Original paper

Mammographic breast density and IGF-1 gene polymorphisms rs1520220, rs2946834 and rs6219 in Polish women

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Aim of the study: The aim of the study was to analyze three single nucleotide polymorphisms – rs1520220, rs2946834, rs6219 – of the *IGF-1* gene in the context of breast mammographic density.

Material and methods: The research material included 202 samples of the peripheral blood of women with increased mammographic breast density and 238 samples of the epithelium from the oral mucosa of women without diagnosed pathological changes of the breast and with no family history of breast and/or ovarian cancer. The real-time polymerase chain reaction method was applied for analysis of polymorphisms.

Results: rs1520220 polymorphism was associated with increased mammographic density of the breasts. The presence of the CC genotype in the *IGF-1* gene increased the risk of developing higher breast density visible in mammography by 2.43-fold. CC homozygotes (rs1520220) correlated with higher Breast Imaging-Reporting and Data System scale (3 vs. 4 and 5) (OR = 5.6; 95% CI: 1.82–16.3, $p = 0.001$). In the present study no relationship was detected between rs6219 and rs2946834 polymorphism and mammographic breast density.

Conclusions: The results suggest that the rs1520220 polymorphism of the *IGF-1* gene plays an important role in the occurrence of increased mammographic breast density.

Key words: mammographic breast density, IGF, polymorphism, breast cancer.

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Introduction

The amount of radiographically dense breast tissue shown on a mammogram varies from woman to woman due to differences in the composition of the breast tissue and is referred to as mammographic density. Mammographic density is a strong risk factor for breast cancer and the risk of breast cancer is 4–5 times higher in women with a higher density in more than 75% of the breast area than in women with little or no breast density. Dense tissue in more than 50% of the breast surface can explain about one-third of breast cancers [1–4].

Our limited understanding of the biology of mammographic density makes the selection of candidate genes difficult. Moreover, mammographic density is a dynamic feature, decreasing with age and menopause, and even changing temporarily during the menstrual cycle. Higher density is observed in premenopausal women, nulliparous with a low body mass index (BMI) or using combination postmenopausal hormone, and lower in postmenopausal women, multiparous, with a high BMI or treated with tamoxifen [5]. Because of these hormonal influences, most candidate gene research has focused on evaluating the pathways that regulate the synthesis and metabolism of steroid hormones, hormone receptors, and proliferative pathways, including the insulin-like growth factor pathway. However, some studies have focused on genes that were previously noted as strongly (*BRCA1/BRCA2*) or hypothetically (*XPD*, *XRCC3* and *ERBB2*) associated with breast cancer risk [5]. Literature data indicate that the most common genes for predisposition to breast carcinoma are: *ATM*, *BARD1*, *BRCA1/2*, *CDH1*, *CHEK2*, *Nf1*, *PALB2*, *PTEN*, *RAD51C*, *RAD51D* and *TP53* [6]. A recent study confirmed the risk of breast cancer in women with neurofibroma type 1 (NF-1) [7]. As for the *Nf1* gene, the so-called first hit mutation guaranteed that further carcinogenesis is inherited germinally in patients with NF-1 [8, 9].

So far, many studies have been carried out on genetic factors that may affect breast density [10–14]. Yet, it has been suggested that some elements of the insulin-like growth factor 1 (IGF-1) pathway are associated with breast density and IGF levels [11, 12]. Scientific experiments show the relationship between single nucleotide polymorphisms (SNPs) in the *IGF-1* and *IGFBP-3* gene region with breast density [15–26]. It is also known that SNPs in insulin receptor substrate 1 (IRS1), insulin-like growth factor receptor 1 (IGF1R) and phosphoinositide-3-kinase, catalytic, β-polypeptide (PI3KCB) genes may affect breast morphology and carcinogenesis [20].

