Weighted gene coexpression network analysis reveals negative regulation of maximum left ventricular wall thickness by carboxylesterase 1 and cathepsin C in hypertrophic cardiomyopathy

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Abstract

Hypertrophic cardiomyopathy (HCM) is a primary cardiomyopathy characterized by hypertrophic cardiomyocytes. It is one of the leading causes of sudden death in adolescents. However, the molecular mechanism of HCM is not clear. In our study, ribonucleic acid (RNA) sequence data of myocardial tissue in HCM patients was extracted from the Gene Expression Omnibus (GEO) database and analyzed by weighted gene co-expression network analysis (WGCNA). A total of 31 co-expression modules were identified. The co-expression black module significantly correlated with maximum left ventricular wall thickness (Maxi LVWT). We screened the differentially expressed mRNAs between normal tissues and HCM tissues using the dplyr and tidyr packet in R3.6.2. The genes in the black module and differentially expressed genes were further intersected. We found that the expression of carboxylesterase 1 (CES1) and cathepsin C (CTSC) was down regulated in HCM tissues, and negatively correlated with Maxi LVWT. We further verified the above conclusion in clinical samples from HCM patients. We found the expression of CES1 and CTSC was down regulated in HCM tissues, and negatively correlated with Maxi LVWT. The above conclusion was further verified in clinical samples from HCM patients. In summary, the study suggests that CES1 and CTSC negatively regulate development of HCM and have potential as a therapeutic and diagnostic target for HCM.

Introduction

Hypertrophic cardiomyopathy (HCM) is a kind of primary cardiomyopathy characterized by hypertrophy of cardiomyocytes. HCM has clear genetic characteristics, which is chromosome dominant inheritance (Maron MS. 2012). The clinical manifestations of HCM are asymmetrical ventricular wall hypertrophy, smaller ventricular cavity, increased or constant ejection fraction, left ventricular outflow tract obstruction, diastolic dysfunction, myocardial ischemia and other symptoms (Marian AJ and Braunwald E. 2017; Canepa M et al. 2016; Lu D-Y et al. 2018; Liew AC et al. 2017). However, the clinical symptoms of HCM are not obvious and the patients are difficultly detected. Severe patients may have heart failure or even sudden death (Liew AC et al. 2017; Efthimiadis GK et al. 2014). At present, HCM has become one of the main causes of adolescent sudden death.

The pathogenesis of HCM reflects the mutation and diversity of pathogenic genes. The occurrence and development of HCM are divided into four interlocking mechanisms: Familial chromosome mutation. At present, it has been confirmed that at least 14 gene mutations are related to the pathogenesis of HCM (Wang Y et al. 2013), and 10 of them are genes encoding sarcomere structural proteins (Marques MdA and de Oliveira. 2016). Mutated genes directly lead to abnormal structure and function of coding proteins, such as sarcomere cell protein, which is the key protein of pathogenesis. (Kamisago M et al. 2000). Gene regulation is a complex network regulation process. The change of gene expression level will lead to the activation or closure of HCM related signaling pathways, such as MAPK (Josowitz R et al. 2016) and TGF-β1 signaling pathway (Vakrou S et al. 2018). The changes of these molecules and pathways lead to the occurrence of HCM. Therefore, HCM is the result of the interaction of pathogenic genes, mutated genes and abnormal signaling pathways.

In our study, Ribonucleic acid (RNA) sequence data of myocardial tissue in HCM patients was extracted from the Gene Expression Omnibus (GEO) database and analyzed by weighted gene co-expression network analysis (WGCNA). We found that the expression of carboxylesterase 1 (CES1) and cathepsin C (CTSC) was down regulated in HCM tissues, and negatively correlated with maximum left ventricular wall thickness (Maxi LVWT). We further verified the above conclusion in clinical samples from HCM patients. The study suggests that CES1 and CTSC negatively regulate development of HCM and have potential as a therapeutic and diagnostic target for HCM.

Materials And Method

Data collection

The RNA expression data (high throughput sequencing) of myocardial tissues from 28 HCM patients and 9 healthy donors were downloaded from the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE130036). The RNA expression matrix was pretreated to remove the genes with more than 20% missing value (value=0). Clinical and demographic data of 28 HCM patients were obtained from the citation (doi: 10.1038/s41597-019-0094-6)(Liu X et al. 2019), including sex, age, smoking, LAD (left atrial diameter), LVST (left ventricular septal thickness), LVEDD (Left ventricular end-diastolic), LVEF (left ventricular ejection fraction), Maxi LVWT (maximum left ventricular wall thickness), Maxi LVOTG (maximum left ventricular outflow track gradient at rest or after exercise) and sample location.

