行政院國家科學委員會專題研究計畫　成果報告

由肝細胞癌差異表現基因之定量分析來進一步了解肝癌致病機轉

計畫類別：個別型計畫
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計畫主持人：許金川

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中　華　民　國 92 年 10 月 30 日

行政院國家科學委員會補助專題研究計畫成果報告

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Insight into hepatocellular carcinogenesis by quantitative analysis of differential expressed genes in hepatocellular carcinoma

計畫類別：■個別型計畫□整合型計畫

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中華民國 92 年 10 月 25 日

（一）研究計劃中文摘要：

關鍵詞：肝細胞癌、即時定量 PCR 系統

肝細胞癌是國人癌症死因的第一位。其發生的主要原因與 B 型肝炎病毒、C 型肝炎病毒的慢性感染有關，其他可能的因素包括食物遭黃麴毒素的污染或遺傳缺陷，然而真正的分子致病機轉目前尚不清楚，有待進一步的研究。

之前我們利用 Incyte Human GEM 1 cDNA 基因晶片（約 9700 基因）來分析肝細胞癌的 mRNA 轉錄輪廓(transcription profile)，比較肝癌病人非腫瘤和腫瘤組織之間差異表現的基因。總共找到 73 個候選基因在肝腫瘤組織表現量上升，58 個候選基因表現量下降。我們將基因晶片結果中找到的差異性表現基因，比對已發表過的 LOH 及 CGH 實驗結果，我們挑選出 3 個基因(11 β-hydroxysteroid dehydrogenase、histidine-rich glycoprotein、CCT-γ chaperonin)作進一步的實驗。首先我們抽取了 44 位肝細胞癌病人的肝癌組織以及其周圍非肝癌組織的 RNA，將 mRNA 反轉錄成 cDNA。為了確認 cDNA 基因晶片實驗結果的正確性，即時定量 PCR（real-time quantitative PCR）系統被用來評估這 3 個基因的 mRNA 差異表現量是否有一致性。實驗結果發現，即時定量 PCR 結果與晶片數據有高度相似性。

另外我們也檢視這 3 個基因在肝癌病人族群表現情形，在 55%（25/44）的 HCC 病人，其周圍非肝癌組織中 HSD11 mRNA 含量大於肝癌組織中兩倍以上。HRG 亦發現在 59%(26/44)病人癌組織中有表現下降情形。而 Cctg 則是有 59%（26/44）的病人，其肝癌組織 RNA 含量是大於周圍非肝癌組織的兩倍以上。即時定量 PCR 系統提供一個大量快速篩檢平台來確認個別基因表現，將可輔助基因晶片技術。我們將更進一步確認這些基因的蛋白表現情形，希望可以對肝癌的致病機轉有更多的了解，進而發現可以當作診斷或是治療標的基因。

（二）研究計劃英文摘要：

Keywords: hepatocellular carcinoma (HCC), real-time quantitative polymerase chain reaction (QPCR)

Hepatocellular carcinoma (HCC) is one of the most common cancer in the world and is the leading cause of cancer death in Taiwan. Chronic hepatitis B and recently the hepatitis C viral infection are thought related to the development of HCC. However, the basic molecular mechanism remained to be clarified.

In 2001, we have analyzed the transcriptional profiles of six HCC specimens using Incyte Human GEM1 cDNA microarray which consists of ~9700 genes. Comparison of expression profiles between HCC tumors and the corresponding nontumor tissues, we identified 73 genes were up-regulated and 58 genes were down-regulated in tumor tissues. The highly concordant overexpressed profile included transcripts involved in the function of respiration control, protein synthesis, degradation, cytoskeleton and carcinogenesis. Down-regulation of genes related to drug metabolism, differentiation and immune response was observed in the majority of the tumors examined.

