Knowledge-based genetic association study of hepatitis B virus related hepatocellular carcinoma

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Abstract

Background: Recent genome-wide association studies (GWASs) have suggested several susceptibility loci of hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC) by statistical analysis at individual single-nucleotide polymorphisms (SNPs). However, these loci only explain a small fraction of HBV-related HCC heritability. In the present study, we aimed to identify additional susceptibility loci of HBV-related HCC using advanced gene- and gene-set-based association tests.

Methods: We performed a meta-analysis of two existing GWASs of HBV-related HCC, based on which a series of association analyses at genes and multiple gene sets curated according to current knowledge were carried out for prioritizing potential risk genes. A series of prioritized SNPs were selected to replicate genetic associations in an independent sample of 965 cases and 923 controls.

Results: The gene-based association analysis suggested that five genes are significantly associated with HBV-related HCC risk: RNY4, GOLGA8M, LINC01207, WHAMMP2 and SLC39A8. Through gene-set-based association analysis, we found that the genes in systemic lupus erythematosus pathway may be relevant to development of HBV-related HCC. Three previously reported genes, NAT2, GSTA1 and GSTA2, were also highlighted to be susceptibility genes of HBV-related HCC when genes were stratified in a liver-specific expression set. However, probably due to small sample size, none of the genes prioritized by knowledge-based association analyses are successfully replicated in an independent sample.

Conclusions: This comprehensive knowledge-based association mining study suggested several promising genes significantly associated with HBV-related HCC risk. More experiments or larger samples are needed to validate their contribution to the pathogenic mechanism of HCC.
Background

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide. With 750,000 new HCC cases diagnosed each year, it is the third leading cause of cancer mortality. (1) As many as 30% of patients diagnosed with hepatitis, fibrosis or cirrhosis ultimately develop HCC. In high endemic areas such as Africa and Asia, at least 60% of HCC is associated with hepatitis B virus (HBV). (2) However, only a minority of HBV carriers develops HCC. HBV carriers with a family history of HCC were estimated to have over two-fold risk for HCC compared with those without a family history of HCC. (3) Furthermore, genetic complex segregation analysis suggested that major genes may be involved in the genetic predisposition to develop HCC at an earlier age. (4)

Genome-wide association study (GWAS) is a widely used strategy for identifying risk loci of complex diseases. Recently, several GWASs on risk of HBV-related HCC were conducted using single-nucleotide polymorphisms (SNPs)-based statistical association tests. Multiple susceptibility loci were identified, including rs17401966 in intron 24 of KIF1B at 1p36.22, rs7574865 in intron 3 of STAT4 at 2q32.2–32.3, rs9275319 between HLA-DQB1 and HLA-DQA2 at 6p21.3, rs9272105 between HLA-DQA1 and HLA-DRB1 at 6p21.3, and rs455804 in intron 1 of GRIK1 at 21q21.3. (5–7) However, these susceptibility loci account for only a small fraction of the contribution of genetics to HBV-related HCC. Identifying additional genetic alterations associated with HBV-related HCC may be difficult due to the relatively weak effects of many individual risk SNPs, which may be unidentifiable with the currently available, relatively small sample sizes. (8) SNP-based statistical association tests alone in GWAS do not have enough power to discover most risk loci for human complex diseases.

Gene- and biological pathway-based association analysis has been proposed to have superior statistical power compared with conventional statistical tests, as it relieves multiple testing and enriches signals. (9) Moreover, gene- and biological pathway-based
analysis also lends itself to introducing more disease-specific knowledge into the analysis.

In the present study, we performed a gene-based association analysis with meta-analysis
$p$-values from two independent HBV-related HCC GWASs. The gene-based $p$-values were
further evaluated within multiple gene-sets defined according to knowledge of HCC. SNPs
within prioritized genes were selected for replication in two independent HBV-related HCC
case/control populations.

