our knowledge, the associations between genetic polymorphisms and circulating levels of IGF-I and IGFBP-3 and risk of proliferative BBD have not been evaluated in previous literature. Given the role of IGF-I and IGFBP-3 in cell proliferation and cellular transformation and the increased risk of breast cancer associated with proliferative BBD, we hypothesized that higher circulating levels and genetic variation of IGF-I and IGFBP-3 would be associated with an increased risk of proliferative BBD. We tested this hypothesis in a nested case-control study in predominantly premenopausal women in the Nurses' Health Study II (NHSII). We further examined the relations among IGF-I, IGFBP-1 and IGFBP-3 SNPs and circulating hormone levels of IGF-I and IGFBP-3.

Material and Methods

Study population

The NHSII is an ongoing cohort study that was initiated in 1989, when 116,678 U.S. female registered nurses aged 25–42 years completed a mailed, self-administered questionnaire, including information on a variety of health-related exposures and conditions. The cohort has been followed up every 2 years since 1989.

In 1996–1999, blood samples were collected from 29,611 NHSII participants aged 32–54 at blood draw. Some 18,521 premenopausal women, who had not taken any type of hormones, been pregnant, or breast-fed in the previous 6 months, provided follicular and luteal phase, hereafter called timed blood samples. Women who were not eligible (*i.e.*, perimenopausal, postmenopausal, had a simple hysterectomy, or currently used oral contraceptives (OCs) or other hormones) or who declined to give timed blood samples provided a single untimed blood sample ($n = 11{,}090$). For women who gave both follicular and luteal samples, luteal samples were used in this study, because menstrual cycle variations of IGF are only modest.[24,25]

Follicular plasma was aliquoted by the participant 8–24 hr after collection and frozen. Luteal and untimed samples were shipped *via* overnight courier with an ice-pack to our laboratory where the samples were processed. Whole blood samples were centrifuged and plasma, buffy and red blood cells aliquoted into labeled cryotubes. All samples are stored in the vapor phase of liquid nitrogen freezers (temperature $\leq -130^{\circ}C$), which have alarms and are monitored 24 hr a day. The blood cohort is very similar to the overall NHSII cohort, except that more women in the blood cohort reported having a family history of breast cancer (19% *vs.* 15%). The current study was nested within this subcohort of women who returned a blood sample.

BBD case identification, pathologic classification and control selection

On the 1989 baseline questionnaire and each subsequent biennial questionnaire, all women were asked whether they had ever received a physician diagnosis of fibrocystic or other benign breast disease and whether the diagnosis was confirmed by biopsy or aspiration. Women who reported a first diagnosis of biopsy-confirmed BBD were contacted to confirm the diagnosis and to acquire permission to review their pathology specimens. After permission was granted, benign breast biopsy slides were collected from hospital pathology departments and were coded and submitted to the study pathologists in a blinded fashion for review.

The pathologists reviewed slides independently using the classification of 3 broad categories of histology: nonproliferative, proliferative disease without atypia and atypical hyperplasia (ductal and lobular). Any slide identified as showing atypia or questionable atypia was jointly reviewed by 2 pathologists. Only those women whose benign breast biopsy slides were confirmed as proliferative BBD with or without atypia by the study pathologists were included as cases in this study.

For each case with a blood sample, 1 control subject was selected at random from among the blood cohort who had not reported on previous questionnaires a diagnosis of BBD at the time the case occurred, matching on year of birth, menopausal status (premenopausal, postmenopausal or uncertain) and fasting status ($\geq$8 hr *vs.* not). Cases and controls were further matched on month ($\pm$1 month) and time of day of blood collection due to the circadian variation in hormone levels. If cases were premenopausal and gave timed samples at blood draw, controls were matched to the case on day of luteal sample collection. Postmenopausal cases and controls were matched on current postmenopausal hormone use at blood collection (yes *vs.* no). Women with a prior history of cancer (except nonmelanoma skin cancer) were excluded. Because the majority of BBD is diagnosed through screening mammography, controls selected must have reported breast cancer screening (clinical breast exam or mammography) in the questionnaire cycle of diagnosis of their matched cases.

Among the 29,611 women who provided blood samples in 1996–1999, 730 had reported a first diagnosis of biopsy-confirmed BBD on the 1993, 1995 or 1997 questionnaires. Of the 729 women who were eligible (1 woman deceased), 665 (91% of those eligible) confirmed the BBD diagnosis and granted permission for review of their biopsy records and pathology specimens. Adequate pathology material was obtained from hospitals and reviewed for 621 women (93%

of those who gave permission), and 592 of these (95% of those reviewed) were confirmed by detailed histologic review to be valid BBD cases. Of these, 371 (63%) were classified as proliferative benign breast disease. An additional 12 women were excluded for various reasons, including not having enough plasma reserve, not having white blood cells, or having reported prior cancer before BBD diagnosis, 359 were included as final cases in the current analysis. Because the number of women with atypia was limited ($n = 32$), we did not consider this as a separate outcome in this analysis. The study was approved by the Committee on the Use of Human Subjects in Research at the Brigham and Women's Hospital and the Harvard School of Public Health.

