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干擾素與細胞毒化學治療劑合併處理對人類肝癌細胞株之影響及作用機制之探討

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干擾素與細胞毒化學治療劑合併處理對人類肝癌細胞株之影響及作用機制之探討

計畫類別：☑ 個別型計畫　□ 整合型計畫
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（一）中、英文摘要及關鍵詞(keywords)。

肝癌是世界上常見的惡性腫瘤之一，每年約有四十幾萬的新增病例。慢性 B 型肝炎或 C 型肝炎引起之肝硬化病人是罹患肝癌的高危險族群。肝癌的治療以手術為主，然而術後之復發及轉移是決定病人預後的關鍵因素。因為病毒感染引起的發炎反應以及肝硬化等危險因子持續存在，造成多數病人的復發及死亡。肝癌細胞與正常肝細胞一樣具有藥物代謝及去活化的能力；此外，肝臟癌化過程常伴隨抗藥基因的過度表現及抑癌基因的缺失，大大增加化療的困難性。迄今為止，主要的抗癌藥物都曾用於肝癌的治療，可惜並無令人振奮的結果。因此，未來在肝癌的防治上除了醫療技術的提升（如早期診斷及手術改良），如何避免術後病人的復發應是當務之急。

干擾素是目前治療慢性肝炎的臨床用藥，部分研究指出干擾素之使用可以預防肝炎及肝硬化引起的肝癌發生率。臨床前研究顯示：在施用於細胞培養系統和人類腫瘤移植入動物的實驗中，干擾素顯現了抗病毒，抗增殖，抑制血管新生及免疫調節功能，這些特性也賦予干擾應用於肝癌治療契機。近來有報告指出在其他癌症中，合併使用干擾素及抗癌藥物，其藥效有相加或加成的效果。然而，干擾素如何引起這樣的作用，其詳細機轉仍有待釐清。將干擾素與肝癌治療的首選藥物 Doxorubicin 合併使用來治療肝癌似乎有其潛力。在我們的結果顯示：干擾素對 Doxorubicin 的細胞毒性確實有加強的效果。此外，我們發現干擾素與 Doxorubicin 合併處理的毒性加強現象，與干擾素接受體的基因表現量呈現相關性：單獨處理 Doxorubicin 會抑制該基因的表現，而當干擾素與 Doxorubicin 合併處理時，此抑制現象幾乎可被完全回復，這似乎暗示透過干擾素接受體訊息傳遞途徑的重新啟動，將有助於抗癌藥物引起的細胞毒殺效果。探討 Doxorubicin 之抗癌機轉與干擾素接受器傳遞之訊息途徑的關係時我們發現：單獨處理 Doxorubicin 卻可以稍微增加干擾素接受器下游 Stat 蛋白質的表現，若干擾素與 Doxorubicin 合併處理時，蛋白質的表現亦比單獨處理 Doxorubicin 還多。為了釐清干擾素對 Doxorubicin 的細胞毒性加強的效果是否與干擾素活化接受器產生之傳遞之訊息途徑有關，我們使用了干擾素接受器的中和性抗體來阻斷此訊息傳導途徑；結果顯示：當訊息傳導途徑受到阻礙時，干擾素對藥物的毒性加強作用便降低了，此證據直接指出此訊息傳遞途徑之重要性。然而，上述的阻斷效果並不完全，暗示除了干擾素接受器訊息途徑之外，仍有其他調控之因子參與干擾素對藥物的作用。我們在本研究中也利用 RT-PCR 實驗針對其他基因的表現量進行探討，結果顯示：除了內部控制組β-Actin 的基因表現量在各實驗組別無明顯差異之外，TNFα、IL-1β、IL-18、PPARα、及 PPARγ的基因表現量都與干擾素接受器的變化一致，此結果暗示干擾素作用的確牽涉許多訊息傳導途徑。我們於此研究中亦嘗試使用其他作用機制的抗癌藥物與干擾素合併處理肝癌細胞，來探討是否有如同 Doxorubicin 的現象，結果顯示：即使各種臨床用抗癌藥物的作用機制並不相同，合併干擾素處理細胞都有加強藥效的效果，不過目前的數據並不足以清楚說明干擾素實際的作用機轉，未來應針對各種抗癌藥物的作用機轉加以探究，期望透過我們在抗肝癌藥物作用機轉及分子層次上的認識，為未來肝癌的治療，提供更有力的選擇。

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, with increasing incidence in Western countries. There are 437000 new cases annually, and patients with chronic hepatitis B (HBV) and C (HCV) liver cirrhosis are of the greatest risk. Surgical resection plays an important role in determining prognosis, however, recurrence and metastasis become key issues in survival after curative resection. The risk of developing recurrence remains in the majority of patients, in whom the inflammatory stimulation and cirrhosis continue to exist as background risk factors. HCC, consistent with the metabolic and secretive role of hepatocytes, highly express several genes involved in drug metabolism and inactivation, represent the first major obstacle to chemotherapy. In addition, overexpression of multidrug resistance protein and loss of tumor suppressor gene during hepatic carcinogenesis enhance the resistance of the tumor cells against antineoplastic agents. To date, nearly all the major classes of antineoplastic agents have been tested in patients with HCC; unfortunately, none of them has shown encouraging results. Therefore, future advances; not only in medical technologies improvement, but also a new strategy to prevent recurrence may be required.