Methods

Two existing GWASs on HBV-related HCC

The association $p$-values were obtained from two previous GWASs on HBV-related HCC in
Chinese populations for meta-analysis and knowledge-based association analysis. One
study(7) contained 2,689 chronic HBV carriers (1,212 HBV-related HCC cases and 1,477
controls) recruited from May 2006 to December 2012 by the Qidong Liver Cancer Institute
in Jiangsu Province of Mainland China. The other study(10) consisted of 95 HBV-infected
HCC patients (cases) and 97 HBV-infected patients without HCC (controls) recruited at
Queen Mary Hospital, Hong Kong. The sample inclusion and exclusion criteria were
described in the original papers.(7, 10)

Subjects in replication studies

The subjects in replication, including 965 chronic HBV carriers with HCC as cases and 923
chronic HBV carriers without HCC as controls, were recruited from the affiliated hospitals
of the Second Military Medical University, Shanghai, China. All the samples are of Han
Chinese descent and have participated in previously published studies.(7, 11)

The study was performed in accordance with guidelines approved by the local ethical
committees from all participating centers involved in both the GWAS stage and the
replication stage. An informed consent to participate in the study was obtained from each
subject in accordance with the declaration of Helsinki principles. All study participants
approved the storage of their frozen DNA specimens, for research purposes, in our laboratory.

Genotyping and quality control in replication

Genomic DNA from the peripheral blood of all participants in replication was extracted using the QIAamp DNA Blood Mini Kit (QIAGEN GmbH, Hilden, Germany). Genotyping analyses for replication samples were conducted using the Sequenom MassArray system (Sequenom) according to the manufacturer’s instructions. Genotyping quality was examined by a detailed QC procedure consisting of a 95% successful call rate, duplicate calling of genotypes, and internal positive control samples and two water samples (PCR negative controls) included in each 96-well plate. Genotype analysis was performed by technicians in a blind fashion.

Meta-analysis of variants

The association p-values of untyped SNPs were imputed directly by the tool FAPI (http://grass.cgs.hku.hk/lmix/fapi/)(12) with default settings. The p-values of the two GWASs were then combined by Stouffer’s Z-score method for meta-analysis on FAPI as well:

$$Z_{meta} = \frac{\sum_{i=1}^{N}(w_i*z_i)}{\sqrt{\sum_{i=1}^{N}w^2}} \quad \text{where } w_i = \sqrt{n_i}$$

in which $N$ is the number of GWASs, $z_i$ is the individual z-score of the $i_{th}$ GWAS study, and $n_i$ is the sample size of the $i_{th}$ study.

Gene-based and gene-set-based analysis

The knowledge-based secondary analysis platform KGG (http://grass.cgs.hku.hk/lmix/kgg/) was used to map the SNPs onto reference genes (UCSC RefGene hg19), and to perform gene-based and gene-set-based association analysis with default settings. The phased
genotypes of Eastern Asian samples in the 1000 Genomes Project were used to account for linkage disequilibrium of SNPs through KGG. The Benjamini-Hochberg approach was used to control false discovery rate (FDR) of genome-wide genes at a level, which is a more powerful multiple testing approach than Bonferroni correction when there are multiple susceptibility genes.

**Variants functional annotation**

The genomic annotation tools, HaploReg v4.1 (http://www.broadinstitute.org/mammals/haploreg/haploreg.php)(14) and RegulomeDB Version 1.1 (http://regulomedb.org/)(15), were used to annotate SNPs with epigenomic markers and potential regulatory elements, including regions of DNase I hypersensitivity, binding sites for transcription factors (TFs), promoter regions that have been biochemically characterized to regulate transcription, chromatin states as well as DNase foot printing, PWMs, and DNA Methylation. KGGSeq (Version 1.0)(16, 17) was used to annotate selected SNP with four regulatory or functional prediction scores (including CADD.CScore(18), SuRFR(19), FunSeq2(20) and cepip(21)).

**Results**

We first combined the association p-values of variants by meta-analysis from two independent GWASs. Association analyses at genes and multiple knowledge-based gene-sets were carried to prioritize potential HBV-related HCC susceptibility genes. A series of prioritized variants were selected to replicate their genetic associations in a group of independent case-control samples. The overall workflow is shown in Figure 1.

