Original Article

Differential mRNA Expressions in HCMV infected HUVECs*

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Abstract

Objective The aim was to identify the gene expressions of human cytomegalovirus (HCMV)-infected human umbilical vein endothelial cells (HUVECs) and to study its possible pathogenic mechanism on atherosclerosis using microarray technology.

Methods The gene expression differences in HCMV AD169 strain-infected HUVECs were studied by the microarray technology to explore the potential molecular mechanism of HCMV infection. The qPCRs were performed to verify the transcriptome results.

Results A total of 2,583 differentially expressed genes, including 407 down-regulated genes and 2,176 up-regulated genes, were detected by the systematic bioinformatics analysis. The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses showed that the significantly differentially expressed genes were mainly involved in regulating protein kinase activity, inflammatory response, ubiquitination, protein phosphorylation, cell metabolism, and exosomes, among which 12 genes had significant changes and were screened by protein-protein interaction (PPI) analysis and verified by qPCR. The experimental qPCR results were consistent with the microarray results.

Conclusion The GO and KEGG analyses revealed that the regulation of protein kinase activity, inflammatory response, ubiquitination, protein phosphorylation, and cell metabolism played important roles in the process of endothelial cell infection. Furthermore, 12 genes were involved in the process of HCMV infection of endothelial cells and contributed to the current understanding of the infection and pathogenic mechanisms of atherosclerosis.

Key words: Cytomegalovirus; Endothelial cells; Atherosclerosis; Gene expressions; Cell injury; RNA microarrays

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INTRODUCTION

Human cytomegalovirus (HCMV) is a ubiquitous β-herpesvirus that causes opportunistic infection and is present throughout life in most of the world's population[1,2]. HCMV has long been considered relatively non-pathogenic to immunocompetent adults. However, under the appropriate conditions, HCMV infection can cause serious complications in immunocompromised individuals; In newborns, primary HCMV infection can lead to life-threatening diseases and may be associated with certain congenital defects in the nervous system[3-5]. In addition, chronic vascular infection of HCMV is related to the development of cardiovascular diseases, such as atherosclerosis, restenosis, and graft sclerosis, which may be related to the activation of immune and inflammatory pathways by CMV infection[6,7].

Cardiovascular and cerebrovascular diseases caused by atherosclerosis have gradually become the leading cause of death worldwide[8]. An increasing number of studies have shown that HCMV is an important risk factor for atherosclerosis. Endothelial dysfunction is an early event in atherosclerosis pathophysiology, and endothelial cells (ECs) are the *in vivo* targets of HCMV infection and play a key role in viral pathogenesis[9]. Besides, ECs are the first point of HCMV infection and are a potential viral reservoir for virus persistence and propagation[10]. HCMV infection can cause endothelial cell dysfunction and vascular wall damage, which then induces inflammation and leads to the formation of atherosclerosis. The importance of ECs in the occurrence and development of atherosclerosis has long been recognized, that is, the condition of the endothelium determines the adhesion state of blood vessels, the tension and permeability of blood vessels, and the potential for thrombosis[11].

Numerous studies have revealed that HCMV infection plays an important role in affecting the status of ECs. HCMV infection can cause ECs injury and promote the expression of Intercellular Cell Adhesion Molecule-1 (ICAM-1), Vascular Cell Adhesion Molecule-1 (VCAM-1), and Endothelial Leukocyte Adhesion Molecule-1 (ELAM-1)[12]. The expression of these factors in vascular ECs enhances the adhesion of ECs, mediates the binding of inflammatory cells such as neutrophils and monocytes to ECs, activates and damages ECs, causes local vascular inflammation, and promotes the pathological process of atherosclerosis. It has been suggested that cytomegalovirus infection of ECs results in the secretion of viral proteins and mature viral particles, which are then presented to smooth muscle cells by adjacent ECs[13]. In addition, HCMV infection can increase the permeability of endothelial cells and induce monocyte recruitment and migration across ECs[14]. Recent studies have shown that HCMV can affect the expression of matrix metalloproteinases in ECs and smooth muscle cells, and affect the stability of atherosclerotic plaques[11]. Furthermore, HCMV infection induces associated inflammation, leads to an imbalance of thrombotic/ antithrombotic function of ECs, increases platelet adhesion to ECs, and increases the risk of thrombosis[11,15]. However, the molecular mechanisms of endothelial cell injury and atherosclerosis caused by HCMV infection are still poorly understood.

In this study, we established an HCMV infection model with human umbilical vein ECs (HUVECs) to simulate the changes in arterial endothelium caused by HCMV infection during atherosclerosis; notably, there was no significant difference between venous and arterial-derived ECs. We aimed to identify genes that were significantly differentially expressed in ECs infected with HCMV by microarray. Using bioinformatics analysis, the differential expression of genes before and after HCMV infection in ECs was systematically analyzed to explore possible mechanisms of viral infection and endothelial cell injury, as well as possible molecular targets for treatment.

