Human Molecular Genetics, 2006, Vol. 15, No. 1 **1–10**
doi:10.1093/hmg/ddi398
Advance Access published on November 23, 2005

IGF1 and IGFBP3 tagging polymorphisms are associated with circulating levels of IGF1, IGFBP3 and risk of breast cancer

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Received September 1, 2005; Revised and Accepted October 14, 2004

Experimental and observational studies in humans and animals suggest that insulin-like growth factor 1 (IGF1) and its principal binding protein, IGFBP3, may influence breast cancer susceptibility. We have examined the association of nine and four single nucleotide polymorphisms (SNPs) in the IGF1 gene and in the IGFBP3 genes, respectively, with circulating levels of their gene products in a population-based study of 600 middle-aged men and women, and in a breast cancer case–control study, comprised 4647 cases and 4564 controls. All study participants are from the East Anglian region of UK. SNPs were specifically chosen to tag all other known SNPs in each gene. Several SNPs in each gene are associated both with circulating levels of their respective proteins and with risk of breast cancer. In particular, the *c* allele of IGF1 SNPrs1520220 is associated with increased circulating IGF1 ($r^2 = 2.1\%$, *P*-trend = 0.003) in females and an increased risk of breast cancer: odds ratio (OR) (*cc*/*gg*) = 1.41; 95% confidence intervals (95% CI) 1.11–1.79, *P*-trend = 0.03. The *a* allele of IGFBP3 SNP rs2854744 is associated with increased circulating IGFBP3 ($r^2 = 9.7\%$, $P < 10^{-9}$) and a decreased risk of breast cancer: OR (*aa*/*cc*) = 0.87; 95% CI 0.77–0.99, *P* = 0.03. Our data indicate that common variants in the IGF1 and IGFBP3 genes are associated with differences in circulating levels of IGF1 and IGFBP3 and with breast cancer risk. More specifically and consistent with experimental models, our data suggest that higher IGF1 levels may increase the risk of breast cancer but higher IGFBP3 levels may be protective.

INTRODUCTION

Insulin-like growth factor 1 (IGF1) is produced predominantly by the liver and has actions similar to both a circulating peptide hormone and a tissue growth factor. Circulating IGF1 is sequestered by insulin-like growth factor binding proteins (IGFBP), principally IGFBP3, which regulate the biological activity of IGF1. Circulating levels of both proteins vary substantially between individuals. Although these variations are dependent, to some extent, on levels of growth hormone, gender, age and nutritional status, levels of both proteins are also under genetic control. Twin studies have indicated that 38% of the variance in IGF1 levels and 60% of that in IGFBP3 is attributable to genetic effects (1).

Experimental evidence indicates that IGF1 has anti-apoptotic and mitogenic actions and may promote tumour growth (2–4). Transgenic mice, over-expressing IGF1, show increased rates of mammary tumour development (5). In contrast, IGF1-deficient (LID) mice have a lower incidence of chemically induced mammary tumours (6). These LID mice

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also exhibit compensatory hyper-secretion of growth hormone and hyper-insulinaemia, which suggests that their decreased rates of tumour development and metastases may be directly attributable to their lower circulating IGF1 levels.

In humans, prospective observational studies have suggested that relatively higher circulating IGF1 levels may be associated with an increased risk of breast cancer. Specifically, a recent meta-analysis of six cohort and case–control studies (7) reported that women with IGF1 levels in the highest quartile may have double the risk of developing pre-menopausal breast cancer compared with those in the lowest. On the basis of this evidence, one would expect that variants in the IGF1 gene that are associated with circulating levels of IGF1 might also be associated with breast cancer risk. As IGFBP3, in part, acts as a sequestering agent for IGF1, higher circulating levels of IGFBP3 might protect against cancer development. Consistent with this biological role, meta-analytical summaries suggest that there is an inverse association between IGFBP3 levels and risk of colorectal and lung cancer, but the same association is not clear for breast cancer. Indeed, the meta-analysis (7) reports a contrary 2-fold increased risk of breast cancer for individuals with IGFBP3 levels in the top quartile compared with those in the lowest. This unexpected positive association between IGBPB3 levels and breast cancer risk has generated speculation that IGFBP3 may have additional IGF-I-independent functions and recent studies even indicate that it may encourage breast cell proliferation (8).

By identifying single nucleotide polymorphisms (SNPs) that are associated with circulating levels of IGF1 and IGFBP3, and evaluating their association with breast cancer risk, it may be possible to evaluate whether the observed associations between circulating protein levels and risk are likely to be causal, rather than due to the effects of confounding or reverse causation—an approach known as Mendelian randomization. We, therefore, assessed the association between tagging SNPs in the IGF1 and IGFBP3 genes with circulating IGF1 and IGFBP3 levels in a cohort of middle-aged women and men, and in a case–control study of breast cancer, comprised 4647 cases and 4564 controls.

RESULTS

IGF1 gene—SNP selection

From the NIEHS re-sequencing data set of 133 SNPs in the IGF1 gene (density: 1 SNP per 0.66 kb), we identified 36 SNPs with a minor allele frequency of >0.05 in the 62 subjects from the polymorphism discovery resource with least evidence of African ancestry. A GOLD plot (9) of these data indicates that all 36 SNPs fall into a single linkage disequilibrium (LD) block: there is strong LD between SNPs across the entire length of the gene with no clear hot-spots for recombination (Supplementary Material, Fig. S1A). The output of the TagSNPs program run on these data is provided in Supplementary Material, Figure S1B. Based on these data, there are 19 predicted haplotypes with frequencies >0.01 and, of these, three have frequencies >0.05. We selected eight SNPs (IGF1-01–IGF1-08, Table 1) as tags for 34 of the full set of 36 SNPs (minimum $R^2_p > 0.77$). Two of the 36 SNPs were imperfectly tagged (dbSNP: rs5742623 and rs3032449) and might be at hypermutable sites. They appear to have arisen on multiple unrelated haplotypes, and are not well tagged by any of the other SNPs. Neither of these SNPs lies in coding regions of the gene and, because carriers of their rare alleles do not necessarily share a common ancestor, we did not undertake further analyses of these SNPs. All haplotypes were tagged with minimum $R^2_h > 0.8$, with the exception of the two haplotypes carrying the potentially hypermutable SNP rs5742623. We also included an additional SNP (IGF1-09), which lies 3′ of the IGF1 gene locus. The positions and assay details of these SNPs are given in Supplementary Material, Table S2.

