Genetic Polymorphisms Involved in Insulin-like Growth Factor (IGF) Pathway in Relation to Mammographic Breast Density and IGF Levels

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Abstract

The insulin-like growth factor (IGF) pathway is believed to play a role in carcinogenesis of the mammary gland. Single nucleotide polymorphisms (SNPs) of *IGF-I*, *IGF-binding protein-3* (*IGFBP-3*), *IGF receptor 1*, *insulin receptor substrate 1*, and *phosphoinositide-3-kinase, catalytic, β polypeptide* genes, which are members of the IGF pathway, have been associated with risk of common cancers, breast density, and/or IGF levels but results remain inconclusive. Thus, we evaluated the association of 11 targeted IGF pathway SNPs with circulating IGF levels and mammographic breast density. Among 741 white premenopausal women, blood samples were collected at time of screening mammography, and plasma IGF-I and IGFBP-3 levels were measured by ELISA. Percent and absolute breast density were estimated using a computer-assisted method. Multivariate linear models were used to examine the associations. Women carrying increasing number of copies of the rare allele of *IGF-I* rs1520220 and rs6220 SNPs had increased percent breast density (P_{trend} = 0.04 and 0.06, respectively). Carriers of increasing number of copies of the rare allele of *phosphoinositide-3-kinase, catalytic, β polypeptide* rs361072 SNP had decreased percent (P_{trend} = 0.04) and absolute (P_{trend} = 0.02) breast density. An association of *insulin receptor substrate 1* rs1801278 SNP with absolute density (P_{trend} = 0.03) was also observed. All four *IGFBP-3* SNPs (including rs2854744) were associated with IGF-I and IGFBP-3 levels. This study shows that several components of the IGF pathway are associated with breast density or IGF levels. Our findings provide additional support for the idea that several components of the IGF pathway may affect breast cancer risk and that this effect on breast cancer development may be mediated, at least in part, through its influence on the morphogenesis of breast tissue. (Cancer Epidemiol Biomarkers Prev 2008;17(4):880–8)

Introduction

Mammographic breast density reflects the proportion of the breast occupied by epithelial and stromal tissue and is strongly associated with breast cancer risk (1). Moreover, several breast cancer risk factors that affect the growth (proliferation and apoptosis) and/or differentiation of breast tissue, such as pregnancy, menopause, hormone replacement therapy, and hormones levels, are also associated with breast density (1, 2). Family history of breast cancer in first-degree relatives is also associated with an increase in breast density (reviewed in ref. 2), and, a large proportion of population variation in breast density is highly heritable (3).

Members of the insulin-like growth factor (IGF) pathway are believed to play an important role in the regulation of cell proliferation and apoptosis (Fig. 1). IGF-I, a polypeptide ligand, is a well-established mitogen for various cell types and may contribute to the progression of several human cancers (4, 5), including breast cancer (6). IGF-I is the primary ligand for the cell surface tyrosine kinase signaling molecule, IGF receptor 1 (IGF1R), which regulates apoptosis and cell proliferation through activation of downstream signaling molecules, including insulin receptor substrate 1 (IRS1) and phosphoinositide-3-kinase, catalytic, β polypeptide (PI3KCB) (4, 6). IGF-binding protein-3 (IGFBP-3) is the principal carriers of circulating IGF-I. IGFBP-3 has been proposed as an anticancer protein (7) because it may promote an IGF-I–independent action on apoptosis (7, 8). Some epidemiologic studies suggested that premenopausal women with high levels of IGF-I and/or low levels of IGFBP-3 may have higher breast density (9-12) and increased breast cancer risk (13-16).

More recently, polymorphic variants such as single nucleotide polymorphisms (SNPs) located in genes of the IGF pathway have been reported to be associated with risk of common cancers and/or circulating levels of growth factors (Table 1; refs. 17-22). In the *IGF-I* gene,

