MS were observed in the present study, including a low frequency of CSF IgG abnormalities and poor response to IFN-β. Zéphir *et al*[16] also reported an absence of CSF oligoclonal IgG bands in five cases with CCPD. Collectively, these findings suggest that at least some mechanisms are distinct from MS function in CCPD.

Most patients with CCPD responded well to immunotherapies, regardless of acute or chronic onset, suggesting a contributory immune/inflammatory mechanism. Although we found only one paper reporting on the efficacy of the combined use of intravenous immunoglobulin and plasma exchange in a case of CCPD,[12] the present study disclosed for the first time a high efficacy for plasma exchange in CCPD, which may suggest humoral immunity involvement. A female preponderance in CCPD is also consistent with the nature of autoimmune diseases, although common systemic autoantibodies and antiganglioside antibodies were infrequent, as in previous reports.[8–12 16 23] The unresponsiveness or even disease exacerbation following IFN-β therapy found in our study was consistent with previous reports of CIDP development after IFN-β introduction in patients with MS.[24 25] Such a phenomenon may also support an autoantibody-mediated mechanism because type I IFNs potently stimulate the production of all subclasses of IgG antibodies.[26] These findings suggest the involvement of specific autoantibodies reactive to antigens that are commonly present in CNS and PNS tissues in CCPD. Additional large-scale studies will be needed to clarify the relevant antigens in this condition.

There were several distinctive features between cases of simultaneous and temporarily separated onset of CCPD. A relapsing remitting course was observed more often in the latter than in the former, whereas a monophasic course was observed more often in the simultaneous onset. This difference may be because of the classification criteria as well as the shorter observation period of the simultaneous onset group. However, Adamovic *et al*[17] reported that among 13 paediatric patients with acute simultaneous inflammatory demyelination of both the CNS and PNS, recurrence was seen only in 2 (15.4%) cases. Accordingly, as in our series, the simultaneous onset cases were followed-up nearly 4 years on average. Therefore, simultaneous onset CCPD may be less likely to recur. In addition, the difference in clinical and laboratory manifestations cannot be explained solely by the difference in observation times. For example, visual disturbance and VEP abnormalities were observed more frequently in the temporarily separated onset group than in the simultaneous onset group, in which none of the cases showed apparent visual impairment. By contrast, frequencies of other cranial nerve involvements did not differ between the simultaneous onset and temporarily separated onset groups. Thus, frequent optic nerve involvement appears to be one characteristic feature of CCPD with temporarily separated onset. This suggestion is consistent with previous case reports examining relapsing CIDP with optic nerve lesions,[27–29] as well as the relatively high frequency of VEP abnormalities in relapsing or progressive patients with CIDP (8/17, 47%).[23] Therefore, this may be a useful laboratory test for suspected CCPD cases, especially those with relapsing CIDP as a presenting feature. By contrast, the simultaneous onset group had a significantly higher frequency of patients with PNS involvement and isolated brain involvement than the temporarily separated onset group, and no patients in the simultaneous onset group had PNS involvement and isolated spinal cord involvement, whereas 20% of the temporarily separated group patients did. Collectively, such differences in the CNS sites of involvement further indicate that distinct mechanisms are operating in these two subgroups.

It is interesting to note that LESCLs were exclusively found in the simultaneous onset group and extensive cerebral lesions were also more common in the simultaneous onset group than in the temporarily separated onset group. Since no AQP4 antibodies were detected in any CCPD cases examined, LESCLs are likely produced by a mechanism distinct from that in neuromyelitis optica (NMO). Indeed, Devic type (optic–spinal) involvement was seen only in the temporarily separated group but not in the simultaneous onset group, further suggesting that LESCLs in the simultaneous onset group are produced by mechanisms distinct from those in NMO. Although the mechanisms for such extensive lesions remain unknown, it is important to raise CCPD as a differential diagnosis for LESCLs and extensive brain lesions.

