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Pathway and network-based analysis of genome-wide association studies in multiple sclerosis

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Genome-wide association studies (GWAS) testing several hundred thousand SNPs have been performed in multiple sclerosis (MS) and other complex diseases. Typically, the number of markers in which the evidence for association exceeds the genome-wide significance threshold is very small, and markers that do not exceed this threshold are generally neglected. Classical statistical analysis of these datasets in MS revealed genes with known immunological functions. However, many of the markers showing modest association may represent false negatives. We hypothesize that certain combinations of genes flagged by these markers can be identified if they belong to a common biological pathway. Here we conduct a pathway-oriented analysis of two GWAS in MS that takes into account all SNPs with nominal evidence of association (P < 0.05). Gene-wise P-values were superimposed on a human protein interaction network and searches were conducted to identify sub-networks containing a higher proportion of genes associated with MS than expected by chance. These sub-networks, and others generated at random as a control, were categorized for membership of biological pathways. GWAS from eight other diseases were analyzed to assess the specificity of the pathways identified. In the MS datasets, we identified sub-networks of genes from several immunological pathways including cell adhesion, communication and signaling. Remarkably, neural pathways, namely axon-guidance and synaptic potentiation, were also over-represented in MS. In addition to the immunological pathways previously identified, we report here for the first time the potential involvement of neural pathways in MS susceptibility.

INTRODUCTION

The usefulness of genome-wide association studies (GWAS) to discover common genetic variants associated with susceptibility to complex diseases has been empirically demonstrated (1). The aim of these studies is to characterize the genetic architecture of complex genetic traits through the identification of such disease variants against the background of random variation seen in a population as a whole. In a typical GWAS, hundreds of thousands of markers are tested simultaneously in cases and controls and the allelic frequencies of each marker are compared between the two groups. However, because of the exceedingly large multiple testing involved in these studies, very few exceed the genome-wide significance threshold and those that do not exceed this stringent statistical requirement are generally neglected. In many cases where loci with small but measurable genetic effects are involved, it is likely that, accepting the null hypothesis...
of no association represents a type II error. A notable example of this situation can be illustrated by the confirmed association of PPARG variants in type 2 diabetes (T2D) (2). Due to its modest effect on disease susceptibility (odds ratio 1.2), this true association was overlooked by four out of five studies designed to replicate the initial finding. A similar scenario was more recently found with IL7R in multiple sclerosis (MS) (3). In this paper, we aim to show that while individual modest genetic effects are difficult to ascertain, they can be collectively identified by combining nominally significant evidence of genetic association with current knowledge of biochemical pathways.

MS is the most common acquired neurological disease of young adults with a prevalence of approximately 1:1000 in population groups of northern-European ancestry. MS is characterized by a variable state of relapsing or progressive neurological disability that ensues as the consequence of an autoimmune attack against myelin in the central nervous system (CNS) (4). Compelling data indicate that susceptibility to MS is in part inherited (5–8). In addition to the strong effect of HLA-DRB1, the recently reported GWAS in MS identified and confirmed the involvement of the genes IL2RA and IL7RA in disease susceptibility (9). However, as in other studies of this kind, many associations with markers that were nominally significant but did not reach the genome-wide significance threshold were not pursued any further. It is likely that a significant proportion of these rejected associations are false negatives, and methods of interpretation are needed that allow such associations to be recognized.

We hypothesize that meaningful combination of genes harboring markers with only modest evidence of association can be identified if they belong to the same biological pathway or mechanism. In addition to the single-locus associations identifiable by standard genome-wide analysis, this type of analysis can reveal a statistical enrichment of associations within known biological pathways. The methods presented here may be useful to identify pathways and networks whose involvement in disease susceptibility are consistent with current models of pathogenesis, but most importantly may also identify statistically over-represented but unexpected pathways revealing novel disease mechanisms.

Inspired in part by analytical advances in the study of gene expression, we propose a pathway-oriented analysis for GWAS. We apply a method similar to the one that uses gene ontologies (10) to analyze a list of differentially expressed genes, but replacing the measure of differential expression for each gene by a P-value. Modest significant P-values are computed: one set for assessment of the association of each SNP with the trait and another set for comparison of the observed and expected number of moderately associated genes in a GO or biological pathway. The second stage requires that evidence of association at all the marker loci genotyped within each gene be reduced to a single, gene-wise P-value.

A limited number of studies have used network-based algorithms to prioritize candidate loci in genetic studies (11–14). However, these studies either do not use actual genetic (genotypic) data or are applied to model organisms. The only study to date that uses pathway-based analysis of GWAS data does not consider a protein interaction network (PIN) to further restrict the possible combinations of causal genes (15).