**Genome-wide meta-analysis of two HBV-related HCC GWASs in Chinese populations**

Association p-values were imputed based on the linkage disequilibrium (LD) pattern in the Eastern Asian Panel from the 1000 Genomes Project. A genome-wide meta-analysis was
then performed with SNP p-values from two existing Chinese HCC GWASs using the tool FAPI.(12) After quality control (QC), 5,375,073 meta-analysis p-values of SNPs were obtained. The Manhattan plot and QQ plots of p-values are shown in Supplementary Figure 1 and Supplementary Figure 2, respectively. At the upper tail of the QQ plot, there is a deviation from the 95% confidence level of the non-hypothesis line, suggesting the existence of association signals at some SNPs.

**Gene-based association analysis**

We then used the meta-analysis p-values for gene-based association analysis by GATES(22) on a tool called KGG (version 3.5).(23) In addition to SNPs within the untranslated regions, introns and exons, the meta-analysis p-values of SNPs within 5kb upstream and downstream of a gene were also included in the gene-based association test. SNPs in overlapping regions of multiple genes were assigned to all involved genes. The QQ plots of gene-based p-values are shown in Figure 2.

According to the gene-based p-values, three genes, *RNY4, GOLGA8M* and *LINC01207* passed the multiple testing correction by FDR, 0.05 (Table 1). Interestingly, the *RNY4* and *LINC01207* are non-coding RNA genes, which have not been previously well studied. In addition, two genes, *WHAMMP2* and *SLC39A8*, have nearly significant p-values on the genome, corrected $p = 0.054$ (Table 1). We further annotated the two RNA genes (*RNY4* and *LINC01207*) and the pseudogene *WHAMMP2* with known regulatory elements and epigenomic markers by the UCSC genome browser (http://genome.ucsc.edu). While the *RNY4* has no known regulatory factors (See Supplementary Figure 3), the *LINC01207* and *WHAMMP2* genes have many regulatory factors and epigenomic markers (See Supplementary Figure 4 and Supplementary Figure 5). These annotations imply that the latter two genes are functionally active despite not encoding proteins.

**Prioritization of genes in different gene-sets**
To select more promising genes for replication in independent samples, we resorted to a series of gene-set resources to prioritize genes with suggestive association p-values. We first examined the association with HCC in 1,057 canonical pathways curated in the Molecular Signatures Database (MSigDB V 4.0), after removing the pathways containing too few (<5) or too many (>300) genes. According to the gene-set-based association p-value by the Wilcoxon test on KGG, one pathway, the systemic lupus erythematosus (SLE) pathway, passed the significance level (nominal $p = 1.63 \times 10^{-5}$, corrected $p = 0.017$). Seven genes have a gene-based $p$-value below 0.05 in the pathway.

Then, we investigated whether the genes highly and specifically expressed in human liver were associated with HCC. In the database, Tissue-specific Gene Expression and Regulation (TiGER, http://bioinfo.wilmer.jhu.edu/tiger/), 309 genes preferentially expressed in liver were retrieved. In the human proteome atlas (http://www.proteinatlas.org/humanproteome), 433 genes showing elevated expression of proteins in liver compared to other tissue types were retrieved as well. To reduce potential false positives, we only used overlapping genes in the two sets. As a result, a total of 189 genes were obtained. The gene $\text{NAT2}$ had the lowest gene-based $p$-value of 0.01, the genes $\text{GSTA1}$ and $\text{GSTA2}$ had the second and third smallest $p$-values (See the genes and $p$-values in Table 2 and Supplementary Table 1).

We also examined the association of recurrent integrated genes by HBV reported in previous studies,(24–27) the genes reported to be genetically associated with HBV-related HCC risk in previous studies, and HCC risk genes defined by COSMIC database (http://cancer.sanger.ac.uk/cosmic). However, none of the genes had a promising association $p$-value with HCC in our samples (see the genes and $p$-values in Supplementary Table 2–4).

**Replication study in independent samples**
We replicated genetic association at genes prioritized by the above gene-based and gene-set-based associations in a group of independent HBV-related HCC case-control samples. In total, 21 SNPs of the prioritized genes were selected according to the stability of their allele sequences in ancestry matched reference panel in the 1000 Genomes Project and/or their predicted functional importance by RegulomeDB (http://regulomedb.org/) with regulatory elements. After the genotype quality assessment, two SNPs were excluded because they failed to pass the Hardy-Weinberg equilibrium test ($p<0.001$).

