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Lack of Support for a Role for RLIP76 (RALBP1) in Response to Treatment or Predisposition to Epilepsy

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Summary: *Background:* Multidrug transporters are postulated to contribute to antiepileptic drug (AED) resistance. The transporter best studied is P-glycoprotein, an ATP-Binding Cassette (ABC) transporter superfamily member. RLIP76 is suggested to be an energy-dependent non-ABC transporter, reducing AED blood–brain barrier penetration, with a more important role than P-glycoprotein. Knowledge of which transporters may be critical in drug resistance is important for design of potential therapies. We tested the hypothesis that RLIP76 mediates AED resistance using methods complementary to those in the original report.

Methods: Double-labeling fluorescent immunohistochemistry localized RLIP76 expression. Population genetics was used to explore association of variation in the RLIP76-encoding gene with drug-response and epilepsy phenotypes. Comparative protein structure modeling and bioinformatic annotation were used to predict RLIP76 structure and features.

Results: In normal and epileptogenic brain tissue, immunoreactivity for RLIP76 was cytoplasmic, with colocalization with a neuronal, but not an endothelial, marker. Genotyping of six tagging SNPs, representing common genetic variation in RLIP76, in patients with epilepsy responsive (n = 262) or resistant (n = 107) to AEDs showed no association with phenotype at any level. RLIP76 genotypic and haplotypic frequencies in 783 patients with epilepsy and 359 healthy controls showed no association with epilepsy susceptibility. RLIP76 is not predicted to have transmembrane localization or ATPase activity.

Conclusions: No support for RLIP76 itself in directly mediating resistance to AEDs nor in increasing susceptibility to epilepsy was found. More evidence is required before either a role for RLIP76 in drug resistance can be accepted or focus directed away from other transporters, such as P-glycoprotein.
Key Words: RLIP76—Antiepileptic—Drug transporter—Tagging SNPs—Genetic association.

Recently, the phenomena and potential bases of resistance to AEDs have attracted considerable interest (Schmidt and Loscher, 2005). Whilst drug resistance is difficult to define precisely (Berg and Kelly, 2006), it is generally accepted that some 20–30% of patients with epilepsy have resistance to AEDs. Two broad hypotheses have emerged as potential explanations of at least part of the phenomenon of drug resistance: the transport hypothesis and the target hypothesis (not considered further here). The transport hypothesis holds that the concentration of AEDs at their target(s) is reduced below a clinically effective threshold by the abnormal overactivity of brain-expressed multidrug transporter proteins. Investigations using immunohistochemistry, in vitro models, whole animal models, and genetic association studies lend some credence to the transporter hypothesis. However, there is undoubtedly a need for careful evaluation of the candidacy of any proposed mechanism (Kwan and Brodie, 2005; Schmidt and Loscher, 2005; Sisodiya and Bates, 2006).

The transporter proteins studied to date have been mainly members of the ATP-Binding Cassette (ABC) superfamily of transporter proteins. P-glycoprotein, multidrug-resistance associated proteins 1 and 2 and breast cancer resistance protein, have all been postulated to have a role in mediating resistance to AEDs. All have been found in human epileptogenic brain, overexpressed in various patterns in various cell populations; some have been shown to have possible transport capacity for AEDs in at least some systems or models (Loscher and Potschka, 2005), while others would appear not to be able to transport AEDs at all or at therapeutic concentrations (Owen et al., 2001; Weiss et al., 2003; Cerveny et al., 2006). Though important data are emerging from animal models (Brandt et al., 2005; van Vliet et al., 2006), no transporter

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has yet been proven to have a role in generating resistance to AEDs in humans with drug-resistant epilepsy.

Most of the prior knowledge in this field has come from studies of transporter biology in oncology (Scheffer and Scheper, 2002). Studies of the survival of cancer cells in vitro and in vivo have been the most fertile source of putative new transporters that might mediate resistance to AEDs in patients. For example, major vault protein, strongly associated with, but probably not itself mediating resistance in cancer, has come to be studied in drug-resistant epilepsy, with evidence that it is at least present in human epileptogenic brain tissue (Aronica et al., 2003; Sisodiya et al., 2003).

Extending parallels with cancer biology, a recent study proposed that RLIP76, also known as RALBP1, had a greater effect in mediating resistance to AEDs in patients with drug-resistant epilepsy than the canonical transporter P-glycoprotein (Awasthi et al., 2005). It was proposed that RLIP76 was present in the luminal capillary endothelial surface in patients with drug-resistant epilepsy, and that it was a non-ABC, energy-dependent, multispecific transporter capable of transporting both carbamazepine and phenytoin. We sought to test this hypothesis using additional methods.

MATERIALS AND METHODS

The study was approved by the Joint Research Ethics Committee of the Institute of Neurology and the National Hospital for Neurology and Neurosurgery. All subjects gave written informed consent.

Brain tissue

We studied formalin-fixed paraffin-embedded human brain tissue from our neuropathological archive. Case tissue ($n = 3$) was from therapeutic surgical resection for epilepsy resistant to multiple AED treatment and was pathological tissue surplus to diagnostic requirements. Routine staining (Nissl, hematoxylin, and eosin) was performed to confirm the histological diagnosis and to provide anatomical detail. Disease surgical resection specimens had ideal fixation conditions, suffering no significant preresection hypoxia, being immersed in formalin immediately and embedded within a week. Cases and controls had been previously studied and reported (Sisodiya et al., 2006) and were typical of cases of hippocampal sclerosis (HS; $n = 2$) or focal cortical dysplasia (FCD; $n = 1$). Histologically normal control specimens were used, from therapeutic lobectomies for relief of post-traumatic raised intracranial pressure.

Antibodies and immunohistochemistry

Seven micrometer paraffin-embedded sections were dewaxed, rehydrated through graded alcohols and taken to water. Endogenous peroxidase activity was blocked in 2% hydrogen peroxide and methanol for 10 min. Sections were microwaved for 15 min in 0.05M EDTA pH 7.5 and cooled for 15 min, followed by protein blocking with normal rabbit serum (Peroxidase Vectastatin Elite ABC Kit, Vector, Burlingame, CA, U.S.A.). Endogenous avidin and biotin blocking (Vector) was carried out (15 min for each blocking) before sections were incubated with polyclonal primary anti-RLIP76 antibody (sc-1527, Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:500) for 2 h at room temperature. This polyclonal antibody has no reported cross-reactivity. Labeling was detected with biotinylated secondary antibody and ABComplex (Vector). Staining was visualized with DAB (3,3'-diaminobenzidine; Dako, Denmark). Between each step, sections were washed with PBS containing 0.05% Tween 20. Nuclei were counterstained with hematoxylin. The sections were dehydrated, cleared and coverslipped. Negative controls were treated identically, omitting the primary antibody and using irrelevant isotype-specific antibodies.

