

POLYMORPHIC VARIATION AT THE -202 LOCUS IN *IGFBP3*: INFLUENCE ON SERUM LEVELS OF INSULIN-LIKE GROWTH FACTORS, INTERACTION WITH PLASMA RETINOL AND VITAMIN D AND BREAST CANCER RISK

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Previous reports have suggested an association between circulating IGFBP-3 levels and the risk of premenopausal breast cancer, and a single nucleotide polymorphism (SNP) in the promoter region of IGFBP-3 (nucleotide -202) was shown to influence transcription. There is prior evidence that the action of antiproliferative agents such as retinoids and selective estrogen receptor modulators (SERMs) act in part by upregulating *IGFBP3* gene expression. We identified 677 women with incident breast cancer and 834 matched controls from the Nurses' Health Study (NHS) and genotyped them at the -202 locus. For 943 of these women, we had previously measured IGF-I and IGFBP-3 plasma levels, and for 861 of these subjects, plasma retinol levels were available. Age-adjusted mean circulating IGFBP-3 levels were highest in the individuals with the AA genotype and decreased significantly in a stepwise manner in the presence of 1 or 2 copies of the C allele (4,426 ng/ml, 4,060 ng/ml and 3,697 ng/ml, respectively). We found a positive relation between age-adjusted IGFBP-3 levels and plasma retinol (14% difference in IGFBP-3 in top vs. bottom tertiles of retinol, p for trend < 0.001; Spearman correlation coefficient $r = 0.25$), which was similar across genotypes at the -202 *IGFBP3* locus (interaction term, $F = 0.10$, $p = 0.91$). Breast cancer risk was not significantly related to genotype at the -202 locus in our prospective analyses. We confirmed a relation between the -202 *IGFBP3* polymorphism and IGFBP-3 serum levels and observed a positive correlation between circulating retinol levels and circulating IGFBP-3 levels, providing further evidence that retinoids may influence IGF physiology. Our data do not demonstrate a significant influence of this locus on breast cancer risk, but we cannot exclude a minor influence or an influence confined to subgroups.

Key words: insulin-like binding protein 3 (IGFBP3) polymorphism; breast cancer; retinol; vitamin D; epidemiology

Insulin-like growth factor binding protein-3 (IGFBP-3) is 1 of 6 currently identified IGFbps that, by binding IGF peptides, prolong their half-lives and maintain the reservoir of IGF. Insulin-like growth factors regulate cellular proliferation and differentiation,¹ thereby potentially controlling cancer growth. Evidence from observational studies for an association between IGFBP-3 and breast cancer risk is limited and inconsistent.²⁻⁶ There is increasing evidence, however, for a relatively strong, positive association between IGF-I and breast cancer risk among premenopausal women.^{3,4,6-8}

The gene for IGFBP-3 is located at 7p14-p12.⁹ Twin studies^{10,11} have shown about half of the interindividual variability in circulating IGF-I and IGFBP-3 levels to be genetically determined. The promoter region of *IGFBP3* has been previously examined for polymorphic variants in the gene, and 5 polymorphic loci were detected.^{12,13} Only one of these loci, the -202 locus, has been related to circulating IGFBP-3 levels in a previous report.¹² To date, 2 studies assessed the relation between this *IGFBP3* polymorphism and plasma IGF levels. The first¹² found a strong positive relation between genotype and circulating IGFBP-3 levels in men (highest IGFBP-3 levels among individuals with the AA

genotype, stepwise significantly declining with each additional copy of the C allele;¹² mean IGFBP-3 levels 4,390, 4,130, 3,840 ng/ml for the AA, AC and CC genotype, respectively), and in addition describes a positive correlation between circulating retinol and IGFBP-3 levels. The second study¹⁴ reported very similar findings for premenopausal women, where higher IGFBP-3 levels were associated with the AA genotype at a locus at the -202 position relative to the CAP site. Retinoic acid and vitamin D are known regulators of the IGF system and have been shown to upregulate IGFBP3 expression.¹⁵⁻¹⁷ The only population-based study to date supports these findings.¹⁸

We utilized a nested case-control design within the large, prospective Nurses' Health Study (NHS) cohort to investigate an association between a genotype at the -202 locus and breast cancer, for the first time to our knowledge in the literature. We further asked if variability in circulating IGF-I and IGFBP-3 levels might be related to polymorphic variants of the promoter region of the gene and whether we could replicate the previously reported correlation between retinol and IGFBP-3 levels in women.

