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Research Article

INTEGRATED NETWORK ANALYSIS OF THE POTENTIAL MOLECULAR BIOMARKERS AND KEY PATHWAYS IN CLEAR RENAL CELL CARCINOMA (ccRCC)

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ABSTRACT. Clear cell renal cell carcinoma (ccRCC) is the most prevailing subtype of renal cancer with the highest death rates. The objective of our study is to discover more reliable biomarkers and key pathways mostly related to ccRCC. The publicly reachable GSE168845 datasets were accessed from the Gene Expression Omnibus database. Firstly, we identified differentially expressed genes (DEGs) in ccRCC and control samples by the GEO2R tool. Second, we performed Gene Ontology (GO) and KEGG pathway analysis of determined DEGs by the DAVID database. Then, we established protein-protein interaction (PPI) networks of the identified DEGs using the STRING database and Cytoscape software. Finally, we identified the hub genes by Cytoscape software. We identified 3,935 genes as DEGs. GO function analysis in the biological process, molecular function, and cellular component category showed that DEGs were mostly involved in the inflammatory response, receptor activity and integral component of the plasma membrane, respectively. Based on KEGG pathway analysis results the identified DEGs were mostly associated with Staphylococcus aureus infection, phagosome and natural killer cell-mediated cytotoxicity. We discovered 10 hub genes that have roles in the molecular etiology of ccRCC (*OXGR1, MAPK1, GNG2, LCK, ITGB2, HLA-DRB1, KIF20A, GNG10, GNB4,* and *HLA-DRA*). In conclusion, our study discovered DEGs and associated functional terms pathways. Our results would help to reveal the pathological mechanisms of ccRCC and precise targets for the treatment of ccRCC.

Keywords: *Clear cell renal cell carcinoma, biomarkers, pathway analysis, bioinformatic analysis, gene expression omnibus*

INTRODUCTION

Renal cell carcinoma (RCC) is the most frequently seen kidney cancer type and it includes several histological subtypes [1]. Clear cell renal cell carcinoma (ccRCC) is defined as the most prevalent histological type of renal tumors [2] and ccRCC is responsible for about 80% of all RCC cases [3]. The ccRCC includes clear cytoplasmic cells and a fine vascular network [4]. The clinical characteristics of ccRCC are hematuria, renal mass, pain, and fever and it is more common in adults than children.

Thanks to medical imaging the diagnosis of ccRCC has enhanced in recent years [5], however, there are still some patients with lack of early ccRCC symptoms. Current treatment strategies are limited in surgery, because radiotherapy and chemotherapy are ineffective in many ccRCC cases [6] and [7]. Identifying sensitive, specific and reliable biomarkers to diagnose the ccRCC at an early stage would help the patients to be treated

at the early stages effectively. Several genes have been identified which are associated with ccRCC including *VHL* [8] *AL-1/4.1B* [9], *HIF1A* [10], *FOXM1* [11], and *KISSIR* [12], however, the molecular mechanisms causing the ccRCC still needs more research.

Microarray technology examines gene expression and DNA methylation profiles of several different genes to identify the molecular pathways of several diseases, including various cancer types. Several studies have identified different genes, biomarkers and key pathways for ccRCC by analyzing the gene microarray data. Another study identified *UBE2C*, *BUB1B*, *RRM2* and *TPX2* may be potential prognostic and treatment targets for ccRCC [13]. ABCG1 is another potential biomarker for the diagnostic and therapeutic targets for ccRCC [14]. One recent study also showed that *CXCL12*, *BDKRB2*, *ADYC7*, *KNG1*, and *LPAR5* are effective prognostic and diagnostic biomarkers of ccRCC [15].

Although there are several biomarkers have been discovered recently, it is still important to identify new sensitive and precise biomarkers for therapeutic and diagnostic targets of the ccRCC disease. It is an urgent need to identify more sensitive and specific biomarkers to diagnose the ccRCC to help the diagnosis at an early stage, so this study aims to identify biomarkers analyzing the one of the most recent dataset GSE168845 deposited to NCBI GEO dataset in silico in ccRCC and normal samples.

