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Heterozygous Loss-of-Function Variants in CYP1B1 Predispose to Primary Open-Angle Glaucoma

Francesca Pasutto,1 Gabriela Chavarria-Soley,1 Christian Y. Mardin,2 Karin Michels-Rautenstrauss,1 Magnus Ingelman-Sundberg,3 Lorena Fernández-Martínez,1 Bernhard H. F. Weber,4 Bernd Rautenstrauss,1,5 and André Reis1

PURPOSE. Although primary congenital glaucoma (PCG)–associated CYP1B1 mutations in the heterozygous state have been evaluated for association with primary open-angle glaucoma (POAG) in several small studies, their contribution to the occurrence of POAG is still controversial. The present study was conducted to determine whether heterozygous functionally characterized CYP1B1 mutations are associated with the disease in a large cohort of German patients with POAG.

METHODS. The frequency of CYP1B1 variants on direct sequencing of the entire coding region was compared in 399 unrelated German patients with POAG (270, POAG; 47, JOAG; and 82, NTG) and 376 control subjects without any signs of glaucoma on ophthalmic examination. In vitro functional assays were performed and relative enzymatic activity of the CYP1B1 variants embedded in their respective background haplotypes and not previously unambiguously classified were determined, to assess their possible causative role.

RESULTS. Apart from known polymorphic variants, 11 amino acid substitutions in CYP1B1 reported before, both in PCG and POAG cases, were identified. After in vitro functional assay, variants P52L and R368H showed marked reduction of activity, confirming their role as loss-of-function mutations similar to previously determined variants G61E, N203S, and G329V. In contrast, variants G168D, A443G, and A465V showed no relevant effects and were thus classified as polymorphisms. Overall, seven functionally impaired variants were present in 13 (3.6%) patients and in 1 (0.2%) control subject (P = 0.002, OR = 5.4). Reanalysis of previous studies reporting CYP1B1 mutations in patients with POAG based on updated functional validation showed a significant excess of carriers among patients compared to controls (OR = 3.85; P = 2.3 × 10⁻⁵).

CONCLUSIONS. Heterozygous CYP1B1 mutations with absent or reduced relative enzymatic activity can be considered a risk factor for POAG. (Invest Ophthalmol Vis Sci. 2010;51: 249–254) DOI:10.1167/iovs.09-3880

G laucoma represents a heterogeneous group of complex neurodegenerative diseases and is the second leading cause of blindness worldwide.1,2 The most common form of glaucoma is primary open-angle glaucoma (POAG; OMIM 137760) which affects more than 35 million people worldwide.3,4 POAG is characterized by loss of retinal ganglion cells, specific atrophy of the optic nerve, progressive loss of the neuroretinal rim of the optic disc, with corresponding progressive visual field loss, and, if untreated, eventual blindness.5 Elevated IOP appears to be a major risk factor, although glaucoma develops in some patients even in the absence of elevated IOP.6 In this case, glaucoma is defined as normal-tension glaucoma (NTG). Based on age at onset, a juvenile form (JOAG, age at onset before 40 years) is differentiated from adult-onset POAG.6 A family history of the disease has long been recognized as a risk factor, suggesting that specific genetic variants contribute to pathogenesis of POAG. In some families, glaucoma segregates as a Mendelian trait with reduced penetrance and variable expressivity, but most cases are sporadic, suggesting a multifactorial contribution to its etiology.7

To date, 14 genetic loci for POAG have been identified through linkage studies (GLC1A–GLC1N). However, only three causative genes have been described: myocilin (MIM 601771), optineurin (OPTN/GLC1E), and WDR36/GLC1G). Altogether, mutations in these genes account for less than 10% of POAG cases.8 In addition, association studies have implicated more than 20 other genes.9 Although most of these have been reported in single studies, a few have been investigated in multiple-association studies, but findings have been inconsistent. One of these POAG-associated genes is cytochrome P450 1B1 (CYP1B1; MIM 601771 (Mendelian Inheritance in Man; National Center for Biotechnology Information, Bethesda, MD). Cytochrome P450 proteins are monooxygenases, which catalyze many reactions involved in the metabolism of drugs as well as steroids and other lipids.10 Mutations in CYP1B1 are the predominant cause of primary congenital glaucoma (PCG), which although generally rare, is the most common form of glaucoma in infants, with more than 80% of cases observed within the first year of life.11 This disorder is most likely due to developmental defects in the trabecular meshwork and the anterior chamber angle. In PCG, elevated IOP can rapidly lead to axonal loss and permanent loss of vision in untreated individuals. Inheritance is primarily autosomal recessive with variable penetrance.12 Although the role of CYP1B1 in congenital glaucoma is not well understood, the enzyme is probably responsible for the metabolism of compounds that are critical for the developing eye.10