An association was observed between the rs1520220 and rs6220 polymorphisms of the *IGF-1* gene and rs1801278 of the *IRS* gene and high breast density, while the rs361072 polymorphism of the *PI3KCB* gene was associated with low breast density [27]. Four SNPs of the *IGFBP-3* gene (rs2132572,

rs2854744, rs2471551 and rs3110697) were related to the levels of IGF-1 and IGFBP-3 [27]. The association of the following SNPs of the *IGF-1* gene with increasing IGF-1 levels was demonstrated: rs9989002, rs2033178, rs7136446, rs978458 and rs6220. These polymorphisms were slightly associated with breast density in premenopausal women. A much stronger correlation was found between polymorphisms and mammographic breast density in postmenopausal women. The most statistically significant results were obtained for the rs6220 polymorphism and the IGF-1 level. Research suggests a link between *IGF-1* genetic variants and fluctuating protein levels. However, their relationship with breast density has not been fully confirmed [27].

Research indicates that the IGF pathway may influence the risk of breast cancer by affecting the morphogenesis of breast tissue [28]. The studies of Verheus *et al*. showed that the rs9989002, rs2033178, rs7136446, rs978458, rs6219 polymorphisms were significantly associated with an increase in IGF-I levels, but they did not correlate with breast density in the studied women [28].

IGF-1 gene SNPs may affect the level of IGF1 protein in tissues [29, 30]. It has been shown that the rs1520220 or rs6220 polymorphisms in the *IGF-1* gene region correlate with a high level of the IGF1 protein encoded by this gene, and thus with a high risk of breast cancer [29].

The aims of the study were:

– to analyze the SNPs rs1520220, rs2946834, rs6219 of the *IGF-1* gene in a group of women diagnosed with increased mammographic breast density and in a control group,
– to correlate the obtained results with clinical and pathological data,
– to determine the significance of the obtained results in the context of increased breast mammographic density.

Table 1. Clinical-pathological characteristics of patients

	Increased mammographic breast density (%)	Control (%)	*p* test χ^2 breast density vs. control
Number of samples	202	238	
Age group			
35–44	0 (0)	3 (1.26)	0.003
45–54	92 (45.5)	78 (32.8)	
55–64	95 (47.1)	119 (50.0)	
65+	15 (7.4)	38 (15.9)	
BMI			
< 24.9	137 (67.8)	102 (42.8)	< 0.001
25–29.9	52 (25.7)	67 (28.1)	
> 30	13 (6.5)	69 (29.1)	
Age of menarche			
≥ 14	97 (48.0)	118 (49.6)	0.56
12.13	80 (39.6)	98 (41.2)	
< 12	25 (12.4)	22 (9.2)	
Number of pregnancies			
0–1	101 (50.0)	101 (42.4)	0.24
2–3	83 (41.1)	116 (48.7)	
≥ 4	18 (8.9)	21 (8.9)	
Hormonal treatment			
Yes	56 (27.7)	71 (29.8)	0.62
No	146 (72.3)	167 (70.2)	
BIRADS scale			
Category 3	26 (12.9)	–	–
Category 4	167 (82.7)		
Category 5	9 (4.4)		
Menopausal status			
Premenopausal	25 (12.4)	15 (6.3)	0.07
Peri-menopausal	45 (22.3)	51 (21.4)	
Post-menopausal	132 (65.3)	172 (72.3)	
Cigarettes			
Yes	78 (38.6)	84 (35.3)	0.47
No	124 61.4)	154 (64.7)	

BMI – body mass index, BIRADS – Breast Imaging-Reporting and Data System

Material and methods

Patients

The research material included 202 samples of peripheral blood of women with increased mammographic density and 238 preparations of the epithelium of the oral mucosa in women without diagnosed pathological changes of the breast and no family history of breast and/or ovarian cancer. The severity of mammographic breast density in women was classified according to the six-point Breast Imaging-Reporting and Data System (BIRADS) scale [31, 32]. The clinical and pathological characteristics of the studied group of women are presented in Table 1. The Polish Mother's Memorial Hospital Research Institute Ethical Committee approved the design of the study (No. 10/2012).

