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IGFBP1 and *IGFBP3* polymorphisms predict circulating IGFBP-3 levels among women from high-risk breast cancer families

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Abstract

Purpose: The insulin-like growth factor (IGF) pathway has been implicated as risk modifier in premenopausal breast cancer. In this study, we investigated associations between single nucleotide polymorphisms (SNPs) and diplotypes in the *IGFBP1* and *IGFBP3* genes and circulating IGFBP-3 levels, *BRCA* family status and breast cancer among women from high-risk breast cancer families. **Methods:** Nine *IGFBP1* and *IGFBP3* SNPs were genotyped with PCR based methods in 323 women. Nine *IGFBP1* and ten *IGFBP3* diplotypes were identified. Plasma IGFBP-3 levels obtained during cycle day 18-23 were available for 231 women, 87 current users of combined oral contraceptives and 144 non-users. **Results:** *IGFBP1* (rs1995051 and rs4988515) and *IGFBP3* (rs2471551 and rs2854744) SNPs were associated with circulating IGFBP-3 levels ($P<0.05$). $IGFBP1^{low}$ diplotypes were associated with lower IGFBP-3 levels and were more common in *BRCA2* families OR 2.05 (95%CI 0.97-4.30). $IGFBP3^{high}$ diplotypes were associated with higher IGFBP-3 levels and were more common in *BRCAX* families OR 1.68 (95%CI 1.04-2.74). After adjusting the models for *BRCA* family status, both the *BRCA1* and *BRCA2* family status ($P\leq0.006$) and the *IGFBP1* diplotype GTAC/ACAT ($P=0.004$) were associated with lower IGFBP-3 levels. Similarly, both the *BRCA1* and *BRCA2* family status ($P\leq0.03$) and the IGFBP-3 diplotypes GCA/GCG ($P=0.007$) and GCG/CCG ($P=0.002$) were significantly associated with lower IGFBP-3 levels, adjusted for age, weight, OC use, and other *IGFBP* diplotypes. No individual SNP was associated with breast cancer. There were 23 cases of breast cancer and one *IGFBP1* diplotype was associated with a decreased risk of breast cancer after age 18 (log rank $P=0.05$). **Conclusion:** We observed independent effects from *IGFBP1, IGFBP3* diplotypes and *BRCA* family status on IGFBP-3 levels. These factors may influence the risk of breast cancer among women from high-risk breast cancer families.

Introduction

Breast cancer is the most commonly diagnosed cancer among women in the Western world and the lifetime risk is approximately 12%. Women with one first-degree relative with breast cancer have an approximately 2-fold greater risk compared to women in the general population. Mutations in the high-penetrance *BRCA1* or *BRCA2* genes confer 60-80% lifetime risk of breast cancer [1]. Still, >50% of the genetic predisposition to hereditary breast cancer remains unexplained and the influence of other low-penetrance genetic or non-genetic factors is under intense investigation [2].

Insulin-like growth factor I (IGF-I) is a polypeptide growth hormone that promotes cell proliferation and inhibits apoptosis of both normal and malignant breast epithelial cells [3]. Given the high circulating IGF-I concentration and wide tissue distribution, tight regulation and control of its actions is required to maintain homeostasis. The vast majority of circulating IGF-I is bound to IGF binding proteins, predominantly IGFBP-3 in complex with an acid-labile subunit, which restrict the bioactive IGFs and limit their interaction with receptors. Dysregulation of this control resulting in increased IGF-I levels or altered IGF-I/IGFBP-3 ratios may contribute to an increased risk of breast cancer. IGFBP-3 has a well-defined role in sequestering IGF-I and thereby attenuating its mitogenic actions. However, in addition to this regulatory role on IGF-I actions, IGFBP-3 may exert IGF-I independent effects promoting cellular growth [4,5]. High circulating IGFBP-3 levels have been associated with proliferative benign breast disease and increased breast cancer risk [6,7].

Non-genetic factors such as oral contraceptives (OC) have been shown to increase IGFBP-3 levels in most women [8,9]. In addition to lifestyle and environmental factors, IGFBP-3 plasma levels are likely to be influenced by genetic variation in the *IGFBP1* and *IGFBP3*

genes [10]. The gene for *IGFBP3* is located at chromosome 7p14-p12, in a tail-to-tail configuration with *IGFBP1* at 7p13-p12 [11]. Twin studies indicate that genetic variants may account for up to 60 % of the inter-individual variation in circulating IGFBP-3 levels [12-14]. Our group has previously reported significant associations between the AA genotype of the *IGFBP3* SNP rs2854744 (A-202C) in the promoter region of *IGFBP3* and higher circulating IGFBP-3 levels, especially in women from *BRCAX* families, in the present study population [8]. This polymorphism may directly influence *IGFBP3* gene promoter activity [15,16]. To our knowledge, associations between multiple genetic polymorphisms and diplotypes in *IGFBP1* and *IGFBP3* and circulating IGFBP-3 levels among women from *BRCA1, BRCA2* or *BRCAX* families have not been previously investigated. The aims of present study were to examine the associations between genetic variation in the *IGFBP1* and *IGFBP3* genes and IGFBP-3 plasma levels, *BRCA* status, and breast cancer risk among women from high-risk breast cancer families.