Double immunofluorescence using the TSA system and Alexa fluor fluorescent antibodies was undertaken using the same procedures as for immunohistochemistry, except at the final stage instead of DAB, sections were incubated with Fluorescein TSA Plus (Tyramide Signal Amplification, PerkinElmer, Boston, MA, U.S.A.). All procedures following this stage were light-protected. Negative controls were treated identically except that primary antibody was replaced with normal goat IgG (Autogen Bioclear) applying the same dilution as for anti-RLIP76 antibodies. Sections labeled for RLIP76 were then double-stained with four different primary antibodies (Table 1) overnight at 4°C. Sections were then washed with PBS and incubated with Alexa Fluor goat anti-mouse and goat anti-rat 594 (red; 1:100 Molecular Probes, Leiden, The Netherlands) for 1–2 h at 30 °C. Sections were washed and mounted on Prolong Gold antifade with DAPI (Molecular Probes). All Alexa Fluor antibodies were diluted in Dako Chem-Mate Diluent. Sections were visualized with a Leica Laser DMRB-SP2 (MP UV) confocal microscope.

TABLE 1. *Details of primary antibodies used in colocalization experiments*

Antibody	Source	Species	Isotype	pAb
Anti-CD34 QBEnd10	DAKO	Mouse	IgG1	1:20
Anti-P-gp JSB-1	VU	Mouse	IgG1	1:40
Anti-MRP1 MRPr1	VU	Rat	IgG2a	1:10
NeuN	Chemicon	Mouse	IgG1	1:200

P-gp, P glycoprotein; MRP1, multidrug resistance-associated protein 1; NeuN, Neuron-specific nuclear protein; VU, (Vrije Universiteit Medical Centre, Amsterdam, The Netherlands); Chemicon (Temecula, U.S.A.); pAb, primary antibody dilution.

Patients and controls

All patients were recruited through the epilepsy clinics of the National Hospital for Neurology and Neurosurgery. There are no generally accepted definitions for AED response (Berg and Kelly, 2006): we used a classification system we previously employed (Siddiqui et al., 2003) to categorize patients according to AED response: *drug-resistant*: four or more seizures a year at recruitment, with trials at maximum tolerated doses of three or more different appropriate AEDs; *drug-responsive*: seizure-free on AEDs for at least a year at recruitment. Patients having had epilepsy surgery were considered drug-resistant. For association with drug resistance, 369 patients were studied. For susceptibility studies, an additional 415 patients with epilepsy, from the same clinics, were also studied. Healthy controls were 364 unrelated individuals from a twin registry. DNA was extracted using standard methods and genotyping carried out using TaqMan technology according to standard conditions (Applied Biosystems, Foster City, CA, U.S.A.). Primer and probe sequences are given in Table 2.

Genetic analyses

Design of tagging SNPs

For determination of linkage disequilibrium surrounding the RLIP76-encoding gene, we retrieved genotype data for 19 SNPs with minor allele frequency >5% in a population of Northern European ancestry (CEPH) from the HapMap project (www.HapMap.org). The region investigated spans 89.5 kb (from rs4797379 to rs167897), and includes the entire gene and about 33 kb of upstream region. Tagging single nucleotide polymorphism (tSNP) inference was done using the haplotype r^2 function implemented in the TagIT software package (Weale and Goldstein, 2005). The criteria for the choice of the tSNP set were the following: (i) average r^2, weighted by allele frequency, between the tSNPs and all the other SNPs greater than 0.8; (ii) minimum r^2 between the tSNPs and each individual SNP in the gene is 0.85 (determined using a SNP dropping procedure implemented with the performL function, also in the TagIT software package). We considered different possible numbers of tSNPs (H = 5, 6, or 7 tSNPs), and constrained the analysis to exclude SNPs which fell into repetitive sequences, as this would preclude genotyping using TaqMan assays. The set of minimum size that satisfies these criteria was for H = 6, corresponding to SNPs rs1979368, rs1561998, rs2028660, rs1813100, rs329007, and rs167897. Details of the assays are given in Table 2. All analyses were carried out using the TagIT software package (Weale et al., 2003; Weale and Goldstein, 2005), available at http://www.genome.duke.edu/resources/Tagit˙v3˙03.zip/download.

Data analyses

Genotypic and allelic significance levels were assessed with contingency tables, using the chi-square distribution. Haplotypes were inferred by applying the EM algorithm as implemented in the program PLEM (http://www.people.fas.harvard.edu/junliu/plem/click.html). Haplotypic association was assessed through the log-likelihood ratio method as implemented in the EH and PM programs (Zhao et al., 2000, available at: http://www.iop.kcl.ac.uk/IoP/Departments/PsychMed/GEpiBSt/software.shtml). This

TABLE 2. *List of RLIP76 tagging SNPs and TaqMan assay characteristics*

tagID	dbSNP ID	Alleles	Position	Localization	MAF[a]	Primer	Primer sequence	Probe	Probe sequence
1	rs1979368	C/T	9444478	upstream	0.33	F	TGCCTCTGG CTGCAACA	VIC	CACAAATAG CGTATCTAC
						R	TGAAGTGAATGT TTCTGGCTGAGT	FAM	CACAAATAG CATATCTAC
2	rs1561998	A/C	9468293	RLIP76	0.25	F	TGTATGTTCACAC CAATAATTGCCTACTT	VIC	CAGAGGCTA ATAAGACAAA
						R	CCAATACAGACA CCATCACTCAGTT	FAM	CAGAGGCTA ATACGACAAA
3	rs2028660	C/T	9498997	RLIP76	0.22	F	ATTGAAATACAGT ATGGTAAGTGCTGTGAA	VIC	CATCCTGAA CGTACCTC
						R	CCCCATCCAGTAT AAGCCACAAATAC	FAM	ATCCTGAA CATACCTC
4	rs1813100	A/G	9502985	RLIP76	0.42	F	CTTGGTGTTA GGTGACTCTGCTA	VIC	TTTTTCTCT TGGTACAGGTG
						R	GGACGAGGTCT TCTCTGTTACC	FAM	TTCTCTTG GTGCAGGTG
5	rs329007	A/G	9512606	RLIP76	0.24	F	CATATACCCTCT GGTACTGACATTGG	VIC	TGAACTTGA GAAGATAGG
						R	AAAGGAAAAGA CCCGTATTCAAGCT	FAM	AACTTGAGG AGATAGG
6	rs167897	C/T	9517100	RLIP76	0.13	F	GGCACATTCAT GGGACTTTTACAC	VIC	ATTACATATC CTGGAGCGCA
						R	GAAAAATACGC AGAGGGCTTCTG	FAM	CATATCCT GAAGCGCA

[a]Minor allele frequency in CEPH caucasian.

