Identifying and exploiting gene-pathway interactions from RNA-seq data for binary phenotype

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Abstract

Background: RNA sequencing (RNA-seq) technology has identified multiple differentially expressed (DE) genes associated to complex disease, however, these genes only explain a modest part of variance. Omnigenic model assumes that disease may be driven by genes with indirect relevance to disease and be propagated by functional pathways. Here, we focus on identifying the interactions between the external genes and functional pathways, referring to gene-pathway interactions (GPIs). Specifically, relying on the relationship between the garrote kernel machine (GKM) and variance component test and permutations for the empirical distributions of score statistics, we propose an efficient analysis procedure as Permutation based gEne-pAthway interaction identification in binary phenotype (PEA).

Results: Various simulations show that PEA has well-calibrated type I error rates and higher power than the traditional likelihood ratio test (LRT). In addition, we perform the gene set enrichment algorithms and PEA to identifying the GPIs from a pan-cancer data (GES68086). These GPIs and genes possibly further illustrate the potential etiology of cancers, most of which are identified and some external genes and significant pathways are consistent with previous studies.

Conclusions: PEA is an efficient tool for identifying the GPIs from RNA-seq data. It can be further extended to identify the interactions between one variable and one functional set of other omics data for binary phenotypes.

Keywords: Gene-pathway interaction, Garrote kernel machine, Variance component testing, Binary phenotype

Background

RNA sequencing (RNA-seq) technology has identified amounts of significant genes and given some evidence for the diagnosis and treatment of complex disease, especially cancers [1, 2]. Most of existing statistical methods focus on identifying the differentially expressed (DE) genes and heritability estimation by the RNA-seq count data [3–7]. However, with the assumption that only minority of genes associate with phenotypes, these models inevitably lose the regulation information from DE genes, thus are hard to elucidate the etiology and mechanism [8–10]. Systematic characterization of the biological function of genes represents an important step for investigating the molecular mechanisms underlying the identified disease associations. Enrichment analysis methods are based on different ideas: some only including genes participating in pathways and some considering the regulations between genes in networks [11–14]. Furthermore, omnigenic model assumes that disease may be driven by genes with indirect relevance to disease and be propagated by functional pathways. These external genes may cause the disease by distantly regulating significant pathways and they may explain most heritability [15]. For transcriptome data, one common
sense is that the core gene effects can be understood by their interactions within underlying pathways or any expressed gene [16, 17]. As a result, identifying the interactions between external genes and significant pathways (GPIs) holds for further understanding of etiology and improving the prediction ability [18].

Considering the potential importance of interactions in defining the genetic architecture of complex traits and inefficiency of traditional methods in high-dimensional data, emerging statistical methods have been implemented to identify interactions with low calculation resources [19]. Different algorithms use different ideas, such as set tests to identify interactions with low calculation resources [19].

Data, emerging statistical methods have been implemented to improve the prediction ability [18]. (GPIs) holds for further understanding of etiology and

We apply our method to analyze platelet RNA-seq data. In addition, the parameter estimation is more accurate. Under curve (AUCs) with controllable type I error rates. (LRT) for generalized linear models, PEA has higher areas compared to the traditional likelihood ratio test. Extensive simulations vvide a method overview of PEA, including the parameter estimation and hypothesis testing. Extensive simulations show that compared to the traditional likelihood ratio test (LRT) for generalized linear models, PEA has higher areas under curve (AUCs) with controllable type I error rates. In addition, the parameter estimation is more accurate. We apply our method to analyze platelet RNA-seq data from a case-control study (GSE68086) [1]. PEA can also be applied to analyze other interactions in binary phenotypes, such as pathway-environment interactions. PEA is implemented as a Rcpp function, freely available at https://github.com/biostat0903/RNAseq-Data-Analysis.