In this article, we describe a network-based pathway analysis of two GWAS in MS (3,9), where evidence for genetic association is combined with evidence for protein–protein interaction. The rationale for performing a pathway-based analysis in a GWAS lies in the assumption that several genes, each modestly associated with the disease, may interact synergistically to confer susceptibility. We carry out extensive statistical validations and apply the same approach to other published GWAS to demonstrate the potential utility of this method.

RESULTS

GWAS results from two MS studies were analyzed to identify modestly associated variants within genes with related biological functions. The first dataset was produced by the International MS Genetics Consortium (IMSGC), and comprises 931 family trios genotyped with the GeneChip® Human Mapping 500K Array Set (Affymetrix) (9). Quality control for this dataset included sample genotyping efficiency, assessment of marker heterozygosity and allelic frequency, departure from Hardy–Weinberg equilibrium, gender consistency, reproducibility and population genetic structure. A total of 334 923 SNPs survived the quality control protocol and were tested for association with the trait. As expected, a number of markers in the HLA region were strongly associated with the disease phenotype. In addition, 78 markers outside the HLA region were found to exceed the $P < 1 \times 10^{-4}$ genome-wide threshold of significance. The second dataset (the GeneMSA study, (3)) was generated using the Sentrix® HumanHap550 BeadChip (Illumina). After a similar quality control protocol, 551 642 SNPs were used to conduct an association analysis using the genotypic test in 978 cases and 883 controls (3). In addition, the association of each individual marker with the disease was tested by fitting a logistic regression genotypic model in which gender, Center of sample origin and HLA-DRB1*1501 status were included as covariates. In the GeneMSA study, 87 SNPs outside of the HLA region exceeded the genome-wide significance threshold of $P < 1 \times 10^{-4}$. Although there was no full overlapping of associated markers between the two studies, several genes showed evidence of association in both (3). A meta-analysis is being conducted and will be reported in the near future.

To carry out the protein interaction network-based pathway analysis (PINBPA), we computed a single $P$-value for each gene (the gene-wise $P$-value, Fig. 1) and overlapped these onto a curated PIN. Many markers map within gene deserts or unannotated genes, and these were excluded from the present analysis. This process resulted in gene-wise $P$-values for 14 442 and 17 342 genes for the GeneMSA and IMSGC GWAS, respectively. We next conducted sub-network searches on the two MS GWAS using the Cytoscape plugin jActive modules. jActive modules combines the network position and association $P$-value of each gene to extract potentially meaningful sub-networks or modules. In addition to searching for significant modules using both datasets together,
we also conducted individual searches for each study (data not shown). Although the same basic modules were identified in both strategies, higher scores were obtained when both datasets were used together, suggesting a real power gain when larger datasets are used. To assess the specificity of the modules associated with MS, we also performed equivalent analyses on recent GWAS from other autoimmune diseases (rheumatoid arthritis, RA; Crohn’s disease, CD; type 1 diabetes, T1D), neurological diseases (Alzheimer’s disease, AD; bipolar disorder, BP) and unrelated diseases (coronary artery disease, CAD; hypertension, HT; T2D) (16). We observed statistically significant modules in all diseases (Fig. 2). Interestingly, the largest number of significant modules was observed in MS, suggesting greater genetic heterogeneity in this disease when compared with others. To test to what extent significant network modules could be obtained by chance, we conducted 10 searches randomizing the P-values among the same set of genes (those with association P-values <0.05). With the notable exception of MS, RA and AD, the module scores obtained from the randomized P-values were equal to or even higher than those obtained using the real P-values (Fig. 2A). This observation suggests that many of these modules do not represent bona-fide biological networks and that their high scores may have been obtained by chance. In contrast, significantly fewer modules were identified in the searches based on randomized P-values for MS, RA and AD suggesting that the significant modules obtained from the real P-values in these diseases represent biologically meaningful networks. To examine in detail the magnitude of the scores for real and randomized P-values, we plotted those of the top 20 modules for each set of P-values for each disease (Fig. 2B). As expected from the previous analysis in MS, RA and AD, most of the top 20 modules obtained with real P-values showed higher scores than the average score of the randomized searches. In the case of RA, only the top two modules show significantly higher scores than the average of their randomized searches (Fig. 2B). Notably, these two (partly overlapping) modules are composed exclusively of HLA genes, in which association with the disease is highly significant. Although the total number of modules obtained using real P-values in BD and CD do not differ significantly from those obtained with the randomized P-values (Fig. 2A), 18 of the top 20 scores were higher for the real P-values (Fig. 2B). Altogether, these results suggest that the significant modules found with the original data may represent real effects of interacting proteins on each disease phenotype.

