Efficient pathway enrichment and network analysis of GWAS summary data using GSA-SNP2

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ABSTRACT

Pathway-based analysis in genome-wide association study (GWAS) is being widely used to uncover novel multi-genic functional associations. Many of these pathway-based methods have been used to test the enrichment of the associated genes in the pathways, but exhibited low powers and were highly affected by free parameters. We present the novel method and software GSA-SNP2 for pathway enrichment analysis of GWAS *P*-value data. GSA-SNP2 provides high power, decent type I error control and fast computation by incorporating the random set model and SNP-count adjusted gene score. In a comparative study using simulated and real GWAS data, GSA-SNP2 exhibited high power and best prioritized gold standard positive pathways compared with six existing enrichment-based methods and two self-contained methods (alternative pathway analysis approach). Based on these results, the difference between pathway analysis approaches was investigated and the effects of the gene correlation structures on the pathway enrichment analysis were also discussed. In addition, GSA-SNP2 is able to visualize protein interaction networks within and across the significant pathways so that the user can prioritize the core subnetworks for further studies. GSA-SNP2 is freely available at https://sourceforge.net/projects/gsasnp2.

INTRODUCTION

Improving the power of genome-wide association study (GWAS) has been a major challenge for the last decade. The multiple testing correction in GWAS typically resulted in only a handful of single nucleotide polymorphism (SNP) markers. Analysis of such top-ranked SNPs discarding all except 'the tip of the iceberg' was capable of revealing only a small number of associated functions. As the sequencing cost keeps dropping, whole genome sequencing data have come to be used for GWAS (1), which poses a much greater multiple testing burden. A number of multi-loci (gene or pathway)-based association analysis methods were developed to address the problem. These methods substantially increased the statistical power, and revealed many novel genes and pathways that were not found by the SNP-based approach (2–4). In particular, pathway-based association analysis methods directly provide biological interpretations and are capable of detecting aggregate association of multiple genes even when the individual genes are only moderately associated.

In earlier efforts, most of the pathway-based GWAS analysis methods incorporated the *competitive* null hypothesis (5), and tested the relative enrichment of the associated genes in each pathway gene set. GenGen (6), GSEA-SNP (7), iGSEA4GWAS (8), SSEA (9) and MAGENTA (10) implemented modified GSEA algorithms which were originally developed for the pathway analysis of gene expression data; GSA-SNP (11) implemented a modified Z-test as well as two GSEA algorithms; Aligator (12) and Gowinda (13) provided Gene Ontology over-representation analysis accounting for the gene size (or SNP count); INRICH (14) tested enrichment of pathway gene-sets across independent genomic intervals; and MAGMA (15) exploited multiple regression models for gene and gene-set analysis. Whereas competitive methods for GWAS data provide fast and simple implementations, many of them exhibit low powers and are susceptible to some free parameters.

The pathway-based association analysis methods were then developed for the *self-contained* null hypothesis in re-

cent years (3,5,16,17). Competitive methods directly target pathway-level aberrations by testing the *enrichment* of the associated genes in each pathway, whereas self-contained methods test the *existence* of an associated gene therein (18). Thus, self-contained methods are in general highly sensitive and therefore are useful in discovering novel pathways. However, genes typically have multiple functions and the mere existence of an associated gene does not always imply a *pathway-level* aberration. Thus, both approaches are useful and complement each other.

Furthermore, protein–protein interaction (PPI) networks have also been considered for analyzing GWAS summary data to identify large modules of associated proteins beyond the pre-defined pathway gene-sets (19,20). Overall, interrogation of GWAS data from different levels of biologic objects (SNP, gene, pathway and network) has proven useful for revealing novel associations with a phenotype of interest.

Here, we present the novel method and C++ standalone tool GSA-SNP2. It accepts GWAS SNP *P*-values and implements a powerful competitive pathway analysis as well as PPI network visualization in the significant pathways. Compared with the previous version (11), GSA-SNP2 provides a greatly improved type I error control by using the SNP-count adjusted gene scores, while nevertheless preserving high statistical power. The gene scores are adjusted for the SNP counts in each gene using monotone cubic spline trend curve. It was critical to remove high scoring (potentially associated) genes before the curve fitting to achieve high power. The performance of GSA-SNP2 was compared with those of six existing competitive pathway analysis methods and two recently developed self-contained methods using GWAS data simulated under linear model (21) and three publicly available GWAS summary datasets. GSA-SNP2 exhibited high power and surpassed other methods in prioritizing the curated *gold standard* pathways. Based on these results, the different pathway analysis approaches for GWAS data are compared and discussed. Furthermore, the difference in the gene correlation structures between GWAS and gene expression data, and their effects on competitive pathway analysis are investigated. In addition, GSA-SNP2 is able to visualize the PPI networks within (local) and across (global) the significant pathways. These networks suggest how the key proteins interact with each other and affect their neighbors in the aberrant pathways. The global network, in particular, shows the core PPI structure that cannot be captured by single pathways guiding a mechanistic study. GSA-SNP2 is freely available at https://sourceforge.net/projects/gsasnp2.

MATERIALS AND METHODS

Methods used in GSA-SNP2

GSA-SNP2 employs the Z-statistic of the random set model (22) for evaluating gene-sets (pathways). The critical improvement from the previous version (11) is obtained from the usage of the gene scores adjusted for the SNP counts for each gene using a monotone cubic spline trend (23).