MATERIALS AND METHODS

Cell Culture and Virus Infection

HUVECs were purchased from Procell Life Science & Technology Co., Ltd and cultured in F12K basic medium (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA), 1% streptomycin (Solarbio, China), and 1% endothelial growth factor (Science cell, USA) at 37 °C, 5% CO_2, and 95% air humidity. The HCMV used in this study was the AD169 experimental strain, which has been widely propagated *in vitro* as a standard strain. After the cell confluence reached 80%–90%, HCMV virus suspension with a multiplicity of infection (MOI) of 0.01 was inoculated into HUVECs. After 2 hours of CO_2 incubation with the cells, the culture medium was changed to the medium containing 2% foetal bovine serum (FBS); After 24 hours of incubation, the cells were collected and centrifuged at 3,000 rpm for 5 min twice with phosphate buffer saline (PBS). The PBS was used as the mock-treated group

as a control versus the virus.

RNA Extraction and Microarray Hybridization

The Trizol reagent (Invitrogen, USA) extracted total RNA from samples. Nanodrop 2000 (Thermo, USA) and Agilent 2100 Bioanalyzer were used for quantitative and quality evaluation of RNA, respectively. RNA samples with the optical density of 1.7 < A260/A280 < 2.2 and biological analyzer results with RIN ≥ 7.0 and 28S/18S > 0.7 were used for further experiments. According to the manufacturer's protocol, the cells were sequenced on the Clariom S array (Affymetrix, USA). The GeneChip scanner 3000 (Affymetrix, USA) was used for signal detection and quality checking of expression Console Software (Affymetrix, USA). The differential expression between the two groups (three biological replicates per group) was compared using DESeq2 software (1.6.1). A model based on the negative binomial distribution was used to determine differential expression in digital gene expression data. Benjamin and Hochberg's method adjusted the resulting *P*-value to control the false detection rate (FDR)[16]. The *P*-value < 0.05 and |Fold Change| ≥ 2.0 were used as screening criteria for significantly different genes.

Gene Ontology (GO) and Pathway Enrichment Analysis

The David database (https://david.ncifcrf.gov) was used to analyze differential genes on Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) after screening (https://www.kegg.jp). The GO analysis includes a biological process (BP), molecular function (MF), and cellular component (CC).

Differential Gene and Protein Network Interaction Analysis

The interaction between significantly differentially expressed genes was analyzed through the STRING (a search tool for the retrieval of interaction gene/proteins) database and the protein interaction network was constructed based on the known and predicted interactions between proteins.

Validation of Gene Expression Microarray Data Using qPCR

We designed the primers to validate the transcriptome results by qPCR for 12 significantly altered genes (Table 1). Total RNA was extracted using the Trizol method, and RNA was reverse transcribed into cDNA using ReverTra Ace qPCR RT Kit. The SYBR® Green Realtime PCR Master Mix system and a 7500 real-time PCR system (Thermo, USA) were applied for detection. Reactions were performed under the following amplification conditions: initial denaturation at 95 °C for 60 s, followed by 40 cycles of reaction at 95 °C for 15 s and 60 °C for 60 s. The $2^{-\Delta\Delta CT}$ method was used to analyze the expression level of related genes, and the β-Actin gene was used as an internal reference to compare the differential expression of different selected genes with the control group.

RESULTS

Differential mRNA Expression Profiles of HUVECs Infected with HCMV

To determine the differential mRNA expression

Table 1. Primers used for qPCR in this study

Genetic element	qPCR product size (bp)	Sequence (5'–3')
ESCO1 (F)	121	AACGCAAGGTAGAACATCAGACAGC
ESCO1 (R)		TCAGAAGTTACAGGCACAGGTTTCG
SMC3 (F)	104	AAGGAGATGTGGAGGGCAGTCAG
SMC3 (R)		AAACTGGTCAACTGATGGGACACTG
SMC4 (F)	101	CCGCCTCCAGCAATGACCAATG
SMC4 (R)		CCCAGAATTTTCTCCCCAGCATAGG
RAD50 (F)	87	TTTGGCATTAGGGCGACAGAAAGG
RAD50 (R)		TCAGCATCCCGAAATTGTGGTTCTC
KIF18A (F)	92	TGTCACCATTTGCACCTCCAGAAC
KIF18A (R)		TGCCTGTGTTGCTGTTCCTGAAG
RPS9 (F)	114	GCTGAAGCTGATCGGCGAGTATG
RPS9 (R)		TTCTCATCAAGCGTCAGCAGTTCC
IMP3 (F)	140	GGACTACACGCGCTACAACCAG
IMP3 (R)		GCACCAAGCCGAGAGCATACAG
ENO1 (F)	81	GTACCGCCACATCGCTGACTTG
ENO1 (R)		GAACCGCCATTGATGACATTGAACG
EIF3G (F)	91	CCGTGTCACCAACTTGTCAGAGG
EIF3G (R)		AGCCAGGTAGATGCGGGAGATG
ALDOC (F)	105	GTTCATCAAGCGGGCTGAGGTG
ALDOC (R)		TGGTTGGCAATGTAGAGTGACTGTG
PGAM1 (F)	115	AGCATCTGGAGGGTCTCTCTGAAG
PGAM1 (R)		AGAAACTGCATGGGCTTGATAGGC
RECQL4 (F)	121	GCCTTTCACAGCGTAGCCTTCC
ECQL4 (R)		GCTCCTGCCCTTCCTCTTCCTC