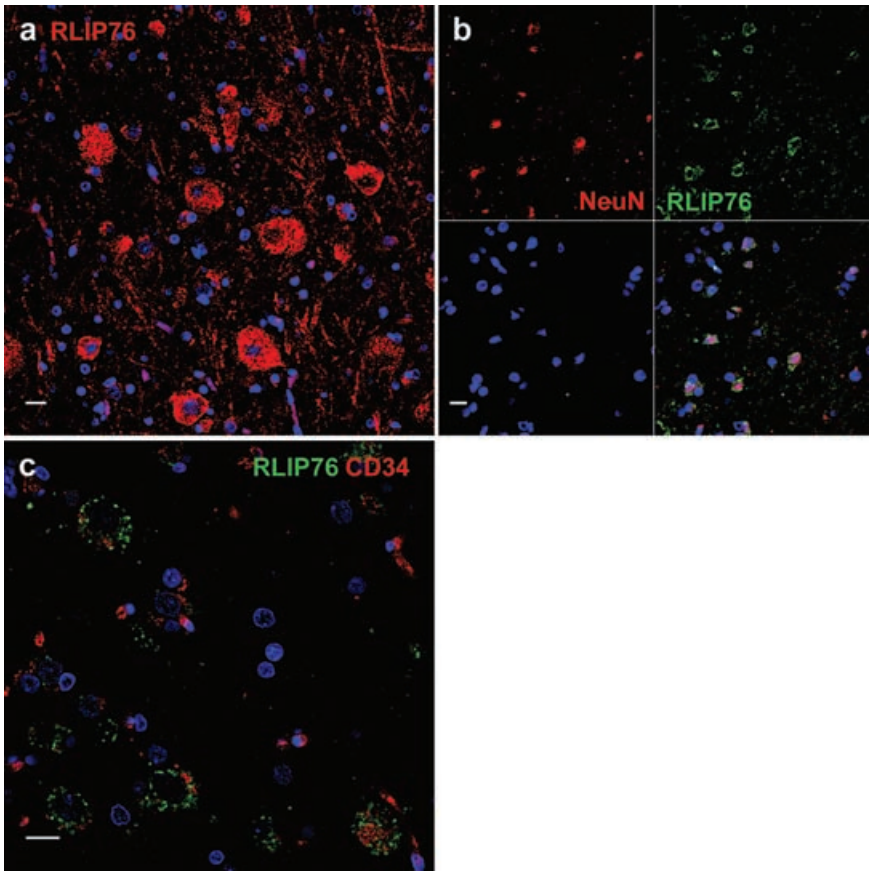

FIG. 1. Immunohistochemistry for RLIP76 in histologically normal brain tissue from a subject without epilepsy. In **(A)** immunolabeling appears neuronal; this is confirmed by double labeling with NeuN **(B)**; no vascular immunopositivity for RLIP76 was observed, with no colocalization with CD34 immunoreactivity **(C)**. Scale bars = 20 μM.

involves the calculation of the log-likelihoods of estimated frequencies for each cases, controls and case and controls combined. Significance for association is then calculated using the test statistic 2 × (ln(L case) + ln(Lcontrol) – ln(Lcase/Lcontrol), which has a χ^2 distribution with n–1 degrees of freedom (where n = number of inferred haplotypes).

Sequence and structure analysis

Transmembrane helix predictions for the RLIP76 sequence (UniProt ID: Q15311) were made using TMHMM (Krogh et al., 2001) and HMMTOP (Tusnady and Simon, 2001). Putative functional domains were predicted by sequence comparison with annotated protein family motifs in the Prosite database (http://ca.expasy.org/prosite/) (Hulo et al., 2006). Protein models were generated using ModPipe, an automated pipeline that uses the MODELLER package to identify structural homologs and build comparative protein structure models. All protein models are available in the ModBase database (http:/modbase.compbio.ucsf.edu/modbase-cgi-new/index.cgi).

RESULTS

Immunohistochemistry

We investigated expression and co-localization of RLIP76 with two multidrug transporter proteins, P-glycoprotein (P-gp) and multidrug resistance-associated protein 1 (MRP1), and with the neuronal marker NeuN and the endothelial marker CD34, in two cases of HS, one case of FCD and one histologically normal brain tissue sample (Figs. 1–3). For control and disease cases, transporter expression appeared uniform in the microvasculature throughout the studies in histologically normal or pathological areas.

In control tissue, P-gp immunoreactivity was microvascular in localization, as expected. RLIP76 had a predominantly neuronal localization, being localized mainly in the cytoplasm of pyramidal cells (Fig. 1A). Double-labeling with the neuronal marker NeuN confirmed the predominantly neuronal cytoplasmic expression of RLIP76 (Fig. 1B). Double-labeling with the endothelial marker CD34 did not show any colocalization of immunoreactivity for RLIP76 in vascular structures (Fig. 1C).We did not detect instances of colocalization of RLIP76 with either P-gp or MRP1 immunoreactivity (data not shown).

Analysis of disease specimens revealed similar patterns of expression for RLIP76. RLIP76 immunoreactivity in HS and FCD was cytoplasmic, and colocalized to cells labeling with NeuN, ie neurons (HS, FCD: Figs. 2A and 3A, respectively). There was no colocalization with CD34 immunoreactivity (Figs. 2B and 3B), or with P-gp (Figs. 2C and 3C) or MRP1 immunoreactivity (Figs. 2D and 3D).