Genomic DNA isolation

Genomic DNA extraction was performed from peripheral blood using the commercially available EXTRACTME DNA Blood Kit (Blirt, Poland) according to the manufacturer's instructions. DNA was extracted from oral mucosa swabs, using a commercially available Extractme DNA Swab & Semen Kit (Blirt, Poland), according to the manufacturer's instructions.

Single nucleotide polymorphism genotyping

Three SNPs in the *IGF-1* gene were selected according to the National Center for Biotechnology Information SNPs database: rs6219, rs1520220 and rs2946834. All SNPs were believed to have minor allele frequency (MAF) > 5%. Genotyping of 3 SNPs was performed with the allelic discrimination method using TaqMan probes labeled with VIC and FAM according to the manufacturer's instructions (C__11495137_10, C__2801118_10, C__2861121_10). Polymerase chain reaction (PCR) amplifications were conducted in a total volume of 10 µL and consisted of 5 µL

Table 2. Associations between IGF-1 single nucleotide polymorphisms and mammographic breast density

SNP genotype	Cases (%), controls (%)	OR (95% CI)[a]	*p*	OR (95% CI)[b]	*p*
rs6219					
CC	52 (25.7), 62 (26.0)	1.00 (reference)	0.98	1.00 (reference)	0.92
CT	96 (47.5), 114 (47.9)	1.00 (0.63–1.58)	0.88	1.03 (0.58–1.57)	0.86
TT	54 (26.8), 62 (26.1)	1.04 (0.61–1.74)		1.07 (0.69–1.82)	
p trend[c]	0.89		0.94		
CT or TT vs. CC	150 (74.2), 176 (73.9)	0.98 (0.64–1.50)	0.78	0.98 (0.63–1.50)	0.94
CT or CC vs. TT	148 (73.2), 176 (73.9)	1.06 (0.25–9.17)		1.04 (2.24–9.12)	0.71
rs1520220					
GG	30 (14.8), 41 (17.2)	1.00 (reference)		1.00 (reference)	0.35
GC	68 (33.7), 119 (50.0)	0.78 (0.44–1.37)		0.62 (0.38–1.37)	0.024
CC	104 (51.5), 78 (32.8)	2.41 (1.04–4.19)	0.38	2.43 (1.11–4.52)	
p trend[c]	0.002		0.033		0.007
GC or CC vs. GG[d]	172 (85.1), 197 (82.8)	2.65 (0.73–9.38)	0.012	2.25 (0.73–11.34)	0.25
GC or GG vs. CC[e]	98 (48.5), 160 (67.2)	1.44 (0.71–2.76)	0.31	1.42 (0.72–2.86)	
rs2946834					
GG	69 (34.6), 95 (39.9)	69 (34.6), 95 (39.9)		1.00 (reference)	
GA	102 (34.6), 108 (45.4)	102 (34.6), 108 (45.4)	0.21	1.32 (0.86–1.97)	0.48
AA	31 (30.8), 35 (14.7)	31 (30.8), 35 (14.7)	0.50	1.26 (0.71–2.23)	0.07
p trend[c]	0.08	0.08			
GA or AA vs. GG[d]	132 (65.3), 143 (60.1)	132 (65.3), 143 (60.1)	0.11	2.35 (0.75–4.01)	0.12
GA or GG vs. AA[e]	140 (69.3), 203 (85.2)	140 (69.3), 203 (85.2)	0.15	1.75 (0.87–2.12)	0.15

SNP – single nucleotide polymorphisms, [a] – crude, [b] – adjusted for age, and smoking status, [c] – testing additive genetic model (Cochran-Armitage test for trend), [d] – testing dominant genetic model, [e] – testing recessive genetic model

2x TaqMan Genotyping Master Mix buffer (Thermo Fisher Scientific, USA), 0.25 µL 40x TaqMan Genotyping Assay (Thermo Fisher Scientific, USA) and 10 ng of genomic DNA. Thermal conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of sequential incubation at 95°C for 15 s and 60°C for 1 min final point measurement of fluorescence [33]. Real-time PCR amplifications and allelic discrimination were performed using Mastercycler ep realplex (Eppendorf, Germany).