IGF1 SNPs and plasma IGF1 levels

The nine tagging SNPs were genotyped in the Ely study population. As expected, mean circulating IGF1 levels are statistically significantly higher in males than in females (respective means: 169.3; 95% CI 163.0–175.6 ng/ml versus 147.1; 95% CI 142.9–152.1 ng/ml; $F = 31.7$; $P < 10^{-6}$). For six of the nine SNPs, there is evidence for interaction between IGF1 genotype and gender on IGF1 levels; thus levels are presented for the women and men separately (Table 1).

Five of the nine SNPs (IGF1-02, IGF1-03, IGF1-05, IGF1-06 and IGF1-09) show statistically significant associations with circulating levels of IGF1 in females, but not in males (Table 1). For all five of these SNPs, the rare allele is associated with higher IGF1 levels. These five SNPs are strongly correlated and they tag a tightly overlapping set of haplotypes (Supplementary Material, Fig. S1B). In a multivariate analysis, using the five SNPs that are statistically significant in univariate analyses, only IGF1-05 remains so. SNPIGF1-05 accounts for 2.1% of the total variance (r^2) in circulating IGF1 levels. Relative to the *gg* homozygotes, *gc* heterozygotes have 11% higher circulating IGF1 levels, whereas *cc* homozygotes have 17% higher levels (P-trend $= 0.003$). Thus the effect of the *c* allele on circulating levels fits an additive (co-dominant) model although, using a likelihood ratio test, we cannot formally exclude it from having a dominant mode of action.

SNPs IGF1-01 and IGF1-04 are not associated with significant differences in circulating IGF1 in females (Table 2) but, unexpectedly, both are associated with significantly higher levels of circulating IGF1 in males (P-trend $= 0.02$, $r^2 = 2\%$ and P-trend $= 0.002$, $r^2 = 3\%$, respectively). SNPs IGF1-07 and IGF1-08 are not associated with circulating IGF1 levels in either sex.

IGF1 SNPs and breast cancer susceptibility

The same nine SNPs were genotyped in stage 1 of our breast cancer case–control study. The genotype distributions of seven of these nine SNPs met our criteria for continued evaluation (described in Materials and Methods) and we genotyped these seven SNPs in the full breast cancer case–control study (stages 1 and 2). Five of the seven SNPs (IGF1-02, IGF1-03, IGF1-04, IGF1-05 and IGF1-06) show statistically significant association with breast cancer risk (P-heterogeneity or P-trend < 0.05 after the two stages, Table 2). These associations

Table 1. Mean plasma IGF1 level (ng/ml) by IGF1 genotype

IGF1		Females				Males[a]			
		n	Mean	95% CI	*P*-trend (r^2)[b]	*n*	Mean	95% CI	*P*-trend (r^2)[b]
rs5742615	*cc*	337	147.5	142.0–153.0	0.9	232	167.4	159.6–175.2	0.002
IGF1-01	*ca*	11	161.1	130.7–191.5		11	225.5	189.7–261.4	(3.0%)
	aa	2	115.5	44.1–186.9		0			
rs5742678	*cc*	190	142.6	135.9–149.4	0.009	130	172.7	161.3–184.1	0.5
IGF1-02	*ct*	129	154.9	145.8–163.9	(1.7%)	88	166.1	154.2–177.9	(0.2%)
	tt	27	163.8	137.0–190.6		23	166.1	141.1–191.0	
rs5742694	*tt*	200	143.2	136.6–149.9	0.02	141	175.8	164.6–187.0	0.06
IGF1-03	*tg*	123	151.9	142.5–161.3	(1.4%)	87	163.3	152.0–174.7	(1.0%)
	gg	27	166.6	139.3–193.9		16	153.1	126.1–180.0	
rs1549593	*tt*	255	150.8	144.6–157.1	0.4	177	164.2	155.4–173.1	0.02
IGF1-04	*tg*	82	142.3	130.6–154.1	(0.1%)	56	180.2	163.8–196.5	(2.0%)
	gg	5	163.9	104.8–223.1		6	213.5	124.3–302.7	
rs1520220	*gg*	225	142.9	136.5–149.2	0.003	163	169.8	160.0–179.6	0.9
IGF1-05	*gc*	107	158.8	148.1–169.4	(2.1%)	66	170.6	156.7–184.3	(0.4%)
	cc	13	167.2	134.9–199.6		13	163.4	122.9–203.8	
rs6220	*aa*	208	141.5	135.0–148.0	0.002	128	172.0	160.8–183.1	0.9
IGF1-06	*ag*	162	153.0	145.0–161.0	(2.1%)	114	168.3	157.8–178.9	(0.4%)
	gg	34	168.4	142.3–194.5		27	179.0	146.8–211.2	
rs6214	*gg*	147	143.4	135.1–151.6	0.6	101	168.3	155.1–181.4	0.5
IGF1-07	*ga*	205	153.4	145.8–161.0	(0.2%)	123	167.8	158.3–177.3	(0.2%)
	aa	67	142.9	131.0–154.7		56	175.4	156.3–194.6	
rs6219	*cc*	338	147.3	141.6–152.9	0.8	234	169.9	161.6–178.2	0.8
IGF1-08	*ct*	78	153.8	142.5–165.2	(0.2%)	46	169.0	153.9–184.1	(0.3%)
	tt	4	109.1	65.0–153.2		2	202.5	−769.5–1174	
rs2946834	*gg*	154	140.1	132.9–147.4	0.02	103	166.6	153.7–179.3	0.6
IGF1-09	*ga*	151	155.0	145.9–164.1	(1.3%)	114	175.0	164.0–186.0	(0.3%)
	aa	38	154.8	136.9–172.7		24	166.1	142.6–189.7	