Laboratory assays for circulating levels of IGF-I and IGFBP-3

IGF-I and IGFBP-3 were assayed by ELISA in the Departments of Medicine and Oncology at McGill University, using reagents from Diagnostic Systems Laboratory (Webster, TX). All samples were sent in 1 batch but were assayed in multiple runs in the lab. Cases and their matched controls were assayed in the same runs. Masked split 84 quality control plasma samples were included to calculate the coefficient of variation (CV). The lab assay personnel were blinded to case, control and quality control status. The intrabatch and interbatch CVs were 3.5 and 5.3% for IGF-I and 1.6 and 3.3% for IGFBP-3, respectively. Intraclass correlation coefficients (ICC) for hormone levels over 3 years were also calculated in premenopausal women in NHSII.[26] The ICCs were 0.83 (0.77–0.87) for IGF-I and 0.76 (0.70–0.82) for IGFBP-3 for luteal samples,[26] respectively, suggesting that a single sample is adequately representative of long-term hormone levels.

SNP selection and genotyping methods

We focused on tagging SNPs for common haplotypes occurring at a frequency of 5% or greater in IGF-I, IGFBP-1 and IGFBP-3 genes identified from a National Cancer Institute Breast and Prostate Cancer Cohort Consortium (BPC3) study. As part of the BPC3, SNP discovery and htSNP selection was conducted in the Multiethnic Cohort (MEC).[27,28] To identify missense SNPs not in the standard database, the coding exons in these 3 genes were resequenced in 95 aggressive prostate cancer and 95 advanced breast cancer cases from 5 major ethnic groups (Caucasian, Latino, Japanese, native Hawaiian, and African American, $n = 19$ per ethnic group).[29] To identify regions of high linkage disequilibrium (LD), 64 SNPs in IGF-I and 36 SNPs in IGFBP-1 and IGFBP-3 genes were genotyped in a panel of 349 cancer-free women from the MEC.[20,30] The D' statistic was used to determine pairwise linkage disequilibrium between SNPs.[31] Regions of strong LD (*i.e.*, haplotype blocks) were defined using criteria from Gabriel *et al.*[32] HtSNPs were selected for a Caucasian population using the program TAGSNPS (TagSNPs Program) and based on R^2_H, a measure of the correlation between observed haplotypes and those estimated by the tagging SNP genotypes.[33] Among Caucasians, 14 SNPs tag the common haplotype patterns in 4 haplotype blocks of high LD in the IGF-I gene with 2 common (>5%) haplotypes in block 1, 4 in block 2 and 6 in blocks 3 and 4, respectively, and 13 SNPs tag the common haplotypes in 3 blocks across IGFBP-1 and IGFBP-3 genes with 5 haplotypes in block 1, 4 in block 2 and 5 in block 3. Three additional IGFBP-3 SNPs (rs6670, rs2453839 and rs2960436) did not fall into these blocks.

These 30 SNPs were genotyped in the cases and controls on DNA extracted from buffy coat fractions using the Qiagen QIAmp Blood kit (Qiagen, Chatsworth, CA). The principal genotyping technique was Taqman SNP allelic discrimination in 384-well format on the ABI 7900HT and genotyping was done at the Harvard Genotyping Core. Case-control pairs were genotyped in the same runs and the genotyping personnel were blinded to case-control and quality control status.

Statistical analysis

To estimate the associations between circulating hormone levels and the ratio of circulating IGF-I to IGFBP-3 and proliferative BBD risk, conditional logistic regression models were used to calculate odds ratios (ORs) and corresponding 95% confidence intervals (CIs). The ratio of IGF-I to IGFBP-3 was calculated by dividing IGF-I by IGFBP-3 values. We adjusted for matching factors and potential risk factors of proliferative BBD [body mass index (BMI), first-degree family history of breast cancer, age at menarche, parity and age at first birth, alcohol consumption, OC use and height], defined on the questionnaire prior to the cycle when cases first reported BBD. Circulating IGF-I, IGFBP-3 and the ratio of IGF-I to IGFBP-3 values were categorized into quartiles based on the distribution of each variable among controls. Tests for trend were performed by calculating the Wald statistics using the medians of each quartile as a continuous variable in the model.

For genetic analysis, χ^2 tests were conducted to assess departures of the genotype distribution from Hardy-Weinberg equilibrium among controls. Conditional logistic regression was used to assess the associations between each individual htSNP and proliferative BBD risk under different assumptions of inheritance modes (additive, dominant and recessive), and test for trend was performed using additive models.

Generalized linear models were used to assess the associations between IGF-I, IGFBP-1 and IGFBP-3 SNPs and circulating IGF-I and IGFBP-3 levels among controls. All models were adjusted for matching factors [age (30–37, 38–40, 41–42, 43–45 or ≥46 years), menopausal status (premenopausal, postmenopausal or uncertain), fasting status (≥8 hr *vs.* not), date (≤06/1997, 07/1997–12/1997, 01/1998–09/1998 or ≥10/1998) and time of day of blood draw (1–7, 8, 9 or 10–24)]. Models with circulating IGF-I levels as the dependent variable were further adjusted for BMI (<21, 21–22.9, 23–24.9, 25–28.9, or ≥29 kg/m^2), family history of breast cancer (yes *vs.* no), parity and age at first birth (nulli-parous, parous and age at first birth <25 years, parous and age at first birth 25–