**Significant modules for MS**

We identified 346 significant modules on the basis of their aggregate degree of genetic association with MS. Due to the nature of the search algorithm, several of these modules overlap extensively in their component genes. Thus, to describe modules representative of association with MS, we selected those with the highest scores which also displayed a minimum degree of overlap (Fig. 3). Consistently with all previous genetic studies in MS, the most significant module (MS_I) included several HLA genes (Fig. 3A). Although the only gene consistently found associated to MS in this region is HLA-DRB1, the module shown lists another gene, HLA-DRA, as its most significant node. It is possible that because HLA-DRB1 is highly polymorphic, most SNP markers included in large-capacity arrays are not targeting this gene directly. Indeed, there are three times as many SNPs in HLA-DRA as there are in HLA-DRB1 in the Illumina 550 k platform and the DRA SNP rs313588 tags with high sensitivity the HLA-DRB1*1501 allele. The observed associations with other HLA genes like HLA-DMA/B and HLA-DOA/B may be also due to the extensive linkage disequilibrium (LD) seen in this region. Interestingly, HLA-DRB5, present in the most significant module, is part of the DR15 haplotype and has been identified as a potential modifier of the disease (17,18). The other two HLA genes that are part of the DR15 haplotype (DQA and DQB) are not present in the module shown. Although the P-values for each of these genes exceed the threshold of significance used for this analysis, they are not part of module MS_I because there is no evidence that they physically interact with any of its components. Not surprisingly, a KEGG pathway search with these genes identified the terms ‘antigen processing’, ‘cell adhesion molecules’ and ‘Immune system’ as the most significantly over-represented,
relative to the number of genes in these pathways expected in the module by chance (Table 1).

Figure 3B shows another highly significant module characteristic of MS. In this module (MS_II), several HLA genes are also prominent members, but by virtue of highly connected molecules such as CD4, CD82 and ITGB2, a more extensive immune pattern emerged. Interestingly, two non-HLA susceptibility genes previously associated with MS (IL2Ra and CD58) also appear in this module. We hypothesize that in addition to its own significance, the presence of IL2Ra in this module may result from its physical interaction with STAT3 and ITGB2, which themselves show modest association with MS. CD58 was initially identified as a susceptibility gene in the IMSGC study (9) and its expression was recently found to be upregulated in peripheral blood cells during disease relapses (19). In contrast, IL7Ra, another gene recently identified as a susceptibility factor in MS (20) is not part of this module. We speculate that it may act through an independent pathway. Although several of the other immune-related genes in this module have not been formally associated with MS, their involvement in disease pathology seems plausible. These include several cell adhesion (ITGGB2, ITGA4, ITGA6, ITGAM and ICAM1) and signaling molecules (TGFBR2, TNFRSF10A and STAT3). Notably, ITGAM (CD11b) has been recently associated with susceptibility to systemic lupus erythematosus, another autoimmune disease (21). KEGG pathways analysis with genes from module MS_II revealed statistically significant over-representation of the processes of cell adhesion, leukocyte transendothelial migration and antigen processing (Table 1).
Interestingly, the other two modules characteristic of MS (MS_III and MS_IV) suggest a neural component in the susceptibility to the disease. Module MS_III is highly enriched with genes typically expressed in neurons and glia (NCK2, EPHA3, EPHA4, FYN, EFNB1, EFNB2 and EPHB2). Similarly, module MS_IV includes seven glutamate receptors (GluRs) (GRIK1, GRIK2, GRIK4, GRIA1, GRIA4, GRIN2A and GRID2) in addition to HOMER1, DLG1 and DLG2. HOMER1 regulates group 1 metabotropic GluR function, and DLG1 and DLG2 interact at postsynaptic sites to form a multimeric scaffold for the clustering of receptors, ion channels and associated signaling proteins. The identification of the latter two modules in MS suggests for the first time that modestly significant associations in genes involved in neural pathways may contribute to the overall susceptibility to this disease. Indeed, when members of these modules were tested for membership to KEGG pathways, highly significant enrichment in axon guidance pathways (module MS_III) and long-term depression and potentiation pathways (module MS_IV) were detected (Table 1).