IFN is a family of cytokines originally identified as enhancers of antiviral host defense, and is now widely used for the eradication of HCV from patients. Other studies also showed that IFNα

has preventive effects on the development of HCC in patients with hepatitis and cirrhosis. Several properties of IFNs, such as antitumor, antiproliferative, antiangiogenic, and immunomodulatory activity, make them potentially active against HCC. Recent reports demonstrated that IFN has additive to synergistic effects on a broad range of anticancer agents, including alkylating agents, antimetabolites, anthracycline antibiotic, and drugs that interact with microtubules. However, little has revealed the mechanism of IFN's actions to the synergistic effect of IFN in combination chemotherapy.
The combination of IFN and doxorubicin, the most effective single agent against HCC, offers a promising therapeutic approach for the treatment of HCC. Our preliminary data showed that IFNα2b acts synergistically with doxorubicin. Is this effect due to the inducing apoptosis or the suppressing cell growth? Further investigation in several aspects of cell biology, including cell proliferation, apoptosis, and changes in the cell cycle after doxorubicin treatment with or without IFNα2b will be performed. Our results also showed that the synergistic effect from IFNα2b addition correlated with different expression patterns of IFN receptors; with a down-regulation in treating doxorubicin alone and an up-regulation in the combined treatment. What's the relationship between the synergistic effect and the expression of IFNRs? Accordingly, does the phenomenon occur at translation levels still? If it does, will incubation with neutralizing IFNR antibodies abolish the synergy? How about the molecules involving in signal transduction pathway? These results may provide evidence and rationale associated with the underlying mechanism for this combination therapy and further guide therapies by advancing of our understanding on HCC at the molecular level in the future.
Keyword: interferons, interferon receptors, doxorubicin, Stat.

（二）報告內容：

INTRODUCTION:

Hepatocellular carcinoma (HCC) accounts for approximately 6% of all human cancer. It is estimated that half a million cases occur annually worldwide, making HCC the fifth most common malignancy in men and the ninth in women (1). HCC is rapidly fatal in most patients, which makes its incidence rate very close to its mortality rate and its prevalence rates essentially the same as the incidence rates (2). The primary risk factor for the development of HCC is cirrhosis. Even patients without cirrhosis who develop HCC are typically found to have some underlying hepatic abnormality, such as steatohepatitis or chronic viral hepatitis. Although cirrhosis of any cause increases the risk of developing HCC, cirrhosis associated with chronic hepatitis B or C virus infection or hemochromatosis carries the greatest risk. HCC is closely related to hepatitis viral infections, and commonly arises in the liver with chronic inflammation. The annual incidence of HCC reaches approximately 3% in type B (HBV)- and 7% in type C (HCV)-hepatitis virus-infected cirrhotic patients (3). Moreover, the incidence leaps up to approximately 20-25% a year in cirrhotic patients who underwent curative treatment of the primary HCC (4). Additional factors such as patient age and sex, duration and severity of liver disease, concurrent alcohol or aflatoxin exposure, liver histology, and alpha-fetoprotein levels also contribute to the relative risk of developing HCC (2-5).

The treatment options and prognoses of the HCC patients largely depend not only on the characteristics of the tumor but also on the severity of the underlying chronic hepatic disease that affects most of the patients. Prognosis is relatively better for the subset of patients eligible for surgical treatments (tumor resection, orthotopic liver transplantation) or other potentially curative locoregional treatments (radiofrequency ablation, percutaneous ethanol injection, transcatheter arterial chemoembolization, systemic chemotherapy, hormone therapy, external and targeting radiotherapy, or immunotherapy). A worse outcome is expected in those patients who can be treated only with palliative locoregional treatments (e.g., transarterial embolization) or who are not suitable for any of

the above options (6). Although surgical managements or non-surgical therapeutic modalities have been employed, either separately or in combination, treatment for HCC is rarely curative. The prognosis for HCC is generally poor, and the 5-year survival rate is limited to 25–58% after surgery (7,8). The risk of developing another malignant focus (recurrence) remains in the majority of patients, in whom the inflammatory stimulation and cirrhosis continue to exist as background risk factors (9). Thus, despite extensive clinical advances in the therapy of HCC, many patients show disease recurrence and finally progress to the advanced stages with vascular invasion and multiple intrahepatic metastases. Therefore, future advances, merely in medical technologies, in both the early detection and therapy of HCC may not serve for the further improvement of therapeutic outcome of HCC, but a new strategy to prevent post-therapeutic recurrence of HCC may be required for such purpose.

A great number of chemotherapeutic agents have been tested in patients with advanced HCC; unfortunately, none of them has shown encouraging results. Liver cancer cells, consistent with the metabolic and secretive role of hepatocytes, highly express several genes involved in drug metabolism and inactivation, which represent the first major obstacle to the success of cytotoxic treatments. In addition, several molecular changes, such as overexpression of the multidrug resistance protein (MDR) and the loss of tumor suppressor gene p53, have been described during hepatic carcinogenesis. All these modifications are likely to play a role in the resistance of the tumor cells against commonly used antineoplastic agents (10). Moreover, treatment with these drugs is often associated with unacceptable toxicity. This is not surprising if we consider that the recommended doses of antineoplastic agents are usually derived from phase I studies carried out on patients who have no impairment of hepatic function. The same doses may not be adequate for patients with HCC, who usually show various degrees of hepatic failure caused by the underlying chronic liver disease (6). To date, nearly all the major classes of antineoplastic agents have been tested in patients with HCC. Neither single agent nor combination chemotherapy demonstrate a clear reproducible advantage in terms of overall survival, and might be due to innate resistance or poor tolerance, systemic chemotherapy has played only a minor role in the treatment of HCC.