In the present series, compared with the temporarily separate onset cases, the simultaneous onset cases exhibited more severe disabilities at the peak of illness, such as higher frequencies of respiratory disturbance and greater Hughes functional scale scores, which were likely a reflection of the high frequency of extensive brain and spinal cord MRI lesions. These findings were consistent with those of Adamovic *et al*,[17] who showed that among 13 paediatric patients with acute simultaneous inflammatory demyelination of the CNS and PNS, 6 were bedbound or wheelchair users and one remained on mechanical ventilation at discharge. In our series, however, the simultaneous onset group showed improvements similar to or better than the temporarily separated onset group after immunotherapy, suggesting a high efficacy of immunotherapy for simultaneous onset CCPD, despite severe manifestations. Further studies and characterisation of simultaneous onset and temporarily separated onset CCPD cases may support the existence of two CCPD subtypes and help to shed light on the distinct mechanisms between the two subtypes.

In conclusion, CCPD exhibits distinctive features from those of the classical demyelinating diseases and, therefore, may be a distinct disease, but it is not a simple coexistence of MS and CIDP. Simultaneous onset CCPD is characterised by severe disability with a relatively high frequency of respiratory disturbance, as well as extensive brain and spinal cord lesions observed in MRI scans. By contrast, temporarily separated onset CCPD features a high frequency of optic nerve involvement. Although CCPD is extremely rare, awareness of this condition is important because responses to disease-modifying drugs, such as IFN-β, for patients with CCPD are different from those in patients with MS, and appropriate immunotherapies may well produce satisfactory outcomes with minimal disabilities.

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Contributors HO, DM, TY and JK conceived the study, supervised the analyses and wrote the paper. RY, NK, TM, MH and HM participated in procedure development and collated the data.

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A nationwide survey of combined central and peripheral demyelination in Japan

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Genetic associations with brain cortical thickness in multiple sclerosis

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Abstract

Multiple sclerosis (MS) is characterized by temporal and spatial dissemination of demyelinating lesions in the central nervous system. Associated neurodegenerative changes contributing to disability have been recognized even at early disease stages. Recent studies show the importance of gray matter damage for the accrual of clinical disability rather than white matter where demyelination is easily visualized by MRI. The susceptibility to MS is influenced by genetic risk, but genetic factors associated with the disability are not known. We used MRI data to determine cortical thickness in 557 MS cases and 75 controls and in another cohort of 219 cases. We identified 9 areas showing different thickness between cases and controls (regions of interest, ROI) (8 of them were negatively correlated with Kurtzke's expanded disability status scale, EDSS) and conducted genome-wide association (GWA) in 464 and 211 cases available from the two data sets. No marker exceeded genome-wide significance in the discovery cohort. We next combined nominal statistical evidence of association with physical evidence of interaction from a curated human protein interaction network, and searched for subnetworks enriched with nominally associated genes and searched for commonalities between the two data sets.

This network-based pathway analysis of GWAS detected gene sets involved in glutamate signaling, neural development and an adjustment of intracellular calcium concentration. We report here for the first time gene sets associated with cortical thinning of MS. These genes are potentially correlated with disability of MS.

Introduction

Multiple sclerosis (MS) [OMIM: 126200] is the most prevalent cause of nontraumatic neurologic disability in young adults in Europe and North America (Trapp & Nave, 2008), and is characterized by temporal and spatial dissemination of demyelinating lesions in the central nervous system (CNS)(Hauser & Oksenberg, 2006). Most patients with MS have a relapsing-remitting course with progressive neurological disability being common at the late stages of the disease. Recent studies, however, show that the neurodegenerative changes contributing to the sustained clinical deficits are manifest even at an early phase in the natural history of the disease.

On the basis of demyelination patterns found by magnetic resonance imaging (MRI) and post-mortem studies, searches for the etiology of MS have been largely confined to the white matter (WM) (Noseworthy *et al.*, 2000). However, advances in neuroimaging and neuropathological techniques have enabled the localization of damage to the gray matter (GM) as well, manifested as both focal demyelination and more generalized cortical tissue thinning (Bo *et al.*, 2003, Calabrese *et al.*, 2007, Geurts *et al.*, 2005, Kutzelnigg *et al.*, 2005, Nakamura & Fisher, 2009, Patenaude *et al.*, 2011). Furthermore, both cortical lesions and volume loss appear to occur already at early stages of MS (Calabrese *et al.*, 2007, Chard *et al.*, 2002, Dalton *et al.*, 2004, De Stefano *et al.*, 2003, Giorgio *et al.*, 2011, Henry *et al.*, 2008, Tiberio *et al.*, 2005) and are more correlated with physical disability and cognitive impairment than WM lesion load (Calabrese *et al.*, 2010b, Chen *et al.*, 2004, Fisniku *et al.*, 2008). Finally, GM damage could serve as a potential prognostic factor for disease progression (Calabrese *et al.*, 2011, Calabrese *et al.*, 2010b, Filippi *et al.*, 2010). Altogether, these findings suggest that identifying the causes of GM pathology is a

valid strategy to advance our understanding of the neurodegenerative processes underlying MS.