Materials and methods

Study Population

The study population included 323 women from 192 families from two inclusion arms, all belonging to high-risk breast cancer families in the South Swedish Health Care Region with DNA available for genotyping. The first inclusion arm has been described in detail elsewhere [17]. Briefly, 267 young healthy women with no previous cancer history or prophylactic mastectomy or bilateral oophorectomy were enrolled in the study between 1996-2006. Eligible participants had to belong to high-risk breast cancer families and be either 1) known *BRCA1* or *BRCA2* mutation carriers or 2) first or second-degree relatives of a breast cancer case or 3) first or second-degree relatives of a known male or female *BRCA1* or *BRCA2* mutation carrier. In the second inclusion arm an additional 40 *BRCA1* and 16 *BRCA2* mutation carriers born between 1950-1988 were included irrespective of cancer status, in order to include all *BRCA1/2* mutation carriers in the South Swedish Health Care Region with DNA available for SNP testing. Information on *BRCA* mutation status was obtained through medical records from the Oncogenetic Clinic at the Department of Oncology, Lund. Written informed consent was obtained from all participating women and the study was approved by the local ethics committee at Lund University. Extensive questionnaire data with information on reproductive factors, the use of combined oral contraceptives (OC) and other medications etc. were obtained from the majority of cohort members. Characteristics of the women are presented in Table 1.

IGFBP-3 plasma levels

IGFBP-3 levels were measured in plasma samples obtained between 07:15 am and 12:15 pm 5-10 days prior to the predicted onset of the next menstrual period, i.e. during cycle day 18-23 in most women, using the IMMULITE 2000 IGFBP-3 enzyme-labeled chemiluminiscent

immunometric assay (Siemens) in Uppsala University Hospital (Uppsala, Sweden) as previously described [8]. The assay sensitivity was 0.02 μg/ml. The intra-assay and inter-assay variation was 4.1% and 7.3%, respectively.

SNP selection and genotyping

Four *IGFBP1* (rs1995051, rs3763497, rs1065780, and rs4988515) in one haplotype block and three *IGFBP3* haplotype-tagging (ht)SNPs (rs2471551, rs2854744, and rs2132572) in another haplotype block were selected to capture 95% of the genetic diversity of the *IGFBP1* and *IGFBP3* genes in a Swedish population, based on personal communication with Dr Mattias Johansson (International Agency for Research against Cancer, IARC, Lyon, France) and data from a Swedish cancer cohort [18]. Two additional *IGFBP3* SNPs (rs6670 and rs2453839) between the blocks were also included.

Genomic DNA was extracted from peripheral blood using Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). Genotyping was performed in the Department of Genotyping and Sequencing at Region Skåne's Competence Center of Clinical Research (RSKC, Malmö, Sweden).

The SNP (rs3763497, rs1065780, rs4988515, rs6670, rs2471551, rs2854744, and rs2132572) analyses were performed on a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry on a Sequenom MassARRAY® platform (Sequenom, San Diego, CA, USA), using iPLEX reagents according to the manufacturers' protocol. The Sequenom MassARRAY® designer software was used for multiplex SNP analysis design. The SNPs (rs1995051 and rs2854744) were genotyped using a Taqman SNP allelic discrimination assay in 384-well format on ABI PRISM 7900 Sequence Detection System (Applied Biosystems,

Foster City, CA, USA). Six out of 323 women were not successfully genotyped for *IGFBP3* rs2854744 SNP using TaqMan. For those six women, rs2854744 genotypes were available from iPLEX and DNA sequencing from the previous study [8] (100% concordance) and used in the study models. For quality control of genotype data, more than 10% of the samples were run in duplicates, with 100% concordance. The genotyping success rate was ≥ 95% for all SNPs.