As a control for our interpretation of these genes in MS, we next conducted similar analyses on the modules identified for other diseases. Interestingly, for two of the three autoimmune diseases tested (RA and T1D), the most significant modules were exclusively composed of HLA genes (Fig. 4). On the other hand, only genes involved in the JAK-STAT signaling pathway (GRB2, JAK1, STAT3 and IFNAR1), and extracellular matrix-receptor interactions (CD44, COL4A2, COL1A1 and FN1), but not HLA were identified in the third autoimmune disease (CD). The two genes most robustly associated with CD (NOD2 and IL23R) are not part of the selected module. As described for module MS_II, this may...
Table 2 shows the genes and pathways contained in the significant modules for AD and BD were neural networks, lacking. As expected, the great majority of pathways identified in the significant modules for T2D, CAD and HT are also shown in Table 2. The top statistically significant modules identified for RA, T1D, AD and BD.

Although possibly representing false discoveries, the top modules identified for T2D, CAD and HT are also shown for comparison (Fig. 2B). In T2D, the most significant module contained genes involved in intracellular signaling pathways.
(EGFR and BCR), apoptosis (IGF1R, AVEN and APAF1) and insulin receptor signaling pathway (IGF1R and IGF2). In HT, the top scoring module listed genes are almost exclusively involved in cell communication (EGFR, VAV3 and RAC1).

To assess module specificity, we compared the performance of each of them in the disease in which they were identified against its performance across all other diseases. This was accomplished by tabulating the gene-wise P-value of association of each gene in the module with every disease. If a module reached significance just because it was composed of large-sized genes, for which relatively low P-values could be obtained by chance, it would be expected that the same module be also significant in several or all other diseases, but modules are significant only in the disease in which they were originally identified, suggesting they were identified because they were disease-specific and not due to chance. As demonstrated in Figure 5, the four most significant modules identified in MS show almost no association with any other disease. However, there are some genes that show strong association with other diseases in addition to MS. In particular, the HLA genes also show highly significant associations with both RA and T1D, two autoimmune diseases. Most notably, several significant genes from modules in AD and BD are also significant in MS. For example, SNCA, CDC42EP3, FHL2 and CRMP1 all show P-values < 1 × 10^{-3} in MS and AD or BD, but consistently higher P-values for all the non-neurological diseases. The maximum number of SNPs tested in these genes ranged from 49 (CRMP1) to 79 (CDC42EP3), slightly above the median number of 40 SNPs/gene across the Illumina 550 k array. In contrast, genes such as PARK2, VAV3, PAK7 and NTRK3 yielded relatively low P-values (P < 1 × 10^{-3}) across most or all diseases, possibly because a larger number of SNPs were tested for these genes, and some achieved significance by chance. Indeed, the number of SNPs for these genes in the Illumina platform ranges from 149 (PAK7) to 455 (PARK2).

