計畫編號：NHRI-EX92-9116BP-2

國家衛生研究院整合性醫藥衛生科技研究計畫

B型肝炎 pre-S 突變蛋白在肝癌發生的角色 (Prot.3)

計畫名稱

九十二年度成果報告

執行機構：國立成功大學

計畫主持人：黃溫雅

本年度執行期間：92年1月1日至92年12月31日

＊＊本研究報告僅供參考用，不代表本院意見＊＊
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計畫主持人：黃溫雅
研究人員：黃溫雅
關鍵字：B 型肝炎病毒，肝癌，pre-S 突變蛋白，DNA 修復

壹、九十二年度計畫研究成果摘要

中文摘要

本研究為"B 型肝炎 pre-S 突變蛋白在肝癌發生的角色"兩年群體計畫下的第三子計畫：pre-S2 mutant HBS 突變蛋白所導致的基因體不穩定性。本計畫在 2003 年以個人計畫執行。本年度計畫研究成果摘要如下：

一：pre-S B 型肝炎表面突變蛋白所引起的基因突變機率
我們利用人類肝癌細胞 Huh-7 穩定性轉染誘發表現的 HBS 基因 (inducible HBS)來研究 pre-S B 型肝炎表面突變蛋白誘發氧化壓力而的導致 DNA 損傷。此外，老鼠肝癌細胞 ML-1 持續性的表現不同種形式的 HBS 基因也被應用在實驗中。利用 DCFH-DA 染色和流式細胞分析儀來研究在 Huh-7 細胞中，pre-S B 型肝炎表面突變蛋白誘發的氧化性物質。利用彗星分析以及 DNA 修復酶 FPG 來偵測表現 HBS 的 Huh-7 細胞中氧化性鹼基 8-hydroxyguanine 的量。我們的結果清楚的證實 pre-S2 B 型肝炎表面突變蛋白會誘發較高的氧化壓力，且 pre-S1 型所誘發的氧化力較 pre-S2 型高；但是 pre-S2 型卻導致較嚴
重的 DNA 氧化性損傷。因此 pre-S2 HBS 助長肝癌的形成是藉由內質網壓力以外的其他機制所造成。

為了研究是否 pre-S mutant 的表現會影響整體的基因突變機率，我們發展了一套方法去偵測 hppt 基因的突變，我們的結果顯示出 pre-S mutant HBS 的存在會增加基因的突變進而導致基因體的不穩定。

二：pre-S2 B 型肝炎表面突變蛋白對 DNA 修復的影響

此實驗我們利用表現不同種類 HBS 基因的 Huh-7 細胞和 ML1 細胞來進行。

實驗結果表示在表現 pre-S mutant B 型肝炎表面突變蛋白的細胞中，ogg1 的表現量受刺激而增加。ogg1 為修復氧化性 DNA 損傷的重要 DNA 修復酶。Host cell reactivation assay 也證實鹼基修復路徑（BER）受活化。這些結果表示 pre-S mutant 的表現會刺激修復氧化性 DNA 損傷，進而刺激 DNA 修復路徑（BER）的活化，而且 pre-S2 型所誘發的 DNA 修復更勝於 pre-S1 型。實驗中偵測多種的 DNA 損傷反應性基因包含 PARP-1 和 p53。PARP-1 參與 BER 和細胞凋零，我們發現在表現 pre-S B 型肝炎表面突變蛋白的 Huh-7 細胞及 ML1 細胞中 PARP-1 的表現量有大幅度的上升，pre-S2 型也如預期有較高的表現量。

除了 in vitro 的細胞實驗外，我們也利用轉殖基因老鼠的肝臟組織來進行實驗。利用免疫組織化學染色法探測 p53 突變性蛋白的存在，初步的結果顯示表現 pre-S2 突變型 HBS 的轉殖基因老鼠的肝臟組織中含有 p53 突變性蛋白。

此外，我們也以雷射捕獲顯微技術(LCM) 分離六位 HCC 病人的肝臟組織，RT-PCR 的結果顯示出表現 pre-S B 型肝炎表面突變蛋白的細胞中ogg1 的
表現量上升，尤其是表現 pre-S₂ 突變型 HBS 的細胞 oggl 的表現量更是大幅度的上升。此結果意味著在慢性 B 型肝炎感染中，表現 pre-S₂ 突變型 HBS 的肝臟細胞存有較高量的氧化壓力。這些以雷射捕獲顯微技術分離出表現不同突變形式 HBS 的肝臟細胞並分析 p53 突變的情形，我們的結果顯示有一半以上的患者在 p53 密碼子 (exon) 5-8 的位置有突變發生，這表示 pre-S B 型肝炎表面突變蛋白與腫瘤抑制基因 p53 的突變有關，未來將利用這些雷射捕獲顯微技術分離出的肝臟細胞偵測 Rb 和 BRCA1 基因的表現量。

整體而言，我們大致上已完成特定的實驗目標，我們的結果提供了一模式連接了 pre-S₂ B 型肝炎表面突變蛋白與 HCC 的相關性。
English Abstract

This study is the second-year project for the component project 3 of the previous PPG "The role of pre-S mutant in hepatocarcinogenesis". This component project was entitled "Genomic instabilities induced by the pre-S₂ mutant HBS Ag". In the year of 2003, this project runs as an IRG. The research results are summarized here:

1: DNA mutation rates induced by the pre-S₂ HBS mutant antigen

The DNA damages conferred by the oxidative stress, induced by the pre-S HBsAgs, have been studied in the human Huh-7 cells that were stably transfected with the inducible HBS genes. Also, the mouse hepatoma IL-1 cells, in which various types of HBS genes were constitutively expressed, were applied to this study. Using the Huh-7 cells, the ROS induced by the pre-S HBsAgs was assayed by DCFH-DA staining and flow cytometry analysis. The comet assays, coupled with the use of the FPG DNA repair enzyme, were performed to detect the amounts of 8-hydroxyguanine DNA lesions in the Huh-7 cells over-expressing the pre-S HBS genes. Our data have clearly shown that the pre-S HBsAgs induce significantly higher oxidative stresses than the wild-type HBS protein does. The pre-S₁ type exhibited stronger oxidative stress than the pre-S₂ type did. On the contrary, the pre-S₂ type resulted in higher level of oxidative damages. This indicates that the pre-S₂ HBS contributes to hepato-carcinogenesis through the mechanism(s) beyond ER stress.

