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Original Research

Transcriptomic analysis of human non-small lung cancer cells A549 treated by one synthetic curcumin derivative MHMD

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Abstract: In our previous studies, we have identified one curcumin analog MHMD could induce apoptosis of lung cancer cells A549 via extrinsic and intrinsic pathways in our previous studies. But the specific regulatory genes and molecular mechanisms remain poorly understood. Here, the transcriptomic profile of A549 cells was detected with RNA-seq technique after MHMD treatment at 48 h. A total of 16584651 clean data from 21831774 sequence reads were obtained and 80.75% of them could be mapped on the human test genome. 18635 unigenes with the mean length of 4027 bp were finally assembled. 850 up-regulated and 855 down-regulated genes were differently expressed in MHMD-incubated A549 cells, which were involved in many cellular pathways of MHMD-treated A549 cells. Furthermore, the major genes involved in the apoptotic and NSCLC pathways were analyzed. mRNAs of four genes (casp7, p53, tgfa, prkar1b) were validated by RT-PCR, which suggested that MHMD indeed activated the apoptotic pathway of A549 cells.

Key words: Curcumin analog; Transcriptome; Differently expressed genes; Apoptosis.

Introduction

In view of low bioavailability and extensive metabolism of curcumin (an antioxidant polyphenol from the rhizonme of curcumin *longa Linn*), curcumin analogs or derivatives had been synthesized and identified for their antitumor activities in many experimental studies and could be good alternatives of curcumin in future (1,2). Research showed that a large number of them often led to the cancer cells death via apoptotic or (and) autophagic pathways, such as double carbonyl analog of curcumin A17 (3), Hydrazinobenzoyl curcumin (HBC) (4), 3,5-bis(2-hydroxybenzylidene)tetrahydro-4*H*-pyran-4-one glutathione conjugate (EF25-(GSH)2) (5).

Until now, more studies had been carried out to study molecular or pathogenic processes during chemical molecules treatment or pathogens dissemination processes with high-throughput sequencing technologies (6). As a recently developed approach to transcriptome profiling, RNA-sequencing technology has been widely used as a revolutionary tool for transcriptomics in many studies (7). It allows researchers to quantify, discover and profile RNAs. A large number of sequence data, which represent the gene expression pattern of tissues or cells at a specific condition, can be easily obtained using this technology. In the past few years, some interesting papers had been published in our research area. For example, Liu et al. (8) employed the RNA-seq to analyze the transcriptome changes in Tetranychus cinnabarinus treated with curcumin or the solvent. Colacino et al. (9) also applied RNA-seq analysis to identify novel genes and pathways targeted by curcumin in breast cancer cells. These studies could help to elucidate the mechanism by which curcumin may act as a cancer-preventive compound and provide novel targets for cancer prevention and treatment. However, reports on the RNA-seq analysis of cancer cells treated with curcumin analogs are rare. To our knowledge, only Wu et al. (10) applied RNA-seq analysis to identified 57 androgen-regulated genes with high significance altered in HBC-treated prostate cancer cells. Different analogs may possess different chemical effects and affect particular cellular pathways of cancer cells inhibition and hence much more work need to be done to develop new cell signal targets or pathway with the deep sequencing technology.

We previously showed that one synthetic curcumin analog MHMD could induce apoptosis of A549 lung cancer cells via extrinsic and intrinsic pathways (11,12). But the possible apoptotic related factors and specific molecular mechanism of the pathways are still not known. The purpose of the present work is to provide a global view of the gene expression of A549 cells after MHMD treatment at 48 h using the Illumina sequencing technology. Based on the transcriptome data, the gene expression patterns of test group and control were compared. The current gene expression profile analysis of MHMD-treated A549 may aid in the identification of curcumin analogs resistance-associated genes and development of possible potential clinical control of lung cancer cells in future work.

Materials and Methods

Cell culture and sample preparation

The non small lung cancer cell line A549 were stored

and cultured in our lab recently, which were maintained in DMEM medium (Gibco) supplemented with 10% calf serum at 37 °C CO_2 (5%) incubator. The curcumin analog MHMD were got from Dr. Gangchun Sun's lab in Henan University of Technology (12). The A549 cells were incubated with MHMD for final concentration at 4 μM for 48 h and then cellular RNA was extracted for the next treatment according to the following procedures.