Diplotype assignment

The htSNPs were used to predict the most likely haplotypes and their corresponding diplotypes, i.e. paired haplotypes. Each of the htSNPs within *IGFBP1* or *IGFBP3* was cross-tabulated against the remaining htSNPs within their respective genes to generate possible linkage combinations and to identify non-existing or unlikely combinations. The most likely haplotypes were created and compared with previously published data [18]. The women were subsequently assigned to their most likely diplotypes. Diplotypes with an assignment of five women or fewer were combined together into a composite "rare diplotypes" category.

Follow-up

Women were considered at risk for breast cancer from age 18 and were followed until the development of a first breast cancer according to the Regional Cancer Registry, until the date of a self-reported prophylactic mastectomy or oophorectomy, or until May 31 2009, whichever came first. The women in the study who were considered to have a high-risk of developing breast cancer were offered clinical follow-up including annual mammograms, ultrasounds or magnetic resonance imaging and physical examination of the breasts. The report rate of the Swedish cancer registries is close to 100%.

Statistical Analysis

The statistical software SPSS 17.0 was used for most analyses and the multivariate linear regression models were also adjusted for family clustering by using the cluster option of the regress command in STATA. For analyses of IGFBP-3 levels, we excluded women who were breast-feeding at blood draw (n=4), women using hormonal contraceptives other than combined OCs (n=19), or both (n=1), as well as one woman who had not answered the question on hormonal contraceptive use, leaving 87 current OC users and 144 non-users. For SNP and diplotype analyses, frequencies and associations with circulating levels were calculated for all women as well as stratified according to OC status. Multivariate linear regression models were used to estimate the standardized [age(29 years), weight(ln67 kg) and no current OC use] mean IGFBP-3 levels for each SNP. Non-linear additive effects for the minor allele were accounted for in the analyses through the use of dummy variables for each copy of the minor allele and the wild type allele as reference. An interaction term between OC status and cumulative number of SNP alleles were created.

Non-standardized means for circulating IGFBP-3 levels for each diplotype were obtained using one-sample t-tests. Kaplan-Meier survival analyses were used to investigate incident breast cancers after 18 years of age in relation to htSNPs or diplotypes. For healthy women, we censored at the woman's age on May 31 2009, or at the age of a prophylactic mastectomy. Nominal *P*-values are presented. All *P*-values were two-tailed.

Results

Frequencies of htSNPs in IGFBP1 and IGFBP3 and associations with IGFBP-3 levels

Several SNPs in *IGFBP1* and *IGFBP3* were associated with circulating IGFBP-3 levels (Table 2). The strongest associations with IGFBP-3 levels were observed with *IGFBP1* (rs1995051 and rs4988515) and *IGFBP3* (rs2471551 and rs2854744) ($P<0.05$ for all). The minor alleles of *IGFBP1* SNPs rs1995051 and rs4988515 were associated with lower IGFBP-3 levels. These minor alleles segregated at a higher frequency among women from *BRCA2* families (35.5% and 20.6%, respectively) than among women from *BRCA1* (25.4% and 7.5%, respectively) or *BRCAX* families (27.3% and 10.9%, respectively) (additional data given in Online Resource 1). Similarly, the heterozygous variant of the *IGFBP3* rs2471551 was associated with lower IGFBP-3 levels. In contrast, the minor allele of the *IGFBP3* rs2854744 was associated with higher IGFBP-3 levels. Women from *BRCA2* families were less frequently homozygous for this variant allele (5.9%) than women from *BRCA1* or *BRCAX* families (12.9% or 18.2%, respectively) (additional data given in Online Resource 1). These four SNPs were associated with changes in mean circulating IGFBP-3 levels ranging from 4% (rs2471551) to 12% (rs2854744). However, only rs2854744 remained significantly associated with circulating IGFBP-3 levels (nominal $P=0.0002$) after adjustment for multiple testing. The differences in standardized mean IGFBP-3 levels according to rs2854744 genotype were 271 ng/ml (6%, $P=0.009$) for heterozygotes (*CA*) and 547 ng/ml (12%, $P=0.0002$) for homozygotes (*AA)* compared with the wild type (*CC*).

OC use influences IGFBP-3 levels and was considered as effect modifier of the SNP and IGFBP-3 level relationship. Hence, analyses were also stratified for OC use (Table 2). The mean IGFBP-3 levels were lower in the 144 non-users 4768 ng/ml (95%CI: 4636-4899) than

in the 87 current OC users 5190 ng/ml (95%CI: 5039-5341). An interaction was observed between cumulative number of *IGFBP1* SNP (rs3763497) and OC use ($P_{interaction}$=0.034).

