Expression profile analysis of Differentially Expressed Genes in Human Coronary Artery Endothelial Cells Induced by Serum from Kawasaki Disease: A preliminary study

Xue Fan
Shenzhen Children's Hospital
Meng Li
Shenzhen Children's Hospital
Min Xiao
Shenzhen Children's Hospital
Cong Liu
Shenzhen Children's Hospital
Mingguo Xu (18938690175@163.com)
Shenzhen Children's Hospital

Research article

Keywords: DEGs, Kawasaki disease, human coronary artery endothelial cells, RNA-seq, bioinformatics analysis

Posted Date: August 13th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-54163/v1

Abstract

Background: Kawasaki disease (KD) leads to coronary artery damage and the etiology of KD is unknown. The present study was designed to explore the differentially expressed genes (DEGs) in KD serum-induced human coronary artery endothelial cells (HCAECs) by RNA-sequence (RNA-seq).

Methods: HCAECs were stimulated with serum (15% (v/v)), which were collected from 20 healthy children and 20 KD patients, for 24 hours. DEGs were then detected and analyzed by RNA-seq and bioinformatics analysis.

Results: The expression of SMAD1, SMAD6, CD34, CXCL1, PITX2, and APLN was validated by qPCR. 102 genes, 59 up-regulated and 43 down-regulated genes, were significantly differentially expressed in KD groups. GO enrichment analysis showed that DEGs were enriched in cellular response to cytokines, cytokine-mediated signaling pathway, and regulation of immune cells migration and chemotaxis. KEGG signaling pathway analysis showed that DEGs were mainly involved in cytokine–cytokine receptor interaction, chemokine signaling pathway, and TGF–β signaling pathway. Besides, the mRNA expression levels of SMAD1, SMAD6, CD34, CXCL1, and APLN in the KD group were significantly up-regulated compared with the normal group, whilePITX2 was significantly down-regulated.

Conclusion: 102 DEGs in KD serum-induced HCAECs were identified, and six new targets were proposed as potential indicators of KD.

Background

Kawasaki disease (KD) is also known as mucocutaneous lymph node syndrome and predominantly occurs in young children that under the age of 5 years old[1].The main manifestations of KD are persistent fever, cervical lymphadenopathy, polymorphous skin rashes, bilateral non-purulent conjunctivitis, peripheral extremity alterations, diffuse mucosal inflammation, and non-specific inflammatory syndrome of small and medium blood vessels in the body[2].KD caused permanent vascular complications, especially coronary artery damage including coronary artery dilation, coronary artery aneurysms (CAAs), and myocardial infarction, are the major causes of its morbidity and mortality[3, 4].CAAs progress in 25% of untreated cases, which can be reduced to 4% with treatment of intravenous immunoglobulin and aspirin[5].However, the etiology of KD-induced coronary artery damage is unknown. And these highlight the importance and urgency of unravelling the mechanisms of KD-induced coronary artery damage. Endothelial cells play an indispensable role in maintaining the normal functions of vessels. And dysfunction or inflammatory activation of coronary artery endothelial cells lead to the disruption of endothelial cell homeostasis, which are the initiating factors for the damage of coronary artery in patients with KD[6–8].Therefore, a better understanding of alterations in coronary artery endothelial cells underlying KD is required to develop targeted treatments. RNA-sequencing (RNA-seq) has been widely used to analyze gene expression offering a cheap, efficient and high throughput method to investigate global gene expression patterns. This method isalso widely used to predict and

offer new potential gene targets for disease pathogenesis. However, there is a limited report about the transcriptomic study of coronary artery endothelial cells under the condition of KD.

In the present study, differentially expressed genes (DEGs)of human coronary artery endothelial cells (HCAECs)induced by KD serum were detected and analyzed using RNA-seq and bioinformatics analysis. And six DEGs were validated, which may assist in the prevention and treatment of coronary artery injury in the progress of KD.

Materials And Methods

Human subjects and serum sample collection

A total of 40 subjects, including 20 normal children (average age:1.90 years and the ratio of male/female: 11/9) and 20 KD patients (average age:1.80 years and the ratio of male/female: 11/9), were recruited from Shenzhen Children's Hospital. Serum samples were separated by centrifugation at 1,000 × g for 10 min and aliquots were stored at − 80℃.The diagnostic criteria of KD were according to the Japanese Circulation Society Joint Working Groups performed in 2012.

Hcaecs Culture And Treatment

HCAECs were brought form ScienCell (San Diego, CA, USA) and cultured with endothelial cell growth medium containing growth factors, supplements, and 10% fetal bovine serum at 37℃ in 5%CO_2. Cells were then stimulated with culture medium containing 15% serum (15% (V/V)) from KD patients or healthy donors for 24 h. The cells were then used in subsequent studies. Each measurement was performed in triplicate (n = 3).