MATERIAL AND METHODS

Study population

In 1976, 121,700 female registered nurses ages 30-55 years and living in 11 large U.S. states were enrolled in the Nurses' Health Study. Since baseline they have completed biennial mailed questionnaires that include items about their health status, medical history and known or suspected risk factors for cancer¹⁹ and heart disease.²⁰ Every 2 years, follow-up questionnaires have been sent to cohort members to update the information on potential risk factors and to identify newly diagnosed case subjects of cancer and other major medical events. Follow-up data are available for > 90% of the cohort. Incident breast cancers are identified by self-report and confirmed by medical record review. From 1989 to 1990, blood samples were collected from 32,826 women. Approximately 97% of the blood samples were returned within 26 hr of blood draw, immediately centrifuged, aliquoted into plasma, RBC and buffy coat fractions and stored in liquid nitrogen freezers. The

Grant sponsor: National Cancer Institute (NCI); Grant numbers: CA49449, CA87969; Grant sponsor: NCI Specialized Program of Research Excellence (SPORE) in breast cancer at the Channing Laboratory.

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follow-up has been >98% for this subcohort. Further details of the Nurses' Health Study are described elsewhere.^{21,22}

From all women with no cancer diagnosis (with the exception of nonmelanoma skin cancer) prior to blood collection, there were 728 cases of breast cancer reported anytime after blood collection up to June 1, 1996. All of those were confirmed by review of pathology report. In total, 621 were pathologically confirmed invasive breast cancers and 107 were *in situ* cancers. Two controls were matched to each premenopausal and postmenopausal case not using hormone replacement therapy at blood, and one control was matched to all other women on their year of birth, menopausal status at blood draw (postmenopausal vs. not), recent postmenopausal hormone (PMH) use defined as use within 3 months of blood collection vs. not, time of day of blood draw, month of blood collection for a particular year and fasting status at blood draw (≥ 10 hr since a meal vs. not). Samples were not available for 40 women, thus, there were 721 incident breast cancer cases and 884 matched controls available for these analyses. Our study was approved by the Committee on the Use of Human Subjects in Research at the Brigham and Women's Hospital.

We evaluated *IGFBP3* polymorphism genotype and breast cancer risk both overall and by menopausal status. Menopausal status was assessed at the time of breast cancer diagnosis for cases and their matched controls. We defined women as premenopausal if they reported having at least one natural menstrual cycle within the previous year or if they reported a surgical menopause without bilateral oophorectomy and were under age 48 years (if a nonsmoker) or under age 46 years (if a current smoker). These ages were defined based on the age when <10% of the cohort had gone through natural menopause. Women were categorized as postmenopausal if they reported having a natural menopause or a bilateral oophorectomy. Women who reported a hysterectomy with either one or both ovaries remaining were defined as postmenopausal if they were at least 56 years (if a nonsmoker) or 54 years (if a current smoker). Similarly, these ages were derived from when a natural menopause had occurred in 90% in the cohort. We excluded women with uncertain menopausal status from all menopause-specific analyses. We also separately examined women who were premenopausal and younger than 50 years of age at blood collection—a subgroup where we previously observed the strongest relation between IGF and breast cancer risk.⁷

Genotyping of the -202 single nucleotide polymorphism

A single polymorphism at position -202 relative to the CAP site was selected for further study because of prior evidence that genotype at this locus was related to circulating IGFBP-3 levels.^{12,14} In a subset, there was agreement between the previously described RFLP method,¹² Taqman, and direct sequencing methods.

