A Novel Val286Ala Polymorphism in the NPXXY Motif of the Sphingosine-1-Phosphate Receptor S1PR2 Associates with the Incidence and Age of Onset of Diabetes

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Abstract

Aim: S1PR2 is one of five known sphingosine-1-phosphate receptors and has recently been discussed to be implicated in the development or progression of diabetes. Our aim was to identify S1PR2 polymorphisms in the coding region of the human S1PR2 gene and perform association analyses between S1PR2 polymorphisms leading to amino acid exchanges and phenotypic parameters in a clinical study cohort.

Method: We screened the coding region of the S1PR2 gene for polymorphisms resulting in amino acid exchanges in the S1PR2 protein in 40 Caucasian DNA samples from a CEPH control panel. The resulting polymorphisms were then analyzed in more than 3400 patients of the Ludwigshafen Risk and Cardiovascular Health (LURIC) study cohort.

Results: In addition to the known Asn→Lys S1PR2 polymorphism at position 10 of the S1PR2 protein, we identified a novel polymorphism in the NPXXY motif of S1PR2, resulting in the amino acid exchange Val→Ala at position 286. The S1PR2 genotype Val286Ala showed strong association with the incidence and age at onset of diabetes in the studied patient cohort.

Conclusion: With our analysis we substantiate previous reports on the possible implication of S1PR2 in the development of diabetes and identified the S1PR2 Val286Ala genotype to be significantly associated with incidence and age at onset of diabetes likely due to an altered function of S1PR2.

Keywords: Clinical diabetes; S1PR2; Genetics; Epidemiology; Cohort study

Abbreviations: S1PR2: Sphingosine-1-Phosphate Receptor 2; S1P: Sphingosine-1-Phosphate; EDG: Endothelial Differentiation Gene; Val: Valine; Ala: Alanine; Asn: Asparagine; Lys: Lysine; T2D: Type 2 Diabetes

Introduction

Sphingosine-1-phosphate (S1P) is an important bioactive phospholipid with a wide range of cellular functions. Levels of S1Ps are tightly regulated via their synthesis, controlled by sphingosine kinases 1 and 2 (SphK1 and SphK2, respectively), and degradation, regulated by S1P phosphatases and S1P lyase. The primary sources for S1P in the circulation are platelets, endothelial cells, erythrocytes and mast cells [1,2]. S1P exerts its extracellular biological effects through binding to G protein-coupled receptors; five different S1P receptors (S1PRs) have been identified so far, S1PR1-5. The differential expression pattern of S1PRs and coupling to distinct G protein subunits confers to the great variety of biological effects of S1P. Most of the biological functions of S1PRs are known by the generation of S1PR knock-out mice and the study of expression patterns in the developing or adult organism. S1PR1 (EDG1/S1P1) knock-out mice die in utero between day 12.5 and day 14.5 due to incomplete vascular maturation and hemorrhage [3]. S1PR2 (EDG5/S1P2) mice exhibit no obvious gross abnormalities [4]. However, a variety of vascular phenotypes has been described in S1PR2 deficient mice. Lorenz and co-workers have shown that S1PR2 mice display decreased vascular resistance and a blunted vascular response after agonist challenge in an aortic ring model giving rise to the assumption that S1PR2 has important modulator function in the vasculature [5]. Furthermore, S1PR2 has been discussed to play a role in alveolar and vascular barrier function [6]. Interestingly, in zebrafish a mutation in the s1pr2-related miles a part gene leads to abnormal heart development [7]. The most striking phenotype for S1PR2−/− mice was described independently by two groups. Both groups reported a central role for S1PR2 in the auditory and vestibular system, with S1PR2−/− mice being profoundly deaf [8,9]. Apart from cellular abnormalities in the inner ear, an abnormal morphology of the vasculature in the stria vascularis was discussed to be related to the observed phenotype [9]. Additionally, S1PR2 deficiency leads to inhibition of atherosclerosis in apoE-deficient mice [10]. No phenotype abnormalities were observed in S1PR3−/− (EDG3/S1P3) mice [11]. S1PR4 (EDG6/S1P4) shows high expression in dendritic cells and was discussed to play an important role in immune response [12]. However, no S1PR4−/− mice have been described so far. High expression of S1PR5 (EDG8/S1P5) has been
shown on natural killer cells and a deficit in homing of NK cells to blood, spleen, lung and inflamed liver was shown in S1PR5−/− mice [13]. In contrast to mice, very little is known about S1PRs function from studies or analysis in humans. The only study published so far, is a comparative analysis of variants of the S1PR4 gene between 406 ulcerative colitis cases and 531 controls. In this study, a higher frequency of mutations was identified in the case group compared to controls [14]. Recently, S1PR2 was identified in a complex analysis of modulators of the insulin signaling pathways as a novel candidate gene for the development of type II diabetes (T2D) [15]. In addition, S1PR2 was shown to be down-regulated in platelets of individuals with T2D [16]. The aim of our work was to screen the human S1PR2 gene for polymorphisms leading to amino acid exchanges and to study associations of these polymorphisms in patients of the Ludwigshafen Risk and Cardiovascular Health (LURIC) study cohort.