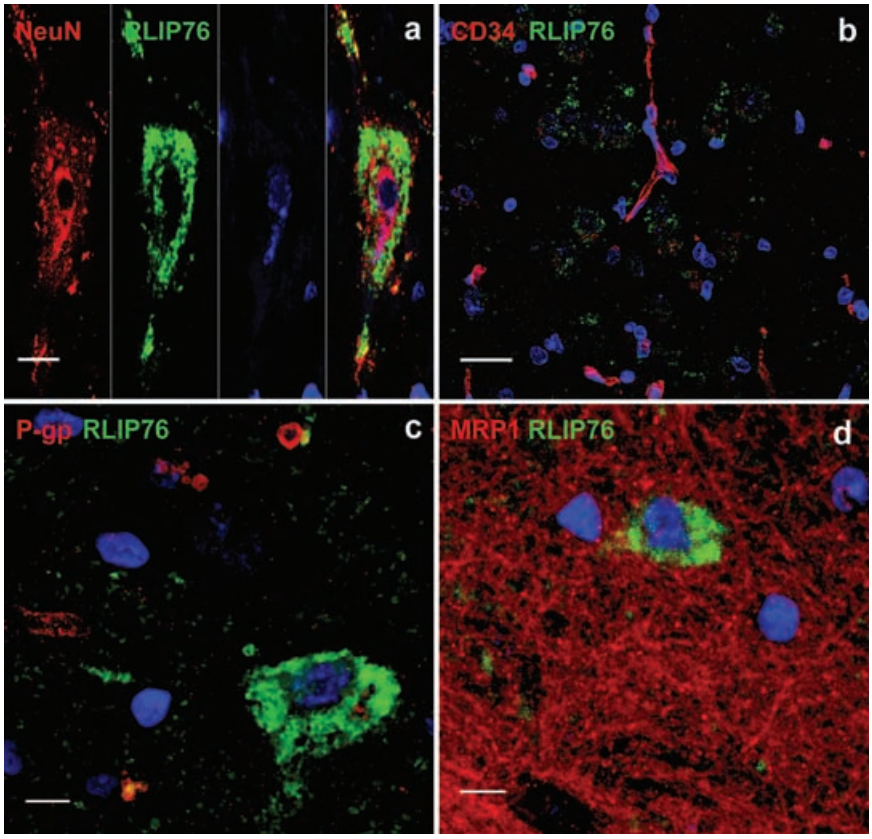

FIG. 2. In the CA1 region of a resected sclerotic hippocampus, the morphology and colocalization of immunoreactivity for RLIP76 and NeuN suggests neuronal expression of RLIP76 **(A)**. No colocalization is seen with CD34 **(B)**, P-glycoprotein (P-gp) (**C**, seen in vascular endothelium) or MRP1 **(D)**. Scale bars = 20 μm for **A–C**; 10 μM for **D**.

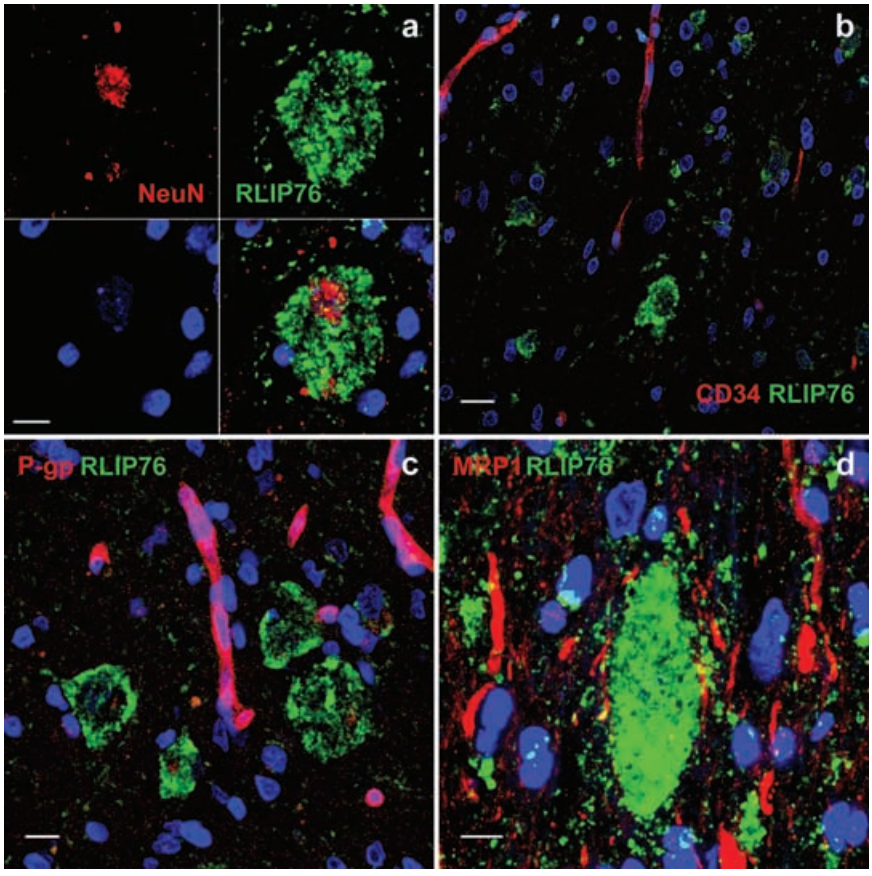

FIG. 3. Cytoplasmic immunopositivity for RLIP76 is seen in dysplastic neurons **(A–D)** in sections of focal cortical dysplasia, colocalization with NeuN **(A)**, but not with P-gp (**C**, seen in vascular endothelium) or MRP1 (**D**, seen in glia). Scale bars = 20 μM for **B** and **C**; 10 μM for **A** and **D**.

Genetic association

We first investigated the contribution of the gene encoding RLIP76 to the drug response phenotype. The results for association with individual SNP loci are shown in Table 3. No association was observed for individual loci, with the highest allele frequency difference between drug-resistant and drug-responsive patients being 0.4% (locus rs1813100; $\chi^2 = 1.19$; p-value = 0.27). There were no significant differences for genotypic frequencies between drug-resistant and drug-responsive patients (Table 3).

There was no significant association between RLIP76 genetic variants with the overall predisposition to epilepsy. We compared the entire set of epilepsy patients, irrespective of their response status, to healthy controls, finding no significant association of allelic or genotypic frequencies with disease (Table 4). Analysis of multilocus haplotypes did not reveal significant differences between drug-resistant and drug-responsive patients or between patients with epilepsy and controls (Table 5).