profile of ECs before and after HCMV infection, we performed principal component analysis (PCA) on 21,448 gene expression data obtained by microarray and analyzed the dispersion of gene expression in each group. The results showed significant separation between the infected and control groups, and the intra-group repeatability was valid (Figure 1A). Using the criterion of *P*-value < 0.05 and |Fold Change| ≥ 2.0, we detected 2,583 differentially expressed genes, including 407 down-regulated and 2176 up-regulated genes (Figure 1B). Thus, the data suggest that 24 hours of HCMV infection leads to altered gene expressions of HUVECs.

GO Enrichment Analysis of Differentially Expressed Genes

To observe the functional changes of ECs infected with HCMV, we performed GO enrichment analysis from three different aspects: biological process (BP), cell composition (CC), and molecular function (MF). The up-regulated genes in BP were significantly enriched in transcription, regulation of transcription, protein phosphorylation, cell cycle, and epidermal growth factor receptor signaling pathway (Figure 2A); the down-regulated genes were mainly enriched in canonical glycolysis, SRP-dependent co-translational protein targeting to membrane, viral transcription, and cell-cell adhesion (Figure 2B). The CC up-regulation genes were mainly enriched in the nucleus, cytoplasm, membrane, intracellular, and microtubule cytoskeleton (Figure 2C); the down-regulated genes were mainly enriched in extracellular exosome and cytosol (Figure 2D). MF up-regulated genes were enriched in nucleic acid binding, protein binding, poly(A) RNA binding, transcription factor activity, and ATP binding (Figure 2E); down-regulated genes were enriched in cytochrome-c oxidase activity and cell adhesion (Figure 2F).

Pathway Enrichment Analysis of Differentially Expressed Genes

To explore the molecular mechanism of HCMV infection, we performed enrichment analysis using the KEGG database. KEGG is a comprehensive database that integrates genomic, chemical, and systemic functional information better to understand the molecular response network of coding genes. The data showed that these differentially expressed genes were mainly enriched in ubiquitination, cell cycle, FOXO signaling pathway, mTOR signaling pathway, intercellular junctions, TNF signaling pathway, oxidative phosphorylation, and carbon metabolism. The KEGG results indicated ubiquitination was an important process in viral infection (Figure 3C).