Three genetic models (additive, dominant and recessive) were considered under a logistic regression framework in which the HCC status was adjusted for sex and age. Generally, the independent sample failed to replicate a significant association in the discovery sample after correcting multiple testing. Only two SNPs, rs389883 and rs17343667, had an association $p$-value below 0.05. The rs389883, which is in intron region of STK19, had $p$-values of 0.026 and 0.032 for HCC association under additive and recessive models, respectively, with a protective effect at the minor allele G. However, in the original Qidong GWAS sample and Hong Kong GWAS sample, G was estimated to have a risk effect. The other SNP, rs17343667, which is located in the first intron of EIF2AK1, had an association $p$-value equal to 0.02 under the additive model with an odds ratio of 1.27 for the minor allele, which was found to have a risk effect in both original Qidong and Hong Kong GWAS samples (Table 3). In addition, the regulator potential of rs17343667 was supported by expression quantitative trait locus (eQTL) and TF binding/ DNase peak (scored 1f) in RegulomeDB (See details in Supplementary Figure 6).

Discussion

This study utilized knowledge-based approaches to mine new susceptibility loci of HBV-related HCC in existing HBV-related HCC GWAS data sets. The gene-based association analysis suggested five statistically significant genes including *RNY4*, *GOLGA8M*,

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LINC01207, WHAMMP2 and SLC39A8. The gene-set-based association analysis implied that
genes in the SLE pathway may be relevant to the development of HCC. In addition, three
genes, NAT2, GSTA1 and GSTA2, were also highlighted when genes were stratified in some
functional sets. Furthermore, our analysis also suggested that the germline susceptibility
loci of HBV-related HCC are unlikely to be enriched in recurrent targeted genes of HBV
infection, or HCC risk genes with many somatic mutations. However, probably due to small
sizes in our replication samples, no associations prioritize by the knowledge-based
association analysis are successfully replicated in an independent sample. The
rs17343667 of EIF2AK1 is the only one with suggestive significance.

Our study is the first to indicate that these five genes (RNY4, GOLGA8M, LINC01207,
WHAMMP2 and SLC39A8, which were discovered by gene-based association analysis) are
relevant to the development of HBV-related HCC. For RNY4, GOLGA8M and WHAMMP2,
there are no publications, to our knowledge, about their roles in risk of HCC or other
cancers until this study. LINC01207 has been implicated as a biomarker for survival of
colorectal adenocarcinoma(28) and promoting proliferation of lung adenocarcinoma.(29)
SLC39A8 has been reported to regulate IFN-γ level in T cells(30) and influence trace
element homeostasis in liver,(31, 32) which may be relevant to the development of HCC.
Functional studies are warranted to explore the mechanisms of the potential roles of these
genes in risk of HBV-related HCC.

Interestingly, our finding that the SLE pathway-related genes may be relevant to the
development of HBV-related HCC is supported by a recent meta-analysis involving 59,662
SLE patients, which suggested that SLE had a relative risk of 3.21 (95% CI, 1.70–6.05) for
liver cancer.(33) In addition, studies have found that a number of risk genes are shared by
SLE and HBV-related HCC, such as STAT4 and genes in the HLA region.(7, 34) Our results
may further explain the comorbidity of the two diseases from a genetics aspect.
The three genes, \textit{NAT2}, \textit{GSTA1} and \textit{GSTA2}, that are highly expressed in liver have been previously suggested to be relevant to HCC risk. Both Gelatti et al.\textsuperscript{(35)} and Yu et al.\textsuperscript{(36)} observed a significant association between \textit{NAT2} genetic polymorphisms and HCC susceptibility among chronic HBV carriers who were smokers. Huang et al.,\textsuperscript{(37)} found that the \textit{NAT2} gene polymorphisms may confer different susceptibilities to the effect of red meat intake on HCC. \textit{GSTA1} polymorphism was suggested to be associated with an increased risk of occurrence of HCC, and decreased expression of \textit{GSTA1} was considered as a marker of advanced and highly aggressive HCC.\textsuperscript{(38)} \textit{GSTA1} polymorphism was also reported to correlate with both \textit{GSTA1} and \textit{GSTA2} expression in the liver, which is expected to be of significance for individual risk of cancer or individual response to chemotherapeutic agents.\textsuperscript{(39)}

The negative findings in all curated gene sets were unexpected. Particularly, three gene sets (recurrent targeted genes of HBV infection, HCC risk genes with many somatic mutations and genes highly and specifically expressed in human liver) appeared to be very biologically relevant to the development of HCC. In the analyses, there were no trends that genes with smaller HCC association \textit{p}-values were enriched in the gene sets. These results suggest that the biological context or connection of underlying susceptibility genes is elusive, and that it is difficult to use our current knowledge to identify the unknown susceptibility genes of HCC. Using larger sample sizes for hypothesis-free GWASs is likely the only reliable way for identification of HCC risk genes at present.