[a]Six of the nine SNPs showed borderline or significant interaction of genotype and gender on IGF1 levels, $P < 0.1$—IGF1-06; $P < 0.05$—IGF1-02, IGF1-05 and rs; $P < 0.01$—IGF1-01, IGF1-03 and IGF1-04.
[b]r^2 values are from univariate analyses of genotypes on mean levels. Mean levels remain very similar after adjustment for age (data not shown).

Table 2. IGF1 SNP associations with breast cancer

SNP		Stage 1					Stages 1 and 2				
IGF1		Cases	Controls	OR (95% CI)	*P*-trend	*P*-het	Cases	Controls	OR (95% CI)	*P*-trend	*P*-het
rs5742615	*cc*	2122	2215	1.00	0.3	0.6					
IGF1-01	*ca*	65	57	1.19 (0.83–1.71)							
	aa	4	4	1.30 (0.35–4.87)							
rs5742678	*cc*	1238	1290	1.00	0.3	0.05	2498	2605	1.00	0.03	0.05
IGF1-02	*ct*	786	850	0.96 (0.85–1.09)			1660	1671	1.04 (0.95–1.13)		
	tt	163	129	1.32 (1.03–1.68)			322	271	1.24 (1.04–1.47)		
rs5742694	*tt*	1202	1303	1.00	0.2	0.03	2476	2660	1.00	0.09	0.04
IGF1-03	*tg*	703	788	0.97 (0.85–1.10)			1485	1588	1.00 (0.92–1.10)		
	gg	143	112	1.38 (1.07–1.79)			274	233	1.26 (1.05–1.52)		
rs1549593	*tt*	1511	1588	1.00	0.08	0.1	3185	3213	1.00	0.02	0.05
IGF1-04	*tg*	493	561	0.92 (0.80–1.06)			1064	1165	0.92 (0.84–1.01)		
	gg	33	51	0.68 (0.44–1.06)			73	99	0.74 (0.55–1.01)		
rs1520220	*gg*	1388	1525	1.00	0.08	0.02	2938	3099	1.00	0.04	0.02
IGF1-05	*gc*	569	617	1.01 (0.89–1.16)			1223	1251	1.03 (0.94–1.13)		
	cc	79	52	1.67 (1.17–2.39)			163	122	1.41 (1.11–1.79)		
rs6220	*aa*	1077	1169	1.00	0.1	0.008	2267	2406	1.00	0.035	0.03
IGF1-06	*ag*	763	868	0.95 (0.84–1.08)			1678	1741	1.02 (0.94–1.12)		
	gg	188	147	1.39 (1.10–1.75)			368	314	1.24 (1.06–1.46)		
rs6214	*gg*	706	705	1.00	0.4	0.15					
IGF1-07	*ga*	987	1130	0.87 (0.76–1.00)							
	aa	347	356	0.97 (0.81–1.17)							
rs6219	*cc*	1693	1820	1.00	0.5	0.06	3580	3706	1.00	0.4	0.1
IGF1-08	*ct*	325	359	0.97 (0.83–1.15)			706	730	1.00 (0.89–1.12)		
	tt	25	12	2.24 (1.12–4.47)			49	32	1.59 (1.01–2.48)		
rs2946834	*gg*	940	966	1.00	0.7	0.9	1931	2050	1.00	0.4	0.7
IGF1-09	*ga*	877	930	0.97 (0.85–1.10)			1825	1889	1.03 (0.94–1.12)		
	aa	213	224	0.98 (0.79–1.20)			451	452	1.06 (0.92–1.22)		

Table 3. Mean plasma IGFBP3 level (mg/l) by IGFBP3 genotype

IGFBP3		Females				Males			
		n	Mean	95% CI	*P*-trend (r^2)[a]	*n*	Mean	95% CI	*P*-trend (r^2)[a]
rs2132571	*gg*		4.051	3.938–4.164	0.0003	136	4.012	3.859–4.165	0.07
IGFBP3-01	*ga*	153	3.816	3.697–3.934	(3.0%)	116	3.842	3.697–3.987	(0.8%)
	aa	40	3.648	3.438–3.857		21	3.762	3.426–4.098	
rs2132572	*gg*	235	4.055	3.956–4.154	0.001	170	4.021	3.889–4.154	0.01
IGFBP3-02	*ga*	150	3.777	3.646–3.907	(2.4%)	91	3.844	3.690–3.998	(1.9%)
	aa	18	3.772	3.348–4.196		12	3.467	2.968–3.965	
rs2854744	*cc*	125	3.614	3.495–3.734	$<10^{-9}$	72	3.628	3.469–3.787	0.00004
IGFBP3-03	*ca*	189	3.941	3.824–4.057	(9.7%)	129	3.964	3.821–4.106	(5.6%)
c-202a	*aa*	87	4.316	4.155–4.478		64	4.202	3.959–4.444	
rs2471551	*cc*	239	3.967	3.865–4.068	0.8	155	3.985	3.844–4.126	0.2
IGFBP3-04	*cg*	96	4.018	3.850–4.185	(0.3%)	82	3.792	3.614–3.969	(0.2%)
	gg	21	3.810	3.404–4.215		9	4.000	3.492–4.508	

[a]r^2 values are from univariate analyses of genotypes on mean levels. Mean levels remain very similar after adjustment for age (data not shown).

appear to fit a recessive genetic model (only the rare homozygotes display an increased risk of breast cancer), although we cannot formally reject a co-dominant model.