Adjusted gene scores. SNPs that are located in the range of a gene [gene start − padding, gene end+padding] are as-
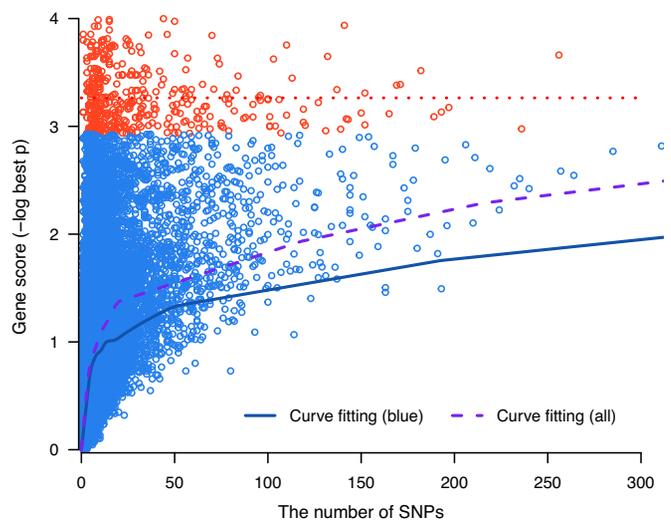

Figure 1. The monotone cubic spline trend curves. Red circles represent high scoring genes that have zero correlation coefficient (red dotted line). Both the trend curves with (purple dash) or without (blue solid) red circles are depicted. The blue curve is used for calculating the adjusted gene scores.

signed to the gene. The padding size of a gene is chosen among 0, 10 000 and 20 000 (default) and the same padding size is applied to all genes. According to Pickrell *et al*. (24), 90% of SNPs affecting expression quantitative trait loci were observed within 15 kb from the 5′ and 3′-end of a gene. Thus, padding sizes around 15 kb (10 or 20 kb) may be reasonable. Then, the initial gene score is given as the maximum of –log(SNP *P*-value) for those SNPs. These gene scores in general tend to increase as the number of assigned SNPs is increased. Thus, the initial gene scores are adjusted for the number of assigned SNPs using monotone cubic spline trend as shown in Figure 1. Note that a number of genes have very high scores irrespective of the increasing trend for the SNP counts. Therefore, such high scoring (presumably associated) genes are removed before fitting the trend curve. In other words, a range of top gene scores where their correlation coefficient becomes zero (red circles) is searched and the corresponding genes are removed. And then, a monotone cubic spline curve (blue solid curve) is fitted for the remaining genes (blue circles). We note that if such high scoring genes are not removed, the trend curve rather moves up (purple dash), which considerably lowers the power of our method. The meaning of these high scoring 'uncorrelated' genes is discussed in Supplementary Data. The adjusted gene score for *i*th gene g_i is given as:

$$Adj(g_i) = -\log(p_i) - C(g_i),$$

where p_i is the best *P*-value among the SNPs assigned to g_i and $C(g_i)$ is the estimated gene score on the trend curve. Note that the removal of the high scoring genes is only for the curve fitting and they are all restored when calculating the adjusted gene scores. See 'Supplementary Data' section for the detailed description of the algorithms for the outlier treatment, the novel algorithms for data sampling and conversion to monotonic data using *dual cubic splines* for the final curve fitting.

Pathway statistic. Each pathway gene-set P_j ($1 \leq j \leq K$) can be assessed by Z-statistic as follows:

$$Z\left(P_j\right) = \frac{\overline{P_j} - m}{\sigma/\sqrt{N_j}},$$

where $\overline{P_j}$ is the average of the adjusted gene scores in the gene-set P_j, m and σ are respectively the mean and standard deviation of all the adjusted gene scores, N_j is the number of genes in P_j. In this method, each pathway gene-set is assumed to be a random collection of genes from the genome; hence the pathway statistic $Z(P_j)$ is assumed to have standard normal distribution. The pathway P-value is obtained from the right-tailed test and Benjamini–Hochberg method is used for the multiple testing correction (25). It was also shown that one-tailed Z-test (used for GWAS data) better controls false positives compared with two-tailed Z-test (used for gene expression data), rendering our Z-test-based approach more attractive (26). GSA-SNP2 uses a modified Z-statistic (22) to capture more closely the impact of the set size on the set statistic by replacing σ with $\sigma^* = \sigma \cdot (\frac{|G|-N_j}{|G|-1})^{\frac{1}{2}}$, where $|G|$ is the total number of genes analyzed. This modification slightly increases the power of our method by amplifying the original Z-statistic. This Z-statistic indeed had standard normal distribution (Supplementary Figure S1).

Adjacent gene filtering. Some genes in a pathway can be closely located on the genome or highly overlapping family genes, and some of those genes may also belong to the same linkage disequilibrium (LD) block. Such genes exhibit a positive correlation in their P-values and may contribute to increasing false positive pathways. To prevent this possibility, the adjacently located genes within a pathway are alternatively removed if they also have high positive genotype correlations (>0.5) in the 1000 Genomes data. See 'Supplementary Data' section for the detailed algorithm and test results for different correlation thresholds (Supplementary Table S4). However, in practice, only a small portion of genes in a pathway were adjacently located while at the same time having high correlations (<1% on average even for lower correlation threshold 0.3). Thus, this filtering process seems to have a limited effect in reducing false positives.

Pathway analysis methods compared with GSA-SNP2

The type I error rate control and statistical power of GSA-SNP2 were compared with those of six existing competitive pathway analysis methods that analyze GWAS summary data as follows: Z-test of GSA-SNP (denoted GSA-SNP1), iGSEA4GWAS, MAGMA, INRICH, Gowinda and MAGENTA. MAGMA was tested for mean, top 1 SNP statistic, as well as their combination (denoted MAGMA-mean, MAGMA-top1 and MAGMA-multi, respectively). For MAGENTA, two default enrichment cutoffs (75 and 95 percentiles of all gene scores) were used. For INRICH, the SNP intervals were constructed for top 1% association P-values. Using other larger percentages resulted in a very long computation time in the simulation test. $R^2 = 0.5$ was used for the LD-clumping parameter. Gowinda was tested for gene-mode, and candidate SNPs were selected for the

top 1, 5 or 10% of P-values. For other parameters, the default values were used.

In the power simulation test, the relative 'enrichment' of associated genes in the target pathway was simulated; therefore, only the competitive methods described above were compared. In analyzing public GWAS datasets, however, two self-contained methods, sARTP and self-contained versions of MAGMA were also compared.

