

行政院國家科學委員會專題研究計畫 成果報告

B 型肝炎病毒之自然 preS2 蛋白突變種在肝癌發生過程中之 重要性

計畫類別：個別型計畫

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計畫主持人：粟發滿

計畫參與人員：梁有志、鄭靜茹

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行政院國家科學委員會補助專題研究計畫 ☐ 成果報告
☐ 期中進度報告

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成果報告類型(依經費核定清單規定繳交)：☒ 精簡報告 ☐ 完整報告

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執行單位：臺北醫學大學

中 華 民 國 94 年 10 月 30 日

Abstract:

Hepatitis B virus (HBV) is the causative agent of acute and chronic hepatitis B. Individual with chronic HBV infection has a greater risk of progressing into cirrhosis and hepatocellular carcinoma (HCC). Epidemiological study showed that the risk of developing HCC in HBV chronic carriers is approximately 200 folds higher than that in normal population. Indeed, HBV vaccine has been proven to be significantly reduces the incidence of HCC in children. Thus, HBV is one of the few viruses that is actually involved in human cancers.

Although HBV is a DNA virus, it replicates by reverse transcription of an RNA pregenome. Due to the lack of proofreading activity of its reverse transcriptase, HBV polymerase tends to generate more mutations than other DNA polymerases. The surface protein (envelope) gene of HBV is subdivided into preS1, preS2, and S regions, which encode three different envelope proteins. Naturally occurring deletion mutation within the preS2 region has frequently been identified in patients with HCC.

We have characterized the preS2 deletion variants identified from two HCC patients, demonstrating that preS2 deletion variants were replication and virion secretion competence. Moreover, we also observed the accumulation of surface protein of HBV within the cells for preS2 deletion variants. Other studies have reported that the hepatocytes harboring the preS2 internal deletion mutant showed a clonal proliferation and growth advantage of hepatocytes. Endoplasmic reticulum (ER) loaded with an excessive quantity of envelope proteins could result in the “ground glass” morphology and physiological stress. The ER stress can activate several cellular transcriptional pathways, which in turn induce a high level of cell proliferation. These data thereby support indirectly the hypothesis that preS2 deletion protein could be able to stimulate proliferation of cells, and preS2 deletion protein may be a potential oncoprotein which could alter cell cycle through an ER stress signal cascade pathway, which lead to an excessive proliferation of cells, program the cells for unrestrained growth and eventually lead to malignant transformation.

To understand the effect of HBV preS2 protein deletion mutant on the cell cycle progression, we transfected both HBV preS2 wild-type and deletion variant tandem dimer replicons into hepatoma cells. Flow cytometry experiments indicated that preS2 wild-type and deletion proteins did not change the cell cycle profile in both Huh-7 and HepG2 cells. Trypan blue dye exclusion assay showed that no any toxicity and no significant different in cell number between mock and various preS2 variants expression cells. However, we found that preS2 wild-type and deletion proteins could induce cyclooxygenase-2 expression and CTGF secretion in different degree. During the experimental periods, we detected low expression level of preS2 wild-type and deletion proteins by transfection of original expression vector, which had SV40 promoter. Therefore, we re-cloned preS2 wild-type and deletion gene into pIND(SP1)/V5-His vector, and successfully obtained the pIND(SP1)/V5-His-based constructs. On going experiments, we use the stable and overexpression cell lines by transfection of various pIND(SP1)/V5-His-preS2 constructs, and investigate the relationships between preS2 variant

and liver fibrosis.

Key words: Hepatitis B virus, Surface protein, preS2 deletion variants, Hepatocellular carcinoma.