Susceptibility to MS is thought to be influenced by both environmental and genetic factors (Hauser & Oksenberg, 2006). In addition to the well-documented effect of certain human leukocyte antigen (HLA) alleles, genome-wide association studies (GWAS) carried out on large sample sizes have shown that DNA polymorphisms at more than 100 non-HLA loci influence MS risk ((Imsgc) *et al.*, 2011, Beecham *et al.*, 2013, Patsopoulos *et al.*, 2011). However, with the notable exception of the HLA system, genetic factors associated with clinical phenotype or quantitative neuroimaging outcomes have not been yet identified ((Imsgc) *et al.*, 2011, Beecham *et al.*, 2013, Okuda *et al.*, 2009, Tur *et al.*, 2014, Yates *et al.*, 2014). On the other hand, brain structure is also influenced by genetic factors. For example, comparison of monozygotic and dizygotic twins highlighted the genetic influence on cortical thickness and even with standardized measures of intellectual ability (Eyler *et al.*, 2012, Eyler *et al.*, 2011, Joshi *et al.*, 2011, Kochunov *et al.*, 2011, Kremen *et al.*, 2010, Rimol *et al.*, 2010). One study reported that siblings with MS had similar patterns of cortical thinning and lesion numbers (Calabrese *et al.*, 2012). Although small and not yet replicated, these observations suggest that genetic factors may influence GM pathology in MS.

The aim of this study is to identify genes correlated with GM pathology measured as brain cortical thinning in two large MS cohorts. We conducted a GWAS using cortical thickness as the outcome variable in both groups independently and conducted a pathway analysis using protein interaction networks. Loci identified through this effort

will shed light on a novel aspect of genetic influence on MS, which may have an effect on the clinical course.

Materials and Methods

Subjects

A total of 851 subjects evaluated at the Multiple Sclerosis Center of the University of California, San Francisco (referred to as UCSF) (n=557 cases and 75 healthy controls) and University Hospital Basel, Switzerland (referred to as Basel) (n=219) were recruited to participate in a prospective study of phenotype–genotype-biomarker associations using identical diagnostic and inclusion/exclusion criteria (Baranzini *et al.*, 2009b). Individuals affected with all disease types were included: clinically isolated syndrome (CIS), relapsing remitting MS (RRMS), secondary progressive MS (SPMS), primary progressive MS (PPMS), and progressive relapsing MS (PRMS) (Table 1). CIS was defined as the first well-established neurological event lasting ≥ 48 h, involving the optic nerve, spinal cord, brainstem or cerebellum. In CIS patients, the presence of two or more hyperintense lesions on a T2-weighted MRI sequence was also required for enrollment into the study. The diagnosis of RRMS was made utilizing the revised McDonald's criteria (Polman *et al.*, 2005). SPMS was defined by 6 months of worsening neurological disability not explained by clinical relapse. PPMS was defined both by progressive clinical worsening for more than 12 months from symptom onset without any relapses, and abnormal cerebrospinal fluid as defined by the presence of ≥ 2 oligoclonal bands or an elevated IgG index. Individuals were excluded if they had experienced a clinical relapse or received treatment with glucocorticosteroids within the previous month of enrollment. The concomitant use of disease modifying therapies for MS was permitted. For all subjects, the expanded disability status scale (EDSS) score (Kurtzke, 1983) was assessed. In this study, age of onset was defined as the first episode of focal neurological dysfunction suggestive of CNS demyelinating disease.

This information was obtained via individual recall and verified through review of medical records.

The control group consisted of genetically unrelated individuals, primarily spouses/partners, friends and other volunteers. Control subjects were of northern-European ancestry and matched as a group, proportionally with cases according to age (± 5 years) and gender. A family history or current diagnosis of MS as well as a relation to another case or control subject in the cohort were sufficient reasons for exclusion. The protocols were approved by the Committee on Human Research in San Francisco and the ethics committee in Basel. Informed consent was obtained from all participants.