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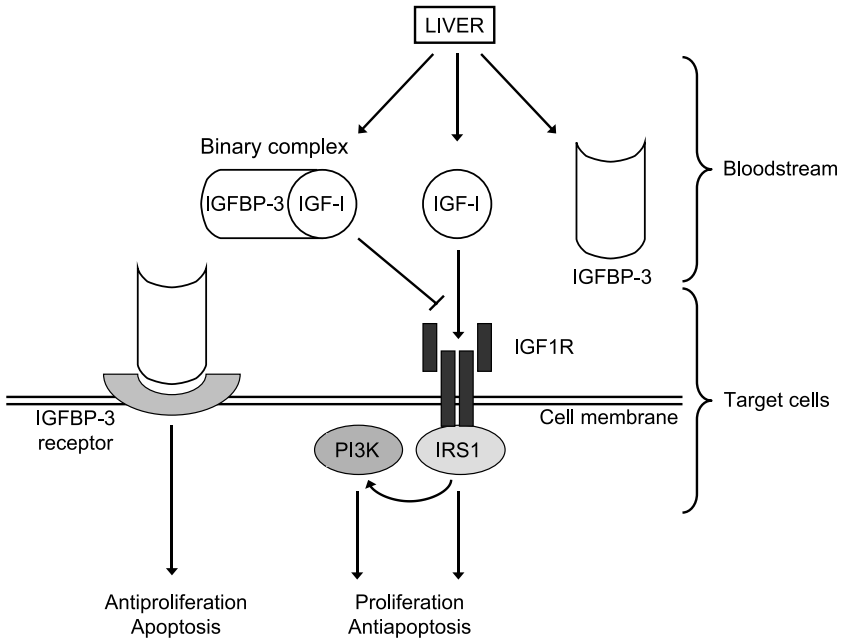

Figure 1. Overview of simplified IGF pathway. In the bloodstream, IGF-I is bound to >95% of IGFBP-3. In addition to prolonging IGF-I half-life and modulating its biological activities in serum, tissue IGFBP-3 can promote apoptosis independently of IGF-I via its own receptor. In tissue, IGF-I binds to IGF1R, a tyrosine kinase cell surface receptor, and exerts growth-promoting and antiapoptotic effects. IRS1, the major substrate for IGF1R, acts as docking protein between IGF1R and further downstream signaling molecules. IRS1 is required for the activation of PI3K, which stimulates proliferation and inhibits apoptosis.

a novel SNP located in the promoter region (rs5742612) has been associated with colorectal cancer (18), whereas two SNPs located in the intron 3 (rs1549593 and rs1520220) and one in the 3′-untranslated region of the exon 4 (rs6220) have been associated with levels of IGF-I and breast cancer risk (17). In addition to the well-studied polymorphic locus (rs2854744; A-202C) in the promoter region of the *IGFBP-3* gene, another SNP in the promoter region (rs2132572) plus two others located in the intron 1 (rs2471551) and intron 3 (rs3110697) have been associated with breast cancer risk and IGFBP-3 levels (17, 19). Moreover, SNPs located in exon 16 (rs2229765) of *IGF1R* gene and in the promoter region (rs361072) of *PI3KCB* gene were found associated with levels of free IGF-I (20). Lastly, in the *IRS1* gene, SNP located in exon 1 (rs1801278) has been associated with colorectal and prostate cancer risks (21, 22).

Up to now, only three studies have examined the association of some SNPs located in *IGF-I* and/or *IGFBP-3* genes with breast density, but results were inconsistent (23-25). SNPs in *IGF1R, IRS1*, and *PI3KCB* genes may affect breast morphology and carcinogenesis, but to our knowledge, their association with breast density has not been reported. The purpose of this study was to examine the association of 11 IGF pathway polymorphisms with mammographic breast density, as well as with IGF-I and IGFBP-3 levels, among white women recruited during screening mammography examinations.

Materials and Methods

Study Population and Recruitment Procedures. Details of the study design and methods have been published elsewhere (11). Briefly, study subjects for the present analysis were premenopausal women who underwent screening mammography between February and December 2001 at the Clinique Radiologique Audet (Quebec, Quebec, Canada). The study focused only on premenopausal women because, in previous analyses of these data, the associations of IGF-I and IGFBP-3 levels with breast density were observed only among these women (11). Women were classified as premenopausal if they had at least one natural menstrual cycle within 12 mo or were younger than 48 y (if a nonsmoker) or 46 y (if a smoker) after hysterectomy without bilateral oophorectomy or use of hormonal derivatives (26). The eligibility was restricted to women not taking hormonal derivatives within 3 mo of the mammography, not pregnant, never having used tamoxifen or raloxifene, without a history of cancer at any site or breast surgery, and without endocrine system diseases.

A total of 787 premenopausal women were found eligible. Among these women, 2 declined to be interviewed, and the mammograms of 2 women could not be retrieved for review. In the remaining 783 women, 37 accepted to participate in our initial study (11) but did not give authorization for blood banking of samples for further study. Therefore, a total of 746 premenopausal women were included in the present analysis.

This study was reviewed and approved by the research ethics committee of the Centre Hospitalier Affilié Universitaire de Québec (Quebec, Quebec, Canada). All study participants provided written informed consent.