Statistical data analysis

Genotype distributions were evaluated for agreement with Hardy-Weinberg equilibrium by the χ^2 test. All genotype distributions of *IGF-1* fit the Hardy-Weinberg equilibrium. Unconditional multiple logistic regression models were used to calculate odds ratios and 95% confidence intervals (CI) for the association of genotype with breast mammographic density. Genotype data were analyzed with the homozygote of the common allele as the reference group. Variants of homozygotes and heterozygotes were combined to evaluate the dominant effect. For each SNP, trend tests were conducted by assigning the values 1, 2, and 3 to homozygous wild type, heterozygous, and homozygous variant genotypes, respectively, and by adding these scores as a continuous variable in a logistic regression model. Reported *p* values were two sided. Probabilities were considered significant whenever the *p* value was lower than 0.05. All analyses were completed using Statistica software (version 13.0, StatSoft Poland).

Results

The number of patients in age ranges, both in the study and control groups, is summarized in Table 1. All of the studied individuals, patients and controls, were Caucasians. Some additional clinical data of the patients in the studied group are presented in Table 1. Genotype and allele distributions in the *IGF-1* gene in 202 MD patients and 238 controls are summarized in Table 2. All genotype and allele frequencies (rs6219, rs1520220, rs2946834) were confirmed as compatible with Hardy-Weinberg equilibrium among the case and controls (all $p > 0.05$). As a result, only rs1520220 SNP in the *IGF-1* gene was significantly related to higher mammographic breast density in this population-based case-control study. The frequency of detected G allele carriage was 31.7% and 42.2% in cases and controls, respectively. C allele carriage was detected in 68.3% of mammographic breast density (MBD) patients and 57.8% of control subjects. The genotype frequency distribution of the rs1520220 SNP was as follows: 17.2% (GG), 50.0% (GC), and 32.8% (CC) in healthy controls and 14.8% (GG), 33.7% (GC), and 51.5% (CC) in patients. Likewise, genotype distribution concerning BMI and using hormonal replacement therapy (HRT) was found to be very similar. *IGF-1* gene SNPs in relation to age, BMI, menarche, pregnancy, HRT, menopausal, and smoking status in cases and controls are shown in Table 3. Significant interactions with age, pregnancy, and menopausal status were observed in cases. It should also be emphasized that MBD individuals ≥ 54 years of age had heterozygosity (GC) at the rs1520220 SNP

Table 3. IGF-1 gene rs1520220 polymorphism in relation to demographic and clinical parameters