Image acquisition

Brain MRI scans were performed on all subjects upon entry into the study, and analyses were carried out without knowledge of disease subtype, duration or treatment history. MRI images at UCSF were acquired using an 8-channel phased array coil in reception and a body coil in transmission on a 3T GE Excite scanner (GE Healthcare Technologies, Waukesha, WI). For the UCSF cohort, MR imaging examination included axial dual-echo spin echo sequences (TE at 20 and 90ms, TR=2000ms, 512 × 512 × 44 matrix, 240 × 240 × 132 mm^3 FOV, slice thickness=3mm, interleaved). A high-resolution inversion recovery spoiled gradient-echo T1-weighted isotropic, volumetric sequence (3D IRSPGR, 1 × 1 × 1mm^3, 180 slices) was also performed (TE/TR/ TI = 2/7/400ms, flip angle = 15º, 256 × 256 × 180 matrix, 240 × 240 × 180 mm^3 FOV, NEX = 1). In Basel, MRI scanning was performed using a 1.5 T Magnetom Avanto scanner (Siemens Medical Solutions, Erlangen, Germany). High resolution T1 weighted MPRAGE images were acquired in sagittal plane (TR/TI/TE=2080/1100/3.0 ms; =15°, 160 slices, isotropic voxel of 1x1x1 mm).

Axial 3 mm proton density-weighted (PDw) and T2-weighted (T2w) images were also acquired to determine the white matter lesion load (double spin echo: TR/TE1/TE2=3980/14/108 ms; 40 slices with an in-plane resolution of 1x1 mm).

Brain lesions were identified by medical doctors specializing in MS after analyzing high-resolution T1-weighted, T2-weighted, and proton density-weighted images. To create T1 lesion masks, lesions were outlined based on semi-automated thresholding with manual editing utilizing in-house software in San Francisco and using Amira 3.1.1 (Mercury Computer Systems Inc.) in Basel (Blum *et al.*, 2002). Both intra and inter-observer variability analyses were performed to ensure the accuracy of data acquired. T2-lesion volumes from ROI selections were calculated by multiplying the area of the lesion by the slice thickness and the number of slices penetrated using in-house software.

Measurement of cortical thickness

FreeSurfer Image Analysis Suite (http://surfer.nmr.mgh.harvard.edu/) (Dale *et al.*, 1999, Fischl *et al.*, 1999) was used for cortical reconstruction, volumetric segmentation, subcortical (Fischl *et al.*, 2004) and cortical parcellation (Desikan-Killiany Atlas). Volumes were normalized for intracranial volume (Desikan *et al.*, 2006). Segmentation results were assessed by an experienced operator to ensure accuracy and manually edited when needed. Both white matter and juxtacortical lesions were filled before running Freesurfer segmentation of the cortical thicknesses. Two cases were removed for having more than 2 times the interquartile range of median cortical thickness across all cortical regions. Thickness values were confined to a range of the mean ± 3 standard deviations in each region.

Defining cortices of interest for genome wide association analysis

In order to define regions of interest (ROI) for the GWAS, cortical thickness adjusted by age, gender and a volumetric scaling factor (used for standardizing head size) in each of the 34 regions parcellated by FreeSurfer was compared between cases and controls in UCSF using a linear regression model. The Bonferroni method was used to adjust significance for multiple hypotheses testing in each hemisphere. ROI were defined as cortex areas showing a statistically significant ($P_{corr} < 0.05$) difference between cases and controls in either hemisphere (corrected for 34 tests) and a nominal p value of 0.05 in the contralateral hemisphere. The cortical thickness in the ROI were independently measured in patients from the Basel cohort in order to confirm the genetic associations observed. This analysis was performed using the R statistical package.

Single nucleotide polymorphism (SNP) genotyping

DNA was isolated from blood cells using standard protocols. Genotyping of subjects was performed at the Illumina facilities using the Sentrix® HumanHap550 BeadChip as described elsewhere (Baranzini *et al.*, 2009b). SNPs that did not satisfy the following quality control criteria were excluded: genotype call rate < 95%, significant deviation from Hardy–Weinberg equilibrium $p < 0.001$, minor allele frequency < 0.005.

