Oncologie
DOI: 10.32604/oncologie.2020.012436
Article

Tech Science Press

Sinomenium Inhibits the Viability of Hepatoma Carcinoma Cells through Activating IFNA2

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Abstract: Hepatocellular carcinoma (HCC) ranks the sixth place of most common cancers. Meanwhile, it is the tertiary mortality cause of cancer. There is no effective therapeutic method to prevent and treat the liver cancer. Sinomenine is a kind of Chinese traditional medicine herbal, it is reported that it can inhibit the viability of several cancer cells. The study is to explore whether sinomenine is also able to inhibit the cell viability of HCC and its potential mechanism. The IC50 of sinomenine in BEL-7402 cells was 5.351 mmol/L, and the IC50 of sinomenine in SMMC-7721 cells was 6.204 mmol/L. The gene expression results showed the relative expression of FGF2, CCND2, DCN, F3, MMP7, NRG1, HMGB1, TRIM29, HAS2, EHF, CTGF, PLK2 were down-regulated, and the relative expression of VEGF A, CITED2, NUPR1, DDX58, IRF9, NAMPT, MMP1, NDRG1, HMGA2, PPARGC1A, IFIT2, PARP9, HEY1, LOX, ETV1, ISG15, BACH, CYLD were up-regulated. Moreover, the IPA analysis results suggested that IFIT3, IFIT1, OAS1, MX1, IRF9, IFI6, IFITM1, ISG15 were up-regulated in BEL-7402 cells treated with sinomenine by activating IFNA2. The findings presented in this study may provide a promising method for the prevention and treatment of liver cancer.

Keywords: Hepatocellular carcinoma; sinomenine; ingenuity pathway analysis

1 Introduction

Liver cancer is also called hepatic cancer or primary hepatic cancer, which originates in human livers [1]. Liver metastasis means that cancer has spread from elsewhere to the liver. It is more common than that which originates in the liver. There are many possible symptoms of liver cancer, such as mass or pains under right ribs, abdominal swelling, yellowing of skin, easy bruising, and weight loss [2]. Globally, primary liver cancer ranks the sixth place of most common cancers (6%), which is also the secondary mortality cause of cancer (9%) [3]. It occurred in 782,000 people in 2012 and killed 810,500 people in 2015. In 2015, hepatitis B caused 263,000 deaths from liver cancer, alcohol caused 245,000 deaths, and hepatitis C caused 167,000 deaths [4]. Liver cancer has a high morbidity in regions with hepatitis B and C popularity, including Asia and sub-Saharan Africa. Men are more susceptible to HCC infection than women. The largest number of diagnoses are between 55 and 65 years of age. Five-year survival rates are 18% in the United States [5]. Unfortunately, there are no very effective treatments for liver cancer, so more effective therapeutic methods are under urgent need.

Chinese traditional herbal medicine (also known as Herbalism) studies the herbal treatments, which have been used by humans since ancient times. Today, this traditional medicine is still widely used. Modern medicine uses many of the compounds derived from plants as the basis for evidence-based drugs [6]. Sinomenine or cocculine is an alkaloid, existing in the root of the local climbing plant *Sinomenium acutum* in Japan and China. In these countries, the plant is traditionally used for the treatment of rheumatism and arthritis [7]. The release of histamine mainly mediates its anti-rheumatic effect [8]. But other effects may also be involved, such as inhibiting the synthesis of prostaglandins, leukotrienes and nitric oxide [9]. For the cancer treatment, Sinomenine was able to inhibit the cell viability of breast cancer [10], lung cancer [11], colon cancer [12], gastric cancer [13], and bladder cancer [14]. However, the inhibition of sinomenine on liver cancer has rarely been reported.

In our study, we hypothesis that sinomenine can inhibit the viability of liver cancer cells. The study selected BEL-7402 and SMMC-7721 cells as in vitro models to determine the impacts of sinomenine on their proliferation. The ingenuity pathway analysis was also use to explore its potential mechanism.

2 Methods and Materials

2.1 Cell Culture of SMMC-7721 and BEL-7402 Strains

Both DMEM (Dulbecco's Modified Eagle Medium) and RPMI (Roswell Park Memorial Institute) 1640 (Thermofisher Scientific, Waltham, MS, USA) were added with 10% FBS (fetal bovine serum) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). DMEM was applied to culture SMMC-7721 cell strain while RPMI 1640 were applied to culture Bel-7402 cell strain in a 37°C humid environment with 5% CO_2.

2.2 IC50 of Sinomenine in SMMC-7721 and BEL-7402 Cell Strains

Under normal conditions, SMMC-7721 and BEL-7402 cell strains (Invitrogen, Carlsbad, CA, USA) were inoculated in 96-well plates, with a density of 1,000 cells/well. After adhering for 12 hours, they were incubated in the sinomenine with different concentration. Following, all cells were treated by 10% TCA (tricarboxylic acid cycle) for 1 hour on the ice. Then, they were rinsed and dyed with 50 μl 0.4% SRB (Sulfate-Reducing Bacteria) (Invitrogen, Carlsbad, CA, USA) for 30 minutes. Next, cells were repeatedly rinsed with 1% acetic acid for the removal of the unbound dye. Then, these cells were dried and 10 mM Tris solution was used for the dissolution of the protein-bound stain. The PowerWave microplate reader (BioTek, Whiting, VT, USA) was used for the measurement of the SRB absorbance at 560 nm. The SRB assay was used to determine the concentration inhibiting cell survival in 50% (IC50) and the concentration inhibiting cell growth in 50% (GI50).

