Molecular Characterization of the Oncogenic Potential and Mechanisms of Cytomegalovirus Infecting MRC-5 Cells

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Abstract

Background Human cytomegalovirus (HCMV), a member of herpes virus family, is associated with different cancers in humans, including breast and colorectal cancers.

Objectives To shed light on the possible oncogenic mechanisms exerted by HCMV on tissues at the genetic level.

Methods HCMV was propagated in medical research council-5 (MRC-5) cell line and cytopathic effects of HCMV were checked for confirming infection. Oncogenic mechanism that exerted by HCMV was screened using high-throughput real-time qPCR microarray to investigate the mRNA expression of 983 genes in 16 gene families associated with oncogenesis in human cells.

Results Twenty differentially expressed genes. Upregulated genes (SCGB1A1, MAP2K3, ITGB3, TGFB1, PTN, MMP2, AKT1, AKT2, BCL2L10, CKS1B, SERPINB4, CASP4, TOLLIP, BCL-2, SERPINB5) were mostly of oncogenic or oncopromoting activity, involved mainly in MAPK, PI3K/Akt, inflammation, and angiogenesis pathways while downregulated genes (STAT3, BAK1, BLM, RB1, and IGF2R) were mostly tumor suppressor, proapoptotic, and DNA damage repair genes.

Conclusion The current study revealed that HCMV might have an oncogenic or oncomodulating activity on normal infected cells by different intracellular pathways.

Keywords Cytomegalovirus, oncogenic viruses, mrc-5, Waffergen, SmartChip Real-Time PCR System.

List of Abbreviation: HCMV =Human cytomegalovirus, MRC-5 = Medical research council-5

Introduction

Human cytomegalovirus (HCMV) is a ubiquitous herpes virus that leads to a life-long persistence. The frequency of infection ranges from 50-100% in the community [1]. HCMV, species *Human herpes virus 5*, belongs to genus *Cytomegalovirus*, subfamily *Betaherpesvirinae*, family *Herpesviridae*. HCMV is a leading agent of complications of organ transplantation, congenital malformations, non-heterophil infectious mononucleosis, and life-threatening morbidities in immunocompromised individuals [2]. In addition, active and/or latent infection of HCMV has been recently linked with different human cancers including breast, colorectal, prostate, and malignant glioma cancers [3].

In vivo, HCMV is capable of infecting many cell types including fibroblasts, endothelial cells, smooth muscle cells, hepatocytes, macrophages and lymphocytes, and tumor cells [4]. Medical research council-5 (MRC-5) cells are human diploid fibroblast cell line approved for vaccine production and favored for the routine propagation of HCMV [5]. MRC-5 cells have been used as a standard for over 30 years in basic research for HCMV infection. Moreover, MRC-5 cells have the ability of 46 cells doublings, enabling prudent exploit of these cells in assessing intracellular changes after exposure to HCMV [5].

The relationship between HCMV infection and cancer has been investigated for decades. Presence of HCMV DNA, mRNA, and its proteins in tumor tissues indicated a probable role of HCMV infection in the causation of several human malignancies [2]. Moreover, sero-epidemiologic evidence added more linkage between HCMV and human cancers [6]. It was shown that HCMV can transform normal human embryonal cells in vitro [7]. Other studies reported that HCMV infection in normal rodent cells affects cellular genes that are critical for malignant transformation [8,9]. However, there is controversy on the carcinogenic effect of HCMV on normal cells. It was suggested the oncomodulation concept of HCMV, which refers to the capability of HCMV to preferentially infect tumor cells and enhance their malignant profile lead to a shift to a more malignant phenotype of tumor cells and tumor progression [10]. Several studies showed that the presence of HCMV in tumor cells but not in adjacent normal tissue was more than 90% of patients with certain malignancies, such as colon cancer, malignant glioma, prostate carcinoma, and breast cancer [11-14]. Nevertheless, in the context of the oncogenic or oncomodulation scenarios, by all means, HCMV, seems to have a direct or indirect role in carcinogenesis.

There have been numerous studies on the oncogenic and oncopromoting potential of HCMV on tumor cell lines such as neuroblastoma cell lines [15-17]; however, no such coverage was done on the oncogenic potential of HCMV, if any, on normal cell lines such as MRC-5 cells using high throughput quantitative PCR microarray. Therefore, this study aimed at covering a different aspect of HCMV relationship with cancers as this study focused on the changes at genetic expression of normal cells, MRC-5 cell line, as a response to HCMV infection. This approach is believed to shed light on HCMV oncogenic/ oncomodulating potential on normal cells rather than previously researched tumor cells. Hence, this study was designed to investigate the mechanisms of oncogenesis, or oncomodulation, if any, by HCMV on normal diploid cells by using a high throughput SmartChip quantitative real-time PCR microarray. This study explored 983 genes that may have a role in carcinogenesis and transformation of human cells and tried to give a complete map for the target genes which might be up- or down- regulated by this virus. This will facilitate the understanding of the possible oncogenic and/or oncomodulating mechanisms exerted by HCMV on human cells.

Methods

This study was conducted in multidiscipline laboratories of Kent State University. It is a single authored project funded by Kent State University, Ohio, USA and Fulbright organization and was conducted during the period from July 5th to September 15th 2012.