To assay the global gene mutation rates caused by pre-S expression, we also developed an experimental system to assay gene mutation rates in the hp rt gene. Our results have shown that the pre-S mutant HBS proteins enhance gene
mutations and result in genomic instability.

2: DNA repair activities induced by the pre-S2 HBS mutant antigens
This study was conducted in both of the human Huh-7 and the mouse hepatoma ML-1 cells, consisting of the various types of HBS gene. Results in this study indicate that the pre-S expressions induce the expression of the \textit{ogg1}, an important DNA repair enzyme for oxidative DNA damages. The enhanced BER activities in these cells were also demonstrated by the results of the host cell reactivation (HCR) assays. These results have found that the pre-S expression stimulates BER efficiency for the oxidative DNA damage repair. And the pre-S$_2$ exhibits a higher stimulatory effects than the pre-S$_1$ type does.

Various DNA damage response genes, including the PARP-1 and p53, have been tested. In the Huh-7 cells stably transfected with the inducible HBS genes, the PARP1 gene expression was examined. The PARP-1 is involved in base excision repair as well as apoptosis. We found that the PARP1 was greatly induced in both of the human Huh-7 and the mouse ML-1 cells expressing the pre-S HBS antigens. As expected, the pre-S$_2$ type exhibited the highest levels among the HBS genes tested.

In addition to the \textit{in vitro} cell lines used, the animal liver tissues have been employed to this study. The immunohistochemistry studies, using the specific antibody against the mutant p53 protein, have been initiated. The preliminary results show that the pre-S$_2$ transgenic mice exhibit the p53 mutations in the cells expressing the pre-S$_2$ HBS.

The liver tissues from HCC patients were isolated by laser capture microscopy. Six patient cases have been analyzed. 3 to 4 LCM sections each expressing a
specific type of HBS antigen was isolated from each patient. By RT-PCR we have found that the DNA repair ogg1 gene was induced in the pre-S HBS-expressing cells. The pre-S2 cells exhibited highest level of ogg1, as compared with the wild-type HBS or the pre-S1 cells did. These data suggested that the pre-S2 HBS antigen causes strong oxidative stress in human hepatocytes with chronic HBV infection.

The LCM tissues with specific expression patterns of the HBS antigen have been analyzed for the p53 mutations. Our data have identified that more than half of the cases exhibited gene mutations in p53 exons 5 to 8. This has shown that the pre-S mutations are associated with the mutations in the p53 tumor suppressor gene. The tests on the Rb and BRCA1 gene expressions will be carried out in these LCM tissues.

In summary, we have accomplished most of the specific aims proposed. The genomic instabilities caused by the pre-S2 mutant HBS antigen have been well characterized. Our research results offer a model for the association of the pre-S2 mutant HBS antigen with the HBV-related HCC. The research results have been submitted for publication (see Appendix for manuscript).
### 貳、九十二年度計畫著作一覽表

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〔註〕

期刊論文：指在學術性期刊上刊登之論文，其本文部份一般包含引言、方法、結果、及討論，並且一定有參考文獻部分。未在學術性期刊上刊登之文章（研究報告等）與博士或碩士論文，則不包括在內。

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肆、九十二年度計畫重要研究成果

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內容必須包括：

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(2) pre-S : B 型肝炎表面突變蛋白會影響細胞的 DNA 修復能力

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三、 計畫對民衆具教育宣導之研究成果
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慢性 B 型肝炎患者罹患肝癌的機率是正常人的 100 倍以上。至今對於 HBV 感染如何誘發肝癌的機制仍不明。我們的研究是去探討 HBV 的 pre-S mutant HBsAgs 是否引發基因體的不穩定性，因而導致癌症。我們證實 pre-S mutant HBsAgs 會誘發較高的氧化壓力，導致嚴重的 DNA 氧化性損傷，而 DNA 修復路徑(BER)也被活化。在基因突變分析顯示 pre-S mutant HBS 的存在會增加基因突變率進而導致基因體的不穩定。此外，在 pre-S 轉殖基因老鼠肝臟組織中，初步發現有腫瘤抑制基因 p53 突變性蛋白產生，這表示 pre-S mutant HBsAg 與腫瘤抑制基因 p53 的突變有關。最後我們利用六位肝癌病人的肝臟組織，偵測出細胞中 oggl(DNA 修復酶)的表現量上升，意味著表現 pre-S mutant HBS 的肝臟細胞存有較高的氧化壓力。我們的結果提供了一個模式連接了 pre-S mutant HBsAgs 與肝癌發生的關係。
四、 簡述全程計畫成果之討論與結論，如有技術移轉、技術推廣或業界合作，請概述情形及成效
(1) pre-S B 型肝炎表面突變蛋白會造成基因產生突變
(2) pre-S2 B 型肝炎表面突變蛋白會影響細胞的 DNA 修復能力

五、 成效評估（技術面、經濟面、社會面、整合綜效）
本研究成效良好，已達成所有的既定目標

六、 下年度工作構想及重點之妥適性

七、 檢討與展望
本計畫進行順利，相關研究結果以投稿至國際期刊接受審查
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〔註〕

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柒、參與九十二年度計畫所有人力之學歷分析

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玖、九十二年度之著作抽印本或手稿

依「貳、九十二年度計畫著作一覽表」所列順序附上文獻抽印本或手稿
The Pre-S mutant surface antigens in chronic hepatitis B virus infection induce oxidative stress and genomic instability

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Keywords

Hepatitis B virus
Pre-S mutant
hepatocellular carcinoma
DNA damage
DNA repair
Genomic instability
ABSTRACT