Bioinformatic analysis of RLIP76

The RLIP76 sequence was run through two transmembrane helix prediction tools, TMHMM (Krogh et al., 2001) and HMMTOP (Tusnady and Simon, 2001), both found by an independent analysis to be high-performing transmembrane helix predictors (Moller et al., 2001). TMHMM predicts no transmembrane helices, while HMMTOP predicts two, the first in the residue range 312-328 and the second in the range 355–372. The sequence also contains a GTPase-activating protein domain for Rho-like GTPases (RhoGAP) and a leucine zipper motif, among others, as annotated in the Prosite database (Moller et al., 2001). Additional evidence that this sequence contains a RhoGAP domain is that the protein shows 28% sequence identity in the 183–367 residue range to the 1tx4 crystal structure, which is a 1.65 Å structure of RhoA and its GTPase-activating protein in complex with a transition-state analogue (Rittinger et al., 1997). A protein model based on the 1tx4 template was generated automatically by ModPipe and can be accessed through the ModBase protein model database (Pieper et al., 2006). The model covers residues 183–367 in the human RLIP76 sequence. It is worth noting that the modeled structure includes two alpha helices in the regions predicted to be transmembrane helices by HMMTOP.

In closely related sequences defined by the NCBI homologene database (Wheeler et al., 2006), sequences from *C. familiaris*, *M. musculus*, *R. norvegicus*, *G. gallus*, *D. melanogaster*, *A. gambiae* and *C. elegans* are also predicted as having no transmembrane helices by TMHMM. These sequences are between 90% and 30% identical to human RLIP76 sequence. All of these sequences share the RhoGAP domain. Finally, none of these sequences share known ABC transporter motifs, as shown in Fig. 4.

DISCUSSION

Drug resistance in epilepsy is an important clinical problem, aggravating risks of morbidity and mortality caused by epilepsy. Much attention has focused on the

TABLE 3. *Summary of single-locus statistics for genetic association between the six RLIP76 tSNPs and response to antiepileptic drugs*

Locus	Genotype number			Genotype frequency (%)					Allele number		Allele frequency (%)			
rs1979368	CC	TC	TT	CC	TC	TT	χ^2	P-value	C	T	C	T	χ^2	P-value
Drug-resistant	23	127	112	0.09	0.48	0.43	0.36	0.83	173	351	0.33	0.67	0.33	0.57
Drug-responsive	8	50	49	0.07	0.47	0.46			66	148	0.31	0.69		
Total	*31*	*177*	*161*	*0.08*	*0.48*	*0.44*			*239*	*499*	*0.32*	*0.68*		
rs1561998	AA	CA	CC	AA	CA	CC			A	C	A	C		
Drug-resistant	10	89	161	0.04	0.34	0.62	0.02	0.99	109	411	0.21	0.79	0.01	0.9
Drug-responsive	4	36	67	0.04	0.34	0.62			44	170	0.21	0.79		
Total	*14*	*125*	*228*	*0.04*	*0.34*	*0.62*			*153*	*581*	*0.21*	*0.79*		
rs2028660	CC	TC	TT	CC	TC	TT			C	T	C	T		
Drug-resistant	12	94	153	0.05	0.36	0.59	0.69	0.71	118	400	0.23	0.77	0.43	0.51
Drug-responsive	5	34	68	0.05	0.32	0.63			44	170	0.21	0.79		
Total	*17*	*128*	*221*	*0.05*	*0.35*	*0.60*			*162*	*570*	*0.22*	*0.78*		
rs1813100	AA	GA	GG	AA	GA	GG			A	G	A	G		
Drug-resistant	80	123	59	0.31	0.47	0.22	1.27	0.53	283	241	0.54	0.46	1.19	0.27
Drug-responsive	39	47	21	0.36	0.44	0.20			125	89	0.58	0.42		
Total	*119*	*170*	*80*	*0.32*	*0.46*	*0.22*			*408*	*330*	*0.55*	*0.45*		
rs329007	AA	GA	GG	AA	GA	GG			A	G	A	G		
Drug-resistant	160	88	10	0.62	0.34	0.04	0.05	0.98	408	108	0.79	0.21	0.04	0.84
Drug-responsive	67	35	4	0.63	0.33	0.04			169	43	0.80	0.20		
Total	*227*	*123*	*14*	*0.62*	*0.34*	*0.04*			*577*	*151*	*0.79*	*0.21*		
rs167897	CC	TC	TT	CC	TC	TT			C	T	C	T		
Drug-resistant	182	72	9	0.69	0.27	0.04	0.06	0.97	436	90	0.83	0.17	0	0.97
Drug-responsive	74	28	4	0.70	0.26	0.04			176	36	0.83	0.17		
Total	*256*	*100*	*13*	*0.69*	*0.27*	*0.04*			*612*	*126*	*0.83*	*0.17*		

TABLE 4. *Summary of single-locus statistics for genetic association between the six RLIP76 tSNPs and epilepsy predisposition*

Locus	Genotype number			Genotype frequency (%)			χ^2	p Value	Allele number		Allele frequency (%)		χ^2	p Value
rs1979368	CC	TC	TT	CC	TC	TT			C	T	C	T		
Controls	40	147	177	0.11	0.4	0.49	2.96	0.23	227	501	0.31	0.69	0.3	0.58
Patients	74	357	350	0.09	0.46	0.45			505	1057	0.32	0.68		
Total	114	504	527	0.1	0.44	0.46			732	1558	0.32	0.68		
rs1561998	AA	CA	CC	AA	CA	CC			A	C	A	C		
Controls	21	132	209	0.06	0.36	0.58	1.64	0.44	174	550	0.24	0.76	1.64	0.2
Patients	37	263	479	0.05	0.34	0.61			337	1221	0.22	0.78		
Total	58	395	688	0.05	0.35	0.6			511	1771	0.22	0.78		
rs2028660	CC	TC	TT	CC	TC	TT			C	T	C	T		
Controls	11	134	219	0.03	0.37	0.6	4.55	0.1	156	572	0.21	0.79	0.8	0.37
Patients	46	268	465	0.06	0.34	0.6			360	1198	0.23	0.77		
Total	57	402	684	0.05	0.35	0.6			516	1770	0.23	0.77		
rs1813100	AA	GA	GG	AA	GA	GG			A	G	A	G		
Controls	130	163	70	0.36	0.45	0.19	1.29	0.52	423	303	0.58	0.42	0.83	0.36
Patients	252	370	155	0.32	0.48	0.2			874	680	0.56	0.44		
Total	382	533	225	0.34	0.47	0.2			1297	983	0.57	0.43		
rs329007	AA	GA	GG	AA	GA	GG			A	G	A	G		
Controls	210	133	21	0.58	0.37	0.06	1.37	0.5	553	175	0.76	0.24	1.33	0.25
Patients	475	261	39	0.61	0.34	0.05			1211	339	0.78	0.22		
Total	685	394	60	0.6	0.35	0.05			1764	514	0.77	0.23		
rs167897	CC	TC	TT	CC	TC	TT			C	T	C	T		
Controls	240	111	13	0.66	0.3	0.04	1.82	0.4	591	137	0.81	0.19	1.64	0.2
Patients	548	211	25	0.7	0.27	0.03			1307	261	0.83	0.17		
Total	788	322	38	0.69	0.28	0.03			1898	398	0.83	0.17		