Rna Isolation And Rna-seq Analysis

After treatments, total RNA was extracted using a Trizol reagent (Illumina, San Diego, CA, USA) according to the manufacturer's protocol and isolated RNA was used for following studies. The MGI Easy mRNA library was used to construct the mRNA library and the MGI0seq 2000 platform was used for sequencing. Data with less than 17 bases will be eliminated. The data were compared with the human mRNA database (reference database: human_hg19-refMrna20150317as) by the FANse2 algorithm. After being standardized by the RPKM (read per kilo bases per million mapped reads) method, the differentially expressed genes were analyzed from two aspects of different multiple and significant level. The threshold value was set up as follows: |log2 (Fold Change) | > 1 and p value < 0.01. The DEGs was drawn into heat map using R language.

Bioinformatics Analysis

To further elucidate the role of DEGs in KD-induced coronary artery endothelial cells, GO (Gene Ontology) enrichment analysis was performed to predict the function of DEGs and KEGG (Kyoto encyclopedia of genes and genomes) pathway analysis were performed to determine the involvement of DEGs in different

biological pathways. FDR < 0.01 were selected as threshold values for GO and KEGG pathway enrichment. And $P < 0.05$ were considered statistically significant.

Quantitative Real Time-polymerase Chain Reaction (qpcr)

qPCR was conducted to measure the mRNA expression levels of SMAD1, SMAD6, CD34, PITX2, CXCL1, APLN, and GAPDH (used as the internal control), and primer information listed in the Table 1. cDNA was synthesized from isolated RNA using the PrimeScript RT Reagent Kit according to the manufacturer's instructions. qPCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems) in the ABI 7500 RT-PCR System (Applied Biosystems).Thermal cycling conditions were as follows: pre-denaturation for the 30 s at 95℃, followed by 40 cycles and denaturation for 5 s at 95℃, annealing for 30 s at 60℃.

Table 1
Primer sequences of SMAD1, SMAD6, CD34, PITX2, CXCL1 and APLN.

Genes	Primer sequence (5'-3')
SMAD1	Forward: 5'-CAGAGGAGATGTTCAGGCGGT-3'
	Reverse:5'-AGGCATGGAACGCTTCACCC-3'
SMAD6	Forward: 5'-CGCGACGAGTACAAGCCACT-3'
	Reverse:5'-GGAGACATGCTGGCGTCTGA-3'
CD34	Forward: 5'-GAAAGGCTGGGCGAAGACCC-3'
	Reverse:5'-GTCCCGTTTTCCTGAGCCCC-3'
PITX2	Forward: 5'-CGAGTCCGGGTTTGGTTCAAGA-3'
	Reverse:5'-TGGTGGATAGGGAGGCGGAT-3'
CXCL1	Forward: 5'-ACCGAAGTCATAGCCACACTCA-3'
	Reverse:5'-TTCAGGAACAGCCACCAGTGA-3'
APLN	Forward: 5'-GAATGGGCTGGAAGACGGCA-3'
	Reverse:5'-TTCAGTCCTGCTTCAGAAAGGCA-3'
GAPDH	Forward: 5'-CCTTCCGTGTCCCCACT-3'
	Reverse:5'-GCCTGCTTCACCACCTTC-3'

Statistical analysis

Results are presented as the mean ± standard deviation (SD). To confirm a significant difference between specific treatment groups, group comparisons were performed using the Student's t-test. In all cases, P-value < 0.05 was considered a statistically significant difference.

Results

Analysis of DEGs expression profiles in normal serum or KD serum-treated HCAECs

DEGs expression profiles are presented in the form of a heat map (Fig. 1a). And the differences in DEGs expression profiles between normal and KD groups are further shown as a volcano plot including 59 up-regulated and 43 down-regulated mRNAs(Fig. 1b) (supplementary table 1).Venn diagram comparisons for all DEGs respectively illustrate the overlap of expressed genes among normal (N) serum or KD (A) serum-stimulated HCAECs and each group was repeated three times (Fig. 1).Results showed that there were 12159, 11058 and 11676 DEGs in A1, A2 and A3 of normal group, respectively and a total of 10933 genes were expressed in all three normal groups (Fig. 2a). And there were 11729, 10995 and 11556 DEGs in A1, A2 and A3 of KD group, respectively a total of 10859 genes were expressed in all three KD groups (Fig. 2b). Furthermore, 10509 putative genes were shared by all comparison samples (Fig. 2c).

Enrichment Analysis Of The Biological Function Of Degs

The GO analysis showed that the DGEs were significantly enriched in the biological process including cellular response to chemical, organic substance or cytokines, cytokine-mediated signaling pathway, and regulation of immune cells migration and chemotaxis (Fig. 3a). And the most significantly enriched pathways of the DEGs in the KEGG analysis were cytokine-cytokine receptor interaction, chemokine signaling pathway, TNF signaling pathway, influenza A, malaria, and TGF-β signaling pathway (Fig. 3b).