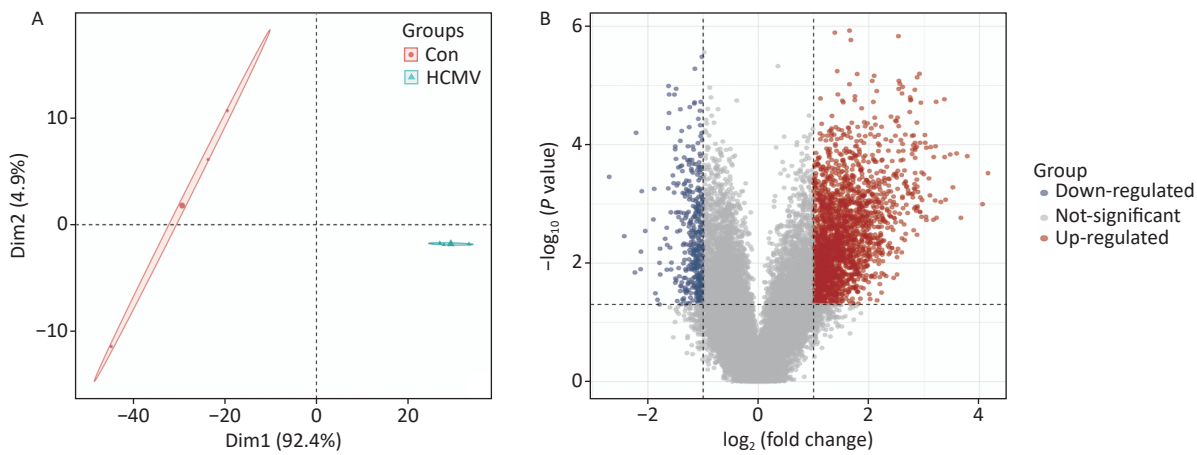

Figure 1. HCMV infection alters the mRNA expression profile of HUVECs. Visualization of differential gene data on endothelial cells infected with HCMV was performed by principal component analysis (PCA) and volcanic mapping. (A) PCA. Red dots indicate the results of blank control, and blue dots indicate the results after 24 hours of HCMV infection. (B) Volcanic map. The abscissa is the multiple of the difference (logarithmic transformation with base 2), and the ordinate is the *P*-value of the significance of the difference (logarithmic transformation with base 10). The genes were screened with the criteria of |Fold Change| ≥ 2.0 and *P*-value < 0.05. The red dots indicate a significant up-regulation of the genes; the blue dots indicate a significant down-regulation of the genes, and the gray dots indicate no significant difference.

PPI Network of Differentially Expressed Genes

To explore and predict the interactions of significant differentials, we analyzed the protein-protein interaction network (PPI) of the 30 largest differentials of up-regulation using the STRING database and selected the five most important genes as establishment of sister chromatid cohesion N-acetyltransferase 1 (*ESCO1*), structural maintenance of chromosomes 3 (*SMC3*), structural maintenance of chromosomes 4 (*SMC4*), RAD50 double strand break repair protein (*RAD50*), and kinesin family member 18A (*KIF18A*) (Figure 4A). The PPI analysis was performed on the 50 genes with the largest down-regulation changes, and seven important genes were selected, i.e.,