potential role of transporters in mediating such resistance (reviewed in Sisodiya, 2003; Kwan and Brodie, 2005), not least because of the emerging availability of agents that might inhibit some transporters postulated to have a role. For example, experimental animal studies have suggested that presumptive inhibition of P-glycoprotein, the best-studied transporter, may improve seizure control in specific models (Brandt et al., 2005; van Vliet et al., 2006). The existence of a large number of other multispecific transporters is well appreciated (Hediger et al., 2004; Kusuhara and Sugiyama, 2005): some of these may be present in human brain, and may possibly contribute to the phenomenon of multiple drug resistance. It is clearly important to know which transporters might be involved, if rational therapies are to be developed based on proof of such involvement—particularly if research activity is directed away from one transporter, such as P-glycoprotein, to another. Therefore it is important to establish with as much certainty as possible whether a molecule does or does not mediate resistance.

In the first instance, it must be demonstrated that a candidate transporter is to be found in the human brain in an appropriate distribution (Sisodiya, 2003). It remains unclear whether an appropriate distribution need be endothelial, glial or neuronal, or indeed a combination or variety depending on the substrate specificity and AED transport capacity (if any) of the promulgated transporter. It seems reasonable to suppose that expression in brain microvascular endothelial cells would be appropriate for most AEDs, as passage across the blood–brain barrier is a sine qua non for any AED as currently conceptualized. With respect to RLIP76, we were unable to replicate the original finding of its endothelial localization, both in terms of morphological appearance on microscopy and co-localization with an established endothelial cell marker, CD34. Issues of possible nonspecific reaction or cross-reaction of antibodies have been well rehearsed and in the oncology field, guidelines exist for immunohistochemical detection of candidate proteins (Beck et al., 1996). The chief recommendation is that two separate antibodies, directed against separate epitopes, are used for immunohistochemistry. The antibody we employed targets a different epitope from that to which the antibody employed in the original report reacts, so that these two studies together might be considered to address the need for dual antibody detection. In addition, double labeling fails to show colocalization of RLIP76 with either CD34 or P-glycoprotein, immunoreactivity for both of which is found in brain microvascular endothelium (Navratil et al., 1997; Sisodiya et al., 2006). On these grounds, it would seem that the brain endothelial localization of RLIP76 could not be assumed proven at this point. It should also be noted that technical issues may complicate immunohistochemical studies (Volk et al., 2005).

TABLE 5. *Multilocus associations between RLIP76 tSNPs and the two traits studied*

Trait	Ln (case)	Ln (control)	Ln (case+ control)	d.f.	χ^2	p value
Drug resistance	–820.56	–352.78	–1,179.6	9	12.52	0.19
Epilepsy predisposition	–1256.96	–569.32	–1,833.24	9	13.92	0.13

Ln, natural log.

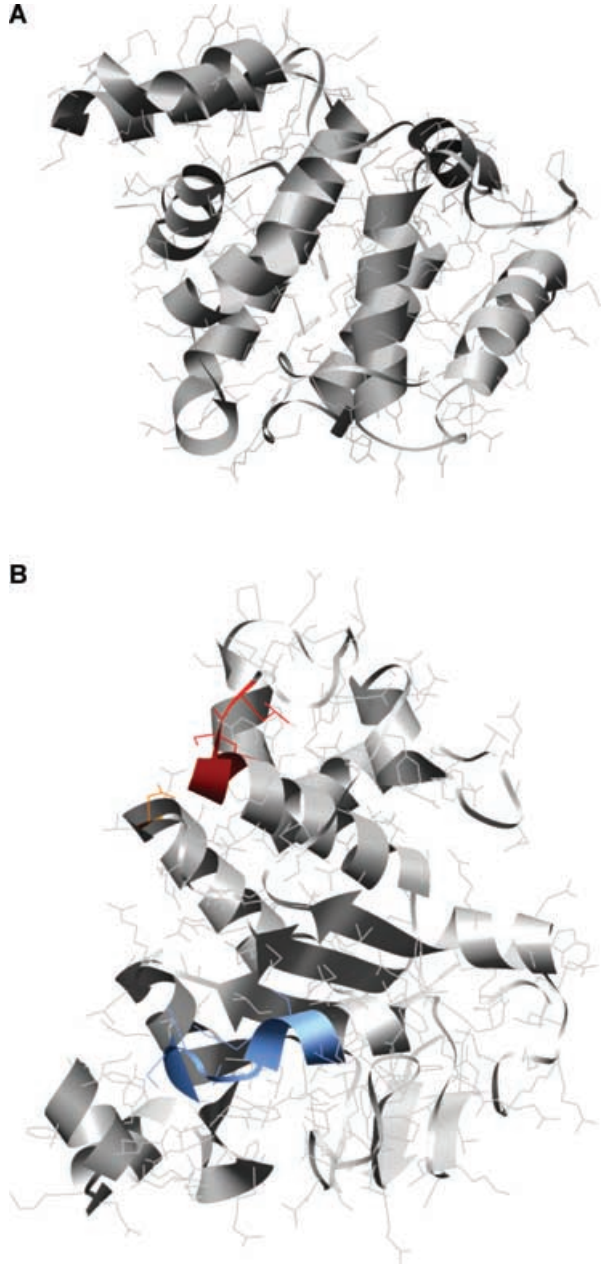

FIG. 4. (A) Comparative model of human RLIP76 residues 183–367 based on the template structure of human RhoA and its GTPase-activating protein in complex with a transition-state analogue (PDB ID: 1tx4). **(B)** Comparative model of human ABC transporter ABCB1 (P-glycoprotein) nucleotide binding domain 2, residues 1028-1271. The Walker A motif is shown in light blue, the Walker B motif in orange and the signature (LSGGQ) sequence is shown in red. None of these motifs are present in the RLIP76 sequence. Figures created with UCSF Chimera. Models available via the ModBase comparative model database (Pettersen et al., 2004; Pieper et al., 2006).