From 1,605 eligible women, 94 were not genotyped because of low volume and problems with PCR amplification, which left DNAs from 677 breast cancer cases and 834 controls for genotyping. We employed the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) in 384-well format. The 5' nuclease assay (TaqMan[®]) was used to distinguish the 2 alleles of the A→C transition at position -202 in the *IGFBP3* gene. The *IGFBP3* PCR amplification was carried out on 5–20 ng DNA using 1× TaqMan[®] universal PCR master mix (No Amp-

TABLE I—AGE-ADJUSTED, MEAN IGFBP-3 LEVELS IN ALL SUBJECTS ACCORDING TO GENOTYPE AT NUCLEOTIDE -202 (MEAN ± SE)

Genotype (nt -202)	No. ¹	Mean levels of IGFBP-3 (ng/ml)	SE
AA	204	4,426	68.8
AC	464	4,060	45.8
CC	275	3,697	59.2

p for trend < 0.001

¹568 people less than the total study population (no IGFBP-3 levels available).

TABLE II—RELATIVE RISK OF BREAST CANCER BY POLYMORPHIC VARIATION IN *IGFBP3* AT THE -202 LOCUS¹

Genotype at the -202 locus	All women ¹ (677 cases, 834 controls)		Postmenopausal (536 cases, 687 controls)		Premenopausal, all ages ⁴ (82 cases, 85 controls)		Premenopausal, age 50 ⁴ (70 cases, 70 controls)	
	Simple RR (95% CI) ²	MV RR (95% CI) ³	Simple RR (95% CI) ²	MV RR (95% CI) ³	Simple RR (95% CI) ²	MV RR (95% CI) ³	Simple RR (95% CI) ²	MV RR (95% CI) ³
AA	0.97 (0.72–1.30)	0.94 (0.69–1.29)	1.01 (0.73–1.39)	0.94 (0.78–1.35)	1.06 (0.40–2.80)	0.93 (0.33–2.62)	0.87 (0.29–2.60)	0.67 (0.20–2.20)
AC	0.99 (0.77–1.26)	1.03 (0.80–1.33)	1.04 (0.79–1.35)	1.02 (0.68–1.31)	0.97 (0.46–2.02)	0.91 (0.40–2.07)	0.95 (0.43–2.14)	0.96 (0.37–2.46)
CC	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

¹Conditional logistic regression models were used in analyses among all women. Unconditional logistic regression, controlling for the matching factors (age, month and time of blood collection, fasting status) was used in all other analyses.²Simple RR adjusted for age and matching variables.³In the multivariate models (MV), covariates were categorized as follows: body mass index at age 18 (<21, 21–22.9, 23–24.9, >25 kg/m²); weight change from age 18 (<5 kg, 5–20 kg, >20 kg); menopausal status: age at menarche (<12, 12, 13, >14 years); parity/age at first birth (nulliparous, 1–2 children/age at first birth <25 years, 1–2 children/age at first birth >25 years, >2 children/<25 years, >2 children/>25 years); first-degree family history of breast cancer (in mother/sister); personal history of benign breast disease (yes/no); duration of postmenopausal hormone use; and age at menopause (continuous); for postmenopausal women only.⁴Numbers do not add up because women with uncertain menopause status were excluded.

TABLE III – AGE-ADJUSTED, MEAN IGFBP-3 LEVELS ACCORDING TO TERTILE OF RETINOL (LOWER TERTILE, MEDIAN = 313 MG/L; MIDDLE TERTILE, MEDIAN = 448 MG/L; UPPER TERTILE, MEDIAN = 622 MG/L)