Diplotype associations with circulating IGFBP-3 levels

Eight *IGFBP1* and nine *IGFBP3* diplotypes were created from four *IGFBP1* and three *IGFBP3* htSNPs, respectively, based on assignment to >5 women (Figure 1). One additional composite group of each rare *IGFBP1* and rare *IGFBP3* diplotypes (≤5 women) were included in the analyses. Two *IGFBP3* SNPs outside of the haplotype blocks (rs6670 and rs2453839) were excluded from the diplotype analyses.

Four *IGFBP1* diplotypes and four *IGFBP3* diplotypes were associated with changes in mean IGFBP-3 levels of >200 ng/ml relative to the mean level of 4927 ng/ml for all women (Figure 1). Among the four *IGFBP1* diplotypes, GTAC/ACAT and the composite rare *IGFBP1* diplotypes group were associated with decreased mean IGFBP-3 levels (-654 and -427 ng/ml, respectively), while two *IGFBP1* diplotypes (GCGC/GCGC and GTAC/GTAC) were associated with increased mean IGFBP-3 levels (201 and 227 ng/ml, respectively). Among the *IGFBP3* diplotypes, GCG/CCG and GCA/GCG were associated with decreased IGFBP-3 levels (-488 and -301 ng/ml, respectively), while GAG/GAG and the composite rare *IGFBP3* diplotypes group were associated with increased IGFBP-3 levels (314 and 673 ng/ml, respectively). Using linear regression models, the adjusted mean IGFBP-3 level associated with the *IGFBP1* diplotype GTAC/ACAT was significantly lower compared with the reference diplotype GCGC/GTAC (*P*=0.004), adjusted for age, weight, OC use and other *IGFBP1* diplotypes. Similarly, the adjusted mean IGFBP-3 levels associated with the *IGFBP3* diplotypes GCA/GCG and GCG/CCG were significantly lower compared with the reference diplotype GAG/GCA (*P*=0.004 and *P*=0.002, respectively). The associations between

diplotypes and IGFBP-3 levels differed between OC users and non-users (Figure 1). The *IGFBP1* diplotype GCGC/ACGC had opposing associations on IGFBP-3 levels dependent on OC use, while the IGFBP-3 levels in GTAC/ACGC carriers was similar irrespective of OC status.

After adjusting the models for *BRCA* family status, both the *BRCA1* and *BRCA2* family status ($P \leq 0.006$) and the *IGFBP1* diplotype GTAC/ACAT ($P=0.004$) were associated with lower IGFBP-3 levels, adjusted for age, weight, OC use, and other *IGFBP* diplotypes. Similarly, both the *BRCA1* and *BRCA2* family status ($P \leq 0.03$) and the *IGFBP3* diplotypes GCA/GCG ($P=0.007$) and GCG/CCG ($P=0.002$) were significantly associated with lower IGFBP-3 levels, adjusted for age, weight, OC use, and other IGFBP diplotypes. This suggests independent effects from *IGFBP1*, *IGFBP3* diplotypes and *BRCA* family status on IGFBP-3 levels. After adjustment for family clustering, all statistical associations between *IGFBP1* and *IGFBP3* diplotype data and IGFBP-3 levels remained.

Diplotype co-segregation with BRCA1/2 mutation status

We examined possible co-segregation of diplotype frequencies and *BRCA* family status in all women (Figure 2a and 3a) and in the first included woman from each family (Figure 2b and 3b). GCGC/GTAC was the most common *IGFBP1* diplotype across the *BRCA1*, *BRCA2* and *BRCAX* families (Figure 2a and b). As previously reported IGFBP-3 levels were higher in *BRCAX* than in *BRCA1/2* families [8]. Based on the corresponding IGFBP-3 levels the diplotypes were sub-grouped into $IGFBP1^{low}$, $IGFBP1^{high}$, $IGFBP3^{low}$ and $IGFBP3^{high}$ compared with the overall mean IGFBP-3 level. No evident co-segregation of *IGFBP1* or *IGFBP3* diplotypes was observed among the *BRCA1* families. Women from *BRCA2* families more frequently carried *IGFBP1* diplotypes associated with lower IGFBP-3 levels (60.6%)

OR 2.05 (95%CI 0.97-4.30) than women from *BRCA1* or *BRCAX* families (40.8% or 45.5%, respectively) (Figure 2a). In contrast, *IGFBP3* diplotypes associated with higher IGFBP-3 levels (*IGFBP3*^{high}) co-segregated among *BRCAX* families (57.3%) OR 1.68 (95%CI 1.04-2.74) compared with women from *BRCA1* or *BRCA2* families (45.1% or 41.2%, respectively) (Figure 3a).