Data Collection. At the time of mammography, a trained nurse measured the women's weight (kg), height (cm), and waist and hip circumferences (cm) and collected 20 mL of blood. Information on known or suspected breast cancer risk factors was collected by trained interviewers and included menstrual and reproductive histories, family history of breast cancer, personal history of breast biopsies, past use of hormonal derivatives, smoking status, alcohol intake, education, and physical activity, which was expressed as metabolic

equivalent (MET-h/wk). Finally, diet over the previous year was assessed with a self-administered 161-item semiquantitative food frequency questionnaire (97GP copyrighted at Harvard University), which was translated into nutrient intake, including energy intake (kcal/d), at the Channing Laboratory of Harvard University.

Digitization of Mammograms and Assessment of Mammographic Breast Density. All mammograms were scanned at 260 μm/pixel with a Kodak Lumiscan85 digitizer. Then, for each woman, the proportion of the breast showing tissue density (percent density, %) and the absolute amount of dense tissue (absolute density, cm^2) were assessed by one trained author (C.D.) from the craniocaudal view of a randomly chosen breast. This assessment was done without any information on women using a computer-assisted method (27). Variability in the assessment of breast density was as follows: the within-batch intraclass correlation coefficients (n = 210 duplicate images) were 0.98 and 0.98 and the between-batch coefficients of variation (n = 10 images repeated 21 times) were 4% and 5% for percent and absolute breast density measurements, respectively.

Analysis of Plasma IGF-I and IGFBP-3. At the time of blood collection, blood constituents were rapidly aliquoted and stored at −80°C until analysis. Under the supervision of one of us (M.P.), plasma levels of IGF-I and IGFBP-3 (ng/mL) were blindly assayed by ELISA with reagents from Diagnostic Systems Laboratory. The intra-batch coefficients of variation (4 samples per batch of 39 samples for a total of 46 batches) were 10.5% and 13.2%, and the between-batch coefficients of variation were 7.9% and 10.5% for IGF-I and IGFBP-3, respectively.

DNA Extraction and SNP Genotyping. DNA was extracted from buffy coat using the Puregene DNA extraction kit (Gentra, Inc.) following the manufacturer's protocol. Genomic DNA of five women could not be obtained. Then, DNA samples were blindly genotyped for 11 selected SNPs located in five genes of the IGF pathway: *IGF-I, IGFBP-3, IGF1R, IRS1*, and *PI3K* (Fig. 1). In the present study, SNPs were not chosen based on a tagging approach. Instead, to be included in the study, SNPs had to be located in promoter regions or exons (including untranslated regions) of an IGF pathway gene (4) and found to be associated with breast density, common cancer risk, and/or levels of growth factors in previous studies (17-23).

To this list, four SNPs located in introns of *IGF-I* or *IGFBP-3* genes were added because they have been found to be associated with breast cancer risk (17, 19). From the 13 SNPs selected, 11 have been successfully genotyped and are described in Table 1. SNP analyses were done using the GenomeLab SNPstream (Beckman Coulter) or Sequenom MassArray genotyping platforms according to the manufacturer's protocols. Each 96-well plate included negative (no DNA) and positive controls to ensure accuracy of genotyping. Genotyping call rates for both technologies ranged between 98.7% and 99.5%. In this study, genotyping from new Sequenom MassArray platform was compared with fluorescent

Table 1. SNPs evaluated in the present study

Gene name	Encoded product	*n*	Alleles (major > minor)	SNP reference ID*	Position in gene	Codon	Panel	Call rate	Diseases or factors associated to SNP
IGF-I	Insulin-like growth factor-I	737	A > G	rs5742612	Promoter	—	SNPstream	99.5%	Colorectal cancer (18)
		732	C > A	rs1549593	Intron 3	—	Sequenom	98.8%	Breast cancer (17); levels of IGF-I (17)
		733	C > G	rs1520220	Intron 3	—	Sequenom	98.9%	Breast cancer (17); levels of IGF-I (17)
		731	A > G	rs6220	Exon 4; 3′-untranslated region	—	SNPstream	98.7%	Breast cancer (17); levels of IGF-I (17)
IGFBP-3	Insulin-like growth factor–binding protein-3	735	G > A	rs2132572	Promoter	—	SNPstream	99.2%	Breast cancer (17); levels of IGFBP-3 (17)
		731	A > C	rs2854744	Promoter	—	Sequenom	98.7%	Breast cancer (17, 19); breast density (23); levels of IGFBP-3 (17, 19)
		733	G > C	rs2471551	Intron 1	—	Sequenom	98.9%	Breast cancer (19); levels of IGFBP-3 (17, 19)
		736	C > T	rs3110697	Intron 3	—	SNPstream	99.3%	Breast cancer (19); levels of IGFBP-3 (19)
IGF1R	Insulin-like growth factor receptor 1	734	G > A	rs2229765	Exon 16	$Glu^{1013}Glu$	SNPstream	99.1%	Levels of free IGF-I (20)
IRS1	Insulin receptor substrate 1	736	G > A	rs1801278	Exon 1	$Gly^{972}Arg$	SNPstream	99.3%	Colorectal cancer (22); prostate cancer (21)
PI3KCB	Phosphoinositide-3-kinase, catalytic, β polypeptide	733	T > C	rs361072	Promoter	—	SNPstream	98.9%	Levels of free IGF-I (20)