Ground glass hepatocytes (GGHs) are the historic hallmarks for the hepatocytes in the late and non-replicative stages of hepatitis B virus infection. We have recently identified the type I and type II GGHs, which contain two novel mutant types of large HBV surface antigens with deletions over the pre-\(S\)\(_1\) and pre-\(S\)\(_2\) regions, respectively. The mutant pre-S mutant HBV surface proteins accumulate in endoplasmic reticulum (ER), resulting in a strong ER stress. Because of the clustering proliferation of type II GGHs, we speculate that these mutant pre-\(S\)\(_1\)/\(S\)\(_2\) proteins may be implicated in the HBV-related hepatocarcinogenesis. In this study, we investigated the potential DNA damage and genomic instability imposed by the mutant pre-S-induced ER stress. By comet assay, we found that the pre-\(S\)\(_1\) and pre-\(S\)\(_2\) mutant HBV surface antigens cause oxidative stress and DNA damages. The DNA repair genes \(ogg1\), \(xrcr-1\) and \(parp-1\) were induced by over-expression of the pre-S mutant HBS proteins. The DNA repair activity was also stimulated, as shown by the host cell reactivation assay. The enhanced mutation rates in the \(hprt\) gene were also observed in the mouse hepatoma ML-1 cells which constitutively expressed the pre-\(S\)\(_1\) and pre-\(S\)\(_2\) HBsAgs. The \(ogg1\) over-expression was detected in the transgenic mice carrying the pre-S\(_2\) HBS antigen as well as the hepatic tissues from the HCC patients. These results indicate that the pre-\(S\)\(_1\) and pre-\(S\)\(_2\) mutant HBV surface antigens, which compose the GGHs, induce oxidative DNA damages and genomic instability in hepatocytes in the late stages of HBV infection.
INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common histological form of primary liver cancer, the tumor cells having retained features of hepatocytic differentiation. Chronic hepatitis B virus (HBV) infection is a major global cause of HCC. Individuals who are chronic HBV carriers have a greater than 100-fold increase of the relative risk of developing HCC. Expression of the HBV surface proteins is highly associated with hepatic injury. The HBV surface antigen is able to up-regulate the transforming growth factor-alpha (TGF-α) gene, the most important growth factor of hepatocytes. In the phase of chronic HBV infection, the expression of HBV surface antigen is associated with hepatic nodular formation, which is the pre-requisite for hepatocellular carcinogenesis.

Expressions of the various types of HBS antigens are dependent on the replicative status of the HBV genome. In the acute phase of HBV infection, the major (i.e., small) HBS is the predominant form, which composes the envelope of the virion. In the chronic phase, the HBV genome integrates into the host genome, and the large form becomes dominant. The large form of HBS antigen includes an additional pre-S region that is the upstream promoter regions for the small HBS antigen. In the past years, we have identified a number of truncated surface gene mutants partially deleted of the pre-S regions. The pre-S mutant HBS antigens were found to contribute to two novel histological patterns, which were designated ground glass hepatocyte (GGH) types I and II. The type I GGHs display inclusion-like pattern of HBsAg, which is truncated in the pre-S1 promoter region. The type II GGHs display HBS antigen at the margins of hepatocytes. This type of mutant HBS gene is deleted of the nucleotides 2 to 55 over the pre-S2 region and contains a point mutation at the
start codon of middle S region, leading to a dramatic decrease of the synthesis of major and middle surface antigens. Both types of GGHs present accumulation of the HBS antigens in endoplasmic reticulum (ER). Thus, the type II GGH has high correlation with progression of cirrhosis and HCC. This mutant form of HBS gene, designated pre-S2 mutant HBS, emerges only at the late or non-replicative phase of chronic HBV infection and eventually becomes a dominant HBV gene product in hepatocytes. Hepatocytes expressing this type of mutant consistently cluster into groups due to clonal and integrated expansion (Fan et al. 2000; Fan et al. 2001).

Ground glass hepatocyte (GGH) is a historic hallmark of HBS antigen-containing liver cells in chronic HBV infection. The GGHs containing specific mutant pre-S1 or pre-S2 proteins exhibit significant ER stress, and possibly other aspects of cellular effects as well. Here we characterize the molecular and genetic changes caused by these mutant HBS forms. Our findings show that the pre-S1 and pre-S2 mutant HBsAgs confer oxidative stress-induced DNA damages and genetic instability in hepatocytes.
MATERIALS AND METHODS

Cell lines and reagents

The cell lines used in this study are listed in Table 1. The human Huh-7 and mouse ML1-4a hepatoma cell lines were stably transfected with the large wild-type, pre-S₁ or pre-S₂ HBS genes for the in vitro cell culture studies. These cells were maintained in regular Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum, 2mM L-glutamine, 1× non-essential amino acid, and 1× of the antibiotic/antimycotic mixtures (GibcoBRL, Grand Island, NY). The cells were grown at 37°C with 5 % CO₂.

Most of the reagents used for the reverse transcription and PCR amplifications were purchased from Promega (Madison, WI). The low-melting-point agarose, used for the comet assays, was purchased from GibcoBRL (Grand Island, NY). SYBR Green II, the staining reagent for comet DNAs, was purchased from Molecular Probes (USA). The DNA repair enzyme FPG, used for detection of 8-hydroxyguanine DNA lesions, was purchased from Trevigen, inc. (USA). The HBS Ag ELISA assay kit, used for quantification of the HBS proteins in the Huh-7 as well as the ML1-4a cells, was purchased from Abott Chemical Co. (IL, USA). Antibodies used for immunological stainings were purchased from DAKO (Carpinteria, CA). Common chemicals used were purchased from Sigma Chemical Co. (St. Louis, MO). Other reagents for specific uses will be mentioned in the following sections where the experiments are described.

Comet assays

Comet assays by single-cell alkaline electrophoresis were employed to measure the
levels of oxidative DNA damages. The Huh-7 cells in which the inducible wild-type or pre-S₁/S₂ mutant HBS genes were transfected were employed for the study. After induction of each type of the HBS gene by ponasterone A for 48 hours, the cells were harvested by trypsinization, centrifuged, and re-suspended in PBS, then counted for cell numbers using a hemacytometer. To prepare a glass slide for comet assays, 1% of agarose in PBS (pH7.4) was first coated on the slides, then covered with 1.5% of low-melting-point agarose gel in which $4 \times 10^4$ cells were embedded. After the coatings of cell agarose layer, another layer of 1% low-melting agarose gel was coated on top. After the coatings, the slides were covered with coverslips and placed on ice for 5 minutes. The cells on slides were then lysed with the ice-cold lysis buffer (5 M NaCl, 100 mM EDTA, 100 mM Tris-HCl, 1% triton X-100, 10% dimethylsulfoxide) at 4°C for 1 hour. After the lysis, the slides were immersed in PBS (pH 7.4) for 10 minutes then subjected to enzymatic digestion.