GWAS with cortical thickness as outcome variable

For the MS patients, allelic effects for cortical thickness were calculated based on a linear regression model including age at examination, gender, disease course, a volumetric scaling factor and T2 lesion load as covariates. Disease course was

aggregated into two categories: non-progressive MS (CIS + RRMS), and progressive MS (SP + PP + PRMS). The genetic effects for thickness were tested using minor allele dominant/recessive/additive linear regression models and the most significant p value among the three models was selected for each SNP. To keep minor allele homozygous sample size, minor allele frequency (MAF) threshold was set at 0.15 in the recessive model. All calculations were performed in PLINK (Purcell *et al.*, 2007).

Computing gene-wise p values and association blocks

The first step in any pathway analysis is to compute gene-level p-values. To that end, we used Versatile Gene-based Association Study (VEGAS), a previously described method that takes into account gene sizes and patterns of linkage disequilibrium (Liu *et al.*, 2010). VEGAS assigns SNPs to each of 17,787 autosomal genes according to positions on the UCSC Genome Browser (hg18 assembly). To capture regulatory regions and SNPs in linkage disequilibrium (LD), gene boundaries are defined as 50 kb beyond the 5' and 3' untranslated regulatory regions (UTRs) of each gene. VEGAS factors in LD patterns between markers within a gene by using Monte-Carlo simulations from the multivariate normal distribution on the basis of the LD structure of a set of reference individuals (the HapMap2 CEU population). An empirical p value of 0 from 10^6 VEGAS simulations can be interpreted as $p < 10^{-6}$, which exceeds a Bonferroni-corrected threshold of $p < 2.8 \times 10^{-6}$ (= 0.05/ 17,787; this threshold is likely to be conservative given the overlap between genes).

We defined association blocks as those groups of sequential genes with a p value < 0.05. A block_id was assigned to each association block along the genome for each region. To evaluate the likelihood that these nominally significant genes were replicated in two data sets by chance, a hypergeometric test (one-sided Fisher's exact test) was conducted in each region.

Protein interaction network based pathway analysis (PINBPA)

We downloaded the entire iRefIndex database (Razick *et al.*, 2008), a collection of 15 human protein interaction network (PIN) data sets from different sources, and computed the union data set. This set comprised more than 400,000 interactions among ~25,000 proteins. However, many of these interactions were either predicted or backed up by a single experiment (i.e., a single publication). In order to minimize the rate of false positives, we then filtered this large network to keep only those interactions that were described in at least two independent publications. This resulted in a network of 8,960 proteins (nodes) and 27,724 interactions (edges). We used this high-confidence network for all subsequent analyses. The network was uploaded into Cytoscape 3.0.1 and annotated with genomic position, gene-wise p value, and block_id (loaded as node attributes) for all analyzed regions.

For each region and data set, we computed significant first-order interactions by filtering the main network so as to keep only those genes (i.e. their encoded proteins) with VEGAS p values < 0.05. Then, the number of resulting nodes and edges and the size of the largest connected component were computed within Cytoscape. To evaluate the likelihood that these numbers were obtained by chance (as a consequence of the sheer number of interactions), we computed 1,000 simulations by assigning p values at random from the same network and creating subnetworks of similar size. These simulations were used as background for estimating the significance of the subnetworks obtained with the real gene-wise p values. We then used the program (plugin) jActiveModules to conduct searches of subnetworks enriched with (but not necessarily composed of) genes with significant p values (Ideker *et al.*, 2002). jActiveModules starts by converting each gene p value into a Z score by using the inverse normal cumulative distribution function. Then, it

produces an aggregate Z score (Z_A) for an entire subnetwork A of k genes by summing the Zi over all genes in the subnetwork $Z_A = 1/\sqrt{k} \sum_{i \in A} Z_i$.

That score is later corrected for network size and topology and an S_A value is produced. As in previous studies, we took an $S_A > 3$ as evidence of a biologically active subnetwork ((lmsgc), 2013, Baranzini *et al.*, 2009a, Ideker *et al.*, 2002). To evaluate the likelihood that these enriched networks were replicated in the two data sets by chance, a hypergeometric test (one-sided Fisher's exact test) was performed in each region and the significance was estimated by a permutation test, in which we iteratively shuffled gene-wise p values, mapped the shuffled p values to the PIN, extracted the enriched subnetworks for 50 times, then we calculated an enrichment between any two out of the 50 independent data sets. Based on an enrichment distribution of the 1,225 pair-wise combinations, we calculated the fraction of permutations that showed a more extreme value than the enrichment observed in each original data set

Functional annotation enrichment analysis

The reported biological significance of gene sets was evaluated by gene ontology (GO) analysis (biological process FAT set) by using the Cytoscape plug-in ClueGO (Bindea *et al.*, 2009) with the following parameters: statistical test = enrichment (right-sided hypergeometric test), correction method = Bonferroni step down, minimum level = 3, maximum level = 8, number of genes = 3, minimum percentage = 5.0, Kappa score threshold = 0.3.