MATERIALS AND METHODS

Microarray data

GSE168845, publicly available dataset retrieved from National Center for Biotechnology Information Gene Expression Omnibus (NCBI-GEO) database (https://www.ncbi.nlm.nih.gov/geo/) [16]. GSE168845 dataset is preferred because it was one of the newest ccRCC datasets in NCBI-GEO database. This dataset had been obtained using the Platform GPL21185 Agilent Microarray Platform and was deposited by Raimonda Kubiliute. The dataset includes 4 ccRCC and 4 non-cancerous renal tissue samples (Figure 1).

Identification of DEGs

DEGs between ccRCC and noncancerous samples were analyzed by GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/). DEGs were considered statistically significant with p- *value* < 0.05 and log2FC≥ 1 or p value < 0.05 and log2FC ≤1. GEO2R is an interactive dataset analysis tool that compares two classes of samples to find out the DEGs under the same experimental conditions.

GO and KEGG pathway enrichment analyses

The Database for Annotation, Visualization and Integrated Discovery (DAVID 6.8, https://david.ncifcrf.gov/summary.jsp) is an online biological information database to analyze the identified DEGs in terms of biological meaning and pathway relations [17]. All DEGs were characterized based on their biological processes, molecular functions, and cellular components of GO. GO enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were carried out with a *p-value* <0.05 accepted as significant at the statistical level.

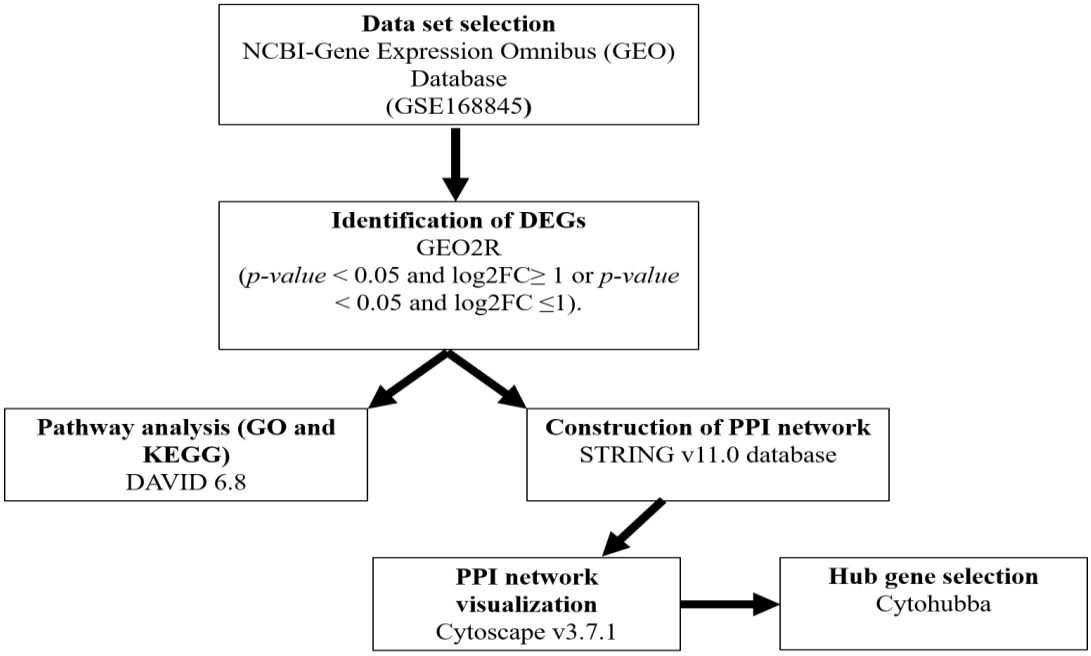

Figure 1. *Bioinformatics analysis steps of the study*

PPI network analyses and hub genes selection and analysis

Identified DEGs were imported into the Search Tool for the Retrieval of Interacting Genes (STRING) v11.0 database (https://string-db.org/) to analyze protein-protein (PPI) interactions. PPI scores are defined based on one or more scores (0 to 1) that shows the confidence level. PPI combined score of > 0.7 (high confidence) was selected to construct the protein interaction network. CytoHubba plugin in Cytoscape software (Cytoscape v3.7.1) was used to identify hub genes. The CytoHubba plugin calculates protein interactions using 12 (Maximal Clique Centrality (MCC), Density of Maximum Neighborhood Component (DMNC), Maximum Neighborhood Component (MNC), Degree, Edge Percolated Component (EPC), BottleNeck (BN), EcCentricity, Closeness, Radiality, Betweenness, Stress and Clustering Coefficient) different scoring methods. These scores are then used to select hub genes. Top 10 hub genes were selected based on the highest degree of value in the PPI network, because "degree" term topological term shows the connection with other genes.