Diplotypes associated with breast cancer

Against a background of a limited number of breast cancer cases (n=23), there was a non-significant tendency towards increased breast cancer incidence among women carrying a combination of *IGFBP1*^{high} and *IGFBP3*^{high} diplotypes (9/88, 10.2%) compared with *IGFBP1*^{low} and *IGFBP3*^{low} diplotypes (4/68, 5.9%). The incidences of breast cancer found among women carrying *IGFBP1*^{low}/*IGFBP3*^{low} diplotypes (4/69, 5.8%) and *IGFBP1*^{high}/*IGFBP3*^{low} diplotypes (6/91, 6.6%) were similar to the incidence of breast cancer among women with the *IGFBP1*^{low}/*IGFBP3*^{low} diplotypes. Among the 23 breast cancer cases, nine (39.1%) carried a *IGFBP1*^{high}/*IGFBP3*^{high} diplotype and only four (17.1%) carried a *IGFBP1*^{low}/*IGFBP3*^{low} diplotype (Figure 4a). One *IGFBP1* diplotype (GTAC/ACGC) showed a tendency towards being associated with decreased risk of breast cancer (log rank *P*=0.050) (Figure 4b).

Discussion

To our knowledge, this is the first study to examine genetic variants (SNPs) and diplotypes in the *IGFBP1* and *IGFBP3* genes in relation to both circulating IGFBP-3 levels and risk of breast cancer among women from *BRCA1, BRCA2* or *BRCAX* families. Two SNPs in *IGFBP1* and two SNPs in *IGFBP*-3 were associated with circulating IGFBP-3 levels, but no associations between individual SNPs and breast cancer incidence were found. Several diplotypes in *IGFBP1* and *IGFBP3* were associated with circulating IGFBP-3 levels. Breast cancer incidence was non-significantly higher among women carrying a combination of *IGFBP1*^{high}/*IGFBP3*^{high} diplotypes associated with higher circulating IGFBP-3 levels. The frequency of *IGFBP1*^{high} and *IGFBP3*^{high} diplotypes differed between women from *BRCA1, BRCA2* or *BRCAX* families, which were further reflected in significant differences in mean circulating IGFBP-3 levels.

The IGF axis plays important roles in normal physiology by regulating cell proliferation, differentiation and apoptosis [3]. In addition, considerable evidence from laboratory, clinical and epidemiological research demonstrates important roles of the IGF-I and its major binding protein IGFBP-3 in the development and progression of several tumor types, including breast cancer [3]. Large inter-individual variations in circulating levels of IGF-I and IGFBP-3 exist, which in part may be related to different genotypes. Previous studies have evaluated associations between circulating IGFBP-3 levels and risk of sporadic breast cancer in relation to individual *IGFBP1* and *IGFBP3* SNPs or haplotypes and reported conflicting results. Although many SNPs have been associated with the corresponding IGFBP-3 plasma levels, their respective associations with breast cancer risk have been inconsistent across studies. Reports from the Nurses Health Study II and from the NCI Breast and Prostate Cancer Cohort Consortium found associations between *IGFBP3* polymorphisms and circulating IGFBP-3

levels, but no substantial influence on breast cancer risk [19,20]. In some studies, genetic variants and IGFBP-3 levels were inversely associated with breast cancer risk [21,22], while in other studies IGFBP-3 levels were positively associated with breast cancer risk [7,23]. In addition, meta-analyses report that high circulating IGFBP-3 levels were associated with increased risk of pre-menopausal breast cancer [24,25].

Our results suggest that women carrying the heterozygous variant of *IGFBP1* SNPs rs1995051, rs4988515 or *IGFBP3* SNP rs2471551 have lower IGFBP-3 levels, consistent with results observed by others [26]. Notably, these heterozygous variants all co-segregated among women from *BRCA2* families. A recent study reported positive associations for the rare allele of the extensively studied *IGFBP3* rs2854744 (A-202C) with both IGFBP-3 levels and proliferative benign breast disease, a marker of increased breast cancer risk [6]. The higher circulating IGFBP-3 levels observed with the AA genotype are consistent with the functional differences between the A and C alleles indicated *in vitro*, describing significantly higher promoter activity of the A allele compared to the C allele [15,16]. Consistent with these findings, we have previously reported that women carrying the AA genotype have higher IGFBP-3 levels and that this genetic variant segregated at a higher frequency among women from *BRCAX* families in the present study population [8], while the CC genotype segregated among women from *BRCA1* families [8]. However, there were residual effects of BRCA family status on IGFBP-3 levels beyond the effect of the rs2854744 genotype and beyond the effects of diplotypes.

