Original Paper

HORMONE RESEARCH IN PÆDIATRICS

Horm Res Paediatr 2015;83:177–182
DOI: 10.1159/000369013

Received: August 5, 2014
Accepted: October 9, 2014
Published online: December 18, 2014

GPR30 Gene Polymorphisms Are Associated with Gynecomastia Risk in Adolescents

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Key Words
Gynecomastia · Estrogen · GPR30 · Polymorphism

Abstract
Aim: The G protein-coupled receptor, GPR30, which is a third estrogen receptor, has been shown to mediate estrogenic effects on the essential features of human breast cancer cells. The aim of this study was to evaluate the association between GPR30 single nucleotide polymorphisms and gynecomastia in males. ***Methods:*** This study included 109 male adolescents with gynecomastia and 104 controls. Follicle stimulating hormone, luteinizing hormone, total testosterone, estradiol (E2), dehydroepiandrosterone sulfate (DHEAS), and prolactin levels were measured. DNA was extracted from whole blood using a GeneJET Genomic DNA purification kit. The genotypes of the GPR30 gene (rs3808350, rs3808351 and rs11544331) were studied using a tetra-primer ARMS (amplification refractory mutation system) PCR approach. ***Results:*** The median E2 (11.80 vs. 16.86 IU/l, $p < 0.001$) and DHEAS levels (116.8 vs. 146.5 μg/dl, $p = 0.044$) were higher in the gynecomastia group. The G allele of rs3808350 and the A allele of rs3808351 were frequently observed in patients with gynecomastia. Gynecomastia was more common in patients with the GG genotype of rs3808350 and in patients with the AA genotype of rs3808351. ***Conclusions:*** Our results suggest that increased E2 levels, the G allele of rs3808350 and the A allele of rs3808351 might explain why certain adolescents are affected by gynecomastia.

Introduction

Gynecomastia, which is the most common breast condition in males, is characterized by generalized enlargement of the breast tissue with the presence of a rubbery or firm mass extending concentrically and symmetrically from the nipple [1] accompanied by a histopathologically benign proliferation of glandular male breast tissue [2, 3]. The most important cause of gynecomastia is an imbalance between the actions of estrogen and androgen, and this imbalance can result from an absolute increase in estrogen production, a relative decrease in androgen production or a combination of the two [1–4]. Excess estrogen in men causes breast growth by inducing ductal

1663–2818/14/0833–0177$39.50/0

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epithelial hyperplasia, ductal elongation and ductal branching and by increasing the proliferation of periductal fibroblasts and vascularity. Local tissue factors, such as increased aromatase activity, decreased estrogen degradation and changes in the levels or activity of estrogen or androgen receptors, could also play a role in breast enlargement [4]. Studies on the expression of estrogen receptors in patients with gynecomastia have been reported only through histological analyses [5, 6], and few studies have demonstrated a relationship between estrogen receptor-α 454-351A/G polymorphisms, estrogen receptor-β rs4986938 gene polymorphisms and gynecomastia [7, 8]. The G protein-coupled receptor GPR30, a seven-transmembrane receptor, has been shown to mediate the estrogenic effects on the essential features of human breast cancer cells [9]. GPR30 expression in breast cancer cells lacking estrogen receptor-α and estrogen receptor-β was associated with estrogen responses [10]. GPR30 was found to be a third estrogen receptor activated by estrogens or G1 [11, 12]. However, several studies have demonstrated that G1 is an important GPR30 ligand [13, 14]. In estrogen receptor-α/β-negative breast cancer cells, estradiol (E2) binding to GPR30 was shown to increase the expression of connective tissue growth factor (CTGF), thereby stimulating both cell growth and migration [13]. Chevalier et al. [15] showed that GPR30 was overexpressed in seminomas, was localized at the membrane of human seminoma cells and was able to mediate the promotive effect on seminoma cell proliferation. To the best of our knowledge, no study has been conducted to assess the relationship between GPR30 expression and gynecomastia in males. The purpose of this study was to determine whether three GPR30 single nucleotide polymorphisms (SNPs) were related to the risk and the characteristics of gynecomastia. One of these SNPs is located in the GPR30 gene promoter, the second is located in the 5′-untranslated region (UTR) and the third is a missense exon SNP that leads to the amino acid exchange Pro16Leu. We compared the genotype and allele frequencies of these SNPs in 109 males with gynecomastia and in 104 healthy males.