Clinical sample collection

In this study, 20 peripheral blood samples were obtained from HCM patients or healthy people between November 2020 and May 2021 in the Kunming Yan'an hospital (Kunming, China). This study was approved by the Ethics Committee of the Kunming Yan'an hospital (Kunming, China; approval No. 2019-058-01) and all patients signed informed consent. We collected clinical information for all patients. The clinical features of the patients, including sex, age, LAD, LVST, LVEDD, LVEF, Maxi LVWT and sample location were collected from their medical records.

Quantitative reverse transcription PCR

Total RNA was extracted from the peripheral blood using GenElute ™Total RNA Purification Kit (Product Number: RNB100; Sigma-Aldrich LLC.) according to the manufacturer’s instruction. The concentration of total RNA was detected by a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNAs and quantitative real-time PCR (qPCR) was performed using TaqMan One Step qRT-PCR Kit (cat. no. T2210; Beijing Solarbio Science&TechnologyCo., Ltd) in the real-time fluorescent quantitative PCR instrument (model: ABI7500; Thermo Fisher Scientific, Inc.). The following primer sequences were used forqPCR: CES1 forward, 5’- CTGTGTAACGCTCCTCCTGTG-3’ and reverse, 5’-CCCAGCACAGGGATCACATC-3’; CTSC forward, 5’- CACAGATGGCCCTCTCAAGG -3’ and reverse, 5’-CAGGGGCTGATACCAAGGAC -3’; GAPDH forward, 5’-ATGACATCAAGAAGGTGGTGAAGCAGG-3’ and

reverse, GCGTCAAAGGTGGAGGAGTGGGT. The difference of mRNA expression between thenormal and HCM groups was calculated using the $2^{-\Delta\Delta Cq}$ method (Schmittgen TD and Livak KJ. 2008). GAPDH was used asinternal reference for standardization.

Weighted correlation network analysis of mRNAs

Weighted gene co-expression network analysis (WGCNA) aims to find co-expressed gene modules and explore the relationship between gene network and concerned phenotypes (Langfelder P and Horvath S. 2008). The RNA expression matrix was preprocess to remove the genes with more than 20% missing value (value= 0) and the rest was $\log_{10}^{(TPM + 0.001)}$ standardized. We then screened the protein-coding genes from processed RNA expression data using the *dplyr* and *tidyr* packet in R3.6.2 software (https://www.r-project.org/), according to *Homo sapiens GRCh38.94.* A total of 14348 genes were included in the WGCNA analysis. First, a sample tree was established and the outlier samples were removed. A sample dendrogram and heatmap of clinical trait were visualized by *WGCNA* package. A soft-thresholding power (β) was selected to establish an adjacent matrix according to the degree of connectivity so that our gene distribution fit into the scale-free network. The proximity matrix and topological matrix were obtained according to the β value, and the memory network was verified to be close to scale free under the selected β value. The genes were clustered, and then the tree was cut into different color modules using the dynamic tree cut method. 400 genes were randomly selected to draw topological overlapping heatmap. The Pearson's correlation coefficient (PCC) between module eigengenes and clinical traits was analyzed. According to correlation and pvalue, the modules related to specific clinical traits were explored. The mRNAs involved in the key modules were considered to be highly interconnected with the specific clinical traits.

Screening for differentially expressed mRNAs (DEmRNAs)

Differentially expressed mRNAs between normal samples and HCM samples in GSE130036 and GSE68316 were screened by using *limma* package in R3.6.2 software. Pvalue < 0.01 and $|\log^{\text{fold change}}{}^{(FC)}| > 1.5$ were set as the strict thresholds. The visualization of DEmRNAs was using *Volcano map* and *pheatmap* package in R3.6.2.

Statistical analysis

Statistical analysis of all data was used the R programming language (Version 3.6.2) and statistical product and service solutions (SPSS) 15.0 software. The experimental data were expressed as means ± standard deviation (SD), and the results were repeated at least three times. The student's two tailed t-test were used for statistical analysis. Pearson correlation coefficient was used to calculate correlation between relative gene expression and Maxi LVWT. P<0.05 showed statistical significance.

