Transcriptome analysis of sinensetin-treated liver cancer cells guided by biological network analysis

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Abstract. Hepatocellular carcinoma is recognized as one of the most frequently occurring malignant types of liver cancer globally, making the identification of biomarkers critically important. The aim of the present study was to identify the genes involved in the anticancer effects of flavonoid compounds so that they may be used as targets for cancer treatment. Sinensetin (SIN), an isolated polymethoxyflavone monomer compound, possesses broad antitumor activities in vitro. Therefore, the identification of a transcriptome profile on the condition of cells treated with SIN may aid to better understand the genes involved and its mechanism of action. Genomic profiling studies of cancer are increasing rapidly in order to provide gene expression data that can reveal prognostic biomarkers to combat liver cancer. In the present study, high-throughput RNA sequencing (RNA-seq) was performed to reveal differential gene expression patterns between SIN-treated and SIN-untreated human liver cancer HepG2 cells. A total of 43 genes were identified to be differentially expressed (39 downregulated and 4 upregulated in the SIN-treated group compared with the SIN-untreated group). An extensive network analysis for these 43 genes resulted in an in vitro model (3). The major way to explore the molecular mechanism involved in the anti-cancer effect of flavonoids is by determining gene activities and functions using transcriptome analysis (9). Genomic findings through a sequencing approach help to develop strategies targeting these genes for treating liver cancer.

Introduction

Liver cancer is one of the most common cancers with an increasing death rate. Approximately 0.56 million new cases are reported annually (1). Approximately 50% of patients during treatment with chemotherapy develop metastasis, thus reducing their survival rate (2). Difficulties in chemotherapeutic techniques have led to other treatment options such as the use of nutraceuticals and natural therapies for combating this disease with prior knowledge about their mechanisms of action (3). Thus, an emerging theme in cancer biology is targeting genes and/or proteins that can link to the progression of cancer and factors that directly or indirectly affect the proliferation and metastasis (4). Natural herbs have gained attraction in the current era due to their roles in controlling cancer growth by targeting oncogenes and proteins that are altered in cancers. Isolated monomer polymethoxyflavones (PMFs) have shown a broad spectrum of anti-cancer activities by inhibiting cell proliferation and cancer prevention (5). Sinensetin (SIN) is one such polymethoxyflavone present in citrus fruits that has notable anti-inflammatory and anti-tumor activities (6). Although the anti-cancer effect of SIN in cancer cells has been studied recently, gene expression changes and molecular mechanisms associated with its anti-cancer activities remain largely unknown.

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Key words: liver cancer, sinensetin, RNA sequencing, transcriptome involved in cancer after SIN treatment in HepG2 cells and may help to develop strategies targeting these genes for treating liver cancer.
Furthermore, gene expression obtained from transcriptome data can lead to the discovery of novel key genes associated with the related pathway (11). The advent of next-generation sequencing (NGS) data provides a detailed cancer profile that can explain the relationship and connection of genes involved in a disease. In addition, the analysis of differential patterns of genes can help us understand biological processes, cellular components, and interacting pathway network related to cancer pathogenesis for each one (12). Furthermore, bioinformatics analysis of differential gene profiles is an attractive strategy to identify novel therapeutic biomarkers for treating a disease (13). In this regard, taking an integrated approach to identify mRNA targets using next-generation sequencing data can reveal specific biomarkers for cancer types.

Developed almost a decade ago, RNA-seq is a potent tool for understanding genomic functions. Differentially expressed genes (DEG) remains the primary application of RNA-seq (14). DEGs are genes whose expression levels are significantly altered between two or more conditions such as before and after treatment with a drug. In this regard, the concept of hub genes is gaining interest. Hub genes are highly interconnected genes in a protein-protein interaction (PPI) network. Their associated biological process gene ontology terms and pathways might improve our understanding of their roles in carcinogenicity (15).