After comparing the LOH, CGH, and microarray data, we pick 3 differential expression genes for further analysis. First, we extracted 44 pair of HCC RNA from tumor part and corresponding nontumor part. RNA reverse transcribed to cDNA. In order to validate cDNA microarray data, real-time quantitative polymerase chain reaction (QPCR) was used to evaluate 3 gene (11 β-hydroxysteroid dehydrogenase, histidine-rich glycoprotein and chaperonin CCT-γ) expressions. Highly correlation was observed between these two methods. We also screen these 3 gene expression levels in a panel of 44 HCC specimens. 11 β-HSD and HRG were down-regulated in 55% (25/44), and 59% (26/44) HCC cases respectively. CCT-γ was up-regulated in 59% (26/44) HCC cases. The expression profiles of the 6 HCC cases provide valuable information for elucidation of hepatocarcinogenesis, and also represent the potential biomarkers implicated in this malignancy.

（三）成果內容:

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide, mainly in South Africa and Southeast Asia. Major risk factors are chronic hepatitis resulting from infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) and exposure to various carcinogens including alfatoxin B1 (1). However, the molecular mechanisms underlying HCC in most patients remain unclear.

Over the past few years, systematic efforts had been made to approach the genetic abnormality in human cancers mainly in two strategies: one was screening for chromosomal regions with frequent allelic imbalance using loss of heterozygosity (LOH) analysis and comparative genome hybridization (CGH) and the other was gene expression profiling. These techniques have been successfully applied to HCC studies, and the results focus on chromosome 1p, 4q, 5q, 6q, 8p, 8q, 10q, 11p, 13q, 16q, 17p and 22q. More recently, systematic survey of overall transcriptome became feasible because of the completion of the human genome sequence.

cDNA microarray is a newly developing powerful gene expression tool. It could simultaneously and rapidly monitor tens of thousands of mRNA expressions, and get large information about a cell or tissue transcriptional status. Currently, cDNA microarray has been used to classify some heterogeneous cancers into tumor subtypes, and to identify new prognostic markers, for example, diffuse large B-cell lymphoma (DLBCL), breast cancer and prostate cancer. Expression profiling using cDNA microarrays not only provides a measurement of the quantity of each mRNA species but also can be used to analysis of regulatory pathways (2). These studies imply that cDNA microarray is a very powerful tool in investigating the carcinogenesis. Therefore, we used the Human Genome GEM1 cDNA microarray containing 9766 human cDNA elements to determine globally which cellular genes were altered in HCC specimens. Our data show 131 transcripts that were differentially expressed in HCC tumors.

In order to confirm cDNA microarray result, in this study, we use real-time quantitative polymerase chain reaction (RT-QPCR) to determine 3 gene expressions.

Materials and Methods

Real-time Quantitative PCR

RT-QPCR analysis was performed on an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster city, CA, USA). The reverse transcription reaction and PCR was carried out using SYBR Green master mix reagents kit (Applied Biosystems, Foster city, CA, USA). The match primers were designed by Primer Express v2.0 software. The sequences of primers used for RT-PCR as follows: HSD11-F,

5’-GGAATGTGCCCTGGAGATCA-3’, HSD11-R,
5’-ACTTCTTCTTGGCGCAGAGC-3’, Cctg-F,
5’-GGAAGCCAGACTGACATTGAGAT-3’, Cctg-R,
5’-AATTCGGGTGAAGTCCTCCTCT-3’, HisG-F,
5’-TCCGGTCATAGATTTCTTTGA-3’, HisG-R,
5’-GCTTGTTTTCTGTAGCGCTCAGT-3’, Actin-F,
5’-AAGGAGAATGGCCCAGTCCT-3’, Actin-R,
5’-TGCTATCACCTCCCCTGTGTG-3’. The thermal cycle condition was set as 95℃ for 10 min and 45 cycles at 95℃ for 15 sec and 60℃ for 1 min. Initial template concentration was derived from the cycle number at which the fluorescent signal crossed a threshold in the exponential phase of the PCR reaction. Relative gene expression was determined based on the threshold cycles of the gene of interest and of the internal reference gene. Use of a reference gene avoids the need to direct quantify the RNA, which could be a major source of error for analysis. cDNA from two hepatoma cell line, HepG2 and Hep3B, were used as reference samples. Two reference samples were included on each assay plate to verify plate-to-plate consistency. Plates were normalized to each other using these reference samples, if necessary. Contamination of the RNA samples by genomic DNA was excluded by an analysis of all RNA samples without prior cDNA conversion. Quantitative PCRs were performed in duplicate for each sample-primer set, and the mean of the 2 experiments was used as the relative quantification value. The mRNA levels of the interest genes were expressed as the ratio of the interest gene to human β-actin mRNA for each sample. The level of each interest gene mRNA in each cancer was compared to the level in the corresponding nontumor part.