Table 1. Age and age-standardized means and percentages for selected characteristics according to quartiles of circulating igf-i and igfbp-3 levels among controls in the nurses' heath study II[1]

	IGF-I				IGFBP-3			
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
No. participants	89	90	90	90	89	90	89	90
Means								
Age at case diagnosis (years)	43.4	42.0	40.4	39.1	41.8	41.8	40.7	40.5
Age at blood draw (years)	46.2	44.5	42.6	41.6	44.5	44.2	42.9	43.3
BMI (kg/m^2)	28.6	25.4	24.1	23.6	26.4	24.4	24.9	25.0
Height (inches)	65.5	65.2	65.6	64.8	65.3	65.4	65.5	65.3
Alcohol consumption (g/d)	2.6	3.8	3.2	3.3	2.8	3.9	3.2	4.3
Age at menarche (years)	12.3	12.7	12.5	12.9	12.3	12.6	12.6	12.5
Parity[2]	2.2	2.3	2.6	2.0	2.3	2.2	2.3	2.2
Age at first birth (years)[2]	24.9	26.8	26.2	28.0	25.4	26.4	26.0	27.7
Percentages (%)								
Nulliparous	25.2	17.8	24.2	25.7	21.8	14.1	30.1	22.4
Age at first birth $\geq$ 30 years[2]	13.5	24.5	15.2	41.8	17.5	21.0	18.6	30.2
First-degree family history of breast cancer	7.2	9.9	5.3	1.7	7.6	9.6	2.0	3.6
Ever used oral contraceptive	84.9	82.8	84.2	84.7	80.7	83.7	88.7	78.2
Postmenopausal women	13.6	2.8	3.5	4.5	8.0	4.3	5.9	5.2

[1]Except for the data on mean age, all data shown are values at cycle prior to first report of BBD diagnosis and standardized to the age distribution at diagnosis. [2]Among parous women.

29 years or parous and age at first birth $\geq$30 years), height (63, 64–65, 66–67, $\geq$68 inches) and OC use (never, ever use $<$3 years, or ever use $\geq$3 years) defined at or prior to blood draw. Models with circulating IGFBP-3 levels as the dependent variable were additionally adjusted for parity and age at first birth, family history of breast cancer defined as previously, age at menarche ($\leq$11, 12, 13, $\geq$14 years) and alcohol intake (0, $>$0–$<$1.5, 1.5–$<$4.5, $\geq$4.5 grams/day). These covariates explained the largest amount of variability in the circulating levels of these hormones. For IGF-I levels, we used the log-transformed values to improve normality of the data.

Results

Distributions of selected characteristics of control participants are presented in Table 1, according to circulating IGF-I and IGFBP-3 levels. Age and BMI were inversely correlated with IGF-I values. Women with the highest circulating IGF-I levels were the youngest and leanest. Women with the highest circulating IGF levels were more likely to have an older age at first birth than women with lowest values. Mean height and age at menarche did not vary substantially across quartiles of circulating biomarker levels. There was some random variability for alcohol consumption, menopausal status, family history of breast cancer and ever OC use.

Cases and controls were identical for age at diagnosis [cases (n = 359): 41.3 years (SD = 4.4); controls (n = 359): 41.2 years (SD = 4.2)] (p for paired t-test = 0.86). The mean circulating IGF-I and IGFBP-3 values were not significantly different between cases and controls [log-transformed IGF-I: cases (n = 358): 5.4 ng/mL (SD = 0.4), controls (n = 359): 5.3 ng/mL (SD = 0.3), p = 0.32; IGFBP-3: cases (n = 358): 5205.3 ng/mL (SD = 853.5), controls (n = 358): 5153.4 ng/mL (SD = 807.2), p= 0.42; the p-values were from mixed-effects regression models]. Circulating IGF-I and IGFBP-3 were highly correlated (Spearman correlation: 0.57, $p <$ 0.0001 among controls). No significant association was observed between plasma IGF-I levels and proliferative BBD (Table 2). The OR comparing the highest quartile to the lowest quartile of circulating IGF-I was 1.17 (95% CI (0.69–1.99), p-trend = 0.40). No association was observed after additional adjustment for IGFBP-3 levels [highest *vs.* lowest quartile OR (95% CI): 0.85 (0.46–1.56), p-trend = 0.83]. Higher circulating IGFBP-3 levels were significantly associated with increased proliferative BBD risk (highest *vs.* lowest quartile OR (95% CI): 1.70 (1.06–2.72), p-trend = 0.03). The association remained after adjustment for circulating IGF-I levels [OR (95% CI): 1.81 (1.06–3.10), p-trend = 0.04]. No association was observed for the ratio of IGF-I to IGFBP-3 [highest *vs.* lowest quartile OR (95% CI): 1.23 (0.71–2.15), p-trend = 0.45]. Results were essentially the same when the analyses were restricted to premenopausal women at diagnosis (n = 318 cases/339 controls, data not shown).

For the genetic analysis, all genotype frequencies were in Hardy-Weinberg equilibrium among controls ($p >$ 0.05), with the exception of SNP IGF-I rs1520220 (p exact test = 0.03).

