Analysis of the Common Vasoactive Intestinal Peptide Receptor 1 Polymorphism in Gallstone Patients

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Abstract

Background and aim: Cholesterol gallstone disease is caused by both genetic and environmental factors (e.g., deranged motility of the gallbladder wall). Recently, a single nucleotide polymorphism (SNP) of the vasoactive intestinal peptide receptor 1 (VIPR1) gene has been linked to late onset of achalasia, a lower esophagus dysmotility disorder. As VIPR1 is expressed in the gallbladder wall as well, and patients with achalasia exhibit extraesophageal motility disorders, the influence of VIPR1 SNP on cholelithiasis was investigated. Methods: We analyzed 254 gallstone-free controls (confirmed by ultrasound, age 21-78 years, 88% women, BMI 16-43 kg/m²) and 226 individuals from 107 families with gallstones (age 24-80 years, 87% women, BMI 17-55 kg/m²). All individuals were genotyped for the VIPR1 rs437876 SNP (intron 4) with PCR-based 5’- nuclease and fluorescence detection assays (TaqMan). We performed nonparametric linkage (NPL) analysis in affected sib-pairs (ASP), association tests, and regression analyses. Results: Controls were significantly younger (P < 0.01) and leaner than ASP and cases (P < 0.01), and both age as well as BMI significantly increased the risk of developing gallstones (P < 0.001). Allele frequencies were in line with database entries and no deviation from Hardy-Weinberg equilibrium was detected. Neither allele and genotype distributions nor NPL scores or the restriction of analysis to individuals older than 50 years provided evidence for association or linkage of the VIPR1 SNP and cholelithiasis. Conclusion: The VIPR1 polymorphism, previously linked to gastrointestinal dysmotility disorders, does not represent a common risk factor for gallstones in the general or in an elderly population.

Key words


Introduction

One of the most frequent and costly digestive diseases in Western countries is gallstone disease, with a prevalence in adults ranging from 10% to 15% [1, 2]. Indeed, this polygenic disease affects more than 30,000,000 Americans and accounts each year for 750,000 cholecystectomies in the United States [3] and over 190,000 in Germany [4, 5]. Nevertheless, over two-thirds of gallstone patients remain asymptomatic.

Several independent risk factors involved in gallstone formation have been described. Besides environmental influences, such as infection [6] and diet [1, 7], there is also a genetic component that favours a lithogenic bile composition with cholesterol supersaturated bile and an excess of pro-nucleating factors including biliary glycoproteins and mucin [1]. This complex interaction between multiple environmental and genetic factors is also observed in other polygenic diseases, e.g. diabetes and obesity [8]. Indeed, the analysis of the Swedish Twin Registry showed that genetic factors contribute to about 25% of the gallstone phenotype [9].

A genome-wide association scan [10] and an analysis of sib-pairs with gallstones [11] demonstrated that a genetic variant of the hepatocanalicular cholesterol transporter ABCG5/G8 substantially increased the risk of developing gallstones. Since the p.D19H ABCG8 polymorphism only partially accounts for the genetic background of gallstone disease, other studies were performed. In 2008, Kovacs et al. published the analysis of farnesoid X receptor (FXR) haplotypes (i.e. combinations of alleles at multiple loci on the same chromosome transmitted together) in three independent cohorts of patients with gallstones [12]. Interestingly, one of the haplotypes proved to be associated with the onset of the disease in Mexican patients, whereas it was protective in Chilean women and was not associated with gallstone disease.
in a German cohort. On the other hand, a recent study by Renner et al. [13] showed a strong association between an apical sodium-dependent bile acid transporter variant and gallstone formation in two independent German cohorts. Nevertheless, these studies do not fully explain the genetic background of gallstones in humans [4, 9].

It has been shown that deranged motility of the gallbladder either in the fasting and postprandial period confers a high risk of cholelithiasis [14-16]. Moreover, we have previously demonstrated that a subgroup of gallstone patients have impaired gallbladder and gastric motility as well as abnormal gastro-oesophageal pH-profiles, pointing to multiple functional defects of the upper gastrointestinal tract in gallstone disease [17].