Finally, we identified the 400 genes in which the gene-wise P-values varied most widely across diseases, and performed one-way hierarchical clustering on these P-values to produce a dendrogram identifying diseases with similar patterns of genetic association (Fig. 6). The two MS and the two RA studies clustered almost perfectly with each other and they, in turn, were grouped together in a looser cluster which also included T1D (autoimmune) and AD (neurological), but did
| Pathway                                      | Annotated genes in module | Observed | Expected | P-value |
|----------------------------------------------|---------------------------|----------|----------|---------|
| **RA**                                       |                           |          |          |         |
| Cell adhesion molecules (CAMs)               | SELH[LDA-DA1P1|CD4H[FDA-DBP121]|          |          |          |
| HLA-DBMBSEL|HLD-DQDA2HDLA-DA2|          |          |          |          |
| Antigen processing and presentation         | HLA-DA1P1CD4HLA-DBP1HLA-DBMB          | 8/18 (44.4%) | 91/2361 (3.8%) | 6.06E-06 |
| HLA-DQDA2HLA-RAA                          |          |          |          |          |          |
| Type 1 diabetes mellitus                    | HLA-DA1P1DBP1HLA-DBMBHLA-DQDA2HLDARA | 6/18 (33.3%) | 43/2361 (1.8%) | 1.07E-05 |
| Human diseases                              | CBLBRETMAPK12[GRB2H[DA-DA1P1|          |          |         |
| HLA-DBP1PRNPABLIL[DA-DBM]          |          | 12/18 (66.6%) | 411/2361 (17.4%) | 6.00E-05 |
| Metabolic disorders                         | HLA-DA1P1HLA-DBP1HLA-DBMB          | 6/18 (33.3%) | 78/2361 (3.3%) | 1.54E-04 |
| Immune system                               | CBLB[MAPK12GRB2HDA-DA1P1|          |          |         |
| HLA-DBP1HLA-DBMP1HLA-DQAR]          |          | 10/18 (55.5%) | 425/2361 (18.0%) | 3.14E-03 |
| Chronic myeloid leukemia                    | CBLBGRB2ABP1PK3R1 |          |          |         |
| Signaling molecules and interaction         | SELH[L DA-DA1P1|CD4HDLA-DBP1|          |          |         |
| HLA-DBMBSEL|HLD-DQDA2HLDRA|          |          |          |         |
| T-cell receptor signaling pathway           | CBLBGRB2CD4PK3R1 |          |          |         |
| Environmental information processing        | MAGIK2[CD4HLA-DQ2]KDR                      | 4/18 (22.2%) | 86/2361 (3.6%) | 2.00E-02 |
| VEGF signaling pathway                      | MAPK12PK3R1KDR |          |          |         |
| **TID**                                     |                           |          |          |         |
| ECM-receptor interaction                    | LAMA1COL4A2COL4A1HSPG2COL1A2ITGA2|          |          |         |
| ITGALITG1C[OL11A1]|          | 9/31 (29.0%) | 67/2419 (2.7%) | 3.66E-06 |
| Antigen processing and presentation         | TAP1HLA-DOAHLA-DBPHLA-DBP1|          |          |         |
| HLA-DQ2HLA-DRAHLA-F                      |          | 7/31 (22.5%) | 47/2419 (1.9%) | 2.25E-05 |
| Type 1 diabetes mellitus                    | HLA-DOAHLA-DBPHLA-DBMHLA-DQ2A|          |          |         |
| Cell Communication                          | TNL1MAG1COL4A2COL4A1ITGA10ITGA2|          |          |         |
| ITPR3GRM1ITG1ITPR1PXN[LAMA1TUBB]|          | 15/31 (48.3%) | 351/2419 (14.5%) | 8.68E-05 |
| Cellular processes                          | TNL1ITGA10BCL21HLA-DBM[ITGB1]PXN|          |          |         |
| TUBB[PRNP][TAP1HLA-DOA[COL11A1]|          | 26/31 (83.8%) | 1098/2419 (45.3%) | 1.10E-04 |
| Focal adhesion                              | LAMA1COL4A2COL4A1MAG1COL4A1ITGA2|          |          |         |
| ITP3GRM1ITG1ITG2A2ITPR1HLA-F |          | 10/31 (32.2%) | 170/2419 (7.0%) | 2.43E-04 |
| Cell adhesion molecules (CAMs)              | HLA-DOAHLA-DBPH[ITGB1]|          |          |         |
| HLA-DQ2HLA-DRAH[HLA-F]                      |          | 7/31 (22.5%) | 93/2419 (3.8%) | 9.26E-04 |
| Metabolic disorders                         | HLA-DOAHLA-DBPHLA-DBMHLA-DQ2A|          |          |         |
| HLA-DRAHLA-FA                          |          | 6/31 (19.3%) | 79/2419 (3.2%) | 2.55E-03 |
| Signaling molecules and interaction         | COL4A2COL4A1HSPG2ITGA2ITGA10|          |          |         |
| HLA-DBMHLA-DQ2A[GRM1ITG1]PSXN|          | 16/31 (51.6%) | 563/2419 (23.2%) | 3.05E-03 |
| Environmental information processing        | COL4A2COL4A1HSPG2ITGA2ITGA10BCL21|          |          |         |
| ITPR3ITG1HSP2[ITGA1|          | 23/31 (74.1%) | 1077/2419 (44.5%) | 3.88E-03 |
| Human diseases                              | LAMA1APP[CAP8PSXN][P21]PRNP|          |          |         |
| Neurodegenerative disorders                | LAMA1APP[CAP8P21]PRNP |          |          |         |
| Gap junction                                | TUBB[ITPR3GRM1ITPR1] |          |          |         |
| Prion disease                               | LAMA1PRNP |          |          |         |
| AD                                          |                           |          |          |         |
| Neurodegenerative disorders                | APP[SNAIP][MAG1]SNA[PAK2][ITCH][APB1A|          |          |         |
| SNCA[SNCA][PAK2]                          |          | 7/26 (26.9%) | 103/2419 (4.2%) | 3.77E-03 |
| EGFRA[AK6]CD42[PAK7][RB2][SRGAP1]           |          | 3/26 (11.5%) | 13/2419 (0.5%) | 7.93E-03 |
| Human diseases                              | EGFRCDC42APP[SNAIC][MAG1][TFB][GNA12]|          |          |         |
| PRK2[ITCH][APB1][TFB]                      |          | 11/26 (42.3%) | 423/2419 (17.4%) | 3.46E-02 |
| BD                                          | EGFRI[GF1]MPP1[CRBB][ITPN1]INSR |          |          |         |
| MAPK signaling pathway                      | EGFRI[ME2]MPP1[RPS62K][ITRN2][GNA12]TFPR[NFATC2][CDC25B]|          |          |         |
|                                             | 6/28 (21.4%) | 68/2419 (2.8%) | 5.67E-03 |
|                                             | 9/28 (32.1%) | 219/2419 (9.0%) | 1.40E-02 |
not include any of the unrelated diseases. Intriguingly, the study on CD did not cluster with MS and RA, but with the unrelated diseases. One possible explanation for this difference is the lack of a strong association with HLA genes in CD compared with the other autoimmune diseases. Instead, genetic susceptibility to CD appears to be more widely spread across the genome (16,22).