RNA extraction, mRNA purification

Total RNA was treated with RQ1 DNase (Promega, USA) to remove DNA. The quality and quantity of the purified RNA were determined by measuring the absorbance at 260 nm/280 nm (A260/A280) using smartspec plus (BioRad). RNA integrity was further verified by 1.5% Agarose gel electrophoresis.

For each sample, 10 μg of total RNA was used for RNA-seq library preparation. Polyadenylated mRNAs were purified and concentrated with oligo (dT)-conjugated magnetic beads (Invitrogen) before used for directional RNA-seq library preparation. Purified mRNAs were iron fragmented at 95 °C followed by end repair and 5' adaptor ligation. Then reverse transcription was performed with RT primer harboring 3' adaptor sequence and randomized hexamer. The cDNAs were purified and amplified and PCR products corresponding to 200-500 bps were purified, quantified and stored at -80 °C until used for sequencing.

cDNA sequencing

For high-throughput sequencing, the libraries were prepared following the manufacturer's instructions and applied to illumina Nextseq 500 system for 151 nt pair-end sequencing by ABlife. Inc (Wuhan, China).

Sequence assembly and gene annotation

Raw reads were firstly cleaned by removing adaptor sequences and low quality sequences (Q>20), and then assembled into EST clusters. All contigs were annotated with GetORF from the EMBOSS package and the ORF of each predicted protein was used for Blastp searches. GO annotations were also derived based on sequence similarity. The KEGG pathway annotations were performed against the Cluster of Orthologous Groups database and the Kyoto Encyclopaedia of Genes. In this study, we used the human genome database for reference genome.

Quantification of selected unigenes by RT-PCR

To validate the expression pattern of the selected genes, five unigene-specific primer pairs were used to identify the different unigenes in the final volume of 20 μl containing 0.5 μl diluted first-strand cDNA, 1 μl sense primer (10 μM), 1 μl anti-sense primer (10 μM), 7.5 μl double-distilled water, and 10 μl 2 × Power Taq PCR Master Mix® (BioTeke). The PCR program was separated into two stages. The first stage involved 35 cycles of a reaction at 94 °C for 35 s, at 52-58 °C for 35 s for different genes, at 72 °C for 90 s, and an extension at 72 °C for 5 min as the final stage. The amplified PCR products were separated using 1% agarose gel stained with Golden View™. The gel was then screened with the Tanon 3500 Gel imaging system (Tanon, Shanghai, China). The actin gene was selected for the endogenous control.

Results

Transcriptomic sequences assembly and analysis

For test sample, a total of 16584651 clean data from 21831774 sequence reads (clean percent 75.97%) with a mean read length of 100bp were obtained. Furthermore, about 80.75% of clean reads (13392626) could be mapped on the human test genome and the percentage of uniquely mapped reads is 84.26% (11284020), which suggested that much of polyA RNA detected are mRNA or ncRNA in this study. These raw data were further assembled into different contigs and finally assembled into a total number of 18635 unigenes with the mean length of 4027 bp. The unigene size distribution was shown in Figure. 1.

Further gene ontology (GO) analysis was performed with these contigs. A total of 17237 unique proteins mapped to 28518 GO terms: 10893 unigenes mapped to biological processes, 2799 unigenes mapped to molecular function, and 4193 unigenes mapped to cellular components (Figure. 2).

To obtain more information for predicted functions of the unigenes, the genes from the A549 cells were categorized basing on the Kyoto Encyclosedia of Genes and Genomes (KEGG) database. As shown in Figure3, a total of 1943 unigenes was obtained the KO number. The largest KEGG group was "diseases overview" (498 unigenes), which showed that many kinds of environmental pathogenic factors could regulate the gene expression of A549 cells. Besides that, much of unigenes were mapped onto "signal transduction", "signaling molecules and interaction" and "metabolic pathways".