Recently, a single nucleotide polymorphism (SNP) of the vasoactive intestinal peptide receptor 1 (VIPAC1), encoded by the VIPR1 gene, has been linked to late onset of achalasia, a dysmotility disorder of the lower oesophagus [18]. In this condition, the relaxation of the lower oesophageal sphincter is impaired as a result of a defect in NO and vasoactive intestinal peptide (VIP) releasing neurons [18]. It has also been suggested that patients with primary achalasia might display an extraoesophageal manifestation of the disease, which causes incomplete relaxation of the gallbladder. As a result, these patients have smaller gallbladders [19]. Of note, VPAC1 has been localised in the biliary tract [20]. In humans, VIP stimulates the secretion of the bicarbonate-rich bile; it increases bile flow and bile salt output, thus favouring a protective milieu against gallstones. In addition, it has been shown that transcriptional regulation of VIPR1 expression is induced by bile salts through FXR [21, 22]. The identification of VIP as a novel modulator of immune responses further adds to its possible role in gallstone formation [23].

With our current study, we aimed to verify whether the VIPR1 variant SNP rs437876, previously associated with late-onset achalasia [18], contributes to gallstone formation. In this respect, we performed an analysis of a well-defined cohort of gallstone affected sib-pairs (ASP) as well as gallstone free-controls. Genotyping of both cohorts allowed us to perform nonparametric linkage analysis in ASP and association (case-control) studies.

**Methods**

**Patients**

We analysed a prospectively recruited cohort of Romanian gallstone-affected sib-pairs (ASP) and unrelated gallstone-free controls, who were described previously (Table I) [11]. In short, the presence of gallstones in ASP was confirmed by abdominal sonography or history of cholecystectomy. Accordingly, individuals who did not meet these criteria were included in the study as controls. In total, the cohort consisted of sib-pairs from 107 families (n = 226, history of cholecystectomy n = 171, 24 - 80 years old, 87% females, body mass index (BMI) 17 - 55 kg/m²) and 254 unrelated gallstone-free, age-, gender- and BMI-matched controls (21 - 78 years old, 88% females, BMI 16 - 43 kg/m²). Only individuals with a documented Caucasian ethnicity living in the same geographical area were included in the study [11] in order to ensure homogeneous population samples.

All participants signed an informed consent form, and the study was conducted according to a study design approved by the local ethical committee.

**Genotyping**

Genomic DNA was isolated from EDTA anticoagulated blood according to the membrane-based QIAamp DNA extraction protocol (Qiagen, Hilden, Germany). The VIPR1 coding SNP rs437876 (intron 4) was genotyped using solution-phase hybridization reactions with 5'-nuclease and fluorescence detection (TaqMan assays) in a 7300 real-time PCR system (Applied, Norwalk, CT). The following primers and probes were used: forward primer GCCCAAATGGGACATACTAGTG, reverse primer TTTCATGTCCTCCAGCATGTG, VIC TTTCATGTCCTCCAGCAT GTG, FAM TTTCATGTCCTCCAGC A A, FAM TTTCATGTCCTCCAGCATGGC. PCR reactions contained 20 ng DNA, 900 nM of each primer, 1× TaqMan Universal Master Mix, and 200 nM of VIC-labelled and FAM-labelled probes in 25 µL-reactions. Amplification conditions were 95°C for 10 min, 40 cycles of 92°C for 15 s, and 60°C for 1 min.

**Statistics**

Unless stated specifically, statistical analysis was performed with SPSS 18.0 (SPSS, Munich, Germany), and phenotypic quantitative data were expressed as medians and ranges. For all tests, two-sided P values < 0.05 were regarded as significant.

Student’s t-test was employed to compare mean age and BMI values between controls and ASPs as well as between controls and cases. The effects of the VIPR1 SNP and the other potential prolithogenic factors (age, gender, BMI) [1] on the development of gallstones were estimated by logistic regression analysis.

To investigate the role of the VIPR1 variant in the development of gallstones, we performed an association case-control analysis and non-parametric linkage (NPL) tests. In the association analysis, all controls and a randomly selected single member from each sib-pair family (cases) were included. Association was tested in contingency tables (alleles: chi² test; genotypes: Armitage’s trend test). Hardy-Weinberg equilibrium (HWE) was checked by exact tests (http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl). Subsequently, association analysis was restricted to older patients (age > 50 years), since the VIPR1 polymorphism was associated with the trait among older patients in a previous study [18]. In addition, we analysed data from 107 sib-pairs, which allowed us to calculate the NPL score using GENEHUNTER-MODSCORE v2.0.1 (http://www.staff.uni-marburg.de/~strauch/software.html) [24]. In short, the NPL score estimates the significance of alleles shared among the family members for the development of the disease.