RESULTS AND DISCUSSION

Identification of DEGs

The GSE168845 dataset was downloaded from the GEO database and DEGs were determined between ccRCC and normal renal tissues using the GEO2R tool. 3,935 DEGs were identified including 1,991 upregulated genes and 1,944 downregulated genes from 58,192 probes (*p-value* < 0.05 and log2FC≥ 1 or *p-value* < 0.05 and log2FC ≤1). The volcano plot for all up-regulated, down-regulated DEGs and unchanged genes was shown in Figure 2.

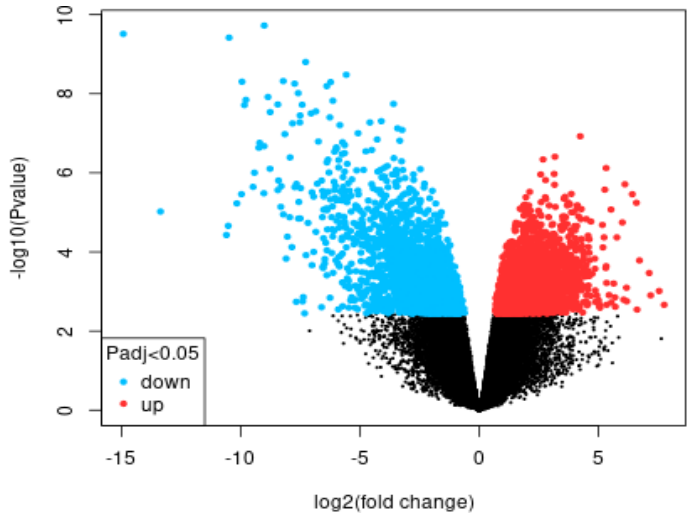

Figure 2. *Volcano plot of gene expression in tissue samples from 4 ccRCC and 4 non-cancerous patients. The blue, red, and black dots represent down-regulated, up-regulated, and unchanged DEGs, respectively. (based on p- value < 0.05 and log2FC≥ 1 or p value < 0.05 and log2FC ≤1. DEGs: differentially expressed genes.*

GO and KEGG pathway enrichment analysis of DEGs

All DEGs were characterized based on their biological processes, molecular functions and cellular components of GO using DAVID, with a *p-value* < 0.05. The top 10 GO terms in the biological process category are listed in Table 1. The most significant GO term in the biological process category is "inflammatory response" with a *p-value* = 6.5E-15. The most important GO term in the molecular function category is "receptor activity" with a *p-value* = 1.8E-6 (Table 2). The most significant GO term in the cellular component category is "integral component of plasma membrane" (*p-value* = 3.0E-15) (Table 3). The most 10 significant KEGG terms are listed in Table 4. The most significant KEGG term is found" Staphylococcus aureus infection" (*p-value* = 3.0E-7).

Table 1. *The 10 most significant GO terms associated with biological process terms*

GO term	Pathway	Count	*p*-value
GO:0006954	Inflammatory response	87	6.5E-15
GO:0007165	Signal transduction	170	3.5E-09
GO:0006955	Immune response	11	2.4E-08
GO:0050900	Leukocyte migration	9	2.1E-07
GO:0045087	Innate immune response	12	2.3E-07
GO:0031295	T cell costimulation	10	1.9E-06
GO:0007588	Excretion	11	3.1E-06
GO:00421104	T cell activation	11	3.1E-06
GO:0007166	Cell surface receptor signaling pathway	7	3.2E-06
GO:0060333	Interferon-gamma mediated signaling pathway	3	3.8E-06

Table 2. *The 10 most significant GO terms associated with molecular function terms*