From the 1Institute of Human Genetics and the 2Department of Ophthalmology, Friedrich-Alexander-University Erlangen-Nuremberg, Erlangen, Germany; the 3Section of Pharmacogenetics, Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden; and the 4Institute of Human Genetics, University Regensburg, Regensburg, Germany.

1Present affiliation: MGZ-Medical Genetics Centre, Munich, Germany.

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Corresponding author: André Reis, Institute of Human Genetics, Friedrich-Alexander-University Erlangen-Nuremberg, Schwabachanlage 10, 91054 Erlangen, Germany; reis@humgenet.uni-erlangen.de.
Initially, \(CYP1B1\) was suggested to be a modifier gene for the expression of \(MYOC\) in patients with JOAG.\(^1,2\) However, some studies have indicated that \(CYP1B1\) may play a causative role in JOAG, with possible monogenic association in French,\(^3,4\) Indian,\(^5,6\) and Spanish\(^7,8\) patients. Furthermore, heterozygous variants in \(CYP1B1\) have been proposed as potential factors governing severity in patients with POAG.\(^9,10\) In these reports, however, heterozygous \(CYP1B1\) variants have also been found in healthy subjects, which raises questions about their role as disease-causing mutations.

In a recent study, we reported a measurement method for relative enzymatic activity (combining net enzymatic activity and protein abundance) and found variation in activity between the major \(CYP1B1\) haplotypes as well as either a drastic or an intermediate to mild reduction in activity of four different \(CYP1B1\) mutations (G61E, Y81N, N203S, and E229K), compared with that of the corresponding background haplotype.\(^11\) A marked decrease of the relative activity was seen for variants N203S and G61E, which were classified as non-fide mutations. In contrast, variants Y81N showed an intermediate and E229K a mild reduction in activity, thus leading to a classification as hypomorphic alleles.\(^12,13\)

In the present study, we evaluated whether heterozygous \(CYP1B1\) mutations showing absent or reduced relative enzymatic activity are associated with the disease in a large cohort of German patients with POAG.

**Materials and Methods**

**Patients and Control Subjects**

The study was approved by the ethics review board of the Medical Faculty of the University of Erlangen-Nuremberg and was conducted in accordance with the tenets of the Declaration of Helsinki. All subjects gave informed consent before entering the study.

The group of patients with glaucoma consisted of 399 subjects of German (European) origin. Two hundred seventy had POAG (high-pressure POAG), 47 had JOAG, and 82 had open-angle NTG. All individuals underwent standardized clinical examinations for glaucoma at the Ophthalmology Department of the University of Erlangen-Nuremberg. These comprised slit lamp biomicroscopy, gonioscopy, automated visual field testing (model G1; Octopus, Interzeg, Switzerland), fundus photography (fundus camera; Carl Zeiss Meditec, Jena, Germany), optional laser scanning tomography (HRT I and II; Heidelberg Engineering, Heidelberg, Germany), and a 24-hour Goldmann applanation IOP tonometry profile with five measurements. Manifest high-tension POAG was defined as the presence of glaucomatous optic disc damage (in at least one eye), visual field defects in any eye without therapy, and visual acuity at least 0.8 or 20/25 and IOP below 20 mm Hg, no glaucomatous disc damage, and no family history of glaucoma. Visual acuity was at least 0.8 or 20/25 and the media were clear for examination.

All patients were screened for the myocilin (MYOC), optineurin (OPTN) (data not shown), and WD repeat domain 36 (WDR36)\(^22\) mutations, as determined by direct sequencing of all coding regions.