IGF-I tagging SNPs were not associated with proliferative BBD or circulating IGF-I levels (Table 3). The only

Table 2. Relative risk of proliferative benign breast disease by quartiles of circulating IGF-I, IGFBP-3, and IGF-I/IGFBP-3 ratio, 1993–1997

	Quartile 1	Quartile 2	Quartile 3	Quartile 4	P for trend[1]
IGF-I (ng/mL)[2]	135	186	237	304	
Cases/controls	89/89	84/90	90/90	95/90	
Model 1[3]	1.00 (ref)	0.93 (0.59–1.45)	1.00 (0.63–1.59)	1.06 (0.65–1.73)	0.70
Model 2[4]	1.00 (ref)	0.90 (0.55–1.46)	1.05 (0.64–1.72)	1.17 (0.69–1.99)	0.40
Model 3[5]	1.00 (ref)	0.76 (0.46–1.28)	0.85 (0.49–1.46)	0.85 (0.46–1.56)	0.83
IGFBP-3 (ng/mL)[2]	4211	4841	5386	6084	
Cases/controls	71/89	89/90	91/89	107/90	
Model 1[3]	1.00 (ref)	1.22 (0.81–1.85)	1.31 (0.85–2.01)	1.51 (0.97–2.34)	0.07
Model 2[4]	1.00 (ref)	1.29 (0.83–2.00)	1.34 (0.85–2.13)	1.70 (1.06–2.72)	0.03
Model 3[5]	1.00 (ref)	1.37 (0.87-2.17)	1.44 (0.87-2.37)	1.81 (1.06-3.10)	0.04
IGF-I/IGFBP-3[2]	0.03	0.04	0.05	0.06	
Cases/controls	85/89	88/89	89/90	96/90	
Model 1[3]	1.00 (ref)	1.04 (0.66–1.64)	1.05 (0.65–1.70)	1.16 (0.70–1.92)	0.57
Model 2[4]	1.00 (ref)	1.08 (0.66–1.75)	1.10 (0.65–1.86)	1.23 (0.71–2.15)	0.45

[1]Tests for trend were *p* values of the Wald statistics using the medians of each quartile of circulating IGF-I and IGFBP-3 levels and the ratio of IGF-I to IGFBP-3 as a continuous variable in the model. [2]Values are medians of each quartile of circulating IGF-I and IGFBP-3 levels and the ratio of IGF-I to IGFBP-3 among controls. [3]Conditional logistic regression model adjusted for matching factors. [4]Conditional logistic regression model adjusted for matching factors and additionally adjusted for BMI (<21, 21–22.9, 23–24.9, 25–28.9 or ≥ 29 kg/m^2), first-degree family history of breast cancer (yes *vs*. no), age at menarche (≤ 11, 12, 13 or ≥ 14 years), parity and age at first birth (nulli-parous, parous andage at first birth <25 years, parous and age at first birth 25–29 years, or parous and age at first birth ≥ 30 years), alcohol consumption (0, >0–<1.5, 1.5-<4.5 or ≥ 4.5 grams/day), oral contraceptive use (never, ever use ≤ 3 years or ever use >3 years) and height (≤ 63, 64–65, 66–67 or ≥ 68 inches). [5]For circulating IGF-I, adjusted for the same covariates in model 2 and additionally adjusted for circulating IGFBP-3; for circulating IGFBP-3, adjusted for the same covariates in model 2 and additionally adjusted for circulating IGF-I.

significant IGF-I SNP was SNP rs4764695, with the minor allele associated with lower circulating IGFBP-3 levels (p-trend = 0.02).

Two IGFBP-3 SNPs were significantly associated with proliferative BBD risk [homozygous variant *vs*. homozygous wild-type OR (95% CI): IGFBP-3 rs3110697: 0.6 (0.4–0.9), p-trend = 0.02; IGFBP-3 rs2132570: 0.2 (0.1–0.6), p-trend = 0.02] (Table 4). These 2 SNPs were in the same haplotype block and in strong LD (D' = 0.94). The results were more consistent with a recessive mode of inheritance for both SNPs (p recessive = 0.01). These 2 SNPs were also significantly associated with circulating IGF-I levels (p-trend < 0.01), but not with circulating IGFBP-3 levels (p-trend = 0.40 and 0.84, respectively).

Significant associations were observed between several IGFBP-1 and IGFBP-3 SNPs and circulating hormone levels. Minor alleles of 3 IGFBP-3 SNPs, the most extensively studied SNP rs2854744 and SNPs rs2960436 and rs2854746, were significantly associated with higher circulating IGFBP-3 levels (p-trend < 0.01). All 3 SNPs were in strong LD (pairwise D' = 0.98–0.99) and SNPs IGFBP-3 rs2854744 and rs2854746 were in the same haplotype block. Two SNPs were also marginally associated with proliferative BBD risk [homozygous variant *vs*. homozygous wild-type OR (95% CI): IGFBP-3 rs2854746: 1.8 (1.1–3.0), p-trend = 0.07; IGFBP-3 rs2960436: 1.4 (0.9–2.2), p-trend = 0.09]. Results of BBD risk for these 2 SNPs became essentially null after adjustment for circulating IGFBP-3 (data not shown). The minor allele of SNP IGFBP-3 rs2854746 was associated with lower circulating IGF-I levels (p-trend = 0.02), whereas the minor allele of SNP IGFBP-3 rs2270628 was associated with higher IGF-I levels (p-trend < 0.01). SNP IGFBP-1 rs2201638 was associated with both circulating IGF-I and IGFBP-3 levels (p-trend = 0.05 and 0.04, respectively).