Functional annotation of the DEGs

A GO term analysis was conducted through hypergeometic test in order to identify DEGs with specific biological functions in disease response processes and pathways. Of the 849 A549 cell DEGs in the comparison, they could be assigned a significant GO classification. Among these, 271 DEGs were mapped to GO biological processes, 126 to molecular functions, and 452 to cellular components (Figure. 4). At 48 h, highly represented GO terms included 'response to stimulus', 'nucleobase, nucleodide, nucleotide', and 'pathogenesis', suggesting that A549 cells were undergoing rapid

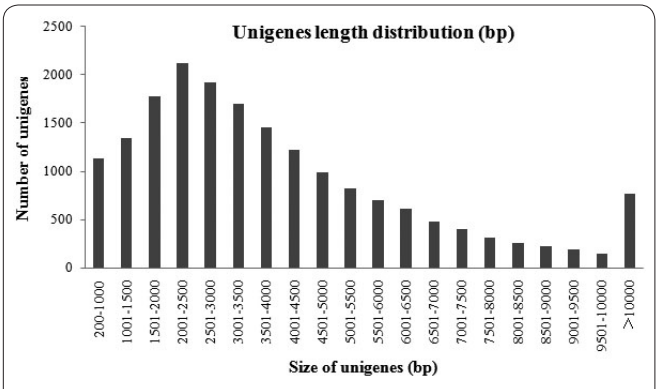

Figure 1. Overview of MHMD-treated A549 cell transcriptomic sequence length distributions for all unigenes. The x-axis indicates unigene size and the y-axis indicates the number of unigenes with different lengths.

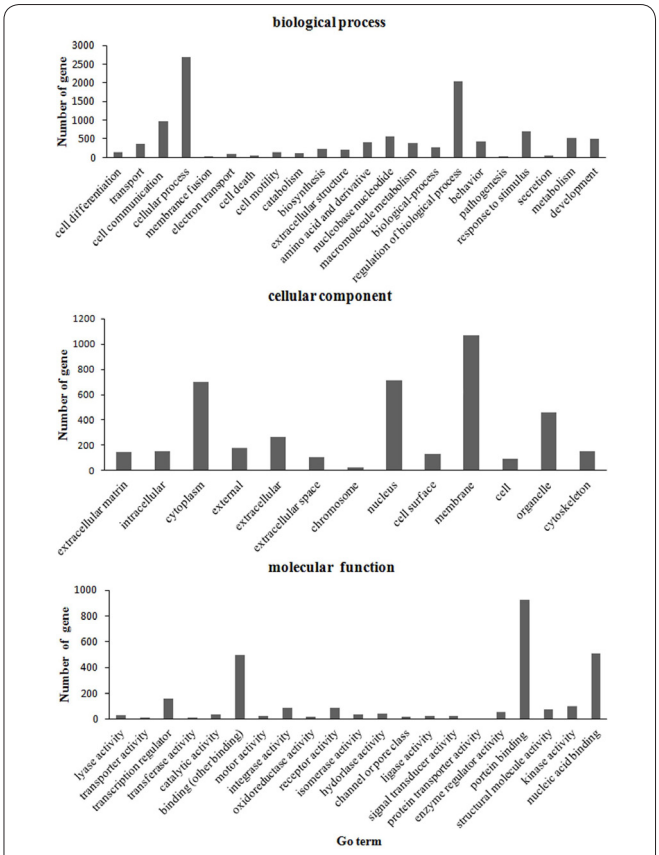

Figure 2. Gene ontology (GO) classification of unigenes for MHMD-treated A549 cells at 48 h. The annotated contigs were classified into the three gene functional categories of different terms (GO), including biological process, cellular component, and molecular function. The x-axis indicates the GO terms and the y-axis shows the number of genes mapped to the different GO term.

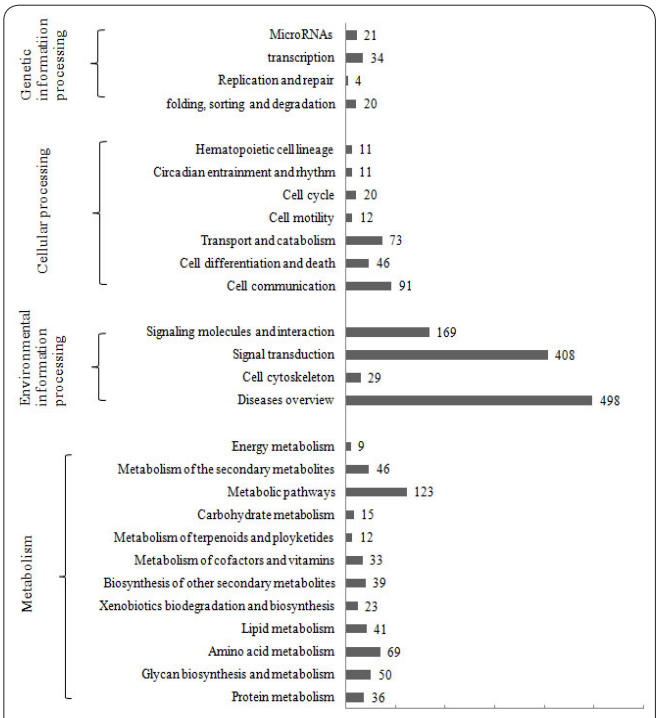

Figure 3. KEGG classification of the MHMD-treated A549 cells transcriptome. The x-axis indicates the number of predicted proteins and the y-axis indicates the pathway.

changes at the molecular level in response to MHMD.