Results

Construction of co-expression modules of HCM

A total of 14348 mRNAs were included in the WGCNA analysis. According to mRNA expression matrix, we clustered the samples and eliminated the outliers. Among the 28 HCM samples, there is no obvious outlier, which can be used for subsequent WGCNA analysis (Fig. 1A). Clinical and demographic data of 28 HCM patients were included sex, age, smoking, LAD, LVST, LVEDD, LVEF, Maxi LVWT, Maxi LVOTG and sample location (Table I). Based on mRNAs expression data and clinical data, we grouped the sample dendrogram and trait heatmap (Fig. 1B). According to the power value (β) and scale R^2 value, the independence and the average connectivity of the co-expression module were defined. We checked whether the memory network approximated scale free under the selected β value. As show in figure 2B, K was negatively correlated with P (K) (R^2=0.86), indicating that the selected β value could establish a scale-free gene network. As shown in Fig. 2A, the best value of the power value was 6 and the corresponding scale R^2 was 0.9. Therefore, we defined the adjacency matrix with β=6 and constructed the co-expression gene module. There were 31 co-expression modules of co-expressed genes in WGCNA analysis (Fig. 2C). The number of genes in the corresponding module was shown in Table II. The gene network was visualized using a heatmap which depicted the Topological Overlap Matrix (TOM) among top 400 genes in the analysis (Fig. 2D).

Co-expression modules related to clinical traits

GSE130036 provides the following clinical data, including sex, age, smoking, LAD, LVST, LVEDD, LVEF, Maxi LVWT, Maxi LVOTG and sample location. We drew the heatmap of the correlation between gene co-expression module and clinical traits. As shown in Fig. 3, the co-expression black module was significantly associated with Maxi LVWT (maximum left ventricular wall thickness, R =0.61, $p=5\times10^{-4}$). We further plotted the scatter plot of gene significance for Maxi LVWT and module membership in the black module. The high correlation can be revealed that gene significance for Maxi LVWT was highly associated with module membership in black module (cor=0.74, $p=2.8\times10^{-101}$, Fig. 4C). Therefore, we chose the genes in black module to further analysis.

The core genes were further screened by differential expression analysis

There were total of 291 mRNAs expressed differentially between HCM samples and the normal samples (pvalue <0.05 and $|\log^{FC}|$>1.5). Compared with the expression of mRNAs in HCM samples, 239 mRNAs (82.13%) were upregulated in the normal samples, while the 52 mRNAs (17.87%) were downregulated (Table S1). The differences in the expression of mRNAs between the normal samples and HCM samples were hierarchical clustering analysis and visualized by hierarchical heatmaps and volcano plots (Fig. 4A, B). CES1 and CTSC were obtained from the intersection of the genes with different expression and in black module (Fig. 4D). Compared with the expression of mRNA in normal tissues, mRNA expression of CES1 and CTSC was down regulated in HCM tissues (P<0.001, Fig. 5A). To further verify the lower expression of CES1 and CTSC in HCM, we analyzed another RNA sequencing data from GEO (GSE68316). As shown in Fig. 5B, compared with the expression of mRNA in normal tissues, mRNA expression of CES1 and CTSC was also down-regulated in HCM tissues (P<0.001). Pearson correlation coefficient was used to calculate correlation between relative gene expression and Maxi LVWT. As shown

in Fig. 5C, mRNA expression of CES1 and CTSC was negatively correlated with Maxi LVWT (cor=-0.48, P<0.001; cor=-0.31, P<0.001). In summary, mRNA expression of CES1 and CTSC was down-regulated in HCM and was negatively correlated with Maxi LVWT.

Clinical sample validation

In this study, 20 peripheral blood samples were obtained from HCM patients or healthy people between November 2020 and May 2021 in the Kunming Yan'an hospital (Kunming, China). We collected clinical information for all patients. The clinical features of the patients including sex, age, LAD, LVST, LVEDD, LVEF, Maxi LVWT and sample location were collected from their medical records (Table III). As shown in Fig. 6A, mRNA expression of CES1 and CTSC in HCM groups was decreased to 60.3±22.07% (P<0.001) and 82.1±25.43% (P<0.001) of the normal groups respectively. We further analyzed that mRNA expression of CES1 and CTSC was negatively correlated with Maxi LVWT in HCM patients (cor=0.54, p<0.001; cor=-0.34, p<0.001, Fig. 6B). In summary, the study confirms the abnormal low expression of CES1 and CTSC in HCM through clinical samples from HCM patients, and their expression was negatively correlated with Maxi LVWT.