Tertile of retinol	No.	Mean levels of IGFBP-3 (ng/ml)	SE
Lower	291	3,755	56.3
Middle	282	4,015	65.6
Upper	288	4,280	55.2

p for trend < 0.001

erase UNG), 900 nM forward (CACCTTGGTTCTTGACGACAA) and reverse (GGCGTGCAGCTCGAGACT) primers, 200 nM of the FAM-labeled probe (CTCGTGC^TCACGCC) and 200 nM of the VIC-labeled probe (TCCTCGTG^CCACG) in a 5 μ l reaction (the polymorphic base is shown underlined). Amplification conditions on an ABI 9700 dual plate thermal cycler (Applied Biosystems) were as follows: 1 cycle of 95°C for 10 min, followed by 50 cycles of 92°C for 15 sec and 58°C for 1 min. TaqMan[®] primers and probes were designed using the Primer Express[®] Oligo Design software v2.0 (ABI PRISM). Laboratory personnel who performed genotyping were blinded to case-control status. Furthermore, to validate genotype identification procedures, 10% of the total samples were blinded quality-control samples. For those, concordance was 100%.

IGF-I, IGFBP-3, vitamin D and retinol plasma levels

IGF-I and IGFBP-3 were assayed by ELISA with reagents from Diagnostic Systems Laboratory (Webster, TX). Measurement of retinol, 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D levels were performed by high-performance liquid chromatography. All samples were masked to case or control status. Masked split specimens included within each batch were used to calculate the coefficient of variation within batches; for IGF-I these ranged from 4.1–8.7% and for IGFBP-3 from 5.1–9.3%. Between-batch CVs were 15.6 for IGF-I and 19.4 for IGFBP-3, respectively. Within-batch CVs for retinol ranged from 7.7–19.5% and between-batch CVs from 16.0–25.8%. For 1,25-dihydroxyvitamin D and 25-hydroxyvitamin D, within-batch CVs ranged from 4.3–10.6% and between-batch CVs from 7.8–23.7%, respectively.

Statistical analyses

To assess significant departures of the genotype distributions from the Hardy-Weinberg equilibrium, we used the χ^2 test. We estimated the percent variation in the IGFBP-3 that can be explained by the -202 polymorphism alone or by the genotype and age, using a general linear regression model (ANOVA). Analysis of covariance models were fitted using PROC GLM of SAS to compare the age-adjusted levels of IGFBP-3 according to the genotype. We further assessed (age-adjusted) mean levels according to the genotype and tertile of plasma retinol, tertile of height or five groups of body mass index among cases and controls. We used both cases and controls for these analyses because results restricted to controls were similar although less precise, particularly in the stratified analyses. To evaluate the presence of an interaction between circulating retinol levels (in tertiles) and genotype, we tested if there were different regression coefficients for the 3 genotype groups, adding an interaction term into our general linear model.

Previous work suggested a modest association between current hormone replacement therapy (HRT) and plasma IGFBP-3 levels,²³ with even stronger associations between HRT and plasma IGF-I levels.^{23–25} In our own data set, mean plasma IGFBP-3 levels did not significantly vary according to postmenopausal hormone use, and we therefore did not exclude current HRT users from our analyses. To assess whether plasma IGF-I levels varied by the -202 polymorphism, however, we analyzed the subset of control women who were not postmenopausal hormone users at blood collection. Women with an *IGFBP3* polymorphism genotype of CC were the referent group in all analyses. Conditional logistic regression was used to estimate relative risks (RRs) and

95% confidence intervals (95% CIs) in the total data set. Because results were similar with either method and to increase statistical power, we used unconditional logistic regression, controlling for the matching factors, for all subset analyses (according to menopausal status). The RRs were also calculated adjusting for body mass index (BMI) at age 18, weight change since age 18, age at menarche, parity/age at first birth, first-degree family history of breast cancer, personal history of benign breast disease, duration of postmenopausal hormone use and age at menopause. Because relations between the -202 polymorphism and breast cancer risk did not differ substantially when we excluded *in situ* cancers, we used both invasive and *in situ* cancers for all analyses. We did not adjust for IGF-I levels in our models because plasma levels were not available for all women. Moreover, in a subset of women for whom plasma IGF-I had been assessed, further adjustment for IGF-I did not materially alter the RRs. All *p*-values are 2-sided, and all analyses used the SAS program package (SAS Institute, Cary, NC).