To detect the 8-hydroxyguanine DNA lesions, the FPG DNA repair enzyme was employed to specifically cleave the DNA strand at the sites of 8-hydroxyguanine. For these slides, 2 units of FPG was incubated with the slides at 37°C for 1 hour. After the FPG treatment, the slides were washed for a few times in PBS (pH 7.4). For single-cell alkaline electrophoresis, the slides were first immersed in alkaline electrophoresis buffer (0.3 M NaOH, 1 mM EDTA) for 20 minutes, followed by electrophoresis at 23 volts and 30 mAs for 25 minutes. After the alkaline electrophoresis, the slides were transferred to the neutralization solution of 0.4 M Tris-HCl (pH7.5) then stained with SYBR green II for 3 minutes in dark. The slides were examined by fluorescence microscopy. 100 cells on each slide were examined and scored for the fluorescence tail length from the cell nuclei. Each cell was assigned a score on an arbitrary scale of 0-4 (i.e. ranging from 0= no DNA damage, to
4=extensive DNA damage), based on comet tail length migration. A total damage score for each slide was derived by multiplying the number of cells assigned to each grade of damage by the numeric value of the grade and summing over all grades (maximum possible score: 400).

**RT-PCR of DNA repair genes**

The human Huh-7 cells expressing inducible HBS and the mouse ML1 cells constitutively expressing HBS genes were analyzed for expressions of DNA repair genes. For the Huh 7-derived cell lines, cells were treated with ponasterone A or mock-treated for 48 hour then subjected to RNA extraction. The reactions of reverse transcriptions were performed in a mixture of 1 µg of cellular RNA, 0.5 µg of random hexamer, 2 mM of each dNTP, 0.6 µl of RNase inhibitor (Takara, Tokyo, Japan), 50mM Tris-HCl (pH8.3), 75mM KCl, 3mM MgCl₂, 10mM dithiothreitol, and 200 units of Moloney Murine Leukemia virus reverse transcriptase (MMLV-RT). The reaction was performed at 37°C for 90 minutes then stopped at 95°C for 5 minutes.

For each PCR reaction, 5 µl of cDNA product of the reverse transcription reaction was used as the template. The PCR primers used were as follows. *ogg1*, forward: 5'-cactgcactgtgtacgccagg-3', and reverse: 5'-gcgtgcagcgccaggtage-3'; *xrccl*, forward: 5'-atgcggagatccgctccg-3', and reverse: 5' -catcatacctcaatgctcaca-3'; *parp1*, forward: 5'-ccgagtacagtgcgagctcag-3', and reverse: 5' -ctcgctcaagatgcgccgac-3'; and the housekeeping gene β-actin, forward: 5'- atcatgttggagaccttca-3', and reverse: 5'- catctcttgctgtaagtc-3'. For the GGH cells isolated by LCM from the HCC patients, the nested PCR was performed. The primers for the nested PCR were forward: 5'-gttgtgcgactgtgccgacaa -3', and reverse: 5'-gcgtgcagcgccaggtage-3'. The PCR
mix contained 25 mM N-[tris (hydroxymethyl) methyl]-3-aminopropanesulfonic acid (TAPS, pH 9.3), 50 mM KCl, 2 mM MgCl₂, 1 mM β-mercaptoethanol, 200 μM each dNTP, 0.25 μg/μl activated calf thymus DNA and 2 units of SuperTherm Fold DNA polymerase (JMR Holdings, Kent, UK). The PCR reaction was performed in the following order: ogg1: 94°C for 10 minutes, then 35 cycles (xrccl, 26 cycles; parp1, 26 cycles with annealing temperature at 57°C) with each cycle of 94°C for 60 seconds, 54°C for 45 seconds and 72°C for 120 seconds. Finally, the PCR tubes were incubated at 72°C for 10 minutes. The PCR products were examined by agarose gel electrophoresis.

**Host-cell reactivation assay**

The host-cell reactivation activities on the H₂O₂-damaged plasmids were employed to detect the DNA repair activity in cells. To construct the plasmid substrate, the pCMV₅₅ DNA was treated with 5 % H₂O₂ at room temperature for 30 minutes in dark. The treated plasmid DNA was purified by ethanol precipitation. To test the DNA repair activity in Huh-7 cells expressing various types of HBS genes, the H₂O₂-damaged pCMV₅₅ as well as the wild-type, pre-S₁ or pre-S₂ HBS genes in the p(3A)-S plasmid vector were co-transfected into the Huh-7 cells. After transfection for 48 hours, cell lysates were collected and the luciferase activities were detected using the luciferase assay kit (ABI Applied Biosystems).

**Random gene mutation assays**

ML1-4a cells constitutively expressing the wild-type, pre-S₁ or pre-S₂ HBS genes were analyzed for gene mutation rates in the hypoxanthine phosphoribosyl transferase (hpert) gene. Cells were seeded into 96-well plates in the concentrations of 2 x 10³ cells per well containing 5 μg/ml 6-thioguanine (Sigma, inc.) for selection of the hpert
mutants. After selection for 14 days, the numbers of surviving colonies were counted for the mutation rates of the \textit{hprt} gene. These cell colonies resistant to 6-thioguanine treatments were further grown and sub-cultured in the DMEM growth medium in the presence of 3 \( \mu \)g/ml 6-thioguanine. After the cells became confluent in the culture wells, the cellular RNAs were isolated. The \textit{hprt} cDNA was amplified by RT-PCR and then sequenced to identify the mutations in the gene. The PCR primers used are: forward, atgccggccgcagtcagctcg, and reverse, ttaggtttttatgtctttcctc.