For haplotype analysis,[34,35] common haplotypes were constructed using 30 haplotype tagging SNPs in IGF-I, IGFBP-1 and IGFBP-3 genes within regions of strong LD (Supplemental Table 1). Overall, no associations were observed between common haplotypes in these 3 genes and proliferative BBD risk (Supplemental Table 2).

Discussion

To our knowledge, this is the first study to examine the associations between circulating levels and common genetic variation of IGF-I, IGFBP-1 and IGFBP-3 and proliferative BBD risk. Circulating IGFBP-3 levels were positively associated and 2 IGFBP-3 SNPs were inversely associated with proliferative BBD risk. Three other IGFBP-3 SNPs were strongly associated with circulating IGFBP-3 levels. IGF-I circulating levels and SNPs were not associated with proliferative BBD. Overall, no associations were observed between IGF-I and IGFBP-1 SNPs and circulating IGF-I or IGFBP-3 levels.

Circulating IGF-I levels were not associated with proliferative BBD in this study or with breast cancer in a previous

Table 3. Associations between IGF-I SNPS and proliferative benign breast disease risk and circulating IGF-I and IGFBP-3 levels in NHSII

SNP rs no.	Geno type	Cases ($N = 359$)	Controls ($N = 359$)	BBD risk[1] OR (95% CI)	p for trend[4]	IGF-I level (ng/mL)[2]	p for trend[4]	IGFBP-3 level (ng/mL)[3]	p for trend[4]
rs12821878	GG	203	194	1.0 (ref)	0.60	208	0.62	5154	0.91
	GA	126	121	1.1 (0.8–1.5)		202		5109	
	AA	17	26	0.7 (0.3–1.3)		210		5252	
rs1019731	CC	256	262	1.0 (ref)	0.26	205	0.65	5120	0.73
	CA	85	72	1.3 (0.9–1.9)		211		5203	
	AA	6	7	0.9 (0.3–2.8)		201		4948	
rs2195239	CC	203	209	1.0 (ref)	0.75	208	0.59	5198	0.28
	CG	132	118	1.2 (0.8–1.6)		199		5009	
	GG	15	18	0.8 (0.4–1.7)		217		5303	
rs10735380	AA	188	186	1.0 (ref)	0.67	210	0.61	5173	0.99
	AG	138	145	0.9 (0.7–1.3)		201		5065	
	GG	26	18	1.4 (0.7–2.8)		221		5462	
rs2373722	GG	302	300	1.0 (ref)	0.83	205	0.28	5114	0.16
	GA	38	42	0.9 (0.6–1.4)		211		5242	
	AA	3	1	2.0 (0.2–22.1)		333		6422	
rs1549593	GG	257	269	1.0 (ref)	0.18	204	0.99	5135	0.45
	GT	82	70	1.2 (0.8–1.7)		210		5104	
	TT	7	4	1.8 (0.5–6.2)		158		4659	
rs1520220	CC	235	236	1.0 (ref)	0.52	206	0.75	5153	0.73
	CG	105	107	1.0 (0.7–1.3)		204		5086	
	GG	11	4	2.5 (0.8–7.9)		256		5508	
rs7965399	TT	321	322	1.0 (ref)	0.29	205	0.42	5123	0.41
	TC	31	25	1.3 (0.7–2.2)		215		5260	
	CC	1	0	NE		NE		NE	
rs35767	GG	239	234	1.0 (ref)	0.94	203	0.27	5108	0.38
	GA	92	95	1.0 (0.7–1.5)		207		5188	
	AA	9	10	0.9 (0.3–2.4)		227		5231	
rs5742665	CC	266	261	1.0 (ref)	0.65	206	0.50	5157	0.52
	CG	76	76	1.0 (0.7–1.4)		207		5062	
	GG	7	9	0.8 (0.3–2.1)		226		5186	
rs2946834	GG	155	167	1.0 (ref)	0.64	203	0.52	5119	0.62
	GA	166	147	1.1 (0.8–1.6)		209		5133	
	AA	28	29	1.0 (0.6–1.7)		207		5215	
rs4764876	GG	180	200	1.0 (ref)	0.13	205	0.69	5178	0.55
	GC	148	130	1.3 (0.9–1.7)		209		5112	
	CC	24	20	1.3 (0.7–2.4)		206		5141	
rs4764695	AA	77	89	1.0 (ref)	0.24	210	0.16	5331	0.02
	AG	179	163	1.4 (1.0–2.0)		204		5043	
	GG	86	79	1.3 (0.8–2.0)		197		5040	
rs1996656	AA	232	236	1.0 (ref)	0.50	209	0.09	5194	0.06
	AG	107	102	1.1 (0.8–1.5)		201		5027	
	GG	10	8	1.3 (0.5–3.3)		180		4949	

[1]Conditional logistic regression adjusted for matching factors. [2]Generalized linear models adjusted for matching factors: age, menopausal status, fasting status, date and time of day of blood draw and additionally adjusted for BMI, family history of breast cancer, parity and age at first birth, height and OC use. [3]Generalized linear models adjusted for matching factors: age, menopausal status, fasting status, date and time of day of blood draw and additionally adjusted for parity and age at first birth, family history of breast cancer, age at menarche and alcohol intake.
[4]Tests for trend are p values from the additive models.
Note: NE: not estimable.