RESULTS

From the 1,961 women from the Nurses' Health Study (breast cancer cases and matched controls 1:1 and 1:2) who were available for analyses, 1,511 women were successfully genotyped at the -202 locus. For 943 of these, we had previously measured IGF-I and IGFBP-3 plasma levels, height and weight, and for 861 of these subjects, plasma retinol levels were also determined. Data for 25-hydroxyvitamin D or 1,25-dihydroxyvitamin D levels, IGFBP-3 levels and *IGFBP3* -202 genotype were available for 329 and 276 subjects, respectively.

Variation at the -202 locus was common in the study population (genotype distribution: AA = 323, AC = 753, CC = 435), and the genotype frequencies were in Hardy-Weinberg equilibrium ($\chi^2 = 0.004$; *p* > 0.90). In accordance with a previous study among men,¹² we found that the A allele (46%) was less frequent than the C allele (54%).

Mean IGFBP-3 levels were lowest in the individuals with the CC genotype at the -202 *IGFBP3* locus and increased significantly in a stepwise manner in the presence of 1 or 2 copies of the A allele (*p* < 0.001 for each pairwise comparison, Table I): age-adjusted mean IGFBP-3 levels according to genotype were 4,426 ng/ml, 4,060 ng/ml and 3,697 ng/ml, respectively (20% difference between AA and CC). The relation between genotype and circulating serum IGFBP-3 levels was apparent with or without adjustment for age. Using a general linear model, we estimated that the percent variation in IGFBP-3 that can be explained by the -202 polymorphism is 6% ($r^2 = 0.06$). The -202 genotype was not associated with the total circulating IGF-I level [AA = 183 \pm 9 ng/ml vs. AC = 182 \pm 6 ng/ml vs. CC = 178 \pm 8 ng/ml, respectively (*p* > 0.5 for all pairwise comparisons)].

We examined the possibility that breast cancer risk might be related to genotype at the -202 *IGFBP3* locus (Table II). There was no statistically significant relation between genotype and RR for breast cancer, either overall or when assessed by menopausal status. Using the CC genotype as the reference (RR = 1.0), the simple RR for all women combined associated with the AC genotype was 0.99 (95% CI 0.77–1.26), and the RR associated with the AA genotype was 0.97 (95% CI 0.72–1.30). Further adjustment for established breast cancer risk factors and additional control for retinol did not materially alter these estimates. We also examined this association in subgroups of menopausal status (Table I). Among the small group of premenopausal women younger than age 50, we observed a modest, nonsignificant, inverse relation between genotype AA and breast cancer risk (multivariate RR = 0.67, 95% CI 0.20–2.20). This trend is in the direction one would predict, assuming that IGFBP-3 functions as a growth inhibitor and that the AA promoter variant is more active.¹² Among postmenopausal women who never used postmenopausal hormones, the RR for the AA genotype was 0.80 (95% CI 0.42–1.52). All associa-

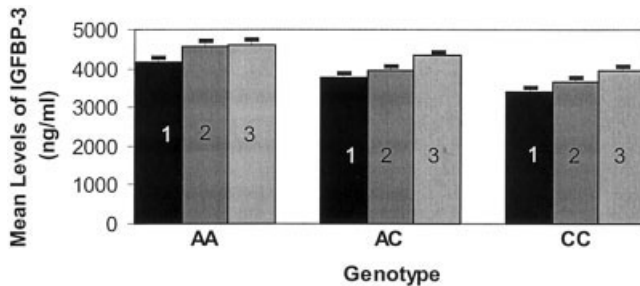


FIGURE 1—Mean IGFBP-3 levels stratified by both genotype at nucleotide -202 and circulating retinol tertile (1, lower tertile; 2, middle tertile; 3, upper tertile).

tions between the -202 polymorphism and breast cancer risk remained essentially unchanged when we assessed them among invasive cancers only (data not shown).