*Polymorphisms are identified by their dbSNP accession number at http://www.ncbi.nlm.nih.gov/SNP/.

Table 2. Associations of SNPs in *IGF-I* and *IGFBP-3* genes with mammographic breast density and levels of growth factors

Gene name	rs number	Genotype	*n* (%)	Percent density (%)*		Absolute density (cm^2)*		IGF-I (ng/mL)†		IGFBP-3 (ng/mL)†	
				Mean	*P*‡	Mean	*P*‡	Mean	*P*‡	Mean	*P*‡
IGF-I	rs5742612	AA	698 (94.7)	42.2		46.3		224.0		4,780	
		AG	37 (5.0)	45.1		51.0		210.1		5,107	
		GG	2 (0.3)	59.6	0.19	92.5	0.08	260.0	0.29	5,240	0.005
	rs1549593	CC	546 (74.6)	42.5		47.0		223.8		4,793	
		CA	175 (23.9)	41.9		45.4		221.6		4,762	
		AA	11 (1.5)	40.0	0.61	40.5	0.35	217.5	0.53	5,041	0.93
	rs1520220	CC	547 (74.6)	41.8		46.4		222.8		4,801	
		CG	168 (22.9)	42.9		45.2		221.2		4,793	
		GG	18 (2.5)	54.7	0.04	60.6	0.48	258.0	0.19	4,413	0.19
	rs6220	AA	431 (59.0)	41.6		46.7		221.9		4,820	
		AG	262 (35.8)	41.9		45.2		223.2		4,771	
		GG	38 (5.2)	51.3	0.06	54.7	0.59	239.8	0.13	4,645	0.14
IGFBP-3	rs2132572	GG	454 (61.8)	42.5		47.4		220.8		4,866	
		GA	242 (32.9)	42.0		44.9		225.9		4,720	
		AA	39 (5.3)	43.3	0.95	49.5	0.63	240.8	0.02	4,507	0.0003
	rs2854744	AA	188 (25.7)	43.0		48.3		212.2		4,996	
		AC	361 (49.4)	41.0		45.0		222.4		4,815	
		CC	182 (24.9)	44.0	0.61	47.1	0.65	236.4	<0.0001	4,525	<0.0001
	rs2471551	GG	481 (65.6)	41.9		46.2		219.5		4,844	
		GC	225 (30.7)	43.2		47.3		230.4		4,681	
		CC	27 (3.7)	43.1	0.42	45.6	0.77	231.1	0.008	4,712	0.009
	rs3110697	CC	253 (34.4)	41.5		46.9		213.3		4,943	
		CT	369 (50.1)	42.8		46.3		227.5		4,748	
		TT	114 (15.5)	42.9	0.46	46.9	0.95	233.0	<0.0001	4,571	<0.0001

*Analyses are adjusted for age, body mass index, waist-to-hip ratio, number of full-term pregnancies, number of breast biopsies, and smoking status. Mean absolute density is presented as back-transformed values.
†Analyses are adjusted for age, body mass index, waist-to-hip ratio, levels of physical activity in the past year, and levels of IGF-I or IGFBP-3 when applicable.
‡*P* value for codominant model is the P_{trend} for the test of gene dosages.

polarization-single-base extension platform on 10% of the samples; concordance was >99.6%. Protocols could be provided on request from the corresponding author.

Statistical Methods. For each SNP, deviation of genotype frequencies among women from the Hardy-Weinberg equilibrium was assessed by a χ^2 test with one degree of freedom. In the present study, no significant deviation from Hardy-Weinberg expectations was observed for any of the polymorphisms ($P > 0.05$). Linkage disequilibrium strength was evaluated for within-gene SNP pairs. The D' values were all >0.92 except for two pairs of SNPs located in *IGF-I*: rs5742612 with rs1520220 ($D' = 0.03$) and with rs6220 ($D' = 0.31$).