In the current study, we performed Illumina NovaSeq6000 sequencing for SIN-treated and -untreated HepG2 liver cancer cells and studied differential gene expression patterns. Highly interconnected genes (hub) genes among these were studied extensively for their roles in cancer. The expression of these hub genes was further validated by qRT-PCR.

**Materials and methods**

**Cell culture.** A human liver cancer cell line (HepG2) authenticated using short tandem repeat (STR) profiling was obtained from the Korean Cell Line Bank (Seoul, Korea). HepG2 cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO₂.

**Isolation of RNA for sequencing.** HepG2 cells were seeded into 6-well plates and treated with 100 µM of SIN for 48 h at 37°C. After 48 h treatment, total RNAs were extracted using TRIzol. The concentration of RNA was determined using a spectrophotometer. Isolated total RNA was then subjected to sequencing to obtain expression data.

**Library preparation and sequencing.** Sequencing was performed by TheragenEtex (Gyeonggi-do) with the following described protocol. RNA-seq libraries were constructed using a TruSeq Stranded mRNA Sample Prep Kit (Illumina, Inc.) for 150-bp paired-end sequencing. Poly-A-containing mRNA molecules were purified and fragmented from 2 µg of DNase-treated total RNA using oligo(dT) magnetic beads. Following the purification step, mRNAs were fragmented and used for synthesis of single-stranded cDNAs by means of random hexamer priming. With the constructed single-stranded cDNAs as templates, second strand cDNA synthesis was carried out to prepare double-stranded cDNAs. These cDNAs were then amplified with a sequential process of end-pair repair, addition of A-tail, and adapter ligation using polymerase chain reaction (PCR). The quality of constructed cDNA libraries was evaluated with an Agilent 2100 BioAnalyzer (Agilent Technologies, Inc.) and quantified with a KAPA library quantification kit (Kapa Biosystems) according to each manufacturer’s protocol. These products were then purified and enriched with cluster amplification using PCR to obtain the final complementary DNA library for high-throughput paired-end (2×150 bp) DNA sequencing using an Illumina NovaSeq6000 (Illumina, Inc.).

**Transcript data analysis.** After filtering out low quality reads, TopHat was used to map quality-filtered reads to a reference genome (hg38) (16). We measured gene expression levels with Cufflinks v2.1.1 using the Ensembl human gene annotation database. We performed differential expression analysis using cuffdiff (17). To improve the accuracy of measurement, we applied frag bias and multi-read correct options for both cuff-links and cuffdiff. All other options were set to default values. DEGs were identified and filtered with the following criteria: false discovery rate <0.05 and log₂ FC >1 (18).

**Identification of hub genes and their analysis.** To identify hub genes among DEGs, a PPI network of 43 DEGs was constructed using STRING (https://string-db.org/) with a ‘minimum required interaction score’ set to medium confidence (0.400) and ‘maximum number of interactors to show’ set to query proteins for both 1st and 2nd shells (19). The PPI network was imported into Cytoscape using CytoHubba’s Maximal Clique Centrality (MCC) scoring method to identify top ten hub genes incorporated in STRING and to discover significantly enriched biological process gene ontology (GO) terms and KEGG pathways (20). Top ten biological process GO terms with the lowest false discovery rate were analyzed using REVIGO (21). Additionally, we performed an extensive literature survey for these genes to uncover their roles in cancer.

**qRT-PCR and data analysis.** To validate hub genes differentially expressed based on transcriptome analysis, we studied mRNA expression levels of these hub genes by quantitative real-time polymerase chain reaction (qRT-PCR). HepG2 cells were seeded into 6-well plates and treated with 100 µM of SIN for 48 h at 37°C. Total RNA was isolated and its concentration was determined using a spectrophotometer. Total RNA (1 µg) was converted to cDNA using an iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Inc.). qRT-PCR was then performed using cDNA and AccuPower® 2X Greenstar™ qPCR Master (Bioneer). qPCR primers used in this study are listed in Table I. All data were analyzed using Bio-Rad CFX Manager Version 3.1. We measured relative quantification using the $2^{-\Delta\Delta Cq}$ method. mRNA expression levels of target genes were normalized against those of β-actin.