In contrast to the classical chemotherapeutical agents that were incorporated into drug combination for their putative effect on a specific biochemical locus, biological agents have pleiotropic cellular actions and have been studied with only a minimal understanding of their biochemical actions. Biological agents might function in a manner analogous to that of biochemical modulating agents, and their multiple cellular effects could contribute to their overall efficacy in combination chemotherapy. For example, the combination of interferon (IFN) and conventional chemotherapeutic agents offers a promising therapeutic approach for the treatment of cancer. Recent *in vitro* studies have demonstrated both direct cytotoxic and cytokinetic effects for IFN (12), an interesting role derives from its ability to synergistically potentiate the activity of cytotoxic agents against human hepatocellular carcinoma cells is of our curiosity. IFN is a family of cytokines originally identified as enhancers of antiviral host defense, and is now widely used for the eradication of HCV from patients with chronic liver diseases. In addition, IFN is also known to exert antitumor activities, and is applied for the therapy of chronic myelogenous leukemia, hairy-cell leukemia, non-Hodgkin’s lymphoma, multiple myeloma, condyloma acumination, malignant melanoma and AIDS-related Kaposi's sarcoma (13). Several biologic properties of IFNs, such as antiproliferative, antiangiogenic, and immunomodulatory activity, make them potentially active against HCC. Clinical evidence has been accumulating regarding preventive effects of IFNα on the development of HCC in patients with chronic hepatitis and cirrhosis associated with HCV infection (12-18). Indeed, IFN have been shown to have not merely additive but also synergistic effects on a broad range of anticancer agents, including alkylating agents, antimetabolites,

anthracycline antibiotic, and drugs that interact with microtubules in models using cell culture, animal tumors, and human xenografts (12,19). However, there is as yet no consensus on optimal strategies for combining this family of compounds with other cancer therapies. The interaction between IFN and cytotoxic agents *in vitro* is complex and depends not only on the choice of cytotoxic agent but also on the concentrations, ratios, duration, and sequence of exposure to the two drugs. In addition, the limited studies have not resolved the significance of IFN's actions to the potentiating effect of IFN in combination chemotherapy. The mechanisms of action of IFNs, especially when combined with cytotoxic drugs, remain to be elucidated.

Among the cytotoxic chemotherapeutic agents used in clinics for the treatment of HCC, doxorubicin remains the most effective single agent, with a tumor response rate of approximately 10-15% (11). Therefore, it may be fruitful to explore the combination with IFN and doxorubicin against the human hepatocellular carcinoma cells. Here, a clinically proven therapeutic agent for chronic hepatitis B and chronic hepatitis C, IFNα2b (Intron A), was used for this purpose. The majority of HCC cells are characterized by overexpressing the multidrug resistance (MDR-1) gene as well as the loss of tumor suppressor gene like p53. Hep 3B cells, a malignant HCC cell line possessing such key features, were investigated.

MATERIALS and METHODS

Chemicals and reagents

Aprotinin, Dulbecco's modified Eagle's medium (DMEM), dimethyl sulfoxide (DMSO), DTT, doxorubicin, EDTA, leupeptin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), penicillin, phenylmethylsulfonyl fluoride, propidium iodide (PI), ribonuclease A (RNase A), sodium chloride, sodium dodecyl sulfate (SDS), streptomycin, trypan blue, tryspin-EDTA, Tween 20 are bought from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, Utah). Recombinant human IFNα2b is obtained from Schering-Plough (Brinny) Company (Ireland).

Cell culture and drug preparation

The human hepatocellular carcinoma cell line, Hep 3B, is obtained from the American Type Culture Collection (Rockville, MD). Cells are grown in 90% DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. Exponentially growing cultures is maintained in humidified atmosphere of 5% CO_2 at 37ºC. Before treatments, culture plates or dishes are left overnight to allow cell to adhere. Thereafter the medium is replaced by fresh culture medium containing the test compounds and cells are allowed to grow for another 24h. Doxorubicin is applied in DMSO to a final concentration of 10 mg/ml and stored at –20ºC. The concentrations used in this study are 0.25, 0.50, 1.00, and 2.00 μg/ml and freshly diluted to the basal medium with a final DMSO concentration at 0.1%. Controls are always treated with the same amount of DMSO as used in the corresponding experiments.

Growth Inhibitory Assays with Doxorubicin and IFNα.

Cells (1×10^4) per well are added in triplicate to a 96-well microplate, and 12 h later, the medium is replaced by 0.1 ml of fresh medium containing IFN alone, doxorubicin alone, or medium containing IFN and doxorubicin. Concentrations of doxorubicin tested are 0.05, 0.5 and 5 μg/ml, and those of IFNα are 50 and 500 units/ml. Tumor cells suspend in complete medium are used as a control for cell viability. The medium is changed every 48 h, and 4 days after the addition of doxorubicin and IFNα, the numbers of viable cells are assessed by MTT assay (36). Briefly, 10 μl (50 μg) of MTT are added to each well. The plate is incubated for 4 h at 37°C. Unreacted MTT is then removed, leaving the resultant formazan crystals at the bottom of the well. Then, 0.1 ml of 2-propanol is added to each well to dissolve the crystal. The absorbance of the plate is measured in a microplate reader at a wavelength of 570 nm. These assays are repeated

until similar results are obtained. Also in other parts of the present study, experiments are repeated at least twice, and no discrepant results are obtained. The cell viability determines by using the MTT assay in increasing concentrations of doxorubicin is compared with that in the culture medium without doxorubicin or in combination with IFN, and the results are expressed as a percentage of the control value. The survival curves are obtained by plotting the cell viability of the treated cells versus the logarithm of the drug concentration. The sensitivities of the anticancer drugs are obtained by determining the IC_{50} of the drugs (the concentration of the drugs that reduced the cell viability to 50% of the control) from the survival curves. The drug-sensitivity assays are repeated more than five times for each drug.