Multivariate-adjusted means of breast density or circulating levels of growth factors by category of genotypes under codominant mode of inheritance were estimated using generalized linear models. In these models, associations between the number of copies of the rare allele entered as a continuous variable (0, 1, or 2) and breast density or levels of growth factors were evaluated by linear regression models (P_{trend}). Assumption of normality of residuals from these analyses was met with untransformed variables except for absolute density, which was square root transformed, and results are presented as back-transformed values.

The strength of associations of genotypes to breast density among women with IGF-I levels above the median was compared with that among women with levels at or below the median by using above models to which an interaction term between levels of IGF-I (≤median/>median) and genotypes was added. The *P* value of the interaction term between levels of IGF-I (≤median/>median) and the genotypes under the codominant mode of inheritance was used to assess the effect modification of IGF-I.

Models using mammographic percent or absolute breast density as dependent variables were adjusted for age at mammography (years), body mass index (kg/m^2), waist-to-hip ratio, number of full-term pregnancies and number of breast biopsies treated as continuous variables, and smoking status (never, former, or current smoker) treated as a categorical variable. Models using IGF-I or IGFBP-3 levels as dependent variables were adjusted for age at mammography (years), body mass index (kg/m^2), waist-to-hip ratio, levels of physical activity in the past year (MET-h/wk), and IGFBP-3 or IGF-I levels, respectively, treated as continuous variables.

Further adjustment for factors potentially associated with breast density and/or levels of growth factors (levels of physical activity in the past year; number of full-term pregnancies; number of breast biopsies and smoking status when applicable; height, energy, and alcohol intakes in the past year; duration of past use of hormone replacement therapy and oral contraceptive; lactation; age at first full-term birth; age at menarche; family history of breast cancer; education; and seasons at time of recruitment) had little or no influence on the

estimates. Therefore, they were not added in the models. Throughout the analysis, statistical significance was based on two-sided P values. All statistical analyses were carried out using the Statistical Analysis System version 9.1 (SAS Institute, Inc.) software system.

Results

Characteristics of the study population are described elsewhere (11). Briefly, the 741 premenopausal women included in the present study were on average (±SD) 46.8 years old (±4.6 years old) and had mean percent and absolute breast density of 42.3% (±24.3%) and 47.1 cm^2 (±28.7 cm^2), respectively. Mean levels of IGF-I and IGFBP-3 were 223.8 ng/mL (±63.7 ng/mL) and 4,798 ng/mL (±906 ng/mL), respectively. Almost all women reported to be Caucasians (99.7%; n = 739).

Relations of *IGF-I* polymorphisms with breast density and levels of growth factors are presented in Table 2. Women carrying increasing number of copies of the rare allele of rs1520220 and rs6220 SNPs had increased adjusted mean of percent breast density (P_{trend} = 0.04 and 0.06, respectively). Although statistical or borderline significance was reached in the codominant models, recessive effect of these SNPs on breast density or IGF-I levels seems more probable (common homozygotes had means similar to those of heterozygotes). Under such recessive models, the multivariate-adjusted means of percent and absolute breast density were higher in the rare homozygous states for rs1520220 and rs6220 compared with other subjects (for percent: P = 0.007 and 0.004; for absolute: P = 0.04 and 0.07).

Moreover, women carrying two copies of the rare allele of rs1520220 and rs6220 SNPs had also greater levels of IGF-I (P = 0.003 and 0.04) than other subjects. To examine if the observed associations between rs1520220 or rs6220 SNP and breast density were attributable to the effects of these SNPs on circulating levels of IGF-I, the models were further adjusted for IGF-I levels. We found that associations were unchanged when this adjustment was made (for percent: P = 0.006 and 0.003; for absolute: P = 0.03 and 0.07). Finally, we observed an association of rs5742612 SNP with IGFBP-3 levels (P_{trend} = 0.005). No association was found of rs1549593 with breast density or levels of growth factors.

Table 2 also shows that all four *IGFBP-3* SNPs were associated with IGF-I and IGFBP-3 levels, but none with breast density. Women carrying increasing number of copies of the rare allele of any *IGFBP-3* SNP had increased adjusted mean levels of IGF-I ($P_{trend} \leq 0.02$) and decreased adjusted mean levels of IGFBP-3 ($P_{trend} \leq 0.009$). When all four *IGFBP-3* SNPs were in the same model, only the associations of rs2854744 SNP with high levels of IGF-I and low levels of IGFBP-3 were still statistically significant (P_{trend} = 0.02 and 0.0001, respectively).