GO term	Pathway	Count	*p*-value
GO:0004872	Receptor activity	44	1.8E-6
GO:0042803	Protein homodimerization activity	99	1.7E-4
GO:0050700	CARD domain binding	6	3.2E-4
GO:0017124	SH3 domain binding	24	6.3E-4
GO:0003779	Actin binding	44	7.5E-4
GO:0005031	Tumor necrosis factor-activated receptor activity	9	1.1E-3
GO:0005261	Cation channel activity	9	1.5E-3
GO:0032395	MHC class II receptor activity	7	1.6E-3
GO:0032403	Protein complex binding	34	1.6E-3
GO:0005178	Integrin binding	21	1.7E-3

Table 3. *The 10 most significant GO terms associated with cellular component terms*

GO term	Pathway	Count	*p*-value
GO:0005887	Integral component of plasma membrane	200	3.0E-15
GO:0005886	Plasma membrane	507	6.0E-14
GO:0070062	Extracellular exosome	350	6.1E-10
GO:0016323	Basolateral plasma membrane	44	4.3E-9
GO:0009986	Cell surface	91	2.6E-8
GO:0016020	Membrane	275	5.1E-8
GO:0009897	External side of plasma membrane	45	2.7E-7
GO:0045121	Membrane raft	44	2.7E-7
GO:0016324	Apical plasma membrane	53	2.6E-6
GO:0005829	Cytosol	374	8.9E-6

Table 4. *The 10 most significant KEGG terms for ccRCC*

KEGG ID	Term	Count	*p*-value	Genes
hsa05150	Staphylococcus aureus infection	21	3.0E-7	*FCGR3A, FCGR2A, C1QA, C1QB, C1QC, CFD, FPR1, ITGAL, ITGAM, ITGB2, HLA-DMA, HLA-DPA1, HLA-DPB1, HLA-DQA2, HLA-DQB1, HLA-DRA, HLA-DRB1, PLG, PTAFR, SELPLG, SELP*
hsa04650	Natural killer cell mediated cytotoxicity	31	9.2E-6	*BID, CD247, FASLG, FCER1G, FCGR3A, LCK, SH2D1A, SHC1, SHC3, TNFRSF10A, GZMB, HCST, ITGAL, ITGB2, IFNAR2, IFNGR1, IFNGR2, KIR2DS2, KLRD1, KLRK2, LCP2, MAPK1, NFATC2, PRKCB, PTPN6, RAC2, RAET1E, VAV1, VAV2, VAV3, ZAP70*

Table 4. *(continued)*

KEGG ID	Term	Count	*p*-value	Genes
hsa04145	Phagosome	35	1.6E-5	*ATP6V0A4, ATP6V0B, ATP6V1B1, ATP6V1C2, ATP6V1G3, ATP6V1H, CLEC7A, FCGR3A, FCGR2A, CANX, CORO1A, ITGAM, ITGB2, MSR1, HLA-E, HLA-DMA, HLA-DPA1, HLA-DPB1, HLA-DRA, HLA-DRB1, MPO, NCF1, NCF2, NOS1, PLA2R, SCARB1, STX18, TLR2, TLR6, TAP1, TUBA1B, TUBAL3, TUBB6*
hsa05152	Tuberculosis	39	1.9E-5	*ATP6V0A4, ATP6V0B, ATP6V1H, BID, CLEC7A, CEBPB, CD74, FCER1G, FCGR3A, FCGR2A, TRADD, CASP8, CARD9, CIITA, CORO1A, CYCS, ITGAM, ITGAX, ITGB2, IFNGR1, IFNGR2, IL10RA, LSP1, HLA-DMA, HLA-DPA1, HLA-DPB1, HLA-DQA2, HLA-DQB1, HLA-DRA, HLA-DRB1, MAPK1, MAPK10, NOD2, PLA2R1, RIPK2, TLR1, TLR2, TLR6, TGFB1*
hsa044612	Antigen processing and presentation	22	3.2E-5	*CD74, CD8A, CD8B, TAPBP, CANX, CIITA, HSPA2, HSPA6, KIR2DS2, KLRC3, KLRC4, KLRD1, HLA-E, HLA-DMA, HLA-DPA1, HLA-DPB1, HLA-DQA2, HLA-DQB1, HLA-DRA, HLA-DRB1, PDIA3, TAP1*
hsa01130	Biosynthesis of antibiotics	43	5.4E-5	*HMGCS2, IDNK, ACAT1, ACAA1, ACAA2, ACADM, AK3, AK5, AK7, ADH5, ALDOB, ACY1, AMT, ARG2, BCAT1, BCKDHB, CAT, CTH, FDFT1, FAXDC2, FBP2, GCSH, GLDC, HK2, HK3, HAO2, HADH, HSD17B7, ISYNA1, LDHB, MVK, OGDHL, PCK1, PFKM, PGM3, PLA2G7, PCCA, RGN, SDS, SHMT2, SDHD, SUCLA2, SUCLG1*
hsa00280	Valine, leucine and isoleucine degradation	16	7.1E-5	*HMGCS2, HIBADH, HMGCL, OXCT1, AUH, ACAT1, ACAA1, ACAA2, ACADM, ALDH6A1, BCAT1, BCKDHB, HADH, MCCC1, MUT, PCC*
hsa05140	Leishmaniasis	20	1.2E-4	*FCGR3A, FCGR2A, ITGA4, ITGAM, ITGB2, IFNGR1, IFNGR2, HLA-DMA, HLA-DPA1, HLA-DPB1, HLA-DQA2, HLA-DQB1, HLA-DRA, HLA-DRB1, MAPK1, NCF1, NCF2, PTPN6, TLR2, TGFB1*
hsa04621	NOD-like receptor signaling pathway	17	1.8E-4	*CCL5, CXCL8, NLRC4, NLRP1, PYCARD, BIRC3, CASP1, CASP5, CASP8, CARD9, MAPK1, MAPK10, NOD1, NOD2, PSTPIP1, PYDC1, RIPK2*
hsa05145	Toxoplasmosis	26	2.0E-4	*CCR5, CD40, GNAI1, BIRC3, CASP8, CIITA, CYCS, HSPA2, HSPA6, ITGA6, IFNGR1, IFNGR2, IL10RA, LAMB4, LY96, HLA-DMA, HLA-DPA1, HLA-DPB1, HLA-DQA2, HLA-DQB1, HLA-DRB1, MAPK1, MAPK10, TLR2, TGFB1*