Qpcr Validation Of The Transcriptomic Data

To validate the RNA-seq results, the RNA expression of 6 DEGs, including three up-regulated (SMAD1, CD34 and APLN) and three down-regulated (SMAD6, PITX2 and CXCL1), were analyzed by qPCR. Five of six DEGs (SMAD1, SMAD6, CD34, CXCL1, and APLN) RNA expression were upregulated under KD condition compared to normal conditions (Fig. 4a-e).While, the expression of PITX2was significantly downregulated (Fig. 4f).

Discussion

KD is the leading cause of acquired heart disease in children, given its coronary artery complications[9, 10], and there is still no effective therapy to prevent or treat the occurrence of coronary artery damage due to the limited information. It has been shown that coronary artery endothelial dysfunction plays an important role in the development of coronary artery damage under KD condition [11, 6]. Therefore, it is meaningful to figure out the changes in endothelial cells with or without KD condition, which might provide novel indicators and potential therapeutic targets for KD. In the present study, DEGs following treatment of coronary artery endothelial cells with KD plasma were investigated by RNA-sEq. Moreover, the biological functions of these DEGs were also analyzed by bioinformatics analysis to unravel the molecular mechanisms underlying KD.

The results of RNA-seq showed that 102 DEGs were significantly expressed, including 59 up-regulated and 43 down-regulated, in the KD group compared with the control group. And these genes are enriched in multiple biological processes and pathways, including cellular response to cytokines, cytokines-mediated signaling pathways, regulation of immune cells migration and chemotaxis, immune responses, cytokine-cytokine receptor interaction, chemokine signaling pathway, and TNF signaling pathway. And these biological processes and pathways is associated with vascular inflammation and activation of immune systems, which are two major contributors of the development of KD [12, 13].Therefore, genes relevant to above mentioned biological processes and pathways expressed in KD serum stimulated coronary artery endothelial cells were expected and partly verified by previous studies. For instance, the severity of TNF-mediated vascular inflammation and concurrent coronary artery damage were found in KD patients [14].

To validate the results of RNA-seq, six DEGs (SMAD1, SMAD6, CD34, PITX2, CXCL1, and APLN), which are significantly differentially expressed in KD group compared with control group and are closely associated with cardiovascular system, were analyzed by qPCR. SMADs family consists of 10 members (SMAD1-10) and activated by transforming growth factor (TGF)-β signaling pathway. TGF-β pathway is involved in inflammation and tissue remodeling mediated by endothelial cells, and immune activation, which support the importance of TGF-βpathway in KD occurrence and outcomes[15, 16].CD34 positive cells with enhanced adhesive and homing properties facilitates immune cells homing to the inflammatory sites and mediates inflammatory and immune responses [17].CXCL1, as the chemokine ligand, presents on endothelial surface and mediates immune responses by recruitment of neutrophils and monocytes [18, 19].APLN(apelin) is a vasoactive peptide and an endogenous ligand for APJ receptors. APLN/APJ receptor system plays a vital role in regulation of vascular function, including vascular tone, angiogenesis, proliferation, and permeability [20, 21].In addition, APLN/APJ system also mediates the anti-inflammatory effects of 1,25(OH)2D3 by reduction of pro-inflammatory mediators and adhesion molecules in LPS-stimulated macrophages [22]. PITX2 plays an important role in regulating the differentiation of endothelium and angiogenesis [23, 24].Although these studies provide insights into these biomarkers involved in regulation of cardiovascular and immune system, it is yet unknown whether these alterations can be considered as potential indicators for prevention and treatment of coronary artery damage in KD, which needs further investigations. Besides, some differences in levels of gene regulation between the RNA-seq and PCR data were noted. For example, the down-regulated expressions of SMAD6 and CXCL1 in RNA-seq results were shown to be reregulated in qPCR results, which were possible due to primer specificity, alternatively spliced forms of the genes, RNA integrity, and/or PCR conditions[25].

Conclusion

In the present study identified the differential expressed genes in KD serum treated coronary endothelial cells using RNA-sEq. And these genes are involved in cellular response to cytokines, cytokines-mediated signaling pathways, regulation of immune cells migration and chemotaxis, cytokine-cytokine receptor interaction, chemokine signaling pathway, and TNF signaling pathway, which have been reported to be associated with development of KD. Besides, 6 DEGs expression levels were verified by qPCR, and these

DEGs may be considered as potential indicators of KD. And this study is much more like a descriptive research and further studies still need to confirm the functions of these DEGs in KD-induced coronary artery damage.