Patients and Methods

Study population

The Ludwigshafen Risk and Cardiovascular Health (LURIC) study is a prospective cohort study. The study design has been described previously [17]. Patients were admitted to hospital because of chest pain and were asked to participate in this study. Study participation was voluntary and written informed consent was obtained from all patients. All study subjects underwent coronary angiography for the determination of the coronary status. Coronary artery disease (CAD) was assessed by angiography with wall irregularities in at least three out of 15 coronary artery segments. Left ventricular hypertrophy was diagnosed by echocardiography with myocardium thickness > 12 mm. Angina pectoris was defined as chest pain in patients with documented CAD. Diabetes mellitus was diagnosed according to the American Diabetes Association (ADA) criteria, with fasting glucose > 126 mg/dl, two hours post-oGT test plasma glucose > 200 mg/dl or a history of hypertension or if the systolic blood pressure > 140 mmHg and/or diastolic blood pressure > 90 mmHg. Stroke was defined as the occurrence of a permanent neurological deficit after cerebral infarction, including transient ischemic attack (TIA) and prolonged ischemic neurological deficit (PRIND). The data were collected as presented in the patient’s medical documents (i.e. hospital discharge letter). The study was performed according to the principles of the declaration of Helsinki and the local ethics committee approved the LURIC study.

Identification of single nucleotide polymorphisms in S1PR2

1306 base pairs of genomic sequence containing S1PR2 were sequenced with 3 PCR amplifiers on 40 Caucasian DNA samples from a CEPH control panel. We used purified PCR products (2 μl) as template for sequencing reactions. We added 20 pmole primer and terminator sequencing mix (Applied Biosystems, Foster City, USA) for a final reaction volume of 10 μl. Cycling conditions were performed as specified by the manufacturer. The sequencing reactions were purified on G50 sephadex columns (Amersham Bioscience, Orsay, France). We analyzed the reactions on an ABI 3730 as recommended by the manufacturer. Assemblies and SNP identification has been performed using PHRED, PHRAP and POLYPHRED software.

S1PR2 genotyping

S1PR2 fragments were amplified by PCR from genomic DNA using the oligonucleotides 5’- TGTAATGGGGATCATGTAGT-3’ (forward primer) and 5’- AGAGACGAAGTATTGGC -3’ (reverse primer) for the analysis of the S1PR2 polymorphism rs56357614 and oligonucleotides 5’- TCCAGTGTGCGCTCTCTAC -3’ (forward primer) and 5’- TCTCCATGACCCCTCTGCC -3’ (reverse primer) for the analysis of the S1PR2 C→T polymorphism at position 968 in the coding region of NM_004230.3 (Val→Ala at position 286). After purification of the PCR fragment, we used sequencing reaction with one base elongation (corresponding to the polymorphism to test) using the sense primer 5’- GTGTTCTCTGACCTTGGTGG-3’ for rs56357614 and the sense primer 5’- GAATTTCCCTGCTCAACCCGGG-3’ and antisense primer 5’- GGCTGCCACAGTGATAGT-3’ for the S1PR2 C→T polymorphism at position 968.

Analysis of reaction products was done in an ABI3700 capillary sequencer followed by Genescan and Genotyper data analysis (Applied Biosystems, Foster City, USA).

Statistical analysis

The genotype distributions obtained for the S1PR2 variant was tested for deviation from Hardy-Weinberg proportions. Pearson Chi² tests were performed to study the association of the S1PR2 polymorphism with clinical endpoints. Kaplan-Meier survival analysis was used to study the association between the age at occurrence of diabetes and the S1PR2 variant Val→Ala at position 286. All statistical analyses were performed with statistical analysis software (SAS) package 8.2 (SAS Institute Inc., Cary, USA). For all calculations a p-value < 0.05 was considered as statistically significant.