Relations of *IGF1R, IRS1,* and *PI3KCB* polymorphisms with breast density and levels of growth factors are presented in Table 3. Women carrying increasing number of copies of the rare allele of *PI3KCB* (rs361072) SNP had decreased adjusted mean of percent (P_{trend} = 0.04) and absolute (P_{trend} = 0.02) breast density. Moreover, we observed a significant association of *IRS1* (rs1801278) SNP with absolute density (P_{trend} = 0.03) but not with percent density (P_{trend} = 0.25). No association of *IGF1R, IRS1,* or *PI3KCB* SNP was observed with IGF-I and IGFBP-3 levels.

The variance explained by SNPs found associated with breast density and levels of growth factors in Tables 2 and 3 has been examined. For the most statistically significant associations, inclusion of rs1520220, rs361072, or rs2854744 SNP in the multivariate model explained an additional 1%, 1%, 2%, and 4% of variation in percent density, absolute density, IGF-I levels, and IGFBP-3 levels, respectively.

Laboratory studies suggest that the effects of IGF1R and its downstream signaling molecules in the breast may vary according to IGF-I levels. IGF1R expression is subject to negative feedback regulation by high IGF-I levels (reviewed in ref. 5). Thus, low IGF-I levels are linked with high expression of IGF1R. Although IGF1R expression was not examined here, the molecules under its regulation, such as PI3KCB (Fig. 1), should be also more expressed at low levels of IGF-I. Therefore, we evaluated if the observed association of *PI3KCB* (rs361072) SNP gene with breast density was stronger

Table 3. Associations of SNPs in *IGF1R, IRS1*, and *PI3KCB* genes with mammographic breast density and levels of growth factors

Gene name	rs number	Genotype	n (%)	Percent density (%)*		Absolute density (cm^2)*		IGF-I (ng/mL)†		IGFBP-3 (ng/mL)†	
				Mean	P‡	Mean	P‡	Mean	P‡	Mean	P‡
IGF1R	rs2229765	GG	239 (32.5)	41.4		46.4		229.3		4,736	
		GA	347 (47.3)	41.9		46.2		220.4		4,830	
		AA	148 (20.2)	45.0	0.10	48.3	0.57	220.5	0.06	4,816	0.22
IRS1	rs1801278	GG	641 (87.1)	42.1		45.8		223.5		4,796	
		GA	93 (12.6)	44.1		52.2		222.4		4,820	
		AA	2 (0.3)	54.5	0.25	59.7	0.03	232.6	0.91	4,325	0.98
PI3KCB	rs361072	TT	199 (27.2)	44.5		50.0		224.1		4,799	
		TC	373 (50.9)	42.2		46.4		222.7		4,752	
		CC	161 (22.0)	40.1	0.04	43.0	0.02	224.8	0.92	4,867	0.44

*Analyses are adjusted for age, body mass index, waist-to-hip ratio, number of full-term pregnancies, number of breast biopsies, and smoking status. Mean absolute density is presented as back-transformed values.

†Analyses are adjusted for age, body mass index, waist-to-hip ratio, levels of physical activity in the past year, and levels of IGF-I or IGFBP-3 when applicable.

‡P value for codominant model is the P_{trend} for the test of gene dosages.

Table 4. Associations of SNP in *PI3KCB* gene with mammographic breast density according to IGF-I levels

Gene name	rs number	Genotype	*n* (%)		Mean percent density (%)*			Mean absolute density (cm^2)*		
			IGF-I		IGF-I			IGF-I		
			≤Median ≤217.75 (ng/mL)	>Median >217.75 (ng/mL)	≤Median ≤217.75 (ng/mL)	>Median >217.75 (ng/mL)	$P_{interaction}$	≤Median ≤217.75 (ng/mL)	>Median >217.75 (ng/mL)	$P_{interaction}$
PI3KCB	rs361072	TT	99 (13.5)	100 (13.6)	47.1	41.8		52.4	47.7	
		TC	190 (25.9)	183 (25.0)	41.4	43.0		45.9	46.9	
		CC	77 (10.5)	84 (11.5)	39.6	40.6		41.5	44.3	
		$P^{\dagger}$			0.009	0.70	0.11	0.008	0.41	0.18

*Analyses are adjusted for age, body mass index, waist-to-hip ratio, number of full-term pregnancies, number of breast biopsies, and smoking status. Mean absolute density is presented as back-transformed values.
$^{\dagger}P$ value for codominant model is the P_{trend} for the test of gene dosages.

among women with low levels of IGF-I. Results are presented in Table 4. We observed that women carrying increasing number of copies of the rare allele of *PI3KCB* (rs361072) SNP had decreased percent or absolute breast density among women with IGF-I levels below or equal to the median (P_{trend} = 0.009 and 0.008) compared with those with IGF-I levels above the median (P_{trend} = 0.70 and 0.41) with a $P_{interaction}$ of 0.11 and 0.18, respectively. The interaction between *IRS1* SNP and IGF-I levels could not be examined due to small number of subjects carrying two copies of the rare allele.