Result

Validation of cDNA microarray result by real time RT-PCR

We used real-time quantitative RT-PCR (RT-QPCR) to verify the differential expression detected by cDNA microarray. To extend our analysis to HCC tumors, 44 pairs of clinical HCC specimens were selected for test. We select 3 genes (11β-hydroxysteroid dehydrogenase, histidine-rich glycoprotein and CCT-γ chaperonin) to determine their relative expression changes in tumor over nontumor cells. Comparison of cDNA microarray and RT-QPCR result was shown in Figure 1. The expression ratios calculated from the microarray data were consistent with RT-QPCR results, although somewhat different in each value of relative expression ratio, and it might due to use of different detection systems and normalization methods. High correlation of cDNA microarray and RT-QPCR data suggests cDNA microarray is a reliable method for RNA profiling.

11β-Hydroxysteroid dehydrogenases (11β-HSDs) are enzymes that catalyse the

interconversion of active glucocorticoids into their inactive 11-keto products. Two isozymes have been identified and established as a crucial mechanism modulating corticosteroid hormone action. Genetic defects or alteration in its activity in each isozyme have been associated with several human diseases, including hypertension, intra-uterine growth retardation and obesity (3). With the ever-increasing study in 11 β -HSD, it has been shown that this enzyme is implicated in cancer. In several neoplastic cells types, there is a high level of expression of 11 β -HSD2 in contrast to normal tissue equivalents, which only synthesize 11 β -HSD1. In our study, quantitative real-time PCR revealed down-regulation of 11 β -HSD1 gene expression in 55% HCC case (Figure 2A). The future work is to determine protein level expression in HCC tissue or serum.

Histidine-rich glycoprotein (HRG) is a relative abundant serum glycoprotein with an unusually high histidine content in the His-Pro rich domain. The physiological role of HRG remains to be elucidated, but it has been proposed to be a modulator of coagulation, fibrinolysis, the immune response, and metal ion transport. In a recent study, HRG has potent antiangiogenic activity mediated through the histidine-proline-rich domain (4). Reduced expression the HRG in HCC may be a parameter of diagnosis, but we need more data to support it.

The TCP-1 ring complex (TRiC; also called CCT, for chaperonin containing TCP-1) is a large (~900 kDa) multisubunit complex that mediates protein folding in the eukaryotic cytosol. The chaperonin encapsulates nonnative proteins in the central cavity and promotes their folding in an ATP-dependent reaction. The substrates of CCT chaperonin include actin, tubulin and several noncytoskeletal proteins. The CCT is essential for Cdc20-dependent cell cycle events such as sister chromatid separation and exit from mitosis. According to a recent study, the CCT chaperonin promotes activation of the anaphase-promoting complex through the generation of functional Cdc20 (5). Up-regulation of CCT chaperonin in 59% HCC cases (Figure 2C) might implicate this molecule in carcinogenesis.