Methods

Model

We model GPIs in binary phenotypes as following:

\[
\text{logit}(P(y = 1)) = \mathbf{x}\mathbf{\beta}_C + \sum_{j=1}^{p} \mathbf{p}_j \mathbf{\beta}_j + \mathbf{G}\mathbf{\beta}_G + \sum_{j=1}^{p} (\mathbf{P}_j \mathbf{G}) \mathbf{\xi}_{Gj}
\]

where \(y\) indicates the binary phenotypes \(i = 1, ..., N\), \(\mathbf{x}\), an \(N \times m\) matrix, is supposed as the covariates, \(\mathbf{p}\), an \(N \times P\) matrix, is assumed as the expression levels in a significant pathway, which can be calculated from some gene enrichment analyses, \(\mathbf{P}_j \mathbf{G}\) indicate the GPIs (Fig. 1). We also suppose \(y = P(y = 1)\). \(\mathbf{\beta}_C\), \(\mathbf{\beta}_j\), \(\mathbf{\beta}_G\), and \(\mathbf{\xi}_G\) are the coefficients of the covariates, functional pathway genes, external gene and GPIs, respectively.

Garrote kernel machine (GKM)

A kernel function is suitable to suggest the complicated relationship, including both linear and nonlinear relations, between genes and phenotypes. Here, we extend the linear GKM for the binary phenotype to identify the GPIs, although many other kernel functions can be selected. The kernel function is shown as following:

\[
K(Z_{k}, Z_{l}) = (1 + \delta G_{k} G_{l})/(1 + \mathbf{P}_{k} \mathbf{P}_{l})
\]

where \(Z_{k} = (G_{k}, P_{k})\), \(K(Z_{k}, Z_{l})\) is the kernel matrix of \(k\)th and \(l\)th individuals. We then test for the effect of the GPIs by considering the null hypothesis \(H_0: \delta = 0\).

Parameter estimation

With the kernel function, the Eq. (1) can be rewritten as a semi-parametric model as follows:

\[
\text{logit}(P(y = 1)) = \mathbf{x}\mathbf{\beta}_C + \mathbf{h}
\]

where \(\mathbf{h} = (h_1, h_2, ..., h_N)^T\) is an unknown centered smooth function vector. \(\mathbf{h}\) can be parameterized for different forms of GPIs, such as the Gaussian kernel and \(d\) th ploynomial kernel. As the similarity between the semi-parametric model and mixed effect model, the \(\mathbf{h}\) can be assumed as the random effects following a multivariate normal distribution \(\mathcal{N}(0, \mathbf{r}K(\delta))\). The relationship between the unknown function and the kernel function is as follows:
where \( Q = \frac{V^{-1} - V^{-1}X(X^TV^{-1}X)^{-1}X^TV^{-1}}{tr(QKQK)}/2 \) is the damping factor. 

\[ L_{REML} = -\frac{1}{2} \left( \log |V| + \log |X^TVX| + (\tilde{y} - X\beta_C)^T V^{-1}(\tilde{y} - X\beta_C) \right) \]

where \( \tilde{y} = X\beta_C + \kappa^T \alpha + D^{-1}(\gamma - \gamma) \) and \( V = D^{-1} + \rho K \). \( \tau \) is estimated by the Newton-Raphson algorithm with damping factor \( \omega = 0.5 \). The iteration formula is as following:

\[ r^{(t+1)} = \frac{Q}{r^{(t)}} \]

where \( Q = V^{-1} - V^{-1}X(X^TV^{-1}X)^{-1}X^TV^{-1} \) and \( t \) is the iteration time.

\( \alpha \) and \( \beta_C \) is estimated by the Newton-Raphson algorithm with normal equations as following:

\[ \begin{bmatrix} X^TD^{(t)}X & X^TD^{(t)}K \ 
D^{(t)}X & \tau^{-1}I + D^{(t)}K \end{bmatrix} \begin{bmatrix} \alpha^{(t+1)} \\
\beta_C^{(t+1)} \end{bmatrix} = \begin{bmatrix} X^TD^{(t)}\hat{y}^{(t)} \\
D^{(t)}\hat{y}^{(t)} \end{bmatrix} \]

Hypothesis testing

As the similarity between the mixed model and semi-parametric model, we propose a score test statistic \( U \) to test \( \delta = 0 \):

\[ U = -\frac{1}{2} - \frac{V}{(\hat{y} - X\beta_C)}^T V^{-1}(\hat{y} - X\beta_C) \]

As the \( \frac{\partial V}{\partial \delta} \) is not a semi-definite matrix, \( U \) is not identically greater than zero. Its distribution is hard to use the mixed chi-squared distribution to approximate. Therefore, we propose a permutation test to obtain the empirical distribution of \( U \). Since the null hypothesis is \( \delta = 0 \), we permute the expression level of the external gene and calculate the test statistic without re-estimation of the \( \beta_C \), \( \alpha \) and \( \tau \). The computation consumption is not large, although we use a permutation test.