Simulation of GWAS data based on linear model

We simulated the genotypes of 10 000 individuals by randomly pairing the haplotypes of 1000 Genomes European samples. The phenotype Y of each individual was calculated based on linear model (quantitative trait) (21). For testing the type I error rate control, the following model was used.

$$Y = \beta_1 X_1 + \cdots \beta_k X_k + \varepsilon$$

where $X_1, \cdots, X_k$ are normalized additive genotypes of k effective SNPs, $\beta_1, \cdots, \beta_k$ are SNP effects (set as one in this study) and ε is the residual with $\varepsilon \sim N(0, \sigma^2)$. In the type I error rate test, 300 effective SNPs were randomly selected within gene regions (including the padding parts). The phenotype variance σ^2 is determined by the narrow-sense heritability (h^2). In this case, the simulation data were generated for $h^2 = 25$ or 50%.

For testing the statistical power, the following model was used.

$$Y = \beta_1 X_1 + \cdots \beta_k X_k + \gamma (G_1 + \cdots + G_M) + \varepsilon$$

where γ is the gene-set effect and $G_1, \cdots, G_M$ are the effects of M causal genes in the target pathway. The target pathway is randomly selected among the pathway gene-set database. The effect of a gene g is defined as $G_g = (X_{g_1} + \cdots + X_{g_L})/\sqrt{L}$ where $X_{g_1}, \cdots, X_{g_L}$ are normalized additive genotypes of L causal SNPs within gene g. In this case, the total heritability was decomposed into the background heritability $h_b^2 = \frac{Var(\beta_1 X_1 + \cdots \beta_k X_k)}{Var(Y)}$ and gene-set specific heritability $h_g^2 = \frac{Var(\gamma(G_1 + \cdots + G_M))}{Var(Y)}$, assuming that $X_1, \cdots, X_k$ and $G_1, \cdots, G_M$ have no correlation. The gene-set effect γ and phenotype variance σ^2 are determined by the combination of h_b^2 and h_g^2. The power simulation data were generated for $h_b^2 = 25\%$ or 50% and $h_g^2 = 4\%$ or 8%. A hundred of background SNPs were randomly selected within gene regions, and 10–40% of causal genes in the target pathway were randomly chosen. For each causal gene, one causal SNP was randomly assigned. The 674 Reactome pathway gene-sets (set size: 10–200) were used for both type I error rate and power simulation tests (27,28).

RESULTS AND DISCUSSION

Simulation results for type I error rate control

False discovery rate (FDR) control simulation was repeated 20 times for two conditions $h^2 = 25$ or 50%, and the numbers of significant gene-sets (FDR < 0.05) detected by each method were depicted in Figure 2. Although the causal SNPs were randomly sampled from the
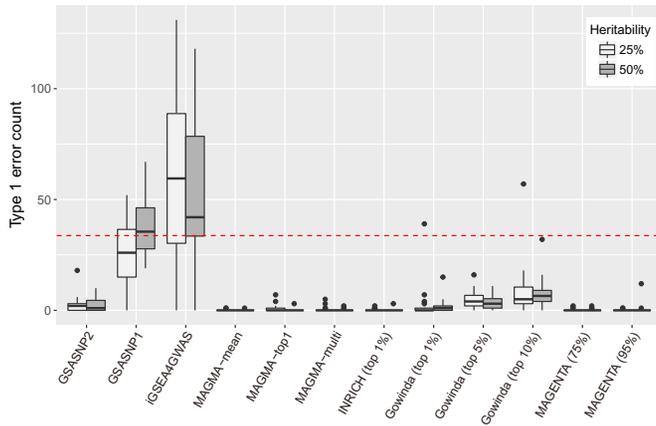
Figure 2. Comparison of false discovery controls. False discovery counts (FDR < 0.05) for competitive pathway analysis methods are shown. The simulation was performed for two heritability values (25 and 50%) and each simulation was repeated 20 times. MAGMA was tested for three gene models; INRICH was tested for approximately top 1% SNPs; Gowinda was tested for approximately top 1, 5 and 10% SNPs; and MAGENTA was tested for 75 and 95% of enrichment cutoffs. The red dashed line indicates the 5% of the total pathways.

genome, iGSEA4GWAS and GSA-SNP1 detected a number of significant gene-sets (median false discovery counts for iGSEA4GWAS: 59.5 for $h^2 = 25\%$, 42 for $h^2 = 50\%$; GSA-SNP1: 26 for $h^2 = 25\%$, 35.5 for $h^2 = 50\%$ out of 674 pathway gene-sets). GSA-SNP2 showed greatly improved FDR control compared with GSA-SNP1 (Median false discovery: 2 for $h^2 = 25\%$ and 1 for $h^2 = 50\%$). INRICH, MAGMA and MAGENTA exhibited strict FDR controls (almost zero false discoveries), whereas Gowinda exhibited rather varied FDR controls depending on the SNP P-value cutoff.