**Mutation Screening**

Genomic DNA was prepared from peripheral blood samples by a standard salting-out protocol. Individual coding exons of the \(CYP1B1\) gene, including flanking intron/UTR sequences were amplified by polymerase chain reaction (PCR) with appropriate amplification protocols. Primer sequences were selected using Primer3 software and are available on request. Purified PCR fragments were sequenced with big dye termination chemistry (ver. 3.1; Applied Biosystems, Inc., [ABI], Weiterstadt, Germany) on a capillary automated sequencer (3730 Genetic Analyzer; ABI). Each variant was confirmed by a second independent analysis. GenBank for \(CYP1B1\) accession NM_022184 was used as cDNA reference sequence and NT_000104 as genomic reference sequence. We used Q16678 (CYP1B1_HUMAN), from the Swiss-Prot/TrEMBL Database as reference protein sequences. URLs of all data sources are provided in the Appendix.

**Plasmids Expression**

Based on wild-type human \(CYP1B1\) cDNA cloned into the pYeDP60 expression plasmid\(^25\) various \(CYP1B1\) constructs were generated by site-directed mutagenesis (QuickChange Site-Directed Mutagenesis Kit; Stratagene, La Jolla, CA) according to the instructions of the manufacturer. Several rounds of site-directed mutagenesis were needed to establish the different SNP haplotypes and the variants embedded in their original haplotype (for more details please refer to Ref. 32). All plasmids were sequenced using the dye-termination cycle sequencing kit ( Prism Big Dye; ABI) and analyzed ( Genetic Analyzer 3730; ABI) to ensure that the constructs were correct.

**Expression of Human CYP1B1 Variants in Yeast**

The different human \(CYP1B1\) variants were coexpressed with human reductase in a Saccharomyces cerevisiae strain, INVS1-HR MAT ahtis341 leu2 trp1-289 ura3-52 (pFL-35 human reductase). Yeast cells were transfected with the pYeDP60-CYP1B1 expression plasmids. Single colonies were inoculated into selective medium (lacking uracil) and grown, followed by microscopy isolation according to a previously established protocol.\(^23\) Microsomes were stored at −80°C until use. Microsomal protein concentration and total P450 content were determined with previously described methods.\(^3,4\) The P450 content is essentially accounted for by the overexpressed \(CYP1B1\).

**Determination of Enzymatic Activity**

\(CYP1B1\) activity in microsome extracts was quantified (P450-Glo CYP1B1 Assay Kit; Promega, Madison, WI) according to the instructions of the manufacturer. To measure enzymatic activity, 1 picomole of each \(CYP1B1\) variant was incubated with a luminogenic substrate and NADPH regeneration system. As a result, a luciferin product (\(\beta\)-luciferin) was generated, and luminescence (proportional to cytoto-
chrome P450 activity) was measured on a microplate reader (GENios; Tecan, Maennderof, Switzerland). The assays were performed in triplicate for all variants, original background haplotypes, and the negative control (microsomal extract from yeast transformed with the empty vector). The luminescence values for the negative control were subtracted from values of the rest of the samples. The mutants were compared against the corresponding background haplotype.

Statistical comparisons were performed with a one-way ANOVA, followed by the least-significant difference (LSD) test (for more details please refer to Ref. 18).

**Statistical Analysis**

Probabilities were calculated with Fisher’s exact test; \( P < 0.05 \) was considered statistically significant. Odds ratio (OR) and 95% confidence interval (CI) were calculated with the free available software written by David J. R. Hutchon.

**RESULTS**

An extensive screening for mutations using direct sequencing of the complete coding sequence of \( \text{CYP1B1} \), including untranslated 5’, 3’, and intronic flanking regions was performed on a well-studied, previously characterized cohort of 399 unrelated patients with glaucoma (270 with POAG, 47 with JOAG, and 82 with NTG) and 376 healthy subjects of German (European) origin.22 Apart from known polymorphic variants, this systematic mutation screen led to the identification of 11 amino acid substitutions in \( \text{CYP1B1} \) (Table 1). These 11 variants have been reported before, both in PCG and POAG cases, and some of them have also been found in healthy subjects.27,28 To delineate variants impairing enzymatic function, we performed in vitro functional assays for \( \text{CYP1B1} \) amino acid changes, P52L, G168D, R368H, A443G, and V465A not previously unambiguously classified. These variants were generated in vitro by site-directed mutagenesis, embedded in their respective background haplotypes. The relative enzymatic activity of each variant was calculated by multiplying its molar enzymatic activity and relative enzyme amount.18 Variants P52L and R368H presented a drastic decrease in relative enzymatic activity (Fig. 1), confirming their role as loss-of-function mutations similar to the previously reported variants G61E, N203S, and G329V.29 In contrast, relative enzymatic activity of the variants G168D, A443G, and V465A was unchanged or even slightly increased in respect to that of their respective haplotypes, but within the normal range seen in wild-type haplotypes resulting in a classification as polymorphisms.