Although mutations in the high-penetrance *BRCA1* and *BRCA2* genes predispose to early-onset breast cancer, considerable individual variation in tumor incidence and onset exist. In addition, only one-third of women from Swedish high-risk breast cancer families carry

disease-causing mutations in *BRCA1* and *BRCA2*. Considering that differences in individual breast cancer susceptibility probably result from the additive effect of multiple genetic variants, where each variant contribute but a modest risk increase [2], we investigated associations between *in vivo* IGFBP-3 levels and *IGFBP1* and *IGFBP3* diplotypes. The diplotype analyses revealed significant variation in circulating IGFBP-3 levels between the highest and lowest *IGFBP1* diplotypes (range ~900 ng/ml). Interestingly, the homozygous *IGFBP1* diplotypes were all associated with the highest IGFBP-3 levels. Similar variation was observed for the *IGFBP3* diplotype associations with circulating IGFBP-3 levels (range ~1100 ng/ml). Two out of three homozygous *IGFBP3* diplotypes, of which one contained the AA genotype of *IGFBP3* SNP rs2854744, were associated with the highest IGFBP-3 levels.

By applying diplotype analyses in preference to individual SNP analyses, we found a tendency towards increased breast cancer incidence among women carrying a combination of *IGFBP1*^{high}/*IGFBP3*^{high} diplotypes. The higher frequency of *IGFBP3*^{high} diplotypes among *BRCAX* families implies that the subgroup of these women who carry a combination of *IGFBP1*^{high}/*IGFBP3*^{high} diplotypes may have an increased breast cancer risk related to high IGFBP-3 levels. This is the first study where *IGFBP1* and *IGFBP3* diplotype analyses have been applied to examine influence on breast cancer risk; previous studies have only examined haplotype data [19-21]. One *IGFBP1* diplotype (GTAC/ACGC) was borderline associated with decreased breast cancer incidence after age 18. However, these observations warrant replication in larger study populations.

Current OC use increases the overall mean circulating IGFBP-3 levels [8,9]. However, the associations between diplotypes and IGFBP-3 levels differed between OC users and non-users. The variations present at position 2 and 3 in the *IGFBP1* GCGC/ACGC and

GTAC/ACGC diplotypes suggest that the CC and GG genotypes of these polymorphisms may causally influence IGFBP-3 levels during OC use, or tag for other unknown functional SNPs. These data suggest that women from high-risk families carrying specific combinations of *IGFBP1* and *IGFBP3* diplotypes may be more susceptible to IGFBP-3 level modulation by OC use, which could further modify their risk of breast cancer. Further studies and the identification of causal mechanisms are needed to better understand how these genotypes and diplotypes may modify non-genetic factors related to IGFBP-3.

In conclusion, this is the first study to investigate associations between multiple genetic polymorphisms and diplotypes in *IGFBP1* and *IGFBP3* and circulating IGFBP-3 levels among women from *BRCA1*, *BRCA2* or *BRCAX* families. Our findings suggest that individual SNPs and diplotypes are associated with circulating IGFBP-3 levels. Theses associations vary according to OC status and may influence the risk of breast cancer among women from high-risk breast cancer families. We observed co-segregation of $IGFBP1^{low}$ diplotypes among *BRCA2* families and of $IGFBP3^{high}$ diplotypes among *BRCAX* families. This study highlights the informative advantage of applying diplotype analyses over individual SNPs or haplotypes. Our findings warrant confirmation in independent study populations.