Simulation results for statistical power

The statistical power of each method was tested for combinations of two background heritability values ($h_b^2 = 25\%$, 50%) and two set-specific heritability values ($h_g^2 = 4\%$, 8%). In each parameter setting, simulations were repeated 50 times and we counted the cases where the target pathway was successfully detected (FDR < 0.05). GSA-SNP1 and iGSEA4GWAS were not included because they exhibited poor FDR controls. Figure 3 shows the powers of each method for the four different parameter settings. GSA-SNP2 exhibited outstanding powers for all the test conditions among the competitive pathway analysis methods (78.0% for $h_b^2 = 25\%$, $h_g^2 = 8\%$; 60.0% for $h_b^2 = 25\%$, $h_g^2 = 4\%$; 65.3% for $h_b^2 = 50\%$, $h_g^2 = 8\%$; 44.0% for $h_b^2 = 50\%$, $h_g^2 = 4\%$). MAGMA exhibited varied powers depending on the gene scoring method and the simulation parameters. For three out of four cases, MAGMA-mean had slightly better powers than MAGMA-top1. Their combination, MAGMA-multi, showed an improved power when $h_b^2 = 25\%$ and $h_g^2 = 8\%$. INRICH and MAGENTA exhibited low powers compared with other methods; the best powers of INRICH, MAGENTA (75%) and MAGENTA (95%) were only 16.3, 12.2 and 14.3%, respectively.
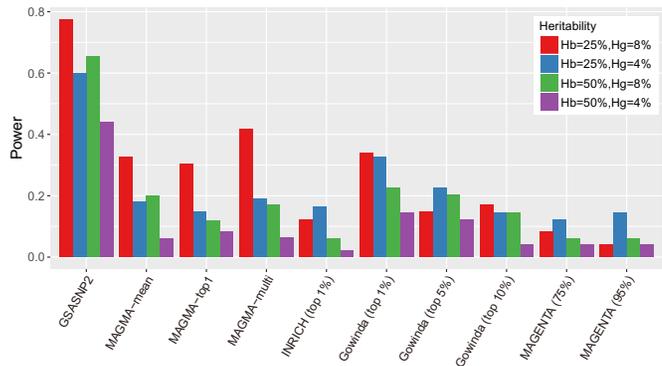

Figure 3. Comparison of statistical powers. Powers of competitive pathway analysis methods under the four different simulation settings ($h_b^2 = 25\%$, $h_g^2 = 8\%$; $h_b^2 = 25\%$, $h_g^2 = 4\%$; $h_b^2 = 50\%$, $h_g^2 = 8\%$; and $h_b^2 = 50\%$, $h_g^2 = 4\%$) are represented. In each method, the same parameters as used in the false discovery control test were used.

Gowinda exhibited varied powers depending on the SNP P-value cutoff.

Performance comparison using real GWAS summary data

GSA-SNP2, six other competitive methods, as well as two self-contained methods were tested for three public GWAS summary datasets (DIAGRAM (29), GIANT height (30) and KARE height (31)), and their performances were compared based on curated gold standard pathways. In addition, we tested hybrid methods where the adjusted gene scores in GSA-SNP2 are replaced with VEGAS (3,32) or GATES (4) gene scores. VEGAS provides empirical P-values for each gene using multivariate normal distribution, and GATES also provides gene-based P-values taking into account the effective number of SNPs in each gene. Between them, only VEGAS provided reasonably good pathway analysis results, so was included in the performance comparison. For VEGAS, two options of using all SNPs in each gene (denoted GS2VEGAS-all) and only the top SNP (denoted GS2VEGAS-top1) were tested. INRICH ($P = 1E-6$ and $P = 1E-8$), Gowinda ($P = 1E-3$, $P = 1E-2$ and $P = 5E-2$) and MAGENTA (enrichment cutoff: 75 and 95% gene scores) were tested for a couple of free parameter values.

First, the type 2 diabetes (T2D) GWAS summary P-values (stage 1 metadata; case 12 171 and control 56 862 samples) were downloaded from the DIAGRAM consortium site (http://www.diagram-consortium.org/). Morris *et al.* have curated 16 hallmark pathways for T2D (29). These pathways as well as those including the word 'diabetes' in the pathway name were regarded as gold standard (GS) positives and were summarized into 15 *GS categories* (Table 1 and Supplementary Data). Among the 1264 MSigDB C2 canonical pathways (28,33), 136 pathways were found to belong to one of these categories (denoted *GS pathways*) and are listed in Supplementary Data. Figure 4A shows the comparison results between different methods; the cumulative GS pathway counts corresponding to the pathway ranks were depicted for each method up to q-value < 0.25. These graphs show how well each method prioritizes
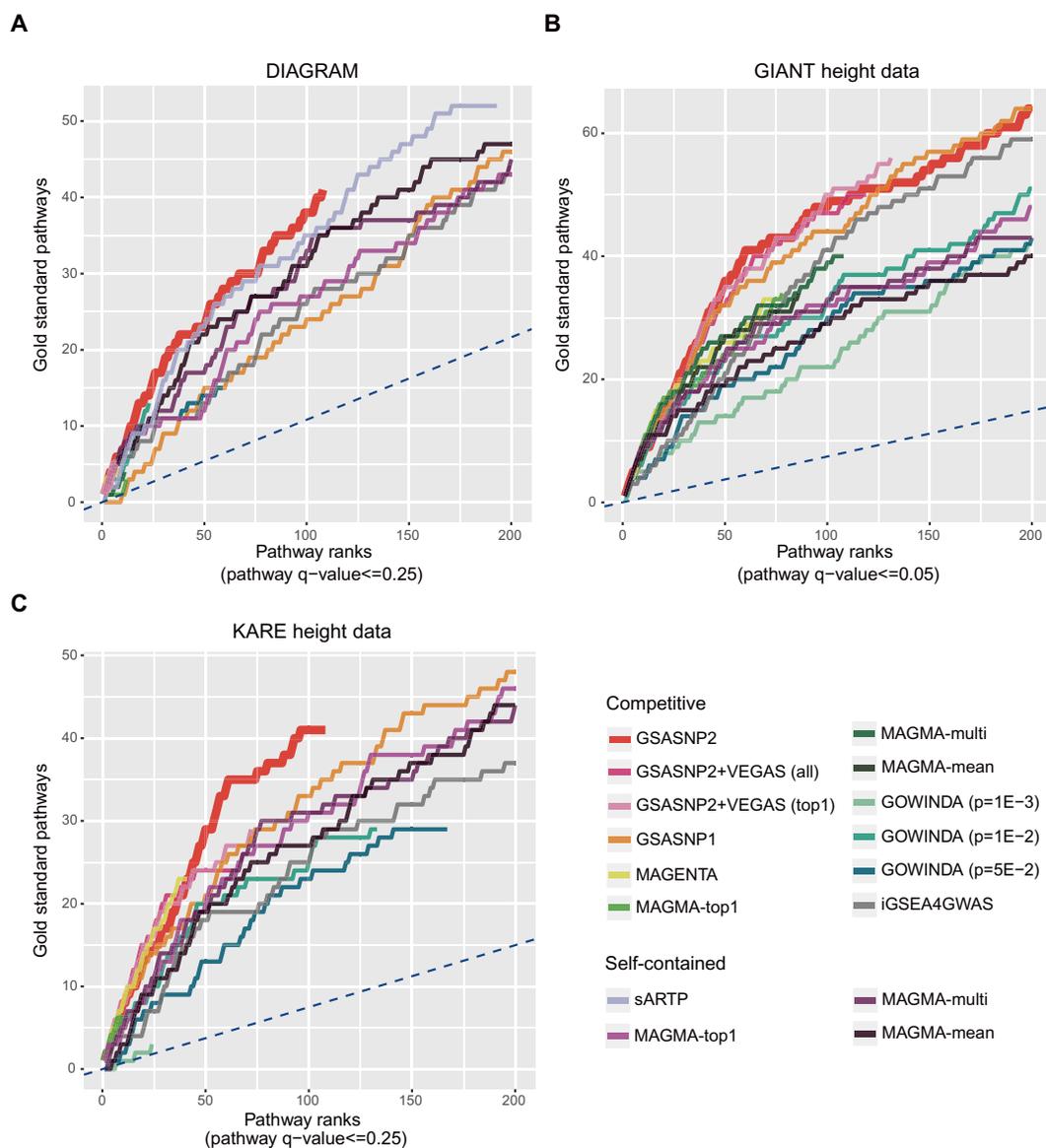
Figure 4. Performance comparison using real data. For three public GWAS summary datasets from (A) DIAGRAM, (B) GIANT height and (C) KARE height data, the cumulative gold standard pathway counts for competitive and self-contained pathway analysis methods were plotted. The results from INRICH were not represented because it detected only one GS pathway. The blue dashed lines indicate the expected cumulative gold standard pathway counts for random prediction.