Western Blot Analysis.

Cells are washed twice with ice-cold PBS and collected with a rubber scraper. After centrifugation, the cell pellets are resuspended in lysis buffer [50 mM HEPES, 150 mM NaCl, 2.5 mM EGTA, 1.0 mM EDTA, 1.0 mM DTT, 0.1% Tween 20, 10% glycerol, and protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin, 1.0 mM phenylmethylsulfonyl fluoride; pH 7.5)]. After sonication, the extracts are clarified at 15000 × g for 5 min at 4°C, and the supernatant fraction is collected. Western blot analysis is performed, as described previously (37).

Antibodies.

The following mouse monoclonal or rabbit polyclonal antibodies (Abs) are used at appropriate concentrations as recommended by the manufacturer. Anti-human IFN receptor α/β Ab and HRP-conjugated anti-mouse or anti-rabbit Abs are obtained from Santa Cruz Biotechnology (38,39). Anti-JAK1, Anti-STAT1 (C-terminus), Anti-STAT (N-terminus), Anti-STAT1 (Y701, phospho-specific), Anti-STAT2, Anti-STAT3, Anti-STAT3 (Y705, phospho-specific), Anti-ISGF3 γ Abs are brought from BD Bioscience Pharmigen. Anti-JAK2 Ab is purchased from Cell Signaling Technology and Anti-tubulin Ab is from Upstate Biotechnology.

RESULTS

Effects of Doxorubicin, Interferon, and Doxorubicin combined with Interferon on hepatocarconoma cell proliferation

To clarify that Doxorubicin, Interferon, or Doxorubicin combined with Interferon exhibited direct or indirect cytotoxic activity against tumor cell, effects of these drugs on human hepatocellular carcinoma cells were examined. Figure 1 showed the viability of Hep 3B cells in the presence or absence of various additives. After 48 hours of incubation, the metabolic activity or viability of Hep 3B cells was evaluated by the MTT assay. We compared the absorbance of MTT results of each groups and defined the cells in the untreated culture was 100%. Treatment with interferon at concentration from 100U to 10000 U did not cause any significant influence on viability (data not shown). However, doxorubicin treatment resulted in a dose-dependant decrease of viability at concentration of 0.5 to 2 μg/ml. In the presence of interferon in the culture of doxorubicin, the damage of cells obviously increased. It seemed that interferon was able to be an additive or synergistic agent to doxorubicin.

Effects of doxorubicin, interferon, or doxorubicin combined with interferon on the transcription level of interferon receptors

Previous study demonstrated that the effects of interferon were through the activation of interferon receptors and the signaling transduction pathway turned on. To explore whether these effects were also in our system, we used the experiments of reverse transcription and polymerase chain reaction to exam the gene transcription of interferon receptors. Figure 2A showed the expression of interferon receptors in Hep 3B cells when the cells treated with various drugs for 12 hours. As shown here, interferon treatment alone did not resulted in any influence on interferon receptors expression. However, doxorubicin treatment alone significant decreased these gene expressions. By contract, expression of interferon receptors in the group of doxorubicin treatment combined with interferon was increased, as compared with the group of doxorubicin treatment

alone. These data indicated that the inhibition of doxorubicin on gene expression was recovered by the addition of interferon. Figure 2B showed the expression of interferon receptors in Hep 3B cells when the cells treated with various drugs for 48 hours. As shown here, expression of interferon receptors was only in the group of doxorubicin treatment in the presence of interferon.

Effects of doxorubicin, interferon, or doxorubicin combined with interferon on the expression of stat-3 protein, the downstream participate of interferon receptors

Previous data showed that the synergistic effect from IFNα2b addition correlated with different expression patterns of IFN receptors; with a down-regulation in treating doxorubicin alone and an up-regulation in the combined treatment. What's the relationship between the synergistic effect and the expression of IFNRs? Accordingly, does the phenomenon occur at translation levels still? To answer the question, immunoblotting of the proteins downstream of IFNRs were tested. Figure 3 showed the expression of Stat-3 after various concentrations of doxorubicin treatments in the presence or absence of interferon. In contrast with the effects on transcription, doxorubicin treatment alone slightly increased the protein expression of Stat-3. Although the increase in protein expression decreased as the drug concentrations increase, the overall increasing effects were obvious. In the presence of interferon, up-regulation of protein expression was also demonstrated. These results indicated that the activation of IFNRs and their signal transduction pathways might participate in the synergistic effect from IFNα2b addition.

Effects of doxorubicin, interferon, or doxorubicin combined with interferon on the transcription level of TNFα, IL-1β, or IL-18

We also used interferon neutralization antibody to block the signal transduction pathway of interferon receptors, the results demonstrated that the blocking did not recover the synergistic effect of interferon on the cytotoxic effect of doxorubicin completely. It indicated that the transduction pathway of interferon receptor was just one possible route for interferon's influence. As shown in Figure 4 and 5, the effects of interferon also worked upon the gene expression of TNFα, IL-1β, IL-18, PPARα, and PPARγ.