Discussion

In this study, we examined whether SNPs of several members of IGF pathway were associated with breast density among premenopausal women. We observed that SNPs located in *IGF-I, IRS1*, and *PI3KCB* genes were associated with percent and/or absolute breast density. Carriers of two rare alleles of these IGF pathway genes had 4.4% to 12.9% absolute mean differences in percent density compared with those carrying two common alleles. Because mammographic breast density is strongly associated with breast cancer risk (1), these 4.4% to 12.9% differences in breast density are meaningful because they may be associated with a significant difference in breast cancer risk. By comparison with nulliparous women, those who had a first child at age 20 years had, in our data, 5.2% lower breast density. Women with such an early first birth have been shown to have a 20% reduction in cumulative incidence (up to age 70) of breast cancer (28). Although circulating levels of growth factors mainly produced in the liver have been linked to breast morphogenesis and breast cancer risk (9-16), our results support the additional idea that some components of the IGF pathway at tissue levels are important in this respect, at least among premenopausal women.

Circulating levels of IGF-I are mainly produced by the liver, but several tissues, including breast tissue, can also express IGF-I (4, 6). Laboratory studies have reported that IGF-I is able to stimulate both epithelial and stromal human breast cell growth (29, 30), whereas epidemiologic findings suggested that premenopausal women with high levels of IGF-I may have increased breast cancer risk (13-16) and higher breast density (9-11). Furthermore, one histologic study suggests that, among women <50 years old, protein levels of IGF-I in breast epithelial and stromal tissue are higher in women with high breast density when compared with women with low breast density (31). Our results suggest that women carrying rare allele of rs1520220 or rs6220 SNP in *IGF-I* gene (two SNPs in close linkage disequilibrium) have higher breast density and higher levels of IGF-I. The associations with breast density remained even after adjustment for circulating levels of IGF-I, suggesting that these *IGF-I* SNPs affect levels of IGF-I in tissue and not only their circulating levels. Two studies showed that women carrying rare allele of rs1520220 or rs6220 *IGF-I* SNP had higher levels of IGF-I (17, 32) and higher breast cancer risk in at least one of these studies (17), although no consensus can be inferred for the mode of inheritance. In contrast, lower breast density has been observed in carriers of rare allele of rs1520220 (25). In the latter study, rs6220 SNP and levels of IGF-I were not measured, limiting the ability to compare findings.

PI3KCB is a well-known enzyme that is often phosphorylated and activated in response to IGF1R binding IGF-I ligand (4, 6). Ho et al. (33) have reported that luminal epithelial cells, myoepithelial cells, and mammary fibroblasts from reduction mammoplasty expressed significant levels of PI3KCB. Moreover, results from Pu et al. (34) suggest that suppression of PI3KCB expression induces the arrest of cell cycle, delays the progression of cell cycle, inhibits the cell proliferation, and promotes cell apoptosis of human malignant glioma cells. The role of rs361072 *PI3KCB* SNP is biologically plausible because it is located 359 bp upstream from the start codon of PI3KCB (35), which may affect gene expression. Thus, we speculate that breast epithelial and stromal tissue of carriers of rare rs361072 allele would express less PI3KCB, which would result in lower breast density. As hypothesized, this association was stronger among women who had lower levels of IGF-I.

IRS1 acts as docking protein between IGF1R and further downstream signaling molecules (4, 6). The common rs1801278 SNP ($Gly^{972}Arg$; exon 1) in *IRS1* has been shown to cause impaired insulin signaling (36) and apoptosis of human pancreatic islets (37). Up to now, previous studies have shown that carriers of at least one rare allele for *IRS1* (rs1801278) were associated with

increased risk of breast (38), prostate (21), and colon (22) cancers. In addition, carriers of two rare alleles of rs1801278 *IRS1* were associated with increased breast cancer risk compared with those who had none (odds ratio, 6.19; $P = 0.12$; ref. 39), although this association was not statistically significant. In the present study, we observed that women carrying AA of rs1801278 *IRS1* SNP had 12.4% and 13.9 cm^2 higher percent and absolute density, respectively, than GG, but the association was statistically significant only for absolute density. However, sample size was small and only two women were carrying two rare alleles of the *IRS1* gene.