\textbf{Immunohistochemical staining}

5-\( \mu \)m sections of the frozen liver tissues of pre-\( S_2 \) HBS transgenic mice were fixed in ice-cold acetone and blocked with 3\% \( \text{H}_2\text{O}_2 \) in PBS, and immuno-stained with monoclonal goat anti-HBS antibody. After washing in PBS, slides were incubated with the biotinylated anti-goat secondary antibody, followed with the peroxidase-conjugated streptavidin. The HBS antigens were then chromogenized with 3-amino-ethylcarbazol, finally counter-stained with Mayer's hematoxylin and observed by microscopy.

\textbf{OGGI immunofluorescent staining in HCC patients}

5-\( \mu \)m sections of the frozen liver tissues from HCC patients were immuno-stained with mouse anti-HBS antibody (DAKO) and rabbit anti-OGGI (Novus Biologicals, Inc., Littleton, CO). The slides were then incubated with anti-mouse secondary antibody conjugated with tetramethylrhodamine (TRITC) as well as the anti-rabbit secondary antibody conjugated with fluorescein isothiocyanate (FITC) (DAKO, inc.). Finally, the cell nuclei were counter-stained with Hoechst 33324. The sections were visualized by confocal microscopy at wavelength 547 nm for TRITC, 480 nm for
FITC, and 345 nm for Hoechst 33324.

RESULTS

Induction of oxidative DNA damages by the pre-S1/pre-S2 HBS mutant antigens

The hepatocytes expressing pre-S1 and pre-S2 HBS mutant antigens present ground glass staining pattern. Our recent studies have shown that these HBS antigens are localized in ER, causing ER stress in cells. The ER stress may induce oxidative stress, which probably results in oxidative DNA damages on the genome. To test for the oxidative DNA damages caused by the pre-S HBS antigens, we performed comet assays on the Huh-7 cells stably transfected with the inducible constructs of the wild-type, pre-S1, or pre-S2 large HBS genes. To specifically detect the amounts of the 8-hydroxyguanine, the major DNA lesion induced by oxidative stress, the DNA repair enzyme FPG was employed. FPG is a DNA glycosylase/lyase that cleaves the DNA strand at the sites of 8-hydroxyguanine. Data depicted in Figure 1a show that with the increasing concentrations of H₂O₂, the cells exhibited higher levels of DNA breakages, presented by the comet scores. With the treatments of FPG, the comet indices were greatly enhanced, indicating that the 8-hydroxyguanine DNA lesions were present in the H₂O₂-treated cells. Data in Figure 1b indicate that the expressions of the wild-type, pre-S1 or pre-S2 HBS mutant antigens all result in higher comet indices in the Huh-7 cells. The comet indices were increased even prior to the addition of FPG, suggesting that some of the 8-hydroxyguanine DNA lesions were processed by endogenous DNA repair activity in hepatocytes and created strand breaks, which represented the intermediate product in DNA excision repair pathways. After treatments with FPG to create strand breaks at the sites of 8-hydroxyguanine, the comet indices were further enhanced. The over-expression of the pre-S2 HBS
antigen revealed the highest comet indices, as compared with the over-expression of the wild-type or pre-S1 HBS antigen, suggesting that the pre-S2 mutant HBS protein would confer higher genomic instabilities than the others.

**Induction of DNA repair by pre-S2 HBS mutant antigen**

The base excision repair (BER) pathway is the major pathway for oxidative DNA damages. We therefore hypothesized that the DNA damages induced by the pre-S HBS mutant antigens, especially the pre-S2 HBS, could activate BER. The 8-oxoguanine glycosylase 1 (*oggl*), the major recognition factor for oxidative DNA lesions, was detected for its expression level by RT-PCR. Results depicted in Figure 2a indicate that after inductions of the large wild-type, pre-S1 and pre-S2 HBS genes by the inducer ponasterone A, an ecdysone analog, the Huh-7 cells exhibited higher levels of the *oggl* mRNAs. The expression levels of *oggl* increased along with the increasing time lengths of induction by ponasterone A; whereas the alcohol, the solvent for ponasterone A, did not exhibit similar effects. The poly(ADP-ribose) polymerase-1 (PARP-1), required for signaling of the DNA damages and re-ligation in BER, was also up-regulated within 12 hours after induction of the wild-type, pre-S1 or pre-S2 HBS antigens. These findings indicate that the accumulations of HBS antigens confer oxidative damages on genome and consequently up-regulate the BER pathway. In the mouse hepatoma ML1-4a cell lines that constitutively express the wild-type large HBS, the pre-S1 or the pre-S2 HBS mutant genes, the BER genes *oggl*, *parp-1* as well as *x-ray cross-complementation 1 (xrccl)* were significantly induced (Figure 2b). The cell line carrying the pre-S2 type of HBS gene exhibited the highest induction level, as compared with the cell lines carrying the wild type or the pre-S1 HBS genes. It indicates that the pre-S1/S2 mutant HBS antigens stimulate BER in the human Huh-7 cells and the mouse ML1-4a hepatoma cells.
The stimulation of BER by the pre-S\textsubscript{1} and pre-S\textsubscript{2} HBS proteins was further confirmed by the host cell reactivation (HCR) assays on the luciferase reporter plasmid pCMV\textsuperscript{huc}, pre-treated with H\textsubscript{2}O\textsubscript{2}. The Huh-7 cells, transiently co-transfected with the H\textsubscript{2}O\textsubscript{2}-damaged pCMV\textsuperscript{huc} plasmid as well as the various types of HBS genes and were applied to the luciferase measurements. Data depicted in Figure 3a indicate that each type of HBS antigen was able to stimulate DNA repair activity for removal of oxidative DNA lesions on the reporter plasmids. And the pre-S\textsubscript{2} type of HBS antigen appears to exhibit the highest stimulatory effect as compared with the wild type or the pre-S\textsubscript{1} HBS antigens. This stimulatory effect was specific to the surface antigens as the envelope (e) antigen was not able to induce the luciferase activity. To assure that the pre-S\textsubscript{2} type of HBS antigen contributes the strongest DNA repair activities, the luciferase activities obtained from cells expressing each type of HBS antigen was normalized with the respective intracellular HBS protein level, measured by ELISA assays. Data shown in Figure 3b indicate that the pre-S\textsubscript{2} type of HBS was indeed capable of inducing highest activity of DNA repair. These data are consistent with our other findings that the pre-S\textsubscript{2} type causes strongest oxidative stress for DNA lesions (Figure 1).