the GS pathways. The graphs corresponding to a stricter cut-off (q-value < 0.05) are also shown in Supplementary Figure S2. See also Supplementary Table S1 for detailed analysis results of each method. GSA-SNP2 exhibited a high power and outperformed the other competitive and MAGMA self-contained methods in the overall GS pathway ranks. It also showed slightly better performance compared with sARTP. Except for GSA-SNP2, GSA-SNP1 and iGSEA4GWAS, most competitive methods detected only a small number of GS pathways (≤ 15) due to their low powers. GSA-SNP2, GSA-SNP1 and iGSEA4GWAS detected 41, 47 and 49 GS pathways out of the 108, 232 and 240 significant pathways (FDR < 0.25), respectively. All the self-contained methods exhibited high powers as expected. sARTP detected 52 GS pathways out of 193 sig-

nificant pathways, and self-contained MAGMA-mean that showed the best result among the MAGMA methods detected 85 GS pathways out of 552 significant pathways.

Then, we compared the GS categories detected by each method. Here, we focused on four methods that detected more than 25 GS pathways within the top 100 ranks (GSA-SNP2, iGSEA4GWAS, sARTP and the self-contained MAGMA-mean). These four methods successfully detected all the three GS pathways in the 'regulation of beta cell' category. Among the other categories, GSA-SNP2 predicted the largest number of GS pathways in the 'diabetes', 'blood glucose regulation', 'branched chain amino acid metabolism', 'inflammation' and 'Notch signaling' categories. iGSEA4GWAS best predicted in the 'unfolded protein response' and 'glycolysis and gluconeoge-

Table 1 Power comparison for the 15 gold standard categories for type 2 diabetes

Category	GSASNP2	GS2VEGAS (best P)	GSASNP1	iGSEA 4GWAS	MAGMA-multi	MAGENTA (75%)	GOWINDA ($P = 0.01$)	sARTP	MAGMA-mean-sc*
Diabetes	**3/4**	1/4	**3/4**	2/4	0/4	1/4	2/4	**3/4**	**3/4**
Regulation of beta cell	**3/3**	**3/3**	1/3	**3/3**	0/3	2/3	**3/3**	**3/3**	**3/3**
Insulin/blood glucose level	**10/25**	0/25	8/25	2/25	0/25	0/25	1/25	4/25	7/25
Adipocytokine signaling	1/6	0/6	0/6	0/6	0/6	0/6	0/6	**2/6**	1/6
Cell cycle	4/22	1/22	1/22	5/22	2/22	0/22	1/22	4/22	**6/22**
Circadian rhythm	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
Unfolded protein response	0/2	0/2	0/2	**2/2**	**2/2**	0/2	0/2	**2/2**	**2/2**
Branched-chain a.a.** metabolism	**1/2**	0/2	**1/2**	0/2	0/2	0/2	**1/2**	0/2	0/2
Fatty acid metabolism	2/10	0/10	3/10	1/10	0/10	1/10	0/10	**5/10**	3/10
Glycolysis and Gluconeogenesis	0/3	0/3	0/3	**2/3**	0/3	0/3	1/3	0/3	0/3
Inflammation	**8/22**	0/22	2/22	3/22	0/22	0/22	0/22	4/22	0/22
NOTCH signaling	**6/14**	0/14	3/14	5/14	0/14	0/14	4/14	5/14	5/14
PPARG signaling	0/1	0/1	0/1	0/1	0/1	0/1	0/1	**1/1**	0/1
WNT signaling	0/11	0/11	**2/11**	1/11	0/11	0/11	0/11	**2/11**	0/11
Mitochondrial dysfunction	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	**1/5**
Total GS pathways	**38/100**	5/7	24/100	26/100	4/11	4/9	13/23	35/100	31/100

Gold standard pathways detected within the top 100 pathways were counted for each category. Insignificant pathways (FDR > 0.25) were excluded. Total counts are shown in the bottom. The best counts in each category were marked in bold. *sc: self-contained method, **a.a.: amino acid.

nesis' categories. Self-contained MAGMA-mean best predicted in the 'diabetes', 'cell cycle', 'unfolded protein response', 'Notch signaling' and 'mitochondrial dysfunction' categories. sARTP best predicted in as many as seven categories such as 'diabetes', 'adipocytokine signaling', 'unfolded protein response', 'fatty acid metabolism', 'PPARG signaling' and 'WNT signaling'. Some pathways showed the characteristics of self-contained methods. For example, the 'KEGG PPAR signaling pathway' contained only one strongly associated gene (PPARG) and was detected only by the self-contained methods (q-value: 0.05 and 0.002 for sARTP and self-contained MAGMA-top1, respectively) demonstrating their high sensitivity. These results indicate that different pathway analysis methods exhibit different preferences for GS categories. Overall, GSA-SNP2 detected the largest number of GS pathways within the top 100 pathways, and also showed a wide coverage of GS categories that is comparable to two powerful self-contained methods.