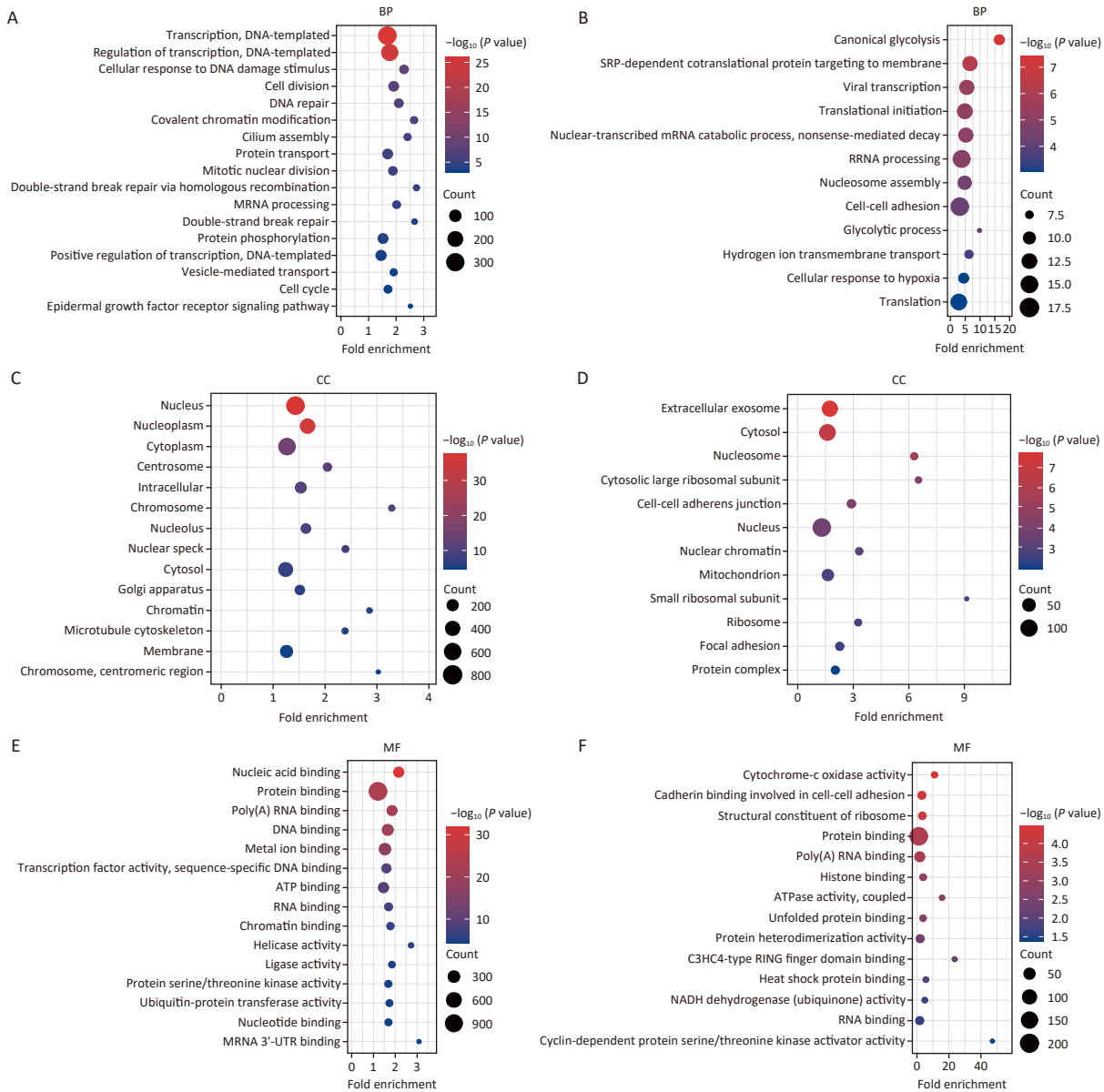

Figure 2. Go enrichment results: (A) BP enrichment results of up-regulated genes. (B) BP enrichment results of down-regulated genes. (C) CC enrichment results of up-regulated genes. (D) CC enrichment results of down-regulated genes. (E) MF enrichment results of up-regulated genes. (F) MF enrichment results of down-regulated genes. The X-axis represents the fold enrichment, and the Y-axis represents Go enrichment results. The size of bubbles represents the number of genes, and the color of bubbles represents -$\log_{10}$ *P*-value. BP, biological process; CC, cell composition; MF, molecular function.