**DISCUSSION**

There is ongoing debate on the exact mechanism of MS pathogenesis. Some theories support the idea that there is a primary immune disorder targeting the CNS, with subsequent neurodegeneration being a consequence of the initial inflammatory process. A competing theory states that neurodegeneration is the primary cause of the disease, leading to an inflammatory reaction within the CNS (23). Most previous genetic studies in MS (by genome-wide or candidate gene approaches) have involved immune-related genes, thus supporting the first scenario. In this article, we employ a novel network-based pathway analysis using data from two independent GWAS and implicate neural pathways in the susceptibility to MS.

Recently reported pathway-based analyses of GWAS data (15,24,25) relied exclusively on classical biological pathways as described in KEGG, Biocarta or gene ontology. The article by Torkamani et al. (15) is of particular interest since they analyzed several of the GWAS datasets we used as control. We also tested a similar approach in which a literature-derived network of biological relationships is first assembled and subsequently, an exhaustive search for subnetworks representing particular pathways is conducted (26). Using the two MS datasets, this analysis yielded significant pathways associated with immune and neurological functions including antigen presentation, axon guidance and neurogenesis (Supplementary Material, Table S1). In order to enhance the potential for discovery of biologically relevant circuits, we introduced the PIN to the analysis. The PINBPA approach focuses on the combined effect of associated genes by restricting the search for pathways to only those gene products that physically interact (as determined by the PIN) are taken into account. The variable number of markers genotyped within each gene for our analysis the gene-wise P-value method, we adjusted the corrected value for LD. However, when these techniques for correcting the gene-wise P-value for the number of SNPs per gene, and adjusting the corrected value for LD. We also applied the Fisher’s method for combining P-values, followed by an adjustment for LD. However, when these

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**Table 2. Continued**

| Pathway | Annotated genes in module | Observed | Expected | P-value |
|---------|----------------------------|----------|----------|---------|
| Nervous system | IGF1R|MAPK1|PRS6KA2|GNAI1|CREBBP|GNA12 | 6/28 (21.4%) | 98/2419 (4.0%) | 1.40E–02 |
| Neurodegenerative disorders | HD|CREBBP|SNCA|PARK2|BCL2L1|INSR | 6/28 (21.4%) | 103/2419 (4.2%) | 1.40E–02 |
| Human diseases | EGFR|IGF1R|MAPK1|HD|INF4A|CREBBP|SNCA| | 12/28 (42.8%) | 423/2419 (17.4%) | 1.78E–02 |
| Long-term depression | IGF1R|MAPK1|GNA11|GNA12 | 4/28 (14.2%) | 61/2419 (2.5%) | 4.92E–02 |
methods were used, a very small number of genes exceeded the threshold of significance, not enough to compute any pathway searches. Although such correction and adjustment are appropriate on the null hypothesis of no associations genome-wide, it may penalize large genes excessively if true (causative) associations are proportionally more common in smaller genes. We also evaluated to what extent larger genes were more likely to be included in our analysis by virtue of being more represented in the array. We found no significant difference in the distribution of gene sizes within a given module when compared with a random set of genes (Supplementary Material, Fig. S1). Altogether, the min P-value method provided the most consistent and balanced results. This method has also been applied by Torkamani et al. (15) in their pathway analysis of the WTCCC dataset with similar results.