With the exception of IGF1-09, all SNPs associated with raised circulating IGF1 levels in women are also associated with increased risk of breast cancer. A fifth SNP (IGF1-04) shows an association with breast cancer susceptibility but is not associated with circulating IGF1 levels in women. The rare allele appears to have a protective effect: relative to the common *tt* homozygotes, *tc* heterozygotes have OR = 0.92 (95% CI 0.84–1.01) and the *cc* group have OR = 0.74 (95% CI 0.55–1.01), *P*-trend = 0.02. In a multivariate analysis of the five SNPs that are statistically significant in univariate analyses, only SNPs IGF1-05 and IGF1-04 remain so, suggesting that these two SNPs may have independent actions.

The haplo.score program predicted nine haplotypes with frequencies greater than 1% in stage 1 (Supplementary Material, Fig. S1A). No individual haplotype showed a significant frequency difference between cases and controls (consistent with no individual SNP or haplotype having a dominant effect on breast cancer risk) and the global test score was 14.2, *P*-value = 0.12 (9 d.f.).

IGFBP3 gene—SNP selection

The IGFBP3 gene has not yet been investigated within NIEHS but the entire coding and promoter regions have been extensively re-sequenced in studies of Silver Russell syndrome. A total of 10 common SNPs have been reported (10–12) on four common haplotypes (12). This gives a density of 1 SNP per 0.9 kb across the gene. We were able to obtain Taqman® assays to tag all four of these haplotypes (Supplementary Material, Fig. S1B). These SNP positions and assay details are given in the Supplementary Material, Table S2.

IGFBP3 SNPs and plasma IGFBP3 levels

All four IGFBP3 SNPs were genotyped in the Ely sample (Table 3). Circulating IGFBP3 levels are not significantly different between males and females (respective means: 3.95; 95% CI 3.84–4.02 mg/l versus 3.96; 95% CI 3.89–4.03 mg/l; $F = 0.21$; $P = 0.6$). Three SNPs (IGFBP3-01, IGFBP3-02 and IGFBP3-03) are associated with circulating levels of IGFBP3.

The most common SNP, IGFBP3-03 (*c-202a*), is most strongly associated with increased circulating IGFBP3 levels ($P < 10^{-9}$, Table 3). In females, the *ac* genotype group have 9% higher and the *aa* group have 19% higher mean IGFBP3 levels than common *cc* homozygotes; thus the effect of the *a* allele best fits a co-dominant model and a recessive mode of action can be formally rejected. This SNP explains 9.7% of the variation in circulating IGFBP3 levels in females and 5.6% in males. The rare alleles of SNPs IGFBP3-01 and IGFBP3-02 are significantly associated with decreased circulating IGFBP3 levels (Table 3) and are again most compatible with a co-dominant mode of action. Supplementary Material, Figure S1B shows that the rare alleles of IGFBP3-01 and IGFBP2-02 are carried exclusively on the haplotypes that do not carry the rare allele of IGFBP3-03. Comparison of models using likelihood ratio tests indicates that the variance in IGFBP3 levels explained by all three associated SNPs could equally well be explained by IGFBP3-03 alone or by IGFBP3-01 and IGFBP3-02 in combination. Thus, the most parsimonious model is that all the genetic variance in circulating IGFBP3 levels, detected in this experiment, can be explained by the effect of SNP IGFBP3-03 (*c-202a*).

IGFBP3 SNPs and breast cancer susceptibility

All four SNPs were tested for association with breast cancer risk in stage 1 of the breast cancer study (Table 4). Three SNPs met our criteria for continuation into stage 2. In the complete study after both stages, two of the four SNPs (IGFBP3-02 and IGFBP3-03) show a statistically significant association with breast cancer risk. The rarer *a* allele of SNP IGFBP3-02 is associated with an increased risk of breast cancer, OR (*ag/gg*) = 1.09 (95% CI 1.00–1.19) and OR (*aa/gg*) = 1.23 (95% CI 1.02–1.48), *P*-trend = 0.01. In contrast, the rare *a* allele of IGFBP3-03(*c-202a*) is associated with a decreased risk. Relative to the common *cc* homozygotes, the *ac* heterozygotes OR = 0.96 (95% CI 0.87–1.06) and the *aa* homozygotes OR = 0.87 (95% CI 0.77–0.99), *P*-trend = 0.03.

Table 4. IGFBP3 SNP associations with breast cancer

IGFBP3		Stage 1					Stages 1 and 2				
		Cases	Controls	OR (95% CI)	*P*-trend	*P*-het	Cases	Controls	OR (95% CI)	*P*-trend	*P*-het
rs2132571	*gg*	982	1065	1.00	0.85	0.95	2061	2161	1.00	0.7	0.9
IGFBP3-01	*ga*	883	955	1.00 (0.88–1.14)			1776	1829	1.02 (0.93–1.11)		
	aa	172	193	0.97 (0.77–1.21)			478	491	1.02 (0.89–1.17)		
rs2132572	*gg*	1186	1355	1.00	0.009	0.03	2507	2709	1.00	0.01	0.03
IGFBP3-02	*ga*	735	747	1.12 (0.99–1.28)			1556	1548	1.09 (1.00–1.19)		
	aa	123	103	1.36 (1.04–1.79)			250	220	1.23 (1.02–1.48)		
rs2854744	*cc*	606	614	1.00	0.09	0.2	1327	1306	1.00	0.03	0.08
IGFBP3-03	*ca*	1023	1087	0.95 (0.83–1.10)			2150	2203	0.96 (0.87–1.06)		
	aa	407	481	0.86 (0.72–1.02)			834	940	0.87 (0.77–0.99)		
rs2471551	*cc*	1307	1436	1.00	0.6	0.5					
IGFBP3-04	*cg*	649	663	1.08 (0.94–1.23)							
	gg	75	88	0.94 (0.68–1.29)							

SNP IGFBP3-01, which is associated with IGFBP3 levels, shows no association with breast cancer risk. However, the upper 95% CI for the OR is 1.11 in heterozygotes and 1.17 in homozygotes, and so we cannot exclude that this SNP is associated with a moderately increased risk.