Abbreviations

CAAs coronary artery aneurysms; DEGs differentially expressed genes; GO Gene Ontology; HCAECs human coronary artery endothelial cells; KD Kawasaki disease; KEGG Kyoto encyclopedia of genes and genomes; qPCR Quantitative Real time-polymerase chain reaction; RNA-seq RNA-sequence; TGF transforming growth factor

Declarations

Acknowledgments

None.

Authors' contributions

Mingguo Xu conceived and designed the research, reviewed and revised the manuscript. Xue Fan performed a preliminary analysis. Meng Li and Fan xue wrote the manuscript. All authors read and approved the final version of the manuscript.

Availability of data and materials

All data are available in the manuscript and they are showed in figures, tables and supplement file.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

Funding

This study was supported by National Nature Science Foundation of China (81870364) , Shenzhen Scientific Plan (JCYJ20190809164004023) and Shenzhen Sanming Project (SZSM20162057).

Ethical approval

This study was approved by the ethics committee of Shenzhen children's Hospital (Ethics No. 201800601).

Informed Consent

The informed consents were signed from the parents of all participants.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Yeung RS. Kawasaki disease: update on pathogenesis. Curr Opin Rheumatol.2010; 22 (5):551-560. doi:10.1097/BOR.0b013e32833cf051
2. McCrindle BW,Rowley AH, Newburger JW, Pahl E. Diagnosis, Treatmentand Long-Term Management of Kawasaki Disease:A Scientific Statement for Health Professionals.Circulation.2017;135(17):e927-e999.doi:10.1161/cir.0000000000000484
3. Wang CL, Wu YT, Liu CA. Kawasaki disease: infection, immunity and genetics. Pediatr Infect Dis J.2005;24 (11):998-1004. doi:10.1097/01.inf.0000183786.70519.fa
4. Liang CD, Kuo HC, Yang KD. Coronary artery fistula associated with Kawasaki disease. Am Heart J.2009;157 (3):584-588. doi:10.1016/j.ahj.2008.11.020
5. Kawasaki T, Kosaki F, Okawa S.A new infantile acute febrile mucocutaneous lymph node syndrome (MLNS) prevailing in Japan. Pediatrics.1974;54 (3):271-276
6. Xu M,Qi Q,Men L. Berberine protects Kawasaki disease-induced human coronary artery endothelial cells dysfunction by inhibiting of oxidative and endoplasmic reticulum stress. Vascular Pharmacology.2020;106660. doi:https://doi.org/10.1016/j.vph.2020.106660
7. He YE, Qiu HX, Wu RZ, Rong X, Xu HT, Xiang RL, Chu MP. Oxidised Low-Density Lipoprotein and Its Receptor-Mediated Endothelial Dysfunction Are Associated with Coronary Artery Lesions in Kawasaki Disease. J Cardiovasc Transl Res. 2019.doi:10.1007/s12265-019-09908-y
8. Armaroli G, Verweyen E, Pretzer C, Kessel K, Hirono K, Ichida F, Okabe M, Cabral DA, Foell D, Brown KL, Kessel C. Monocyte-Derived Interleukin-1beta As the Driver of S100A12-Induced Sterile Inflammatory Activation of Human Coronary Artery Endothelial Cells: Implications for the Pathogenesis of Kawasaki Disease. Arthritis Rheumatol.2019;71 (5):792-804. doi:10.1002/art.40784
9. Cohen E, Sundel R. Kawasaki Disease at 50 Years. JAMA Pediatr.2016; 170 (11):1093-1099. doi:10.1001/jamapediatrics.2016.1446
10. Kawasaki T. [Acute febrile mucocutaneous syndrome with lymphoid involvement with specific desquamation of the fingers and toes in children]. Arerugi = [Allergy].1967; 16 (3):178
11. Xiao M, Men LN, Xu MG, Wang GB, Lv HT, Liu C. Berberine protects endothelial progenitor cell from damage of TNF-alpha via the PI3K/AKT/eNOS signaling pathway. Eur J Pharmacol.2014;743:11-16. doi:10.1016/j.ejphar.2014.09.024