Table 4. Associations between IGFBP-1 and IGFBP-3 SNPS and proliferative benign breast disease risk and circulating IGF-I and IGFBP-3 levels in NHSII

SNP rs no.	Geno type	Cases ($N = 359$)	Controls ($N = 359$)	BBD risk[1] OR (95% CI)	p for trend[4]	IGF-I level (ng/mL)[2]	p for trend[4]	IGFBP-3 level (ng/mL)[3]	p for trend[4]
IGFBP-1									
rs4619	AA	137	153	1.0 (ref)	0.07	201	0.23	5172	0.55
	AG	157	152	1.1 (0.8–1.5)		207		5124	
	GG	54	39	1.6 (1.0–2.7)		213		5105	
rs2201638	GG	331	318	1.0 (ref)	0.30	207	0.05	5154	0.04
	GA	18	24	0.8 (0.4–1.5)		185		4743	
	AA	1	2	NE		174		5134	
rs1065780	GG	137	139	1.0 (ref)	0.60	199	0.19	5115	0.66
	GA	150	158	1.0 (0.7–1.3)		211		5164	
	AA	64	52	1.2 (0.8–1.9)		207		5155	
rs1553009	GG	218	212	1.0 (ref)	0.67	210	0.20	5122	0.78
	GA	113	105	1.0 (0.7–1.4)		202		5195	
	AA	14	11	1.3 (0.6–3.0)		197		4981	
rs35539615	CC	195	193	1.0 (ref)	0.75	205	0.79	5214	0.11
	CG	132	121	1.1 (0.8–1.5)		206		5012	
	GG	21	26	0.8 (0.4–1.4)		208		5121	
rs1908751	CC	165	162	1.0 (ref)	0.50	211	0.10	5210	0.15
	CT	152	159	0.9 (0.7–1.3)		205		5109	
	TT	30	33	0.9 (0.5–1.5)		193		5023	
rs4988515	CC	280	289	1.0 (ref)	0.07	202	0.41	5132	0.32
	CT	32	17	1.9 (1.0–3.7)		220		5471	
	TT	0	1	NE		173		4132	
rs10228265	AA	177	157	1.0 (ref)	0.64	201	0.15	5111	0.93
	AG	139	158	0.8 (0.6–1.1)		209		5187	
	GG	37	30	1.1 (0.7–1.9)		214		4990	
IGFBP-3									
rs2270628	CC	213	222	1.0 (ref)	0.51	199	<0.01	5113	0.60
	CT	112	109	1.0 (0.8–1.5)		217		5174	
	TT	19	15	1.3 (0.6–2.7)		221		5141	
rs6670	TT	222	230	1.0 (ref)	0.30	209	0.42	5150	0.99
	TA	110	103	1.1 (0.8–1.6)		199		5072	
	AA	21	16	1.3 (0.7–2.6)		213		5356	
rs2453839	TT	229	216	1.0 (ref)	0.54	211	0.04	5175	0.22
	TC	109	120	0.9 (0.7–1.2)		199		5063	
	CC	14	14	0.9 (0.4–2.1)		189		5044	
rs3110697	GG	115	103	1.0 (ref)	0.02	195	<0.01	5199	0.40
	GA	178	166	0.9 (0.6–1.3)		206		5115	
	AA	53	78	0.6 (0.4–0.9)		222		5104	
rs2132570	GG	219	195	1.0 (ref)	0.02	198	<0.01	5139	0.84
	GT	128	126	0.8 (0.6–1.2)		208		5056	
	TT	6	20	0.2 (0.1–0.6)		244		5388	
rs2960436	GG	95	111	1.0 (ref)	0.09	212	0.10	4954	<0.01
	GA	179	174	1.2 (0.9–1.7)		204		5192	
	AA	80	64	1.4 (0.9–2.2)		196		5341	

Table 4. Associations between IGFBP-1 and IGFBP-3 SNPS and proliferative benign breast disease risk and circulating IGF-I and IGFBP-3 ?levels in NHSII (continued)

SNP rs no.	Geno type	Cases ($N = 359$)	Controls ($N = 359$)	BBD risk[1] OR (95% CI)	p for trend[4]	IGF-I level (ng/mL)[2]	p for trend[4]	IGFBP-3 level (ng/mL)[3]	p for trend[4]
rs2854744	GG	89	103	1.0 (ref)	0.17	208	0.35	4934	<0.01
	GT	177	181	1.1 (0.8–1.5)		207		5185	
	TT	78	64	1.4 (0.9–2.2)		199		5340	
rs2854746	GG	118	127	1.0 (ref)	0.07	215	0.02	4995	<0.01
	GC	165	168	1.1 (0.8–1.5)		203		5174	
	CC	56	38	1.8 (1.1–3.0)		193		5449	