Discussion

Hypertrophic cardiomyopathy (HCM) is the most common hereditary cardiovascular disease with an incidence rate ranging from 0.16%-0.29% in adult (Despond EA and Dawson JF. 2018). The main clinical symptoms of HCM are dyspnea, chest pain, elevated blood pressure, fatigue, dizziness and palpitation. Severe patients may have heart failure or even sudden death (Hensley N et al. 2015; Ellims AH. 2017). At present, molecular genetics research shows that at least 40 genes and more than 1400 gene mutations have been confirmed to be associated with the clinical phenotype of HCM (Maron BJ and Maron MS. 2013). Gene detection plays an important role in HCM. It can not only be used to identify the cause of disease, but also guide the family members of patients to carry out genetic screening. The research has shown that there are significant differences between abnormal expression of HCM genes and clinical manifestations, such as family history of HCM, family history of sudden cardiac death, maximum left ventricular wall thickness, etc (Li Y et al. 2018). Therefore, studying the association between differentially expressed genes and clinical traits is helpful to recognize the development mechanism of HCM and find new therapeutic targets for HCM.

Some studies investigated the abnormal expression of mRNA, microRNA (miRNA) and long noncoding RNA (lncRNA) in the pathogenesis of HCM and characterize their roles in HCM pathogenesis. For example, Wei Yang et al (Yang W et al. 2015) found that 1426 lncRNAs and 1715 mRNAs were aberrantly expressed in HCM patients and lncRNA-mRNA co-expression systems were mostly enriched in ribosome and oxidative phosphorylation. Some studies constructed the lncRNA-miRNA-mRNA regulatory networks based on differently expressed RNAs. For example, Jiajianghui Li et al (Li J et al. 2019) integrated four expression profiles (GSE36961, GSE36946, GSE68316 and GSE32453) and constructed lncRNA-miRNA-mRNA and protein-protein networks that regulated the process of HCM. In our study, RNA sequence data

of myocardial tissue in HCM patients was extracted from the GEO database and analyzed by WGCNA. We found that the expression of CES1 and CTSC was down regulated in HCM tissues, and negatively correlated with Maxi LVWT. We further verified the above conclusion in clinical samples from HCM patients.

Carboxylesterase 1 (CES1) encodes a member of the carboxylesterase large family. CES1 participates in fatty acyl and cholesterol ester metabolism, and plays a role in the blood-brain barrier system. More studies focus on the role of CES1 in liver diseases and drug metabolism (Her L and Zhu HJ. 2020; Yan M et al. 2019; Shi J et al. 2016). For example, Keun Na. et al (Na K et al. 2020) reported that CES1 exerted an anti-proliferation effect on hepatocellular carcinoma through the PKD1/PKCμ signaling pathway. Cathepsin C (CTSC) encodes a member of the peptidase C1 family and lysosomal cysteine proteinase. It is a central coordinator for activation of many serine proteinases in cells of the immune system. The research on CTSC focuses more on the association between CTSC gene mutation and Papillon Lefevre syndrome (Pap ÉM et al. 2020; Wu Y et al. 2020). For example, Nikoletta Nagy. et al (Nagy N et al. 2014) conducted a retrospective review which summarized the association between gene mutations of CTSC and Papillon Lefevre syndrome. However, CES1 and CTSC have not been studied in HCM. In our study, we found that CES1 and CTSC were abnormally expressed in HCM, and further found that they were negatively correlated with maximum left ventricular wall thickness (Maxi LVWT). The study suggests that CES1 and CTSC negatively regulate development of HCM, and have potential as a therapeutic and diagnostic target for HCM.

Declarations

Funding

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Competing interests

The authors state that there are no conficts of interest to disclose.

Availability of data

Public data are deposited at Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/, GSE130036 and GPL20795, GSE68316 and GPL20113). Gene database of NCBI (National Center of Biotechnology Information, https://www.ncbi.nlm.nih.gov/gene/). All data generated or analyzed during this study are included in this published article.

Authors' contributions

YK and HG were responsible for the overall design of the experiment and the writing of the paper; JW was responsible for the collection of clinical samples and information; YD performed the statistical and bioinformatics analysis; YW and YJ performed the qRT-PCR assay. YK and HG were responsible for the proofreading of experimental data. All authors read and approved the final manuscript.