Analysis of DEGs related to cell apoptosis and NS-CLC pathways

Compared to the non-treated control A549 cells,

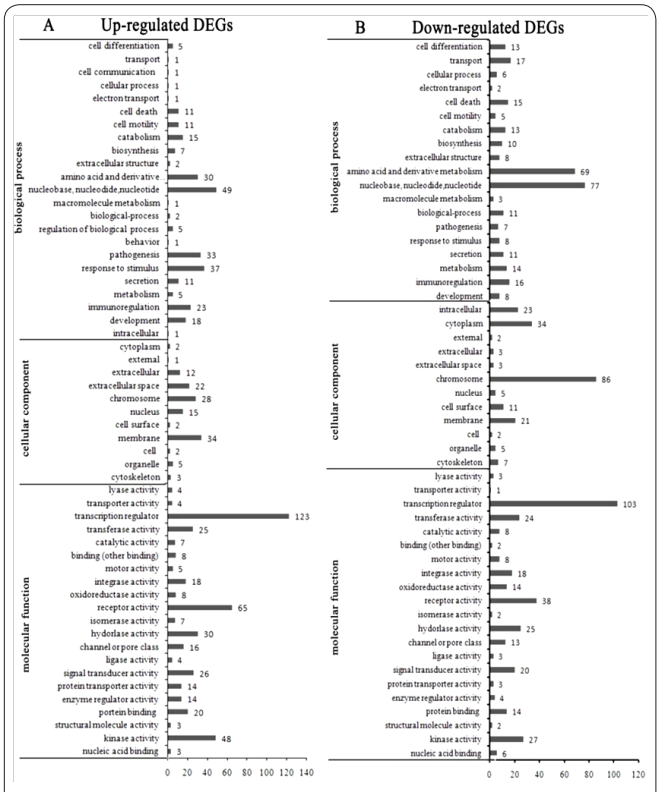

Figure 4. Gene ontology assignments for differentially expressed genes (DEGs) after MHMD treatment. The DEGs upon curcumin treatment were matched the three ontology categories. The x-axis indicates the GO terms and the y-axis indicates the number of genes. A, GO analysis for the up-regulated genes after MHMD treatment. B, GO analysis for the down-regulated genes upon MHMD treatment.

there were 850 up-regulated genes and 855 down-regulated genes after 48 h treatment. In view of MHMD-induced cell apoptosis in previous experiments, in this research we focused on the analysis of major genes related to apoptosis and non-small cell lung cancer pathways, which are shown in the Fig. 5A.Among the 12 major genes, 6 of them were up-regulated after treatment with MHMD, which were apoptosis-related cysteine peptidase (CASP7, 3.2 times), tumor necrosis factor receptor superfamily member 10b and 10d (TNFRSF 10B and 10D, 5.52 and 6.33 times), interleukin 1 alpha and beta (IL1A and IL1B, 3.83 and 11.47 times), transforming growth factor alpha (TGFA, 3.76 times), respectively. In addition to this, other half of genes were down-regulated according to current transcriptional data and tumor necrosis factor (ligand) superfamily member 10 (TNFSF10) possessed the most down-regulation rate (60.33 times), which suggested that it might play important roles in the apoptosis process of A549 induced by MHMD.