**Statistical analysis.** All data were analyzed using GraphPad Prism version 5.0 (GraphPad Software). Results are expressed as means ± SEM. They were evaluated using the Student’s t-test. P<0.05 was considered to indicate statistical significance.
Results

Identification of DEGs. In our previous study on SIN treatment in HepG2 cells, we found that SIN could induce HCC cell death in vitro (22). RNA-seq and Cuffdiff identified 43 DEGs (Table II) between SIN-untreated and SIN-treated HepG2 cells based on the selection criteria (a false discovery rate <0.05 and |log₂ FC| >1). Out of these 43 DEGs, 39 were downregulated and 4 were upregulated. These DEGs were plotted using the heatmap.2 function from R's gplot package.

Table II. Upregulated and downregulated genes in SIN-treated HepG2 cells compared with SIN-untreated HepG2 cells.

|                | A, Upregulated genes | B, Downregulated genes |
|----------------|----------------------|------------------------|
| **No.**        | **Genes**            | **No.**                |
|                |                      |                        |
| 1              | REEP6                | 1                      |
| 2              | CYP1A1               | 2                      |
| 3              | RASGRP2              | 3                      |
| 4              | UGT1A1               | 4                      |

| Gene name      | Primer sequence (5'‑3')                  |
|----------------|------------------------------------------|
| Ceruloplasmin  | F: TGCAAATGGGAGACAGAAGAGA<br>R: TCAGGTGCAAGTGTAAACATTC |
| FGB            | F: CAGGGACATGACATCCAC<br>R: CATCCACCCAGCTCCTCACCA |
| IGF2           | F: CAGGGTTCTACTTCAGCGAG<br>R: GTAGCACTAGTACGTCTCCAG |
| ITIH3          | F: CAGGTGCAAGGCTTCTATAGA<br>R: GCTGTTAAGGTGTTG |
| NR1H4          | F: TACGGAGTTTCAGACCTTGGAC<br>R: CCCAGCAAGGAAGTTCATTGTA |
| ORM1           | F: ACCAAGGTGAGTGCTTGGG<br>R: AGCAACGTAGTGGTCTTGG |
| ORM2           | F: ACCAGTGCTTTGCTAATACTCCAG<br>R: ATCCGTCAGTACGTGCAGA |
| SERPINA7       | F: ATATGACCGTGGAGCCAC<br>R: CTTATGGGCAGCATGCAAGA |
| SERPINE1       | F: GACCACGACACTGCAAGA<br>R: TACAGGTCTCTTGCTGCA |
| THBS1          | F: CCGGACTGGCTGTAGGTTA<br>R: CTGGACTTGGTAGGCAAGA |
| β‑actin        | F: TTCTACAATGAGCTGCTGGTG<br>R: GTGTTGAAAGGTCTCAACATGAT |

F, forward; R, reverse; FGB, fibrinogen β chain; IGF2, insulin-like growth factor 2; ITIH3, inter-α-trypsin inhibitor heavy chain 3; NR1H4, nuclear receptor subfamily 1 group H member 4; ORM1/2, orosomucoid 1/2; SERPINE1, serpin family E member 1; SERPINA7, serpin family A member 7; THBS1, thrombospondin 1.

Hub genes and their enrichment analysis. Hub genes are highly interconnected genes that might be involved in important cancer-related biological processes and functions. Fig. 2 shows the protein-protein interaction network of the ten hub genes identified by Cytohubba's MCC tool.

When these hub genes were subjected to STRING and enrichment analysis to identify significant GO terms and pathways, Table II summarizes the upregulated and downregulated genes in SIN-treated HepG2 cells compared with SIN-untreated HepG2 cells.