Ethics approval

This study was approved by the Ethics Committee of the Kunming Yan'an hospital (Kunming, China; approval No. 2019-058-01) and all patients signed informed consent.

Consent to participate

Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.

Consent to publish

Not applicable

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Tables

Table I: Clinical features of HCM tissue samples from GSE130036

Clinical characteristics	
Age	
Media	32
Mean	33.4
Range	24-54
Sex	
Female	9
Male	19
Smoking	
Yes	10
No	4
Unknown	14
LAD (mm)	
Media	41
Mean	42.6
Range	36-62
LVST (mm)	
Media	20
Mean	20.8
Range	12-29
LVEDD (mm)	
Media	46
Mean	45.7
Range	35-51
LVEF (%)	
Media	69.5
Mean	70.4
Range	50-80
Maxi LVWT (mm)	

Media	26
Mean	25.2
Range	16-38
Maxi LVOTG (mmHg)	
Media	75.7
Mean	70
Range	30-126
Sample location	
Left ventricular septum	28

LAD: left atrial diameter；LVST: left ventricular septal thickness; LVEDD: Left ventricular end-diastolic; LVEF: left ventricular ejection fraction; Maxi LVWT: maximum left ventricular wall thickness;Maxi LVOTG: maximum left ventricular outflow track gradient at rest or after exercise

Table II. Number of genes in 31 co-expression modules.

Module color	Number of genes	Module color	Number of genes
black	578	turquoise	1676
blue	743	darkgreen	485
cyan	505	green	593
steelblue	75	pink	564
lightgreen	479	yellow	691
red	580	darkorange	448
magenta	553	lightcyan	494
white	446	greenyellow	549
purple	553	orange	456
tan	529	grey60	485
salmon	527	skyblue	398
darkgrey	462	midnightblue	505
darkturquoise	463	saddlebrown	377
brown	715	darkred	468
lightyellow	479	royalblue	471
grey	3008		

Table III: Clinical features of samples from HCM patients

Clinical characteristics	
Age	
Media	31
Mean	34.8
Range	24-54
Sex	
Female	10
Male	10
LAD (mm)	
Media	41
Mean	43
Range	33-59
LVST (mm)	
Media	19
Mean	20.2
Range	15-27
LVEDD (mm)	
Media	45.7
Mean	46
Range	32-51
LVEF (%)	
Media	69.5
Mean	69.8
Range	58-79
Maxi LVWT (mm)	
Media	26
Mean	24.2
Range	16-38
Sample location	

Peripheral blood	20

LAD: left atrial diameter；LVST: left ventricular septal thickness; LVEDD: Left ventricular end-diastolic; LVEF: left ventricular ejection fraction; Maxi LVWT: maximum left ventricular wall thickness.