Effects of other chemotherapeutic drugs in combination with or without interferon on the viability of Hep 3B cells

Another supporting results of interferon's widely influence were demonstrated in Figure 6. As the Hep 3B cells treated with other kinds of chemotherapeutic drugs in combination with interferon, the presence of interferon caused an increase of cytotoxicity of treatments, even the mechanism of each drugs on chemotherapy were totally different. These data provided an interesting sight of interferon in combination of different chemotherapeutic drugs.

DISCUSSION

In the present study, we demonstrated that interferon combined with doxorubicin showed more cytotoxic effects on the malignant human hepatocellular carcinoma cells, Hep 3B (Figure 1). The effect was also performed on the other kinds of chemotherapeutic drugs (Figure 6). We found that after 12 hours, the transcription of various genes was slow down in the group of doxorubicin treatment. It was in expectation because that doxorubicin was noticed as an inhibitor of transcription. An interesting finding of our work was that the inhibitory effects of doxorubicin on gene expression seemed to be recovered by interferon addition (Figure 2A). Moreover, the regulatory effects of interferon extended till 48 hours (Figure 2B). The effects of interferon on gene expression, especially as treatments combined with doxorubicin, were obvious on gene transcription. As we further investigated with the effects of interferon combination downstream the signal transduction pathway of interferon receptor, we found that the protein expression pattern of Stat-3 was altered. Although doxorubicin treatment alone cause an increase of Stat-3 expression in Hep 3B cells, interferon combination resulted more degree of influence (Figure 3). We also used interferon neutralization antibody to block the signal transduction pathway of interferon receptors, the results demonstrated that the blocking did not recover the synergistic effect of interferon on the cytotoxic effect of doxorubicin completely. It indicated that the transduction pathway of interferon receptor was just one possible route for interferon's influence. As shown in Figure 4 and 5, the effects of interferon also worked upon the gene expression of TNFα, IL-1β, IL-18, PPARα, and PPARγ. However, the limited data could not explain the exact mechanism of interferon's function, further investigation about the relationship is needed.

（三）參考文獻：

References

1. Di Bisceglie AM. Carithers RL Jr. Gores GJ. Hepatocellular carcinoma. Hepatology 1998; 28:1161–5.
2. Schafer DF. Sorrell MF. Hepatocellular carcinoma. Lancet 1999; 353:1253–7.
3. Shiratori Y. Yoshida H. Omata M. Different clinicopathological features of hepatocellular carcinoma in relation to causative agents. Journal of Gastroenterology 2001; 36:73–8.
4. Kumada T. Nakano S. Takeda I. Sugiyama K. Osada T. Kiriyama S. Sone Y. Toyoda H. Shimada S. Takahashi M. Sassa T. Patterns of recurrence after initial treatment in patients with small hepatocellular carcinoma. Hepatology 1997; 25: 87–92.
5. Nissen NN. Martin P. Hepatocellular Carcinoma: The High-Risk Patient. Journal of Clinical Gastroenterology 2002; 35:S79–85.
6. Massimo DM. Ermelinda DM. Francesco P. Sandro P. Bruno D. Hepatocellular Carcinoma: Systemic Treatments. Journal of Clinical Gastroenterology 2002; 35:S109–14.
7. Chen YJ. Yeh SH. Chen JT. Wu CC. Hsu MT. Tsai SF. Chen PJ. Lin CH. Chromosomal changes and clonality relationship between primary and recurrent hepatocellular carcinoma. Gastroenterology 2000; 119: 431–40.
8. Okuno M. Kojima S. Moriwaki H. Chemoprevention of hepatocellular carcinoma: Concept, progress and perspectives. Journal of Gastroenterology and Hepatology 2001; 16:1329–35.
9. Akriviadis EA. Llovet JM. Efremidis SC. Shouval D. Canelo R. Ringe B. Meyers WC. Hepatocellular carcinoma. The British Journal of Surgery 1998; 85:1319–31.
10. Ng IO. Liu CL. Fan ST. Ng M. Expression of P-glycoprotein in hepatocellular carcinoma. A determinant of chemotherapy response. American Journal of Clinical Pathology 2000; 113:355–63.
11. Nerenstone SR. Ihde DC. Friedman MA. Clinical trials in primary hepatocellular carcinoma: current status and future directions. Cancer Treatment Review 1988; 15:1–31.
12. Wadler S. Schwartz EL. Antineoplastic activity of the combination of interferon and cytotoxic agents against experimental and human malignancies: a review. Cancer Research 1990; 50:3473–86.
13. Gutterman JU. Cytokine therapeutics: lessons from interferon alpha. Proceedings of the National Academy of Sciences USA 1994; 91:1198-205.
14. Nishiguchi S. Shiomi S. Nakatani S. Takeda T. Fukuda K. Tamori A. Habu D. Tanaka T. Prevention of hepatocellular carcinoma in patients with chronic active hepatitis C and cirrhosis. Lancet 2001; 357:196–7.
15. Ikeda K. Saitoh S. Kobayashi M. Suzuki Y. Suzuki F. Tsubota A. Arase Y. Murashima N. Chayama K. Kumada H. Long-term interferon therapy for 1 year or longer reduces the hepatocellular carcinogenesis rate in patients with liver cirrhosis caused by hepatitis C virus: a pilot study. Journal of Gastroenterology and Hepatology 2001; 16:406–15.
16. International interferon-alpha HCC Study group. Effect of interferon-alpha on progression of cirrhosis to hepatocellular carcinoma: a retrospective cohort study. Lancet 1998; 351:1535–9.
17. Kasahara A. Hayashi N. Mochizuki K. Takayanagi M. Yoshioka K. Kakumu S. Iijima A. Urushihara A. Kiyosawa K. Okuda M. Hino K. Okita K. Risk factors for hepatocellular carcinoma and its incidence after interferon treatment in patients with

