The Effect of LIGHT and IFN-γ Gene in the Apoptosis of $HepG_2$ Cells

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The Effect of LIGHT and IFN-γ Gene in the Apoptosis of $HepG_2$ Cells

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Abstract

The Effect of LIGHT and IFN-γ Gene To

the apoptosis of $HepG_2$ cells

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(Directed by Professor Seung Hoon Choi)

【**Objective**】To study the better transfection method in the HepG2 cells mediated by Cationic liposome with LIGHT gene and combined with LIGHT and IFN-γ genes, and the effect of LIGHT and IFN-γ gene in the apoptosis of HepG2 cells.

【**Methods**】To clone full-length ORF of LIGHT and IFN-γ gene. Dividing HepG2 cells into three groups: the control, solo transfection of LIGHT gene and combined transfection of LIGHT and IFN-γ genes, then transfect HepG2 cells mediated by Cationic liposome with pcDNA4C-LIGHT cDNA and pcDNA4His/MaxC-hIFN-γ cDNA extracted from E.coli JM-109, to cellect HepG2 cells on 12 hours,24 hours and 48 hours after transfection respectively .To investigate the apoptosis of HepG2 cells and the expression of cell factors, Fas 、FasL、Survivin、Bcl-2、Caspase-3 and Caspase-8 with flow cytometry.

【**Results**】After transfection, the apoptosis rate of HepG2 cells was increased with the prolonged time , and the apoptosis rate of the solo transfection group was higher than the control group, while combined group were higher than the solo transfections group（$P<0.01$）. The expression of Fas and FasL were increased in HepG2 cells , but the expression of FasL was lower than that of Fas. The expression of Fas in solo transfection group is higher than that in control group, and the combined group is highest（$P<0.01$）.The expression of FasL in the

combined group was higher than that in the solo transfection group on 12 hours and 24 huors, but was lower than that in the solo transfection group on 48 hours after transfection（$P<0.01$）.The apoptosis of HepG2 cells increased as time prolonged ,and the apoptosis of combined group was higher than that of the solo transfection group（$P<0.01$）.The expression of Caspase-3 was gradually increased in the control group、solo group and combined group. On the contratory, the expression of Survivin of decreased gradually. After transfection, the expression of Bcl-2 and Caspase-8 were obviously different than the control group.

【Conclusion】LIGHT and INF-γ genes successfully transfected HepG2 cells mediated by Cationic liposome, pcDNA4/HisMaxC is a stable vector for HepG2 cell apoptosis study. The pcDNA4/HisMax-EGFP identify tansfection efficiency is an ideal method. The possible pathway of LIGHT gene induced HepG2 cell apoptosis is death receptor pathway. LIGHT and INF-γ have synergistic action in HepG2 apoptosis through the down regulation of Bcl-2 expression.

Key words: HepG2 cell; LIGHT; Humen Interferon-γ; Apoptosis; Fas/FasL; Caspase 3; Caspase 8; Survivin; Bcl-2; Transfection

Ⅰ. INTRODUCTION

Primary hepatic carcinoma is one of the frequent malignant tumor, 90% of it was hepatocellualr carcinoma (HCC). Now the incidence of HCC is rising, it is one of the five most common cancers worldwide [1] and is the second malignant tumor which causing death in China. More than 80% of patients with HCC have associated with cirrhosis and impaired liver function making treatment of HCC more difficult than other cancers. Surgical resection is the mainstay of curative treatment for HCC, but prognosis after resection remains unsatisfactory due to a high incidence of postoperative recurrence [2]. Liver transplantation is another potentially curative treatment for early HCC associated with advanced cirrhosis, but its application is limited by the shortage of grafts[3]. For unresectable HCC, various locoregional therapies may be used to palliate symptoms and prolong survival if the tumors remain confined to the liver[4]. Conventional cytotoxic chemotherapy has not been shown to be effective for HCC, and there is no proven effective systemic therapy for HCC patients with metastatic disease[5]. Hence, the search for a novel systemic therapy for HCC is of paramount importance. To overcome these clinical problems, gene therapy has been considered as a new therapeutic approach to treat the HCC.

The carcinogenesis are complex events, including alterations in several genes and, depending also upon external factors, such as infection by hepatitis B or C and chronic alcohol intake etc., can lead to a pre-neoplastic lesion, like the low and high grade displastic nodule/denomatous hyperplasia[6]. Then, these lesions can progress to early cancer and finally, culminate with advanced HCC. The knowledge about the development and progression to HCC have increased in later years with the increasing using of several molecular biology techniques[7]. Thus,

one of the most frequent molecular events in cancer is an aberrant gene expression. The activation of oncogenes and inactivation of tumor suppressor genes (TSG) have an important role in the mechanisms of cancer development. In the case of HCC, the activation of oncogenes has been seen with a low frequency, leading some authors to suggest a secondary role of this genetic event [8].

Recent studies revealed that TNFα, IFNα, β and related cytokines had apoptosis and antitumor effects on HCC through the pathway of TRAIL, Caspases[9], Fas antigen, ISG-encoded protein, etc[10].

Besides other members of tumor-necrosis factor (TNF) family, LIGHT has two effects that might stimulate tumor rejection: first, it binds to the lymphotoxin-β receptor (LTβR), resulting in the expression of cytokines and adhesion molecules that participate in T-cell recruitment; second, it binds to the T-cell HVEM receptor, the activation of which co-stimulates T-cell priming and expansion.

The stromal barrier that forms around tumors keeps the immune system in the dark about cancerous cells, preventing T cells from infiltrating the tumor and becoming activated. Yu and colleagues[13] showed that tumor rejection can be stimulated by priming naive T cells inside tumors with LIGHT-α member of the TNF family.

The definition of gene therapy is the transfer of genes to patients for therapeutic purposes. It becomes one new therapy approach for cancer in the recent years. Introducing the special designed therapeutic gene into target cell, and let the transcription and translation product take the role of treatment is one important way of gene therapy. With the development of gene therapy technology, Gene therapy of HCC becomes the hot spot of HCC therapy.

We have cloned full-length ORF of LIGHT, IFN-γ，new member of TNF family, and transgene expressed into HepG2 cells by recombinant eukaryotic expression vector, pcDNA4/HisMax-hLIGHT, pcDNA4His/MaxC-hIFN-γ to investigate anti-tumor and apoptosis effects of the cytokine on HepG2 cells *in vitro* using the assays of FCM, ELISA, etc.

At the same time, using the assays of FCM, ELISA, we have detected the apoptotic factors expression, such as Fas, FasL, Caspase 3, Caspase 8, Survivin and Bcl-2. Statistic analysis the corelationship between those factors, try to illustrate their role in the pathway of apoptosis.

Ⅱ. MATERIALS AND METHODS

1. Materials

HepG2 cells (from Nanjing Kaiji Molecular Technology Company).

2. Methods

1. Plasmid construction

1.1 Peripheral blood mononuclear cells (PBMC) harvesting

PBMC are isolated from healthy, uninfected donor blood. Whole blood anticoagulated with Sodium Citrate /EDTA-NO_2 were used. The volume drawn is 3 ml. Diluted the whole blood in D-Hanks solution. Carefully pour 15ml of blood and 8 ml leukocyte isolation solution into l centrifuge tubes. Centrifuge the tubes at room temperature at 2000rpm for 20 minutes. After centrifugation, remove cloudy interface (PBMC layer) into a new 15 ml centrifuge tubes, add 5 times volume of D-Hanks solution, centrifuge the tubes at room temperature at 1500 rpm for 10 minutes. Wash cells at this method for two times. Resuspend in 1ml Ham-F12 medium, adding 10 μl PHA-P, incubate at 37℃ for 15 minutes. Centrifuge the tubes at room temperature at 1500rpm for 10 minutes, and completely remove and discard supernatant. Resuspend in 1ml Ham-F12 medium , incubate at 37℃ for 1.5hours. Pipet lysate 10μl and 90μl PBS into 1.5ml microcentrifuge tube vortex or pipet to mix. Counting the lymphocyte cell, the number is 7.5×10^5/ml.

1.2 Total RNA Isolation

Remove the cell in 1.5ml microcentrifuge tube to a new. QIAamp RNA Blood

Mini Kit（QIAGEN, ）were used in the total RNA isolation. According to The Protocol of Purification of Total Cellular RNA from Human Whole Blood.

1.3 Quantification of total RNA

1) RNA concentration measurement: Take RNA sample 5μl, adding RNase-free water 195 μl to dilute. The concentration of RNA should be determined by measuring the absorbance at 260 nm (A260) in a spectrophotometer. RNA concentration =OD260 ×nucleinic acid dilute multiple×40/1000.

2) The purity coefficient: Measuring RNA absorbance at 260 nm and 280nm in a spectrophotometer, and quantitate OD260, OD280, calculate OD260/OD280. The ratio of all the samples are required in the range of 1.75-1.95.

1.4. RT-PCR reaction

Prime Primer 5.0 **DEMO** software were used in Premier design. Primer were made by Shanghai Sangon Company.

Primer 1: 5'-TCTTTGGCTTAATTCTCTCGG-3',

Primer 2: 5'-AATTCAAATATTGCAGGCAGG-3'.

RT-PCR product size: 555bps.

Protocol Using QIAGEN OneStep RT-PCR Kit. Thermal cycler conditions were designed as the following: Reverse transcription: 30min 50℃, Initial PCR activation step: 15 min 94℃, 3-step cycling: Denaturation: 1 min 94℃, Annealing: 1 min 50℃, Extension: 1 min 72℃, Number of cycles: 35. Final extension: 10 min 72℃.

Table 1. RT-PCR Reaction System（50μl）

5×Buffer	10 μl
10mM dNTP	2 μl
Primer 1	5 μl（final concentration 0.6 μM）
Primer 2	5 μl（final concentration 0.6 μM）
Enzyme Mix	2 μl
5×Q-solution	10 μl
Template RNA	8 μl（0.5g）
DEPC-H_2O	8 μl
Total volume	50 μl

1.5 Agarose gel RT-PCR product Quantitation

Making 1.5% agarose gel. This can be done by adding 1.5 grams of agarose to 98.5 ml of 1×TAE buffer and microwaving on medium low for 90 sec until clear. Adding 5μl EB after cold and pour the solution into Electrophoresis box .Set up each digestion by adding 5 μl of PCR product and 1μl of 6×Loading Buffer. Run at 100 volts for approximately 25 minutes in 1×TAE solution. Observing the Electrophoresis DNA band under ultraviolet (UV) rays Analys the gels in Korda Photograph system.

PCR Reaction:

Prime Primer 5.0 **DEMO** software were used in Premier design. Primer were made by Shanghai Sangon Company.

Primer 3：5’-CGCGGATCCATGAAATATACAAGTTATATC-3’

（BamH Ⅰ-G^GATCC）

Primer 4：5’-CCGGAATTCTTACTGGGATGCTCTTCG-3’

（EcoR Ⅰ-G^AATTC）

PCR product size : 519bps

The Protocol of PCR Using HotStarTaq DNA Polymerase were used. Program the thermal cycler as the following: Initial PCR activation step: 15 min 94℃, 3-step cycling: Denaturation: 1 min 94℃, Annealing: 1 min 50℃ Extension: 1 min 72℃, Number of cycles: 35, Final extension: 10 min 72℃.

Table 2 PCR reaction system（50μl）

10×Buffer	5 μl
2.5 μM dNTP	4 μl
5×Q-solution	10 μl
Mg^{2+}	1 μl
Recombinant Taq enzyme（Hot star）	0.25 μl
Primer 3	5 μl（final concentration 0.6 μM）
Primer 4	5 μl（final concentration 0.6 μM）
RT-PCR products	4 μl
DEPC-H_2O	15.75 μl
Total volume	50 μl

1.7 Agarose gel PCR product Quantitation

Making 1.5% agarose gel and solution as previously used. Set up each digestion by adding 10 μl of PCR product and 1 μl of 6×Loading Buffer. Run at 100 volts for approximately 25 minutes in 1×TAE solution. Observing the Electrophoresis DNA band under ultraviolet (UV) rays. Analys the gels in Korda Photograph system.