Figures

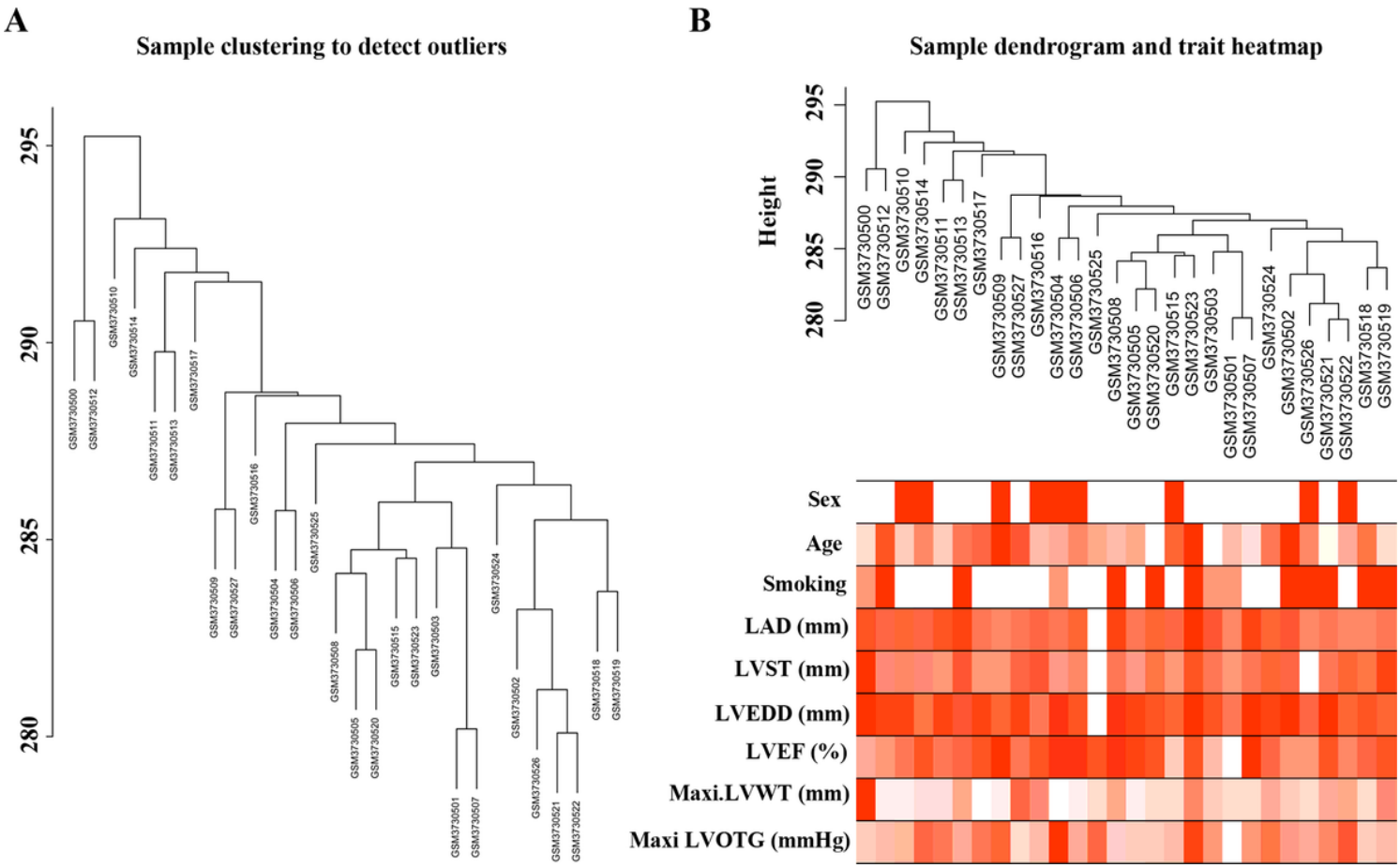

Figure 1

Sample cluster analysis based on RNA expression matrix from GEO database (GSE130036). a Sample cluster based on RNA expression data. **b** The sample dendrogram and trait heatmap based on mRNAs expression data and clinical data. LAD: left atrial diameter; LVST:left ventricular septal thickness; LVEDD: Left ventricular end-diastolic; LVEF: left ventricular ejection fraction; Maxi LVWT: maximum left ventricular wall thickness; Maxi LVOTG: maximum left ventricular outflow track gradient at rest or after exercise.

Figure 2

Construction of co-expression modules in HCM. a The various soft-threshold powers were analysed based network topology. Select the best soft threshold with β=6. The X-axis showed the soft-threshold powers. The Y-axis on the left showed the correlation between connectivity K and P (k). The Y-axis on the right showed the mean connectivity. **b** The verification of constructed scale-free network. R^2=0.86. slope=-1.04. **c** Clustering dendrogram of mRNAs and original modules were present. There were 31 co-expression modules of co-expressed genes in WGCNA analysis. **d** The gene network was visualized using a heatmap plot which depicted the Topological Overlap Matrix (TOM) among top 400 mRNAs in the analysis.