Establishment of PPI network and development of top 10 hub genes

To identify the protein-protein associations between DEGs, we constructed the PPI network using the STRING database. Cytoscape is used for the network visualization and topological calculations of the network were calculated using Cytohubba. The PPI networks for ccRCC were constructed including 1744 nodes and 5377 edges with the p value 1.0e-16. With the use of "degree topological terms" in CytoHubba, the top 10 hub genes were described in our dataset. The top 10 genes are Oxoglutarate receptor 1 (*OXGR1*), Mitogen-activated protein kinase 1 (*MAPK1*), G protein subunit gamma 2(*GNG2*), LCK proto-oncogene, Src family tyrosine kinase (*LCK*), Integrin subunit beta 2 (*ITGB2*), HLA class II histocompatibility antigen, DRB1 beta chain (*HLA-DRB1*), Kinesin family member 20A (*KIF20A*), *GNG10*, G protein subunit gamma 10 (*GNB4*) and HLA class II histocompatibility antigen, DR alpha chain (*HLA-DRA*) (Table 5). The upregulated genes are *HLA-DRB1, HLA-DRA, GNB4, GNG10, KIF20A, ITGB2, LCK, GNG2, MAPK1*. The only down regulated gene is *OXGR1*.

Table 5. *Top 10 hub genes in the PPI network*

Gene ID	Betweenness	BottleNeck	Closeness	Clustering Coefficient	Degree	DNMC	EcCentricity	EPC	MCC	MNC	Radiality	Stress
OXGR1	35637	13	401	0.3	73	0.54	0.06	128.4	9.2	73	8.9	323668
MAPK1	18141	165	435	0.08	66	0.18	0.06	89.9	3.6	57	9.1	1458640
GNG2	28027	13	391	0.3	65	0.58	0.05	128.3	9.2	64	8.9	313278
LCK	53568	16	413	0.2	63	0.33	0.05	115.5	1.7	60	9	715100
ITGB2	38026	14	394	0.2	58	0.29	0.05	99.3	1.3	57	8.9	452548
HLA-DRB1	17939	7	394	0.3	58	0.52	0.05	124	9.2	57	8.9	523728
KIF20A	12327	1	353	0.4	58	0.72	0.05	120.5	9.2	58	8.7	454960
GNG10	8957	4	369	0.4	57	0.69	0.05	121.9	9.2	56	8.8	108962
GNB4	13322	4	382	0.4	57	0.70	0.05	123.3	9.2	56	8.9	143922
HLA-DRA	16360	1	392	0.3	57	0.52	0.05	122.2	9.2	57	8.9	511188

The ccRCC is the most common type of RCC which has a high mortality rate. The development of ccRCC depends on both tumor progression and gene interactions [18]. The diagnosis of ccRCC patients has improved in recent years, however, because some patients don't have early disease symptoms and the scarcity of sensitive ccRRC biomarkers, the treatments for many patients delayed [19].