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Conflict of interest

None of the authors declare any conflict of interest

References

1. Narod SA, Foulkes WD (2004) BRCA1 and BRCA2: 1994 and beyond. Nat Rev Cancer.4:665-676
2. Stratton MR, Rahman N (2008) The emerging landscape of breast cancer susceptibility. Nat Genet.40:17-22
3. Pollak MN, Schernhammer ES, Hankinson SE (2004) Insulin-like growth factors and neoplasia. Nat Rev Cancer.4:505-518
4. Rosendahl A, Forsberg G (2004) Influence of IGF-IR stimulation or blockade on proliferation of human renal cell carcinoma cell lines. Int J Oncol.25:1327-1336
5. Martin JL, Lin MZ, McGowan EM, Baxter RC (2009) Potentiation of growth factor signaling by insulin-like growth factor-binding protein-3 in breast epithelial cells requires sphingosine kinase activity. J Biol Chem.284:25542-25552
6. Su X, Colditz GA, Willett WC, Collins LC, Schnitt SJ, Connolly JL, Pollak MN, Rosner B, Tamimi RM (2010) Genetic variation and circulating levels of IGF-I and IGFBP-3 in relation to risk of proliferative benign breast disease. Int J Cancer.126:180-190
7. Key TJ, Appleby PN, Reeves GK, Roddam AW (2010) Insulin-like growth factor 1 (IGF1), IGF binding protein 3 (IGFBP3), and breast cancer risk: pooled individual data analysis of 17 prospective studies. Lancet Oncol.11:530-542
8. Jernstrom H, Sellberg G, Borg A, Olsson H (2006) Differences in IGFBP-3 regulation between young healthy women from BRCAX families and those belonging to BRCA1/2 families. Eur J Cancer Prev.15:233-241
9. Jernstrom H, Deal C, Wilkin F, Chu W, Tao Y, Majeed N, Hudson T, Narod SA, Pollak M (2001) Genetic and nongenetic factors associated with variation of plasma levels of insulin-like growth factor-I and insulin-like growth factor-binding protein-3 in healthy premenopausal women. Cancer Epidemiol Biomarkers Prev.10:377-384
10. D'Aloisio AA, Schroeder JC, North KE, Poole C, West SL, Travlos GS, Baird DD (2009) IGF-I and IGFBP-3 polymorphisms in relation to circulating levels among African American and Caucasian women. Cancer Epidemiol Biomarkers Prev.18:954-966
11. Ehrenborg E, Larsson C, Stern I, Janson M, Powell DR, Luthman H (1992) Contiguous localization of the genes encoding human insulin-like growth factor binding proteins 1 (IGBP1) and 3 (IGBP3) on chromosome 7. Genomics.12:497-502
12. Harrela M, Koistinen H, Kaprio J, Lehtovirta M, Tuomilehto J, Eriksson J, Toivanen L, Koskenvuo M, Leinonen P, Koistinen R, Seppala M (1996) Genetic and environmental components of interindividual variation in circulating levels of IGF-I, IGF-II, IGFBP-1, and IGFBP-3. J Clin Invest.98:2612-2615
13. Pantsulaia I, Trofimov S, Kobyliansky E, Livshits G (2002) Pedigree-based quantitative genetic analysis of interindividual variation in circulating levels of IGFBP-3. J Bone Miner Metab.20:156-163
14. Pantsulaia I, Trofimov S, Kobyliansky E, Livshits G (2005) Genetic regulation of the variation of circulating insulin-like growth factors and leptin in human pedigrees. Metabolism.54:975-981
15. Deal C, Ma J, Wilkin F, Paquette J, Rozen F, Ge B, Hudson T, Stampfer M, Pollak M (2001) Novel promoter polymorphism in insulin-like growth factor-binding protein-3: correlation with serum levels and interaction with known regulators. J Clin Endocrinol Metab.86:1274-1280
16. Paquette J, Bessette B, Ledru E, Deal C (2007) Identification of upstream stimulatory factor binding sites in the human IGFBP3 promoter and potential implication of adjacent single-nucleotide polymorphisms and responsiveness to insulin. Endocrinology.148:6007-6018