In total we saw six bona fide mutations (P52L, W57X, G61E, R368H, N203S, and G329V) in seven patients, but none in control individuals. In addition, we found two variants previously classified as hypomorphic. The more severely impaired variant Y81N was present in six patients compared with one

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**Table 1. Identified CYP1B1 Mutations and Polymorphisms**

<table>
<thead>
<tr>
<th>Exon</th>
<th>Nucleotide Change</th>
<th>Amino Acid Change</th>
<th>Patients (n = 399)</th>
<th>Controls (n = 376)</th>
<th>Functional Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2</td>
<td>c.155C&gt;T</td>
<td>Pro52Leu</td>
<td>1</td>
<td>0</td>
<td>Mutation</td>
</tr>
<tr>
<td>E2</td>
<td>c.171G&gt;A</td>
<td>Trp57X</td>
<td>1</td>
<td>0</td>
<td>Mutation</td>
</tr>
<tr>
<td>E2</td>
<td>c.182G&gt;A</td>
<td>Gly61Glu</td>
<td>1</td>
<td>0</td>
<td>Mutation</td>
</tr>
<tr>
<td>E2</td>
<td>c.241T&gt;A</td>
<td>Tyr81Asn</td>
<td>1</td>
<td>0</td>
<td>Hypomorphic allele</td>
</tr>
<tr>
<td>E2</td>
<td>c.608A&gt;G</td>
<td>Asn203Ser</td>
<td>1</td>
<td>0</td>
<td>Mutation</td>
</tr>
<tr>
<td>E2</td>
<td>c.986G&gt;T</td>
<td>Gly329Val</td>
<td>1</td>
<td>0</td>
<td>Mutation</td>
</tr>
<tr>
<td>E3</td>
<td>c.567G&gt;A</td>
<td>Arg168His</td>
<td>2</td>
<td>0</td>
<td>Mutation</td>
</tr>
<tr>
<td>E2</td>
<td>c.503G&gt;A</td>
<td>Gly168Asp</td>
<td>1</td>
<td>0</td>
<td>Polymorphism</td>
</tr>
<tr>
<td>E3</td>
<td>c.685G&gt;A</td>
<td>Gly229Lys</td>
<td>8</td>
<td>10</td>
<td>Polymorphism</td>
</tr>
<tr>
<td>E3</td>
<td>c.1328G&gt;C</td>
<td>Ala443Gly</td>
<td>1</td>
<td>0</td>
<td>Polymorphism</td>
</tr>
<tr>
<td>E3</td>
<td>c.1394T&gt;C</td>
<td>Val465Ala</td>
<td>1</td>
<td>0</td>
<td>Polymorphism</td>
</tr>
</tbody>
</table>
TABLE 2. Clinical Features of Patients with Functionally Impaired CYP1B1 Mutations