SIN, sinensetin.
KEGG pathways, a list of 101 biological process GO terms and three significant pathways were retrieved. For the ease of analysis and visualization, we did a REVIGO analysis for top ten biological process GO terms showing the lowest FDR values (23). REVIGO indicated that these genes were mainly involved in immune-system responses, regulation of tumor necrosis factor production, regulation of the apoptotic process, regulation of protein metabolism, and transport and secretion processes as shown in the REVIGO scatter plot (Fig. 3). Additionally, KEGG pathway analysis revealed that SERPINE1 and THBS1 modified wild-type human p53 (TP53), complement, coagulation cascade, and proteoglycans in cancer pathways.

Roles of expression of hub genes in cancer. All our hub genes were downregulated. Therefore, we did a comprehensive literature search to figure out roles of these upregulated hub genes in cancer.

*Insulin-like growth factor-2 (IGF2).* IGF2 is a 67-amino-acid mitogenic peptide hormone involved in the regulation of cell growth, differentiation, and metabolism. It is mainly expressed by the liver, although it can be expressed in many other tissues (24). IGF2 is overexpressed in a variety of cancers. Cancer cells that overexpress IGF2 have a strong tendency to metastasize (25,26). One study has shown that there is a five-fold increase in the expression of IGF2 in HepG2 cells (27). The use of antisense oligodeoxynucleotides (ATON) to halt the translation of IGF2 mRNA has shown
that a decrease of IGF2 can inhibit the growth of HepG2 cells. Another study has also observed an overexpression of IGF2 in hepatocellular carcinoma cells. Similarly, there is a notable decrease in tumor growth and an increase in mice survival when antibodies against IGF1 and IGF2 are administered (28).

**SERPINE1 and SERPINA7.** SERPINE1 and SERPINA7 belong to the human SERPIN gene family, which gets its name from its originally identified function of serine proteinase inhibition. However, many of its members also act as chaperones involved in storage, transport, and other roles (29-31). SERPINE1 encodes for a serine proteinase inhibitor. It can inhibit tissue plasminogen activator (tPA) and urokinase (uPA). High levels of SERPINE1 have been associated with low prognosis rate and survival of lung, breast, oral, stomach, and ovarian carcinoma patients (32-34). In addition, reducing the level of SERPINE1 can decrease cell migration in thyroid cancer (35). In relation to HCC, higher levels of SERPINE1 and increased SERPINE1 proteins associated with invasiveness, metastasis, and prognosis in patients with liver cancer have been observed (36,37). SERPINA7 encodes thyroxine-binding globulin (TBG), a human thyroid hormone protein. SERPINA7 has been found to be upregulated in colorectal cancer patients (38). One study on 30 patients with primary liver cancer has found that 22 of them have higher TBG concentrations. Additionally, in 28 out of 31 patients with liver metastasis, TBG concentration is higher than normal (39).

**Fibrinogen beta chain (FGB).** Fibrinogen is a glycopeptide synthesized by hepatocytes. It is formed by connection of two trimers with each trimer containing a fibrinogen alpha chain that is encoded by the FGA gene, along with the fibrinogen beta chain or gamma chains encoded by FGB or FGG gene, respectively. Increased fibrinogen activity can affect tumor cell growth, progression, and metastasis (40). Moreover, colorectal cancer growth is reduced in fibrinogen-deficient mice (41). The FGB gene is also upregulated in lung carcinomas and breast cancer (42,43). Although we could not find a direct link between upregulation of FGB and HCC, *in vitro* studies have shown that FGG (another gene involved in fibrinogen formation) upregulation can promote the migration and invasion of HCC whereas knockdown of FGG can significantly inhibit phenotypes (44).

**Orosomucoid 1 (ORM1) and orosomucoid 2 (ORM2).** The orosomucoid gene family contains two polymorphic genes (ORM1 and ORM2) primarily secreted by the liver, although they are also abundant in the plasma. They encode for acute-phase proteins that are expressed during stressful conditions such as tissue injury, infections, and inflammations (45). It has been reported that ORM genes are over-expressed in breast cancer, bladder cancer, lung cancer, cholangiocarcinoma (bile duct cancer), colorectal cancer, and HCC (46-50). However, the mechanism of how orosomucoid genes affect cancer cells remains unclear. Of particular interest, knockdown of ORM1 can result in decreased proliferation of HCC cells (46).