摘要:

慢性 B 型肝炎病毒(HBV) 帶原者為肝硬化及肝癌的高危險群。HBV 的繁殖過程中必須利用反轉錄完成病毒 DNA 之複製。因 HBV 的反轉錄酶缺乏校對活性，所以在 HBV 複製過程中，常產生突變現象。HBV 表面蛋白基因可分為 preS1，preS2 及 S 區域，可轉譯出三種不同的表面蛋白。而 preS2 自然突變種常出現於肝癌病患。

在細胞培養實驗中，我們對兩名肝癌病患的 preS2 突變種作研究，發現這突變種不但能複製繁殖，也能分泌病毒顆粒。這突變種能使表面蛋白累積於肝細胞內。其他研究也顯示，若 preS2 突變種存在於肝細胞時，肝細胞會出現同源增殖 (clonal proliferation) 及生長優勢。因 Endoplasmic reticulum, ER 累積過量的表面蛋白時，常導致“毛玻璃(ground glass)”現象及生理上的壓力，這些 ER 壓力能活化某些細胞性轉錄路徑，從而引發細胞增生。這些結果間接顯示 preS2 突變種可能會刺激細胞增殖，同時，preS2 突變蛋白可能是一種致癌蛋白，因它可借由 ER 壓力訊號途徑，導致細胞過度及不正常增殖，最後導致惡性變化。

為了探討 preS2 突變種對細胞週期的影響，我們將 HBV 野生種或 preS2 突變種的 DNA 轉殖至肝癌細胞中。流式細胞儀分析顯示 preS2 野生型及突變型蛋白，不會改變 Hun-7 及 HepG2 細胞週期的分佈。Trypan blue 染料排除試驗顯示 preS2 野生型及突變型蛋白，沒有任何細胞毒性，也不會改變細胞的生長速度。但我們卻發現 preS2 野生型及突變型蛋白會引起不同程度的 COX-2 表現及增加 CTGF 的分泌。實驗期間，利用原始得到的質體進行基因轉殖，但在轉殖細胞內只偵測到非常低量的 preS2 蛋白。因此，我們採用 pIND(SP1)/V5-His 載體，重新構築可調控表現 preS2 野生型及突變型的質體，並且已獲得完整的質體。目前進行中的實驗，我們已經使用穩定過渡表現 preS2 的細胞株，來研究各種 preS2 蛋白與肝硬化的關連性。

關鍵詞：B 型肝炎病毒，表面蛋白，preS2 突變種，肝癌。

INTRODUCTION

Hepatitis B virus (HBV) infection is associated with both acute and chronic liver disease, with the latter often resulting in cirrhosis and hepatocellular carcinoma (HCC).¹ Since the introduction of a nationwide mass-vaccination program against HBV in our country since 1984, the seropositivity rate of hepatitis B surface antigen in children decreased from 9.8% in 1984 to 0.7% in 1999.² However, Taiwan remains an endemic area of HBV infection, and it is estimated that 15%-20% of the total adult population in Taiwan are hepatitis B surface antigen (HBsAg) carriers. A prospective epidemiological study conducted in Taiwan has demonstrated a close relationship between chronic HBV infection and the development of HCC.³ In that study, the risk of developing HCC in HBV chronic carriers is approximately 200 folds higher than that in HBsAg control population. Indeed, HBV vaccine has been proven to be the first successful human cancer vaccine which significantly reduces the incidence of HCC in children.⁴ Thus, HBV is one of the few viruses that is actually involved in human cancers.

HBV is an enveloped, partially double-stranded DNA virus with a genome size of 3.2 kb. Although HBV is a DNA virus, it replicates by reverse transcription of a RNA pregenome.⁵ Because of the lack of proofreading function of its reverse transcriptase, HBV has a low fidelity in replication, and tends to produce sequence variants at a high frequency. Therefore, HBV generally carries more mutations than conventional DNA viruses. Based on sequence analysis of HBV DNA from a chronic carrier, the mutation rate of HBV is estimated to be $1.4\text{-}3.2 \times 10^{-5}$ per nucleotide per year.⁶ This number is about 100-fold higher than that of other DNA viruses.⁷ It is then not surprising to find many naturally occurring mutations in the persistent HBV genome from chronically infected patients. Indeed, we have identified and characterized several mutations in the core (capsid) and surface (envelope) proteins of HBV.⁸