Next, the human height GWAS meta-analysis P-values from the GIANT consortium 2010 (total sample size 183 727) were analyzed (30). In total, 15 GS categories associated with height and bone regulation were curated from three independent studies. First, Pers *et al*. performed DEPICT pathway analysis using the height GWAS meta-analysis summary data from the GIANT consortium 2012–2015 (total sample size: 253 288) (34,35). Because the large sample size increases the statistical power, and DEPICT is shown to properly control the type I error rate and account for confounding factors, we regarded the DEPICT analysis result as a good source for interrogating the height-associated pathways. From 183 significant pathways obtained using a rather strict cutoff FDR < 0.01, we found 12 GS categories that are supported in the literature such as skeletal system development and epigenetics (36,37). Second, Marouli *et al*. analyzed the rare and low-frequency coding variants that affect human height, and suggested several height-associated genes and pathways (38). Among them, 'proteoglycan' and 'reactive oxygen species (ROS)' were experimentally validated in other studies, so we included them in the GS categories (39,40). Third, 'telomerase activity' is known to have an important role in chondrocyte proliferation during bone elongation, and thus was also in-

cluded in the GS categories (41). These 15 height-related GS categories, supports in the literature, as well as all the corresponding GS pathways from the MSigDB C5 gene ontology terms (v 6.0) (28,33) are listed in the Supplementary Data.

Based on these GS pathways, performances of the pathway analysis methods except sARTP were compared; sARTP cannot be applied without effect values. Because of the high powers caused by the large sample size, the cumulative GS pathway counts were plotted up to q-value < 0.05 in each method (Figure 4B). With the GIANT height data, GSA-SNP2 still exhibited a high power and best prioritized the GS pathways. It detected 50 GS pathways within the top 100 significant pathways. The other GSA-SNP methods including GSA-SNP1, GS2VEGAS-all and GS2VEGAS-top1 also showed similar high performances except that GS2VEGAS-all and GS2VEGAS-top1 exhibited relatively less powers (i.e. lower upper bounds of the curves) compared with GSA-SNP1 and GSA-SNP2. Such a power reduction is attributed to the empirical approach of VEGAS which only provides gene P-values > 10E-6. However, their precisions do not seem to be reduced; within the top 100 significant pathways, GS2VEGAS-mean and GS2VEGAS-top1 detected 50 and 53 GS pathways, respectively. Due to the large sample size, most competitive methods including MAGMA, MAGENTA (95%) and Gowinda showed greater powers compared with the T2D case. MAGENTA and MAGMA better prioritized GS pathways compared with the self-contained MAGMA methods (MAGENTA detected 35 GS pathways out of 73 significant pathways; MAGMA-multi detected 40 out of top 100 pathways; and self-contained MAGMA-multi detected 37 out of top 100 pathways).

In addition, there were clear differences in the preference of GS categories between the pathway analysis methods. For example, the competitive MAGMA methods detected the largest number of 'skeletal system development' pathways such as 'cartilage and chondrocyte development' (e.g. MAGMA-multi detected 23 related terms), and many of them were in the top ranks. They were also top-ranked in the MAGENTA result. In contrast, GSA-SNP methods detected relatively more 'epigenetics' pathways (21–22 related terms) in the top ranks. GSA-SNP methods specifi-

cally detected four 'telomerase activity' pathways within the top 100 ranks, whereas the other competitive methods detected only one or no telomerase related term. Additionally, GSA-SNP2 detected two 'ROS' pathways within the top 100 ranks, which were reported from a large-scale rare and low-frequency variant analysis (38), whereas most of the other methods failed to detected these pathways; only GS2VEGAS-top1 and Gowinda ($P = 0.05$) detected one of the ROS pathways. GS2VEGAS-all detected as many as six 'insulin-like growth factor and growth hormone' pathways, whereas the other methods detected three or less corresponding terms. All the detailed analysis results for each method are available in Supplementary Table S2.

Lastly, we analyzed the Korean height GWAS P-values from the KARE consortium where much smaller samples (8842) were used compared with the GIANT height data (31). Comparison of pathway analysis methods using relatively small-sample data is particularly important, because pathway analysis has been applied to GWAS to overcome the low powers of the conventional SNP-based analysis. In this case, the cumulative GS pathway counts were plotted up to q-value < 0.25 due to the lowered powers in each method (See Supplementary Figure S2 for the graphs corresponding to q-value < 0.05). GSA-SNP2 still showed high power and outperformed the other methods in prioritizing the GS pathways. It detected 41 GS pathways out of top 100 terms. Here, GS2VEGAS and MAGENTA (75%) methods showed slightly better rank distributions than GSA-SNP2 in the former part (up to 40th rank). Although MAGENTA had a relatively low power, it exhibited the highest density of GS pathways (25 GS pathways within top 41 pathways: 61.0%) demonstrating its strict false positive control. The powers of MAGMA methods were severely decreased compared with the GIANT height case. MAGMA-mean and MAGMA-multi detected no significant pathways and only MAGMA-top1 detected five 'skeletal system development' and one 'epigenetics' pathways. It seems that MAGMA is very sensitive to GWAS sample size. The preferred GS categories in each method were similar to the GIANT height case. For example, self-contained MAGMA methods detected many 'skeletal system development' pathways (13–16 pathways; GSA-SNP2 detected 11 and the other methods detected eight or less corresponding pathways), whereas GSA-SNP2 and two GS2VEGAS methods detected many 'epigenetics' pathways (17 pathways) and four 'telomerase' pathways. Among the rare and low-frequency variant associated pathways, only GSA-SNP1 and GSA-SNP2 detected four and one 'proteoglycan' pathways within the top 100 ranks, respectively. None of the tested methods detected an ROS pathway within the top 100 ranks. See Supplementary Table S3 for detailed analysis results for each method.