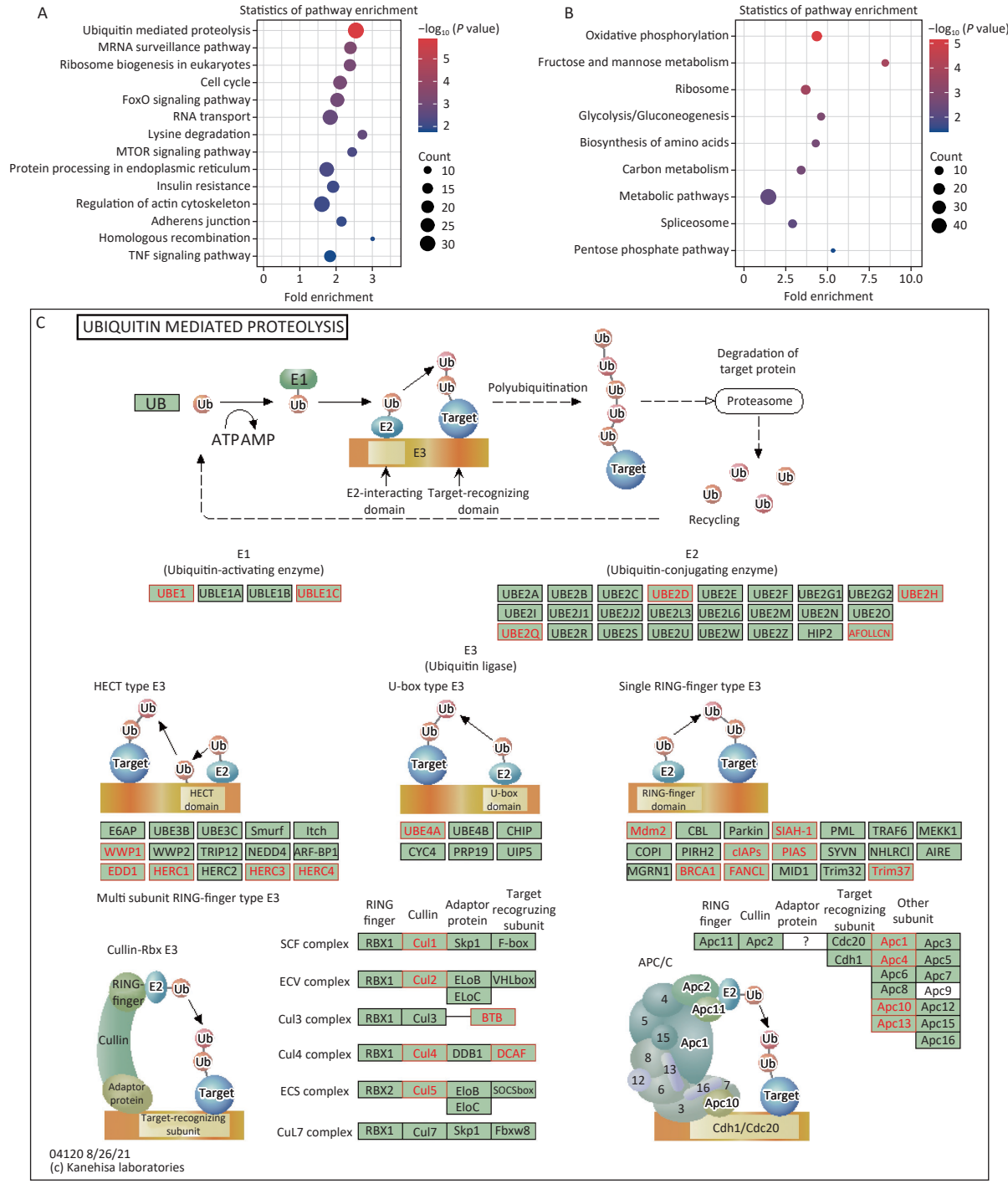

Figure 3. Differential gene analysis using the Genes and Genomes (KEGG) database. The KEGG database was used to analyze the enrichment of up-regulated and down-regulated genes, and the enriched pathways were visualized. (A) KEGG pathway enrichment results of up-regulated genes. The X-axis represents the fold enrichment, the Y-axis represents the KEGG pathway, the size of the bubble represents the number of genes, and the color of the bubbles represents -$\log_{10}$ *P*-value. (B) KEGG pathway enrichment results in down-regulated genes. The X-axis represents the fold enrichment, the Y-axis represents the KEGG pathway, the size of the bubbles represents the number of genes, and the color of the bubble represents -$\log_{10}$ *P*-value. (C) The role of the enriched gene in the Ubiquitin mediated proteolysis process. The red dots are enriched for up-regulated differential genes (www.kegg.jp).

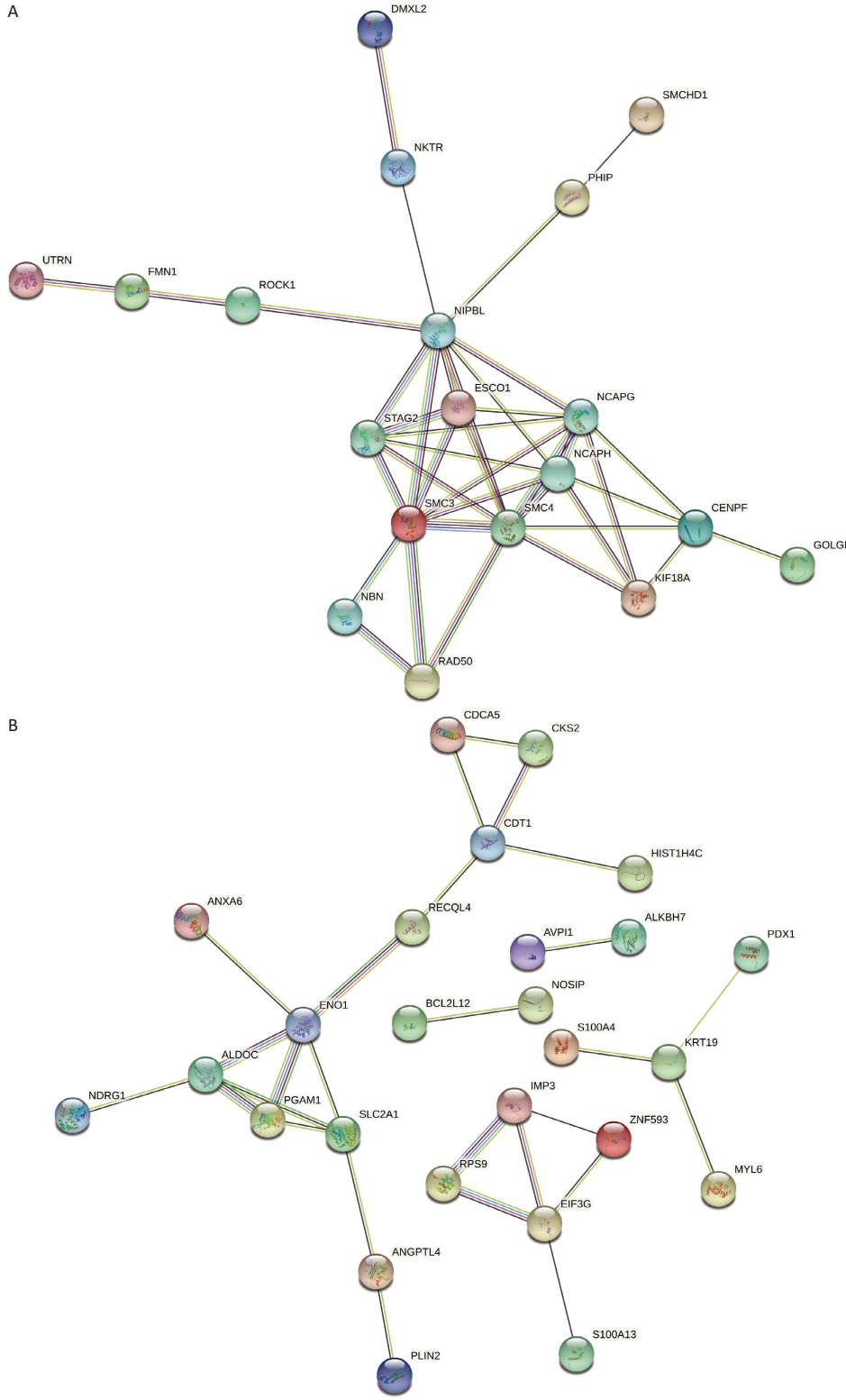

Figure 4. PPI network on HCMV viral proteins. The protein-protein interaction networks of proteins encoded by significant differential genes after HCMV infection in ECs were analyzed by the STRING database to explore and predict the interaction of differential genes. (A) Construction of a PPI network for the 30 up-regulated genes. (B) Construction of a PPI network for the 50 down-regulated genes. PPI, protein-protein interaction; HCMV, human cytomegalovirus; ECs, endothelial cells.