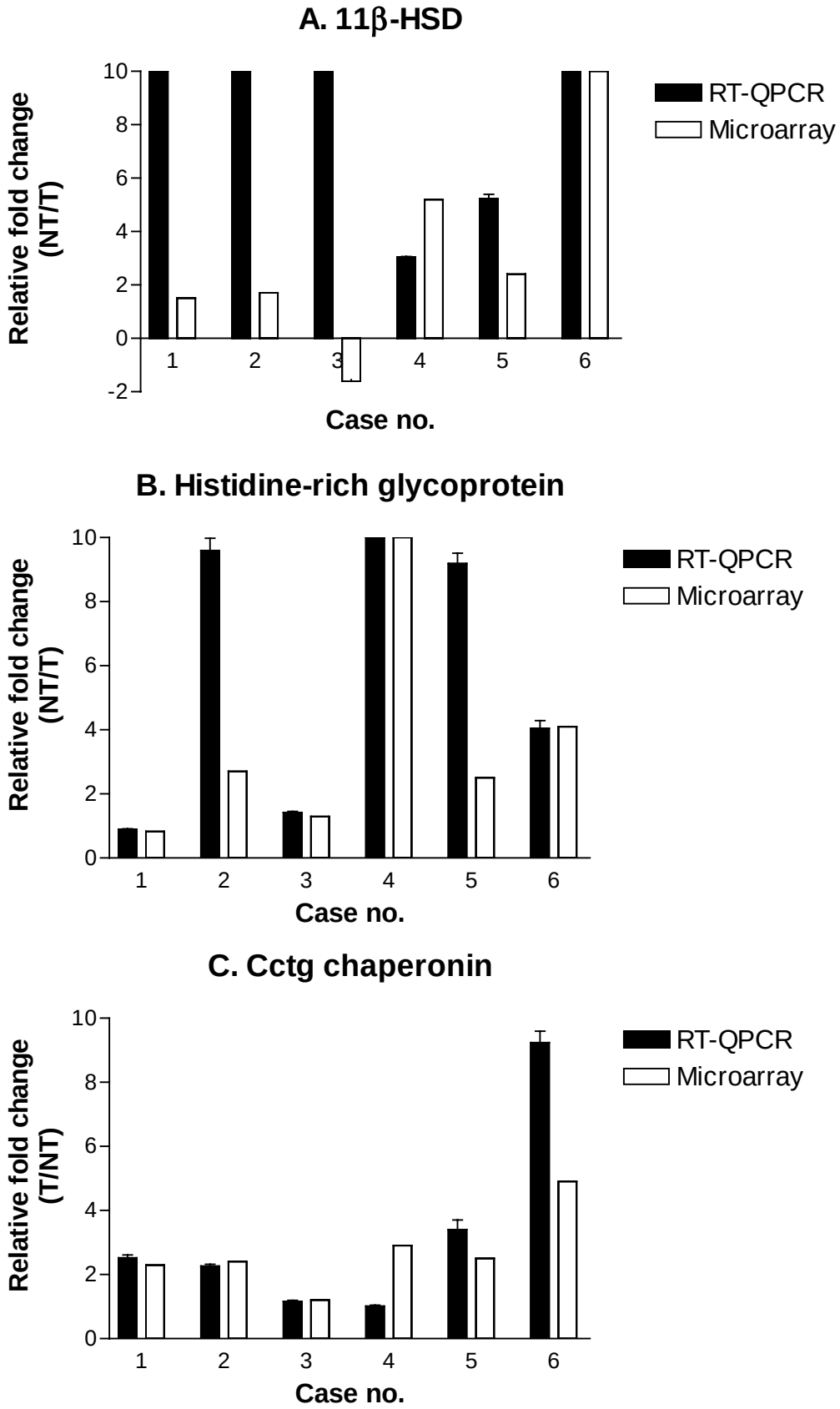

Figure 1 Real-time quantitative PCR and microarray expression analysis of 3 selected genes differentially expressed between HCCs and their nontumor counterparts.