Results

Our investigation for single nucleotide polymorphisms in the S1PR2 gene leading to amino acid exchanges led to the identification of two non-synonymous polymorphisms, the S1PR2 variants Asn→Lys at position 10 and Val→Ala at position 286. The variation at position 10 is listed as rs56357614 in public databases such as NCBI (http://www.ncbi.nlm.nih.gov/snp) or Ensembl (http://www.ensembl.org/Homo_sapiens/Info/Index). Although the S1PR2 variant Val→Ala at position 286 has been identified in the 1000 Genome project (http://browser.1000genomes.org), this polymorphism is described here for the first time in a clinical cohort. The clinical phenotype of individuals of the LURIC study cohort used for our studies is outlined in (Table 1). Out of 4016 patients included in the LURIC study we genotyped the S1PR2 variant Asn→Lys at position 10 and the for S1PR2 variant Val→Ala at position 286 in 3410 patients (Table 2). In our study population, the frequency of Asn10Asn, Asn10Lys and Lys10Lys is 96.66%, 3.31% and 0.03%, respectively. The frequency of Val286Val, Val286Asn and Val286Lys is 97.92%, 2.08%, in that order.

Table 1: Clinical characteristics of LURIC study participants.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Frequency</th>
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<tbody>
<tr>
<td>Female</td>
<td>1201 (29.91%)</td>
</tr>
<tr>
<td>Male</td>
<td>2815 (70.09%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>62.15 ± 10.73</td>
</tr>
<tr>
<td>Smoker</td>
<td>2602 (64.92%)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>2385 (59.48%)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>1271 (31.72%)</td>
</tr>
<tr>
<td>History of myocardial infarction</td>
<td>1623 (40.71%)</td>
</tr>
<tr>
<td>History of CAD</td>
<td>2476 (77.98%)</td>
</tr>
<tr>
<td>History of stroke</td>
<td>177 (7.91%)</td>
</tr>
<tr>
<td>History of VTE</td>
<td>238 (5.85%)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.68 ± 4.22</td>
</tr>
</tbody>
</table>

Frequencies are presented as numbers of patients (n) and percent (%). Data for age and BMI are presented as means ± SD. For definitions of hypertension, diabetes, CAD, stroke and VTE please see Study Population in the Patients and Methods section. CAD=coronary artery disease, VTE=venous thrombosis/ pulmonary embolism, BMI=body mass index.
and determination of frequencies for polymorphisms leading to amino acid exchanges of the sphingosin-1-phosphate receptor 2 (S1PR2) has been performed so far. Therefore we analyzed the S1PR2 gene in 40 Caucasian DNA samples from a CEPH control panel. Two different non-synonymous polymorphisms in the S1PR2 protein have been identified in the course of our analyses, the S1PR2 variant Asn→Lys at position 10 and Val→Ala at position 286 of the S1PR2 protein. The S1PR2 variant Val→Ala at position 286 has not been described previously and was analyzed for the first time in our study. The aim of our study was to explore associations of polymorphisms in the S1PR2 protein with phenotypic and clinical parameters of patients who were recruited to participate in the Ludwigshafen Risk and Cardiovascular Health (LURIC) study. No significant association was calculated for the S1PR2 variant Asn→Lys at position 10 with the clinical phenotypes of patients of the LURIC cohort. However, the S1PR2 variant Val→Ala at position 286 associated significantly with the incidence of diabetes. We found an increased incidence of diabetes in individuals with the Val286Ala genotype relative to individuals with the Val286Val genotype. Furthermore, individuals with the Val286Ala genotype experienced significantly earlier onset of diabetes compared to individuals with the Val286Val genotype. The finding that the S1PR2 variant Val286Ala links to the incidence and onset of diabetes underlines recent reports on a possible role for S1PR2 in the development diabetes. Tu and co-workers attributed a central role in insulin signaling to S1PR2 in a complex analysis of modulators of the insulin signaling pathway. In their analysis, however, an effect on fasting insulin levels was observed only in male S1PR2+ mice compared to wild-type littermates whereas fasting glucose levels remained unaffected in S1PR2− mice. Several reasons might contribute to the fact that no difference in glucose levels can be observed between S1PR2+ and wild-type mice, compared to our study in that we see increased fasting glucose levels (diabetic patients) to be associated with the S1PR2 Val286Ala genotype. This discrepancy might not only relate to the difference between animal models and the human clinical situation but could be a consequence of the differential impact of a modulated S1PR2 function in individuals with the Val286Ala genotype versus a complete deletion of functional activity of S1PR2. In addition, Randriamboavonjy and colleagues have shown altered regulation of S1PR2 expression in blood platelets of patients with type 2 diabetes. The Val→Ala substitution at position 286 of the S1PR2 protein analyzed in our study is at position 7.51 according to Ballesteros-Weinstein nomenclature for GPCRs and lies at the central position of the highly conserved NPXXY motif [18]. This motif plays an important role along the GPCR activation process. Any modification in this motif might influence receptor signaling. The essential functional role of the NPXXY motif in GPCR activation has been studied on a structural basis for bovine rhodopsin by Ernst et al. [19,20], using site-directed mutagenesis studies and comparing the crystal structures of rhodopsin with ligand-free opsin and opsin interacting with the carboxy-termius of Go subunit [21]. Transmembrane (TM) 7 contains the conserved NPXXY motif, which links TM7 and helix 8 (H8) by an aromatic interaction between the aromatic side chains of Tyr7.53 and Phe7.60 (Figure 2). Upon receptor activation, the cytoplasmic end of TM7 reorganizes such that Tyr7.53 rotates to face into the helix bundle. Simultaneous pairing of TM5 and TM6 opens the binding site for the G-protein. Thus, modification of the NPXXY motif must be assumed to impact the activation process. Indeed, mutations in the NPXXY motif in different GPCRs were shown in vitro to affect receptor expression, ligand affinity, receptor sequestration, heterotrimeric G protein coupling, and association with the small G proteins ARF and RhoA [22-25]. The