Cells and viruses

MRC-5 human diploid fibroblasts passage 6 was received from American Tissue Culture Collection (ATCC CCL-171, USA). Cells were cultured in Eagle's minimum essential medium (EMEM) supplemented with Earle's salts containing 10% (v/v) fetal calf serum, $1x10^6$ IU/l penicillin, 120 mg/l streptomycin and 2 mM glutamine. Subcultivation ratio of CCL-171 was 1:2 to 1:5 and the medium renewal once every 5-6 days. Flasks of 75 qcm were used in propagation of MRC-5 cells and incubated at humid 37 °C with 5% CO_2 incubator.

MRC-5 cells were subcultured by removing and discarding culture medium. Briefly by rinsing the cell layer with 0.25% (w/v) Trypsin 0.53 mM EDTA solution to remove all traces of serum, which contains trypsin inhibitor. Then, 2.0 to 3.0 ml of Trypsin-EDTA solution were added to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes). Cells that are difficult to detach may be placed at 37 °C to facilitate dispersal. Afterwards, 6.0 to 8.0 ml of EMEM complete growth medium were added and resultant cell suspension was

aspirated by gently pipetting and appropriate aliquots of the cell suspension were added to new culture vessels and incubated at 37 °C until next subculturing [18].

HCMV infections were performed using high-passage strain AD169, obtained from ATCC. About $1x10^5$ cells of MRC-5 were suspended in 1 ml of cell-free AD169 suspension ($1x10^5$ pfu/ml) for 3 hours at multiplicity of infection (MOI) equal to 1. Afterwards, infected MRC-5 cells were centrifuged at 3500 g for 5 minutes and pellet was resuspended in fresh EMEM medium; washing step was repeated 3 times. Then, HCMV-infected MRC-5 cells were plated onto Corning® T75flasks 75 sq.cm flask for 4 weeks with weekly renewal of EMEM medium and cells subculturing. Cytopathic effects of HCMV were screened routinely for the confirmation of the persistence of HCMV infection.

Two sets of MRC-5 cells were grown for 4 weeks, the first set was infected with HCMV, AD169, virus and the second set was not infected. Stringent measures were pursued to separate two sets apart in order not mix infection. Vital cell count was performed at every freshening step for both sets. The vital count difference between two sets was not more than 7%, being HCMV-infected cells of lower count. So, 7% difference in cell vitality was not considered significant factor that could alter genetic comparison between the two sets of cells.

Flow cytometry analysis

After 4-week interval of growth and viral propagation, HCMV- infected and noninfected MRC-5 cells were harvested and transferred to 15 ml tubes. All of the tubes were centrifuged at 300 g for 8 minutes. The supernatants were discarded and the pellets were washed two times by cold PBS. Then, pellets were resuspended in 70% ice-cold ethanol with PBS, 1:10 v/v. Then, all the supernatants were aspirated after centrifugation at 500 g for 8 min. The washing step by PBS was repeated and the supernatants were aspirated. A cell

count was performed and the number of cells adjusted to $1x10^5$ cell/ml with cold PBS and then, 100% methanol was added and then stored at -20 °C until staining. The method used was according to Jacobbergar and Lehman [19]. Briefly, after removal of fixative, the cells were resuspended in flourochrome-labeled antibody to CMV early nuclear antigen using case-customized kit (Dako, Denmark, No. 1284) diluted in PBS-130 mM NaCl, 15 mM phosphate (pH 7.4), and 1:1 volume of heat-inactivated normal goat serum with 0.004% Triton X-100 and 0.2% sodium azide. The antibody was incubated with the cells for 90 min at 37 °C and washed 3 times in cold PBS. Then, MRC-5 cells were incubated for 30 minutes at 37 °C in 0.5 ml of RNase A (Qiagen, Germany) and samples were then stored in the dark at 4 °C overnight. The assay was measured in duplicates for each sample. The percentage of CMV-infected cells was measured using CyAn ADP apparatus (BECKMAN COULTER, USA). The software Summit (V4.3) was used to analyze the flow cytometry results.

SmartChip Real-Time PCR System

SmartChip Human Oncology Gene Panel version 1.5.1 from (WaferGen, BIOSYSTEMS, Fremont, CA, U.S.A.) enables gene expression profiling of 4,128 reaction wells on a single sample using the SmartChip Nanodispenser (WaferGen, BIOSYSTEMS, Fremont, CA, USA). SmartChip panels are preloaded with pre-optimized PCR primers that have been validated in both microliter- and nanoliter-scale SYBRGreen real-time PCR experiments. The primers used to investigate 983 human oncogenesis-related genes in quadruplicate belonging to 16 functional groups (Table 1), 9 endogenous controls (Table 2) and 6 exogenous yeast controls. The SmartChip Human Oncology Gene Panel was used to measure differential gene expression from 0.5 µg of RNA of HCMV-infected MRC-5 cells versus that of non-infected MRC-5 cells; only

significantly up- and down-regulated genes are displayed in this study.

Table 1. The gene families covered by high throughput Real-Time qPCR System using WaferGen SmartChip Human Oncology Gene Panel

Gene Family
ADME*
Apoptosis
Cancer
Cell Cycle/Proliferation
Cardiovascular Disease
DNA damage repair
Drug Target
G-protein coupled receptor
Growth factor
Homeostasis/Metabolism
Inflammation
Kinase
Proteinase
Signal Transduction
Transcription Factor
NeuroDisease/Phosphotase

*Drug absorption, distribution, metabolism and elimination genes.