The SNP rs17343667 in the \textit{EIF2AK1} is a promising candidate susceptibility variant although it only has a suggestively significant \textit{p}-value in the small replication samples. In RegulomeDB, this SNP is a \textit{cis} eQTL of lymphoblastoid and is located within the DNase peak and histone modifications of multiple tissues and cell types. In the HaploReg (v4.1) database, this SNP is located within multiple regulatory elements, such as histone marks,
DNAse and transcription Motifs. *EIF2AK1* encodes a kinase protein for translation initiation to downregulate protein synthesis in response to stress. Previous studies suggested that *EIF2AK1* mRNA and protein were overexpressed and the kinase activity was enhanced in HCC.\(^{40, 41}\)

**Conclusion**

We performed the first systematic gene- and gene-set-based association study of HCC. Our study suggested several promising genes significantly associated with HCC risk, which may shed insights into pathogenic mechanisms of this fatal disorder. However, the negative associations in multiple curated gene sets also imply that it is difficult to infer gene associations using our current biological knowledge. More hypothesis-free genetic studies with larger sample sizes are needed to elucidate the susceptibility genes and mechanisms of HCC.

**Abbreviations**

eQTL, expression quantitative trait locus; FDR, false discovery rate; GWAS, genome-wide associated studies; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; LD, linkage disequilibrium; QC, quality control; SLE, systemic lupus erythematosus; SNP, single nucleotide polymorphism; TF, transcription factor.

**Declarations**

**Ethics approval and consent to participate**

The study was performed in accordance with guidelines approved by the local ethical committees from all participating centers (The Ethics Committee of Qidong Liver Cancer Institute; the Ethics Committee of the Second Military Medical University; and the Institutional Review Board of Queen Mary Hospital, University of Hong Kong) involved in both the GWAS stage and the replication stage. An informed consent to participate in the
study was obtained from each subject in accordance with the declaration of Helsinki principles. All study participants approved the storage of their frozen DNA specimens, for research purposes, in our laboratory.

Consent for publication

Not applicable.

Availability of data and materials

Please contact author for data requests.

Competing interests

The authors declare that they have no conflict of interest.

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Author Contributions

D. K. J.: study concept and design, material support, obtained funding, analysis and interpretation of the data, and drafting of the manuscript; J. D.: analysis and
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Tables
Table 1. The top 5 genes according to gene-based $p$-values

| Gene   | Locus          | Type                      | Nominal $p$     | Corrected $p^a$ |
|--------|----------------|---------------------------|-----------------|-----------------|
| RNY4   | 7q36           | non-coding RNA            | $1.20 \times 10^{-6}$ | 0.030     |
| GOLGA8M| 15q13.1        | protein-coding gene       | $3.00 \times 10^{-6}$ | 0.037     |
| LINC01207| 4q32         | non-coding RNA            | $5.73 \times 10^{-6}$ | 0.047     |
| WHAMMP2| 15q13.1        | pseudogene                | $8.90 \times 10^{-6}$ | 0.054     |
| SLC39A8| 4q24           | protein-coding gene       | $1.08 \times 10^{-5}$ | 0.054     |

$^a$ The $p$-values are corrected by the Benjamini-Hochberg FDR approach.