In this calculation, allele frequencies at the analysed gene locus are compared with the null hypothesis of no linkage
Results

Age and BMI are independent risk factors for developing gallstones

As shown in Table I, direct comparison of the study cohorts showed that young patients and leaner than cases (both P < 0.01). The analysis of known prolithogenic risk factors (see Methods) by univariate regression analysis provided significant results for both age (OR = 1.032; P = 0.001; 95% CI 1.013 – 1.050) and BMI (OR = 1.124; P < 0.001; 95% CI 1.080 – 1.168). The inclusion of these risk factors in a multivariate analysis demonstrated a significant effect for both age (OR = 1.030; P < 0.001; 95% CI 1.011 – 1.049) and BMI (OR = 1.127; P < 0.001; 95% CI 1.080 – 1.176) as independent risk factors for development of gallstones. On the other hand, neither gender nor VIPR1 polymorphism significantly increased the risk of cholelithiasis in our cohort (both P > 0.05).

Table I. Clinical characteristics of gallstone-free controls, gallstone-affected sib-pairs (ASP) and cases

<table>
<thead>
<tr>
<th>Gender</th>
<th>Controls (N = 254)</th>
<th>Affected Cases (N = 107)</th>
<th>Affected ASPs (N = 226)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>31 (12%)</td>
<td>16 (15%)</td>
<td>29 (13%)</td>
</tr>
<tr>
<td>Female</td>
<td>223 (88%)</td>
<td>91 (85%)</td>
<td>197 (87%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>50 (21 – 78)</td>
<td>55 (24 – 80) *</td>
<td>54 (24 – 80) *</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.0 (16.0 – 43.0)</td>
<td>28.0 (17.9) – 43.0</td>
<td>29.0 (17.9) – 55.0</td>
</tr>
</tbody>
</table>

Provided results were calculated in contingency tables (http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl). Abbreviations: ASPs, affected sib-pairs; BMI, body mass index.

Association (case-control) analysis

The VIPR1 rs437876 polymorphism was successfully genotyped in all ASPs and controls. The genotyping results (Table II) were in line with the frequencies reported in the Entrez SNP database as well as in a previous publication [18]. The genotype frequencies showed no deviation from HWE (P > 0.05), indicating robust genotyping. On the other hand, association tests showed lack of evidence for the involvement of the studied SNP in gallstone formation (P > 0.05). Subsequent restriction of the analysis to individuals > 50 years of age did not provide evidence for association either (Table III).

Table II. Allele and genotype distribution of the VIPR rs437876 SNP in cases and controls and association tests

<table>
<thead>
<tr>
<th>VIPR1 rs437876 allele / genotype</th>
<th>Cases (N = 107)</th>
<th>Controls (N = 254)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>100 (47%)</td>
<td>215 (42%)</td>
</tr>
<tr>
<td>G</td>
<td>114 (53%)</td>
<td>293 (58%)</td>
</tr>
<tr>
<td>AA</td>
<td>27 (25%)</td>
<td>82 (32%)</td>
</tr>
<tr>
<td>AG</td>
<td>46 (43%)</td>
<td>69 (27%)</td>
</tr>
<tr>
<td>GG</td>
<td>34 (32%)</td>
<td>114 (45%)</td>
</tr>
</tbody>
</table>

Association tests: chi² P
Armitrage’s trend test 1.26 0.261
Allele frequency difference test 1.28 0.257

Table III. Allele and genotype distribution of the VIPR rs437876 SNP in cases and controls and association tests in individuals older than 50 years

<table>
<thead>
<tr>
<th>VIPR1 rs437876 allele / genotype</th>
<th>Cases (N = 74)</th>
<th>Controls (N = 122)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>73 (49%)</td>
<td>101 (41%)</td>
</tr>
<tr>
<td>G</td>
<td>75 (51%)</td>
<td>143 (59%)</td>
</tr>
<tr>
<td>AA</td>
<td>21 (28%)</td>
<td>21 (17%)</td>
</tr>
<tr>
<td>AG</td>
<td>31 (42%)</td>
<td>59 (48%)</td>
</tr>
<tr>
<td>GG</td>
<td>22 (30%)</td>
<td>42 (35%)</td>
</tr>
</tbody>
</table>

Association tests: chi² P
Armitrage’s trend test 2.19 0.138
Allele frequency difference test 2.35 0.125

Provided results were calculated in contingency tables (http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl). Abbreviations: CI, confidence interval; OR, odds ratio: VIPR1, vasoactive intestinal peptide receptor 1.
were composed of three affected individuals, whereas in one family we had data for four sibs with gallstones. In line with the association analysis, results of the NPL scores (Table IV) did not support a causative role of the VIPRI polymorphism in gallstone formation (P > 0.05). Narrowing the analysis to individuals > 50 years (Table III), patients with gallstones identified at cholecystectomy, or cases with young age at the onset of gallstone disease did not show association with gallstones and the studied SNP either (all P > 0.05). In line with these results, Table IV demonstrates that the calculation of NPL scores with respect to MAF reported in the Entrez SNP database did not reveal a significant association of the VIPRI polymorphism with cholelithiasis (P > 0.05).