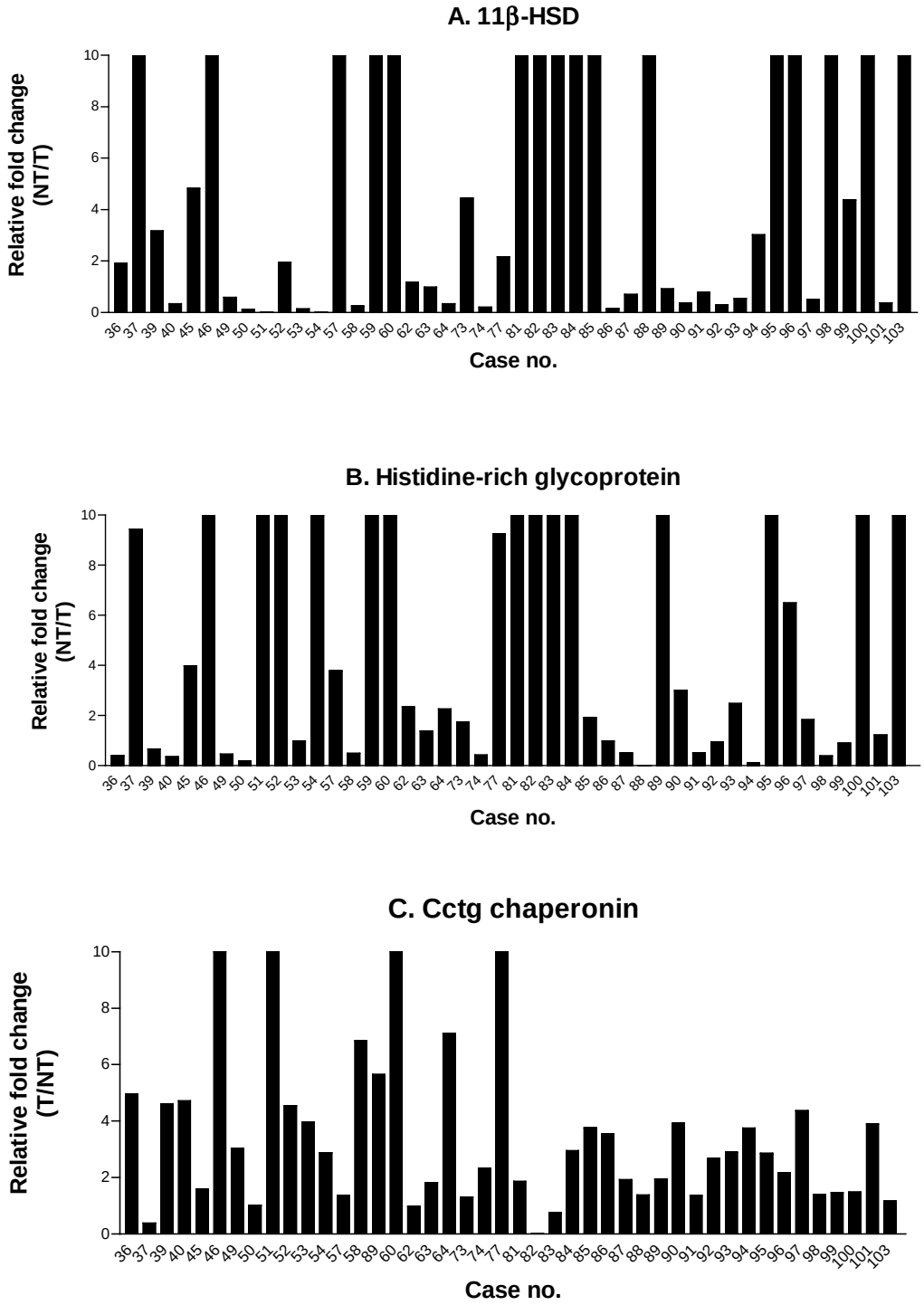

Figure 2 Real-time quantitative PCR analysis of 3 selected gene expressions in 44 pairs of HCC tissues. (A) 11 β -HSD (B) Histidine-rich glycoprotein (C) CCT chaperonin. Down-regulation (2-fold reduced expression) of 11 β -HSD and HRG genes were found in 55% (25/44), and 59% (26/44) HCC cases respectively. Up-regulation (2-fold over-expression) of Cctg chaperonin was found in 59% (26/44) HCC cases.

（四）參考文獻

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（五）計劃成果自評

In this study, we have shown using of real-time quantitative PCR (QPCR) to validate candidate target gene expressions. Due to the advantages of high throughput and sensitivity, it is suitable to apply this technology in post-microarray screening experiments. Because measurements of mRNA do not correlate the protein quantities well, we strongly recommend combination of QPCR with other protein expression analysis to verify target expressions and localizations.