Methods

Study Group

A total of 109 males with pubertal gynecomastia (breast development after the age of 12 years) were enrolled in this study. All of the participants were diagnosed between the ages of 12 and 17 years and were monitored at the outpatient clinic of the Pediatric Endocrinology Unit at the Dr. Behcet Uz Children's Hospital, Izmir, Harran University School of Medicine, Gaziantep Children's Hospital, and Mersin Children's Hospital. The patients were asked to lie supine with their hands placed above their heads. The examination was performed by compressing the breast area between the thumb and forefinger, facilitating the distinction between the presence of breast and adipose tissue in children with suspected gynecomastia. For the purpose of this study, the diagnosis of gynecomastia was confirmed as the presence of a palpable fibroglandular mass that measured at least 0.5 cm in diameter and was located concentrically beneath the nipple-areolar complex. The patients who had a testicular volume greater than 4 ml on the physical examination were accepted as pubertal gynecomastia patients. A testicular examination of the left testis was performed first, followed by an examination of the right testis, with the boy in the supine and crossed-legged position. Prader's orchidometer (a string of different volume 'beads'/testes for comparison by the examiner) was used to assess the testicular size. All of the physical examinations were performed by pediatric endocrinologists. A general and systematic examination was performed, including height and weight measurements. The examiners paid particular attention to the presence of abnormal physical or endocrinological findings to exclude secondary causes of gynecomastia. The auxological parameters of the enrolled patients, including the height, weight and body mass index (BMI), were measured, and the standard deviation scores (SDS) of the data were calculated [16]. A total of 104 unrelated, age- and sex-matched healthy controls were selected from the same geographic area ($p = 0.436$). This group belonged to a cohort of males who visited our hospitals for an annual check-up. The population controls were randomly selected from the same geographical regions and were matched to the cases according to their gender and age. The study was conducted according to the guidelines of the Declaration of Helsinki.

Ethical Standards

The ethics committee of the Dr. Behcet Uz Children's Hospital approved the study. Written informed consent was obtained from the parents of the participants and from each participant when appropriate. The investigations conformed to the principles outlined in the Declaration of Helsinki.

Hormonal Evaluation

The follicle stimulating hormone, luteinizing hormone, total testosterone, E2, dehydroepiandrosterone sulfate (DHEAS), and prolactin levels were drawn from an antecubital vein at 08:00 h and analyzed using an electrochemiluminescence immunometric assay (ECLIA) and a Roche Elecsys E170 immuno-analyzer (Roche Diagnostics, Burgess Hill, UK).

Genotyping

Three SNPs in the GPR30 gene were identified using the Internet sites www.genecards.org and http://www.ncbi.nlm.nih.gov/SNP. The SNPs were selected based on their possible biological relevance. The polymorphisms rs3808350 and rs3808351 are located in the 5′-region of the GPR30 gene (SNP rs3808350 is located in the regulatory 5′-region and SNP rs3808351 is located in the 5′-UTR), whereas the missense SNP rs11544331 is located in exon 3 (coding exon 1) and leads to the amino acid exchange Pro16Leu (fig. 1).

After written informed consent was obtained from all of the participants, 2-ml venous blood samples were collected in vacu-

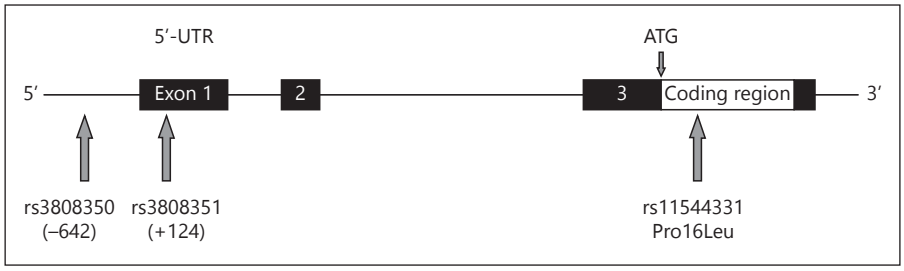

Fig. 1. Location of the genotyped GPR30 SNPs. The numbers below the 5′-SNPs indicate the position relative to the transcription start site (NCBI ref.: NM 001039966.1). Black boxes: UTR; open box: coding region; ATG: translation start site.