chronic hepatitis C. Osaka Liver Disease Study Group. Hepatology 1998; 27:1394–402.
18. Yoshida H. Shiratori Y. Moriyama M. Arakawa Y. Ide T. Sata M. Inoue O. Yano M. Tanaka M. Fujiyama S. Nishiguchi S. Kuroki T. Imazeki F. Yokosuka O. Kinoyama S. Yamada G. Omata M. Interferon therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. IHIT Study Group. Inhibition of Hepatocarcinogenesis by Interferon Therapy. Annals of Internal Medicine 1999; 131:174–81.
19. Kreuser ED. Wadler S. Thiel E. Biochemical modulation of cytotoxic drugs by cytokines: molecular mechanisms in experimental oncology. Recent Results in Cancer Research 1995; 139:371–82.
20. Kalvakolanu DV. Interferons and cell growth control. Histology and Histopathology 2000; 15:523–37.
21. Wadler S. Schwartz EL. Biological agents as biochemical modulators: pharmacological basis for the interaction of cytotoxic chemotherapeutic drugs and interferon. Cancer Chemotherapy and Pharmacology 1994; 35:21–30.
22. Constantinescu SN. Croze E. Wang C. Murti A. Basu L. Mullersman JE. Pfeffer LM. Role of interferon alpha/beta receptor chain 1 in the structure and transmembrane signaling of the interferon alpha/beta receptor complex. Proceedings of the National Academy of Sciences USA 1994; 91:9602–6.
23. Schindler C. Darnell JE Jr. Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. Annual Review of Biochemistry 1995; 64:621–51.
24. Ihle JN. STATs: signal transducers and activators of transcription. Cell 1996; 84:331–4.
25. Bluyssen AR. Durbin JE. Levy DE. ISGF3 gamma p48, a specificity switch for interferon activated transcription factors. Cytokine and Growth Factor Review 1996; 7:11–7.
26. Bluyssen HA. Muzaffar R. Vlieststra RJ. van der Made AC. Leung S. Stark GR. Kerr IM. Trapman J. Levy DE. Combinatorial association and abundance of components of interferon-stimulated gene factor 3 dictate the selectivity of interferon responses. Proceedings of the National Academy of Sciences USA 1995; 92:5645–9.
27. Borden EC. Gene Regulation and Clinical Roles for Interferons in Neoplastic Diseases. The Oncologist 1998; 3:198–203.
28. Meraz MA. White JM. Sheehan KC. Bach EA. Rodig SJ. Dighe AS. Kaplan DH. Riley JK. Greenlund AC. Campbell D. Carver-Moore K. DuBois RN. Clark R. Aguet M. Schreiber RD. Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. Cell 1996; 84:431–42.
29. Durbin JE. Hackenmiller R. Simon MC. Levy DE. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. Cell 1996; 84:443–50.
30. Muller M. Laxton C. Briscoe J. Schindler C. Improta T. Darnell JE Jr. Stark GR. Kerr IM. Complementation of a mutant cell line: central role of the 91 kDa polypeptide of ISGF3 in the interferon-alpha and -gamma signal transduction pathways. EMBO Journal 1993; 12: 4221–8.
31. Levy DE. Kessler DS. Pine R. Darnell JE Jr. Cytoplasmic activation of ISGF3, the positive regulator of interferon-alpha-stimulated transcription, reconstituted in vitro. Genes and Development 1989; 3:1362–71.
32. Decker T. Lew DJ. Cheng YS. Levy DE. Darnell JE Jr. Interactions of alpha- and gamma-interferon in the transcriptional regulation of the gene encoding a guanylate-binding protein. EMBO Journal 1989; 8: 2009–14.
33. Larner AC. Chaudhuri A. Darnell JE Jr. Transcriptional induction by interferon. New protein(s) determine the extent and length of the induction. Journal of Biological

Chemistry1986; 261: 453–9.
34. Kim TK. Maniatis T. Regulation of interferon-gamma-activated STAT1 by the ubiquitin-proteasome pathway. Science 1996; 273:1717–9.
35. Haspel RL. Salditt-Georgieff M. Darnell JE Jr. The rapid inactivation of nuclear tyrosine phosphorylated Stat1 depends upon a protein tyrosine phosphatase. EMBO Journal 1996; 15, 6262–8.
36. Der SD. Zhou A. Williams BR. Silverman RH. Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. Proceedings of the National Academy of Sciences USA 1998; 95:15623–8.
37. Zhou A. Hassel BA. Silverman RH. Expression cloning of 2-5A-dependent RNAase: a uniquely regulated mediator of interferon action. Cell 1993; 72:753–65.
38. Meurs E. Chong K. Galabru J. Thomas NS. Kerr IM. Williams BR. Hovanessian AG. Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon. Cell 1990; 62:379–90.
39. Choubey D. Lengyel P. Binding of an interferon-inducible protein (p202) to the retinoblastoma protein. Journal of Biological Chemistry 1995; 270:6134–40.
40. Chin YE. Kitagawa M. Kuida K. Flavell RA. Fu XY. Activation of the STAT signaling pathway can cause expression of caspase 1 and apoptosis. Molecular and Cellular Biology 1997; 17:5328–37.
41. Chin YE. Kitagawa M. Su WC. You ZH. Iwamoto Y. Fu XY. Cell growth arrest and induction of cyclin-dependent kinase inhibitor p21 WAF1/CIP1 mediated by STAT1. Science 1996; 272:719–22.
42. Carmichael J. DeGraff WG. Gazdar AF. Minna JD. Mitchell JB. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. Cancer Research 1987; 47:936–42.
43. Yamamoto H. Soh JW. Shirin H. Xing WQ. Lim JT. Yao Y. Slosberg E. Tomita N. Schieren I. Weinstein IB. Comparative effects of overexpression of p27Kip1 and p21Cip1/Waf1 on growth and differentiation in human colon carcinoma cells. Oncogene 1999; 18:103–15.
44. Novick D. Cohen B. Rubinstein M. The human interferon α/β receptor: characterization and molecular cloning. Cell 1994; 77:391–400.
45. Domanski P. Witte M. Kellum M. Rubinstein M. Hackett R. Pitha P. Colamonici OR. Cloning and expression of a long form of the beta subunit of the interferon alpha beta-receptor that is required for signaling. Journal of Biological Chemistry 1995; 270:21606–11.
46. Yamamoto H. Soh JW. Monden T. Klein MG. Zhang LM. Shirin H. Arber N. Tomita N. Schieren I. Stein CA. Weinstein IB. Paradoxical increase in retinoblastoma protein in colorectal carcinomas may protect cells from apoptosis. Clinical Cancer Research 1999; 5:1805–15.