ribosomal protein S9 (*RPS9*), IMP U3 small nucleolar ribonucleoprotein 3 (*IMP3*), enolase 1 (*ENO1*), eukaryotic translation initiation factor 3 subunit G (*EIF3G*), aldolase, fructose-bisphosphate C (*ALDOC*), phosphoglycerate mutase 1 (*PGAM1*), and RecQ like helicase 4 (*RECQL4*) (Figure 4B). These 12 genes were selected because they are the hubs genes in the PPI network.

qPCR Verification of the Transcription

To verify the results of the bioinformatics analysis, we screened 12 important genes using the PPI network showing that they play an important role in HCMV infection of ECs, and validated them using qPCR. The results showed that after HCMV infection of ECs, the expression of *ESCO1*, *SMC3*, *SMC4*, *RAD50*, and *KIF18A* increased compared with normal cells, while the expression of *RPS9*, *IMP3*, *ENO1*, *EIF3G*, *ALDOC*, *GPAM1*, and RECQL4 decreased compared with controls, which is consistent with our analysis (Figure 5).

DISCUSSION

Recent studies have shown that CMV infection is closely related to atherosclerosis. The association between atherosclerosis and HCMV was first reported by Adam et al. in 1987. Since then, many epidemiological studies and etiological analyses have been carried out. Increasing evidence has shown that HCMV infection can induce atherosclerosis[11,17,18]. More studies on the mechanism of atherosclerosis caused by HCMV infection have been studied in recent years, but the related mechanism has not been understood. Previous studies have focused on HCMV infection of monocytes, T lymphocytes, smooth muscle cells, and ECs. As the lining layer of blood vessels, ECs are the first barrier to protect blood vessels. Thus, ECs can be infected by viruses firstly and can serve as a reservoir for viruses, which is conducive to virus latency or spread[19]; the vascular ECs also serve as the initial site of atherosclerosis formation, so ECs are considered being the key targets of atherosclerosis caused by HCMV infection.

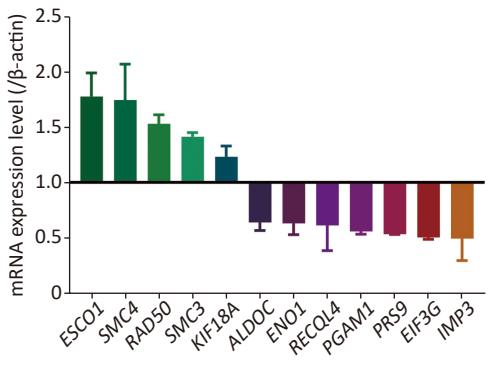

Figure 5. Validation of differential gene expression using qPCR. *ESCO1*, *SMC3*, *SMC4*, *RAD50*, *KIF18A*, *RPS9*, *IMP3*, *ENO1*, *EIF3G*, *ALDOC*, *PGAM1*, and *RECQL4* genes were detected by qPCR after HCMV infection of endothelial cells with MOI for 24 hours. The β-actin was used as the internal reference of the qPCR, and a single bar graph showed the relative expression of the gene compared with uninfected cells, with a mean value of ± SD, $n = 3$.

HCMV infection can lead to endothelial cell damage and affect various intracellular regulatory pathways, but the mechanism of pathogenesis after infection remains unclear. Microarray technology has been widely used in virus research, including exploring the mechanism of early neurodevelopmental abnormalities and cytokine regulation caused by HCMV infection and the molecular mechanism of HCMV infection and systemic sclerosis[20,21]. In this study, an HCMV-infected endothelial cell model was established, and gene expression in ECs after virus infection was detected by the microarray technology. A total of 21,448 gene expression data were detected, and 2,583 differentially expressed genes were identified by bioinformatics analysis, including 407 down-regulated and 2,176 up-regulated genes. HCMV infection has been reported to induce ECs to secrete inflammatory cytokines, such as IL-6, which can promote microvascular formation and a chronic inflammatory environment to promote atherosclerosis[11,22]. In this study, IL-6 expression was also observed dramatically increasing, which was in accordance with the results of previous studies. Yi Ma et al. identified multiple novel biomarkers of pulmonary hypertension, another common vascular disease, based on multi-microarray analysis. Among them, SMC3, SMC4, RAD50, and the Kinesins Family (KIF) genes were the same as those found in this study[23]. In addition, PRS9 was identified as a genetic susceptibility site for Takayasu arteritis in a genome-wide association study[24]. These results suggest these gene changes may co-exist in vascular diseases.