\textit{hprt gene mutations induced by the pre-SHBS antigens}

The attack of oxidative stress on DNA often confers gene mutations. Accumulation of gene mutations is surely a significant factor for carcinogenesis. We therefore detected the global gene mutation rates in the pre-S HBS-overexpressing cells. In the mouse ML-1 cells expressing various types of large HBS genes, the gene mutation rates in the X-linked \textit{hprt} gene was estimated as a marker for the global mutation rates. The 6-thioguanine (6-TG), a nucleotide analog, was used to select for the \textit{hprt} mutant
cells. The cell colonies surviving in the selective medium containing 6-TG were counted for the mutation frequencies of the \textit{hprt} gene. Data depicted in Table 2 indicate that both of the pre-S\textsubscript{1} and pre-S\textsubscript{2} HBS proteins were able to enhance the \textit{hprt} mutation rates. Cells carrying the pre-S\textsubscript{1} and pre-S\textsubscript{2} types of HBS genes exhibited 4.5-fold and 6.2-fold of gene mutation rates, as compared with that in the cells over-expressing the wild-type HBS, respectively. Thus, the pre-S\textsubscript{2} HBS antigen appears to be a stronger mutagen than the pre-S\textsubscript{1} HBS does. The mutation spectra in these 6-TG resistant cells were analyzed. As shown in Table 3, the majority of mutation types were point mutations, one-base additions or deletions. Thus, the majority of them did not present the G to T transversion, the typical mutation type caused by oxidative stress. This finding indicates that the pre-S\textsubscript{2} HBS expression not only induces oxidative stress, it also induces genomic instability.

\textit{Oxidative DNA damages in the transgenic mice of pre-S\textsubscript{2} HBS gene}

The BALB/c mouse model containing the pre-S\textsubscript{2} HBS transgene has been established (Chang \textit{et al.} unpublished). The mice at the age of 14.5 months were sacrificed for the study. The hepatic tissues display around 10\% of HBS-positive hepatocytes in male transgenic mice and lower percentages of HBS-positive cells in females (Figure 4a). In hepatocytes of the both male and female pre-S\textsubscript{2} HBS transgenic mice, the BER factors \textit{ogg1} and \textit{parp-1} were up-regulated, as shown in Figure 4b. This finding indicates that the pre-S\textsubscript{2} HBS antigen induces oxidative DNA damages in the mouse hepatocytes as in the \textit{in vitro} cell cultures.

\textit{Induction of oxidative DNA damages in the GGHs in hepatocellular carcinoma patients}

The pre-S HBS expressions in hepatic nodules are often seen in patients with chronic
HBV infection. Correlations between the pre-S HBS expression and HCC progression have been suggested. And the pre-S2 HBS protein has been reported to display tumor-promoting phenotypes, e.g., enhanced proliferation and clonal expansion abilities. The type II GGHs, expressing the pre-S2 HBS antigen, were isolated by laser capture microdissection. By nested RT-PCR, the ogg1 gene induction was detected in these cells (Figure 5a). The type II cells usually presented as hepatic nodules; while the type I GGHs, expressing the pre-S1 HBS antigen, did not aggregate into nodules. Taken together with the association of pre-S2 HBS and ogg1 induction, we suggest that the pre-S2 HBS expression confers strong oxidative stress on genome, resulting in genomic instability. By immunofluorescence staining on hepatic frozen tissues, the OGG1 protein was not detectable in the normal hepatocytes, and low level of expression in the type I GGHs (Figure 5b). On the contrary, the OGG1 was highly expressed in the type II GGHs. The regions over-expressing ogg1 were those expressing HBS antigen, as seen in the merged image of the individual staining for these two proteins. The OGG1 protein was localized in cell nuclei, as it co-localized with genomic DNA, counter-stained by the Hoechst 33324 fluorescent dye.
DISCUSSION

The HBV-related HCC is a major health problem worldwide. Several mechanisms of HBV-induced HCC have been proposed. The first mechanism is the inactivation of tumor suppressor/protooncogenes through insertional mutagenesis of HBV genome into human chromosomes. The second proposed mechanism is the potential oncogenic role of the viral product pX protein. The pX protein has been shown to consist of in vitro activities for transactivation as well as induction of signal transduction pathways such as Ras/Raf-1. The third and more recently proposed mechanism is the HBsAg-mediated pathway. The HBV surface antigen is now believed not only a structural protein but also a potential oncoprotein. The HBsAg was recently shown to up-regulate the transforming growth factor-alpha (TGF-α) gene, the most important growth factor of hepatocytes. Chisari et al. demonstrated that the overexpression of large S protein in transgenic mice cause hepatocellular injury and nodular regeneration, which potentially lead to genetic instability and neoplasia. Finally, a newly identified mutant pre-S2 HBs product, truncated in the pre-S promoter region, has been shown to induce transforming abilities in the HBV-integrated hepatocytes. This mutant surface antigen accumulates in endoplasmic reticulum (ER) and induces strong ER stress, which appears to be an important potential mechanism of HBV-related hepatocarcinogenesis. Here we demonstrate that these pre-S mutant HBsAgs induce oxidative DNA damages and subsequently stimulates DNA repair. These pre-S mutants, identified in our previous studies on HCC in Taiwan, are partially deleted in the pre-S1 or pre-S2 regions, the promoter regions for the small and middle forms of HBsAg. Such mutations cause dramatic decreases in the levels of middle and small HBsAgs. The large pre-S HBS mutant genes, which are partially deleted at the N-terminal pre-S1/S2 regions, encode
the mutant proteins that are probably folded into improper conformations. We have recently shown that these pre-S mutant HBsAgs accumulate in ER, inducing ER stress signals. Here we show that accumulation of such proteins induce strong oxidative stress, resulting in oxidative DNA damages. The pre-S₂ type of HBsAg emerges at the stages of hepatic cirrhosis nodules, and exhibits growth advantages. It has been suggested to have strong correlations with hepatocellular carcinogenesis. Our data here demonstrate that the pre-S₂ type induces highest mutation frequencies compared with the other types of large HBS proteins, using the hpri gene as a marker. These lines of data indicate that the pre-S mutants cause genomic instability.