（四）附表及附圖：

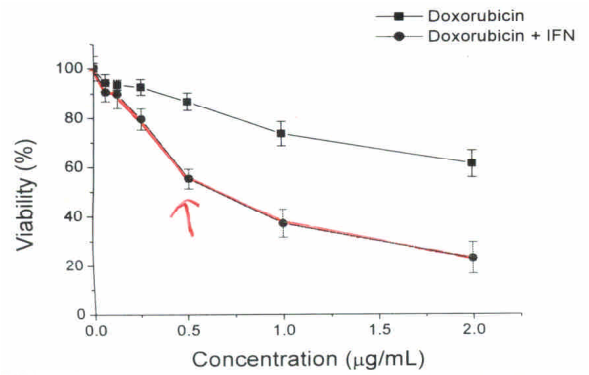

Figure 1. Effects of doxorubicin and doxorubicin combined with IFN on the viability of Hep 3B cells.

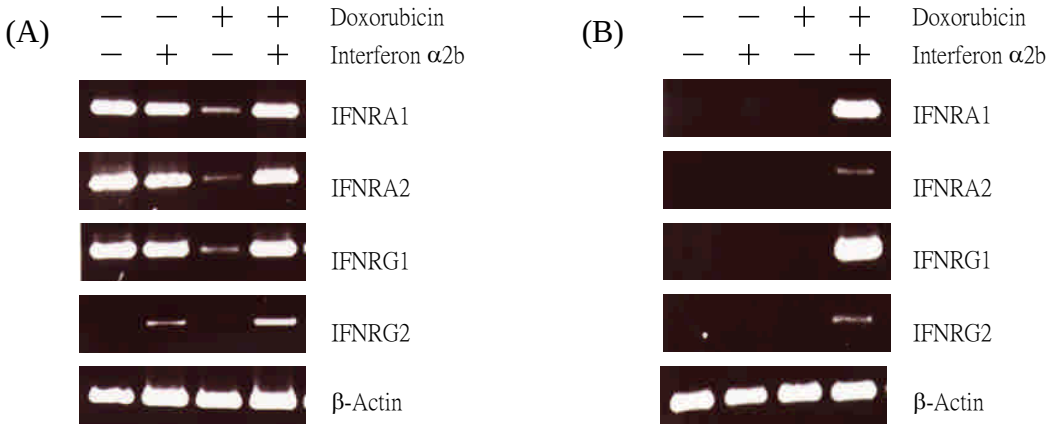

Figure 2. Effects of doxorubicin, interferon, or doxorubicin combined with interferon on the transcription level of interferon receptors in Hep 3B cells. (A) Hep 3B cells treated with doxorubicin, interferon, or doxorubicin combined with interferon for 12 hours. (B) Hep 3B cells treated with doxorubicin, interferon, or doxorubicin combined with interferon for 48 hours. At the indicated time mentioned above, the cells were harvested, and their total RNA was extracted. 5-10 μg of RNA sample of each group was used for reverse transcription. 2μg of RT product was then used for polymerase chain reaction for 25 cycles. Primers for β-Actin gene were included in each PCR reaction to perform as internal control.

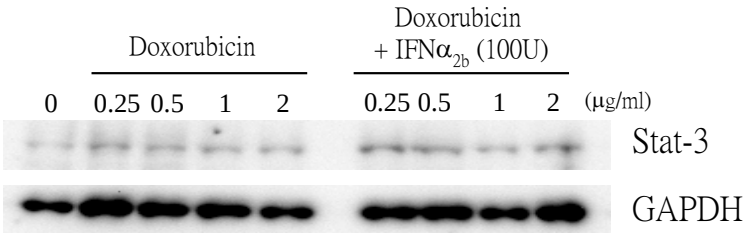

Figure 3. Effects of doxorubicin, interferon, or doxorubicin combined with interferon on the expression of stat-3 protein in Hep 3B cells. The expression of Stat-3 protein in Hep 3B cells was evaluated after 48 hours of drug treatments. Cellular total proteins at 50 μg were loaded. The expression of GAPDH was used as internal control.