Table 2. Endogenous internal control (reference genes) used in SmartChip Real-Time PCR System: all of reference genes were amplified and used successfully for normalization

Primer	Functional	failed	Total	Ct	Ct SD	Tm	Tm SD
TAF10	4	0	4	25.34	0.359	79.0	0.155
GUSB	4	0	4	30.58	0.320	82.2	0.099
TAF11	4	0	4	28.38	0.763	81.4	0.164
HPRT	4	0	4	26.69	0.193	81.9	0.101
PPIA	4	0	4	21.43	0.078	85.0	0.068
HMBS	3	0	3	29.66	1.25	83.2	0.088
ACTB	4	0	4	25.33	0.750	87.2	0.178
B2M	4	0	4	21.94	0.129	85.4	0.085
TAF1L	4	0	4	29.00	1.10	83.1	0.058

RNA extraction

RNA extraction was done according to the guidelines and protocols provided by Wafergen Bio, USA. 1×10^6 MRC-5 cells were harvested and transferred to 15 ml tubes. After centrifuging the tubes at 300 g for 5 minutes, the supernatants were discarded. The pellets were resuspended in PBS and were washed for 3 times. Total RNA was isolated using RNeasy Mini Kit (Qiagen, Germany: Cat No./ID: 74104) Briefly, according to the manufacturer's protocol for RNA isolation from cell culture, cells were resuspended in 700 µl of lysis buffer (Buffer TR) with vigorous vortexing to inactivate cellular RNases together with cell lysis. Later, the lysed cells were transferred to homogenization columns. The columns were centrifuged at 10.000 × g

for 2 min. The flow-though was saved and equal volume of 80% ethanol (700 µl) was added. The lysed cells were transferred into RNA binding columns that were centrifuged at 10.000 × g for 1 min and the flow-through was discarded. Columns were washed using washing buffer and then DNA fragments were removed by DNase I treatment. Further centrifugation at 10.000 × g for 1 min was done to remove any traces of used buffers. Total RNA was collected by placing the columns into new 1.5 ml microtubes with 60 µl RNase-free water addition and standing for 1 min. The microtubes were centrifuged at 10.000 × g for 1 minute.

RNA quality and quantity were determined by Life Science UV/Vis Spectrophotometer, DU Series 700 (BECKMAN COULTER, USA). RNA concentration used in downstream experiments ranged from 503 to 524 ng/µl. Moreover, total RNA quality was checked by Agilent 2100 Bioanalyzer (Agilent, USA) and RNA integrity number (RIN) was measured. RIN of total RNA was 9.1 and passed the quality control limit for running downstream experiments.

Synthesis of cDNA

The reverse-transcription of up to 0.5 µg of the isolated MRC-5 cell's RNA was done using iScriptTM cDNA Synthesis Kit (BIO-RAD, Hercules, Canada). According to the manufacturer's protocol, 4 µl of 5 × iScript reaction mix were mixed with 1 µl iScript reverse transcriptase and 15 µl of RNA template in 1.5 ml microtubes to give final volume of 20 µl per reaction. The complete reaction mix was incubated for 5 min at 25°C then for 30 min at 42 °C. The incubation temperature was increased to 85°C for 5 min. Finally, cDNA was stored at −80 °C for qRT-PCR reaction.

Real-time quantitative RT-PCR

Real-time quantitative PCR reaction was conducted according to the procedure validated by (Wafergen Bio, USA) using the SmartChip human oncology panel version 1.5.1. These chips were preloaded with primer content optimized for performance with the high throughput Real-Time PCR System. The SmartChip Nanodispenser (Wagergen Bio, USA) was used to dispense the sample, combined with master mix and controls, under vacuum into the SmartChip. Five hundred (500) ng of starting sample of cDNA yielded cDNA equivalent of 96 pg of sample per well. Once loaded with sample, the SmartChip was placed into the WaferGen SmartChip Cycler Nanodispenser (Wagergen Bio, USA). The SmartChip Real-Time PCR system employs a qPCR reaction compatible with SYBR green I DNA binding dye. Results were reported in the form of Ct (threshold cycles), Tm (melting temperatures), and amplicon melting curve analyses.

Primers used are based on criteria such as specificity, insensitivity to sequence polymorphisms, amplicon size, and minimization of primer artifacts. Primer specificity was determined using (a) melting curve analysis of amplicon product, (b) gel electrophoresis analysis of amplicons and (c) sequence verification of the amplicons. The up- and down- regulation of genes were analyzed by SmartChip Software (Wafergen Bio, USA). The sensitivity of real-time PCR is 20-30 copies of RNA per well with specificity of genes discrimination <90% homology. To keep precision of readings, standard deviation <0.25 Ct between replicates was used. For valid runs, all primers shared the same or close-by Tm and amplicon size and no primer dimers or primer secondary structures were allowed. Thermal cycling of primers was 95C for 180 sec once, and for 40 cycles: 95C for 60 sec, and 60 C for 70 sec with amplicons size ranged from 80 to 120 nucleotides. Automatic screening for low efficiency wells turned out to be zero and PCR efficiency for all primers was measured and taken into account for calculating fold changes in genes expression. All-means normalization method, where the means of all expressed genes are employed, was used in

the current study. In addition, six exogenous yeast controls were used (WGBS-YCF1-6); at least four of them should be detected and be linear in proportion. At each run two negative tissue controls (NTC), chip no. 34565 and 35935, and two positive tissue controls, brain tissue (PTC), chip no. 34576 and 35939, were used.