tainer plastic tubes containing sodium/potassium EDTA. A DNA sample was extracted using a GeneJET Genomic DNA purification kit (Thermo K0772). DNA was analyzed from whole blood using PCR-RFLP (restriction fragment length polymorphism). We determined the genotypes of the GPR30 gene (rs3808350, rs3808351, rs11544331) using an ARMS (amplification refractory mutation system) PCR approach [9]. For all of the genotyping, PCR was performed in a 25-μl volume with 100 ng of DNA, 100 μm dNTPs, 20 pmol of each primer, 1.5 mM $MgCl_2$, 1× PCR buffer with $(NH_4)_2SO_4$ and 2 U of Taq DNA polymerase (Thermo Scientific EP0401). The amplifications were performed in an automated thermal cycler (Techne Flexigene, Cambridge, UK). These gene polymorphisms were determined by fragment separation at 120 V for 40–50 min on a 1.5% agarose gel containing 0.5 mg/ml ethidium bromide. A 100-bp DNA ladder (Fermentas, Vilnius, Lithuania) was used as the standard size for each gel lane. The gel was visualized under UV light using a gel electrophoresis visualizing system (Vilber Lourmat E-BOX VX5).

Statistical Analysis

All of the statistical calculations were performed using the SPSS software package version 18.0 for Windows (SPSS Inc., Chicago, Ill., USA). The distribution of data was determined using the Shapiro-Wilks test. Continuous variables are expressed as means ± SEM or medians (with ranges), and categorical variables are expressed as frequencies (with percentages). The continuous variables were compared using an independent sample t test or the Mann-Whitney U test for two groups. The Kruskal-Wallis test was used to determine the differences among the three groups. The Bonferroni-adjusted Mann-Whitney U test was used as a post hoc test following the Kruskal-Wallis test. The χ^2 test or Fisher's exact test were used to evaluate the distribution of GPR30 among the patients with gynecomastia and the control subjects. The association between the GPR30 genotypes and the patients with gynecomastia was estimated by computing the odds ratios (ORs) and 95% confidence intervals (CIs) using logistic regression analyses. A p value less than 0.05 was considered statistically significant for all of the tests.

Results

The study populations consisted of 104 healthy unrelated individuals who visited our hospitals for an annual checkup and 109 patients with pubertal gynecomastia. When the healthy controls (median age = 14.17 years) and the patients with gynecomastia (median age = 13.7 years) were compared, no differences in age, height, weight, or BMI ($p > 0.05$) were detected. However, the height SDS, weight SDS and BMI SDS were markedly higher in the gynecomastia group ($p = 0.032$, $p = 0.021$ and $p = 0.007$, respectively). When the hormonal levels were evaluated, there were no differences in the follicle stimulating hormone, prolactin or testosterone levels between the groups. The median serum luteinizing hormone level was higher in the control group (2.25 vs. 1.60 IU/l, $p = 0.023$), and the median E2 (11.80 vs. 16.86 IU/l, $p < 0.001$) and DHEAS levels (116.8 vs. 146.5 μg/dl, $p = 0.044$) were higher in the gynecomastia group. The auxological and hormonal data of the patients and controls are shown in table 1.

The control group was at Hardy-Weinberg equilibrium for all of the analyzed genes ($p > 0.05$). As shown in table 2, the frequencies of the AA, AG and GG genotypes for the rs3808350 GPR30 polymorphism were 33.9, 37.6 and 28.5% in the patients and 42.3, 43.3 and 14.4% in the controls. The distributions of the genotypes of the GPR30 rs3808351 polymorphism were as follows: GG, GA and AA were 33.0, 42.2 and 24.8% in the group with gynecomastia and 50.0, 39.4 and 10.6% in the healthy controls. The relative risk for patients with gynecomastia was more than 2.45 times higher (OR = 2.458; 95% CI: 1.154–5.233) in individuals with the GPR30 rs3808350 polymorphism GG genotype and 3.545 times higher in individuals with the GPR30 rs3808351 polymorphism AA genotype compared with the wild genotype (AA genotype for the rs3808350 polymorphism and GG genotype for the rs3808351 polymorphism; table 2). The proportion of GPR30 rs11544331 polymorphism CC, CT and TT genotypes was 56.0, 39.4 and 4.6% in the patients and 63.5, 35.5 and 1.0% in the controls, respectively (table 2). For the GPR30 rs11544331 polymorphism, the TT genotype was more common in the patients (4.6%) than in the controls (1.0%); however, this difference was not significant (ta-