12. Garcia-Pavon S, Yamazaki-Nakashimada MA, Baez M, Borjas-Aguilar KL, Murata C. Kawasaki Disease Complicated With Macrophage Activation Syndrome: A Systematic Review. J Pediatr Hematol Oncol.2017;39 (6):445-451. doi:10.1097/mph.0000000000000872
13. Schnabel A, Hedrich CM. Childhood Vasculitis. Front Pediatr.2018;6:421. doi:10.3389/fped.2018.00421
14. Okada S, Hasegawa S, Suzuki Y, Matsubara T, Shimomura M, Okuda M, Ichiyama T, Ohga S. Acute pericardial effusion representing the TNF-alpha-mediated severe inflammation but not the coronary artery outcome of Kawasaki disease. Scand J Rheumatol.2015;44 (3):247-252. doi:10.3109/03009742.2014.956140
15. Shimizu C, Jain S, Davila S, Hibberd ML, Lin KO, Molkara D, Frazer JR, Sun S, Baker AL, Newburger JW, Rowley AH, Shulman ST, Burgner D, Breunis WB, Kuijpers TW, Wright VJ, Levin M, Eleftherohorinou H, Coin L, Popper SJ, Relman DA, Fury W, Lin C, Mellis S, Tremoulet AH, Burns JC. Transforming growth factor-beta signaling pathway in patients with Kawasaki disease. Circ Cardiovasc Genet.2011;4 (1):16-25. doi:10.1161/circgenetics.110.940858
16. Peng Q, Deng Y, Yang X, Leng X, Yang Y, Liu H. Genetic variants of ADAM17 are implicated in the pathological process of Kawasaki disease and secondary coronary artery lesions via the TGF-β/SMAD3 signaling pathway. European journal of pediatrics.2016;175 (5):705-713. doi:10.1007/s00431-016-2696-8
17. Buyl K, Merimi M, Rodrigues RM, Agha DM, Melki R, Vanhaecke T, Bron D, Lewalle P, Meuleman N, Fahmi H, Rogiers V, Lagneaux L, Kock J, Najar M. The Impact of Cell-Expansion and Inflammation on The Immune-Biology of Human Adipose Tissue-Derived Mesenchymal Stromal Cells. J Clin Med.2020;9 (3). doi:10.3390/jcm9030696
18. Bai W, Wang Q, Deng Z, Li T, Xiao H, Wu Z. TRAF1 suppresses antifungal immunity through CXCL1-mediated neutrophil recruitment during Candida albicans intradermal infection. Cell Commun Signal.2020;18 (1):30. doi:10.1186/s12964-020-00532-x
19. Vries MH, Wagenaar A, Verbruggen SE, Molin DG, Dijkgraaf I, Hackeng TH, Post MJ. CXCL1 promotes arteriogenesis through enhanced monocyte recruitment into the peri-collateral space. Angiogenesis.2015;18 (2):163-171. doi:10.1007/s10456-014-9454-1
20. Mughal A, O'Rourke ST. Vascular effects of apelin: Mechanisms and therapeutic potential. Pharmacol Ther.2018;190:139-147. doi:10.1016/j.pharmthera.2018.05.013
21. Wysocka MB, Pietraszek-Gremplewicz K, Nowak D. The Role of Apelin in Cardiovascular Diseases, Obesity and Cancer. Front Physiol.2018;9:557. doi:10.3389/fphys.2018.00557
22. Faridvand Y, Bagherpour-Hassanlouei N, Nozari S, Nasiri N, Rajabi H, Ghaffari S, Nouri M.1, 25-Dihydroxyvitamin D3 activates Apelin/APJ system and inhibits the production of adhesion molecules and inflammatory mediators in LPS-activated RAW264.7 cells. Pharmacol Rep.2019;71 (5):811-817. doi:10.1016/j.pharep.2019.04.012
23. Chen L, Martino V, Dombkowski A, Williams T, West-Mays J, Gage PJ. AP-2beta Is a Downstream Effector of PITX2 Required to Specify Endothelium and Establish Angiogenic Privilege During

Corneal Development. Invest Ophthalmol Vis Sci.2016;57(3):1072-1081. doi:10.1167/iovs.15-18103

24. French CR, Seshadri S, Destefano AL, Fornage M, Arnold CR, Gage PJ, Skarie JM, Dobyns WB, Millen KJ, Liu T, Dietz W, Kume T, Hofker M, Emery DJ, Childs SJ, Waskiewicz AJ, Lehmann OJ. Mutation of FOXC1 and PITX2 induces cerebral small-vessel disease. J Clin Invest.2014; 124 (11):4877-4881. doi:10.1172/jci75109
25. Rubin A, Salzberg AC, Imamura Y, Grivitishvilli A, Tombran-Tink J. Identification of novel targets of diabetic nephropathy and PEDF peptide treatment using RNA-seq. BMC Genomics.2016;17 (1):936. doi:10.1186/s12864-016-3199-8