Table 2. Genetic association $p$-values of genes preferentially expressed in liver

| Gene Symbol | $p$  | CHR | Start Position | Length (BP) | Number of SNPs |
|-------------|------|-----|----------------|-------------|----------------|
| NAT2        | 0.010| 8   | 18248754       | 9970        | 69             |
| GSTA1       | 0.011| 6   | 52656177       | 12589       | 50             |
| GSTA2       | 0.013| 6   | 52614884       | 13478       | 36             |
| UGT2B10     | 0.017| 4   | 69870294       | -172558     | 116            |
| UROC1       | 0.027| 3   | 126200007      | 36610       | 89             |
| AQP9        | 0.032| 15  | 58430394       | 47714       | 182            |
| HAO1        | 0.033| 20  | 7863630        | 57464       | 117            |
| TF          | 0.036| 3   | 133464976      | 32875       | 93             |
| SAA2        | 0.037| 11  | 18266774       | 3448        | 51             |
| C3          | 0.041| 19  | 6677845        | 42849       | 138            |

Note. CHR: chromosome; BP: base pairs.

$^a$ Only the genes with a $p$-value less than 0.05 are listed in this table. The whole gene list is shown in Supplementary Table 1.

Table 3. Summary of genetic association results in the replication
| Batch ID | CHR | SNP     | BP            | CADD.CScore | SuRFR | FunSeq2 | HCCCell_Prob | RegulomeDB |
|----------|-----|---------|---------------|-------------|-------|---------|--------------|------------|
| 1        | 1   | rs3813948 | 207269858     | -0.039      | 14.356 | 0.7635  | 0.796        | 5          |
| 1        | 2   | rs60325402 | 16077873      | 0.144       | 17.3   | 0.1852  | 0.370        | 5          |
| 1        | 3   | rs7612684  | 178984575     | -0.163      | 19.334 | 0.8109  | 0.370        | 4          |
| 1        | 3   | rs76863563 | 178987536     | -0.498      | 15.493 | 0.1881  | 0.370        | 5          |
| 1        | 5   | rs116966235 | 57794613    | -0.636      |        | 0.1852  | 0.370        | 3a         |
| 1        | 5   | rs12514619 | 1783655      | 1.741       | 7.556  | 2.705   | 0.370        | 2b         |
| 1        | 6   | rs389883   | 31947460      | 0.142       | 14.213 | 1.623   | 0.370        | 1f         |
| 1        | 6   | rs615672   | 32574171      | -0.162      | 4.627  | 0.7972  | 0.370        | 6          |
| 1        | 7   | rs17343667 | 6065194       | 0.392       | 15.543 | 0.8898  | 0.370        | 1f         |
| 1        | 7   | rs55744175 | 18332396      | 2.275       | 17.195 | 0.6909  | 0.370        | 5          |
| 1        | 8   | rs16898013 | 124138891     | 0.780       | 17.314 | 0      | 0.370        | 3a         |
| 1        | 8   | rs2275959  | 37455059      | 0.245       | 6.377  | 0.3114  | 0.863        | 4          |
| 1        | 8   | rs2736020  | 15714529      | -0.002      | 3.977  | 9.418E-161 | 0.370        | 7          |
| 1        | 10  | rs3001719  | 10409365      | -0.113      | 3.277  | 0.1852  | 0.370        | 5          |
| 1        | 11  | rs10897243 | 62043174      | -0.497      | 15.511 | 4.535E-33 | 0.370        | 6          |
| 1        | 12  | rs79475045 | 39083557      | -0.264      | 15.822 | 0.1881  | 0.370        | 5          |
| 1        | 12  | rs979722   | 118217304     | 0.014       | 15.899 | 0.4365  | 0.370        | 7          |
| 1        | 16  | rs12918376 | 56558181      | -0.025      | 12.043 | 4.562E-74 | 0.370        | 6          |
| 1        | 20  | rs2425046  | 33871661      | 0.090       | 17.787 | 1.78    | 0.918        | 2b         |

Note. CHR: chromosome; BP: base pairs; OR: odd ratio; CI: confidence interval; A1: minor allele; A2: major allele; CADD.CScore, SuRFR and FunSeq2 scores are annotated by KGGSeq (V1.0). HCCCell_Prob: Probability of cell type-specific regulation in GENCODE liver cancer cells (HepG2).

a This model was tested under Logistic regression model with adjustment for age and sex.

b The value is not available.

Figures
Knowledge-based prioritization framework of SNPs’ statistical p-values for association with HCC
Figure 2

Quantile-quantile plot of gene-based p-values and SNP-based p-values

Supplementary Files

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