The surface protein (envelope) gene of HBV is subdivided into preS1, preS2, and S regions, which encode three different envelope proteins. It is because the S opening reading frame (S-ORF) contains three in-frame translational initiation codons, which divide the S-ORF into three regions: preS1, preS2 and S.⁹ The smallest surface protein, termed HBsAg, consists of 226 amino acids encoded only by the S gene. The middle surface protein of 281 amino acids is encoded by the preS2 region and the S gene. In the large surface protein, the preS1 and pre S2 regions in conjunction with the small S gene, produce the 400 or 389 amino acid-preS1 protein, depending on HBV subtypes. All these three surface proteins are synthesized in the rough endoplasmic reticulum (ER) and insert into the membrane cotranslationally as integral membrane proteins.¹⁰ HBV virions and subviral particles are formed at the ER or the pre-Golgi compartment and secreted by the constitutive secretory pathway.¹¹

Naturally occurring deletion mutations of HBV pre-S1 and pre-S2 genes have been found in HBV carriers.¹²⁻¹⁷ The prevalent rate of HBV carriers harboring preS2 deletion mutants ranges from 12% to 83%. This discrepancy of the prevalence rates among these pre-S mutation results was reported to be correlated with different replicative stages of chronic HBV infection.¹⁸ The preS2 deletion mutant appeared to

prevail at low or nonreplicative phase of chronic HBV infection. Indeed, we found 60% of chronically infected patients with HCC contain preS2 deletions, while chronic carriers without cirrhosis and HCC contain no detectable preS2 deletion variants.¹⁹

The preS2 region contains 55 amino acids from codon 120 to 174 (the numbering starts from the preS1 region). Deletion is a frequent event in the N-terminal half of the preS2 region. These deletions were mainly clustered within amino acids 127 and 141 and resulted in the loss of 9-15 amino acids. We have demonstrated that these preS2 deletion variants are replication and secretion competent. Besides, these preS2 deletions coincide with several mapped B and T cell epitopes. We propose that these naturally occurring HBV preS2 deletion variants can escape from host immune surveillance and persist in chronic carriers via loss of immunodominant epitopes.¹⁹

The mechanisms responsible for malignant transformation in HBV infection are not well understood. The exceptionally long carriage of HBV in chronically infected patients suggests that there is a complicated interaction between virus and host. Therefore, viral factors cannot be omitted when trying to understand HBV persistence and carcinogenesis of hepatic tumor. Several mechanisms of HBV-related hepatocarcinogenesis have been proposed in the past years. For example, since the HBV is frequently integrated into host genomes in the advanced stage of chronic HBV infection, the integrated rearranged HBV DNA sequences could directly activate cellular proto-oncogenes.²⁰ On the other hand, X protein and 3'truncated middle surface (preS/S) protein of HBV bears transactivational potential.^{21,22} The integrated HBV X and 3'truncated preS/S sequences can encode functionally active transactivators, indicating that these proteins may play another important mechanism for HBV-related hepatocarcinogenesis.

The other potential candidate will be the surface protein-mediated pathway. The overexpression of surface protein in transgenic mice may lead to injury of hepatocytes, which is followed sequentially by hepatocellular regeneration, dysplasia, adenoma, and eventually hepatocellular carcinoma.²³ In our previous study, we observed a 2-fold decrease in the secretion of all three surface proteins for preS2 deletion variants. Furthermore, these three envelope proteins of preS2 variants were accumulated within the cells.¹⁹ Since surface proteins are targeted to the ER as a transmembrane protein, it was proposed that endoplasmic reticulum loaded with an excessive quantity of envelope proteins could result in the "ground glass" morphology and physiological stress.²⁴ The ER stress can activate several cellular transcriptional pathways, and this ER stress signal cascade pathway has been shown to alter cell cycle by sequential activation of cyclin-dependent kinases. Moreover, translational regulation of cyclin-dependent kinase activities in response to ER stress is a mechanism of checkpoint control that alters cell cycle progression.^{25,26} Therefore, it appears that preS2 deletion variants may potentially play a role in the pathogenesis of HBV-related HCC through an ER stress signal cascade pathway, which lead to an excessive proliferation of cells, program the cells for unrestrained growth and eventually lead to malignant transformation.