1.8 Restriction enzyme digest system of PCR products

1) PCR product enzyme digest: Put the completely mixed tube in 37℃ water bathing, enzyme digest for 1.5-2 hours. (Table 3).

2) Plasmid vector enzyme digest: Put the completely mixed tube in 37℃ water bathing , enzyme digest for 1.5-2 hours. (Table 4).

3) Product Quantitation: the same agarose gel method with PCR product.

Tabale 3 Restriction enzyme digest system of PCR products（40 μl）

IFN-γ cDNA PCR products	10 μl
10×K Buffer	4 μl
EcoRI	2 μl
BamHI	2 μl
DEPC-H_2O	12 μl
Total volume	40 μl

Tabale 4　Restriction enzyme digest system of Plasmid（20 μl）

Plasmid pcDNA4his/max C	1 μl
10×K Buffer	2 μl
EcoRI	1 μl
BamHI	1 μl
DEPC-H_2O	15 μl
Total volume	20 μl

1.9 Ligation of IFN-γ CDNA and pcDNA4his/max C

Ligation reaction: mixed the reaction as table 5 showed, at the temperature 16℃ for 30 minutes.

Tabale 5　Reaction system of Ligation（10 μl）

IFN-γ PCR products	4 μl
pcDNA4his/max C	1 μl
Ligation Mix(containing T4 ligase and buffer)	5 μl
Total volume	10 μl

1.10 Luria-Bertani (LB) Medium making and preparing LB-amp-agar plates

1) Mix the following substances in 1 L of distilled water. Adjust pH to 7.0 with 0.1 N NaOH or 0.1 N HCl. The contents include Tryptone 10 g/L, NaCl 10 g/L and Yeast Extract 5 g/L.

For making solid LB agar for plating, 15 g/L of agar is added to the above mixture

2) If the LB-agar medium is in solid form, make liquid by boling in a microwave oven. Use 30 ml of the stock ampicilin for every 30 ml of the LB-agar medium to pour on one Petri dish plate. Pouring plates must be done in an aseptic laminar hood. The poured plates are left at room temperature till the agar becomes solid. Then, the LB-amp-agar plates are ready to use. The concentration of the ampicilin on the LB-amp-agar plates is 0.1 mg/ml.

1.11 Prepare competent E. coli and Transformation by Heat Shock Method

Competent cells:

Grow an *E. coli* starter on LB, Dilute the starter 1:100 in 200ml/L. Follow A_{600}. Grow to A_{600} =0.3-0.5. Cool cells on ice, 5-15 min. Spin 5,000 rpm, 10 min. Resuspend in 20 ml ice cold 100mM $CaCl_2$. Leave on ice 1 h. Spin 5,000 rpm, 10 min. Resuspend in 2 ml ice cold 100mM $CaCl_2$/10% glycerol. Transfer in 100 µl aliquots to tubes on dry ice. Store at -80℃.

Transformation:

Mix 2.5 µl ligation reaction with 40-100 µl competent cells on ice.Incubate on ice 30 min.Heat shock, 42C, 90 sec.Incubate on ice 5-10 min.Add 250 µl LB. Incubate at 37℃ with shaking, 30-60 min.Plate 150 µl on LBA plates.

2 Identify the recombinant plasmid

2.1 Preparation of high-copy plasmid (QIAGEN plasmid kits)

Pick a single colony from a freshly streaked selective plate and inoculate a starter culture of 2–5 ml LB medium containing the appropriate selective antibiotic. According to the QIAGEN plasmid kits protocol. Carefully decant the supernatant without disturbing the pellet. Air-dry the pellet for 5–10 min, and redissolve the DNA in a suitable volume of buffer(e.g., TE buffer, pH 8.0, or 10mM Tris·Cl, pH 8.5)

2.2 Restriction enzyme digest of recombinant Plasmid

1) Enzyme digest of recombinant plasmid: After mix the reagent to the tube as the following table, put the tube in 37℃ water bathing for 1.5-2 hours.

Tabale 6 Restriction enzyme digest system of recombinant Plasmid（20μl）

Recombinant plasmid	1 μl
10×K Buffer	2 μl
EcoRI	1 μl
BamHI	1 μl
DEPC-H_2O	15 μl
Total volume	20 μl

2) Enzyme digest product identification: Making 1.5% agarose gel and solution as previously used. Set up each digestion by adding 5 μl of enzyme digest product and 1 μl of 6×Loading Buffer. Run at 100 volts for approximately 25 minutes in 1 ×TAE solution. Observing the Electrophoresis DNA band under ultraviolet (UV) rays. Analys the gels in Korda Photograph system. If there is about 500 bps DNA

fragment, it will proved that the recombinant plasmid transformed IFN-γ cDNA. If there is about 700 bps DNA fragment, LIGHT cDNA is cloned successfully.

2.3 Sequencing work

Use the BGH as the reversing primer, and sequencing the enzyme product, If the result is the same with Genebank IFN-γ and LIGHT gene sequence, it will be confirmed that the cloned gene is IFN-γ and LIGHT gene.

3. Plasmid transfection

3 .1Trypsinization of Adherent Cells

Examine the falsk of cultured cells under the inverted microscope to check for contamination, cell density, etc. When a single cell suspension has been obtained, add 10 times volume of complete media as trypsin solution. Transfer the cell suspension to a sterile 15 ml conical centrifuge tube. Spin the cells down at 1,200 rpm for 10 minutes in the tabletop centrifuge. Discard the supernatant carefully, so as not to disturb the cell pellet. Resuspend the cells in 5 ml of fresh complete media and collect 100 µl to count cell number.

Once cells/ml has been determined, seed cells into a fresh T-75 flask to get a final concentration of 2×10^5 cells/ml.

3.2 Subculture of Adherent Cell Lines

View cultures using an inverted microscope to assess the degree of confluency and confirm the absence of bacterial and fungal contaminants. Transfer the required number of cells to a new labeled flask containing pre-warmed medium. Incubate as appropriate for the cell line. Repeat this process as demanded by the growth characteristics of the cell line.

3.3 Cell Quantification

Under sterile conditions remove 100-200 μl of cell suspension. Add an equal volume of Trypan Blue (dilution factor = 2) and mix by gentle pipetting. Clean the haemocytometer. Moisten the coverslip with water or exhaled breath. Slide the cover-slip over the chamber back and forth using slight pressure until Newton's refraction rings appear (Newton's refraction rings are seen as rainbow-like rings under the cover-slip). Fill both sides of the chamber (approx. 5-10 μl) with cell suspension and view under a light microscope using ×20 magnification. Count the number of viable (seen as bright cells) and non-viable cells (stained blue, see below). Ideally >100 cells should be counted in order to increase the accuracy of the cell count (see notes below). Note the number of squares counted to obtain your count of >100. Calculate the concentration of viable and non-viable cells and the percentage of viable cells using the equations below.

3.4 Freezing cells in liquid nitrogen

Take off Media. Trypsinate with 1ml×2 Dulbecco A trypsin. Add 7 ml Media. Pipette up and down to distribute cells throughout media (i.e. not clumped together). Add media to sterile falcon tube (15 ml = 1 flask, 50 ml = 5 flasks). Spin down 1000 rpm, 5 min. Take off media. Resuspend pellet in 9 ml FCS and 1 ml DMSO. Distribute in 1ml aliquots (10 cryovials). Move cells to –84℃ overnight wrapped in cotton wool in a polystyrene box. Finally freeze cells in liquid N_2.

3.5 Transfection of AdherentCells

The day before transfection, seed 2–8×10^5 cells (depending on the cell type) per 60 mm dish in 5 ml appropriate growth medium containing serum and antibiotics. Incubate the cells under their normal growth conditions (generally 37℃ and 5%CO_2).

The dishes should be 40–80% confluent on the day of transfection. The day

of transfection, dilute 1 μg DNA dissolved in TE buffer, pH 7 to pH 8 (minimum DNA concentration: 0.1 μg/μl) with the DNA-condensation buffer, Buffer EC, to a total volume of 150 μl. Add 8 μl Enhancer and mix by vortexing for 1 sec. Incubate at room temperature (15–25℃) for 2–5 min then spin down the mixture for a few seconds to remove drops from the top of the tube. Add 25 μl Effectene Transfection Reagent to the DNA-Enhancer mixture. Mix by pipetting up and down 5 times, or by vortexing for 10 sec. Incubate the samples for 5–10 min at room temperature (15–25℃) to allow transfection-complex formation. For transient transfections, assay cells for expression of the transfected gene. For stable transfections, passage cells 1:5 to 1:10 into the appropriate selective medium 24–48 hour after transfection. Maintain cells in selective medium until colonies appear.

3.6 Fluorescin（EGFP）gene transfect humanHepG2 cell

Nine holes among 24 hole plate were used. 8×10^4 cells were seeding to every holes and cultured overnight. Until 70% cells were confluens, transfect the plasmid (EGFP-pcDNA4 his/max B) into HepG2 cells. Different matching of EGFP plasmid, enhancer and effectene were adding to holes to decide the best transfection efficacy (Table 7).

Table 7 Match of EGFP transfection agent for each well

	1	2	3	4	5	6	7	8	9
EGFP plasmid（μg）	0.1	0.1	0.1	0.2	0.2	0.2	0.4	0.4	0.4
Enhancer （μl）	0.8	0.8	0.8	1.6	1.6	1.6	3.2	3.2	3.2
Effectene（μl）	1	2.5	5	2	5	10	4	10	20

3.7 LIGHT and IFN-γ combinant transfect HepG2 cell

1） Six holes among 24 hole plate were used to seeding 8×10^4 cells one days before transfection.

2） The proportion of adding plasmid ,Enhancer and Effectene to every hole were showed in Table 8.

Table 8 Match of LIGHT and IFN-γ transfection agent for each well

	12h	24h	48h
Plasmid/Ehancer/ Effectene（μl）	1/3.2/10	1/3.2/10	1/3.2/10
Plasmid/Ehancer/ Effectene（μl）	1/3.2/20	1/3.2/20	1/3.2/20

3.8 ELISA Method

Before the assay, both antibody preparations should be purified and one must be labeled. For accurate quantitation, the second antibody should be used in excess. All dilutions should be done in the blocking buffer. Incubate for 2h or more at room temperature in a humid atmosphere. Wash with several changes of PBS. Add substrate as indicated by manufacturer. After suggested incubation time has elapsed, optical densities at target wavelengths can be measured on an ELISA plate reader.

4 Flow Cytometry of HepG2 cells Apoptosis and apoptotic factor expression

4. 1 HepG2 cell apoptosis

Add 50–100 μl of cell suspents to each of three 12×75 mm polypropylene or polystyrene tubes. To the first tube, add the appropriate volume (usually 5–10 L) of fluorochrome- conjugated monoclonal antibody. To the second tube, add the same volume of matched isotyp control antibody. The isotype control antibody should match the isotype of the conjugated antibody of interest. To the final tube of cells, add the equivalent volume of 1×PBS as conjugated antibody. It is critical to mix the cell pellet prior to adding the fixative; otherwise the cells will become fixed into a solid mass that cannot be sent through the flow cytometer. Fixed samples can be stored at 4℃ in the dark until FACS analysis is performed. Fixed samples should be used within 48 hours.

Table 9 Volume of transfection agent for FCM assay

Culture format	DNA（μg）	Enhancer (μl)	Final volume of DNA in Buffer EC (μl)	Volume of Effectence Reagent (μl)	Volume of Medium to Add to cells (μl)	Volume of medium to Add to complexes (μl)
Control	0	0	0	0	2319.8	0
LIGHT	0.6	4.8	100	15	1600	600
LIGHT/IFN-γ	0.6/0.6	4.8	100	15	1600	600

4.2 Apoptotic biomarkers expression

Harvasting the $HepG_2$ cells after transfection12h, 24h, 48h respectively. Fluorescent antibody were added into those cells. Flow Cytometry was used to detect the

expression of Fas, FasL, Bcl-2, surviving, Caspase 3 and Caspase 8.