[1]Conditional logistic regression adjusted for matching factors. [2]Generalized linear models adjusted for matching factors: age, menopausal status, fasting status, date and time of day of blood draw, and additionally adjusted for BMI, family history of breast cancer, parity and age at first birth, height, and OC use. [3]Generalized linear models adjusted for matching factors: age, menopausal status, fasting status, date and time of day of blood draw, and additionally adjusted for parity and age at first birth, family history of breast cancer, age at menarche, and alcohol intake. [4]Tests for trend are p values from the additive models.
Note: NE: not estimable.

case-control study nested in the same NHSII cohort.[36] Earlier studies on circulating IGF-I levels and breast cancer risk reported positive associations among premenopausal, but not postmenopausal women.[10] More recently, 2 large prospective studies reported significant positive associations for both circulating IGF-I and IGFBP-3 concentrations with breast cancer risk among older women (age at diagnosis after age 50[37] or 60[38]), but no evidence of increased risk in the younger group.[37,38] No plausible explanations have been proposed for these discrepant results. Given the role of IGF-I in cell proliferation, cellular transformation, and mammary carcinogenesis in animal models,[7,8] more large prospective studies should be conducted to clarify the role of circulating IGF-I in different stages of breast carcinogenesis, with standardized definitions of menopausal status and age group.

Our result of higher circulating IGFBP-3 levels associated with increased risk of proliferative BBD is inconsistent with an earlier finding of no association of this hormone with breast cancer in the previous prospective breast cancer case-control study in the NHSII.[36] Inconsistent results were observed between circulating IGFBP-3 levels and breast cancer risk. To date, 8 prospective studies have evaluated the association between IGFP-3 levels and breast cancer in premenopausal women; 4 reported positive associations, 3 found no association and a suggestive inverse association was found in one study.[39] A case-control study also observed a positive association among Chinese women.[39]

At first glance, the epidemiological association between higher IGFBP-3 levels and increased breast disease risk is paradoxical to the antiproliferative and proapoptotic activities of IGFBP-3 in breast cancer cells in *in vivo* and *in vitro* studies.[40] However, some biological evidence suggests that depending on the cellular environment, both the IGF-I-dependent and IGF-I-independent effects of IGFBP-3 can be either growth stimulatory or inhibitory.[40,41] In addition to preventing IGF-I from interacting with the type I IGF receptor (IGF-IR), the binding of IGFBP-3 to IGF-I also prolongs the half-life of IGF-I and increases the IGF-I bioavailability to local tissues.[9] Further, IGFBP-3 stimulated the IGF-I-mediated DNA synthesis and IGF-I binding in MCF-7 breast cancer cell lines.[42]

Accumulating evidence suggests that IGFBP-3 could be an intrinsic growth promoter of breast epithelial cells, depending on the cell type (normal *vs.* malignant) involved, the cytokine milieu and the interactions with extra-cellular matrix. In the relatively normal MCF-10A breast epithelial cell line, in an IGF-independent manner, IGFBP-3 inhibited cell growth at low doses but promoted growth at higher concentrations, in contrast to its effects in malignant breast cells.[43,44] Likewise, IGFBP-3 acted as a potent survival factor against ceramide-induced cell death in the normal breast cell line, but enhanced apoptosis induced by ceramide in the breast cancer cells.[43,44] IGFBP-3 has also been reported to enhance epidermal growth factor (EGF)-induced proliferation in the normal breast cell line[45] and breast cancer cell line.[46]

Alternatively, higher circulating IGFBP-3 levels, if they reflect higher expression in tissue, may represent an attempt at homeostatic control of cells that are dividing too quickly. Or, this may also suggest the development of cellular resistance of benign breast tissues to the antiproliferative and proapoptotic effects of IGFBP-3, an explanation which has been hypothesized to explain the correlation between high local expression of IGFBP-3 and poor breast cancer prognosis.[46,47] The observed positive associations between circulating IGFBP-3 and proliferative BBD in this study and breast cancer risk in other epidemiological studies at the population level should motivate more research to better understand the molecular physiology of this protein.

In the genetic analysis, the minor alleles of 2 IGFBP-3 SNPs (rs3110697 and rs2132570) were associated with significantly lower risk of proliferative BBD. These 2 SNPs were not, however, associated with breast cancer risk in previous

studies.[15,20] These SNPs were associated with higher circulating IGF-I levels in this study, and results for SNP rs3110697 are consistent with other studies.[14,15] SNP rs2132570 was not associated with circulating IGF-I levels in the BPC3 study.[15] Previous studies have also reported minor alleles of both SNPs associated with lower circulating IGFBP-3 levels.[13–15] We did observe the minor allele of SNP rs3110697 associated with nonsignificant lower IGFBP-3 levels. Because IGFBP-3 is the major binding protein of circulating IGF-I, binding more than 90% of IGF-I in conjunction with the acid-labile subunit,[6] it is possible that IGFBP-3 SNPs may increase circulating IGF-I levels *via* decreasing IGFBP-3 levels. The lack of significance between IGFBP-3 SNPs and circulating IGFBP-3 could be due in part to the smaller sample size and reduced power in this study. The results that the allele associated with lower proliferative BBD risk were also associated with lower circulating IGFBP-3 levels suggest that the effects of genetic variants may be mediated through their influence on circulating biomarker levels.