2.3 Effects of Sinomenine on SMMC-7721 and BEL-7402 Cell Strain Proliferation

SMMC-7721 and BEL-7402 cell strains were inoculated in 96-well plates (5,000 cells/well) for 24 hours. Then, they were added with different concentration of sinomenine. At each indicated time point, each well was added with CCK-8 solution (10 μl), and the treated cells were incubated for 3 hours at 37°C. A microplate reader (Sunnyvale, CA, USA) was utilized to measure the absorbance (A) at 450 nm.

2.4 RT-PCR

The GeneJET™ RNA Purification Kit (Thermofisher Scientific, Waltham, MS, USA) was used to extract the total RNA. The UV–vis spectroscopy was used for the measurement of the RNA concentration at 260 nm. The High Capacity cDNA Reverse Transcript kit purchased from Applied Biosystems (Applied Biosystems, Foster City, CA, USA) was utilized to process an equivalent amount of RNA to generate cDNA. Taqman gene expression assay ((Thermofisher Scientific, Waltham, MS, USA)) was adopted to perform the quantitative PCR (qPCR) through the Applied Biosystems 7500 Fast Real-Time PCR System ((Applied Biosystems, Foster City, CA, USA)). Triplicates were required for each sample, and the results were normalized to β-actin.

2.5 Gene Ontology and Pathway Enrichment Analysis

R software (clusterprofiler) and DAVID (https://david.ncifcrf.gov/) were used to do GO functional enrichment analysis of differentially expressed genes.

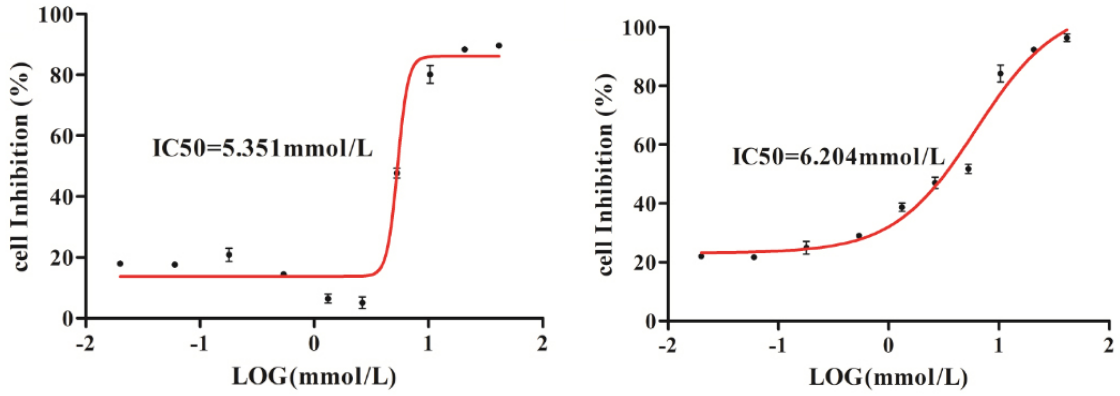
2.6 Analysis of Statistics

For each scaffold section, the obtained data were averaged. Also, they were written as mean ± standard deviation (SD). The one-way analysis of variance (ANOVA) was used to determine the statistical significance between the scaffold sections followed by *t*-test. It was considered that the differences were statistically significant at $p < 0.05$.

3 Results

3.1 Sinomenine IC50 of Hepatocellular Carcinoma (HCC) Cell Strain

Firstly, we tested sinomenine IC50 of HCC cell strain SMMC-7721 and BEL-7402. In Fig. 1A, the IC50 of sinomenine in BEL-7402 cells was 5.351 mmol/L. Also, the IC50 of sinomenine in SMMC-7721 cells was 6.204 mmol/L (Fig. 1B).

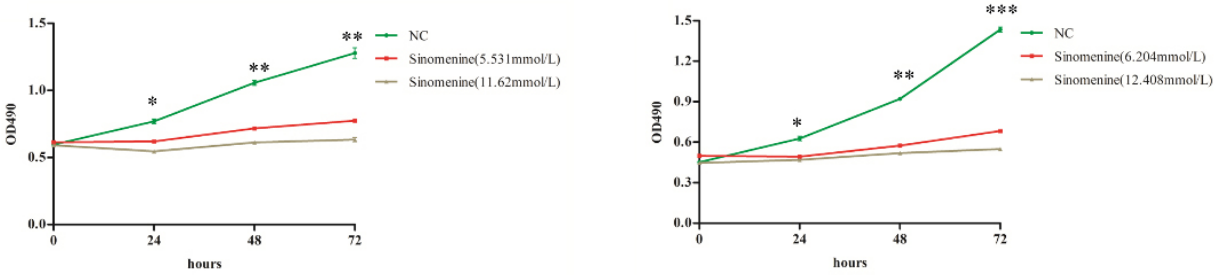


(A) (B)

Figure 1: The IC50 of sinomenine in BEL-7402 (A) and SMMC-7721 (B) cells

3.2 Effects of Sinomenine on HCC Cell Strain Proliferation

As shown in Fig. 2A, sinomenine can significantly inhibit BEL-7402 proliferation compared with the same cells without any treatment at each indicated time point. Moreover, the inhibition effects of 11.62 mmol/L sinomenine was better than 5.531 mmol/L. Similar results were found in SMMC-7721 cell strain (Fig. 2B).