In addition, Fan et al reported that the hepatocytes harboring the preS2 internal deletion

mutant are consistently clustered into groups and are of clonal and integrated expansion, indicating a clonal proliferation of hepatocytes and a growth advantage of hepatocytes expressing pre S2 deletion protein.²⁷ Cell proliferation is important in the fundamental steps of carcinogenesis. When a high level of cell proliferation is coupled with spontaneous DNA damage, the risk of cancer development is greatly increased. Taking together, in addition to the immune escape advantage, we hypothesize that the preS2 deletion protein could be able to stimulate proliferation of cells, and the preS2 deletion protein may be a potential oncoprotein which could alter cell cycle progression via ER stress signal cascade pathway, which lead to an excessive and uncontrolled proliferation of cells.

MATERIALS AND METHODS

Cell Culture

The human hepatoma cell lines, Huh-7 and HepG2, were used for DNA transfection. Cells were cultured in Modified Eagle's medium (MEM) with 10% fetal bovine serum at 37°C in the presence of 5% CO₂.

Plasmid Constructs: HBV wild-type and preS2 deletion protein expression vectors - pECEM (the wild type preS2 protein construct), pECEΔ32, and pECEΔ79 (preS2 deletion protein construct)

The HBV preS2 surface protein expression vector (P30) was kindly provided by Dr. Jame H Ou, USA. P30 was used as a backbone to construct pECEM, pECEΔ32, and pECEΔ79. Briefly, the DNA fragments containing the preS2/S gene from nt 3213 to 1840 was amplified by PCR using liver tumor sample and the wild type HBV as templates. The 1.8-kb HBV fragment containing the preS2/S from either wild-type or preS2 deletion mutant was used to replace the counterpart in P30.

Plasmids and Transfection

The pIND(SP1)/V5-His-preS2 expression plasmid contain wild-type or deletion preS2 gene fragment, was amplified from above pECE, pECE Δ32, or pECE Δ79 vector using the primer 5'-ACTAGCTAGCGCCATGCAGTGGAATTCAC-3' (sense) and 5'-ATTACTCGAGAATGTATACCCAAAGAC-3' (anti-sense). The PCR products were inserted to the pIND(SP1)/V5-His vector (Invitrogen) that had been cut with Nhe I/Xho I. Such that the resulting PCR products contained the Nhe I/Xho I sites, and was ligated in frame into the unique Nhe I/Xho I sites present within the pIND(SP1)/V5-His plasmid. Therefore, we obtained various pIND(SP1)/V5-His-preS2 constructs containing preS2 wild-type and deletion gene. Sequences were confirmed identity by ABI PRISM 377 DNA Analysis System (Perkin-Elmer Corp., Taiwan Branch).

DNA Transfection

The Lipofetamine2000 was used for introducing DNA into cells according to vendor's suggestions (Gibco). Briefly, for each transfection, 3 ug of DNA was mixed with 6 ul of Lipofetamine2000 reagent diluted in 200 ul of DMEM and incubated for 25 min at room temperature. The DNA-Lipofetamine2000 mixture will be then added directly to the cells.

Western Blot

Equal amounts of total cellular protein (50 µg) or conditional medium (25 µl) were resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE), transferred onto Immobilon-P membrane (Millipore, Bedford, MA), and blotted with anti-COX-2, anti- α -tubulin, or anti-CTGF (Santa Cruz Biotechnology, Santa Cruz, CA) antiserum as described previously²⁹.

Flow Cytometry

Cell cycle profile was analyzed by flow cytometry as described previously²⁹. Briefly, cells were trypsinized, washed once with PBS, and fixed in 75% ethanol for 1 h at -20°C. Fixed cells were then washed with PBS, incubated with 0.5 ml PBS containing 0.05% RNase and 0.5% Triton X-100 for 30 min at 37°C, and stained with propidium iodide. The stained cells were analyzed using a FACscan laser flow cytometer and CellFIT DNA analysis software (Becton Dickinson, San Jose, CA).