Here we implicate neural pathways (e.g. axon guidance and long-term potentiation) in susceptibility to MS. Only one other article has reported a neural gene in MS susceptibility to date (32). Due to the power limitations described above, none of the individual genes in these pathways may exceed the genome-wide threshold of significance in tests of association. However, when an entire pathway is considered, even modest associations in several of its component genes contribute to the overall P-value. For example, seven GluR genes were found to be marginally associated with MS (Module MS_IV). Although each marker may not reach significance when tested in isolation, the probability that several GluRs are identified by chance, if none of them is truly associated with the disease, is exceedingly small. Furthermore, not all GluRs are encoded on the same chromosome, thus reducing the likelihood of inflation of the signal due to LD. The identified associations between GluRs and MS are of substantial biological relevance since glutamate is the principal excitatory neurotransmitter within the CNS. Glutamate acts on neuronal and glial ionotropic receptors coupled to specific ligand-gated cationic channels, and mGluRs, coupled to second messengers. Under normal conditions, astrocytes maintain low extracellular glutamate levels by using transporters to take up glutamate rapidly as high extracellular glutamate levels are neurotoxic. Indeed, elevated extracellular glutamate levels can result in the death of neurons and oligodendrocytes through excitotoxic mechanisms and these have been shown to play a role in the pathology of MS and EAE (33,34). Interestingly, susceptibility to excitotoxicity may be under genetic control (35).

A second neural module (MS_III) contained NCK2 and FYN, in addition to two members of the ephrin family of proteins (EFNB1 and EFNB2) and three of their receptors (EPHA3, EPHA4 and EPHB2). These genes are involved in axon guidance, the process during development by which neurons extend their processes and make connections throughout the CNS. Specifically, Eph/ephrin signaling regulates axon guidance through contact repulsion during development of the CNS, inducing collapse of neuronal growth cones (36). Eph receptors and ephrins continue to be expressed in the adult CNS, although usually at lower levels, but have been found to be upregulated in MS lesions on different cell types, including reactive astrocytes, neurons and oligodendrocytes (37). This upregulated expression may directly inhibit regrowth of regenerating axons, but Eph expression also regulates astrocytic gliosis and formation of the glial scar. Therefore, Eph/ephrin signaling may inhibit regeneration by more than one mechanism and modulation of Eph receptor expression or signaling could prove pivotal in determining the outcome of injury in the adult CNS.

Due to the nature of the searches performed, this is a gene-centered analysis, and thus it is possible that true associations with markers that lie in large intergenic regions were neglected. Also, markers within genes not represented in the PIN were not evaluated in this analysis. Finally, it is reasonable to expect that subgroups of patients with shared risk alleles would be identified by this method. We were unable to subclassify patients because our analysis only takes into account the most significant variant for each gene, and more significant markers may be needed to identify such subgroups. Nevertheless, there is scope for the development of related methods to increase the power to detect associations in these regions and genes. In summary, by following a network-based

Figure 5. Module specificity. The P-values of genes from the representative modules shown in Figures 3 and 4 are displayed as a heatmap. Each row corresponds to a single gene. Genes are organized by their membership of modules. Genes corresponding to the four modules described for MS (Fig. 3) are at the top, followed by genes corresponding to modules from all other diseases. Because modules from different diseases may share one or more genes (e.g. HLA in autoimmune diseases), these may be represented more than once in the figure. Color-coded bars next to each module mark the genes that the module comprises. The same color code in the column headers indicates the disease for which the P-values are represented below. In general, genes from modules identified in one disease show the highest P-values for that disease, and less significant P-values for most other diseases. A notable exception is the HLA genes which show overlap between MS, RA and T1D, all of which are autoimmune diseases. Interestingly, some of the genes from the AD and BD modules show significant P-values also in MS.
pathway analysis, we have expanded the immune-related set of genes associated with MS. Furthermore, we have identified neural pathways whose involvement in the disease is biologically plausible. Larger pathway-oriented association studies will ultimately be necessary to validate these findings.

MATERIALS AND METHODS

Genetic association data

In total, 11 GWAS were analyzed (two for MS and nine others as controls). The first of the two studies in MS was a family trio-based analysis recently published by the International MS Genetics Consortium (IMSGC) in which 334,923 SNPs were analyzed in 931 trios by the transmission disequilibrium test (9). The second MS study (GeneMSA) was a multicenter case–control association analysis done collaboratively among the University of California San Francisco, Vrije Universiteit Medical Center in Amsterdam, University Hospital Basel, and the pharmaceutical company Glaxo SmithKline (GSK). The GeneMSA study analyzed 551,642 SNPs in 978 cases and 883 controls (3).