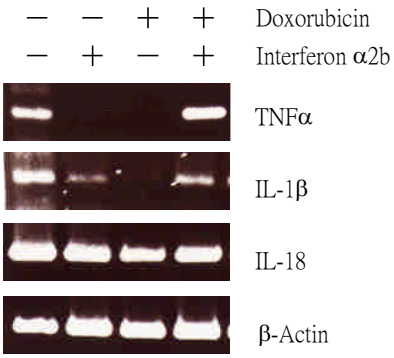

Figure 4. Effects of doxorubicin, interferon, or doxorubicin combined with interferon on the transcription level of TNFα, IL-1β, or IL-18 in Hep 3B cells. Hep 3B cells treated with doxorubicin, interferon, or doxorubicin combined with interferon. 12 hours later, the cells were harvested, and their total RNA was extracted. 5-10 μg of RNA sample of each group was used for reverse transcription. 2μg of RT product was then used for polymerase chain reaction for 25 cycles. Primers for β-Actin gene were included in each PCR reaction to perform as internal control.

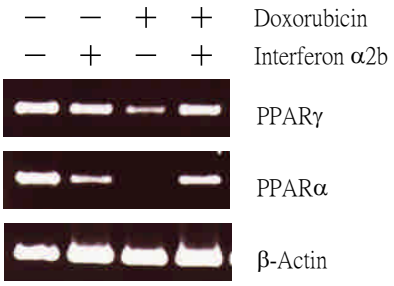

Figure 5. Effects of doxorubicin, interferon, or doxorubicin combined with interferon on the transcription level of PPARα, or PPARγ in Hep 3B cells. Hep 3B cells treated with doxorubicin, interferon, or doxorubicin combined with interferon. 12 hours later, the cells were harvested, and their total RNA was extracted. 5-10 μg of RNA sample of each group was used for reverse transcription. 2μg of RT product was then used for polymerase chain reaction for 25 cycles. Primers for β-Actin gene were included in each PCR reaction to perform as internal control.

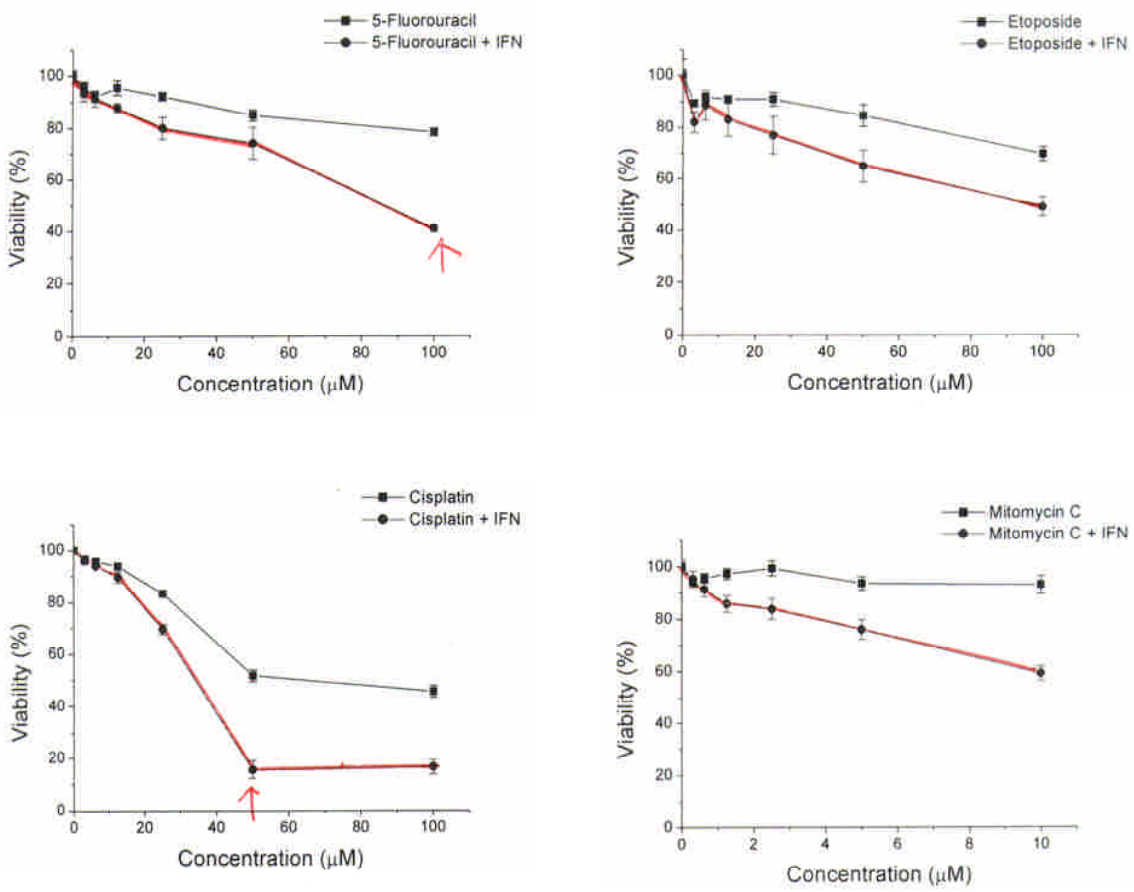

Figure 6. Effects of other chemotherapeutic drugs in combination with or without interferon on the viability of Hep 3B cells. Hep 3B cell treated different kinds of chemotherapeutic drugs combined with or without interferon for 48 hours were used for MTT viability assay.

（五）計畫成果自評：

1. 研究內容與原計畫相符
2. 研究成果達成預期目標且具學術及應用價值。
3. 適合在學術期刊發表。