5. ELISA method to detect the Fas， Fas-L，Survivin ，Bcl-2 ，Caspase3 expression

6. Statistical analysis

SPSS11.5 (America) software were used to do the statistical analysis. Including Pearson Correlation for the correlation between two viables, and one-way ANOVA analysis for the comparison between more than two samples.

Ⅲ. RESULTS

1. Plasmid Construction

1.1 Isolation and Quantification of total RNA

OD260＝0.0373，OD280＝0.0187.

Concentration of RNA stock = OD260×dilution factor×40/1000=0.0373×1.6＝0.0597. OD260/OD280 ＝1.99.

1.2 Quantitation of RT-PCR and PCR product

1.2.1 Agarose Gel of IFN-γ cDNA's RT-PCR product Quantitation.(Fig. 1)

1.2.2 Agarose Gel of Light cDNA's RT-PCR product Quantitation. (Fig.2)

1.2.3 Agarose gel Electrophoresis of PCR product of IFN-γ gene. (Fig. 3)

1.2.4 Agarose gel Electrophoresis of PCR product of LIGHT gene. (Fig. 4)

1.3. Recombinant of plasmid

1）PcDNA4/HisMax C figure.(Fig. 5)

2) PCR product and plasmid BamHI/EcoRI enzyme digest product.(Fig. 6, 7)

3) IFN-γ PCR and PcDNA4/HisMax C ligation and transformation to E.coli JM-109. (Fig. 8).

2. Identify of Plasmid

2.1 Identify the combinant plasmid after Enzyme digestion (Fig. 9).

2.2 IFN-γ cDNA (ORF) sequencing.

The sequence of IFN-γ cDNA (ORF) was the same with the report from Human Interferon-γ cDNA (NM_000619, ORF: 127-627, 501 bps) in GenBank.

2.3. LIGHT cDNA (ORF) sequencing

Compared with the LIGHT cDNA sequence (CR541854, AY893619) in Genebank, the sequence of LIGHT cDNA (ORF) was showed little difference. The 561 base, C mismatched to T. Because it is the third codon, and tgt and tgc are all express cys, belongs to synonym codon，can not change the coding of amino acides.

3. Plasmid Transfection

3.1 HepG2 cell culture (Fig. 10)

HepG2 cell had the characteristic of confluent growth, a distinct outline, ranked tight to each other, conjunct to large pieces. Under the higher power microscopy, it showed big cell nucleus, uniformity caryotin, clearly nucleoli.

3.2 EGFP gene transfecting to human HepG2 cells

1） Transfecting the plasmid which contains EGFP gene. Different matching of EGFP gene plasmid, Enhancer and Effectene were adding to every holes .Under the fluorescence inverted microscope，to observe the expression of intracellular fluorescin at 24h respectively. Then determine the optimization efficiency. **(Fig.11,)**.

2） The expression of fluorescence were higher and no difference in the eighth and ninth hole. The transfection efficiency was higher when the matching ratio of EGFP gene plasmid, Enhancer and Effectene was 0.4μg, 3.2μl, 10μl respectively. The expression of fluorescence at 24 h was higher than12 h.

3.3 ELISA method to detect the IFN-γ expression

Table 10 T test of Regression Coefficient and Intercept

	velume	*Std.error	β	t-test	p
Intercept	-1.7525	0.0794		-22.0783	＜0.001
*Reg. Coefficient	0.5997	0.0364	0.9909	16.4817	＜0.001

*：Reg. Coefficient—regression coefficient，Std.error—standard error

Table 11 IFN-γ Concentration in supernatants of transfected cell

	O.D.	Average	Dilution factor	Concentration (pg/ml)
Control	0.083/0.085	0.084	×1	13.44
12 h	0.759/0.766	0.763	×2	1068.16
24 h	0.812/0.824	0.818	×2	1196.30
48 h	1.188/1.192	1.190	×10	11177.64
3 d	1.083/1.089	1.086	×8	7676.04
5 d	0.996/0.999	0.997	×8	6655.93
7d	0.449/0.457	0.453	×8	1786.19

4 Apoptosis of HepG2 cells after transfection

4.1 Microscopic observing of apoptosis cell

Collecting HepG2 cells after transfection 24 h, Annexin V/FITC and PI antibody were add into the cells, Membrane Annexin V/FITC staining in early apoptosis (Fig.12), Nuclear PI dyeing in end apoptosis (Fig. 13).

4.2 Flow Cytometry of HepG2 cell apoptosis

Collecting the HepG2 cells after transfection 12h, 24h and 48h respectively.

Annexin V/FITC and PI antibody were adding to those cells. The apoptotic ratio of HepG2 cell was increasing with the time prolonged, and the apoptotic ratio of solo LIGHT transfection group was higher than the control group. The combined LIGHT and IFN-γ transfection group was better than solo LIGHT transfection group. (P<0.01, Table 12, Fig. 14, 15).

Table 12 Apoptotic ratio of HepG2 after transfection

	12h	24h	48h
Control	8.54±1.67	9.28±1.87	10.9±1.98
LIGHT	18.75±1.70$^{\#\#}$	25.71±1.62$^{\#\#}$	36.4±2.38$^{\#\#}$
LIGHT / IFN-γ	23.75±1.75$^{\#\#*}$	31.09±1.91$^{\#\#*}$	42.5±2.66$^{\#\#*}$

Compared with control group: $^{\#}$P<0.05, $^{\#\#}$P<0.01;
Combinant group compared with solo group: *P<0.05

4.3 Flow Cytometry of Apoptosis biomarkers

4.3.1 Expression of Fas after transfection

The Flow Cytometry result of HepG2 cells showed that the expression of Fas was increasing with the time prolonged after transfection. The expression of Fas in solo LIGHT gene group was higher than control group, and the combinant group was higher than solo group. Statistics analysis showed height statistic significance (P<0.01, Table 13, Fig. 16).

Table 13 Expression of Fas after transfection

	12h	24h	48h
Control	0.7±0.13	0.57±0.15	1.22±0.34
LIGHT	2.8±0.17$^{\#\#}$	19.6±1.34$^{\#\#}$	28.7±2.63$^{\#\#}$
LIGHT / IFN-γ	10.6±1.25$^{\#\#**}$	24.2±0.67$^{\#\#**}$	39.4±1.57$^{\#\#**}$

Compared with control group: $^{\#}P<0.05$, $^{\#\#}P<0.01$;
Combinant group compared with solo group: $^{*}P<0.05$, $^{**}P<0.01$

4.3.2 The expression of FasL HepG2 cells after transfection

The Flow Cytometry result of HepG2 cells showed that higher expression of FasL after transfection. The expression of FasL in solo LIGHT gene group was lower than combinant group in 12 h and 24 h, and solo LIGHT gene group was higher than combinant group in 48 h. Statistics analysis showed higher statistic significance ($P<0.01$，Table 14，Fig 17).

Table 14 Expression of FasL after transfection

	12h	24h	48h
Control	0.47±0.28	0.58±0.22	0.38±0.3
LIGHT	$1.69\pm0.25^{\#\#}$	$3.5\pm0.39^{\#\#}$	$1.76\pm0.66^{\#\#}$
LIGHT and IFN-γ	$2.2\pm0.51^{\#\#**}$	$3.94\pm0.79^{\#\#**}$	$0.63\pm0.20^{\#\#**}$

Compared with control group: $^{\#}P<0.05$, $^{\#\#}P<0.01$;
Combinant group compared with solo group: $^{*}P<0.05$, $^{**}P<0.01$

4.3.3 The expression of Bcl-2 in HepG2 cells after transfection

The result showed that Bcl-2 expression could be detected by Flow Cytometry. All the transfection group showed down regulation of Bcl-2 expression. The solo transfection group decreased than control group 8.94%, 6.36%, 1.46% at 12h, 24h, 48h respectively，and Statistics analysis showed statistic significance at 12h, 24h (*P<0.05*). The combinant group decreased more obviously than the control group, and decreased 10.6%, 4.32%, 5.52% at 12h, 24h, 48h respectively. Statistics analysis showed statistic significance (*P<0.05*, *P<0.01*). Also the combinant group have a significantly difference with the solo group (*P<0.05*). (Table 15，Fig. 18).

Table 15 Expression of Bcl-2 after transfection

	12h	24h	48h
Control	25.3±6.26	19.8±4.42	10.1±3.80
LIGHT	16.36±4.96$^{\#}$	13.44±3.53$^{\#}$	8.64±2.29
LIGHT and IFN-γ	14.7±3.78$^{\#}$	9.12±2.04$^{\#\#*}$	4.58±1.97$^{\#*}$

Compared with control group: $^{\#}P<0.05$, $^{\#\#}P<0.01$;
Combinant group compared with solo group: $^{*}P<0.05$, $^{**}P<0.01$

4.3.4 The expression of Survivin in HepG2 cells after transfection

The expression change could be seen in the Flow Cytometry graph. The expression of surviving showed a decreased tendency in the solo group and combinant group. No obviously difference was found in the groups. (Table 16，Fig. 19)

Table 16 Expression of Survivin after transfection($\bar{x}$±s %)

	12h	24h	48h
Control	13.5±1.1	9.51±0.79	9.97±0.62
LIGHT	10.1±1.3$^{\#}$	7.92±0.82	8.78±0.89
LIGHT and IFN-γ	7.6±0.97$^{\#\#*}$	8.12±0.91	6.97±0.74$^{\#\#*}$

Compared with control group: $^{\#}P<0.05$, $^{\#\#}P<0.01$;
Combinant group compared with solo group: $^{*}P<0.05$, $^{**}P<0.01$

4.3.5 The expression of Caspase3 in HepG2 cells after transfection

The expression changes of caspase 3 could be seen in the Flow Cytometry graph. The expression of caspase 3 was increased significantly in solo group and combinant group ($P<0.01$). The combinant group was highest among the groups. The expression was different at different time，and 24 h was the highest, gradually decreased with time prolonged. (Table 17，Fig 20).

Table 17 Expression of Caspase 3 after transfection

	12h	24h	48h
Control	0.61±0.16	0.78±0.12	0.48±0.15
LIGHT	2.38±0.13$^{\#\#}$	3.47±0.21$^{\#\#}$	1.03±0.14$^{\#\#}$
LIGHT and IFN-γ	2.73±0.18$^{\#\#}$*	4.49±0.31$^{\#\#}$**	1.91±0.23$^{\#\#}$**

Compared with control group: $^{\#}$P<0.05, $^{\#\#}$P<0.01；
Combinant group compared with solo group: *P<0.05, ** P<0.01

4.3.6 The expression of Caspase8 in HepG2 cells after transfection

The solo transfection group decreased than control group 8.94%, 6.36%, 1.46% at 12h, 24h, 48h respectively, and Statistics analysis showed statistic significance at 12h, 24h (P<0.05). The combinant group decreased more obviously than the control group, and decreased 10.6%, 4.32%, 5.52% at 12h, 24h, 48h respectively. Statistics analysis showed statistic significance (P<0.05, 0.01). Also the combinant group have difference with the solo group. (P<0.05). (Table 2.6， Fig.21).

The result showed that Caspase 8 expression could be detected by Flow Cytometry. All the transfection group showed up regulation of Caspase 8 expression.

Compared with control group, Statistics analysis showed statistic significance (P<0.01). The combinant group had the statistic difference at 12 h with the solo group (P<0.05). No statistic difference was found at 24h and 48h. (Table 18， Fig. 22).