<table>
<thead>
<tr>
<th>Subject ID</th>
<th>CYP1B1 Variant</th>
<th>MYOC Variant</th>
<th>Phenotype</th>
<th>Age at Diagnosis (y)</th>
<th>MAX IOP (mmHg; R/L)</th>
<th>Optic Disc (Jonas)</th>
<th>Disc Area (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20415</td>
<td>P52L</td>
<td>—</td>
<td>JOAG</td>
<td>6</td>
<td>30/30</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>99052</td>
<td>W57X</td>
<td>G367R</td>
<td>JOAG</td>
<td>22</td>
<td>46/38</td>
<td>V/III</td>
<td>2.85/2.89</td>
</tr>
<tr>
<td>10927</td>
<td>G61E/Y81N</td>
<td>—</td>
<td>POAG</td>
<td>46</td>
<td>43/28</td>
<td>IV/IV</td>
<td>ND</td>
</tr>
<tr>
<td>18513</td>
<td>Y81N</td>
<td>—</td>
<td>POAG</td>
<td>74</td>
<td>28/21</td>
<td>ND</td>
<td>2.1/2.1</td>
</tr>
<tr>
<td>99184</td>
<td>Y81N</td>
<td>—</td>
<td>NTG</td>
<td>69</td>
<td>21/21</td>
<td>II/II</td>
<td>2.49/2.48</td>
</tr>
<tr>
<td>17725</td>
<td>Y81N</td>
<td>—</td>
<td>POAG</td>
<td>54</td>
<td>29/26</td>
<td>ND</td>
<td>2.79/2.97</td>
</tr>
<tr>
<td>99004</td>
<td>Y81N</td>
<td>P370L</td>
<td>JOAG</td>
<td>7</td>
<td>30/35</td>
<td>IV/V</td>
<td>ND</td>
</tr>
<tr>
<td>99299</td>
<td>Y81N</td>
<td>—</td>
<td>NTG</td>
<td>60</td>
<td>19/19</td>
<td>III/III</td>
<td>2.8/2.8</td>
</tr>
<tr>
<td>99590</td>
<td>Y81N</td>
<td>—</td>
<td>POAG</td>
<td>51</td>
<td>24/24</td>
<td>IV/V</td>
<td>2.7/2.0</td>
</tr>
<tr>
<td>99168</td>
<td>N203S</td>
<td>E368X</td>
<td>JOAG</td>
<td>39</td>
<td>38/38</td>
<td>ND</td>
<td>1.99/1.87</td>
</tr>
<tr>
<td>17687</td>
<td>G329V</td>
<td>—</td>
<td>POAG</td>
<td>47</td>
<td>25/25</td>
<td>ND</td>
<td>2.59/2.59</td>
</tr>
<tr>
<td>99195</td>
<td>R368H</td>
<td>—</td>
<td>NTG</td>
<td>56</td>
<td>21/21</td>
<td>I/I</td>
<td>2.68/3.55</td>
</tr>
<tr>
<td>11806</td>
<td>R368H</td>
<td>—</td>
<td>POAG</td>
<td>44</td>
<td>32/33</td>
<td>ND</td>
<td>3.6/3.8</td>
</tr>
</tbody>
</table>

Maximum IOP at time of diagnosis (Goldmann applanation tonometry); optic disc atrophy according to Jonas (from normal 0, moderate cupping I, notching of the neuroretinal rim (NRR) II–III, temporal NRR loss IV, to complete atrophy V); optic disc area of left and right eye, respectively, measured with planimetry or laser scanning tomography (HRT; Heidelberg Engineering, Heidelberg, Germany).

TABLE 3. Combined Analysis of Different Studies on Functionally Validated CYP1B1 Mutations in Patients with POAG

<table>
<thead>
<tr>
<th>Study Groups</th>
<th>Ancestry</th>
<th>Patients</th>
<th>Mutations</th>
<th>Undetermined</th>
<th>Controls</th>
<th>Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melki et al.</td>
<td>French</td>
<td>236</td>
<td>6</td>
<td>1</td>
<td>47</td>
<td>1</td>
</tr>
<tr>
<td>Acharya et al</td>
<td>Indian</td>
<td>200</td>
<td>2</td>
<td>6</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Lopez-Garrido, et al</td>
<td>Spanish</td>
<td>119</td>
<td>7</td>
<td>4</td>
<td>92</td>
<td>0</td>
</tr>
<tr>
<td>Kumar et al.</td>
<td>Indian</td>
<td>251</td>
<td>10</td>
<td>3</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td>Chakrabarti et al.</td>
<td>Indian</td>
<td>224</td>
<td>11</td>
<td>6</td>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>This study</td>
<td>German</td>
<td>399</td>
<td>13</td>
<td>—</td>
<td>376</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>1429</td>
<td>49</td>
<td>20</td>
<td>915</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Percentage</td>
<td>100</td>
<td>3.4</td>
<td>1.4</td>
<td>—</td>
<td>0.4</td>
<td></td>
</tr>
</tbody>
</table>