Gene–gene and gene–menopause interaction studies

We find no evidence that any of the IGF1 gene SNPs are associated with circulating levels of IGFBP3 or the converse—that variants in the IGFBP3 gene affect circulating levels of IGF1 (data not shown). We examined the combined effects of SNPs in IGF1 and IGFBP3 on breast cancer risk using a case-only analysis and considered the two SNPs (IGF1-05 in IGF1 and IGFBP3-03 in IGFBP3) that showed the highest individual statistical significances with breast cancer risk. We found no evidence for departure from a multiplicative effect of their combined action (data not shown) although, with alleles at these frequencies, we had 80% power to detect an interaction OR of 1.5 for departure from multiplicativity.

Previous studies (7) have indicated that raised circulating levels of IGF1 or IGFBP3 increase risk of pre-menopausal breast cancer but do not have an effect in post-menopausal women. We have, therefore, compared the genotype distribution of the three most significantly associated SNPs (IGF1-05, IGFBP3-02 and IGFBP3-03) between pre- and post-menopausal breast cancer cases. We find no statistically significant evidence for any differences, indicating that SNPs in both genes that alter circulating levels of their respective gene products have very similar magnitudes of effect on susceptibility to both pre- and post-menopausal breast cancer (see Table 5).

Consistency of association among SNPs, circulating levels and breast cancer risk

From the meta-analytical summary (7), pre-menopausal women with IGF1 levels in the top quartile were estimated to have a 1.65-fold increased risk of breast cancer over those in the bottom quartile but no significant association was found in post-menopausal women. On the basis of the best estimates from this meta-analysis and taking a weighted average of results in pre- and post-menopausal women according to the proportion of cases in our study, the 17% increase in IGF1 levels (167.2 versus 143.9 ng/ml) associated with SNP IGF1-05 should broadly equate to an OR = 1.07 (95% CI 0.92–1.24) for breast cancer. Our observed OR (*cc/gg*) = 1.41 (95% CI 1.11–1.79) for all cases, with OR = 1.49 (95% CI 1.11–2.02) for pre-menopausal disease. The corresponding predicted OR for heterozygotes is 1.03 (0.96–1.10), which is similar to our observed OR = 1.03 (0.94–1.13). The observed OR for homozygote risk is thus somewhat higher than that predicted; however, the two risk estimates are statistically compatible.

One hypothesis is that IGFBP3 may act to reduce IGF1 bioactivity. Thus IGFBP3 genotypes associated with higher IGFBP3 levels may be expected to be associated with a reduced breast cancer risk. This effect was observed for the SNP most significantly associated with IGFBP3 levels (IGFBP3-03). In contrast, the meta-analysis (7) estimated that pre-menopausal women with IGFBP3 levels in the top quartile have a 1.5-fold increased risk of breast cancer over those in the bottom quartile and again, it found no association in post-menopausal women. On the basis of these estimates, we would predict, for SNP IGFBP3-03, an OR of 1.12 (95% CI 1.08–1.17) in *aa* homozygotes and 1.06 (1.01–1.11) in heterozygotes, whereas we actually observed a decreased risk [OR (*aa/cc*) = 0.87 (95% CI 0.77–0.99) in homozygotes and OR (*aa/cc*) = 0.96 (95% CI 0.87–1.06) in heterozygotes]. Again, although the meta-analysis indicated no effect of circulating levels on post-menopausal breast cancer risk, we found no difference in the ORs between pre- and post-menopausal cases.

DISCUSSION

We have identified common variants in the IGF1 that are associated with both higher circulating levels of IGF1 and a moderately increased risk of breast cancer. Additionally, we have found two promoter variants in the IGFBP3 gene with different effects: one (rs2135372) is associated with lower IGFBP3 levels and increased breast cancer risk, whereas the other (rs2854744, *c*-202*a*) is associated with raised levels and decreased risk. Together, they suggest that higher IGF1 levels may increase the risk of breast cancer, whereas higher IGFBP3 levels may be protective against the disease.

Table 5. Genotype distributions of key SNPs in pre- and post-menopausal breast cancer cases

SNP	Genotype	Pre-menopausal		Post-menopausal		χ^2-test (*P*-value, 2 d.f.)
		Observed	%	Observed	%	
IGF1-05	*gg*	1746	68.1	699	68.7	0.089
rs1520220	*gc*	719	28.1	281	27.6	0.96
	cc	97	3.8	38	3.7	
IGFBP3-02	*gg*	1488	58.2	570	56.1	3.9
rs2132572	*ga*	931	36.4	374	36.8	0.14
	aa	139	5.4	72	7.1	
IGFBP3-03	*cc*	796	31.1	306	30.3	1.18
rs2854744	*ca*	1257	49.2	517	51.1	0.55
	aa	503	19.7	188	18.6	

Our evidence indicates that the associated SNPs confer a very similar risk of both pre- and post-menopausal breast cancer, whereas previous observational studies have indicated that circulating levels of IGF1 and IGFBP3, in pre-menopausal women only, are associated with risk. Our findings are not necessarily incompatible with the previous studies, as all post-menopausal women will have been previously exposed to their own pre-menopausal levels and it may be the circulating levels in this earlier time period that are the predictors of cancer risk throughout life.