Figures

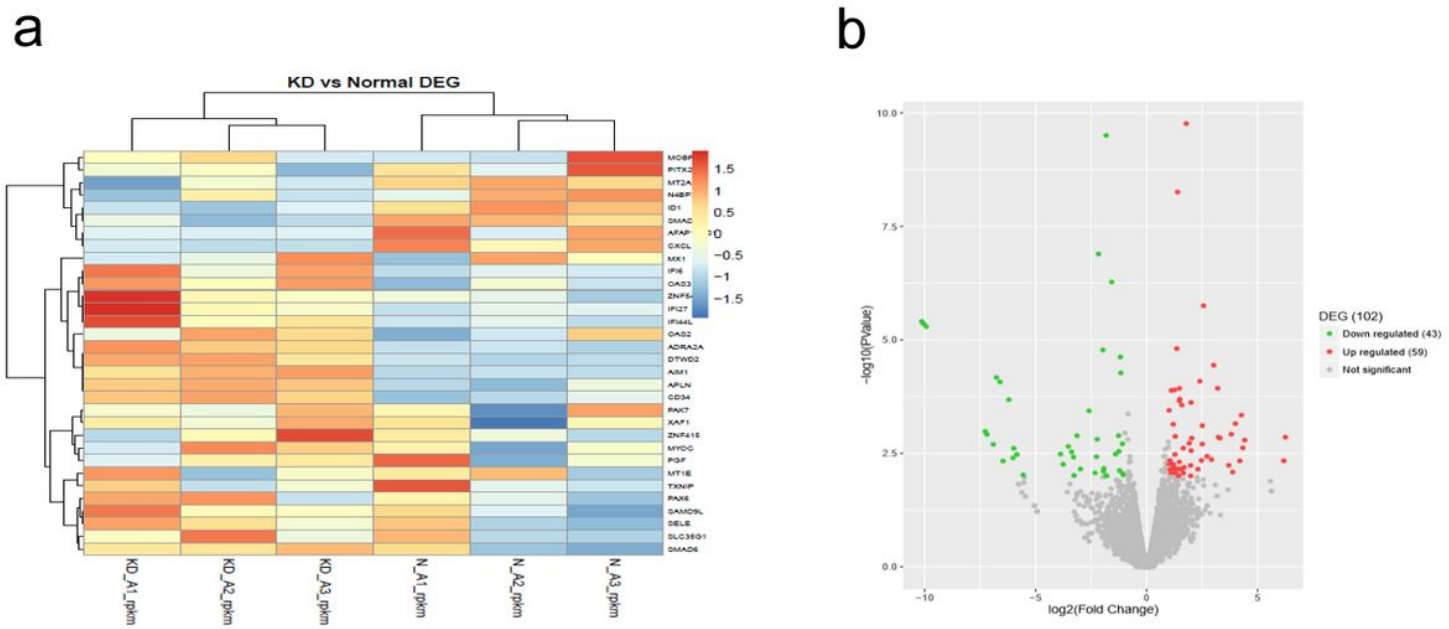

Figure 1

DEGs expression profile in the KD serum treated cells relative to the normal serum treated cells. a. Hierarchical clustering of DEGs. Each group consisted of three individual experiments. DEGs are presented by single rows and samples by single columns. b. Volcano plot of DEGs. The value on the X- and Y-axes represent normalized fold changes and P values, respectively. The color scale demonstrates relative expression, upregulation (red), downregulation (green), and no significant difference (gray). DEGs with fold change <1 and P<0.01 were regarded as differentially expressed.