RESULTS

Here we present a gene-level association study of cortical thickness on 557 MS patients and 75 controls of European ancestry studied at UCSF. An independent cohort of 219 patients studied in Basel was used as replication. The complete workflow of our strategy is depicted in Figure S1. Through this approach, we merge statistical evidence of association and physical evidence of interaction at the protein level to identify associated loci and highlight functional pathways involved in cortical thickness of MS.

Defining regions of interest for GWAS

Cortical thickness was determined in all cases and controls at UCSF for 34 regions bilaterally using FreeSurfer (see methods). We first selected regions of interest (ROI) for GWAS by comparing the thickness of each region between MS and age- and gender-matched controls. Demographic features are summarized in Table 1. Cortical thickness was independently measured in 219 MS patients from the Basel cohort. Cases enrolled in Basel had significantly longer disease duration and more disability than cases in UCSF, and the frequency of progressive MS was also higher in Basel than in UCSF (Table 1). One individual who had more than 2 times the interquartile range of median cortical thickness was excluded from the Basel data set. Differences in the mean adjusted cortical thickness between cases and controls were statistically significant in nine regions (Table 2). Eight of nine regions showed evidence for bilateral pathology (Bonferroni p value < 0.05 in one hemisphere and nominal p value < 0.05 in the contralateral hemisphere); the remaining region (supramarginal cortex) was significant on the left but did not reach significance on the right hemisphere. These nine regions were defined as ROI. As expected, the mean thickness in cases was lower (i.e. thinner) than controls for all ROI (Figure S2).

Based on the bilateral pattern of thinning observed for most ROI, we used an average thickness of right and left hemisphere as a trait for GWAS. We also found that cortical thickness adjusted by age, gender, and head size, was negatively correlated with EDSS in eight of nine ROI (with the exception of banks of superior temporal sulcus) in both UCSF and Basel data sets (Table S1).

SNP-level GWAS

Genotypes (n=553,139) for 464 (out of 557) cases from UCSF, and 211 (out of 219) cases from Basel obtained with the Illumina HumanHap550 BeadChip were available for this analysis. After quality control (see methods), GWAS was carried out on 550,067 SNPs in 464 cases from UCSF and 211 cases from Basel using left-right average thickness at each ROI as phenotype (9 GWAS in total). To this end, a linear regression model was used for each data set including age at examination, gender, volumetric scaling factor, T2 lesion load, and disease course (non progressive or progressive MS) as covariates. After correcting for the number of GWAS performed, no marker was statistically significant at genome wide level in either data set. We explored the possibility of replication at the SNP level by selecting the top 100 SNPs for each ROI in one dataset (UCSF) and examined the corresponding GWAS in the Basel data set. None of the top 100 SNP chosen was replicated for any ROI.

Nominal gene-level associations with cortical thickness

Previous work by us and others suggest that gene-level analysis afford greater power by virtue of aggregation of variants into functionally relevant units ((Imsgc), 2013, (Imsgc) *et al.*, 2010, Liu *et al.*, 2010, Purcell *et al.*, 2009). Thus, we first converted SNP-level p-values into gene-level p values and subsequently conducted a protein interaction network based pathway analysis (PINBPA). We started by computing gene-wise p values using the software VEGAS, which aggregates