Table 18 Expression of Caspase 8 after transfection

	12h	24h	48h
Control	1.23±0.87	1.77±0.64	3.17±1.46
LIGHT	19.3±2.37##	27.2±1.91##	33.7±2.97##
LIGHT / IFN-γ	22.7±2.19##	30.9±3.08##	38.2±3.24##

Compared with control group: #P<0.05, ##P<0.01；

5. ELISA method

5.1. ELISA method to detect the Fas expression

Table 19 T test of Regression Coefficient and Intercept

	velume	*Std.error	β	t-test	p
Intercept	-0.418	0.013		-31.330	＜0.001
*Reg. Coefficient	0.521	0.022	0.997	23.352	＜0.001

*：Reg. Coefficient—regression coefficient，Std.error—standard error

Table 20 Fas Concentration in supernatants of lysated transfected cell without transfection

Control	O.D.	Average	Dilution factor	Concentration (U/ml)
12 h	0.66/0.74	0.70	×1	0.885
24 h	0.56/0.58	0.57	×1	0.757
48 h	1.13/1.15	1.14	×1	1.499

Table 21 Fas Concentration in supernatants of lysated transfected cell with LIGHT transfection

	O.D.	Average	Dilution factor	Concentration (U/ml)
12 h	2.81/2.79	2.80	×1	10.985
24 h	1.958/1.962	1.96	×10	40.10
48 h	2.904/2.898	2.90	×10	124.00

Table 22 Fas Concentration in supernatants of lysated transfected cell with LIGHT/ IFN-γ transfection

	O.D.	Average	Dilution factor	Concentration (U/ml)
12 h	1.01/1.05	1.03	×10	13.14
24 h	2.38/2.42	2.40	×10	67.98
48 h	3.91/3.97	3.94	×10	431.26

5.2 ELISA method to detect the Fas-L expression

Table 23 T test of Regression Coefficient and Intercept

	velume	*Std.error	β	t-test	p
Intercept	-1.218	0.007		-175.09	＜0.001
*Reg. Coefficient	0.120	0.016	0.974	7.515	＜0.001

*：Reg. Coefficient—regression coefficient，Std.error—standard error

Table 24 Fas-L Concentration in supernatants of lysated transfected cell without transfection

Control	O.D.	Average	Dilution factor	Concentration (U/ml)
12 h	0.24/0.28	0.26	×1	0.650
24 h	0.50/0.54	0.52	×1	0.699
48 h	0.21/0.29	0.25	×1	0.649

Table 25 Fas-L Concentration in supernatants of lysated transfected cell with LIGHT transfection

	O.D.	Average	Dilution factor	Concentration (U/ml)
12 h	0.46/0.48	0.47	×1	0.689
24 h	3.48/3.52	3.50	×10	15.92
48 h	1.75/1.77	1.76	×10	9.84

Table 26 Fas-L Concentration in supernatants of lysated transfected cell with LIGHT/ IFN-γ transfection

	O.D.	Average	Dilution factor	Concentration (U/ml)
12 h	1.99/2.13	2.10	×10	10.81
24 h	3.95/3.97	3.96	×10	18.08
48 h	0.61/0.65	0.63	×1	0.720

5.3 ELISA method to detect the Survivin expression

Table 27 T test of Regression Coefficient and Intercept

	velume	*Std.error	β	t-test	p
Intercept	-1.558	0.030		-51.150	＜0.001
*Reg. Coefficient	0.117	0.011	0.984	11.157	＜0.001

*：Reg. Coefficient—regression coefficient，Std.error—standard error

Table 28 Survivin concentration in supernatants of lysated transfected cell with LIGHT transfection

	O.D.	Average	Dilution factor	Concentration (ng/ml)
control	0.319/0.323	0.321	×1	0.0302
12 h	0.053/0.055	0.054	×1	0.0281
24 h	0.061/0.065	0.063	×1	0.0281
48 h	0.067/0.069	0.068	×1	0.0282
3d	0.072/0.076	0.074	×1	0.0282
5d	0.059/0.065	0.062	×1	0.0281

5.4 ELISA method to detect the Bcl-2 expression

Table 29 T test of Regression Coefficient and Intercept

	velume	*Std.error	β	t-test	p
Intercept	-1.312	0.021		-63.600	＜0.001
*Reg. Coefficient	0.134	0.013	0.987	10.454	＜0.01

*：Reg. Coefficient—regression coefficient，Std.error—standard error

Table 30 Bcl-2 concentration in supernatants of lysated transfected cell with LIGHT transfection

	O.D.	Average	Dilution factor	Concentration (U/ml)
control	0.549/0.553	0.551	×1	0.0578
12 h	0.104/0.106	0.105	×1	0.0504
24 h	0.122/0.126	0.124	×1	0.0507
48 h	0.143/0.149	0.146	×1	0.0510
3d	0.136/0.138	0.137	×1	0.0509
5d	0.115/0.117	0.116	×1	0.0505

5.5 ELISA method to detect the Caspase3 expression

Table 31 T test of Regression Coefficient and Intercept

	velume	*Std.error	β	t-test	p
Intercept	0.825	0.010		81.616	＜0.001
*Reg. Coefficient	1.119	0.015	1.000	73.173	＜0.001

*：Reg. Coefficient—regression coefficient，Std.error—standard error

Table 32 Caspase3 concentration in supernatants of lysated transfected cell with LIGHT transfection

	O.D.	Average	Dilution factor	Concentration (U/ml)
control	0.091/0.093	0.092	×1	8.471
12 h	0.104/0.106	0.108	×1	8.828
24 h	0.122/0.126	0.198	×1	11.132
48 h	0.143/0.149	0.330	×1	15.641
3d	0.136/0.138	0.290	×1	14.109
5d	0.115/0.117	0.210	×1	11.481

6 Correlation analysis

By liner correlation analysis, we get the correlation diagrams of the biomarkers (Table 33-36), such as: cell apoptosis and Fas expression (LIGHT transfection, Fig. 23).

Table 33 Correlations Analysis between the apoptosis and the expression of apoptotic factor in Hep G2 cells

		Fas	Bcl-2	Caspase 8
LIGHT transfection	*r*	**0.9515**		**0.9781**
	P	***0.00007***		***0.000005***
LIGHT and IFN-γ Transfection	*r*	**0.9858**	**-0.7501**	**0.9793**
	P	***0.000001***	***0.0199***	***0.000004***
Control group	*r*	**0.83**		**0.9558**
	P	***0.0056***		***0.00005***

Table 34 Correlations Analysis of the Expression of Apoptotic Factor in Hep G2 cells without Transfection

		Survivin	Caspase 3	Caspase 8
Fas	*r*			**0.9089**
	P			***0.000686***
FasL	*r*		**0.8995**	
	P		***0.0009***	
Bcl-2	*r*	**0.7399**	**0.7129**	
	P	***0.0227***	***0.0311***	

Table 35. Correlations Analysis of the Expression of Apoptotic Factors in Hep G2 cells After LIGHT Transfection

		Survivin	Caspase 3	Caspase 8
Fas	*r*			**0.9682**
	P			***0.00001***
FasL	*r*		**0.7846**	
	P		***0.0123***	
Bcl-2	*r*	**0.7212**		
	P	***0.0283***		

Table 36. Correlations Analysis of Apoptotic Factors in Hep G2 cells After LIGHT and IFN- ɣ Transfection

		Bcl-2	Survivin	Caspase 3	Caspase 8
Fas	*r*	**0.837**			**0.9599**
	P	***0.0049***			***0.00004***
FasL	*r*		**0.7953**	**0.9671**	
	P		***0.0104***	***0.00002***	
Bcl-2	*r*				**-0.6787**
	P				***0.0444***

Ⅳ DISCUSSION

Plasmid vector selection

The pcDNA4/HisMa is a eukaryotic expression vector and latest product of Invitrogen company. It has been widespread used in scientific research because of its advantages. This plasmid is the innovated type of pcDNA4/His. It's size is 5.3kb, could express exogenous gene in eukaryotic cell with high efficiency, and could purify and detect the translated protein, could be used in the stable or transient expression after majority eukaryotic cell transfection. Its gene sequence includes human giant cell virus promoter and QBI SP163 transcriptional enhancer, could elevate the expression efficiency. If there no specific antibody expressed by objective gene, it could detect the plasmid itself expressed Xpress and poly histidine polypeptide，and could to determine if there are expression after transfection[11]. It could encode resistance gene of ampicillin and Zeocin that used in positive screening of bacterial transformation[12]. Zeocin is one kind of broad-spectrum antibiotic of BLENOXANE/phleomycin family extracted from streptomycete and have strong toxicity to bacterium, true fungus (includes yeast fungus), plant and mammalian cell. Therefore, Zeocin could be used to pressurize screening of eukaryotic cell tansfected by pcDNA4/HisMax., and get cell line which could stable express target gene. The pcDNA4/HisMax have three subtype: A, B, C. According to the enzyme shear recognition sequence of upstream BamHI, we compare the multiple clone site of three subtype and select pcDNA4/HisMaxC，warrant that no frame shift mutation occurred during translation. Upstream and downstream of Wall premier partake enzyme shear site of BamHI, EcoRI, so the correctness direction of inserted fragment could be guaranteed.

Advantage and disadvantage of liposome transfection

Transgenic technology is the effective method to change cell bionomics in the level of cellular level. Transfer the exogenous gene into hepatocellular carcinoma (HCC) cells and promote the cell to apoptosis. At the same time, enhancing the MHC antigen in tumor cell surface cell to express by Transgenic technology, and profit antigen presentation and immune response. The criticality of success or failure of Transgenic technology is to select the suitable carrier according to different disease therapy. The ideal carrier has the ability of introducing of objective gene into corresponding target organ, and expressing safely, effectively and long-term stably. Some researchers had tried the particle gun and cationic polymerization as vectors, but low transfection efficiency, objective gene expressed transient, repeat application and induce organism immunity are the disadvantage of them. The virus vector has the advantage of high transfection efficiency and strong target tropism[11]. Nevertheless, the disadvantage inhibits its development, such as possible toxigenicity to host cell, even inducing the gene mutation of host cell and wild type virus infection[13].

The ideal effect after transfection is maintaining enough concentration of LIGHT and IFN-γ. So profitting for antigen presentation and enhancing immune response, reducing side effect are obtained. For those reasons, we select the typical liposome vector. It transfection efficiency is relatively high, and exogenous gene could express fastness, also could transfect undivided phase cell. Furthermore, no risk of mutagenic and no unconformity of genome. The theorem of liposome transfection is that liposome interaction with sample DNA, forming Liposome-DNA compounds. This kind of liposome-DNA compounds could sticking at the surface of target cell, transit the Liposome membrane and fusion

with cellular membrane, inducing the exogenous gene into cell. Liposome transfection was conveniently handling, low toxic, reliability transfection efficiency. The main disadvantage of it is that the induced gene has not a long term stably expression.

Transfection efficiency

LIGHT and IFN-γ gene have the similarly molecular weight with EGFP gene, about several hundreds of base pair. But the pcDNA4/HisMax have about 5300 base pair. It is an ideal detecting transfection method with pcDNA4/HisMax-EGFP that have a small result difference. Furthermore, EGFP gene expressed in HCC cells could erupt green fluorescence and could be viewing with convenience under the fluor inverted microscope.

The fluorescence protein expression is the highest and little difference in eighth and ninth well. The transfection efficiency is optimization when the matching ration of EGFP-pcDNA4, Enhancer and Effectene is 0.4μg, 3.2μl, 10/20μl. The expression amount is 48.39% and 52.51% at 24 h. Besides, the transfection efficiency at 24 h is obviously higher than the cells to come from the same culture flask, same cell number at 12 h. The Effectene liposome (QIAGEN Company) were used to mediated the transfection. The HepG2 cells have higher activity, in vitro culture and transfected by EGFP gene combined plasmid pcDNA4/HisMax-EGFP in advance. Different matching ration were installed. At last, the best matching ration is identified. In this study, the transfection efficiency depends on the amount of plasmid when the liposome amount is the same. Similarly, fluorescin expression increased with the increasing amount of liposome when the plasmid amount is the same. Yan’s study found that the cell transfection efficency of positive ion liposome was 2.18%-48.46%. In our study, plasmid 0.4g

and liposome 10μl was selected, because of the transfection efficiency of 10μl group at 24h reaching 48.39%，to get close to the upper limit of liposome transfection efficiency from the previous studies.

Cell apoptosis pathway by LIGHT and IFN-γ induced

Apoptosis is a key process for the development and acts as part of a quality-control and repair mechanism by elimination of unwanted, genetically damaged, or senescent cells and as such is also critically important for the development of multicellular organism. In face, defects in apoptotic pathways contribute to number of human diseases, ranging from neurodegenerative disorders to malignance[14, 15].