17. Jernstrom H, Sandberg T, Bageman E, Borg A, Olsson H (2005) Insulin-like growth factor-1 (IGF1) genotype predicts breast volume after pregnancy and hormonal contraception and is associated with circulating IGF-1 levels: implications for risk of early-onset breast cancer in young women from hereditary breast cancer families. Br J Cancer.92:857-866
18. Johansson M, McKay JD, Rinaldi S, Wiklund F, Adami HO, Gronberg H, Kaaks R, Stattin P (2009) Genetic and plasma variation of insulin-like growth factor binding proteins in relation to prostate cancer incidence and survival. Prostate.69:1281-1291
19. Schernhammer ES, Holly JM, Hunter DJ, Pollak MN, Hankinson SE (2006) Insulin-like growth factor-I, its binding proteins (IGFBP-1 and IGFBP-3), and growth hormone and breast cancer risk in The Nurses Health Study II. Endocr Relat Cancer.13:583-592
20. Patel AV, Cheng I, Canzian F, Le Marchand L, Thun MJ, Berg CD, Buring J, Calle EE, Chanock S, Clavel-Chapelon F, Cox DG, Dorronsoro M, Dossus L, Haiman CA, Hankinson SE, Henderson BE, Hoover R, Hunter DJ, Kaaks R, Kolonel LN, Kraft P, Linseisen J, Lund E, Manjer J, McCarty C, Peeters PH, Pike MC, Pollak M, Riboli E, Stram DO, Tjonneland A, Travis RC, Trichopoulos D, Tumino R, Yeager M, Ziegler RG, Feigelson HS (2008) IGF-1, IGFBP-1, and IGFBP-3 polymorphisms predict circulating IGF levels but not breast cancer risk: findings from the Breast and Prostate Cancer Cohort Consortium (BPC3). PLoS One.3:e2578
21. Al-Zahrani A, Sandhu MS, Luben RN, Thompson D, Baynes C, Pooley KA, Luccarini C, Munday H, Perkins B, Smith P, Pharoah PD, Wareham NJ, Easton DF, Ponder BA, Dunning AM (2006) IGF1 and IGFBP3 tagging polymorphisms are associated with circulating levels of IGF1, IGFBP3 and risk of breast cancer. Hum Mol Genet.15:1-10
22. Qiu LX, Yao L, Yuan H, Mao C, Chen B, Zhan P, Xue K, Zhang J, Hu XC (2010) IGFBP3 A-202C polymorphism and breast cancer susceptibility: a meta-analysis involving 33,557 cases and 45,254 controls. Breast Cancer Res Treat.
23. Yu H, Jin F, Shu XO, Li BD, Dai Q, Cheng JR, Berkel HJ, Zheng W (2002) Insulin-like growth factors and breast cancer risk in Chinese women. Cancer Epidemiol Biomarkers Prev.11:705-712
24. Renehan AG, Zwahlen M, Minder C, O'Dwyer ST, Shalet SM, Egger M (2004) Insulin-like growth factor (IGF)-I, IGF binding protein-3, and cancer risk: systematic review and meta-regression analysis. Lancet.363:1346-1353
25. Shi R, Yu H, McLarty J, Glass J (2004) IGF-I and breast cancer: a meta-analysis. Int J Cancer.111:418-423
26. Diorio C, Brisson J, Berube S, Pollak M (2008) Genetic polymorphisms involved in insulin-like growth factor (IGF) pathway in relation to mammographic breast density and IGF levels. Cancer Epidemiol Biomarkers Prev.17:880-888

Figure legends

Table 1. Characteristics of all women and of women with available IGFBP-3 levels

Table 2. Associations between *IGFBP1* and *IGFBP3* SNP genotypes and standardized mean IGFBP-3 levels

Figure 1. Associations between common diplotypes in the *IGFBP1* and *IGFBP3* genes with the corresponding mean circulating IGFBP-3 levels (ng/ml) and oral contraceptive (OC) use among women from high-risk breast cancer families. The solid line represents the mean IGFBP-3 level for all women (n=231: 4927 ng/ml), the dashed lines represent the mean IGFBP-3 levels among non-users (n=144: 4768 ng/ml) or current OC users (n=87; 5190 ng/ml), respectively. Shaded areas: 95%CI of the mean plasma IGFBP-3 levels for each group. Results are shown as mean values for each diplotype with 95%CI. The stars represent the corresponding standardized [age(29 years), weight(ln67kg) and current OC use] mean IGFBP-3 levels for each diplotype.

Figure 2. *IGFBP1* diplotype distribution among women from *BRCA* families. Frequency (%) of the *IGFBP1* diplotypes among all women (A) and among the first included woman from each family (B). Results are shown as percentage of women among all *BRCA* families (white bars), *BRCA1* families (grey bars), *BRCA2* families (dashed bars) or *BRCAX* families (black bars).

Figure 3. *IGFBP3* diplotypes distribution among women from *BRCA* families. Frequency (%) of the *IGFBP3* diplotypes among all women (A) and among the first included woman from each family (B). Results are shown as percentage of women among all *BRCA* families (white

bars), *BRCA1* families (grey bars), *BRCA2* families (dashed bars) or *BRCAX* families (black bars).

Figure 4. Associations between *IGFBP1* and *IGFBP3* diplotypes and breast cancer incidence. (A) Diplotype distribution among the 23 breast cancer cases. Results are shown as frequency (%) of the four diplotype combination groups associated with high (*IGFBP1*^{high}/*IGFBP3*^{high}; n=9), intermediate (*IGFBP1*^{high}/*IGFBP3*^{low}; n=6, or *IGFBP1*^{low}/*IGFBP3*^{high}; n=4) or low (*IGFBP1*^{low}/*IGFBP3*^{low}; n=4) circulating IGFBP-3 levels among the 23 breast cancer cases. (B) Kaplan-Meier estimates of breast cancer free survival in relation to the *IGFBP1* diplotype GTAC/ACGC. Only one out of the 52 women carrying this diplotype had developed breast cancer (log rank *P*=0.050). Statistics in the lower part of the plot represent the number of women at each decade after age 18.

Supplementary data: Table 1. Distribution of *IGFBP1* and *IGFBP3* SNP genotypes among all women and women from *BRCA1*, *BRCA2* or *BRCAX* families.