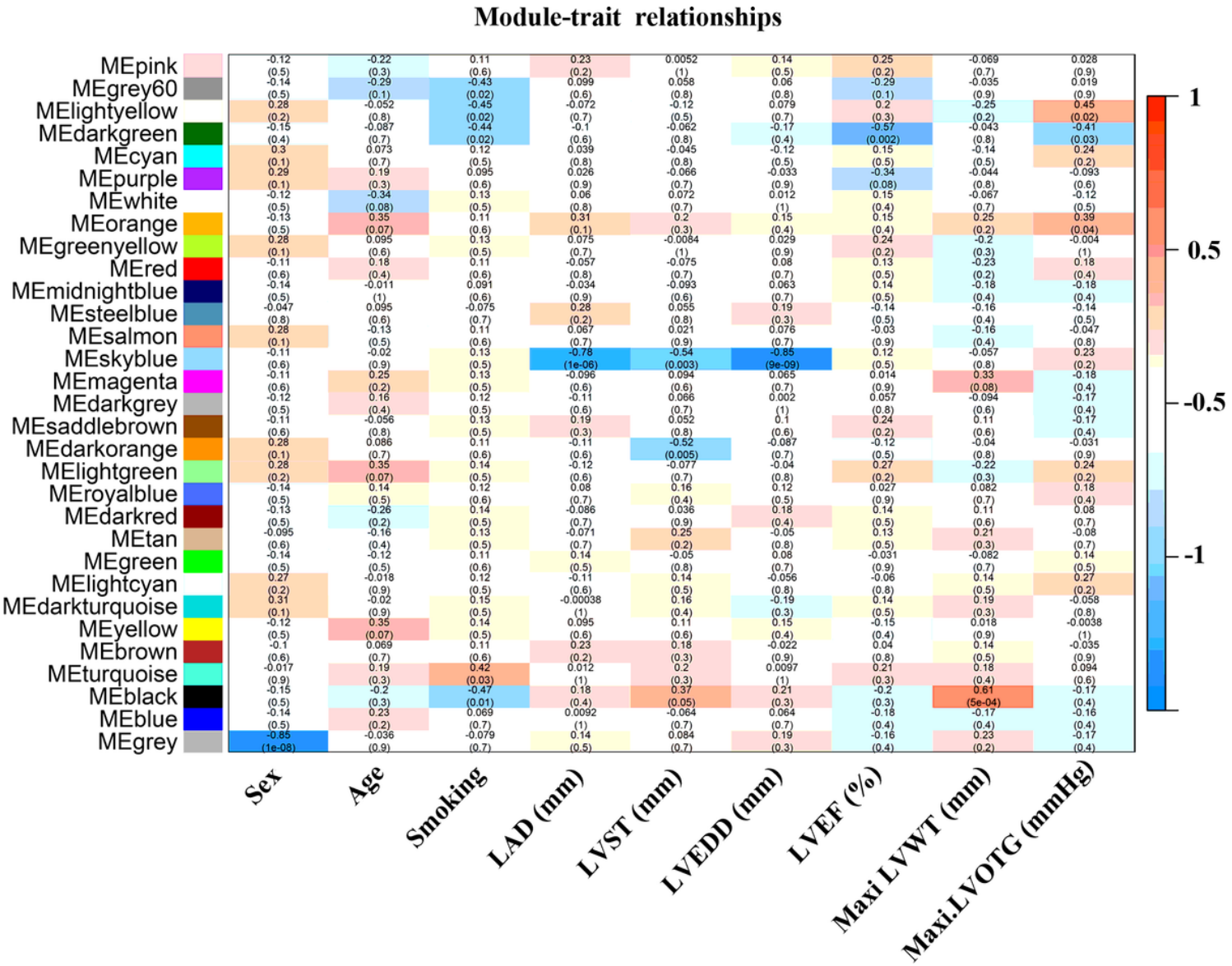

Figure 3

Co-expression modules related to clinical traits. Each rows represented the different co-expression modules. Each columns represented the different clinical traits. Each cells contains the corresponding correlation value and pvalue.

Figure 4

The core genes were further screened by differential expression analysis. a Visualization of differential genes in normal and HCM groups by hierarchical heatmaps. HCM groups vs. normal groups. The blue cells represent the down-regulated genes and the red cells represent the up-regulated genes. **b** Visualization of differentially expressed mRNAs in normal and HCM groups by volcano plot. The dots represented different genes. HCM groups vs. Control groups. The blue dots represent the down-regulated genes, the red dots represent the up-regulated genes. The grey dots represent not differential expressed genes. **c** The scatterplot of gene significance (GS) for Maxi LVWT vs. Module Membership (MM) in the black module. **d** CES1 and CTSC were obtained from the intersection of genes with different expression and genes in black module.