As controls, we used data from four studies in other autoimmune diseases, consisting of two studies in RA, and one each in CD and T1D. Two studies in other neurological diseases included one each in AD and BD. In addition, unrelated diseases included one study each in HT, CAD and T2D. The studies for RA, CD, T1D, BD, HT, CAD and T2D were performed by the Wellcome Trust Case Control Consortium (WTCCC) (16) and the genotypic P-values of association for each tested SNP were obtained from the project’s webpage (www.wtccc.org.uk). A second RA study was performed by Plenge and collaborators in which 317,503 SNPs were tested in 1522 cases and 1850 controls (38). P-values for association were obtained from the Supplementary information provided in that article. Processed data for the AD study performed at GSK is publicly accessible from http://www.imgw.com/public/ (39).

Module (sub-network) searches

We first computed the gene-wise significance for association by choosing the lowest P-value of all SNPs mapping to a given gene (min P-value method) without correction. Although this method potentially introduces biases in favor of larger genes (for which more SNPs are generally typed, thus increasing the chances of type I error), the use of the gene-wise P-value as an input variable in a second analysis step (see Introduction) provides protection against spurious findings caused by such bias. Moreover, we implemented rigorous validation steps that included randomized network searches and comparison with similar datasets from other complex diseases. Other measures of gene-wise significance were considered, including Fisher’s method of combining P-values (40), and a method that corrects for the number of SNPs tested within each gene and subsequently adjusts for LD (41,42) (data not shown). However, since the most biologically significant findings were obtained with the min P-value method, we only report on these results.

Genes with a gene-wise association P-value of 0.05 or less were considered for further study and loaded into the Cytoscape software, a package for visualization and analysis of networks (43). A curated human PIN (n = 7500) was downloaded and visualized in Cytoscape (44,45). The gene-wise P-values for association with each disease were loaded as node attributes of the PIN and the plugin jActive modules (46) was used to identify sub-networks of modestly associated but interacting gene products. The biological interpretation of a statistically significant module (sub-network) is that the products of a set of genes associated to the disease also interact physically, thus raising the possibility that they belong to the same pathway or biological process. jActive modules grows a network from each node by systematically adding one neighbor at a time and computing an aggregate score (S) based on a given statistical significance, in our case, the gene-wise P-value of association with the disease. Specifically, $S = \sqrt{kZ}$, where each gene P-value is converted to a Z-score (using the inverse normal CDF) and k is the number of genes contributing to the score S. Once S ceases to increase significantly, the sub-network stops growing and is reported as a module (46). Next, the test statistic (S) is compared with an appropriate background distribution. As a background distribution, we used the scores of modules randomly selected from the entire PPI network. Since the background distribution is dependent on module size, jActive modules creates a background distribution by scoring 10,000 random modules of each size (in a Monte Carlo procedure). Furthermore, since the scores distribution is a smooth function of module size, jActive modules applies a sliding window average to the background distribution. As in the original publication, modules with $S > 3$ (3 SD above the mean of randomized scores) were considered significant. It is interesting to note that scores of up to 12 were obtained for some diseases. If converted back to P-values and corrected for the multiple
network searches the algorithm performs \((\sim 10^7)\), results would remain highly significant \((10^{-14})\) even after the correction. \(P\)-values from both studies in MS (IMSGC and GeneMSA) and RA (Plenge et al. and WTCCC) were included in the search, resulting in higher confidence in the significance of the modules retrieved. To merge datasets, we included genes with significant \(P\)-values in either study, and when a given gene was present in both, the min \(P\)-value was considered. To evaluate whether the significant modules obtained were biologically meaningful, we computed their enrichment in human biochemical pathways (i.e. the proportion of genes in a specific module that are in a pathway, compared to the overall proportion of genes described for that pathway) using the plugin BINGO (47) with a custom ontology and annotation files derived from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The statistical significance for enrichment of a given module in ontologies and pathways was determined by a chi-square test. Significant KEGG pathways are reported and visualized as directed acyclic graphs similar to those commonly reported for the analysis of GO by many popular gene expression analysis programs. Using the same plugin, we also computed enrichment of significant modules in GO categories. The analysis of several modules resulted in highly significant results with both KEGG pathways and GO. However, although most genes with known functions are categorized in the GO system, this classification is largely based on information retrieved from the literature while KEGG primarily categorizes genes into bona-fide biological pathways. Because biological interpretation of pathways is more straightforward, gene ontology: tool for the unification of biology. The Gene Ontology Society.

To account for the possibility that significant modules were obtained by chance, 10 searches with the gene-based \(P\)-values randomly permuted over the genes were conducted for each disease. The average score of the randomized searches is compared with the scores obtained with the real (i.e. non-randomized) \(P\)-values for each disease.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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