RESULTS

Effects of preS2 variants on the growth and cell cycle progression of hepatoma cells

Both Huh-7 and HepG2 cells were transfected with various preS2 constructs for 3 days, and the DNA content of cells nuclei was measured by flow cytometry. As shown in Table 1, Huh-7 cells were transfected with mock and various preS2 constructs resulted in a same proportion of cells in G1 phase (~50%), S phase (~15 %), and G2/M phase (~33%). Trypan blue dye exclusion assay indicated a little cytotoxicity under transfection with mock and various preS2 constructs (>85% viability after 48 h) compared to untransfected cells (control), but no significant different in transfected cells (Fig. 1). Moreover, there was no significant different in cell growth between mock and various preS2 expression cells. These results suggest that preS2 wild-type and deletion proteins exhibited no cytotoxicity and did not change cell cycle profile in hepatocyte.

Effects of preS2 variants on the protein expression of cyclooxygenase-2 and CTGF secretion in hepatoma cells

Both Huh-7 and HepG2 cells were transfected with various preS2 constructs for 2 days, and

the total cell lysate and conditional medium were detected the COX-2 and CTGF level, respectively, by Western blot. As shown in Fig. 2A, Huh-7 cells transfected with wild-type preS2 construct and resulted in a higher level of COX-2 and CTGF than other transfected cells. Wild-type and deletion preS2 proteins could induce same level of COX-2 expression in HepG2 cells (Fig. 2B). These results suggest that wild-type and deletion preS2 proteins might induce inflammation and fibrosis in hepatocyte.

DISCUSSION

In this study, we found that the expression of preS2 mutant protein in both Huh7 and HepG2 cells did not change the cell cycle profile in hepatocytes. However, it is intriguing to find that transfection of HBV replicons or surface protein expression vectors into the hepatoma cells can induce the expression of COX-2 and CTGF expression. It is well known that CTGF can induce collagen expression in fibroblast and might contribute to fibrosis. It implies that HBV surface protein may contribute to the fibrogenesis of liver during the chronic carriage stage of HBV.

The other important result in this study is that transfection of either the wild type or the mutant protein into the hepatoma cells could increase the level of COX-2 and GEGF. In our plasmid constructs, the wild-type plasmids can express all surface proteins, including PreS1, PreS2 and HBsAg; however, in the context of mutant plasmids, only PreS1 and HBsAg are expressed. Therefore, our finding suggests that PreS1 and/or HBsAg proteins, rather than the PreS2 protien, are the main factor to influence the expression of COX-2 and CGGF.

COX-2, a key enzyme in arachidonic acid metabolism, is overexpressed in the hepatic malignant tumors and it plays a role in inflammation-mediated hepatocarcinogenesis.^{30, 31} Establishment of fibrosis and cirrhosis of liver may be a prerequisite for further development of HCC. The results in this study provide evidence that, in addition to the ER stress signal pathway, HBV surface protein may also contribute to the fibrogensis and inflammation-mediated carcinogenesis of liver. Thus, the role of HBV surface protien in the hepaocarcinogenesis and the mechanisms which COX-2 involves in the development of HCC warrant further investigation.

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Table 1 Effect of preS2 wild-type and deletion proteins on the cell cycle progression in Huh-7 cells.

	Control	Mock	pECEwt	pECEΔ32	PECEΔ79
G1 phase	51.3%	52.1%	53.8%	51.4%	52.0%
S phase	18.3%	15.3%	14.3%	15.1%	13.9%
G2/M phase	26.4%	32.6%	31.9%	33.5%	34.1%

Table 2 Effect of preS2 wild-type and deletion proteins on the cell cycle progression in HepG2 cells.