The transport capacity of candidate transporters is clearly also an important issue. Even for the best studied candidate transporter, P-glycoprotein, there remains some doubt about its AED transport capacity and affinity (Owen et al., 2001; Weiss et al., 2003; Schmidt and Loscher, 2005). Substrate transport against a gradient requires energy. RLIP76 was proposed to utilize hydrolysis of ATP as its energy source (Awasthi et al., 2005). Members of the ABC superfamily of transporters all share three common domains that are absolutely required for ATP hydrolysis, the Walker A, Walker B and signature C motifs (Ambudkar et al., 2006). Our bioinformatic analyses do not provide any evidence that RLIP76 has any of these three functional domains or similarity to other known ATP-binding motifs. Furthermore, protein models of the highly conserved RhoGap domain of RLIP76 and the functional ATP-binding domain of P-glycoprotein show no significant similarity. In light of the fact that these ATPase domains are highly conserved in species ranging from yeast and bacteria up to humans, this analysis provides no evidence that RLIP76 can support ATP-dependent transport by the same mechanism as ABC transporter proteins. It remains possible, however, that novel ATP-binding sites not currently recognized in motif dababases support ATP hydrolysis by RLIP76. Our modeling data also do not provide evidence to support a transmembrane localization for RLIP76. All members of the two large drug transporter superfamilies, ABC and solute carrier (SLC), have the common feature of multiple transmembrane-spanning domains, thought to be essential for facilitating the physical movement of substrates across the lipophilic membrane (Hediger et al., 2004; Tusnady et al., 2006).

The examination of drug transporter activity in vivo in man is problematic. Demonstration of ability to transport drugs in vitro need not equate with a similar capacity in vivo, given potential confounds from the activities of other transporters, known and unknown, the effects of in vitro culture on gene expression, and compensatory changes following gene knockout. Pharmacogenetics association studies offer one method for the exploration of in vivo protein activity (Soranzo et al., 2005), which we have previously utilized in epilepsy drug response (Siddiqui et al., 2003), though they are not without their own difficulties (Tan et al., 2004). Our current studies do not provide evidence that common variations in the gene encoding RLIP76 influences response to AEDs or, indeed, susceptibility to epilepsy. Such data may have implications for prognostication, but do not formally exclude a role for a transporter in mediating resistance, as indeed we have argued for P-glycoprotein (Sisodiya et al., 2005). However, for P-glycoprotein, data from several other types of experiment suggest it may have a role in mediating AED resistance in the absence of proven support from a pharmacogenetic perspective: such data are still lacking for RLIP76.

Our studies do not provide additional supporting data to substantiate a role for RLIP76 in mediating drug resistance in epilepsy. We have not replicated all the experiments undertaken in the original study, and in light of

the complexity associated with drug response it remains possible that RLIP76 does have some role in resistance to AEDs, either directly or by regulation of pathways in which it figures. However, further studies would seem necessary before its role can be considered proven, and before attention is diverted to therapies based on modulation or evasion of RLIP76. The role of transporters in AED resistance is likely to prove complex (Sisodiya and Bates, 2006), and require further cautious investigation before clinical trials of modulation of one or more transporters can be considered for the treatment of drug-resistant epilepsy.

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REFERENCES

Ambudkar SV, Kim IW, Sauna ZE. (2006) The power of the pump: mechanisms of action of P-glycoprotein (ABCB1). *European Journal of Pharmaceutical Sciences* 27: 392–400.

Aronica E, Gorter JA, Van Vliet EA, Spliet WG, Van Veelen CW, Van Rijen PC, Leenstra S, Ramkema MD, Scheffer GL, Scheper RJ, Sisodiya SM, Troost D. (2003) Overexpression of the human major vault protein in gangliogliomas. *Epilepsia* 44:1166–1175.

Awasthi S, Hallene KL, Fazio V, Singhal SS, Cucullo L, Awasthi YC, Dini G, Janigro D. (2005) RLIP76, a non-ABC transporter, and drug resistance in epilepsy. *BMC Neuroscience* 27:6:61–72.

Beck WT, Grogan TM, Willman CL, Cordon-Cardo C, Parham DM, Kuttesch JF, Andreeff M, Bates SE, Berard CW, Boyett JM, Brophy NA, Broxterman HJ, Chan HS, Dalton WS, Dietel M, Fojo AT, Gascoyne RD, Head D, Houghton PJ, Srivastava DK, Lehnert M, Leith CP, Paietta E, Pavelic ZP, Weinstein R. (1996) Methods to detect P-glycoprotein-associated multidrug resistance in patients' tumors: consensus recommendations. *Cancer Research* 56:3010–3020.

Berg AT, Kelly MM. (2006) Defining intractability: comparisons among published definitions. *Epilepsia* 47:431–436.

Brandt C, Bethmann K, Gastens A, Loscher W. (2005) Resistance to antiepileptic drug treatment can be counteracted by inhibition of P-glycoprotein in a rat model of temporal lobe epilepsy. *Epilepsia* 46:(Suppl. 6): 58.

Cerveny L, Pavek P, Malakova J, Staud F, Fendrich Z. (2006) Lack of interactions between breast cancer resistance protein (bcrp//abcg2) and selected antiepileptic agents. *Epilepsia* 47:461–468.

Hediger MA, Romero MF, Peng JB, Rolfs A, Takanaga H, Bruford EA. (2004) The ABCs of solute carriers: physiological, pathological and therapeutic implications of human membrane transport proteinsIntroduction. *Pflügers Archiv: European Journal of Physiology* 447: 465–468.

Hulo N, Bairoch A, Bulliard V, Cerutti L, De Castro E, Langendijk-Genevaux PS, Pagni M, Sigrist CJ. (2006) The prosite database. *Nucleic Acids Research* 34: D227-30.

Krogh A, Larsson B, von Heijne G, Sonnhammer EL. (2001) Predicting trans- membrane protein topology with a hidden Markov model: application to complete genomes. *Journal of Molecular Biology* 305: 567–580

Kusuhara H, Sugiyama Y. (2005) Active efflux across the blood-brain barrier: role of the solute carrier family. *NeuroRx* 2:73–85.