The fold change calculation used was based on the Comparative Ct Method. The comparative Ct Method is also known as the 2-ΔΔCt method and as follows: ΔΔCt = ΔCt treated − ΔCt untreated, where: ΔCt treated = Ct treated − All Mean treated and ΔCt untreated = ΔCt untreated − All Mean untreated. The significant up/down regulated genes are those genes with at least two fold change.

Data analysis

The data in the current study are shown as mean±2SD.The data analysis was conducted by using SPSS software version (11.0.0.1). Simple percentage calculation was used to obtain the % of HCMV-infected cells by Flow cytometry assay. For real-time qPCR, SmartChip qPCR software exerted all data

analysis. P values less than 0.05 were considered significant.

Results

Percentage of MRC-5 cells infected with HCMV

After 4 weeks propagation of HCMV, AD-169 strain, in MRC-5 cells, the percentage of infected cells was measured by using flow cytometry method. The percentage of HCMV-infected MRC-5 cells was shown to be 94.1%. This finding indicated that MOI 1 of MRC-5 cells with AD-169 strain of HCMV is sufficient to attain near complete infection of all exposed cells after 4 weeks of viral propagation. This step is essential to confirm the homogeneity of real-time qPCR results of gene expression profiling.

SmartChip Real-Time PCR System

After the completion of real-time PCR assay, descriptive statistics of SmartChip panel technical results are shown in Table 3 and Figure 1. In addition, the setting values used for SmartChip RT-PCR panel are shown in Table 4.

Table 3. The technical results of the SmartChip oncogenic panel used in the current study

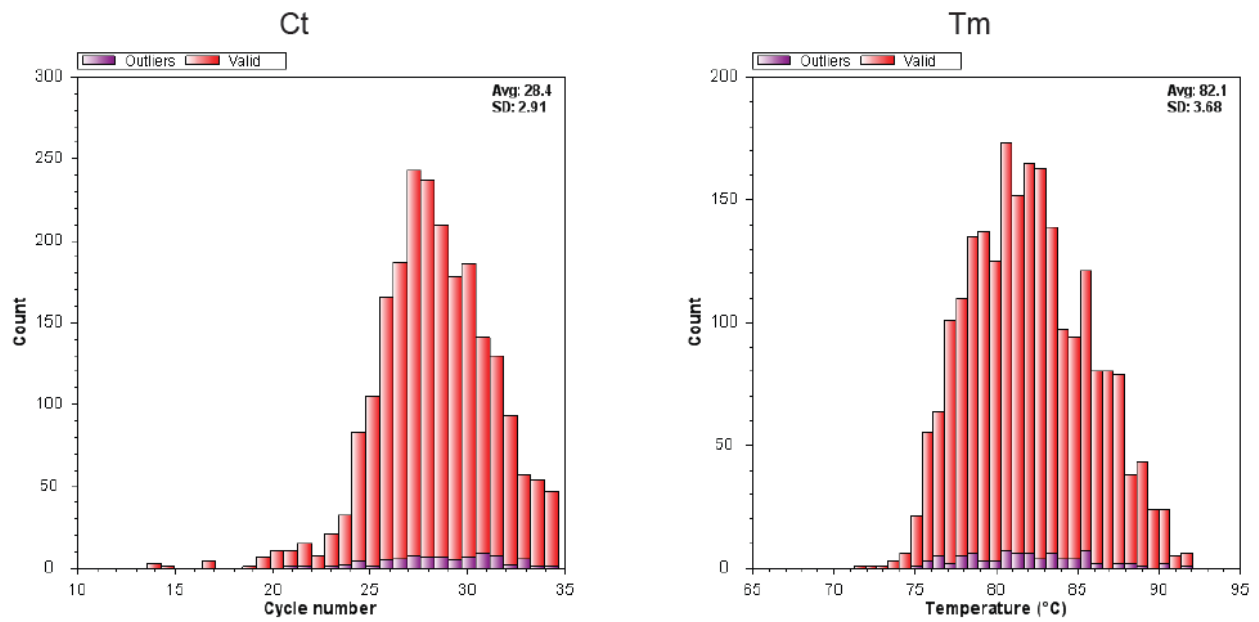| Name	Value
Total wells	4128
No amplification	1648
Curve fit failed	232
With curves	2248
Bad R2	0
Low efficiency	0
High efficiency	0
Ct is small	0
Ct is large	136
Low saturation	0
Saturation/Baseline ratio is low	1
Multiple peaks	62
Multimodal Tm's	14
Informative	302
Replicates without curves	347

Figure 1. Histogram of the frequency of Ct and Tm for SmartChip RT PCR showing that Ct 25 to 30 cycles and Tm 75 to 85 C were dominant among different primer sets used for both HCMV infected and non-infected SmartChiponcopanels

Table 4. The settings and values of the RT-PCR SmartChip assay

Threshold	Value
Ct max deviation	0.5
Fail curve based on R2	0.99
Grubbs confidence	0.05
Grubbs Min SD	0.25
High Ct	33
High efficiency	3
Informative replicates SD	0.6
Low Ct	5
Low efficiency	1.5
Low saturation	1000
Low saturation/baseline ratio	1.8
Max Baseline drift	21
Max Ct	35
Max efficiency	3.5
Melt multimodel	1
Melt peak left	0.6
Melt peak right	0.3
Min amplification	500
Min efficiency	1.5
No amplification ratio	2
Point used for curve fit	15
R2	0.99

Detection of the up- and down-regulation of gene expression by SmartChip Real-time PCR System

The quantitative analysis by real-time PCR revealed a complete picture regarding the up- or down-regulated genes in HCMV-infected MRC-5 cells compared to that of non-infected cells. The results showed that almost all genes were expressed in a very close level (less than 2 folds) while only 15 genes were upregulated and 5 genes were downregulated (Table 5; Figure 2). Although the number of genes that showed differential expression was totally 20, this variation has its own importance due to the association of the affected genes to critical cell functions including cancer, apoptosis and cell cycle regulation. It is noteworthy to mention that most of the genes up- or down-regulated in the current study belong to gene families of multiple functions (Table 6), for example a gene family is related to

inflammation, cancer, signal transduction and apoptosis at the same time.