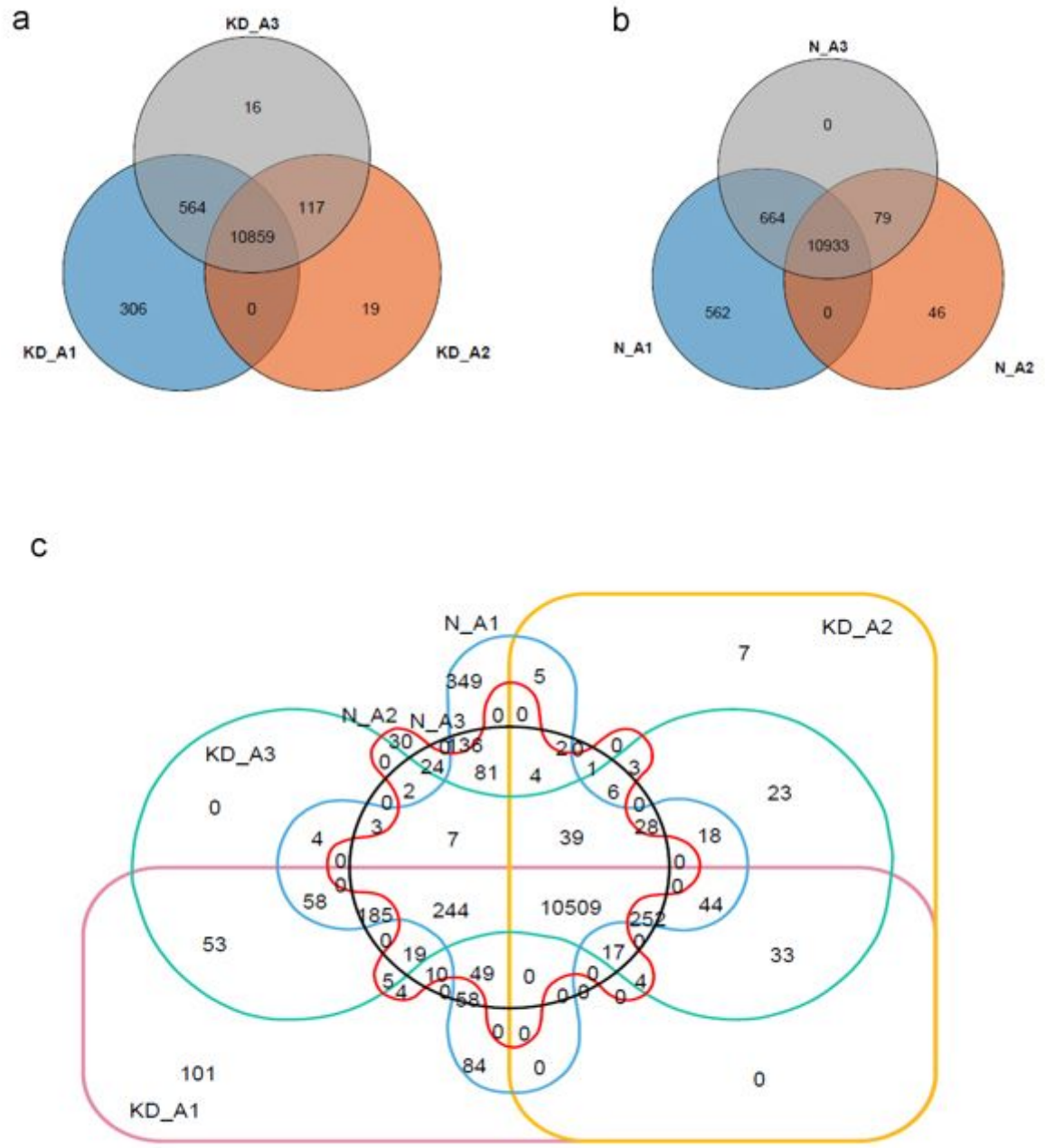

Figure 2

Venn diagram depicting the number of DEGs in KD or normal serum-stimulated HCAECs. a. Comparison of the number of DEGs expressed in all normal (N) samples (A1, A2, and A3). b. Comparison of the number of DEGs expressed in all KD samples (A1, A2, and A3). c. Comparison of the number of DEGs expressed in all six samples. The numbers shown in the Venn diagram indicate the overlap genes expressed in normal and KD samples or genes that only expressed in normal or KD samples.

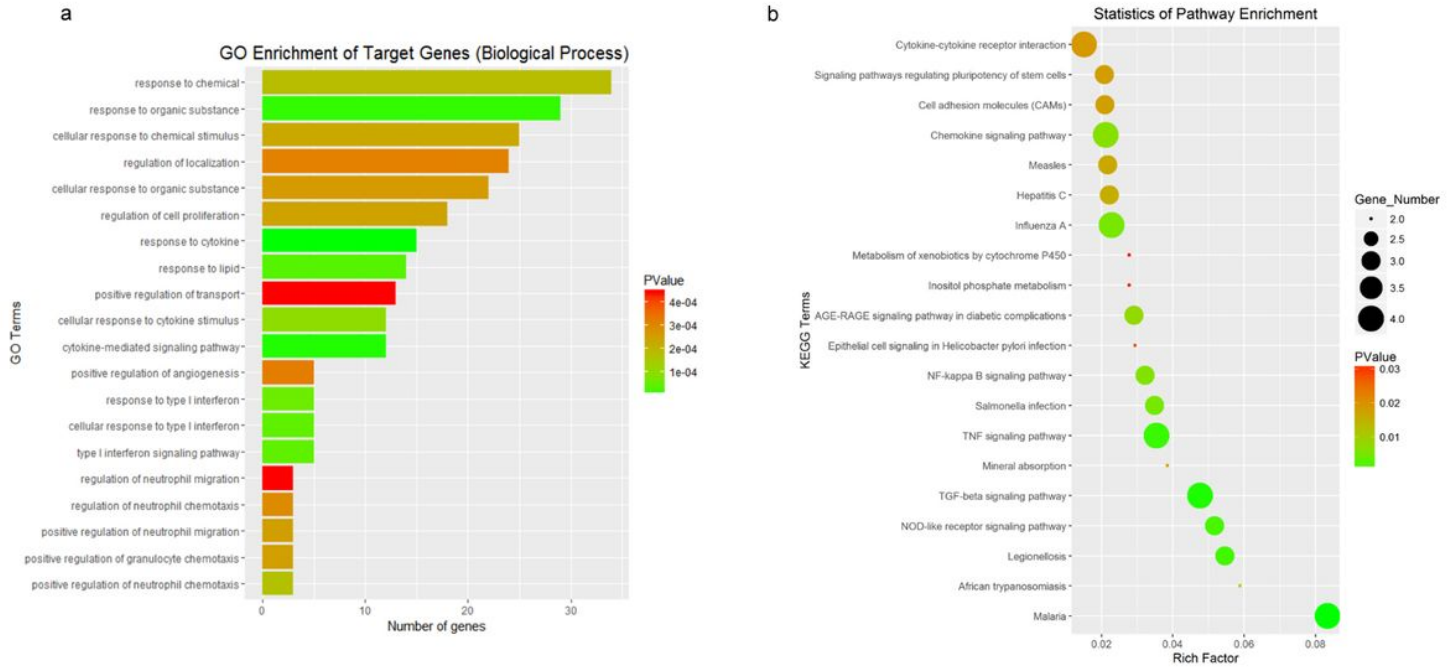

Figure 3

GO and KEGG analysis for DEGs. a. GO enrichment analysis; b. KEGG pathway analysis. GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