Kwan P, Brodie MJ. (2005) Potential role of drug transporters in the pathogenesis of medically intractable epilepsy. *Epilepsia* 46:224–235.

Loscher W, Potschka H. (2005) Blood-brain barrier active efflux transporters: ATP-binding cassette gene family. *NeuroRx* 2:86–98.

Moller S, Croning MD, Apweiler R. (2001) Evaluation of methods for the prediction of membrane spanning regions. *Bioinformatics* 17: 646–653

Navratil E, Couvelard A, Rey A, Henin D, Scoazec JY. (1997) Expression of cell adhesion molecules by microvascular endothelial cells in the cortical and subcortical regions of the normal human brain: an immunohistochemical analysis. *Neuropathology and Applied Neurobiology*. 23:68–80.

Owen A, Pirmohamed M, Tettey JN, Morgan P, Chadwick D, Park BK. (2001) Carbamazepine is not a substrate for P-glycoprotein. *British Journal of Clinical Pharmacology* 51:345–349.

Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE. (2004) UCSF Chimera – A Visualization System for Exploratory Research and Analysis. *Journal of computational chemistry* 25: 1605–1612.

Pieper U, Eswar N, Davis FP, Braberg H, Madhusudhan MS, Rossi A, Marti-Renom M, Karchin R, Webb BM, Eramian D, Shen MY, Kelly L, Melo F, Sali A. (2006) MODBASE: a database of annotated comparative protein structure models and associated resources. *Nucleic Acids Research* 34: D291–5.

Rittinger K, Walker PA, Eccleston JF, Smerdon SJ, Gamblin SJ. (1997). Structure at 1.65 a of RhoA and its GTPase-activating protein in complex with a transition-state analogue. *Nature* 389: 758–762.

Scheffer GL, Scheper RJ. (2002) Drug resistance molecules: lessons from oncology. *Novartis Foundation Symposia* 243:19–31.

Schmidt D, Loscher W. (2005) Drug resistance in epilepsy: putative neurobiologic and clinical mechanisms. *Epilepsia* 46:858–877.

Siddiqui A, Kerb R, Weale ME, Brinkmann U, Smith A, Goldstein DB, Wood NW, Sisodiya SM. (2003) Association of multidrug resistance in epilepsy with a polymorphism in the drug-transporter gene ABCB1. *New England Journal of Medicine* 348:1442–1448

Sisodiya SM, Soranzo N, Wood NW, Goldstein DB. (2005) Association of genetic loci: replication or not, that is the question. *Neurology* 64:1989.

Sisodiya SM, Bates SE. (2006) Treatment of drug resistance in epilepsy: one step at a time. *Lancet Neurology* 5:380–381.

Sisodiya SM, Martinian L, Scheffer GL, Van der Valk P, Cross JH, Scheper RJ, Harding BN, Thom M. (2003) Major vault protein, a marker of drug resistance, is upregulated in refractory epilepsy. *Epilepsia* 44:1388–1396.

Sisodiya SM, Martinian L, Scheffer GL, Van der Valk P, Scheper RJ, Harding BN, Thom M. (2006) Vascular colocalization of P-glycoprotein, multidrug-resistance associated protein 1, breast cancer resistance protein and major vault protein in human epileptogenic pathologies. *Neuropathology and Applied Neurobiology* 32:51–63.

Sisodiya SM. (2003) Mechanisms of antiepileptic drug resistance. *Current Opinions in Neurology* 16:197–201.

Soranzo N, Goldstein DB, Sisodiya SM. (2005) The role of common variation in drug transporter genes in refractory epilepsy. *Expert Opinion on Pharmacotherapy* 6:1305–1312.

Tan NC, Heron SE, Scheffer IE, Pelekanos JT, McMahon JM, Vears DF, Mulley JC, Berkovic SF. (2004) Failure to confirm association of a polymorphism in ABCB1 with multidrug-resistant epilepsy. *Neurology* 63:1090–1092.

Tusnady GE, Simon I. (2001) The hmmtop transmembrane topology prediction server. *Bioinformatics* 17:849–850.

Tusnady GE, Sarkadi B, Simon I, Varadi A. (2006) Membrane topology of human ABC proteins. *FEBS Letters* 580:1017–1022.

van Vliet EA, Van Schaik R, Edelbroek PM, Redeker S, Aronica E, Wadman WJ, Marchi N, Vezzani A, Gorter EA. (2006) Inhibition of the multidrug transporter P-glycoprotein by XR9576 improves seizure control in phenytoin-treated chronic epileptic rats. *Epilepsia* 47:672–680.

Volk H, Potschka H, Loscher W. (2005) Immunohistochemical localization of P-glycoprotein in rat brain and detection of its increased expression by seizures are sensitive to fixation and staining variables. *J Histochem Cytochem.* 53:517–531.

Weale M, Goldstein DB. (2005) TagIT User Guide – Version 3.0.
Weale ME, Depondt C, Macdonald SJ, Smith A, Lai PS, Shorvon SD, Wood NW, Goldstein DB (2003). Selection and evaluation of tagging SNPs in the neuronal-sodium-channel gene SCN1A: implications for linkage-disequilibrium gene mapping. *American Journal of Human Genetics 73*:551–565.
Weiss J, Kerpen CJ, Lindenmaier H, Dormann SM, Haefeli WE. (2003) Interaction of antiepileptic drugs with human P-glycoprotein in vitro. *The Journal of Pharmacology and Experimental Therapeutics*. 307:262–267.
Wheeler DL, Barrett T, Benson DA, Bryant SH, Canese K, Chetvernin V, Church DM, DiCuccio M, Edgar R, Federhen S, Geer LY, Helmberg W, Kapustin Y, Kenton DL, Khovayko O, Lipman DJ, Madden TL, Maglott DR, Ostell J, Pruitt KD, Schuler GD, Schriml LM, Sequeira E, Sherry ST, Sirotkin K, Souvorov A, Starchenko G, Suzek TO, Tatusov R, Tatusova TA, Wagner L, Yaschenko E. (2006) Database resources of the national center for biotechnology information. *Nucleic Acids Research* 34(Database issue):D173–80
Zhao JH, Curtis D, Sham PC. (2000) Model-free analysis and permutation tests for allelic associations. *Human Heredity* 50:133–139.