Cell apoptosis have two signal transmission pathway. One of them is death receptor pathway (also called the extrinsic pathway), priming by the ligand and cell death receptor on cell surface binding; the another way is mitochondrial pathway (also called the intrinsic pathway), participated by mitochondria releasing cytochrome C. The extrinsic and the intrinsic pathways are not mutually exclusive and some cells, hepatocytes included, require mitochondrial involvement to amplify the apoptotic signal initiated from death receptors.

The death receptor includes TNFR family, such as TNF-α receptor, Fas, TGF-β receptor. Death receptor and ligand binding under the effect of death inducing signal, then activating the upstream Caspase，mainly is Caspase 8，and activating the effective Caspase 3. Another way is Cyto-c mediated . Every kind of death signal could induce chondriosome to releasing Cyto-c,and Cyto-c binding with (Apaf-1), activating Caspase 9，thus inducing activation of downstream effective Caspase 3.

Caspases are the central mediators of programmed cell death and comprise a family of at least 14 diferent members in mammalian cells. The apoptotic program is instigated by initiator caspases that in turn trigger an amplifying cascade of elector caspases, such as caspase 3[16]. TNF-alpha is a cytokine that triggers apoptosis through the activation of caspase in a wide variety of cells[17].

Survivin is a recently identified protein of the inhibitor of apoptosis protein (IAP) family which supresses apoptosis[18]. It is undetectable in normal differentiated tissues[19, 20] but becomes notably expressed in most common human cancer *in vivo* [20-22], especially in lung and breast cancer cells[20,23]. Many reports have indicated correlation between survivin expression and either unfavorable prognosis[22,23-26], lack of response to chemotherapy[25, 27] in various tumor tissues. In vivo study has showed that inhibition of surviving and increase of the TNF-alpha, activate caspase 3 and consequently apoptosis occurs[28].

More specifically, the Bcl-2 gene family members have been shown to hold key genetic elements in modulating this homeostasis by means of regulating mitochondrial-dependent programmed cell death. It is now well known that the upregulation of antiapoptotic Bcl-2 family proteins inhibits many forces of apoptosis induced by numerous stimuli. Bcl-2 blocks the mitochondrial release of cytochrome C and prevents the activation of pro-apoptotic caspase proteins[29, 30]. It is influenced by a variety of tumor suppressor pathways, particularly the p53 pathway.

The Fas/FasL system was originally implicated in the maintenance of lymphocyte homeostasis, during which activated peripheral T cells are removed by a Fas-dependent mechanism [31, 32]. The Fas/Fas ligand (FasL) system represents one of the main apoptotic pathways controlling controlling cell proliferation [33]

and tissue remodeling [34]. Both Fas and FasL are type I and type II transmembrane proteins, respectively, and are members of the TNF-receptor family. The interaction between Fas and FasL or activating anti-Fas antibodies results in the trimerization of Fas, followed by the assembly of other intracellular proteins to form the death-inducing signal complex (DISC) [33, 35]. Caspase-8 recruitment to the DISC results in its proteolytic activation, which, in turn, activates other members of the caspase family, eventually ending in apoptosis[36].

LIGHT originated cell function is mediated by the cell surface receptor. LIGHT could interact with HVEM/TR2, LTβR and DcR3/TR6, and those receptor are all TNFR family members. HVEM/TR2 and LTβR mediated signal transmission way is the same way which through TNFR-associated factors TRAFs, such as TRAF1, TRAF3, TRAF5[15]. LIGHT effect to the tumors cell who express that two kind of receptors, could cause cell apoptosis. LIGHT through down regulation to activating caspase 9, causing the activation of caspase 3, 7, inducing the cascade reaction. Activating poly (ADP-ribose) polymerase PARP and DNA fragment factor DFF to result in apoptosis. Besides this, LIGHT could binding with decoy receptor 3 DcR3, activating caspase 8, one after another causing PARP, DFF45 change, inducing cell apoptosis. [16]

Our research found that the transfection efficiency of solo LIGHT transfection group and combinant transfection group (LIGHT and INF-γ combinant transfection) were all higher than the control group. The difference had predominance statistical significance. Furthermore, the Fas expression in three group were positive correlated with the apoptosis rate of HepG2 cell. Also the Caspase 8 had the similar result in FCM. On the contrary, the cell apoptosis rate in the combinant group were negative correlated with the expression of Bcl-2. From

their statistical result, we could deduced that, Fas and caspase 8 anticipated LIGHT induced HepG2 cell apoptosis. The possible signal way is death receptor pathway. However, the expression of Fasl had no significant difference in the three groups. The possible reason to elucidate this phenomenon is that FasL only expressed in the activated matured CD4+, CD8+ T cell, B cell and NK cell surface. While the HepG2 cell line can not express FasL. If there are large amount of FasL in the in vivo enviroment, Fas upregulated by LIGHT inducing, and trimer forming according to Molecular aggregation of those Fas, FasL and LIGHT gene binding, then initiated cell apoptosis.

On the other hand, the expression of Bcl-2 have negative correlation with cell apoptosis rate in the combinant transfection group. Further more, the cell apoptosis rate of combinant group is obviously higher that the solo LIGHT group. Although the solo LIGHT group was expression downregulation, the difference has no significance statistics. But the the expression of Bcl-2 was obviously downregulated in the combinant group, it elucidated that INF-γ and LIGHT have synergistic action which makes the downregulation of Bcl-2 expression. Bcl-2 could inhibiting the releasing of Cyt-c (there are Bcl-2 regulating apoptosis site of action in the upstream and downstram of Cyt-c releasing site), then promoting the apoptosis of HepG2 cell, and this way belongs to the mitochondrial pathway.

Understanding of the molecular mechanisms controlling alternative types of cell death can provide new targets and approaches to the therapy of cancer cells that are often resistant to the standard inducers of apoptosis. Thererfore, therapeutic strategies to inhibit selectively antiapoptotic signals in tumor cells have the potential to provide powerful tools to treat HCC.

Ⅴ CONCLUSION

1. pcDNA4/HisMaxC is a stable vector for HepG2 cell apoptosis study.

2. pcDNA4/HisMax-EGFP identify tansfection efficiency is an ideal method.

3. The possible pathway of LIGHT gene induced HepG2 cell apoptosis is death receptor pathway.

4. LIGHT and INF-γ have synergistic action in HepG2 apoptosis through the down regulation of Bcl-2 expression.

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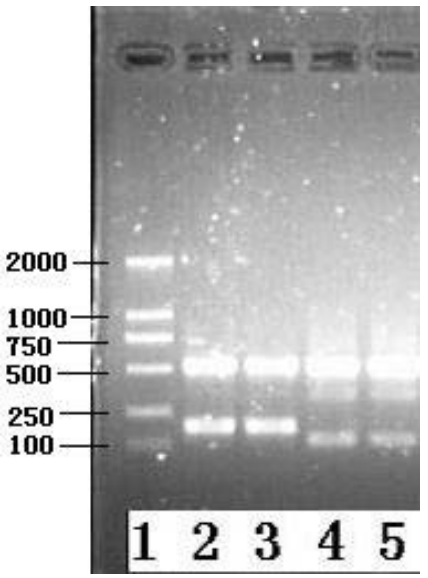
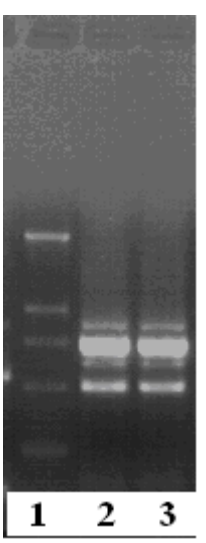


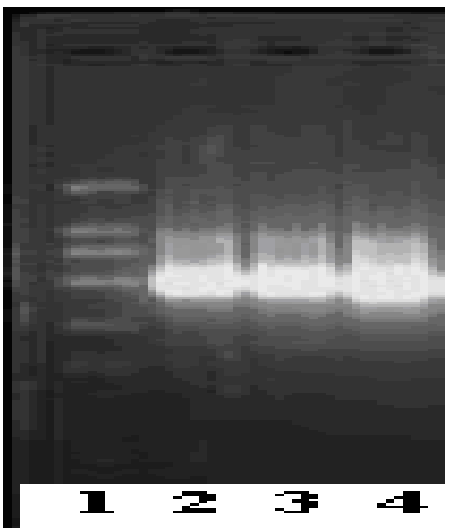
1 : DNA Marker; 2,3,4,5: band in the level of 500 bp is IFN-γ product.

Fig. 1. Agarose Gel of IFN-γ cDNA's RT-PCR product quantitation



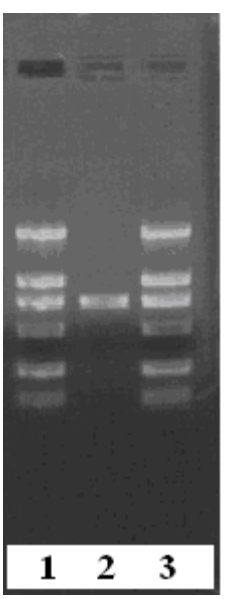
1: DNA Marker; 2,3: LIGHT product .

Fig. 2. Agarose Gel of LIGHT cDNA's RT-PCR product quantitation



1: DNA Marker; 2,3,4: LIGHT product .

Fig. 3. Agarose Gel Electrophoresis of PCR products of IFN-γ gene



1,3 : DNA Marker; 2: LIGHT product .

ig. 4. Agarose Gel Electrophoresis of PCR products of LIGHT gene

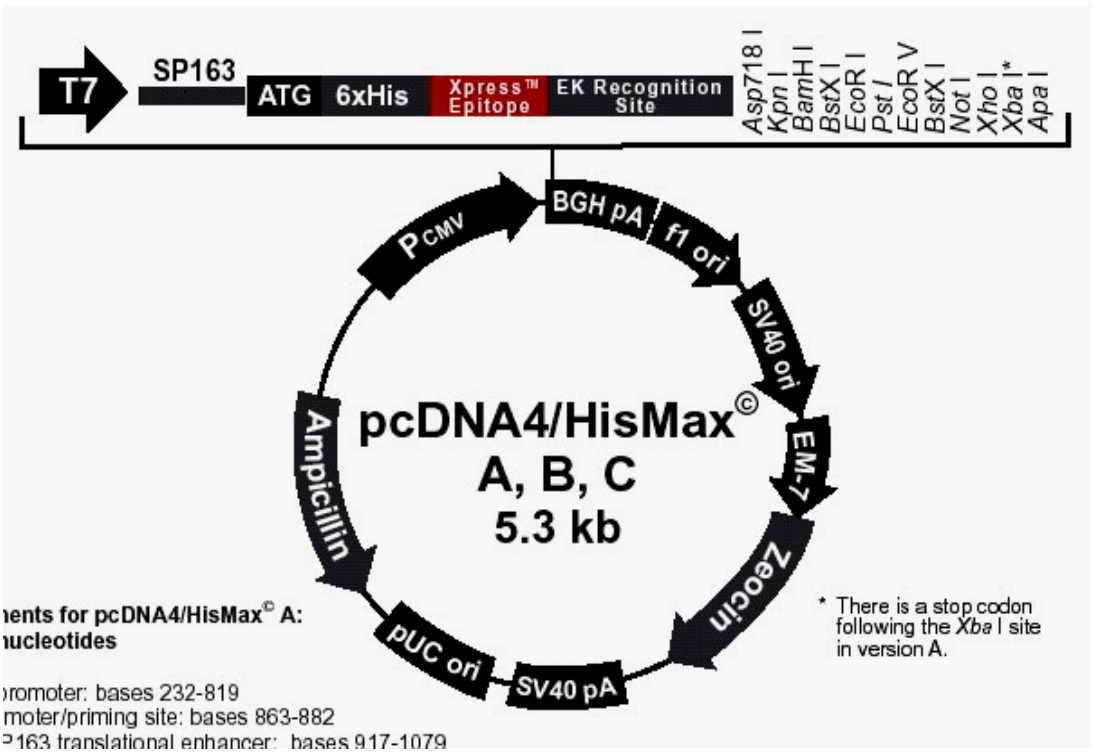
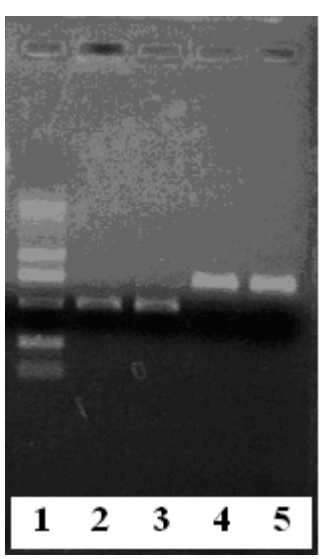
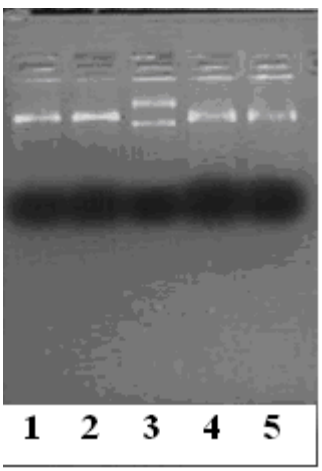


Fig. 5. Graph of PcDNA4/HisMax C



1 : DNA Marker; 2,3: IFN-γ product; 4,5: LIGHT product .