Discussion

To our knowledge, no systematic analysis for the identification

![Table 2: Distribution of S1PR2 variants in patients of the LURIC study. For 3410 patients genotype data were obtained for both polymorphisms.](image)

![Figure 1: Kaplan-Meier curve for age at onset of diabetes in patients of the LURIC study cohort with respect to S1PR2 Val286Ala polymorphism. S1PR2 Val286Ala (blue curve) associates significantly with early age at onset of diabetes compared to patients with S1PR2 Val286Val (red curve).](image)

![Figure 2: Superposition of (inactive) rhodopsin and (active) opsin, viewed from the cytoplasmic side. The arrows indicate differences in the positions of TM5, TM6 and Tyr7.53 (from the NPXXY motif). GPCR activation includes a conformational change of the highly conserved NPXXY motif.](image)
most striking effects of mutations in the NPXXY motif in humans have been reported recently by Roth et al. and Mumford et al. [26,27]. A systematic analysis of the MC4R gene in severe obese children, led to the identification of a Tyr302Phe mutation in a female patient suffering from obesity since early childhood. It was demonstrated that this novel heterozygous mutation led to a decreased cell surface expression of MC4R, in addition to a decreased agonist sensitivity of the mutant receptor [26]. Mumford and colleagues have identified a novel Asp304Asn mutation in the thromboxane A2 receptor in a patient with bleeding diathesis. It was shown ex vivo, that agonist-induced platelet aggregation was disturbed in this patient at agonist concentrations that induced full aggregation in controls [27]. As has been demonstrated for the Tyr302Phe mutation in MC4R, heterozygosity for the Asp304Asn mutation seems to be sufficient for the observed ex vivo effect, however, an additional defect leading to the bleeding phenotype was not excluded.

Because heterozygosity in the described mutations in the NPXXY motif was sufficient to lead to a severe clinical phenotype, these examples outline the essential role of an intact NPXXY motif for proper function of the relevant G protein-coupled receptor. Altered S1PR2 signaling activity might therefore be the underlying molecular mechanism leading to the observed association between the S1PR2 Val286Ala polymorphism and the incidence and age at onset of diabetes. To our knowledge, our study is the first to analyze associations with clinical parameters.

Table 3A: Association analyses between the S1PR2 polymorphisms Aas10Lys (Table 3A) and Val286Ala (Table 3B) and clinical endpoints in patients of the LURIC study. Figures indicate the percentage (%) and number of patients with the given clinical endpoint in the group of individuals with the same S1PR2 genotype. The clinical endpoints listed in the tables are based on questionnaire data, the patient record or have been diagnosed during hospitalization/inclusion phase in the LURIC study. Definitions for disease diagnosis are listed in Patients and Methods under Study Population.

Table 3B: Association analyses between the S1PR2 polymorphisms Asn10Lys and Lys10Lys and clinical endpoints in patients of the LURIC study. Figures indicate the percentage (%) and number of patients with the given clinical endpoint in the group of individuals with the same S1PR2 genotype. The clinical endpoints listed in the tables are based on questionnaire data, the patient record or have been diagnosed during hospitalization/inclusion phase in the LURIC study. Definitions for disease diagnosis are listed in Patients and Methods under Study Population.
Since our study population was composed of individuals with Caucasian origin and were included in this study due to angina, it would be of great interest to analyze whether genotype-phenotype association studies with additional study populations of different ethnic origin or inclusion criteria would come to the same conclusion.

Taken together, we have shown in our study that the novel Val→Ala polymorphism at position 286 in the NPXXY motif of S1PR2 is significantly associated with incidence and age at onset of diabetes in the LURIC study cohort. Thus, our findings substantiate previous reports, attributing to S1PR2 an essential role in the development and progression of diabetes.

References