Data for retinol levels, IGFBP-3 levels and *IGFBP3* -202 genotype were available for 861 subjects (Table III). Age-adjusted IGFBP-3 levels increased across tertiles of plasma retinol (trend analysis: $p < 0.001$; Spearman correlation coefficient: $r = 0.25$; $p = 0.0001$). When the age-adjusted mean levels of IGFBP-3 in each retinol tertile were analyzed after stratification by genotype at the -202 locus, higher levels of IGFBP-3 were consistently observed with the AA genotype regardless of retinol levels (Fig. 1). The IGFBP-3 and retinol relation was similar across categories of genotype (interaction term, $F = 0.10$, $p = 0.91$). BMI and height did not modify the influence of the IGFBP-3 -202 polymorphism on circulating IGFBP-3 levels.

We found no association between the polymorphism and 25-hydroxy vitamin D or 1,25-dihydroxy vitamin D levels [for example, age-adjusted mean levels of 1,25-dihydroxy vitamin D, AA = 33.3 ± 0.9 ng/ml vs. AC = 33.1 ± 0.5 ng/ml vs. CC = 32.8 ± 0.7 ng/ml, respectively ($p > 0.5$ for all pairwise comparisons)].

DISCUSSION

In a matched, nested case-control study of breast cancer, we found that the -202 polymorphism in the promoter region of *IGFBP3* influences circulating IGFBP-3 levels in women. Circulating IGFBP-3 levels vary considerably between normal individuals. The literature remains scant, but estimates from twin studies¹⁰ propose that approximately 40% of the intraindividual variability in IGFBP-3 levels are attributable to genetic variants. Our results suggest that 6% of the variation in plasma IGFBP-3 is due to genotype at the -202 locus.

Consistent with earlier reports that show a similar association in adult men¹² and premenopausal women,¹² we found the AA ge-

notype to be associated with higher circulating levels of IGFBP-3, which were previously described to be associated with reduced cancer risk. We also evaluated the relation between *IGFBP3* polymorphism and breast cancer risk; although the polymorphism would be only one determinant of levels, even modest differences in levels over a lifetime may result in measurable differences in risk. In addition, in contrast to rare mutations (e.g., in *BRCA1*) that have a major influence on risk, modest influences on risk associated with common polymorphisms would apply to a larger number of people and therefore may be important on a population basis. We did not observe a significant relation between this *IGFBP3* polymorphism and overall risk of breast cancer.

Although there is some evidence from laboratory studies that both retinols and vitamin D can upregulate IGFBP3 levels, in our cohort we only found retinol to upregulate IGFBP3 expression. In contrast, there was no association between vitamin D and IGFBP-3 levels in our study.

The design of our study, a case-control study nested within the Nurses' Health Study cohort where blood samples were taken from the cohort before occurrence of disease, eliminates concerns about blood sampling time relative to breast cancer diagnosis. However, we had only a small number of premenopausal women in our cohort, the subgroup where the inverse correlation between IGFBP-3 level and breast cancer risk was originally described,⁷ and these findings will therefore need to be replicated in larger data sets. Furthermore, as the -202 locus accounts for only approximately 6% of the interindividual variability in IGFBP-3 levels, future studies should examine disease risk in relation to haplotypes obtained by simultaneously considering variability at several polymorphic sites in *IGFBP3*.

Overall, our observations do not support an important role of this *IGFBP3* polymorphism alone in the etiology of breast cancer. Our data confirm previous reports of an important relation between circulating IGFBP-3 levels and a -202 polymorphism in the promoter region of IGFBP-3. Furthermore, we found a positive relation between age-adjusted IGFBP-3 levels and plasma retinol. Thus, our data provide support for the laboratory observation that the induction of IGFBP-3 expression is functionally important in the action of chemopreventative agents such as retinoids.

ACKNOWLEDGEMENTS

M.N.P. was partially supported by grants from the Streams of Excellence Program of the Canadian Breast Cancer Initiative. We are indebted to the participants of the ongoing Nurses' Health Study for their continuing outstanding dedication to the study and to K. Corsano for technical assistance. We thank Dr. G. Colditz, Ms. J. Sparrow, Mr. D. Coppola, Dr. H. Ranu and Mr. R. O'Brien for their expert assistance. No authors have declared a financial interest in a company whose product was studied in the work presented here.

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