Table 1. Comparison of auxological and laboratory data between the groups

	Healthy controls (n = 104)	Cases (n = 109)	p
Age, years	14.17 (1.79–17.41)	13.7 (10.10–17.95)	0.372
Height, cm	161.0 (134.5–178.0)	160.5 (143.4–181.4)	0.896
Height SDS	–0.3450 (–1.79–2.35)	0.0250 (–3.50–2.60)	0.032*
Weight, kg	52.8±1.18	55.3±1.32	0.162
Weight SDS	0.03±0.09	0.36±0.11	0.021*
BMI, kg/m^2	19.9 (13.99–27.82)	20.56 (10.58–39.33)	0.198
BMI SDS	0.17 (–2.52–2.00)	0.63 (–3.93–2.66)	0.007*
FSH, IU/l	2.75 (1.04–16.38)	2.35 (0.24–12.3)	0.183
LH, IU/l	2.25 (0.01–16.05)	1.60 (0.07–12)	0.023*
E2, pg/ml	11.80 (5.00–65.4)	16.86 (2.58–78.47)	<0.001**
Prolactin, ng/ml	7.57 (2.17–30.4)	8.02 (1.67–27.12)	0.785
Testosterone, ng/ml	2.07 (0.04–7.4)	1.49 (0.11–12.02)	0.924
DHEAS, μg/dl	116.8 (26.73–385.4)	146.5 (43.6–452)	0.044*

Data are medians (with ranges) or means ± SEM. FSH = Follicle stimulating hormone; LH = luteinizing hormone. * $p < 0.05$; ** $p < 0.001$.

Table 2. Distribution of GPR30 genotypes and the risk of developing gynecomastia

Genotype	Healthy controls, n	Cases, n	χ^2 p value	OR
rs3808350				
AA	44 (42.3)	37 (33.9)		ref.
AG	45 (43.3)	41 (37.6)	0.044	1.083 (0.590–1.991)
GG	15 (14.4)	31 (28.5)		2.458 (1.154–5.233)
rs3808351				
GG	52 (50.0)	36 (33.0)		ref.
GA	41 (39.4)	46 (42.2)	0.007	1.621 (0.891–2.947)
AA	11 (10.6)	27 (24.8)		3.545 (1.562–8.048)
rs11544331				
CC	66 (63.5)	61 (56.0)		ref.
CT	37 (35.5)	43 (39.4)	0.202	1.257 (0.718–2.203)
TT	1 (1.0)	5 (4.6)		5.410 (0.615–47.622)

Values in parentheses are percentages or 95% CI, as appropriate.

ble 2). Furthermore, the individuals with the rs3808350 polymorphism G and rs3808351 polymorphism A allele had a higher risk of gynecomastia compared with the individuals with the rs3808350 polymorphism A and rs3808351 polymorphism G allele (table 3). When the haplotypes for the GPR30 rs3808350, rs3808351 and rs11544331 polymorphisms were determined, the GAT haplotype frequency was higher in the patients (11.5%) than in the controls (4.3%), and the difference was significant (OR = 3.452; 95% CI: 1.514–7.873; table 4).

Discussion

Because estrogens augment the risk of gynecomastia and breast tissue growth, a promising hypothesis is that polymorphisms in the GPR30 estrogen receptor gene might influence susceptibility to gynecomastia. This is the first study to examine the allele and genotype frequencies of GPR30 SNPs in patients with gynecomastia. We studied an SNP located in the promoter region of the GPR30 gene, an SNP in the 5′-UTR and a missense SNP in coding

Table 3. Distribution of GPR30 alleles and the risk of developing gynecomastia

Allele	Healthy controls, n	Cases, n	χ^2 p value	OR
rs3808350				
A	133 (63.9)	115 (52.8)	0.019	ref.
G	75 (36.1)	103 (47.2)		1.588 (1.077–2.342)
rs3808351				
G	145 (69.7)	118 (54.1)	0.001	ref.
A	63 (30.3)	100 (45.9)		1.950 (1.310–2.904)
rs11544331				
C	169 (81.3)	165 (75.7)	0.163	ref.
T	39 (18.7)	53 (24.3)		1.392 (0.874–2.218)

Values in parentheses are percentages or 95% CI, as appropriate.

Table 4. GPR30 rs3808350/rs3808351/rs11544331 haplotypes and the risk of developing gynecomastia

Haplotype	Healthy controls, n	Cases, n	OR	95% CI
rs3808350/rs3808351/rs11544331				
AGC	87 (41.8)	70 (32.1)	ref.	
AGT	12 (5.8)	9 (4.1)	0.932	0.372–2.338
AAC	28 (13.5)	25 (11.5)	1.110	0.594–2.072
AAT	6 (2.9)	11 (5.0)	2.279	0.803–6.468
GGC	34 (16.3)	31 (14.2)	1.133	0.635–2.023
GGT	12 (5.8)	8 (3.7)	0.829	0.321–2.139
GAC	20 (9.6)	39 (17.9)	2.424	1.298–4.524
GAT	9 (4.3)	25 (11.5)	3.452	1.514–7.873

Values in parentheses are percentages.

exon 1. We hypothesized that polymorphisms in the 5′-region might affect the GPR30 expression levels. The third SNP, which was located in coding exon 1 of the GPR30 gene, leads to a Pro16Leu amino acid exchange, which might alter the GPR30 protein structure and function.