The association of the well-studied polymorphic locus (rs2854744; A-202C) in the promoter region of the *IGFBP-3* gene with breast density has been examined in three other studies. In the study conducted by dos Santos Silva et al. (24) and Tamimi et al. (25), no association was found between the number of A alleles of rs2854744 SNP and breast density in premenopausal and postmenopausal women. In contrast, results from Lai et al. (23) study show that increasing number of copies of A allele of rs2854744 SNP was associated with higher breast density in premenopausal women but not in postmenopausal women. Similarly, results of cancer risk studies are also conflicting. Some of them found an association of rs2854744 SNP with breast (17, 19, 38), familial breast (40), and non–small cell lung (41) cancers and advanced disease status in prostate cancer (42), whereas others observed no association with the risk of breast (32, 43-45), familial breast (46), colorectal (18, 22), and prostate (42, 45, 47, 48) cancers. Like Tamimi et al. (25), we also observed no association between rs3110697 SNP in *IGFBP-3* gene and breast density.

Levels of IGF-I and IGFBP-3 have been found to be highly heritable (49). In the present study, we also examined the association of IGF pathway SNPs with levels of growth factors. We observed that rs2854744 *IGFBP-3* SNP was associated with levels of IGF-I independently of IGFBP-3 levels. Our results are consistent with those observed by other groups. Carriers of CC or CA at the well-studied polymorphic locus (rs2854744; A-202C) in the promoter region of *IGFBP-3* had higher IGF-I levels than the others in one (32) of the two studies (32, 50). Moreover, decreasing number of A alleles was associated with increasing levels of IGF-I/IGFBP-3 molar ratio (23, 41, 50-52), which reflects the availability of IGF-I in tissue. One group had examined the association of rs2229765 and rs361072 SNPs of the *IGF1R* and *PI3KCB* genes, respectively, with levels of free IGF-I (20). In that study, the rare rs2229765 genotype of the *IGF1R* gene and the common homozygote genotype of the *PI3KCB* gene were associated with lower levels of free IGF-I. No association of *IGF1R* and *PI3KCB* SNPs with total levels of IGF-I was observed in our data.

We also found that rs5742612 SNP of the *IGF-I* gene and the well-studied polymorphic locus (rs2854744; A-202C) of the *IGFBP-3*, both located in the promoter region, were associated with levels of IGFBP-3 independently of IGF-I levels. This is the first study to examine the relation of rs5742612 SNP in *IGF-I* gene with levels of IGFBP-3. The observed association is biologically plausible because it is known that IGFBP-3 expression is regulated by several hormones, including IGF-I (reviewed in ref. 5), and this regulation depends on the DNA/protein sequence of IGF-I (53). For instance, Oh et al. (53) showed that human breast cancer cells release higher IGFBP-3 levels in conditioned medium following addition of IGF-I but not by IGF-I analogue (synthesized IGF-I mutant), which has significantly decreased affinity for IGFBP-3. Thus, it is possible that *IGF-I* SNP may affect levels of IGFBP-3. We also observed a gene-dose relation of IGFBP-3 levels with the number of rare allele of the *IGFBP-3* rs2854744 SNP. Increasing number of A alleles (rs2854744; A-202C) of the *IGFBP-3* gene has been found to be associated with increasing levels of IGFBP-3 among men and premenopausal and/or postmenopausal women in several studies (17-19, 23, 24, 32, 41, 44, 50-52, 54).

This study has some limitations. Although polymorphisms analyzed here were chosen a priori based on previous data, the possibility that some of the findings could be due to chance cannot be totally excluded because an exploratory approach was used and multiple testing was carried out. Thus, false-positive results can explain why some SNPs are associated with either breast density or levels of growth factors but not with both. However, it is also possible that the absence of statistically significant associations is due to a lack of power of our study. A larger sample size may have detected the association of SNPs located in *IGF-I* and *IGFBP-3* genes with both breast density and levels of growth factors. In addition, in this type of study, population stratification is a major potential source of confounding. However, this problem might not be as important here because our population was mostly composed (99.7%) of Caucasian women and of over 87.7% French-Canadians. Finally, some polymorphisms measured in this study may have no direct influence on the expression and/or may not directly affect the function of the gene. However, these SNPs may in fact be in linkage disequilibrium with one or several unknown functional SNPs and that this may explain some of the observed associations.

In conclusion, our findings suggest that, in premenopausal women, genetic variants of different components of the IGF pathway notably in *IGF-I, IGFBP-3, IRS1*, and *PI3KCB* genes may affect breast density and/or levels of growth factors. These results suggest that several components in the IGF pathway may be involved in breast cancer development.

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