Simulation

Compared to the traditional LRT, simulations investigate the statistical property of \( U \) and the estimation of \( \beta_C \). Type I error rates and AUCs show the statistical property of statistics. Means and standard errors indicate the estimation property. The expressed levels of the functional pathway are generated from correlated uniform distributions.
by a Copula function, and that of the external gene is generated from a uniform distribution \((U(0, 1))\). We study five parameters in simulations: sample size \((N)\), number of genes in the functional pathway \((\nu)\), correlation between genes in the pathway \((c)\), proportion of interaction genes \((p)\) and odds ratio \((O)\) of the external gene \((s)\). Details are shown in the Table 1. The interaction function \(h\) for the \(i\) th individual is defined by a function \(g\) as following:

\[
g(Z_i) = g(Z_{i1}, Z_{i2}, ..., Z_{i4}) = 1 + \sum_{j=1}^{P} \beta_{0j} + \beta_G Z_i + \sum_{j=1}^{P} (\beta_{0j} + \beta_{Gj}) Z_{ij}
\]

\(h\) can be defined as linear and non-linear settings: \(h(Z_i) = g(Z_i)\) and \(h(Z_i) = g(Z_i)^2\).

We simulate 1000 times at the null hypothesis \((s = 0)\) and 100 times at the alternative hypothesis \((s \neq 0)\). Combining the 900 \(P\) values randomly selected from the null and 100 \(P\) values at the alternative, we can calculate the AUC. For each loop, we permute the label 50,000 times to obtain robust results.

**Real data analysis**

We use our method to analyze RNA-seq data of 283 platelet samples (55 healthy donors and 228 tumor samples) [1]. The tumor data is collected from six different cancers, including breast cancer (BrCa, \(n = 35\)), non-small cell lung carcinoma (NSCLC, \(n = 60\)), glioblastoma (GBM, \(n = 39\)), colorectal cancer (CRC, \(n = 41\)), pancreatic cancer (PAAD, \(n = 35\)) and hepatobiliary cancer (HBC, \(n = 14\)). The covariates are gender and age. We delete the individuals with missing data, and the final sample size is 274. The tumor samples are regarded as cases to perform pan-cancer analysis [1].

We only select 5003 genes for subsequent analysis. Second, we use the trimmed mean of M-value (TMM) normalization data for following analysis. Final, edgeR with tagwise and common dispersion is applied to select the external genes with the threshold of false discovery rate (FDR) < 0.001. We can obtain 2500 DE genes, including 1231 de-regulated and 1269 up-regulated genes.

After the filtering and normalizing, we perform three gene enrichment methods to obtain the functional pathways, including DAVID, GSEA and MinePath. DAVID and GSEA are performed by both Gene Ontology (GO) dataset and Kyoto Encyclopedia of Genes and Genomes (KEGG) dataset [34, 35]. MinePath is only based on KEGG dataset. The significant pathways are defined with FDR < 0.001 of DAVID, FDR < 0.25 of GSEA and FDR < 0.05 of MinePath. bioMaRt package is used to map Ensembl ID to corresponding Entrez ID and gene symbol [36]. For the PEA model, we normalize RNA-Seq data to the numbers between 0 and 1.

**Results**

**Simulations**

Simulations evaluate the performances of PEA and traditional LRT by type I error rates and AUCs. In the type I error simulations, we use four different settings: different interaction function settings, \(N, \nu\) and \(c\). In the power simulations, we add \(s\) and \(p\). For the type I error simulations, the response variable is not affected by the effects of the external gene and its interactions. All the results are shown in Figs. 2, 3, 4 and 5 and Additional files 1 and 2. The significant level is set to be 0.05.

As expected, PEA controls type I error rates in all parameter settings, but the traditional LRT fails. Especially, in the non-linear setting, the type I error rates of PEA are close to the significant level, which shows this method properly controls the type I error rates. When \(c = 30\), the traditional LRT is inefficient because of the unbalance between the number of variables and the sample size setting.

As the traditional method is uncontrollable for type I error rates, AUC is used to evaluate the performances of the two methods in the alternative simulations. When \(c = 10\), it is clear that PEA is better than LRT in each setting, especially for the non-linear scenarios. When \(c = 20\) and \(c = 30\), the traditional LRT is better than PEA with the non-linear settings and \(N = 100\). Increasing \(s\) and \(N\) improves power of PEA, but increasing \(p\) and \(c\) decreases power of PEA. The linear settings possibly lead to higher power. The minimum sample size is suggested as 100 by the different \(N\) settings.