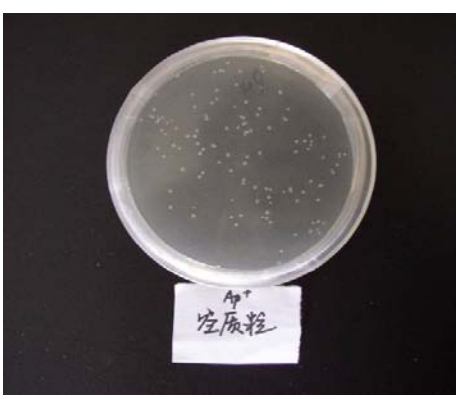
Fig. 6. PCR products after BamHI/EcoRI enzyme digestion



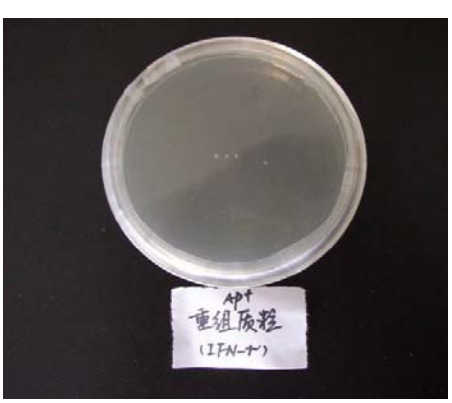
3：empty plasmid without enzyme digestion;

1,2,4,5：Pasmid without enzyme digestion

Fig7. Pasmid PcDNA4C BamHI/EcoRI enzyme digestion;

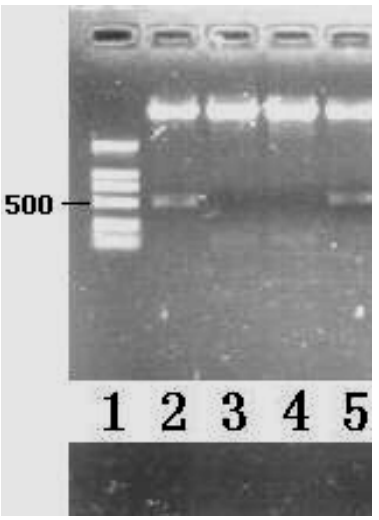


Control group: empty plasmid tranformation

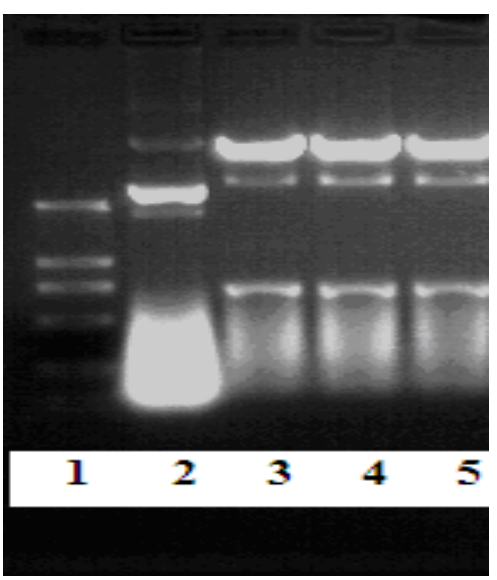


Experiment group：Combined plasmid transformation

Fig8.Positive colon from E. coli after lasmid transformation

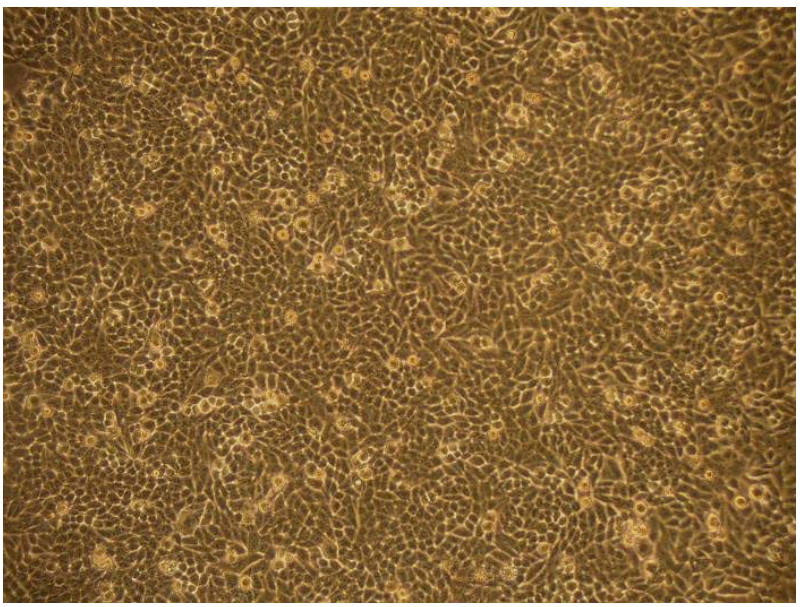


1: DNA Marker;
2,5: The Band in the 500 bp is the IFN- γ cDNA fragment digested from the combinant plasmid.

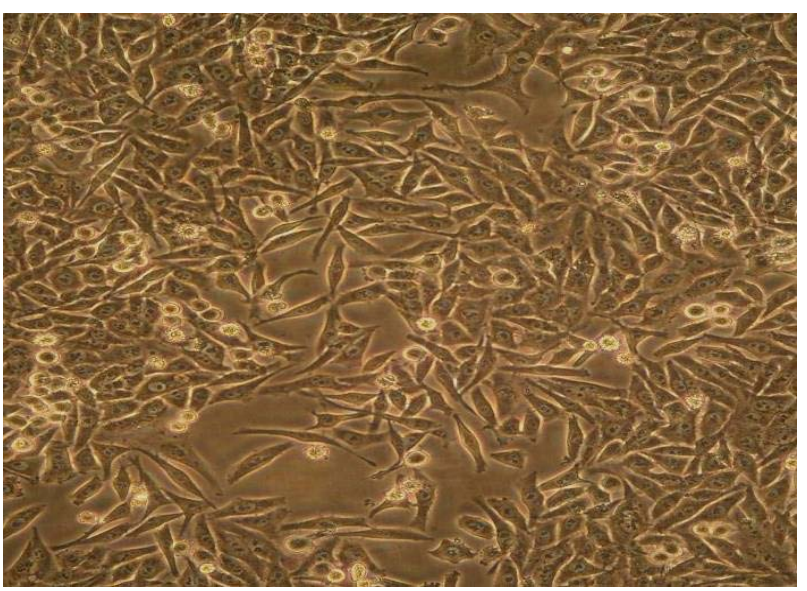


1: DNA Marker;
3,4,5: The band in 750 bp is the LIGHT cDNA fragment digested from the combinant plasmid

Fig. 9. Identification of the combinant plasmid after enzyme digestion



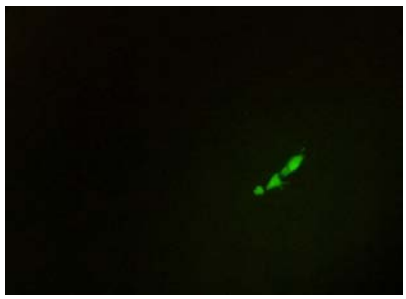
Magnification 10×10



Magnification 10×20

Fig. 10. Human HepG2 cells under microscope

1



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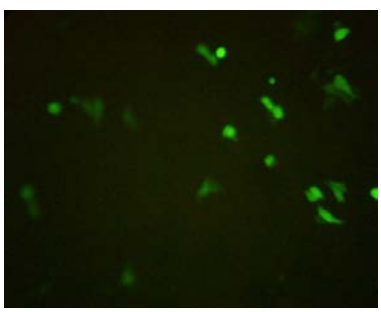

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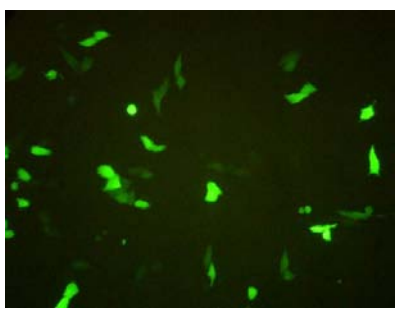

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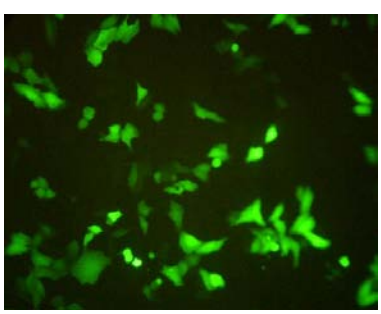

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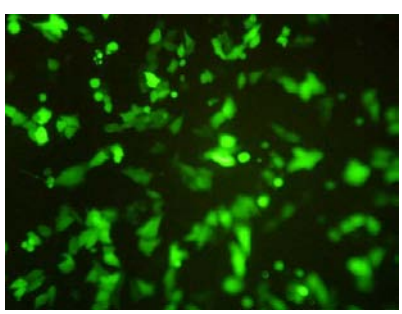

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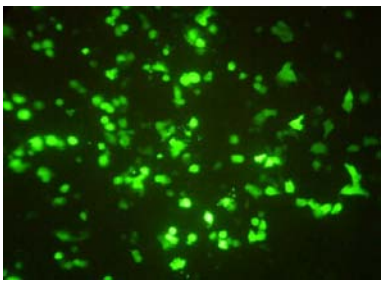

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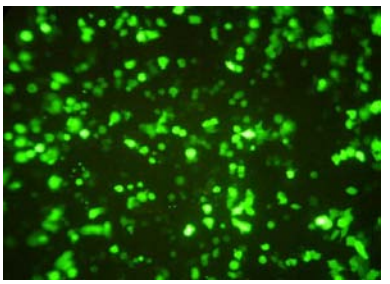

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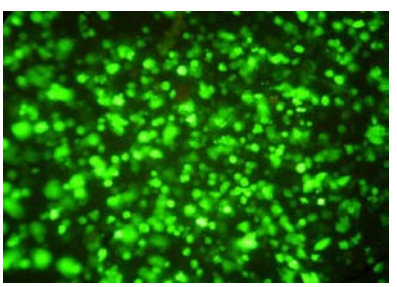


9

Fig. 11. EGF protein expression after 24 hour's transfection under fluorescence inverted microscope