The pre-S₁/S₂ HBsAgs have been shown to induce reactive oxygen species (ROS) production, which is a signal derived from ER stress (Wang et al. submitted). Our data here are consistent with these findings and clearly demonstrate that the DNA lesions occur in these cells. In our studies, the induction of BER gene oggl was consistently shown in all the model systems applied, suggesting that the oggl level be a biomarker for oxidative DNA damages in hepatocytes with chronic HBV infection.

The pre-S₂ has been reported to highly associate with two characteristics for a viral oncoprotein, i.e., growth advantage and immune escape (Fan et al. 2001). Unlike the pre-S₁ HBS, the pre-S₁ HBS has not been shown to exhibit such cancer-prone phenotype; however, in our data it also induces significant amounts of oxidative DNA adducts. Therefore, the exact mechanism by which the pre-S₂ enhances HCC progression remains a mystery. Solutions to these puzzles will only be resolved by long-term studies using the suitable mouse models.

It is likely that the pre-S₂ HBsAg directly regulates the function/expression of a tumor suppressor/oncogene. We have recently found that the p53 gene is mutated in
around half of the patient hepatocytes expressing pre-S2 genes, indicating that the p53 is most likely the target for pre-S2 HBS protein. We have also found that the pre-S2 transgenic mice exhibit higher rates of p53 mutations than the control mice do (data not shown). What remains to be determined would be that whether the pre-S2 HBS directly inhibits the p53 transactivation activity or through activation of another DNA damage responsive factor, such as ataxia telangiectasia (ATM).

Accumulation of the pre-S HBsAgs in ER has caused significant ER stress. ER stress causes oxidative stress. The oxidative stress, associated with autooxidation of the free radicals, can produce a superoxide anion, hydrogen peroxide, and other active oxygen species are extremely cytotoxic and genotoxic. The oxidative DNA damages induced by the pre-S HBsAgs, are surely largely contributed by ER stress induced by the pre-S HBS proteins. Thus, the ER stress can not fully explain the causes of the pre-S2 HBS-related hepatocellular carcinoma, given that the pre-S1 type also exhibit strong oxidative stress. Rather, the pre-S2 HBS protein might directly regulate the function or expression of certain tumor suppressor/oncoprotein that are associated with the DNA damage response pathways. This will be the next important question to be addressed.

The pre-S2 mutant potentially exhibits the two essential features for a viral oncoprotein, i.e., the immune escape and the enhanced transforming ability. The deletion sequence of pre-S2 region coincides with the human leukocyte antigen-restricted T- and B-cell epitopes (Fan et al. 2001). The pre-S mutant HBsAgs distribute in endoplasmic reticulum (ER) in hepatocytes and induce ER stress (Fan et al. 2001). ER stress response induces a series of signal transduction pathways, leading to apoptosis mediated by caspase 12, or alternatively, a growth
proliferation mediated through the NFκB pathway. ER stress also induces oxidative stress in cells (Liu et al. 1997; Serbinova et al. 1989; Wang et al. 1996; Yu et al. 1999). The produced oxygen reactive species may damage intracellular organelles by peroxidation of membrane lipids and modify the cysteine sulphydryl groups that are important to the conformation-dependent activity of several proteins. These include transcriptional regulators, e.g., Fos, Jun and NF-κB, as well as components that are required for the activation of the antioxidant response element.

These data suggest that the pre-S1/S2 HBsAgs emerging at the late phases of chronic HBV infection contribute to hepatocellular carcinogenesis through the oxidative stress-induced genomic instabilities.
REFERENCES

Chang, W-W, Su, I-J, Lai, M-D, Chang, W-T, Huang, W. and Lei, H-Y. The role of inducible nitric oxide synthase in a murine acute HBV infection model induced by hydrodynamics-based in vivo transfection of HBV-DNA. In submission


Wang, H-C, Wu, H-C, Chen, C-F, Lei, H-Y, and Su, I-J. Different types of ground glass hepatocytes in chronic hepatitis B virus infection contain specific pre-S mutants which may induce ER stress and oxidative response. In submission

FIGURE LEGENDS

Figure 1. Oxidative DNA damages in the Huh-7 cells with inducible HBS genes by comet assay. (A) Oxidative DNA damages after treatments with various doses of H$_2$O$_2$. (B) Oxidative DNA damages induced by various types of HBS genes. WT: the wild-type large HBS. Δ1: the pre-S$_1$ HBS. Δ2: the pre-S$_2$ HBS gene.

Figure 2. RT-PCR results of the DNA repair gene *ogg1* in cells expressing the pre-S HBS genes. (A) The Huh-7 cells with inducible HBS genes. Pon A: ponasterone A, the inducer for the HBS gene transcription. Alcohol: the solvent for Pon A. (B) the mouse ML-1 cells with various constitutively expressed HBS genes. WT: the wild-type large HBS. Δ1: the pre-S$_1$ HBS. Δ2: the pre-S$_2$ HBS gene.

Figure 3. Stimulation of BER by the pre-S HBS genes. (A) the luciferase reporter activity in cells transiently transfected with various HBV genes. Cs and Ce: vector controls for the surface (S) and envelope (e) HBV genes. eAg: the envelope HBV gene. WT: the wild-type large HBS. Δ1: the pre-S$_1$ HBS. Δ2: the pre-S$_2$ HBS gene. (B) the luciferase activities normalized with the HBS Ag levels in the cell free extracts.

Figure 4. The pre-S$_2$ HBS transgenic mice. (A) The HBS immunohistochemical stainings in hepatic tissues in the male and female mice. (B) The RT-PCR results of mouse *ogg1* (*mOGG1*) and PARP-1 genes in hepatocytes. C: control, the mock-transfected mice. Δ2: the pre-S$_2$ transgenic mice.