**Real data analysis**

After the enrichment analyses of DAVID and GSEA, we obtain 67 functional pathways (Additional file 3). We

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**Table 1** Parameter settings used for the simulation data

| Parameters                     | Label | Settings          |
|-------------------------------|-------|-------------------|
| sample size                   | \(N\)  | 100, 200          |
| number of genes in potential pathway | \(\nu\)  | 10, 20, 30        |
| correlation between genes in pathway | \(c\)  | 0.25, 0.5         |
| proportion of interaction genes | \(p\)  | 0.8, 1.0          |
| OR of core gene               | \(s\)  | 0, 1.5, 1.8, 2.0  |
| OR of interactions            |       | \(\exp(s)/2\)     |
| OR of genes in potential pathway |       | 1.2               |
identify the GPIs between 2500 DE genes and four biological pathways: RNA processing (GO:0006396, size = 285), RNA splicing (GO:0008380, size = 127), cytosolic ribosome (GO:0022626, size = 80) and cytoplasmic translation (GO:0002181, size = 25). We identified 116, 109, 89 and 124 significant external genes for the different pathways at the nominal level 0.05, respectively. After filtering by the FDR < 0.2, the significant gene numbers are 10, 17, 54 and 83.

After the enrichment analyses of DAVID, GSEA and MinePath, we obtain 2 functional pathways (Additional file 4). We identify the GPIs between 2500 DE genes and two biological pathways: Platelet activation (hsa04611, size = 70) and Fc gamma R-mediated phagocytosis (hsa04666, size = 49). We identified 468 and 475 significant external genes for the two pathways at nominal level 0.05, respectively. After filtering by the FDR < 0.2, the significant gene numbers are 119 and 120.

As the different sizes of the four pathways, the power is different for the four pathways. The result of FDR controlling is similar to that of the simulations. Interestingly, we define multiple same genes for the four GO pathways, such as TUBB, BPI and CA1. Two KEGG pathways also interact with 11 common genes, such as SDCBP, TRAT1 and FABP4. The summary of the result is shown in Table 2.

**Discussion**

Here, we present an effective semi-parametric generalized linear model, together with a computationally efficient parameter estimation method and software implementation of PEA, for identifying potential GPIs of RNA-seq data in binary phenotypes. PEA models the complicated relationship between gene expression and traits using the kernel function. Because the kernel machine can be adaptive for both linear and nonlinear interactions, PEA controls type I error rates in the presence of individual relatedness, and PEA achieves higher power than the traditional method, LRT, across a range of settings. In addition, PEA is available to other interactions of different molecules, such as methylation and gene expression interactions, and biological or technical covariates interactions. We have demonstrated the benefits of PEA using both simulations and applications to recently published RNA-seq datasets.

While PEA is an extension of the GKM, we note that PEA exploits the GPIs in binary phenotypes and estimates the parameters using the damping Newton-Raphson algorithm. As most of medical studies are case-control design, PEA identifies the GPIs for the binary traits. For example, samples are collected from tumor tissue and normal tissue, along with some covariates, such as age, gender and so on. Two Newton-Raphson iterations accurately estimate the coefficients of covariates (Fig. 5). GKM
Fig. 3 AUCs of PEA and traditional LRT in different interaction function settings, \( N, c, s, p \) and \( v = 10 \). a linear interaction function settings with \( p = 0.8 \) (left) and \( p = 1 \) (right); b nonlinear interaction function settings with \( p = 0.8 \) (left) and \( p = 1 \) (right).

Fig. 4 AUCs of PEA and traditional LRT in different sample size settings of different interaction function settings with \( c = 0.5, s = 2, p = 1 \) and \( v = 10 \).
estimates $\tau$ by the *optimx* function in R using the Nelder-Mead method, which is not suitable for PEA.