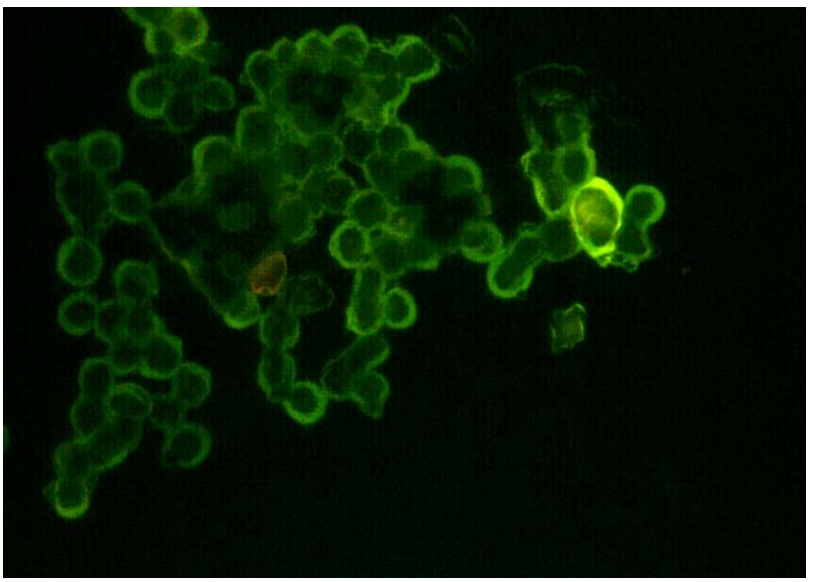


Fig. 12. Apoptosis in early stage (Membrane Annexin V/FITC staining) under fluorescence inverted microscope

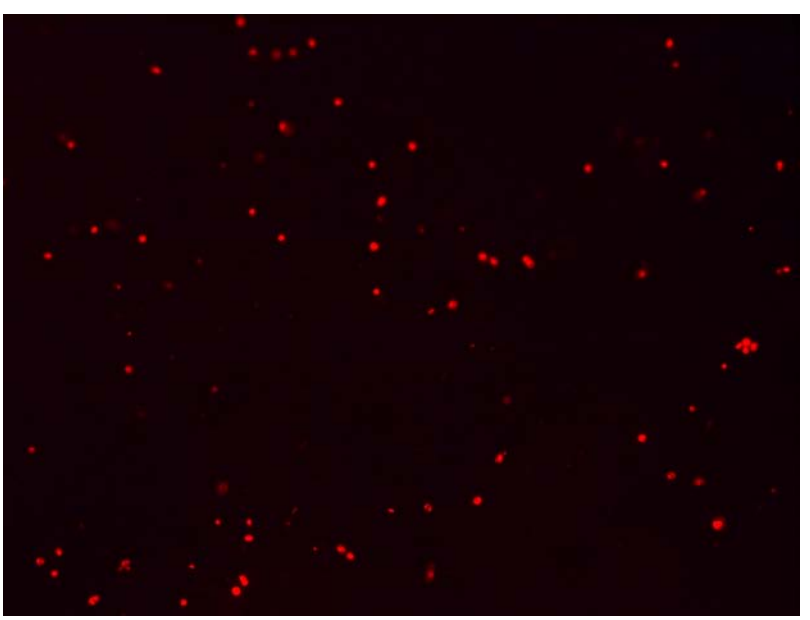
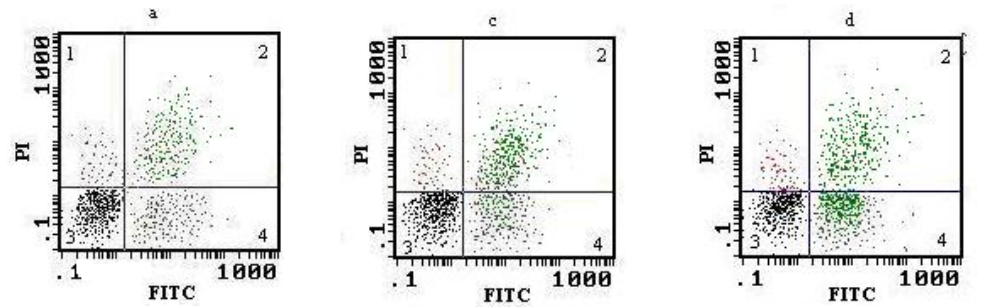
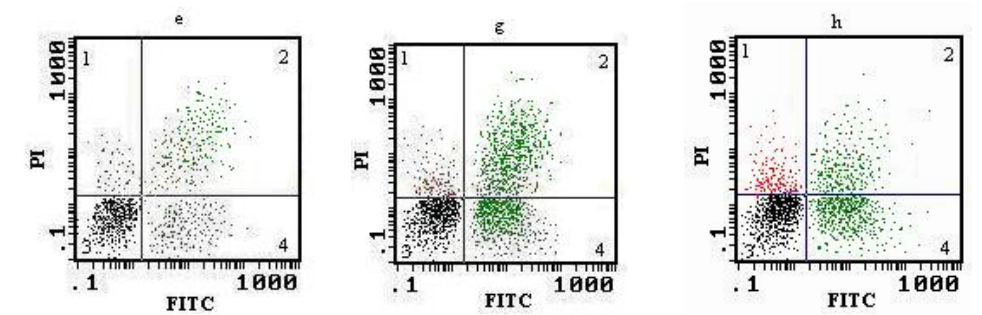


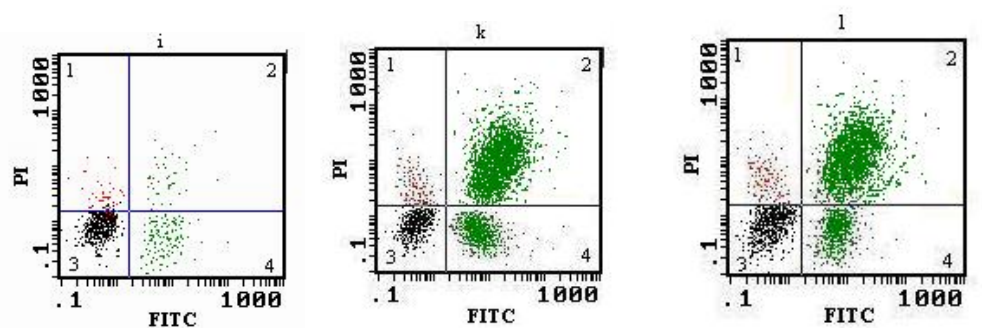
Fig. 13. Apoptosis in end stage (Nuclear PT staining) under fluorescence inverted microscope



Control (12h) LIGHT(12h) LIGHT and IFN- ɣ (12h)



Control (24h) LIGHT(24h) LIGHT and IFN- ɣ (24h)



Control (48h) LIGHT(48h) LIGHT and IFN- ɣ (48h)

The only PI stain in the first quadrant means relative accuracy; The double stain of Annexin V-FITC and PI in second quadrant means end staged apoptotic cell; No Annexin V-FITC and PI stain in the third quadrant means normal cell. The only Annexin V-FITC stain in the fourth quadrant means early stage apoptotic cell. The cells in the second and fourth quadrant are apoptotic cells.

Fig. 14. Scatterplot of Flow Cytometry of HepG2 cells apoptosis after transfection in different time

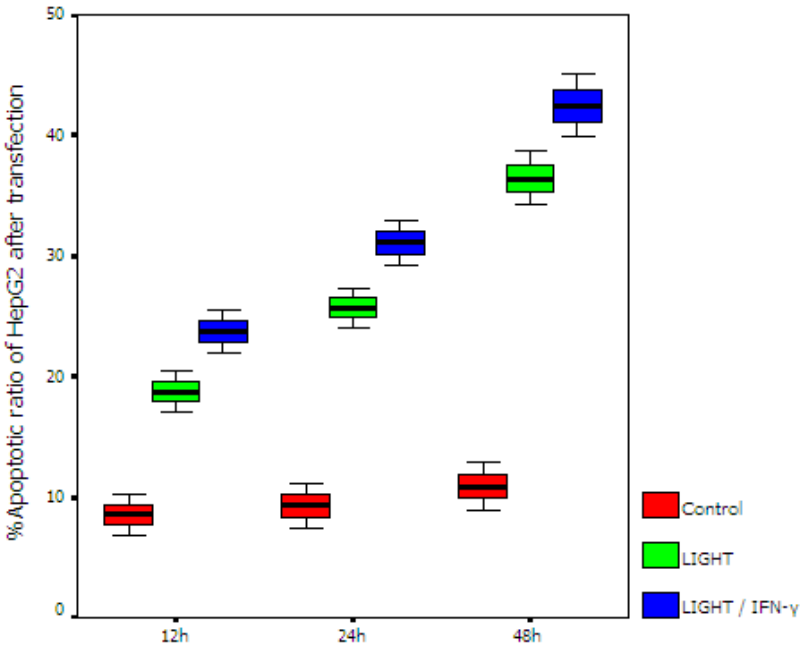


Fig. 15. The apoptotic ratio of HepG2 cells after transfection

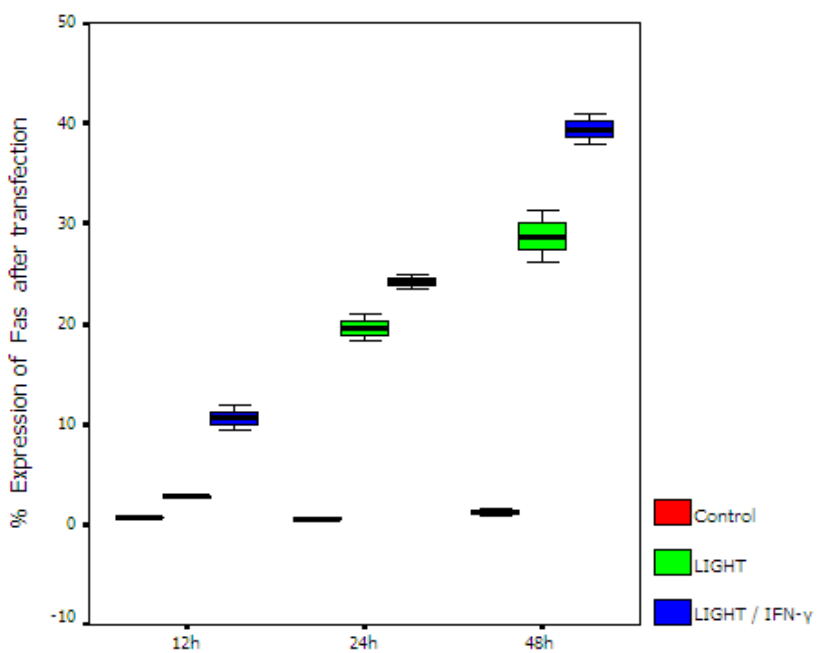


Fig. 16. The expression of Fas in HepG2 cells in different time after transfection

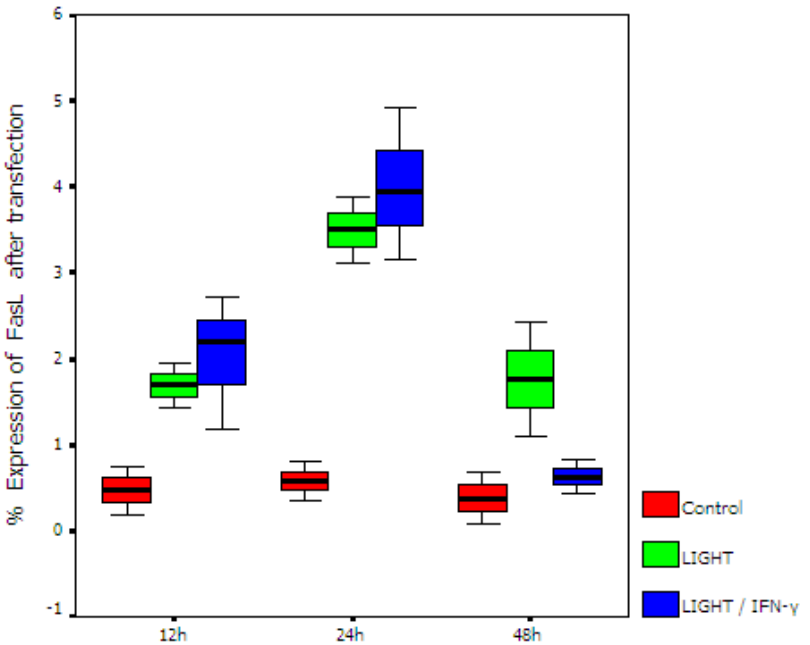


Fig. 17. The expression of FasL in HepG2 cells in different time after transfection