Comparison of competitive and self-contained pathway analysis results

GSA-SNP2 and sARTP results for the DIAGRAM data were further compared by the pathways exclusively detected by either method. The top 10 pathways that were significant with GSA-SNP2 but were not significant with sARTP, and *vice versa* were selected and the distributions of gene P-values (VEGAS best p option) were compared in Figure 5.

In the former case, several genes had similar low P-values which seemed to collectively represent the pathway-level aberrations. On the other hand, in the latter case, most pathways contained one or two extreme gene P-values which seemed to dominate those pathways. If such extreme genes also belong to many other pathways, the association of the corresponding pathway may not be very reliable. We note that competitive methods are also affected by such outlier genes and self-contained methods are also capable of detecting pathways composed of moderately associated genes only; however, this example demonstrates the difference of the two GWAS pathway analysis approaches.

Comparison with the competitive pathway analysis for gene expression data

The core algorithms used for competitive pathway analysis of GWAS data are virtually the same as those used for gene expression data. It is well known that the competitive methods for gene expression data suffer from inflated type I errors caused by the inter-gene correlations in each pathway (5,18,42). Interestingly, in our test for GWAS summary data, many competitive methods yielded little false positives. There is a substantial difference in the inter-gene correlation structure in each pathway between the two data types. In the gene expression case, many genes in each pathway are involved in the same biological process and exhibit positive correlations. However, in the GWAS case, only adjacently located genes in each pathway that belong to the same LD block exhibit positive correlations. Indeed, only a small portion of genes were adjacently located in each MSigDB C2 canonical pathway (median = 1.4%, mean = 4.3%), and only a portion of them had meaningful correlations (median = 0.0%, mean = 0.9% for the correlation threshold 0.3). Therefore, inter-gene correlations in GWAS data seem to exert very limited effect on false positive generation. Note that GSA-SNP2 removes those highly correlated adjacent genes in calculating pathway scores to further reduce false positives (Supplementary Data). Many competitive methods for GWAS data based on GSEA procedure (6,7,9) perform sample label permutation of genotype data in order to control false positives. Our results suggest that the simple competitive methods that randomize gene or SNP labels reasonably control false positives without the heavy permutation of genotype data.

Comparison of running times

At last, the running times for each software were compared for the DIAGRAM data and the C2 canonical pathway sets (Table 2). GSA-SNP1, GSA-SNP2, MAGMA-mean, IN-RICH and Gowinda were quite fast taking only a few minutes, while sARTP took over 10 days run on the same PC (Intel Xeon Processor X5670 @ 2.93GHz, 12 CPUs and 24GB of RAM).

Network visualization

GSA-SNP2 is able to visualize protein interaction networks within individual and across significant pathways. Network plots are generated based on STRING (43) or HIPPIE (44)
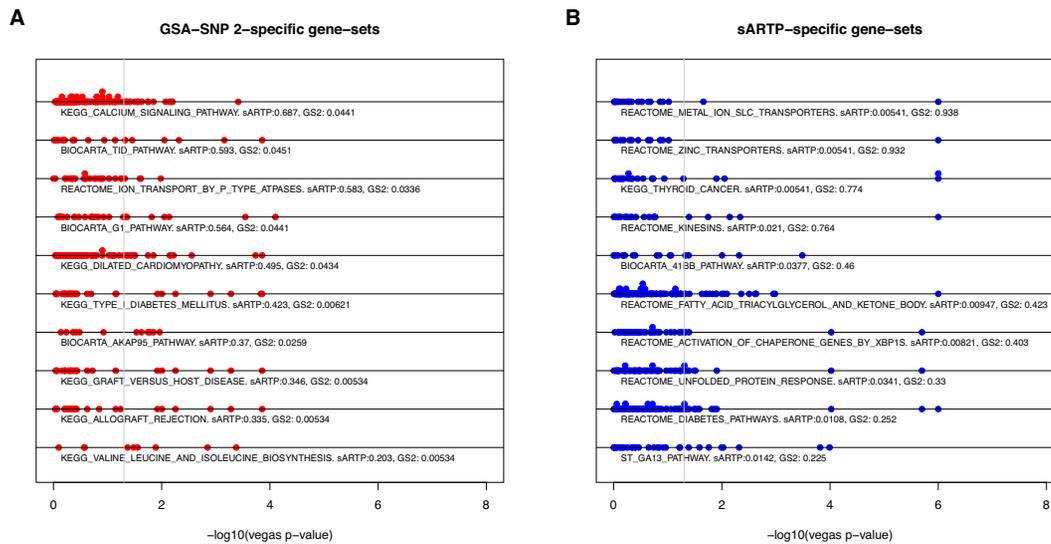
Figure 5. Comparison of gene *P*-value distributions in the pathways that are only significant with (**A**) GSA-SNP2 or (**B**) sARTP. The gray vertical line indicates the significance cutoff (VEGAS2 *P*-value = 0.05). GS2 represents GSA-SNP2. Pathway *P*-values for sARTP and GSA-SNP2 are also represented.