Consistent with results from previous studies,[11–17,19] strong associations were observed among 3 SNPs (rs2960436, rs2854744 and rs2854746) in the 5′ promoter region of IGFBP-3 gene and circulating IGFBP-3 levels. Because of the strong LD between SNPs in this region (pairwise D' $\geq$ 0.98 in this study), the functional polymorphism that affects circulating IGFBP-3 levels remains to be identified. SNP rs2854744, the extensively studied polymorphism located at position −202 from the transcription start site, has been hypothesized as a promising candidate given the evidence from both *in vitro*[48] and epidemiological studies.[15–17,19] Originally, Deal *et al.*[39] demonstrated functional differences between the 2 (C *vs.* A) alleles at this site through *in vivo* transient expression assays, indicating that this polymorphism may directly influence IGFBP-3 gene promoter activity.

Among the 3 IGFBP-3 SNPS associated with circulating IGFBP-3 levels, no significant association was observed between these SNPs and proliferative BBD risk. However, the minor alleles of 2 SNPs (rs2960436 and rs2854746) were marginally associated with increased risk of proliferative BBD. Studies examining these SNPs with breast cancer risk have found inconsistent results. For example, no association[15,19–21,23] and positive association with the C allele of SNP rs2854744[19] have been reported. These results suggest that genetic variation in IGFBP-3 may be important in the earlier breast carcinogenic process possibly through influencing circulating IGFBP-3 levels.

Epidemiologic studies examining polymorphisms in the 5′ promoter region of IGFBP-3 gene have consistently observed strong associations with circulating IGFBP-3 levels but essentially no association with breast cancer. Our results observed suggestive positive associations between 2 IGFBP-3 SNPs in this region and proliferative BBD risk. These findings indicate complex relations between genetic variants, circulating levels of gene products and disease risks. The results of this study suggest that IGFBP-3, but not IGF-I, may influence proliferative BBD risk. Although IGF-I may not be important in the earlier stage of breast carcinogenesis, IGFBP-3 may exert regulatory effects possibly *via* IGF-I independent pathways. Further, the relationship between SNPs and expression of IGFBP-3 in breast tissues is critical to understand the associations among SNPs, circulating hormone levels and disease risks. However, there is very little biologic information on this relationship in the literature and the circulating hormone levels may or may not be a valid proxy for the tissue levels. Taking into account the strong biological support, coupled with the consistent strong evidence of the associations between IGFBP-3 polymorphisms and circulating IGFBP-3 levels, future work should identify the functional SNP(s) in this region and clarify the roles of IGF-I and IGFBP-3 genetic variants and circulating and tissue biomarker levels at each stage in breast cancer development from normal breast tissue to proliferative BBD and then from BBD to breast cancer to further our understanding of breast carcinogenesis.

This study has several strengths and limitations. By restricting the study population to registered nurses and predominantly Caucasian women, the problem of confounding by ethnicity or population stratification is less likely in this study. Although cases included in this study are incident proliferative BBD cases who reported their first diagnosis of BBD before providing blood samples, it is unlikely that this will introduce bias for genetic analysis, because genotype is not affected by disease status, and proliferative BBD is unlikely to affect mortality.

A limitation of the current study is that blood samples were collected after the diagnosis of BBD. Reverse causation may be less of an issue for BBD than for breast cancer, given that there are no published data indicating that BBD influences circulating IGFBP-3 levels and that the biopsy represents full treatment for the disease. However, because a possible effect of the disease on biomarker concentrations cannot be ruled out and circulating levels may reflect benign tissue activity rather than predictors of disease, these results should be interpreted cautiously. For genetic analysis, SNPs examined in this study were chosen using a haplotype tagging SNP approach and not based on a priori biological understanding of the functions of the SNPs. Therefore, the significant SNPs found may not be the causal functional SNPs by themselves but may be because of their being in LD with other unknown functional polymorphisms. Alternatively, given the relatively small sample size and multiple comparisons made, some significant results could be chance findings. However, the associations between IGFBP-3 SNPs and circulating IGF-I and IGFBP-3 are consistent with results from previous studies[11–17,19] and significant even after correction for multiple comparisons using the more conservative Bonferroni method at the gene level.

In summary, we observed significant positive associations between circulating IGFBP-3 levels and inverse associations between 2 IGFBP-3 SNPs and proliferative BBD risk and

confirmed strong associations between several other IGFBP-3 SNPs and circulating IGFBP-3 levels among predominantly premenopausal Caucasian women. Although, the IGFBP-3 SNPs associated with BBD risk were not associated with circulating IGFBP-3 levels, and the SNPs associated with circulating levels were not associated with BBD risk, the directions of these associations were fairly consistent. The alleles associated with reduced BBD risk were associated with lower IGFBP-3 concentrations in the circulation, and the alleles associated with higher circulating IGFBP-3 levels were also associated with higher risk of proliferative BBD. We observed significant albeit modest relative risks of BBD associated with IGFBP-3 genotypes and proteins. Considering that women who have been diagnosed with proliferative BBD have an increased risk of breast cancer and the high proportion of women exposed to high circulating IGFBP-3 levels, the associations observed in this study are meaningful and provide new insights to our understanding of the role of the IGF pathway in breast cancer development and may have implications for prevention of this common malignancy.

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