**Nuclear receptor subfamily 1 group H member 4 (NR1H4).** NR1H4, also known as farnesoid X receptor (FXR), is mainly expressed in the liver, kidney, intestine, and adrenal gland. It is a member of the nuclear receptor superfamily. It is activated upon binding to bile acid for regulating bile acid homeostasis (51-53). *In vitro* studies have revealed that the expression of FXR is significantly increased in non-small-cell lung carcinoma (NSCLC), resulting in cell proliferation. Knockdown of NR1H4 can inhibit cell...
proliferation and xenograft growth in nude mice models. A delay in the G1/S transition of cell cycle has also been reported after knockdown of NR1H4 in NSCLC (54). The expression of FXR in esophageal carcinoma is shown to be highly associated with increased tumor size and lymph-node metastasis, whereas deletion of this gene can suppress tumor-cell growth in both in vitro and in vivo studies (55). Some evidence has highlighted the role of FXR in liver carcinogenesis. How FXR promotes cell proliferation has been elucidated in a HepG2 cell line among others by suppressing the expression of p16/INK4a. Downregulation of FXR also shows proliferation-inhibitory effects (56).

Thrombospondin 1 (THBS1). THBS1, a member of the thrombospondins family of proteins, is an important component of the extracellular matrix (57). Upregulation of THBS1 can increase the invasion and migration of gastric cancer cells, prostate cancer, gliomas, pancreatic cancer, and ovarian cancer (58-62). High expression level of THBS1 is associated with invasiveness and progression of hepatocellular carcinoma cells as well as poor prognosis (63). THBS1 expression is also observed in stromal cells surrounding the cancer (64). A study by Lee et al has highlighted the role of THBS1 in HCC tumor progression because suppression of THBS1 can mediate CD47 signaling and decrease the growth of cancerous liver cells (65).

Ceruloplasmin (CP). CP is an enzyme involved in ferroxdase activity, copper transport, amine oxidase activity, and superoxide dismutase activity (66). In breast cancer, elevated CP levels are associated with metastasis and higher chances of recurrences (67). CP levels are also increased in patients with pancreatic ductal adenocarcinoma, ovarian cancer, bile duct cancer, and cervical cancer (68-71).

Inter-alpha-trypsin inhibitor heavy chain 3 (ITIH3). Inter-alpha-trypsin inhibitors are a family of serine protease inhibitors that are formed by the combination of one light chain and one or two heavy chain proteins. Structurally, two or more heavy chains are covalently linked to hyaluronan acid (HA) which forms the major portion of the cellular matrix (72). Upregulation of ITIH3 expression has been observed in lung cancer and gastric cancer (73-75). The present transcriptome data showed that ITIH3 was downregulated in SIN-treated HepG2 cells than in untreated HepG2 cells. Literature evidence accentuates the role of these hub genes in cancer development when they are expressed at higher than normal levels.

qRT-PCR confirms downregulation of hub genes. Differently expressed, highly integrated hub genes (CP, FGB, IGF2, ITIH3, NR1H4, ORM1, ORM2, SERPINE1, SERPINA7 and THBS1) were quantified for mRNA expression by qRT-PCR. In agreement with transcriptome analysis data, qRT-PCR analysis results validated the downregulation of these hub genes in SIN-treated cells than in untreated HepG2 cells (Fig. 4). These results further confirm the role of SIN in regulating the expression of hub genes in HepG2 cells.