In the real data analysis, the result of PEA can support the assumption of omnigenic model. Although PEA goes beyond the scope of enrichment analysis, efficient enrichment analysis methods, such as MinePath, can essentially provide the robust and reliable pathways before conducting PEA. PEA identify not only some significant GPs, but also the external genes for different pathways. Some significant genes are verified by multiple biological studies. For example, using the data from The Cancer Genome Atlas (TCGA), $TUBB$ expression level influences the survival time of renal and liver cancer [37]. Kelly et al. demonstrate that the mutation of $TUBB$ possibly cause the tumor cell growth and taxane resistance for the patients with NSCLC [38]. From the result of PEA, $TUBB$ might be an important external gene, which associates cancers by the interactions with amount significant pathways.

Currently, despite the newly developed computationally efficient statistical testing method, applications of PEA can still be limited by its relatively heavy computational cost of permutations. Traditional permutation tests increase the computation consumption with the shuffling times, but the permutations of PEA are faster than the standard permutation test because of no parameter re-estimation in each shuffling. PEA can still take close to 7 h with 5 CPUs to analyze a dataset of the size of the GSE68086 which we considered here (274 individuals ~ 2500 genes for one pathway). Therefore, PEA will be used to analyze other datasets that have large sizes.

Table 2 Summary of the external genes for the selected pathways

| Database | Pathway                        | Size | Top genes          |
|----------|--------------------------------|------|--------------------|
| GO       | RNA processing                 | 285  | $TUBB$, $H3F3B$, SDCBP, NTSC2, FKBP5 |
| GO       | RNA splicing                   | 127  | $TUBB$, IL1R2, H3F3B, IFNGR1, PCSK7 |
| GO       | cytosolic ribosome             | 80   | $TUBB$, CAMP, SAMM50, PSMD14, CHD8 |
| GO       | Cytoplasmic translation        | 25   | $TUBB$, SDCBP, H3F3B, CMAHP, SLC44A2 |
| KEGG     | Platelet activation            | 70   | STX7, PCNP, SDCBP, LEF1, TMEM140 |
| KEGG     | Fc gamma R-mediated phagocytosis | 49    | STX7, PCNP, CGRFT1, SHOC2, EFTU2d |

*Top five external genes for the significant pathway*
Conclusions
PEA is an efficient and powerful statistical method for identifying the GPIs from RNA-seq data. It can be further extended to identify the interactions between one variable and one functional set of other omics data for binary phenotypes. Further work is needed to make its widely use in more high-dimensional genomics data analysis practice.

Additional files

Additional file 1: AUCs of PEA and traditional LRT in different interaction function settings, N, c, s, p and v = 20. (A) linear interaction function settings with p = 0.8 (left) and p = 1 (right); (B) nonlinear interaction function settings with p = 0.8 (left) and p = 1 (right). (PDF 27 kb)

Additional file 2: AUCs of PEA and traditional LRT in different interaction function settings, N, c, s, p and v = 30. (A) linear interaction function settings with p = 0.8 (left) and p = 1 (right); (B) nonlinear interaction function settings with p = 0.8 (left) and p = 1 (right). (PDF 27 kb)

Additional file 3: The enriched GO pathways from both GSEA and DAVID. (XLSX 13 kb)

Additional file 4: The enriched KEGG pathways from three algorithms. (XLSX 9 kb)

Abbreviations
AUC: Area under curve; BrCa: Breast cancer; CRC: Colorectal cancer; DE: Differentially expressed; FDR: False discovery rate; GBM: Glioblastoma; GM: Garrotte kernel machine; GQ: Gene Ontology; GPl: Gene-pathway interaction; GSEA: Gene set enrichment analysis; GWAS: Genome wide association study; HBC: Hepatobiliary cancer; KEGG: Kyoto Encyclopedia of Genes and Genomes; LogCPM: Logarithmic counts per million; LRT: Likelihood ratio test; NSCLC: Non-small cell lung carcinoma; PAAD: Pancreatic cancer; PEA: Permutation based gEne-pAthway interaction identification for binary phenotypes; REML: Restricted maximum likelihood; RNA-seq: RNA sequencing; TCGA: The Cancer Genome Atlas; TMM: Trimmed mean of M-value

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Availability of data and materials
The pan-cancer data is publicly available from https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE68086. The PEA software is available from https://github.com/biostat0903/RNaseq-Data-Analysis.

Authors’ contributions
FS, YW, YZ and SY conceived and designed the experiment, FS and SY built the model and wrote the manuscript, SY implemented the software, YW tested the software and prepared the real data. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

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Competing interests
The authors declare that they have no competing interests.

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