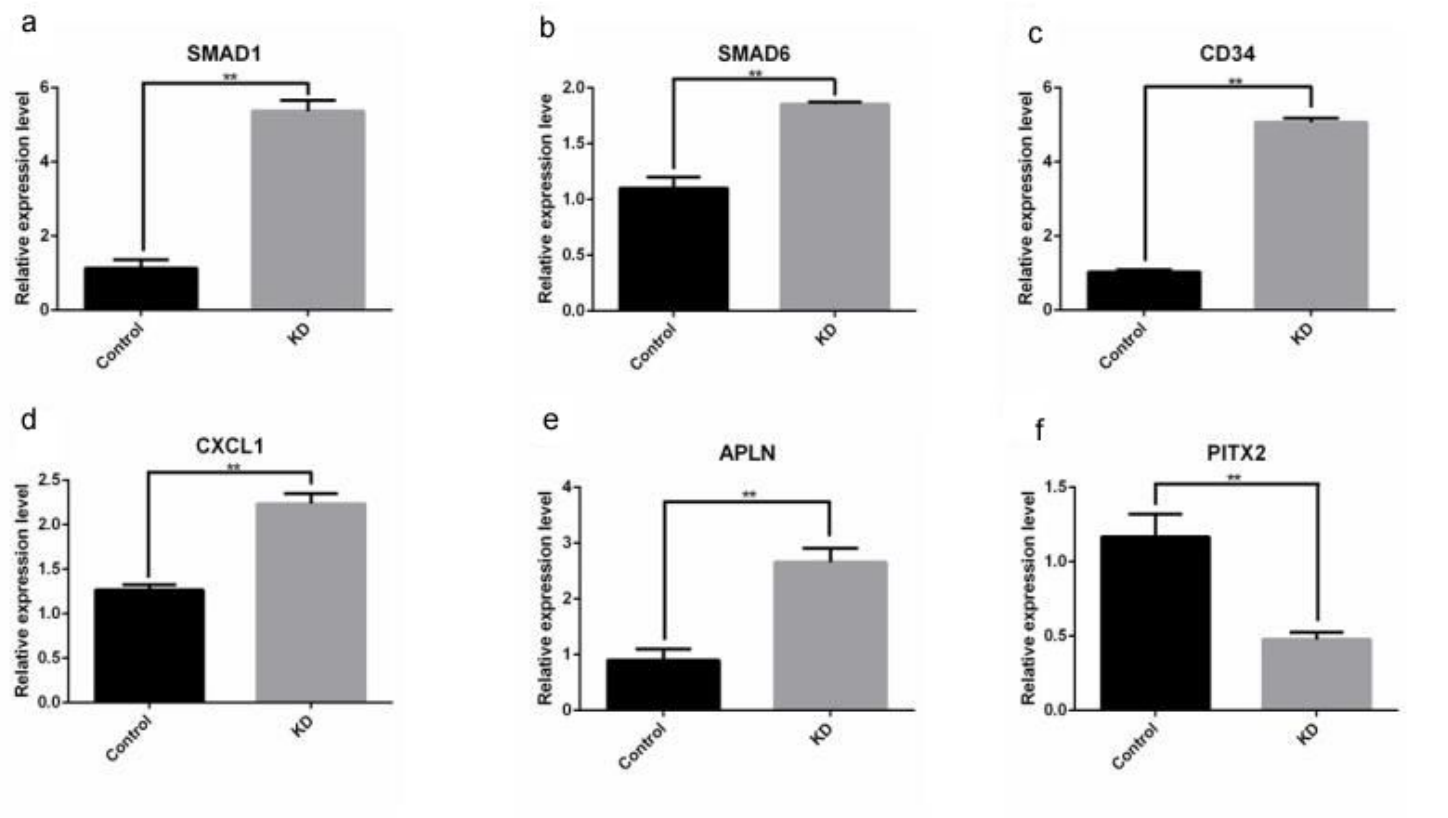

Figure 4

Expression of representative genes in control and KD serum-stimulated HCAECs validated by qPCR. Each experiment was separately repeated three times (n=3). **P<0.01.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarytable1.csv