Table 2 Running times for eight pathway analysis programs for GWAS summary data

Method	Time	Permutation
GSA-SNP2	1.53 min	
GSA-SNP1	1.49 min	
MAGMA-mean	3.03 min	
MAGMA-top1	34.85 min	
MAGMA-multi	41.85 min	
iGSEA4GWAS	30 min	
MAGENTA	114.18 min	10 000
Gowinda ($P = 0.001$)	0.62 min	10 000
Gowinda ($P = 0.01$)	0.80 min	10 000
Gowinda ($P = 0.05$)	2.01 min	10 000
INRICH ($P1 = 1E-6$)	0.85 min	10 000
INRICH ($P1 = 1E-4$)	2.41 min	10 000
sARTP	10.41 days	100 000

networks, and the cut-offs for gene and pathway scores for visualization are selected by the user. Clicking on the gene node pops up a table which shows the gene name, mapped SNPs, the neighboring genes, their association scores as well as further detailed information via the hyperlink to outer databases such as GeneCards (45) and dbSNP (46). The network data are also provided as a text file which also shows the pathways that contain the interacting protein pairs.

In particular, the global networks are able to show interacting protein pairs that do *not* belong to any of the single pathways. Such protein pairs may have drawn relatively less attention, but can provide useful information for mechanistic study. For example, the global networks (extracted from HIPPIE networks) of the significant pathways (FDR < 25%, gene score < 0.01) obtained by analyzing DIAGRAM data contained a sub-network composed of eight genes such as TNF, RAB5A, CHUK, LTA, CARS, IGF2BP2, HSPA1L and HSPA1A (Figure 6). Among them, TNF and RAB5A have been individually studied and both are known to regulate the insulin-responsive glucose transporter (GLUT4) (47–49), a key protein that regulates the

concentration of blood glucose by transporting it to muscle or fat cell. Thus, the deregulation of GLUT4 can lead to insulin-resistance and T2D (50,51). The global networks show the two proteins have a medium level of interaction score 0.63 (affinity chromatography technology), and their interaction may have an important implication in T2D.

The DIAGRAM data were also analyzed using STRING networks. It provided much denser interaction networks among the high scoring proteins than those for HIPPIE networks, and the key T2D proteins TNF and PPARG were represented as hub proteins. Note that many of the interaction edges in the STRING networks were generated from the text-mining of the literature including GWAS papers, and should be carefully analyzed to avoid circular argument.

CONCLUSION

GSA-SNP2 is a powerful and efficient tool for pathway enrichment analysis of GWAS summary data. It provides both local and global protein interaction networks in the associated pathways, and may facilitate integrated pathway and network analysis of GWAS data. Five features of GSA-SNP2 are summarized as follows:

i) **Decent type I error control** by incorporating gene scores adjusted to the corresponding SNP counts using monotone cubic spline trend.

ii) **High power** and **fast computation** based on the random set model.

iii) **Without any critical free parameter**

iv) **Protein interaction networks** are visualized for the significant pathways. This function enables the user to prioritize core sub-networks within and across significant pathways.

v) **Easy to use:** only requires GWAS summary data (or gene *P*-values) and takes *only a minute or two* to get results. Other powerful self-contained pathway analysis
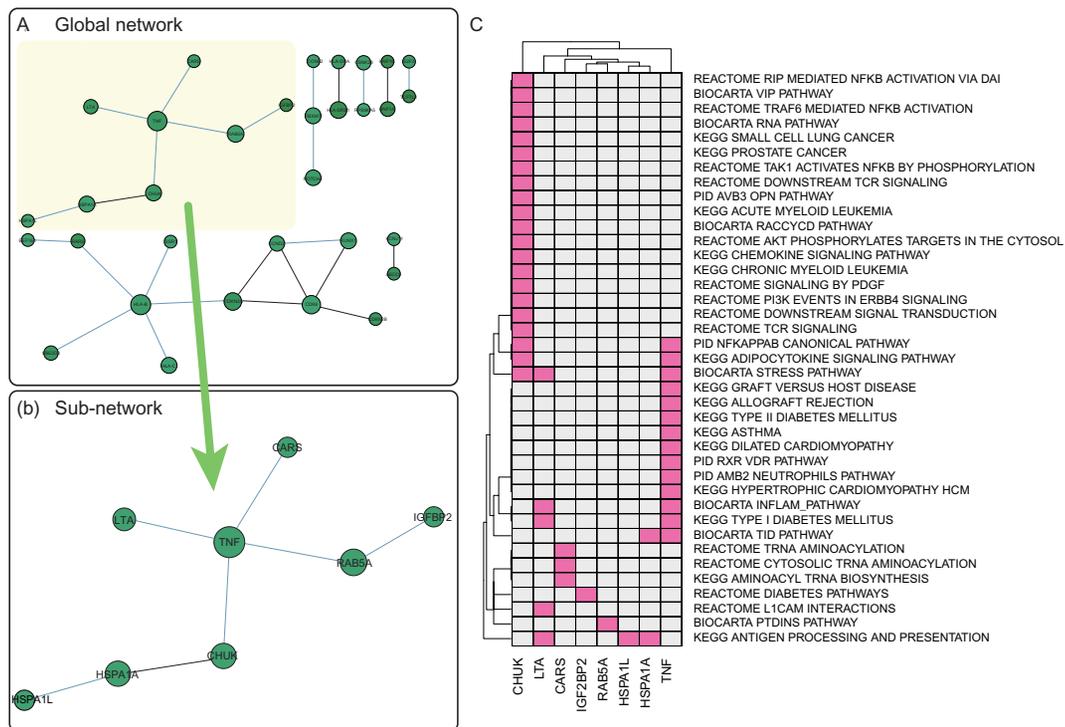
Figure 6. Gene network (HIPPIE) derived from DIAGRAM data analysis. (**A**) Global network from significant gene-sets (FDR < 0.25; gene score < 0.01). (**B**) A sub-network composed of eight nodes from the global network. (**C**) A heatmap representing the membership of each gene node in the significant pathways.

tools also require SNP correlation input and take much longer time.

DATA AVAILABILITY

GSA-SNP2 is available at https://sourceforge.net/projects/gsasnp2.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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