**Table IV. Nonparametric linkage analysis**

<table>
<thead>
<tr>
<th>[A] allele frequency</th>
<th>NPL</th>
<th>LOD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>All ASP</td>
<td>0.44^a</td>
<td>0.613</td>
<td>-5.093</td>
</tr>
<tr>
<td></td>
<td>0.33^b</td>
<td>0.761</td>
<td>-4.836</td>
</tr>
<tr>
<td>ASP &gt; 50 years</td>
<td>0.53^c</td>
<td>0.316</td>
<td>-5.250</td>
</tr>
<tr>
<td></td>
<td>0.33^d</td>
<td>0.357</td>
<td>-5.217</td>
</tr>
</tbody>
</table>

NPL scores were calculated according to the frequencies in the analysed cohort of ASP (a) or in the Entrez SNP database (b), using GENEHUNTER-MODSCORE v2.0.1 (http://www.statf.uni-marburg.de/~strach/software.html [24]. Abbreviations: ASPs, affected sib-pairs; LOD, logarithm of the odds ratio; NPL, nonparametric linkage score.

**Discussion**

Our current study was aimed at dissecting the role of the VIPRI polymorphism in the development of gallstones in humans. We analyzed a cohort of sib-pairs, which led us previously to detect the p.D19H polymorphism of the ABCG8 transporter as first genetic determinant of gallstone formation [11]. Indeed, the analysis of sib-pairs carrying the same condition is a potent model for dissecting novel genetic risk factors [24]. In this respect, the negative results eliminate the studied polymorphism as a major genetic determinant for cholelithiasis either in the general population or in individuals older than 50 years.

Interestingly, the calculation of genotype frequencies in cases and controls showed an overrepresentation of the AA genotype among cases and ASP, yet the difference did not reach significance. Lately, a new linkage score for ASP was derived by Callegaro et al. [25]. In this test, the identical-by-descent sharing is additionally weighted by the age at onset of disease. Although this test would be suitable to investigate the role of the VIPRI polymorphism among patients older than 50 years, estimation of the age at onset is troublesome for most individuals with gallstones. Hence, we decided to perform a classical ASPs analysis and to calculate the NPL score merely for individuals > 50 years at the moment of inclusion.

Of note, in previous studies employing genome-wide association scans [10], ASP analysis [11] and case-control analysis [12, 13], the genetic polymorphisms increasing the risk of cholelithiasis were associated with additional phenotypes as well. It was shown that patients with gallstones are characterised by lower expression of the ASBT protein encoded by the SLC10A2 gene [26] and that both ABCG8 as well as the SLC10A2 risk variants are associated with lower plasma cholesterol levels [13, 27]. Of note, VIP is involved in bile secretion, gallbladder motility and regulation of inflammatory responses [20, 21, 23]. Especially, abnormal gallbladder motility has been delineated as a strong risk factor for gallstone formation. In fact, many gallstone patients exhibit diminished or virtually absent postprandial gallbladder contraction, which increases the risk of cholelithiasis [15], but they may also present with other forms of gallbladder dyskinesia, namely increased gallbladder volume and defective emptying in the fasting state [16]. Additionally, obesity has been shown to impair gallbladder contractility [28, 29], and in our cohort controls were significantly leaner than cases and ASP. Indeed, the association between increased age, BMI and gallstone formation in this study is in line with previous reports [1]. On the other hand, as most ASPs were cholecystectomised at the time of inclusion, we were not able to relate our results to gallbladder motility.

In conclusion, in this study we show that the genetic variant of VIPRI recently linked to impaired motility of the lower oesophageal sphincter does not confer a major risk for gallstone formation. Refined analysis of elderly patients did not prove an association between the risk genotype and late onset of disease either. In this respect, further studies identifying novel genetic polymorphisms that increase gallstone risk are warranted.

**Acknowledgements**

The authors thank all patients for participating in this study and providing blood samples.

**Conflicts of interest**

We declare that we have no conflict of interest.

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