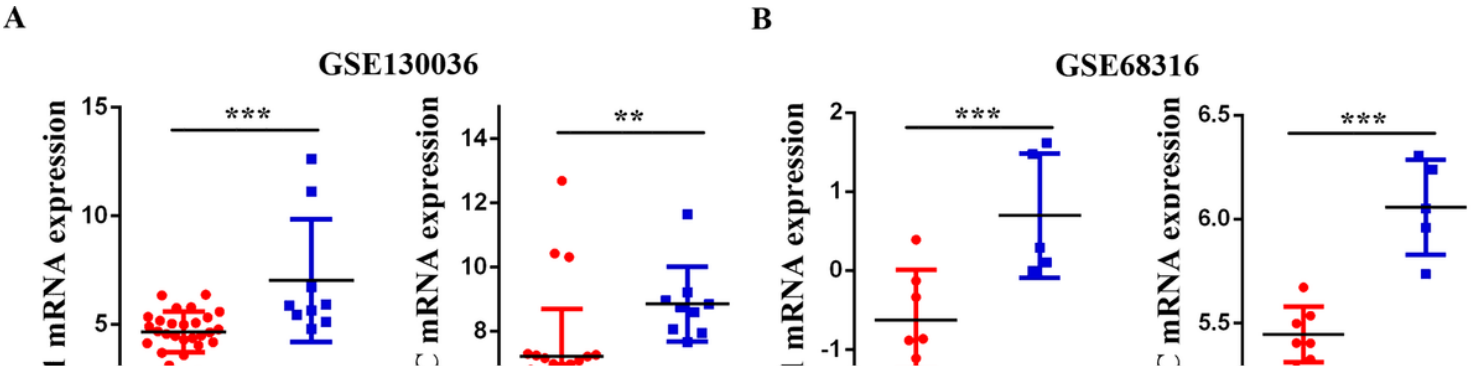

Figure 5

mRNA expression of CES1 and CTSC was down-regulated in HCM and was negatively correlated with Maxi LVWT. a The scatter plot showed mRNA expression of CES1 and CTSC in HCM and normal groups according to GSE130036 data. Normal groups vs. HCM groups, ***P<0.001, **P<0.01. **b** The scatter plot showed mRNA expression of CES1 and CTSC in HCM and normal groups according to GSE68316 data.

Normal groups vs. HCM groups, ***P<0.001. c mRNA expression of CES1 or CTSC was negatively correlated with Maxi LVWT. P<0.001.

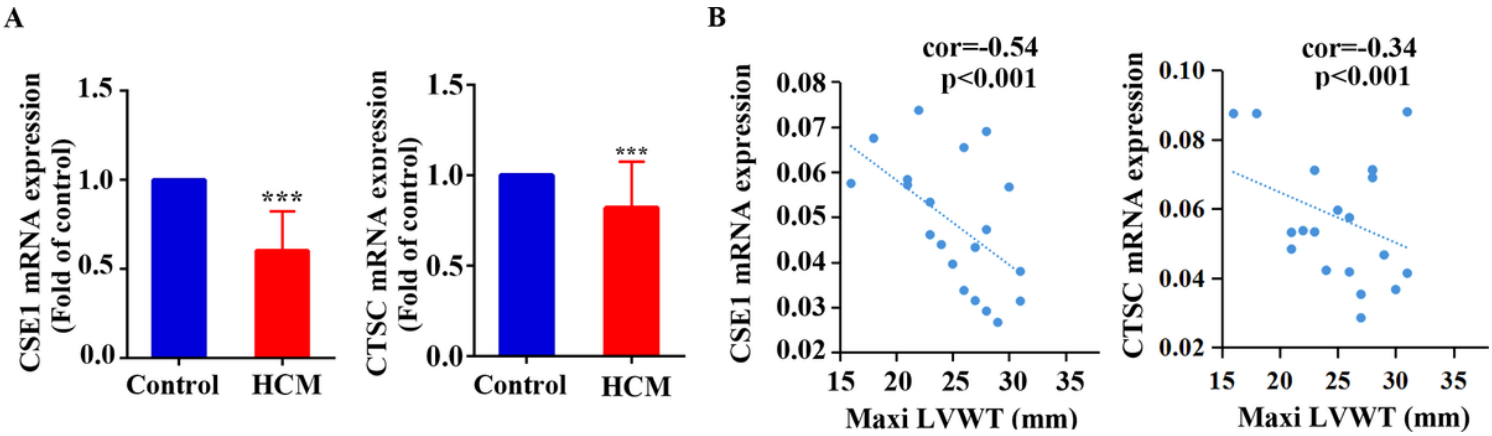

Figure 6

Clinical sample validation. a qRT-PCR assay. mRNA expression of CES1 and CTSC in peripheral blood from HCM patients and healthy people. Control groups vs HCM groups, ***P<0.001. **b** mRNA expression of CES1 or CTSC was negatively correlated with Maxi LVWT. P<0.001.

Supplementary Files

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

- TableS1.doc