We then performed GO and KEGG enrichment analyses on differential genes. The GO BP enrichment found that the up-regulated genes were

mainly enriched in transcription, transcriptional regulation, protein phosphorylation, cell cycle, and epidermal growth factor signaling pathway, among which the EGFR signaling pathway attracted our interesting. Current studies have shown that EGFR is related to the pathophysiological process of atherosclerosis. Tamura et al. found that EGFR is expressed on intimal smooth muscle cells in human atherosclerotic plaque through immunohistochemical localization. The studies on EGFR in rat aortic smooth muscle cells showed that the EGFR signaling pathway mediates cell proliferation and DNA synthesis[25-27]. HCMV infection may promote atherosclerosis by activating the EGFR signal pathway and regulating cell proliferation. The down-regulated genes are mainly enriched in the aspects related to cell metabolism represented by glycolysis. Under normal physiological conditions, ECs produce 85% ATP through anaerobic glycolysis and also promote the glycolysis side pathway, namely the pentose phosphate pathway (PPP), to produce NADPH for the regeneration of reduced glutathione (GSH), a key antioxidant[28].

Interestingly, previous studies have shown that increased glycolysis in ECs in areas prone to vascular atherosclerosis causes inflammation and arteriosclerosis. The research results by Yang et al. explained this situation as follows: The glycolysis of ECs in atherosclerotic prone areas can protect blood vessels from or reduce the onset and development of vascular atherosclerosis, and the reduction of glycolysis will reduce the proliferation rate of ECs, increase the permeability of vascular endothelium, and accelerate the formation of atherosclerosis[29,30]. Therefore, HCMV infection reduces the glycolysis of ECs, increases the permeability of vascular endothelium, and promotes the formation of atherosclerosis. In this study, the down-regulation of ENO1, ALDOC, PGAM1, and other genes can impair cell glycolysis[31-33]. The metabolic dysfunction caused by the virus may become a novel research direction for cardiovascular and cerebrovascular diseases such as atherosclerosis.

The KEGG enrichment analysis showed that many differentially expressed genes were enriched in the FOXO, mTOR and TNF signaling pathway. Shimba et al. found that overexpression of FoxO1 inhibited the progression of atherosclerosis in ApoE KO mice[34]. FoxO1 is a key regulator of endothelial cell metabolism. Modification of FoxO1 by phosphorylation, methylation, glycosylation, acetylation, and ubiquitination can produce different functional properties[35]. Therefore, the FOXO signaling pathway has become a new target for studying HCMV induced atherosclerosis. Studies have shown that mTOR signaling plays an important role in the process of atherosclerosis or atherosclerosis-related diseases. The activation of the mTOR signal promotes endothelial cell dysfunction and foam cell formation by enhancing the process of monocyte to macrophage transformation[36]. Growing evidence suggests that mTOR inhibition has great potential in the treatment of atherosclerosis[37,38]. In addition, mTOR inhibitors can prevent CMV infection after organ transplantation[39]. mTOR pathway has become a dual-target to prevent CMV infection and treat atherosclerosis. Gao et al. showed that HUVECs aggravate inflammation and atherosclerosis through the TNF-α mediated NF-κB pathway, while TNF-α Antagonists can slow down the process of atherosclerosis[40,41]. HCMV infection can promote TNF-α, suggesting that HCMV may activate TNF signaling pathway by infecting ECs, aggravating inflammatory response, and accelerating the process of atherosclerosis[42]. In addition, KEGG enrichment analysis found that 32 differentially expressed genes were enriched in the ubiquitination mediated proteolysis pathway. More evidence has shown that ubiquitination plays an important role in the pathogenesis of atherosclerosis, including regulating vascular inflammation, endothelial and vascular smooth muscle cell function, lipid metabolism, and atherosclerotic plaque stability[43,44]. Liu et al. demonstrated that IE1 is an E3 ubiquitin ligase encoded by HCMV, which establishes a bridge between HCMV and related disease mechanisms and provides a new direction for studying HCMV infection in atherosclerosis[45].

In summary, we screened differentially expressed genes in HCMV-infected ECs by microarray technology and bioinformatics methods. The GO and KEGG enrichment analyses and the construction of a binding protein-protein interaction network helped to understand the genes associated with HCMV infection of ECs and inducing atherosclerosis. It lays a foundation for future research on related mechanisms, puts forward a research direction, and provides a target for preventing and treating atherosclerosis caused by HCMV-infected ECs. It has to be noted that the results were only observed at the transcriptional level and were not always consistent at the protein level. The study only involves *in vitro* experiments that may not fully reflect the *in vivo* environment. Therefore, further studies *in vivo* are needed to

better understand the host-pathogen interaction and pathogenesis.

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