Thanks to microarray technology, understanding disease mechanisms has become easy which helps to identify potential reliable biomarkers for several diseases. The objective of our study is to find out biomarkers and key pathways mostly attributed to ccRCC. The GSE168845 dataset downloaded at NIH/NCBI from the GEO database was analyzed to clarify our study purpose. We identified 3,935 DEGs including 1,991 upregulated genes and 1,944 downregulated genes in the ccRCC vs. control samples. GO function analysis in the biological process category showed that DEGs were mostly involved in the inflammatory response, signal transduction and immune response. GO function analysis

in the molecular function category demonstrated that DEGs were mostly involved in receptor activity, protein homodimerization activity, and CARD domain binding. In addition, GO function analysis in the cellular component category showed that DEGs were mostly involved in integral components of the plasma membrane, plasma membrane, and extracellular exosome. Based on the earlier studies results, immune response affects the tumor development rate in several cancer types such as lung, breast, and ccRCC. One recent study that suggested immune response is enriched in ccRCC which supports our results [20]. Our GO function analysis results are consistent with the previous bioinformatic analysis of ccRCC [21]. Their GO analysis also found out that their identified DEGs are mostly enriched in extracellular exosome, plasma membrane and integral component of the membrane in the cellular component group which support our results [21]. The top KEGG pathways are Staphylococcus aureus infection, natural killer cell mediated cytotoxicity, phagosome and antigen processing and presentation.

We identified 10 hub genes that have roles in the molecular etiology of ccRCC (*OXGR1, MAPK1, GNG2, LCK, ITGB2, HLA-DRB1, KIF20A, GNG10, GNB4*, and *HLA-DRA*). Some of the identified hub genes (*MAPK1, LCK, ITGB2, KIF20*) in our study are consistent with previous bioinformatic analysis studies in ccRCC. The activation of the mitogen-activated protein kinase (MAPK) signaling pathway which has roles in cell proliferation has been shown in several cancers, including renal cell carcinoma [22]. One recent study showed the overexpression of the MAPK and mitogen-activated protein kinases (MKK) in ccRCC [23]. *MAPK1* could be a potential biomarker for the diagnostic and therapeutic target for ccRCC. Another hub gene *LCK*, one recent study also found out that the LCK gene is a reliable biomarker for the prognosis of ccRCC [18]. *ITGB2* gene which has important roles in immune response was another important hub gene that we identified. *ITGB2* gene is also one of the 10 hub genes that Song and colleagues identified in ccRCC pathogenesis [21]. In addition, *ITGB2* mutations might result in leukocyte adhesion deficiency [24]. *KIF20* gene was also one of the 15 hub genes which may contribute to the progression and pathogenesis of ccRCC [25].

Our study has some limitations that we need to point out. The one limitation of our study is the lack of verification of the expression level of the 10 biomarkers we identified in ccRCC cells and tissues. We only performed bioinformatics analysis, so future studies are required to support our in silico results experimentally. The other limitation is the small sample size. Our data set include 4 ccRCC patient samples and further analysis is required with a larger sample size to confirm our results.

CONCLUSION

In conclusion, our study discovered DEGs and associated functional terms pathways. Our study found out that *OXGR1, MAPK1, GNG2, LCK, ITGB2, HLA-DRB1, KIF20A, GNG10, GNB4* and *HLA-DRA* genes may be good candidate therapeutic biomarkers and for ccRCC. Our results would help to understand the pathophysiology and molecular mechanisms of ccRCC. Further biological experimental research is needed to confirm the results of our bioinformatics analysis with larger datasets.

Acknowledgement. The publicly available NIH GEO dataset GSE168845 was used and analyzed in this study (http://www.ncbi.nlm.nih.gov/geo).

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