IGF1

In the association between IGF1 SNPs and circulating IGF1 levels, we found inconsistent results for men and women. Indeed, we found statistically significant genotype-by-sex interactions. These observations may be false-positives and so they require independent replication. However, this interdependency might reflect *trans*-acting regulators of gene expression that are sex-specific. Men are more responsive than women to growth hormone—a positive regulator of IGF1 expression (13). Intervention studies also suggest that sex steroids may have differential effects on circulating IGF1 levels (14) but the mechanisms underlying these differential effects are not yet understood.

Of the nine IGF1 SNPs studied, five showed statistically significant, co-dominant associations with circulating IGF1 levels and recessive associations with breast cancer risk. The magnitudes of the SNP effects on breast cancer risk are small and will require confirmation in further studies. If these recessive effects are confirmed, they might indicate a threshold effect, whereby risk of breast cancer is only increased when circulating, or local, IGF1 reaches a certain trigger level. One IGF1 SNP (IGF1-04) was not associated with IGF1 levels in women, but showed a statistically significant association with breast cancer risk. This finding may be a false-positive or it may represent an effect of this SNP on cancer risk, which is not mediated through changes in circulating IGF1 levels. In contrast, another SNP (IGF1-09) was found to be modestly associated with circulating IGF1 levels in women, but not with breast cancer risk. Owing to limited statistical power, we cannot formally exclude a modest association between this SNP and breast cancer risk, which therefore could represent a false-negative finding. Consistent with our observations of an association between genetic variation at the IGF1 locus and risk of breast cancer, a recent study in the Chinese population reported a statistically significant association between a repeat-length polymorphism in the promoter region of the IGF1 gene and breast cancer risk (15).

IGFBP3

The biological actions of IGFBP3 are still under investigation. Experimental evidence suggests that IGFBP3 may directly stimulate apoptosis and inhibit cellular proliferation of various cell lines, including some human breast cancer cells (16). However, results from experimental studies also suggest that IGFBP3 might enhance the proliferative effects of IGF1 in breast cancer cell lines (8). Thus IGFBP3 has been variously reported to have pro-apoptotic, anti-apoptotic, proliferative and anti-proliferative actions.

In humans, the prospective association between circulating IGFBP3 levels and breast cancer risk is also uncertain. Summary estimates suggested that relatively higher levels of circulating IGFBP3 may be associated with an increased risk of pre-menopausal breast cancer (7). However, a subsequent prospective study, comparing different peptide assays for IGFBP3, found an inverse association between a circulating levels of IGFBP3, as measured by an assay specific to 'intact/functional forms' of IGFBP3, and pre-menopausal breast cancer risk (17). The inconsistent findings from both the case–control and the prospective cohort studies may also be due to variations in the number of individuals with unidentified pre-existing disease who may distort the findings even in the first years of follow-up of prospective studies. Studies assessing the association between circulating levels of IGFBP3 and breast cancer risk may be susceptible to reverse association/causation. Breast tumour cells can express IGFBP3 (18), which could distort any phenotype–disease association, hence the association may be a consequence of the disease rather than a cause.

We found that IGFBP3 variants that were associated with lower IGFBP3 levels were also associated with an increased risk of breast cancer, suggesting that IGFBP3 may be protective. Furthermore, because we used genetic markers, this association is unlikely to be due to reverse causality/association or confounding in contrast to studies assessing the association between circulating levels and breast cancer risk. We also found consistent additive associations between IGFBP3 promotor polymorphisms and circulating IGFBP3

levels in both men and women. At least three other studies, using various biological assays, have shown that SNP IGFBP3-03 (*a-202c*) is associated with circulating IGFBP3 levels in a dose-dependent manner (10,12,19).Results of studies on the effect of this SNP on breast cancer risk have, however, been inconsistent which might be attributed to their relatively small study sizes (12,19,20).

Functional variants

The IGF1 SNPs examined in this study were chosen to maximize SNP-tagging rather than for functionality, but we can speculate on potentially functional variants carried on the disease-associated haplotypes if the NIEHS SNPs are the fully comprehensive set of SNPs in this gene. Although we found SNP IGF1-05 (in intron 3) to have the most significant association with IGF1 levels, it tags several other SNPs (including rs4742653, 972936, 5742667, 5742678 and IGF1-06) with $R_p^2 > 0.85$. Thus, any of these SNPs might be mediating the association with circulating levels. *In vitro* assays will be required to determine which of these are truly functional, but unless there are functional elements in the introns, SNP IGF1-06 in the 3′-UTR would appear to be the best candidate for having a functional role, possibly through altering the RNA stability and thus the amount of IGF1 protein made. For IGFBP3, all four studied SNPs lie within the promoter region of the gene and have the potential to have direct functional effects. SNP IGFBP3-03 explains the greatest effect on IGFBP3 levels in this study and one study (10) has reported that in transient expression assays, constructs containing the *a* allele of this SNP generate twice the chloramphenicol acetyl transferase (CAT) activity of constructs containing the *c* allele, indicating that this SNP directly changes the efficiency of the IGFBP3 gene promotor. However, it also remains possible that there may be other functional variants carried on this IGFBP3 haplotype and a comprehensive search will be required to identify them.

In conclusion, our data indicate that common variants in the IGF1 and IGFBP3 genes are associated with circulating levels of IGF1 and IGFBP3 and with breast cancer risk. More specifically and consistent with experimental models, our data suggest that higher circulating IGF1 levels may increase the risk of breast cancer but higher IGFBP3 levels may be protective.