evidence of association for all SNPs mapping to a given gene taking into account relative genomic position, number of SNPs within a gene, and LD patterns for the appropriate ethnic background. Because our main hypothesis states that even modestly associated genes might participate in biologically plausible pathways, we considered all nominally significant genes (VEGAS $p < 0.05$). As in previous analyses, we observed that the distribution of nominally significant genes was not random across the genome, but it followed patterns resembling LD blocks. We then defined "association blocks" as stretches of contiguous genes showing $p < 0.05$. This resulted in an average of 1,286 blocks across the nine GWAS (range 1,249 – 1,334) for the UCSF dataset and 1,273 blocks (range1,213 – 1,330) for the Basel dataset. An average of 2,762 genes were nominally significant in the UCSF dataset (range 2,629 – 2,913) and 2,654 (range 2,462 – 2,778) in the Basel dataset. While most of these genes may represent false positives (given the low threshold of significance) we computed the overlap between the two datasets and compared that against a background distribution generated with 1,000 permutations of randomly selected genes from the same distribution, as a measure of replication. We found that the GWAS for fusiform (FUS) and inferior parietal (IP) ROI resulted in higher than expected (45% and 51% respectively), statistically significant (one-sided Fisher's exact test, $P = 0.0388$ and 0.0107) overlap (Table S2). It is noteworthy that, unlike the SNP-level analysis, the gene-level analysis was able to suggest global gene-level replication across sites, at least for two ROI. A representative Manhattan-plot visualization of the GWAS on inferior parietal at the gene level shows similar peaks (arrowheads) in both data sets (Figure 1). The remaining plots are available in Figure S3.

Fourteen genes in UCSF and 57 genes in Basel data set were nominally significant in all nine ROI (Table S3), and two genes (*RYR2* and *CDH13*) were replicated in both data sets (55.5 fold enrichment, one-sided Fisher's exact test p = 0.00090).

Pathway analysis using protein interaction networks

In order to identify loci associated with cortical thickness by combining statistical evidence of gene association and physical evidence of interaction of their respective gene products, we assigned gene-wise association p values to a curated human PIN data set consisting of 8,960 proteins (nodes) and 27,724 interactions (edges) in each GWAS data set. All subsequent experiments were performed with Cytoscape, an open-source and extensible tool for network visualization and analysis (Shannon *et al.*, 2003). We first extracted the nodes with p values < 0.05 and computed the number of nodes and edges in the resulting subnetworks for the UCSF and Basel data sets, respectively (Table S4) (we refer to these as first-order interaction networks, as all nodes are at least nominally significant). We next computed the overlap between genes (proteins) in each subnetwork between the UCSF and Basel datasets, as a measure of replication. In both data sets, the subnetworks in most of the regions were more connected than would be expected by chance, as demonstrated by a simulation experiment in which 1,000 networks of similar size were extracted from the same PIN at random (Figure 2A). In each case, subnetworks were composed of a large connected component and several smaller networks or isolated nodes (singletons). We also tested whether the size of the main component was higher than what would be expected by chance (given the number of edges in the first-order network) (Figure 2B). Using these two indices of network complexity, networks identified in seven of the nine ROI were beyond the 75 percentile of at least one index in random networks in either UCSF or Basel data set.

In general, nominally significant genes were more connected and had a larger main connected component than expected. Our results suggest that the properties of these networks are consistent with those displayed by other biologically relevant networks.

Given the small-world topology of the human protein interactome, it is possible that a few highly connected nodes (hubs) bring together several associated genes, even though the hubs themselves are not associated. To explore this possibility, we conducted searches for subnetworks enriched with significant genes by using jActiveModules, a Cytoscape plugin based on a greedy heuristic algorithm with internal cross-validation.(Ideker *et al.*, 2002) Between 23 and 36 significant and minimally overlapping (<20%) modules were identified in each region for both data sets (Table 3). We next computed the union of all modules for each cortical region within each data set, resulting in a single connected network of 1,763.8 nodes on average (range 1,592-1,963) for the UCSF data set and another of 1,898 nodes on average (range 1,707-2,087) for the Basel data set. We next computed the nodes in common between both datasets for each cortical region, resulting in and average overlap of 698.6 nodes (range 610-784).

We then considered only replicated genes and further searched for subnetworks in order to detect networks robustly associated with cortical thickness. In each region, 2-4 enriched subnetworks composed by 9-92 nodes were identified. In each subnetwork, we extracted genes that had nominally significant p values both in UCSF and Basel data sets (Table S5). These genes are of highest importance to our approach because they had significant p values in both data sets and because they were identified as components of significant networks in both data sets. Among these genes, *NPAS3*, *OPCML* and *HS3ST4* were frequently observed across all regions. They were observed in eight, seven and seven regions respectively.