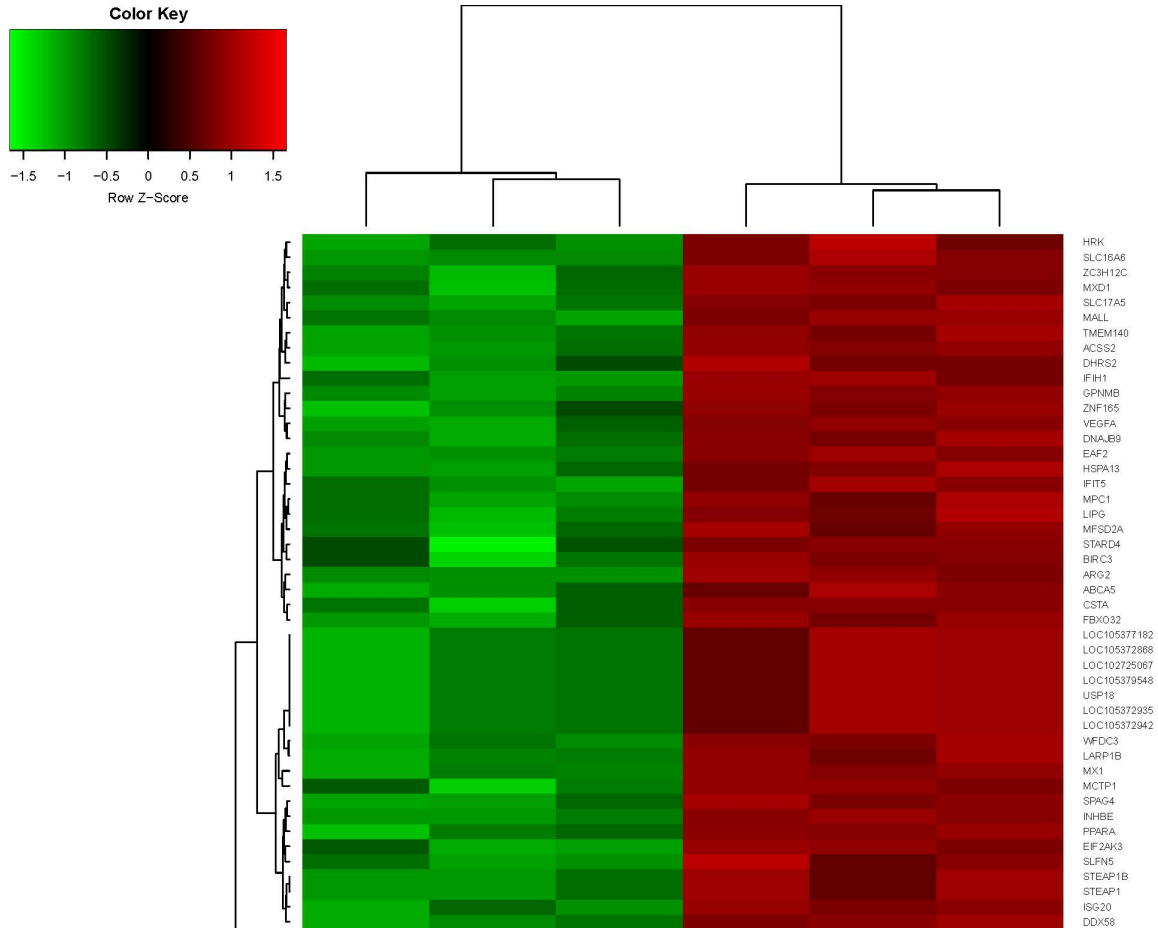


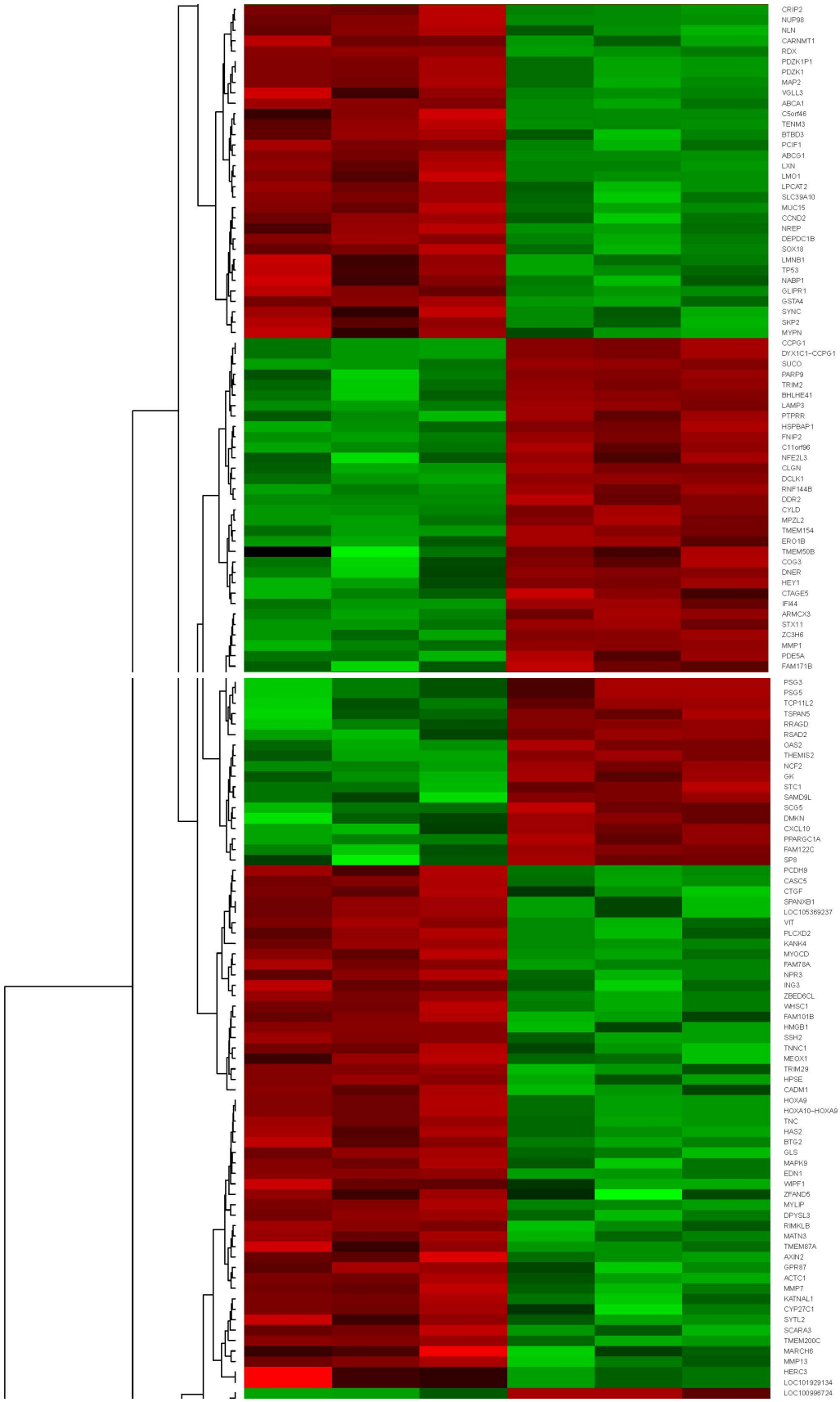
(A) (B)

Figure 2: Proliferation of BEL-7402 cells treated with 5.531 mmol/L and 11.62 mmol/L sinomenine, and SMMC-7721 cells treated with 6.204 mmol/L and 12.408 mmol/L sinomenine

3.3 Gene Expression Patterns

In order to explore the potential mechanism, the gene expression pattern of 5.531 mmol/L sinomenine treated BEL-7402 cells was performed. As shown in Fig. 3, 203 genes were up-regulated and 159 genes were down-regulated. The detailed information please see supplementary information 1. To further verify the above results, the RT-PCR was performed. As shown in Fig. 4, the relative expression of FGF2, CCND2, DCN, F3, MMP7, NRG1, HMGB1, TRIM29, HAS2, EHF, CTGF, PLK2 were down-regulated, and the relative expression of VEGF A, CITED2, NUPR1, DDX58, IRF9, NAMPT, MMP1, NDRG1, HMGA2, PPARGC1A, IFIT2, PARP9, HEY1, LOX, ETV1, ISG15, BACH, CYLD were up-regulated. It showed a similar trend with gene expression pattern.