MATERIALS AND METHODS

Ely population sample

To investigate the associations among IGF1 and IGFBP3 genetic variants and circulating levels of these hormones, we used data from the MRC Ely study (21). In brief, the original sample of 1122 people was recruited between 1990 and 1992, at random from a population-based sampling frame of all adults, aged 40–65 years, free of known diabetes and registered with one general practice in the city of Ely, East Anglia. The initial response rate was 74% and no evidence for systematic differences between the participants and the population from which they were recruited has been found. Ninety-nine percent of the recruited subjects are of European Caucasian ancestry. These individuals underwent a standard 75 g oral glucose tolerance test, after an overnight fast. Blood samples were taken at fasting, 30 and 120 min after the glucose load. All samples were stored at −70°C within 4 hours of collection. Baseline, fasting concentrations of IGF-I were measured in stored plasma using an antibody-based assay (22). This assay had a detection limit of 28 ng/ml and within- and between-assay coefficients of variance (CVs) of <10% in standardized quality control samples. IGFBP-3 was measured by solid-phase enzyme-labelled chemiluminescent immunometric assay using an Immulite Autoanalyser (Diagnostics Products Corporation, California, USA) with an analytical sensitivity of 0.02 mg/l, and within- and between-assay CVs <10%. Consistent with previous studies and the fact that both are stimulated by growth hormone, measured levels of IGF1 and IGFBP3 are positively correlated in these samples (23,24). The data presented here are from a subset of the 937 participants at baseline who additionally had leucocyte DNA extracted at a follow-up clinical assessment (21,25).

Breast cancer case–control series

Cases were drawn from SEARCH (Breast Cancer) Study, an ongoing population-based study with cases ascertained through the East Anglian Cancer Registry (26). All women diagnosed with invasive breast cancer under the age of 55 years between 1 January 1991 and 30 June 1996 and who were alive at the start of the study (prevalent cases, median age 48 years) as well all those diagnosed under the age of 70 years between 1996 and the present (incident cases, median age 54 years) were eligible for inclusion. Sixty-four percent of eligible patients have provided a 20 ml blood sample for DNA analysis and completed a comprehensive epidemiological questionnaire. The total number of cases available for DNA analysis was 4474, of whom, 27% were prevalent cases. It has been possible to determine menopausal status, from the questionnaire data, for 3816 cases (85%) and of these, 2757 were pre-menopausal and 1059 were post-menopausal at diagnosis. Controls were randomly selected from the Norfolk component of EPIC (European Prospective Investigation of Cancer). EPIC is a prospective study of diet and cancer being carried out in nine European countries. The EPIC-Norfolk cohort comprises 25 000 individuals resident in Norfolk, East Anglia—the same region from which the cases and the Ely samples have been recruited. Controls are not matched to cases, but are broadly similar in age, being aged 42–81 years (27). The geographical and ethnic background of both cases and controls is very similar, with over 98% being of north-western European ancestry. When compared with the UK as a whole, East Anglia has seen little migration during the last millennium, and so far it remains a predominantly rural, stable community. No evidence of population genetic sub-structure within our study samples has been detected (28).

The samples have been split into two sets in order to save DNA and reduce genotyping costs (29): the first set (stage 1, $n = 2271$ cases and 2280 controls) is genotyped for all SNPs and the second (stage 2, $n = 2203$ cases and 2280 controls) is tested only for those SNPs that show marginally significant

associations in stage 1 (P-heterogeneity or P-trend < 0.1). This staged approach substantially reduces genotyping costs without significantly affecting statistical power. Cases were randomly selected for stage 1 from the first 3500 recruited, with stage 2 comprising the remainder of these plus the next 974 incident cases recruited. As the prevalent cases were recruited first, the proportion of prevalent cases was somewhat higher in stage 1 than stage 2 (33 versus 20%). Median age at diagnosis was similar in both stages (51 and 52 years old, respectively). There was no significant difference in the morphology, histopathological grade or clinical stage of the cases by set or by prevalent/incident status. Epidemiological details of the subjects in each stage of the study are given in Supplemental Material, Table S1.

Where there is no heterogeneity in the results obtained from the two stages, determined by a χ^2-test on the genotype distributions, the data are combined. Ethical approval was obtained from the Anglia and Oxford Multicentre Research Ethics Committee: MREC 02/5/42 and the Norwich Local Research Ethics Committee: LREC 98CN01. All study participants gave written informed consent.

SNP tagging

The aim of the SNP tagging is to identify a set of SNPs that efficiently tags all the known SNPs with minor allele frequencies (MAF) >0.05 and these are also likely to tag any unknown SNPs in the gene. The selection of tagging SNPs is most reliable where the gene has been re-sequenced in a sample of individuals sufficiently large to identify all common variants.

The NIEHS Environmental Genome Project (http://egp.gs.washington.edu/) has been re-sequencing candidate genes for cancer across panels of individuals representative of US ethnicities. The original polymorphism discover resource (PDR90) panel of 90 individuals consists of 24 European Americans, 24 African Americans, 12 Mexican Americans, six Native Americans and 24 Asian Americans, but the ethnic group identifiers are not available. It is known that there is greater genetic and haplotype diversity in individuals of African origin and so we have identified and excluded 28 of the samples with the greatest African ancestry in this population by comparing the genotypes of the PDR90 sample with genotypes for the same SNPs from the National Heart, Lung and Blood Institute, Variation Discovery Resource Project African American panel (http://pga.gs.washington.edu). Data from the remaining 62 individuals were used to identify tagging SNPs.

The best measure of the extent to which one SNP tags another is pair-wise correlation coefficient R^2_p because the loss in power incurred by using a marker SNP in place of a true causal SNP is directly related to this value. We aimed to define a set of tagging SNPs such that all known common SNPs (MAF > 0.05) had an estimated R^2_p of >0.8 with at least one tagging SNP. Some SNPs are poorly correlated with any other single SNP but are efficiently tagged by multiple SNPs. Therefore, we alternatively aimed for the correlation between each SNP and a haplotype (R^2_h) or a set of tagging SNPs (R^2_s) to be >0.8. We used the tagSNPs program (30) to identify a set of tagging SNPs using the above criteria (http://www-rcf.usc.edu/~stram/tagSNPs.html). This program uses the partition-ligation E–M algorithm to estimate haplotype frequencies based on the full set of SNPs identified from the re-sequencing data.