Discussion

MRC-5 cell line was chosen and AD-169 strain of HCMV was used in order to propagate HCMV in normal diploid cells for 4 weeks ensuring long-term infection that is necessary to observe any variation in genes expression. The limitation of this study is the same as its advantage, namely MRC-5 cells. Breast, colon and prostate tissue cells are more prone to cancers where HCMV is implicated. However, CMV grows slowly in other cell lines and the initial trials of this study for infecting HCMV in cell lines other than MRC-5 turned out with shorter periods of HCMV propagation as well as much higher cell death [data not shown]. Hence, MRC-5 cells were preferred over other types of cells.

Table 5. Fold change and Log2 Fold change of the up- and down-regulated gene expression in HCMV-infected MRC-5 cells compared to HCMV non-infected cells

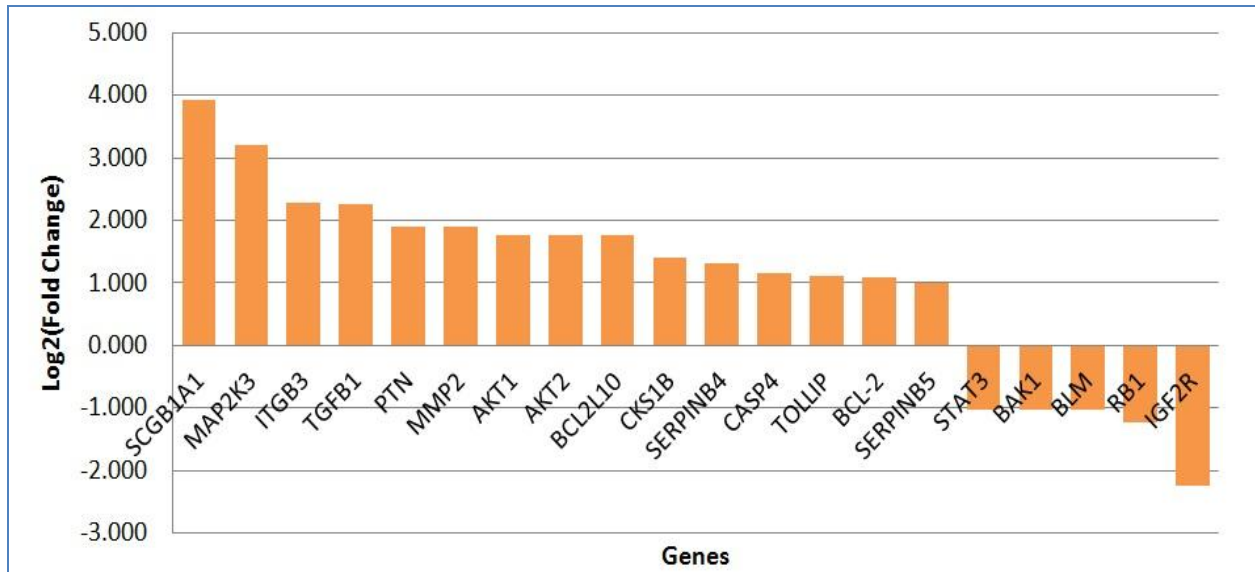| Primer	ΔΔCt	Fold Change	Log2(Fold Change)
SCGB1A1	-3.927	15.211	3.927
MAP2K3	-3.215	9.285	3.215
ITGB3	-2.287	4.879	2.287
TGFB1	-2.253	4.767	2.253
PTN	-1.890	3.708	1.890
MMP2	-1.889	3.704	1.889
AKT1	-1.770	3.410	1.770
AKT2	-1.757	3.381	1.757
BCL2L10	-1.756	3.378	1.756
CKS1B	-1.400	2.639	1.400
SERPINB4	-1.311	2.482	1.311
CASP4	-1.164	2.240	1.164
TOLLIP	-1.108	2.156	1.108
BCL-2	-1.078	2.111	1.078
SERPINB5	-1.003	2.004	1.003
STAT3	1.020	0.493	-1.020
BAK1	1.031	0.489	-1.031
BLM	1.031	0.489	-1.031
RB1	1.152	0.429	-1.223
IGF2R	1.194	0.211	-2.248

Figure 2. A diagram showing 15 up-regulated and 5 down-regulated gene expression in HCMV-infected MRC-5 cells compared to HCMV non-infected cells

Table 6: Gene families and gene functions/pathways involved in the differentially expressed genes between HCMV-infected and HCMV non-infected MRC-5 cells