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NLN
CARNMT1
RDX
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PDZK1
MAP2
VGLL3
ABCA1
C5orf45
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BTBD3
PCIF1
ABCG1
LXN
LMO1
LPCAT2
SLC39A10
MUC15
CCND2
NREP
DEPDC1B
SOX18
LMNB1
TP53
NABP1
GLIPR1
GSTA4
SYNC
SKP2
MYPN
CCPG1
DYX1C1−CCPG1
SUCO
PARP9
TRIM2
BHLHE41
LAMP3
PTPRR
HSPBAP1
FNIP2
C11orf96
NFE2L3
CLGN
DCLK1
RNF144B
DDR2
CYLD
MPZL2
TMEM154
ERO1B
TMEM50B
COG3
DNER
HEY1
CTAGE5
IFI44
ARMCX3
STX11
ZC3H6
MMP1
PDE5A
FAM171B
PSG3
PSG5
TCP11L2
TSPAN5
RRAGD
RSAD2
OAS2
THEMIS2
NCF2
GK
STC1
SAMD9L
SCG5
DMKN
CXCL10
PPARGC1A
FAM122C
SP8
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CASC5
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KANK4
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ING3
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FAM101B
HMGB1
SSH2
TNNC1
MEOX1
TRIM29
HPSE
CADM1
HOXA9
HOXA10−HOXA9
TNC
HAS2
BTG2
GLS
MAPK9
EDN1
WIPF1
ZFAND5
MYLIP
DPYSL3
RIMKLB
MATN3
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AXIN2
GPR87
ACTC1
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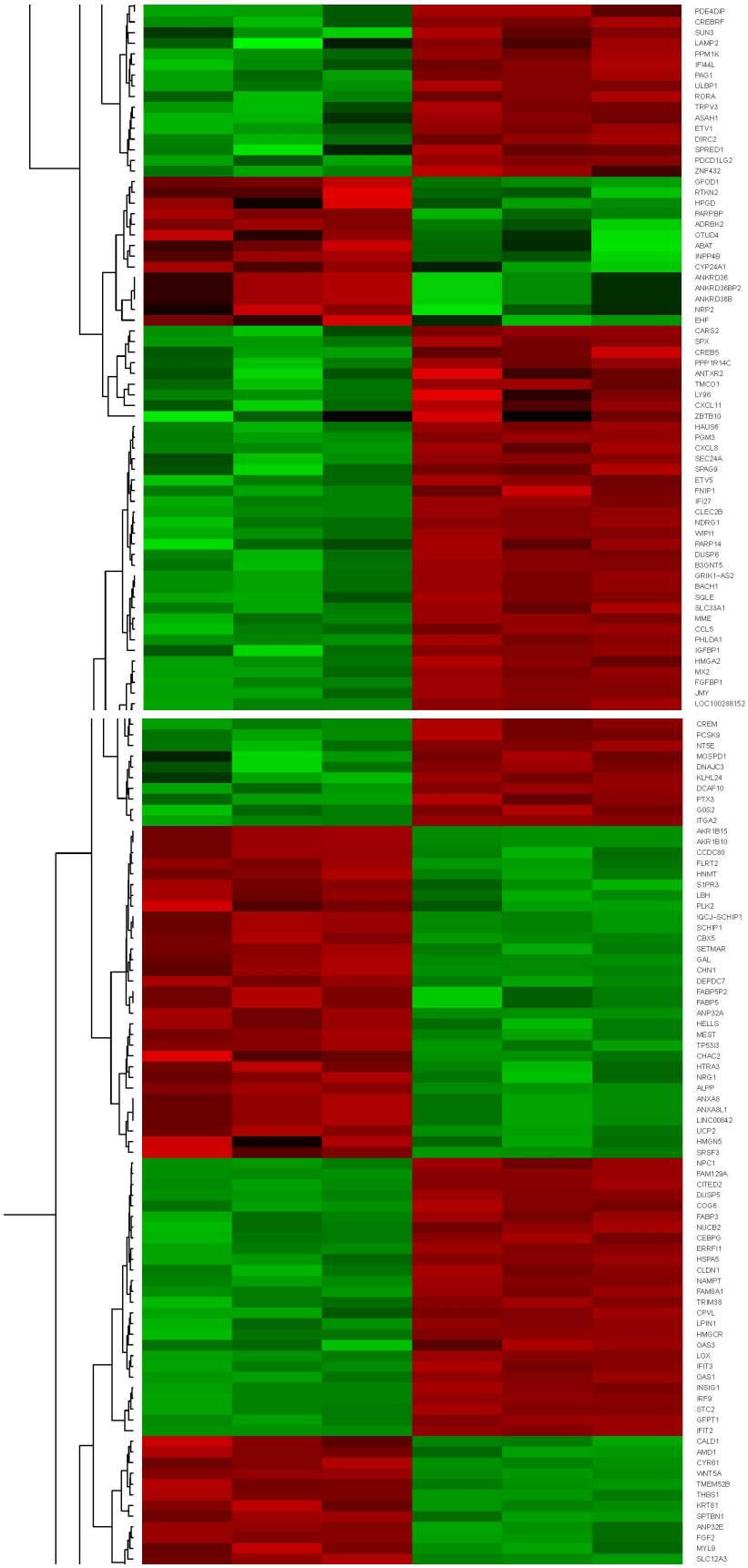
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CREBRF
SUN3
LAMP2
PPM1K
IFI44L
PAG1
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DIRC2
SPRED1
PDCD1LG2
ZNF432
GFOD1
RTKN2
HPGD
PARPBP
ADRBK2
OTUD4
ABAT
INPP4B
CYP24A1
ANKRD36
ANKRD36BP2
ANKRD36B
NRP2
EHF
CARS2
SPX
CREB5
PPP1R14C
ANTXR2
TMCO1
LY96
CXCL11
ZBTB10
HAUS6
PGM3
CXCL8
SEC24A
SPAG9
ETV5
FNIP1
IFI27
CLEC2B
NDRG1
WIPI1
PARP14
DUSP6
B3GNT5
GRIK1-AS2
BACH1
SQLE
SLC33A1
MME
CCL5
PHLDA1
IGFBP1
HMGA2
MX2
FGFBP1
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HNMT
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PLK2
IQCJ-SCHIP1
SCHIP1
CBX5
SETMAR
GAL
CHN1
DEPDC7
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FABP5
ANP32A
HELLS
MEST
TP53I3
CHAC2
HTRA3
NRG1
ALPP
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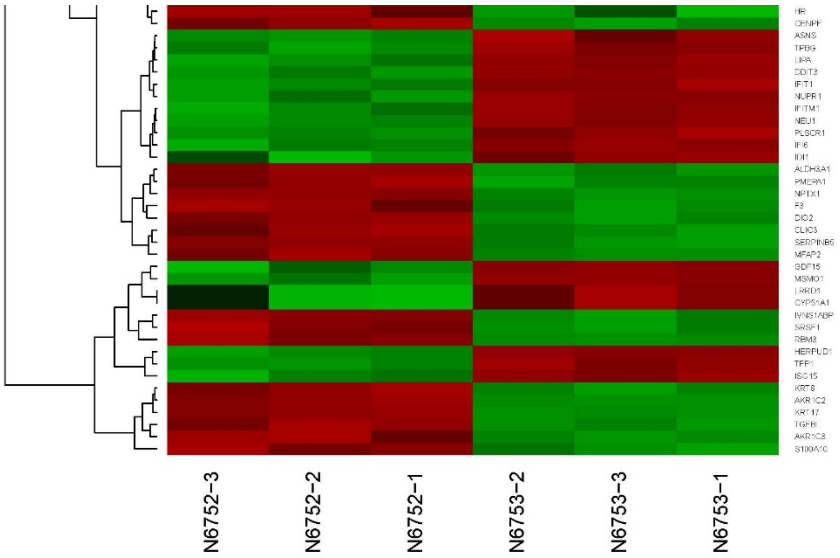