Data from larger studies that reported CYP1B1 mutation frequencies in patients with POAG was reanalyzed based on updated functional validation of mutations (Refs. 18,19,33, and this study). Undetermined refers to mutations, whose functional relevance has not been determined as yet. Overall, 1429 patients of different ancestry were investigated. In each study and overall, patients were more likely to carry a functionally impaired mutation with an associated OR = 3.85 (95% CI = 2.21–6.70), P = 2.3 × 10⁻⁷ (two-tailed Fisher’s exact test).
effects of many variants found, thus presenting an unequivocal classification of them. In these aspects, the CYP1B1 mutation rate in our POAG patient cohort was significantly increased over that expected ($P = 0.0018$, Fisher’s exact test). Further, our reanalysis of the previous studies about CYP1B1 association to POAG$^{11–16,28,31}$ takes into account the functional classification of the CYP1B1 mutations, determined in this and other works$^{18,19,29,55}$ and combined the number of all patient groups screened together against all control subjects. As expected we observed an increase in significance ($P = 2.5 \times 10^{-7}$; two-tailed Fisher’s exact test), thus strengthening the association of functionally characterized CYP1B1 mutations in POAG.

Our results emphasize once more both the importance of functional classification of variants, especially in highly polymorphic genes, and the large number of patients necessary to achieve sufficient power when assessing the contribution of a gene to the pathogenesis of a complex disease such as POAG.

Previous data$^{7–9}$ support the hypothesis that POAG is characterized by a high locus and allelic heterogeneity with many different rare variants in numerous genes. In fact, rare mutations with low frequencies have also been reported for the other known glaucoma genes such as MYOC$^{34}$ and WDR36$^{22}$.

Taken together, these observations suggest that POAG belongs to the same category of traits under the frequent disease–rare variant hypothesis, such as epilepsy$^{35}$ and macular degeneration.$^{36}$ Characteristics of these diseases are rare, with highly penetrant variants found in numerous genes. Some of these variants are familiar, but most occur sporadically.$^{37,38}$ This sporadic occurrence could explain the so far elusive quest to identify more glaucoma genes and has important consequences for designing future studies aimed at unraveling the molecular basis of this devastating disease.

To our knowledge, this is the largest association study linking POAG and rare CYP1B1 variants to date and the first to systematically classify variants based on relative enzymatic activity. Our study and the reanalysis of previous studies supports the hypothesis that CYP1B1 has a broader significance for glaucoma pathogenesis than initially thought, ranging from a causal effect in autosomal recessive PCG and other anterior segment dysgenesis disorders, to a risk factor in POAG.

Acknowledgments

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References


33. Choudhary D, Jansson I, Sarfarazi M, Schenkman JB. Characterization of the biochemical and structural phenotypes of four CYP1B1 mutations observed in individuals with primary congenital glaucoma. *Hum Mutat.* 2008;29(9):1147–1153.


**APPENDIX**

**URLs of Data Sources**

- National Center for Biotechnology Information (NCBI), Bethesda, MD; http://www.ncbi.nlm.nih.gov/
- Primer3 software, Whitehead Institute, Massachusetts Institute of Technology, Cambridge, MA; http://frodo.wi.mit.edu/primer3/
- Genome Browser of the University of California Santa Cruz (UCSC), Santa Cruz, CA; http://genome.ucsc.edu/cgi-bin/hgTracks (reference sequences used: NT_022184 and NM_000104)
- Swiss-Prot/TrEMBL Database, Sanger Centre, Hinxton, UK; http://www.sanger.ac.uk/
- Expert Protein Analysis System (ExPasy) proteomic server, Swiss Institute of Bioinformatics, Geneva, Switzerland; http://www.expasy.org (Reference sequence Q_Human)
- ClustalW, European Bioinformatics Institute, European Molecular Biology Laboratory, Heidelberg, Germany; http://www.ebi.ac.uk/clustalw/
- Calculator for Odds Ratios and Confidence Intervals, written by David J. R. Hutcheon; http://www.hutchon.net/ConfidOR.htm