Up-regulated Gene	Gene family	Gene product related function/pathway/ By Genecards ([21])	Down-regulated gene	Gene family	Gene product related function/pathway/ By Genecards ([21])
SCGB1A1	Inflammation	SCGB1A1 (Secretoglobin, Family 1A, Member 1. Among its related pathways are FOXA1 transcription factor network and Prostaglandin Synthesis and Regulation. this gene product binds to phospholipase A2 inhibitor activity	STAT3	Cancer; Inflamation; Signal Transduction; Transcription Factor;	STAT3 (Signal Transducer And Activator Of Transcription 3 (Acute-Phase Response Factor)) is a Protein Coding gene. Diseases associated with STAT3 include hyper ige syndrome and hyper-ige recurrent infection syndrome. Among its related pathways are Immune System and Signaling by GPCR
MAP2K3	Kinase; Signal Transduction;	MAP2K3 (Mitogen-Activated Protein Kinase Kinase 3) is a Protein Coding gene. Among its related pathways are MAPK signaling pathway and Immune System. GO annotations related to this gene include transferase activity, transferring phosphorus-containing groups and protein tyrosine kinase activity	BAK1	Apoptosis	BAK1 (BCL2-Antagonist/Killer 1) is a Protein Coding gene. Diseases associated with BAK1 include benign mammary dysplasia and chronic gonococcal salpingitis. Among its related pathways are Apoptotic Pathways in Synovial Fibroblasts and ERK Signaling
ITGB3	Cancer; Cardiovascular Disease	ITGB3 (Integrin, Beta 3, Glycoprotein IIIa). Among its related pathways are PI3K-Akt signaling pathway and Immune System	BLM	DNA Damage Repair	BLM (Bloom Syndrome, RecQ Helicase-Like) is a Protein Coding gene. Diseases associated with BLM include bloom syndrome and Werner syndrome. Among its related pathways are Cell Cycle, Mitotic and Integrated Pancreatic Cancer Pathway. GO annotations related to this gene include nucleic acid binding and ATPase activity
TGFB1	ADME; Apoptosis; Cancer; Cardiovascular Disease; Inflamation; Signal Transduction;	TGFB1 (Transforming Growth Factor, Beta 1) is a Protein Coding gene. Diseases associated with TGFB1 include camurati-engelmann disease and cystic fibrosis. Among its related pathways are MAPK signaling pathway and Signaling by GPCR	RB1	Cell cycle checkpoint	RB1 (Retinoblastoma 1) is a tumor supressor protein Coding gene. Diseases associated with RB1 include retinoblastoma and familial retinoblastoma. Among its related pathways are Pathways in cancer and Glioma.

Gene	Category	Description	Gene	Category	Description
PTN	Cancer; Cardiovascular Disease; Inflamation;	PTN (Pleiotrophin) is a Protein Coding gene. Among its related pathways are NF-KappaB Family Pathway and Apoptotic Pathways in Synovial Fibroblasts.	IGF2R	Growht factor	IGF2R (Insulin-Like Growth Factor 2 Receptor) is a Protein Coding gene. Diseases associated with IGF2R include hepatocellular carcinoma. Among its related pathways are Apoptotic Pathways in Synovial Fibroblasts and GPCR Pathway.
MMP2	Cancer; Cardiovascular Disease	MMP2 (Matrix Metallopeptidase 2) is a Protein Coding gene. Among its related pathways are Developmental Biology and Pathways in cancer.			
AKT1	ADME; Apoptosis; Cancer; Cardiovascular Disease; G-Protein Coupled Receptor; Inflamation; Kinase; Signal Transduction;	AKT1 (V-Akt Murine Thymoma Viral Oncogene Homolog 1) is a Protein Coding gene. Diseases associated with AKT1 include breast adenocarcinoma and proteus syndrome, somatic. Among its related pathways are PI3K-Akt signaling pathway and PI3K/AKT Signaling in Cancer			
AKT2	Signal Transduction	AKT2 (V-Akt Murine Thymoma Viral Oncogene Homolog 2) is a Protein Coding gene. Among its related pathways are PI3K-Akt signaling pathway and PI3K/AKT Signaling in Cancer. An important paralog of this gene is AKT1.			
BCL2L10	Apoptosis	BCL2L10 (BCL2-Like 10 (Apoptosis Facilitator)) is a Protein Coding gene. Diseases associated with BCL2L10 include prostatic cyst. Among its related pathways are Apoptosis (WikiPathways) and Apoptosis and Autophagy.			
CKS1B	Kinase	CKS1B (CDC28 Protein Kinase Regulatory Subunit 1B) is a Protein Coding gene. Among its related pathways are Pathways in cancer and Cell Cycle, Mitotic.			
SERPINB4	Angiogenesis;	SERPINB4 (Serpin Peptidase Inhibitor, Clade B (Ovalbumin), Member 4) is a Protein Coding gene. Diseases associated with SERPINB4 include inverted papilloma and tongue squamous cell carcinoma. Among its related pathways are Amoebiasis			
CASP4	Apoptosis, Drug target	CASP4 (Caspase 4, Apoptosis-Related Cysteine Peptidase) is a Protein Coding gene. Diseases associated with CASP4 include hepatitis c virus. Among its related pathways are Immune System and Apoptotic Pathways in Synovial Fibroblasts			
TOLLIP	Inflamation; Signal Transduction	TOLLIP (Toll Interacting Protein) is a Protein Coding gene. Diseases associated with TOLLIP include lymphoproliferative syndrome, x-linked, 1. Among its related pathways are Immune System and Interleukin receptor SHC signaling.			
BCL-2	Apoptosis	BCL2 (B-Cell CLL/Lymphoma 2) is a Protein Coding gene. Diseases associated with BCL2 include follicular lymphoma and intravascular large b-cell lymphoma. Among its related pathways are PI3K-Akt signaling pathway and Immune System.			
SERPINB5	Angiogenesis	SERPINB5 (Serpin Peptidase Inhibitor, Clade B (Ovalbumin), Member 5) is a Protein Coding gene. Diseases associated with SERPINB5 include mandibular cancer and syringoma. Among its related pathways are MicroRNAs in cancer and Angiogenesis (CST)			