Previous studies clearly demonstrated that GPR30 plays an important role in breast cancer cells [9, 17]. One of the molecular mechanisms for the action of GPR30 in breast cancer cells involves epidermal growth factor receptor (EGFR) signaling. GPR30 was demonstrated to activate EGFR [17, 18]. Additionally, EGF was found to upregulate GPR30 expression [17, 18], inducing cellular proliferation and breast enlargement. E2 binding to GPR30 was also reported to increase the expression of CTGF and to disrupt TGF-β signaling, thereby stimulating breast cancer cell growth and migration [13, 14]. The GPR30 estrogen receptor gene causes breast enlargement, most likely by inducing EGFR transactivation and increasing the expression of CTGF. Despite their estrogen receptor-α-dependent functions, the selective estrogen receptor modulator hydroxytamoxifen and the pure antiestrogen ICI 182.780 are thought to activate GPR30 signaling [13, 14]. Our study could lead to the emergence of new medications that might be used in place of selective estrogen receptor modulators to treat gynecomastia because GPR30 was found to be a third estrogen receptor activated by estrogens or G1. Additionally, our study could play an important role in the development of new medications that could be used to treat patients with gynecomastia who are resistant to tamoxifen.

In addition to these observations, an association between GPR30 and breast cancer was reported by Giess et al. [9]. Their study, which included 257 females with breast cancer, demonstrated that the A allele of SNP rs3808351 exhibited significantly lower frequencies in patients with large or G3 tumors, the T allele of SNP rs11544331 was significantly less frequent in patients with positive nodal status and the homozygous GG genotype of the promoter SNP rs3808350 and the T allele of the missense SNP rs11544331 were inversely associated with progesterone receptor negativity. These findings suggested that both SNPs exert protective effects against aggressive breast cancer entities. Because previous studies reported that high GPR30 expression levels were related to aggressive cancer phenotypes and to responsiveness to breast cancer therapies, each SNP could lead to a different phenotype in breast cancer [17, 19]. In our study, the two G protein-coupled estrogen receptor (GPER) SNPs that were evaluated were strongly associated with gynecomastia. A relative risk of gynecomastia frequently occurred in patients with the GG genotype of rs3808350 and the AA genotype of rs3808351. The G allele of rs3808350 and the A allele of rs3808351 frequently occurred in patients with gynecomastia. These data are consistent with the location of these two SNPs, which allows them to directly modulate GPER protein expression. Similarly, Chevalier et al. [15] reported in their study of 150 Caucasian male patients that the homozygous AA genotype of the SNP rs3808350 in the 5′-UTR and the SNP rs3808351 in the 5′-regulatory region were significantly more common in seminoma patients than in the control population, which suggests that the homozygous ancestral genotype GG could exert relative protective effects on tumor development [15].

Although our study group was relatively small for detecting the genotype and allele frequencies of these SNPs,

we observed for the first time that significant genotype-phenotype associations exist between GPER SNPs and gynecomastia. Examining the association between the evaluated SNP genotypes and the GPR30 expression levels or receptor function in patients with gynecomastia would be interesting. Another limitation of our study was the absence of an evaluation of GPR30 expression levels and receptor function in gynecomastia. It is also necessary to verify whether these three SNPs affect the function of this receptor in order to develop new medications that could be used to treat patients with gynecomastia. Several hormone-dependent cancers such as breast, ovarian and endometrium cancers express GPR30. This expression also exhibits prognosis utility in such cancers [19–21], and GPR30 is able to modulate the growth of hormonally responsive cancer cells in vitro [12, 22]. GPR30 expression has not been documented in either the normal male breast or gynecomastia tissues. Thus, it is important to investigate GPR30 expression in the normal male breast and gynecomastia and to assess its role in benign proliferation of glandular male breast tissue.

Our results suggest that increased E2 levels, the G allele of rs3808350 and the A allele of rs3808351 might explain why some adolescents experience gynecomastia. The results of this study support the presence of a genetic factor for susceptibility related to the association of GPR30 with gynecomastia.

Disclosure Statement

The authors have no conflicts of interest to disclose.

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