Variable		Genotype *n* (%)			*p*[a]
		GG	GC	CC	
MBD cases					
Age	< 54 years	18 (8.9)	23 (11.4)	51 (25.2)	0.03
	≥ 54 years	12 (5.9)	45 (22.3)	53 (26.3)	
BMI	< 24.9	18 (8.9)	50 (24.7)	69 (34.1)	0.37
	≥ 25.0	12 (5.9)	18 (8.9)	35 (17.1)	
Menstruation	< 14	13 (6.4)	32 (15.8)	52 (25.7)	0.79
	≥ 14	17 (8.4)	36 (17.8)	52 (25.9)	
Pregnancy	0–1	21 (10.3)	17 (8.4)	63 (31.2)	0.003
	> 1	9 (4.4)	31 (15.3)	41 (30.4)	
HRT	Yes	13 (6.4)	15 (7.4)	28 (13.9)	0.09
	No	17 (8.4)	53 (26.3)	76 (37.6)	
Menopausal status	Pre- and peri-	16 (7.9)	29 (14.3)	25 (12.4)	0.002
	Post-	14 (6.9)	39 (19.3)	79 (39.2)	
Smoking status	Smokers	15 (7.4)	31 (15.3)	32 (15.8)	0.06
	Non-smokers	15 (7.4)	37 (18.5)	72 (35.6)	
non-MBD controls					
Age	< 54 years	27 (11.3)	48 (20.2)	6 (2.5)	< 0.001
	≥ 54 years	14 (5.9)	71 (29.8)	72 (30.3)	
BMI	< 24.9	16 (6.7)	55 (23.1)	31 (13.0)	0.57
	≥ 25.0	25(10.5)	64 (26.9)	47 (19.8)	
Menstruation	< 14	8 (3.4)	86 (36.1)	26 (10.9)	< 0.0001
	≥ 14	33 (13.9)	33 (13.9)	52 (21.8)	
Pregnancy	0–1	13 (5.5)	70 (29.4)	18 (7.6)	< 0.0001
	> 1	28 (11.8)	49 (20.6)	60 (25.1)	
HRT	Yes	16 (6.7)	37 (15.5)	18 (7.6)	0.18
	No	25 (10.5)	82 (34.4)	60 (25.3)	
Menopausal status	Pre- and peri-	11 (4.6)	37 (15.5)	18 (7.6)	0.46
	Post-	30 (12.8)	82 (34.4)	60 (25.1)	
Smoking status	Smokers	16 (6.7)	29 (12.2)	39 (16.4)	0.002
	Non-smokers	25 (10.5)	90 (37.8)	39 (16.4)	

[a] – χ^2 *test, BMI – body mass index, MBD – mammographic breast density, HRT – hormonal replacement therapy*

Table 4. Associations between clinicopathological characteristics (Breast Imaging-Reporting and Data System) and breast mammographic density

Variable	rs1520220		OR (95% CI)[a]	*p*	OR (95% CI)[b]	*p*
	GG or GC *n* (%)	CC *n* (%)				
BIRADS 3 vs. 4 and 5	21 (10.4), 77 (38.1)	5 (2.5), 99 (49.0)	5.4 (1.94–15.0)	0.001	5.6 (1.82–16.3)	0.001

[a] *– crude,* [b] *– adjusted for age, and smoking status, BIRADS – Breast Imaging-Reporting and Data System*

(22.3%), while in younger women this genotype was observed in 11.4%. Homozygosity (GG) was observed in 4.4% of patients with > 1 pregnancy and 10.3% of patients with ≤ 1 pregnancy. However, in the case of the GC genotype, we found an inverse relationship (15.3% and 8.4% respectively). The CC genotype was most common in postmenopausal MBD patients (39.2%). In the control group, statistically significant differences in the distribution of genotypes were observed with regard to age, menarche, number of pregnancies, and smoking status (Table 4).

Our findings reinforce the association of *IGF-1* (rs1520220) with mammographic breast density. In the study population, a pathogenic link between the rs6219 and rs2946834 variants and MBD was not detected. The genetic association analysis revealed that the presence of CC genotype at the *IGF-1* gene was at higher risk with an approximately 2.43-fold increase for the development of higher mammographic breast density. Moreover, confirmation of one copy of the risk allele C at this locus (rs1520220) of the IGF-1 gene conferred an estimated increase risk of MBD of almost 2.25-fold in the model adjusted for age and smoking status (OR = 2.25; 95% CI: 0.73–11.34), $p_{dominant} = 0.007$). The atypical homozygotes (CC rs1520220) appeared to have a higher BIRADS category (3 vs. 4 and 5) (OR = 5.6; 95% CI: 1.82–16.3, $p = 0.001$).

Discussion

In the Introduction we cited a variety of current literature to present compelling evidence that polymorphisms

of *IGF-1* play a considerable role in breast morphology [34–36]. Some genotypes of these polymorphisms have already been studied in breast density, but this area of research remains mostly unexplored [37–44].