	Control	Mock	pECEwt	pECEΔ32	pECEΔ79
G1 phase	45.7%	44.5%	42.1%	42.3%	40.3%
S phase	22.8%	25.1%	24.7%	25.9%	23.3%
G2/M phase	31.6%	30.4%	33.2%	31.8%	36.4%

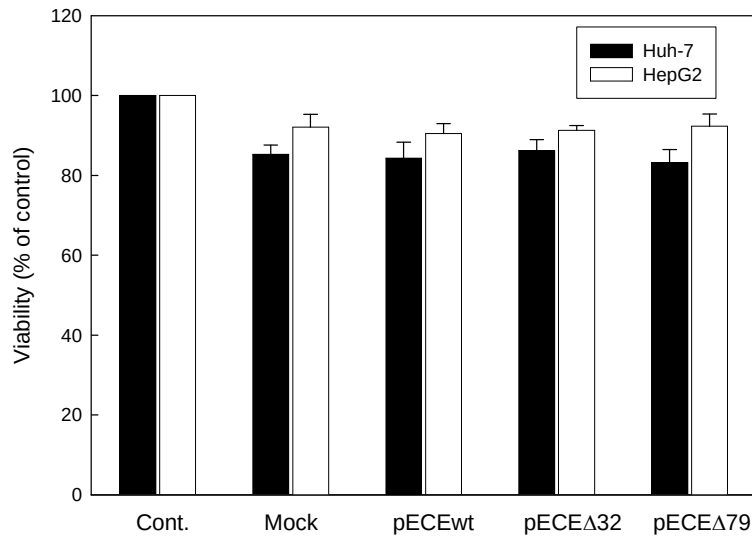
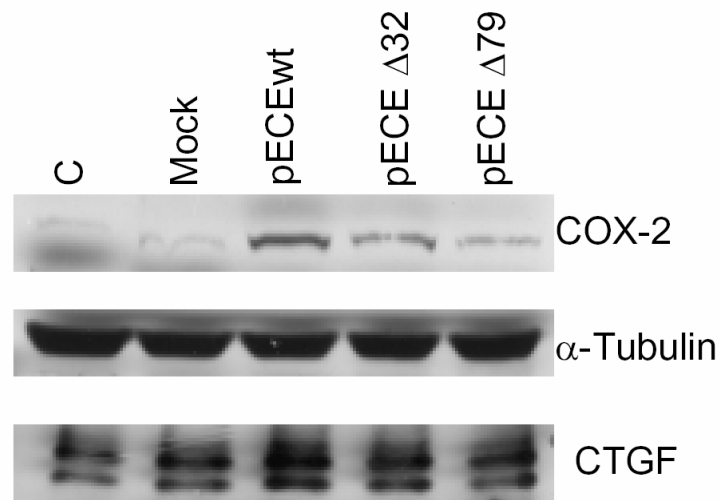


Fig. 1 Effect of preS2 wild-type and deletion proteins on the cell viability in Huh-7 and HepG2 cells. Cells were transfected with mock, preS2 wild-type, or deletion plasmid for 3 day, and determined cell viability by trypan blue dye exclusion assay. Data were presented as the mean $\pm$ S.E. of triplicate tests.

A.



B.

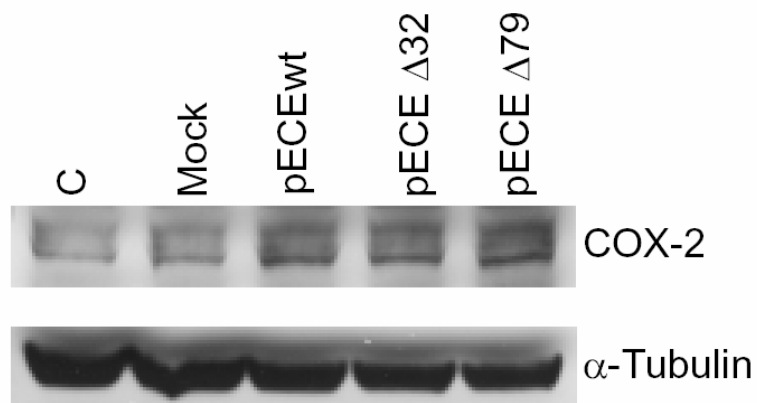


Fig. 2 Effect of preS2 wild-type and deletion proteins on the protein expression of COX-2 and CTGF in Huh-7 cells (A) and HepG2 cells (B). Cells were transfected with mock, preS2 wild-type, or deletion plasmid for 2 day, and determined the protein expression by Western blot as described in “Materials and Methods”.