Using high throughput platforms of real-time qPCR is superior on using gene expression microarrays. The reason is that high throughput real-time qPCR is highly quantitative and precise, unlike that in gene expression microarrays which are precise but not quantitative [20]. The current study screened which genes are differentially expressed when MRC-5 cells are infected with HCMV. Results showed that 15 genes were upregulated in HCMV-infected cells belonging to collectively 11 gene families namely, inflammation, kinase, signal transduction, cancer, cardiovascular disease, ADME, apoptosis, cancer, G-protein coupled receptor, angiogenesis, and drug target. In addition, expression of 5 genes were downregulated in HCMV-infected cells belonging collectively to 9 gene families namely, kinase, apoptosis, DNA damage repair, cell cycle checkpoint, cancer, inflammation, signal transduction, transcription factor, and growth factor.

Taking each gene family separately for the upregulated genes, five genes have inflammation downstream effect including SCGB1A1, TGFB1, PTN, AKT1, and TOLLIP and six genes showing kinase/signal transduction effect namely, MAP2K3, TGFB1, AKT1, AKT2, CkS1B, and TOLLIP and five genes belong to cancer promoting family of genes including ITGB3, TGFB1, PTN, MMP2, and AKT1 and five genes related to apoptosis including TGFB1, AKT1, BCL2L10, CAPS4, BCL-2 and two genes related to angiogenesis namely SERPINB4 and SERPINB5.

Gene function analysis of the upregulated genes shows an important observation that the majority of the gene functions are oncogenic and/or oncopromoting directly or indirectly. SCGB1A1 gene is a Secretoglobin, Family 1A, Member 1; among its related pathways are FOXA1 transcription factor network and Prostaglandin Synthesis and Regulation [21]. So, this gene is pro-inflammatory and was upregulated more than 15 folds indicating HCMV induce inflammation through this gene.

It is well known that inflammation is one mechanism of cancer promotion and initiation [22] especially in long lasting infections [23]. Another gene is associated with inflammation and cancer, PTN which is a Pleiotrophin; among its related pathways are NF-KappaB Family Pathway and Apoptotic Pathways [21]. Pleiotrophin, PTN, gene was found to elicit pro-tumorigenic effects through its receptor, protein tyrosine phosphatase receptor, Z1 (PTPRZ1) [24]. Moreover, mitogen-activated protein kinase (MAPK) family were found to be likely effectors of PTN signaling and is associated with ovarian cancer [24]. Another gene, TOLLIP (Toll Interacting Protein), is also associated with inflammation and signal transduction; among its related pathways are immune system and interleukin receptor SHC signaling [21]. TOLLIP has been linked strongly with lung, prostate, breast cancers and melanoma [25]; and related to activation of interleukin-1 (IL-1) receptor (IL-1R), Toll-like receptor 2 (TLR2), and TLR4 which trigger NF-kappaB and MAPK-dependent signaling, thereby initiating immune responses and maybe tumors [26].

MAP2K3 gene is a MAPK, Kinase 3; involved in MAPK signaling pathway and immune system. MAPK pathway has a clear role in carcinogenesis [21]. This kinase is activated by mitogenic and environmental stress, and participates in the MAPK-mediated signaling cascade [27,28]. Expression of K-ras oncogene is found to result in the accumulation of the active form of this kinase, which thus leads to the constitutive activation of MAPK14, and confers oncogenic transformation of primary cells [29]. It is well known that K-ras expression is strongly associated with carcinogenesis [30]. Therefore, HCMV might acts like a K-ras oncogene in inducing transformation. Another upregulated gene involved in MAPK pathway was TGFB1, which also triggers local immune suppression [31] and induces cancer [32]. Enhanced expression of TGFB1 was found to enhance several cancer-related events such as

cell division, cell motility, and the development of new blood vessels, angiogenesis. The TGFβ-1 protein is abnormally overexpressed in certain types of prostate, breast, colon, lung, and bladder cancers [33].

One of the interesting findings of the current study is that three upregulated genes are involved in PI3K/Akt pathway, which has a well-known role in carcinogenesis [34]. These genes are ITGB3, AKT1 and AKT2. Phosphatidylinositol 3 kinase, or PI3K activates Akt, which modulates the function of numerous substrates involved in the regulation of cell survival, cell cycle progression and cellular growth [21,34]. It has been shown that PI3K/Aktsignalling pathway components are frequently altered in human cancers [35]. It was found that survival signals counteracting apoptosis induced by several receptors are mediated mainly by PI3K/Akt; hence this pathway was found to decisively contribute to the resistant phenotype of many cancers [34,36]. The PI3K/Akt pathway is involved in many of the mechanisms targeted by new anticancer drugs such as Everolimus by Novartis, which is an immunosuppressant and recently approved in treatment of hormone therapy-resistant estrogen receptor-positive breast cancer by blocking some links in the activated PI3K/Akt pathway [37].