SNPs in IGF1 gene were identified by the above method. One hundred and thirty-three SNPs have been identified in the PGR90 set of individuals (http://egp.gs.washington.edu/). After exclusion of the 28 PGR90 subjects who clearly carry African-specific alleles, there remained 36 SNPs with MAF > 0.05. The Graphical Overview of Linkage Disequilibrium (GOLD) package (http://www.sph.umich.edu/csg/abecasis/GOLD/) was used to create a summary of pair-wise linkage disequilibrium patterns on the NIEHS individual genotyping data for these 36 SNPs (Supplemental Material, Fig. S1A). The IGFBP3 gene has not yet been investigated within NIEHS but the entire coding and promoter regions have been extensively re-sequenced in studies of Silver Russell syndrome. A total of 10 common SNPs have been reported (10–12).

Taqman genotyping

Genotyping was carried out using Taqman according to the manufacturer's instructions. Primers and probes were either supplied directly by Applied Biosystems as Assays-by-Design™ (dbSNP: rs5742615, rs5742678, rs5742694 and rs1549593) and Assays-on-Demand™ (rs1520220: C_2801118 and rs2946835: C-2801121) or designed in-house using Primer Express Oligo Design Software v2.0 (Applied Biosystems) (rs6220, rs6214 and rs6219, details in Supplementary Material, Table S2). All assays were carried out in 384-well plates. Each plate included negative controls (with no DNA) and positive controls duplicated on a separate quality control plate. Plates were read on the ABI Prism 7900 using the Sequence Detection Software (Applied Biosystems). Failed genotypes were not repeated. Assays where genotypes in duplicate samples did not show $>95\%$ concordance were discarded and replaced with alternative assays with the same tagging properties.

Power

The statistical power of the study depends on the at-risk allele frequency, the risks conferred and the genetic mode of action (dominant, recessive and co-dominant). The staged approach substantially reduces genotyping costs without significantly affecting statistical power. Supplemental Material, Table S3 compares the power of the two different approaches. For example, assuming that the causative SNP is tagged with $R^2 = 0.8$, a type I error rate of 0.0001 and genotyping success rate of 0.95, the staged/full study has 86/88% power to detect a dominant allele with MAF of 0.05 that confers a relative risk of 1.5 or 87/89% power to detect a dominant allele with MAF of 0.25 that confers a relative risk of 1.3. Power to detect recessive alleles is less—53/60% for an allele with MAF of 0.25 and risk 1.5 and 71/75% for an allele with MAF 0.5 and risk 1.3.

Statistical methods

For each SNP, deviation of genotype frequencies in controls from the Hardy–Weinberg equilibrium was assessed by a χ^2-test with one degree of freedom (1 d.f.). No significant deviations were found in this study. We used linear regression analysis to assess the association between circulating levels of IGF-1 and IGFBP-3 with genotypes. In the primary analyses, we used a general genetic model C. We used log-likelihood ratio tests to compare recessive, co-dominant (additive) and dominant models of association with the general model. Genotype frequencies in cases and controls were compared by χ^2-test for heterogeneity (2 d.f.) and test for trend (1 d.f.). Genotype distributions were also compared between prevalent and incident cases and between subjects in stage 1 and stage 2 with χ^2-tests (2 d.f.). No statistically significant differences were found in this study (data not shown) and results have been combined. Genotype-specific risks were estimated as ORs using logistic regression. We used log-likelihood ratio tests to assess any interactions between IGF1 and IGFBP3 SNPs on breast cancer risk, and to assess whether menopausal status modified the main SNP associations with breast cancer. We also tested for interaction between SNPs and menopausal status on breast cancer risk using a case only design (χ^2-test with 2 d.f.) (31).

Testing for consistency among the associations for SNPs, circulating levels and breast cancer risk

We used the estimated associations between IGF1 and IGFBP3 levels and SNPs, combined with published estimates from a meta-analysis of the association between circulating levels of IGF1 and IGFBP3 and breast cancer risk, to calculate expected ORs for breast cancer for IGF1 and IGFBP3 SNPs. Using results from the meta-analysis, and assuming a linear relation between plasma levels of IGF1 and IGFBP3 and breast cancer risk, and normally distributed plasma levels of these hormones, we calculated the gradient and intercept of the line, which predicted the OR between the highest and lowest quartiles. On the basis of this best fitting line and the observed mean for plasma levels by genotype, we calculated the predicted genotypic ORs for breast cancer risk. CIs for the predicted ORs were calculated from the standard errors of the mean plasma levels for each genotype, and the standard error of the log (OR) for the association between circulating levels and breast cancer risk for from the meta-analysis.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS

The authors are indebted to Joanna Camus, Clare Jordan, Joan Russell and Mitul Shah for sample identification and collection and to Amit Bhaniani for help with statistical analysis, also to the staff of the St Mary's Street Surgery, Ely, and to H. Shannasy, S. Curran, S. Hennings and J. Mitchell for help with the fieldwork for the Ely Study. We would also like to thank Martin Gibson, Adrian Heald and Ramudan Abushufa, Endocrine Sciences Research Group, University of Manchester, UK, for assistance with IGF assays. This work was funded by Cancer Research-UK (CR-UK). B.A.J.P. is a Gibb Fellow of CR-UK, and D.F.B. is a Principal Fellow of CR-UK. The Ely sample collection and IGF assays were funded by the Medical Research Council (MRC). A.A.-Z.'s PhD scholarship was supported by the King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia. Strangeways Research Laboratory has received a UK National Lottery Award (MC00412).

Conflict of Interest statement. The authors know of no financial interests or connections, direct or indirect, or other situations that might raise the question of bias in the work reported or the conclusions, implications, or opinions stated.

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