Discussion

A transcriptome of an organism describes a small proportion of its genetic codes that can be transcribed into RNA molecules. Post-transcriptional processing of RNA plays a crucial role in terms of producing more variant forms of mRNA (76). Thus, it is clear that studying the whole transcriptome and understanding their modifications can provide an extensive knowledge for developing novel strategies to control diseases. Transcriptome profiling has gained extensive attention in cancer research because it enables disease condition analysis and treatment outcomes. The analysis of RNA-seq data to obtain gene expression and transcriptional changes in cancer supports novel approaches (77). Genes and signaling pathways involved in cancer and treatment outcomes have been identified by microarray and RNA-seq approaches. With the advent of second- and third-generation sequencing technologies, RNA-seq is a significant method owing to its low false-positive results and increased reproducibility rate compared to microarrays (78). It also gives an accurate expression change of transcripts and their isoforms that can help us discover novel transcripts. Transcriptome profiling via RNA-seq has discovered many genes that are potentially involved in the anti-cancer effect of natural compounds like flavonoids. A recent report on
integrated whole-transcriptome profiling of genes in HCT-116 cancer cells by quercetin treatment has revealed pathways that can regulate cancer progression (79).

Sinensetin (SIN), a polymethoxyflavone present in the citrus family, can inhibit several cancers by regulating oxidative stress of cells (80). Our previous study has displayed an autophagy-mediated anti-cancer potential of SIN in liver cancer cells. In the current research, we performed RNA-seq analysis for SIN-treated and SIN-untreated liver cancer cells (HepG2) to identify critical genes associated with the anti-cancer potential of SIN.

In vitro and in silico techniques have been widely used to study anti-cancer effects of natural compounds and their mechanisms of action. In vivo animal models have also demonstrated different mechanism involved in their abilities to prevent diseases such as cancer, neurodegenerative diseases, and cardiovascular disorders (81). Such studies can help us understand effects of chemotherapeutics on different enzymes, cell signaling protein cascades, and gene expression.

In the current study, a total of 43 differentially expressed genes were identified between SIN-treated and untreated samples in HepG2 cells. Interestingly, most (39/43) of these genes were downregulated while only four were upregulated by treatment with SIN. With the help of STRING and Cytohubba, we identified ten hub genes from the DEG list. Enrichment analysis indicated that these hub genes were mainly involved in immune-system responses and regulation of tumor necrosis factor production, apoptosis, and protein metabolism. As presented in detail in the results section, we did an extensive literature survey on these identified hub genes, highlighting their roles in tumor growth, tumor invasiveness, poor prognosis, and recurrence in various cancers upon upregulation of their expression. RNA-seq analysis of HepG2 cells treated with SIN showed downregulation of these hub genes. Literature analysis sheds light on how downregulation of these hub genes might mediate anti-cancer processes. qRT-PCR data confirmed that expression levels of these hub genes were consistent with RNA-seq data. Hub genes CSP, FGB, IGF2, ITIH3, NRH14, ORM1, ORM2, SERPINE1, SERPINA7 and THBS1 are highly expressed in several cancers, including liver, lung, pancreatic, and cervical cancers. Significant downregulation of these genes upon SIN treatment showed its prominent capacity in suppressing these genes in HepG2 cells. The confirmation of expression data revealed that these genes could emerge as attractive therapeutic targets in the treatment of liver cancer. Furthermore, RNA-seq and relative expression data strengthened the argument that SIN is a strong anti-cancer agent in HCC.

In conclusion, in the current study, transcriptome analysis of SIN-treated HepG2 cells by next generation sequencing supported its anti-cancer effect. Analysis of DEGs provided a strong insight on the involvement of hub genes related to cancer progression. Results of this study indicate that SIN might induce HCC cell death by regulating the expression of these genes. The objective of this study was to identify the related gene on SIN anti-cancer effect using transcriptome analysis. This paper highlights the necessity for further studies to support anti-cancer effect of those genes.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GSK and KW L conceived, designed and performed the experiments. SMK, SR and AMK organized focus group discussion, collected and analyzed all study data, and prepared the final manuscript. SMK, PV, SEH and HHK performed some experiments and revised the final manuscript. GSK and SMK confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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