Figure 3: The heatmap of 5.531 mmol/L sinomenine treated BEL-7402 cells

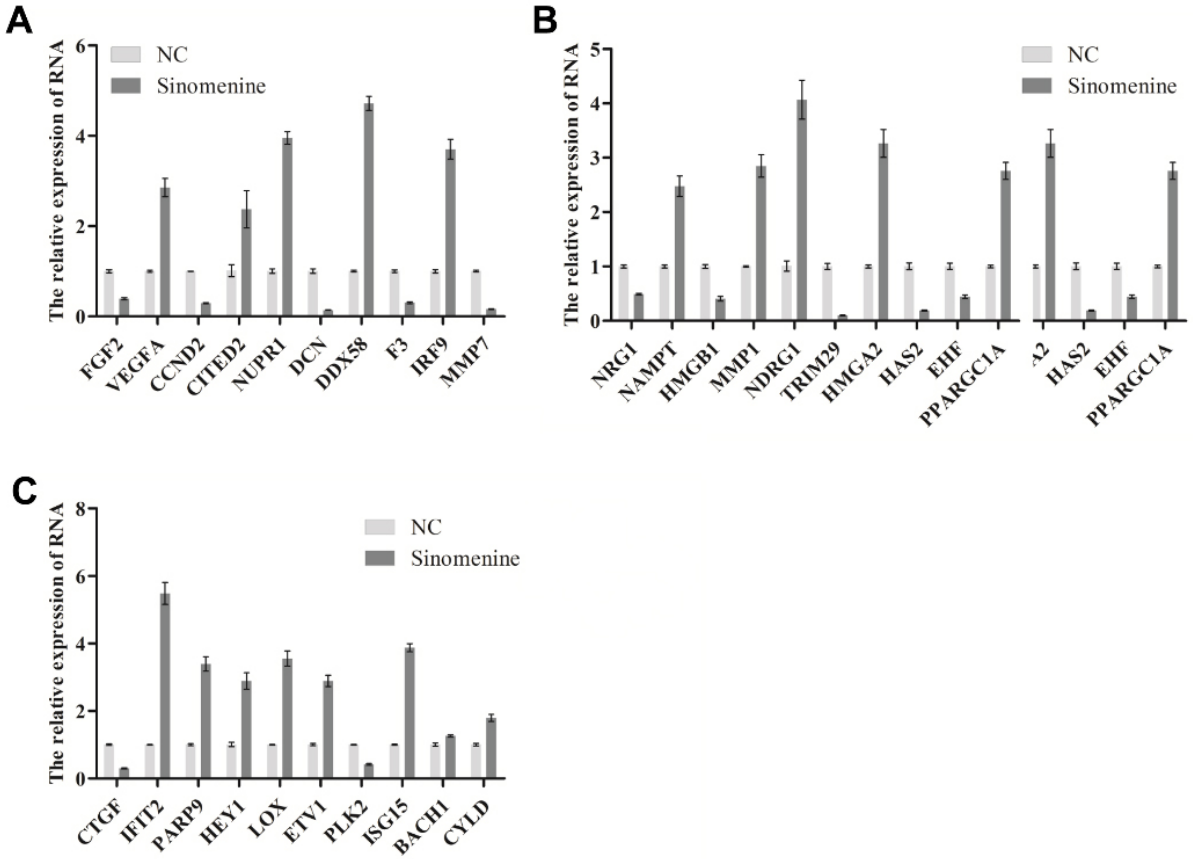

Figure 4: The relative expression of FGF2, CCND2, DCN, F3, MMP7, NRG1, HMGB1,TRIM29, HAS2, EHF, CTGF, PLK2, VEGF A, CITED2, NUPR1, DDX58, IRF9, NAMPT, MMP1, NDRG1, HMGA2, PPARGC1A, IFIT2, PARP9, HEY1, LOX, ETV1, ISG15, BACH, CYLD on BEL-7402 cells treated with 5.531 mmol/L sinomenine