Regarding apoptosis-related genes that were upregulated after HCMV infection, the picture is complex and not so clear. Two upregulated genes induce apoptosis process, which are CASP4 and BCL2L10 while the third one is anti-apoptotic, BCL-2. This needs further investigations by separate experiments in order to elucidate the net effect of apoptosis exerted by HCMV on normal cells.

Another upregulated gene was Matrix Metallopeptidase 2 (MMP-2) gene encoding type IV collagenase. Among its related pathways are developmental biology and pathways in cancer [21]. MMP-2 and MMP-9 secretion is elevated in several types of human cancers and their elevated expression has been associated with poor prognosis [38]. The major implications of MMPs in cancer progression is their role in extracellular matrix degradation, which allows cancer cells to migrate out of the primary tumor to form metastases [39].

Serpin Peptidase Inhibitor, SERPIN, B4/B5 are two genes mildly upregulated by HCMV infection and both are related to enhanced angiogenesis. SERPINB4 is associated with inverted papilloma and tongue squamous cell carcinoma. SERPINB5 (Serpin Peptidase Inhibitor, Clade B Member 5) is associated with mandibular cancer. Among its related pathways are MicroRNAs in cancer and angiogenesis [21]. In addition, elevated levels of these two genes are associated with inflammatory reactions in atopic dermatitis and psoriasis [40]. Hence, these genes exert two mechanisms that might promote cancer status namely induction of inflammation and angiogenesis.

The last of the upregulated genes is CKS1B which is CDC28 protein kinase regulatory subunit 1B; among its related pathways are pathways in cancer and cell cycle, and mitosis [21]. The expression of CKS1B is elevated in multiple cancers, including breast cancer, lymphoma, myeloma, colon cancer, prostate cancer, and lung cancer [41]. Amplification and overexpression of CKS1B are strongly associated with lymph node metastasis and poor prognosis in breast and salivary cancer [42]. Generally, CKS1B is an essential factor in facilitating Skp2-dependent degradation of p27 [41].

There are only 5 downregulated genes after exposure to HCMV infection, which are: STAT3 (cancer, inflammation, signal transduction and transcription factor), BAK1 (pro-apoptotic), BLM (DNA damage repair), RB1 (cell cycle checkpoint), and IGF2R (growth factor) [21]. Downregulation of expression of all above mentioned genes except for STAT3 is of oncogenic, oncopromoting or oncomodulating effect [21]. Downregulation of BAK1 (BCL2-antagonist/killer 1) lowers the antagonism of BAK1 to BCL-2 leading to less apoptotic potential of the cells favoring cell

carcinogenesis indirectly [43]. For RB1 gene, it encodes Retinoblastoma 1which is an essential tumor suppressor protein that regulates cell cycle progression; lowered expression of RB1 product is strongly associated with cancer [44] as cell proceeds to mitosis without rate limiting control by the tumor suppressor protein, retinoblastoma. For BLM gene, it provides instructions for making RecQ helicases, enzymes that attach to DNA and unwind the double helix of the DNA molecule. This unwinding is necessary for several processes in the cell nucleus, including copying DNA in preparation for cell division and repairing damaged DNA [21]. Because RecQ helicases helps maintain the structure and integrity of DNA, they are known as the "caretakers of the genome". Hence, decreased expression of BLM helps damage DNA molecule and facilitates carcinogenesis in long term infection of HCMV [45,46]. For IGF2R (Insulin-Like Growth Factor 2 Receptor), diseases associated with IGF2R include hepatocellular carcinoma and in breast cancer [21]. IGF2R is a negative regulator of cell growth and a putative tumor suppressor gene and the regulation of M6P/IGF2r levels is critical in breast physiology and lowered expression of IGF2R is associated with various aspects of breast cancer [47]. The role of the differential expression of IGF2R between HCMV infected and non-infected seems important as it was downregulated by more than 4 folds and because of its important role in normal physiology of breast tissue [47] where HCMV is strongly linked to breast cancer.

In regard to STAT3 gene (signal transducer and activator of transcription 3), it comprises a family of cytoplasmic transcription factors that mediates intracellular signaling and nucleus transcriptional activity [21]. Constitutive activation of STAT3 was shown in a wide variety of human tumors, including hematological malignancies, head and neck, breast, lung, and gastric cancers [48]. Unlike other differentially expressed genes of this study, lowered expression of this gene seems of protective role against cancer. However,

what is the exact role of the lowered STAT3 in HCMV infection is not yet clear. This needs further investigation. Nevertheless, one gene with anticancer activity versus numerous genes with cancerous activity seems of inferior effect. So taken together, this study has concluded for the first time, that the effect of HCMV infection on the level of expression of 983 genes related directly or indirectly to oncogenesis was studied in normal non-cancerous cells. In this study, HCMV infection stimulated expression of several genes that might trigger or promote cancer by different mechanisms. Most important mechanisms observed were activating MAPK and PI3K/AKT pathways, inducing inflammation, inhibiting apoptosis, inducing angiogenesis, and inhibiting tumor suppressor proteins. Activation of MAPK and PI3K/AKT pathways and lowered tumor suppressor activity implies to tumor initiating more than tumor promoting effect, while inducing angiogenesis, inflammation, inhibiting apoptosis, and inducing extracellular matrix proteases implies to tumor promoting/modulating effect more than tumor initiating effect. This study is believed to direct the coming studies on investigating deeply the role of differentially expressed genes by HCMV infection. This can shed light on the exact mechanisms and pathways that HCMV might exert to initiate or promote/modulate human cancers.

Acknowledgement

Conflict of Interest

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