In experimental research by Al-Zahrani *et al*., five *IGF-1* gene polymorphisms – rs5742678, rs5742694, rs1520220, rs6220 and rs2946834 – showed a statistically significant relationship with the change of IGF-1 level in women, but not in men [45]. The less frequent alleles of these polymorphisms were associated with high IGF-1 levels. The two SNPs rs5742615 and rs1549593 did not correlate with IGF-1 levels in menopausal women, but were associated with high levels of this protein in the age-matched group of men. The rs6214 and rs6219 polymorphisms were not related to IGF-1 levels in either sex [45].

Research has shown that gene polymorphisms in the IGF pathway are related to breast density or IGF-1 levels. Genetic analyses have provided support for the hypothesis that some elements of the IGF pathway may influence the risk of breast cancer and this effect is a result of their influence on the morphogenesis of breast tissues [27]. Genetic variants of *IGF* polymorphisms play a significant role in mammographic breast density development [46–50].

In our study we found a relationship between the rs1520220 polymorphism and the presence of mammographic breast density. The rs1520220 polymorphism is located in intron 3 of the *IGF-1* gene [51]. Literature data indicate that rs1520220 affects the level of IGF-1 protein, encoded by this gene in breast tissues [29, 52]. The rs1520220 polymorphism in intron 3 is associated with high IGF-1 levels and hence a high risk of breast cancer [53–55]. A significant relationship of this polymorphism with IGF-1 levels has been demonstrated in a population of women from the United Kingdom [21, 52]. It has been shown that the rs1520220 polymorphism of the *IGF-1* gene is associated with high breast density [23, 51]. However, there are reports that in women who are carriers of the rs1520220 polymorphism, the breast density is low [24, 30, 56].

World literature data indicate that in premenopausal women polymorphic variants of the *IGF* pathway genes such as *IGF-I*, *IGFBP-3*, *IRS1*, and *PI3KCB* may affect breast density and the level of growth factors. This suggests that components of the IGF system may be involved in the development of breast cancer [55].

In the present study the rs2946834 polymorphism located in the 3′ untranslated region of the *IGF-1* gene was analyzed. This is an important region where there are various signal sequences such as sequences of the signal for polyadenylation, usually AAUAAA, sequences affecting the location of mRNA in the cell, sequences affecting the stability of mRNA (e.g. AURE sequences rich in adenine and uracil), and sequences affecting translation and miRNA binding sites [57–61].

The rs2946834 polymorphism is related to the level of IGF-1 in women with breast cancer in the UK population [21]. However, it has not been shown to be directly related to the risk of breast carcinoma developing. Kelemen *et al*. found that the frequency of the A allele was inversely proportional to the breast density in pre- and postmenopausal women [62].

In the present study, the rs2945834 polymorphism was not associated with mammographic breast density.

Moreover, no relationship between the rs6219 polymorphism and mammographic breast density was observed. A significant relationship with IGF1 levels was found for the rs6219 polymorphism in a population of 2,395 European women (EPIC study) and the rs5742678 polymorphism in a group of 420 women from the United Kingdom [21, 52, 63].

Finally, there are some obvious limitations of our study that need to be mentioned and clarified. The dominant shortcoming of our analysis is the group size: inclusion of 202 cases and 238 controls (440 assays in total) makes an experienced researcher draw final conclusions with care and scepticism. Especially in genetics, our groups may be quantitatively insufficient to make a reliable conclusion.

Having in mind all the above-mentioned findings and being aware of the restrictions of our study, we believe that this research has shed some new light on mammographic breast density.

Conclusions

In conclusion, accumulating evidence indicates that the genetic variation of *IGF-1* may contribute to the pathogenesis of mammographic density. The variant genotype of rs1520220 in the *IGF-1* gene is strongly associated with the percentage mammographic density in the Polish population.

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