3.4 Ingenuity Pathway Analysis

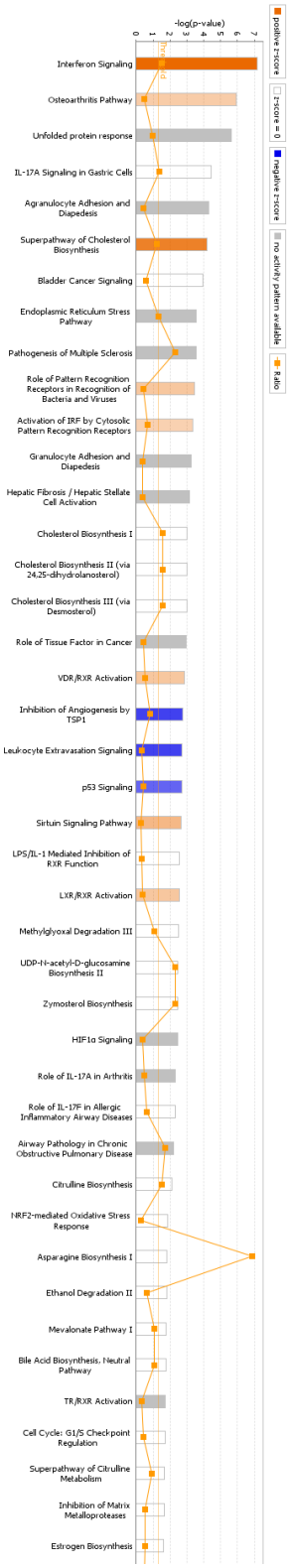

Figure 5: The IPA analysis of signaling pathway involved in BEL-7402 cells treated with 5.531 mmol/L sinomenine

Fig. 5 shows that the differential genes significantly enriches in the classical pathway. In the figure, the X-axis demonstrates the name of the path, and the Y-axis demonstrates the level of the enriching significance. The orange sign represents activated pathway (Z-score > 0), while the blue sign represents the inhibited pathway (Z-score < 0). The color depth of orange and blue (or the absolute value of Z-score) represent the degree of activation or inhibition (according to IPA's internal algorithms and standards, Z-score ≥ 2 is drastically activated pathway, Z-score ≤–2 is drastically inhibited pathway). In this project, the Interferon Signaling had a Z-score of 2.646, i.e., it was drastically activated. In the experimental results, red represents notably up-regulation of gene, while green represents notably down-regulation of gene. This figure visually shows the trend of expression of each gene in the pathway in the experimental results. IFIT3, IFIT1, OAS1, MX1, IRF9, IFI6, IFITM1, ISG15 were up-regulated in BEL-7402 cells treated with 5.531 mmol/L sinomenine (Fig. 6). Furthermore, sinomenine may regulate downstream differentially expressed genes by activating IFNA2. The downstream differentially expressed genes including: CCL5, CXCL10, CXCL11, DDX58, FGF2, IFI27, IFI44, IFI44L, IFI6, IFIH1, IFIT1, IFIT2, IFIT15, IFIT20, LAMP3, MX1, MX2, NT5E, OAS1, OAS2, OAS3, PARP9, PLSCR1, RSAD2, THEMIS2, TP53, USP18, VEGFA (Fig. 7).

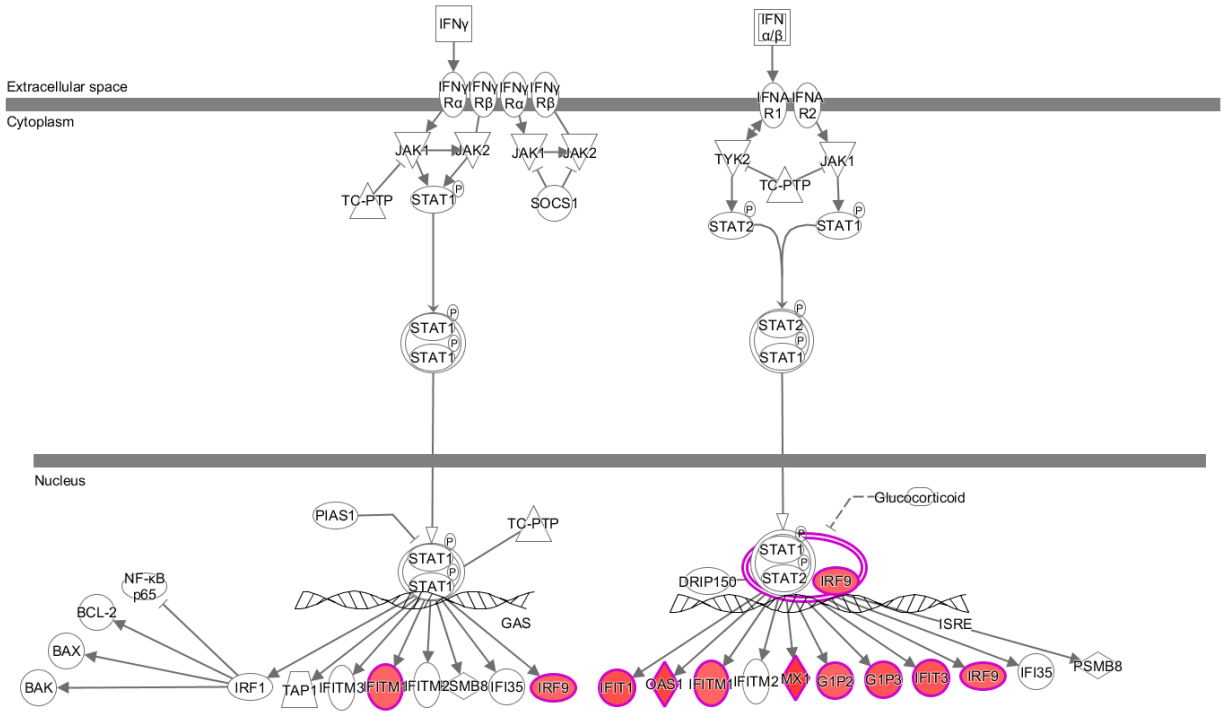

Figure 6: The IPA analysis of genes involved in BEL-7402 cells treated with 5.531 mmol/L sinomenine