Verification of transcriptomic data by RT-PCR

To further evaluate current DEG library, the mRNAs of two up-regulated genes and two down-regulated genes with different fold changes in the apoptotic or NS-CLC pathway were randomly selected to be measured by RT-PCR (The primer sequence of the selected genes and actin gene are listed in the Figure. 5A). As shown in the Figure. 5B, tgfa and caspase-7 showed increased expression tendency, while the tp53 and prkar1b exhibited lower expression in the MHMD-treated groups, which

A

Gene name	Oligonucleotide sequences of primers
TGFA	Forward: CACTCAGTTCTGCTTCCAT Reverse: GATGATAAGGACAGCCAGG
CASPASE-7	Forward: CTTCCTCTTCGCCTATTCC Reverse: GAGTTCCTTGGTGAGCAT
TP53	Forward: CAAGCAGTCACAGCACAT Reverse: TACAGTCAGAGCCAACCT
PRKAR1B	Forward: ACTGTATCGTCCACCTCTG Reverse: GCGACACCTCCTCATCAT
ACTIN	Forward: AGCGAGCATCCCCCAAAGTT Reverse: GGGCACGAAGGCTCATCATT

B

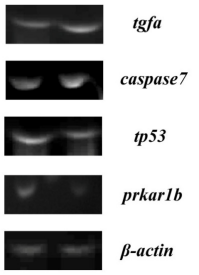

Figure 5: A. Oligonucleotide sequences of primers of five genes. B. RT-PCR analysis of the selected genes. The tgfa and caspase-7 genes were upregulated and the other two genes were downregulated. Actin was as control.

kept similar with high-throughput sequencing data.

Discussion

Curcumin analogs have been studied for their high anti-inflammatory, anticancer, antiviral and other pharmacological effects (13,14). Our previous study had exhibited the curcumin analog MHMD possessed higher antiproliferative effect than curcumin and induced the expression of typical apoptotic proteins of A549 cells (11,12).But whether there are other factors involving in the antiproliferative process are not clear yet. Here, we employed the RNA-seq technique to analyze the transcriptomic profiles of A549 cells after MHMD treatment at 48 h. Current results got a transcriptomic library of 18635 unigenes, most of which could be mapped on the human test genome. More than 1700 genes exhibited differential expressions in treated cells and many of them were involved in the antiproliferative process of MHMD in A549 cells.

Because of the myriad effects of MHMD on key biomolecules that inhibits A549 cells growth, this study continues to identify the major genes which were related to apoptosis and non-small cell lung cancer pathways. As an essential process in cell homeostasis and eukaryotic development, there is considerable interest in targeting the molecular pathways of apoptosis as a component of cancer therapy (15,16). In our study, as expected, casp7 was up-regulated. Two TNFR superfamily members (10b and 10d) exhibited up-regulated expression pattern. Ramachandran et al.(17) found that TNFR and TNFRSF5 were both down-regulated in curcumin-treated MCF-7 human breast cancer cell line, while TNFR was activated in human pancreatic cancer cell line BxPC-3 and MiaPaCa-2 cells after curcumin treatment (18). However, Hashem et al. (19) showed that administration of curcumin decreased TNF-α and TNFR2without affecting TNFR1 levels in rats. This suggested that curcumin or its analogs might make different impacts on the expression of TNFR family members in particular cancer cell lines. In addition, expression of IL-1β and IL-8 expression could also be modulated after curcumin analog (2). LPS induced production of IL-6 was often inhibited by the most curcumin analogs (20,21). So, it is not surprising that our transcriptomic analysis showed the upregulation of IL-1α and IL-1β in MHMD-treated A549 cells. Besides above, our transcriptomic upregulation of TGF-α was also different from previous report on the TGF-β in curcumin-treated human HK-2 cells (22). Hence, still more work need to be carried out to identify the apoptosis-related molecules and their function.

Retinoic acid receptor beta (RARB) and E2F transcription factor (E2F2) were down-regulated in current transcriptomic data. Retinoic acid facilitates the growth of mammary carcinoma cells which express high levels of fatty acid-binding protein 5 (FABP5) and curcumin could restore sensitivity to retinoic acid in triple negative breast cancer cells (23), which facilitated to cell growth suppression. Here, down-regulation expression of RARB might similarly prevent the interaction of RARB and retinoic acid, and then help to MHMD-induced A549 cell death. While E2F transcription factors regulate cell cycle, apoptosis and other wide range of biological processes and E2F2 could act as an activator in tumor progress of non-small cell lung carcinoma (24). Conversely, down-regulated expression of E2F2 may contribute to the MHMD-induced A549 cell death.

In conclusion, a comprehensive RNA-Seq expression characterization of DEGs from MHMD-treated A549 cells was carried out, which generated new data relating to the molecular and cellular signaling factors induced by the MHMD. Future functional studies will further confirm the role of these candidate genes in the anticancer process of MHMD.

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