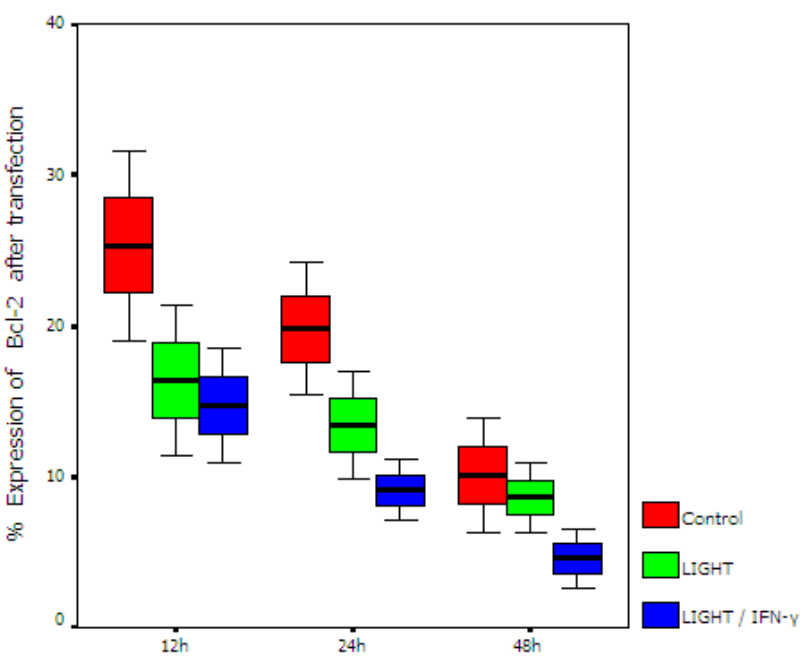


Fig.18. The expression of Bcl-2 in HepG2 cells in different time after transfection

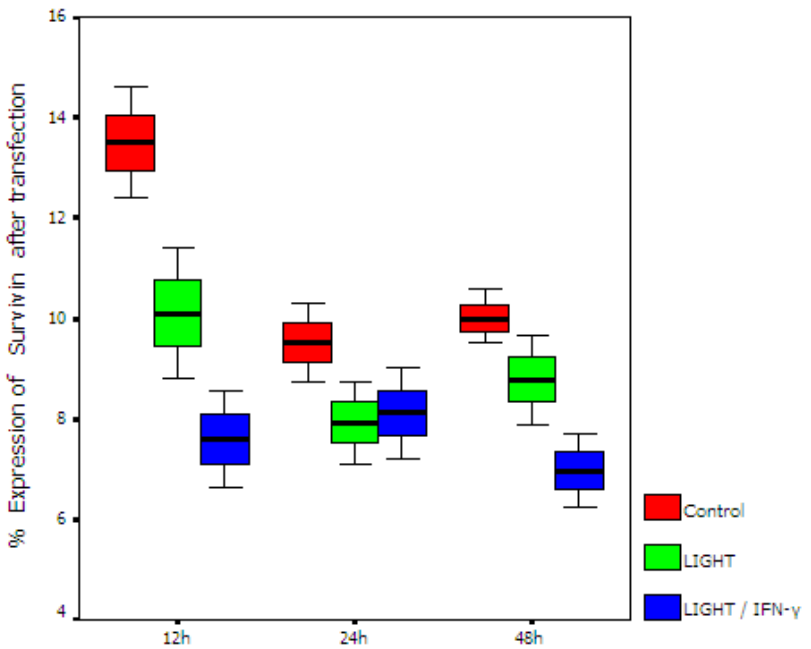


Fig. 19. The expression of Survivin in HepG2 cells in different time after transfection

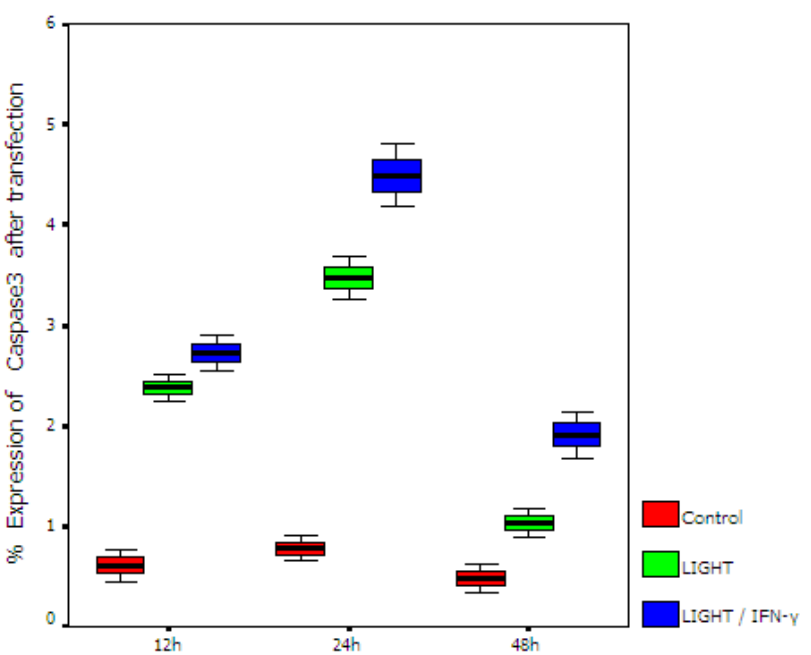


Fig. 20. The expression of Caspase 3 in HepG2 cells after transfection

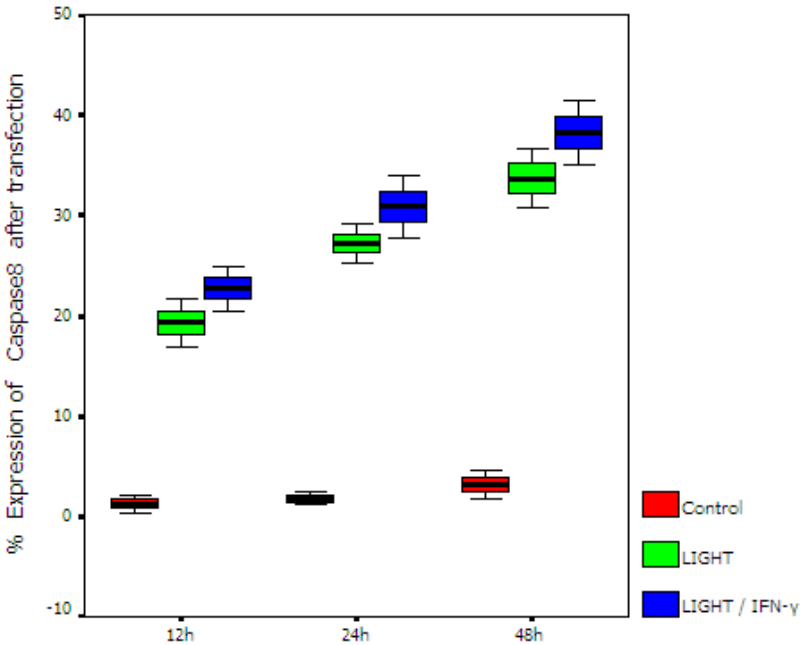


Fig. 21. The expression of Caspase 8 in HepG2 cells in different time after transfection

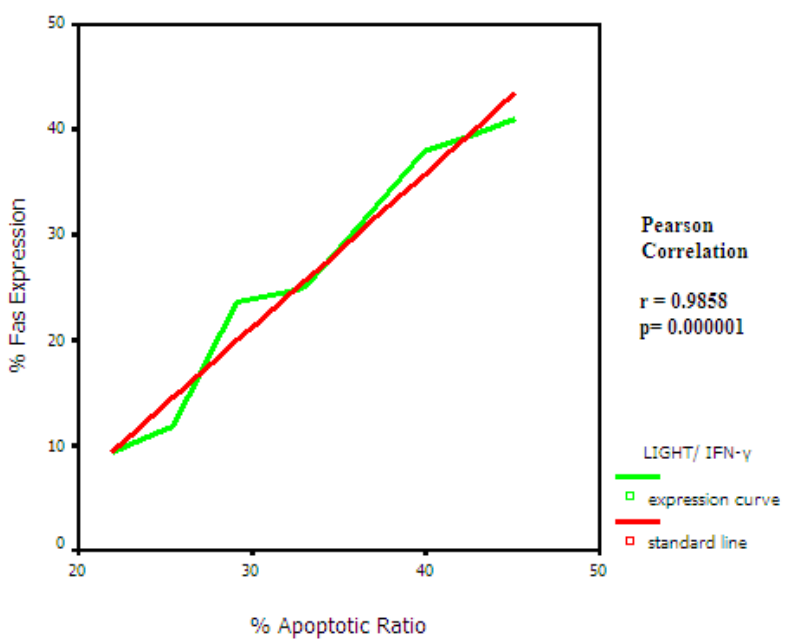


Fig. 22. Correlation between the apoptosis cells and the Fas expression

（LIGHT and IFN- ɣ transfection ）

Abstract (in korean)

$HepG_2$ 세포의 세포사멸에 미치는 LIGHT 유전자와 IFN-ɣ유전자의 영향

목적: $HepG_2$ 세포에 리포좀을 이용한 LIGHT 유전자나 LIGHT 유전자와 IFN-ɣ유전자 를 같이 전사시키는 방법을 개발하고 이들 유전자가 $HepG_2$ 세포의 세포사멸에 작용하는 효과를 연구한다

방법: $HepG_2$ 세포를 세군으로 나누어 대조군, LIGHT 유전자군, LIGHT 유전자와 IFN-ɣ유전자 를 같이 전사시킨 군으로 나눈다. 각 군은 E.coli JM-109 에서 추출한 pcDNA4C-LIGHT cDNA 와 pcDNA4HisMaxC-hIFN- ɣ cDNA 를 리포좀으로 처리한 후 $HepG_2$ 세포에 전사시키고 12,24,48 시간 배양 후에 세포를 얻는다. 각군에 속한 $HepG_2$ 세포의 세포사멸은 플로 사이토메트리를 이용하여 Fas 、FasL、Survivin、Bcl-2、Caspase-3 및 Caspase-8 를 측정하여 알아 본다.

결과: 유전자로 처리 후 $HepG_2$ 세포의 세포사멸은 시간이 지날수록 많아짐을 볼 수 있었다. 두가지 유전자로 처리한 군이 한가지 유전자로 처리한 군에 비하여 세포사멸이 심하였고 ($P<0.01$), 대조군에 비하여 크게 높았다. $HepG_2$ 세포의 Fas 와 FasL 의 발현은 증가하였고 이 중 FasL 의 발현이 Fas 의 발현에 비하여 낮았다. Fas 의 발현은 대조군,

한가지 유전자로 처리한 군, 두가지 유전자로 처리한 군의 순서로 점점 높아졌다. 반대로 Survivin 의 발현은 Fas 의 발현과 반대로 뒤로 갈수록 낮아졌다.

결론: $HepG_2$ 세포에서 리포좀 pcDNA4/His MaxC 를 이용한 LIGHT 유전자와 IFN-ɣ유전자의 전사는 성공적이었다. 따라서 pcDNA4/His Max-EGFP 의 전사 효율법은 이상적인 방법이었다. $HepG_2$ 세포에서 LIGHT 유전자가 세포사멸을 일으키는 것은 사멸 수용체를 통한 방법으로 사료되며 LIGHT 유전자와 IFN-ɣ유전자가 세포사멸을 일으키는 것은 Bcl-2 를 억제를 통하여 되는 것으로 사료되었다.