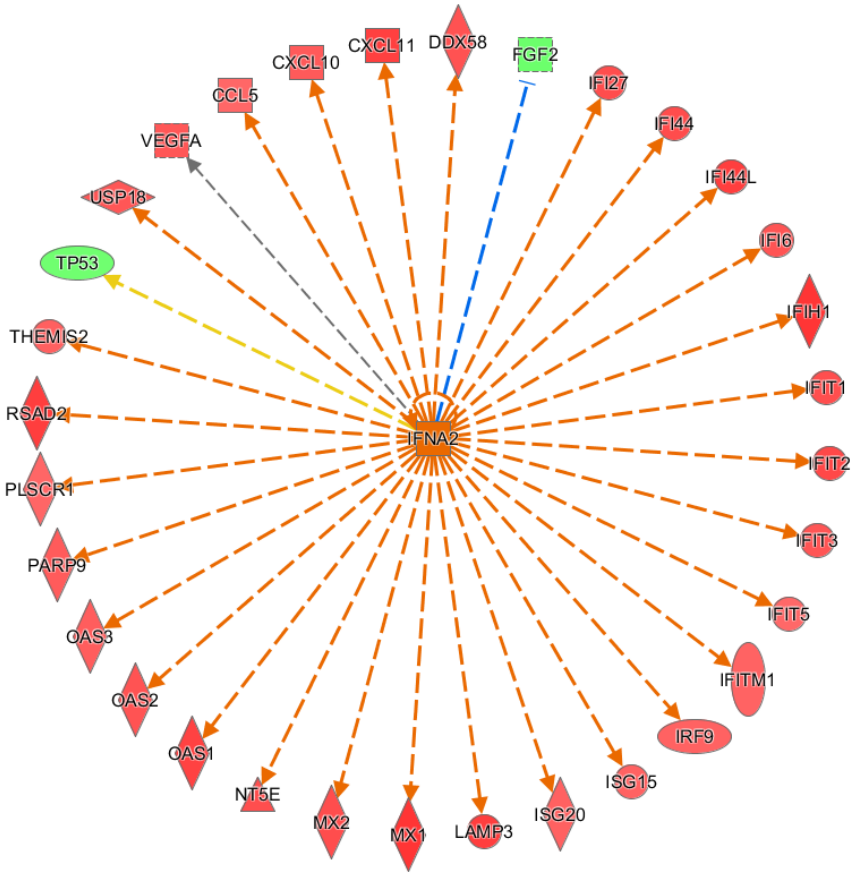

Figure 7: Sinomenine regulates the downstream differentially expressed genes by activating IFNA2

4 Discussion

HCC ranks the sixth place of most common cancers. Meanwhile, it is the tertiary mortality cause of cancer. In 2008, 748,000 new HCC cases were estimated to occur, while 696,000 patients died from HCC globally [15]. However, the therapeutic effect is still limited, and more effective drugs are expected to be developed [16]. In our study, we reported sinomenine showed a great potential to inhibit the viability of hepatoma carcinoma cells.

Since ancient times, as a Chinese herbal medicine, Sinomenium has been used to treat diseases. In recent years, many studies report the anti-cancer potential of sinomenium in vitro, such as breast cancer, lung cancer, colon cancer, gastric cancer, and bladder cancer. The anti-cancer effects of various drugs include inhibiting proliferation and inducing apoptosis [17,18]. It has been shown that sinomenium is critical in cell proliferation inhibition and cell apoptosis induction in various tumor cells of human [19–21]. It is consistent with our study, we also found sinomenium can inhibite the proliferation of the HCC cells.

Moreover, the potential mechanism of sinomenium inhibits viability of hepatoma carcinoma cells was discovered by IPA analysis. The gene expression patterns result showed the relative expression of FGF2, CCND2, DCN, F3, MMP7, NRG1, HMGB1, TRIM29, HAS2, EHF, CTGF, PLK2 were down-regulated, and the relative expression of VEGF A, CITED2, NUPR1, DDX58, IRF9, NAMPT, MMP1, NDRG1, HMGA2, PPARGC1A, IFIT2, PARP9, HEY1, LOX, ETV1, ISG15, BACH, CYLD were up-regulated. In addition, the IPA analysis results suggested that IFIT3, IFIT1, OAS1, MX1, IRF9, IFI6, IFITM1, ISG15 were up-regulated in BEL-7402 cells treated with sinomenine. Furthermore, sinomenine may regulate these downstream differentially expressed genes by activating IFNA2. In summary, the findings presented in this study may provide a promising method to prevent and treat liver cancer.

Funding Statement: This study was supported by grants from Gansu Youth Science and Technology Fund Program (17JR5RA196) and Hospital Project of the First Hospital of Lanzhou University (ldyyyn2018-40); Cui Ying scientific research and training program of the Second Hospital of Lanzhou University (2018 CYXZ-20).

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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