Figure 5. The OGG1 induction in the GGHs of the HCC patients. (A) The
nested RT-PCR results of the ogg1 gene in the GGHs isolated by laser capture microscopy. (B) fluorescence staining of the HBS and OGG1. C: control, the hepatocytes without HBS expression.
<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type</th>
<th>HBS genotype</th>
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<td>Huh-7</td>
<td>human hepatoma</td>
<td>wild-type large HBS</td>
</tr>
<tr>
<td>Huh-7/HBS\textsuperscript{WT}</td>
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<td>nt. 3040-3111 deletion in the pre-\textsubscript{S}\textsubscript{1} region</td>
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<tr>
<td>ML1-4a</td>
<td>mouse hepatoma</td>
<td>wild-type large HBS</td>
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<td>nt. 2-55 deletion in the pre-\textsubscript{S}\textsubscript{2} region</td>
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Table 2. *hprt* mutation frequencies in the ML1-4a cells over-expressing the pre-S HBS antigens

<table>
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<tr>
<th>Cell</th>
<th><em>hprt</em> cells</th>
<th><em>hprt</em> mutation frequency</th>
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<tr>
<td>ML1-4a</td>
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<td>8 / 6 x 10^6 cells</td>
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<tr>
<td>ML1-4a&lt;sub&gt;HBS/A2&lt;/sub&gt;</td>
<td>11 / 6 x 10^6 cells</td>
<td>1.8 x 10^-6 cells</td>
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Table 3. Mutation spectrum for the hprt genes in the ML1-4a cells over-expressing the HBS genes

<table>
<thead>
<tr>
<th>Cell</th>
<th>Clone No.</th>
<th>Mutation position (CDS)</th>
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<th>Amino acid change</th>
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<td>186</td>
<td>ATT → ATC</td>
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<td></td>
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<td>292</td>
<td>GAT → AAT</td>
<td>Asp → Asn</td>
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<td></td>
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<td>301, 302</td>
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<td>Arg → Pro</td>
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<td>TTT → TTG</td>
<td>Phe → Leu</td>
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<tr>
<td></td>
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拾、九十二年度計畫執行情形

註：群體計畫（PPP）者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料
若為群體計畫，請勾選本表屬於： □ 子計畫； 或 □ 總計畫（請自行整合）

1. 請簡述原計畫書中，九十二年預計達成之研究內容

本研究預計達成以下之研究內容：

目標一：研究在表現 pre-S₂ mutant HBsAg 的細胞中發生基因突變的機率
目標二：研究在表現 pre-S₁ HBsAg 的 Huh-7 細胞中，DNA 修復活性上的影響
目標三：研究在表現 pre-S₂ HBsAg 的細胞中 DNA 損傷反應性基因（DNA damage response gene）的表現情形
目標四：利用 HBV 感染末期的 HCC 病人組織來確認隨機 DNA 突變
目標五：檢測在慢性 B 型肝炎感染晚期或急性 HCC 病人中的腫瘤抑制基因／原致癌基因的表現情形

2. 請詳述九十二年度計畫執行情形，並評估是否已達到原預期目標（請註明達成率）

我們大致上已完成所有的預期目標：

目標一：研究在表現 pre-S₂ mutant HBsAg 的細胞中發生基因突變的機率
利用人類肝癌細胞 Huh-7 穩定性轉染誘發表現的 HBS 基因（inducible HBS）
來研究 pre-S HBsAgs 誘發氧化壓力而導致 DNA 損傷；此外，老鼠肝癌細胞
ML-1 持續性的表現不同種形式的 HBS 基因也被應用在實驗中。利用
DCFH-DA 染色和流式細胞分析儀來研究在 Huh-7 細胞中，pre-S HBsAgs 誘
發的氧化性物質。利用彗星分析以及 DNA 修復酶 FPG 來偵測表現 HBS 的 Huh-7 細胞中氧化性鹼基 8-hydroxyguanine 的量。我們的結果清楚的證實 pre-S2 mutant HBsAg 會誘發較高的氧化壓力，且 pre-S1 型所誘發的氧化力較 pre-S2 型高；但是 pre-S2 型卻導致較嚴重的 DNA 氧化性損傷，因此 pre-S2 HBS 助長肝癌的形成是藉由內質網壓力以外的其他機制所造成。

為了研究是否 pre-S mutant 的表現會影響整體的基因突變機率，我們發展了一套方法去偵測 hprt 基因的突變，我們的結果顯示出 pre-S mutant HBS 的存在會增加基因的突變進而導致基因體的不穩定。

目標二：研究在表現 pre-S2 HBsAg 的 Huh-7 細胞中，DNA 修復活性上的影響

我們利用表現不同種類 HBS 基因的 Huh-7 細胞和 ML1 細胞來進行。實驗結果表示在表現 pre-S mutant HBsAgs 的細胞中，ogg1 的表現量受刺激而增加，oggl 爲修復氧化性 DNA 損傷的重要 DNA 修復酶。Host cell reactivation assay 也證實鹼基修復路徑 (BER) 受活化。這些結果表示 pre-S mutant 的表現會刺激修復氧化性 DNA 損傷的 DNA 修復路徑 (BER) 的活化，而且 pre-S2 型所誘發的 DNA 修復更勝於 pre-S1 型。

目標三：研究在表現 pre-S2 HBsAg 的細胞中 DNA 損傷反應性基因 (DNA damage response gene) 的表現情形

實驗中偵測多種的 DNA 損傷反應性基因包含 PARP-1 和 p53。PARP-1 參與 BER 和細胞凋零，我們發現在表現 pre-SHBsAgs 的 Huh-7 細胞及 ML1 細胞中 PARP-1 的表現量有大幅度的上升，pre-S2 型也如預期有較高的表現量。除了 in vitro 的細胞實驗外，我們也利用轉殖基因老鼠的肝臟組織來進行實
驗。利用免疫組織化學染色法探測 p53 突變性蛋白的存在，初步的結果顯示表現 pre-S₂突變型 HBS 的轉殖基因老鼠的肝臟組織中含有 p53 突變性蛋白。

目標四：利用 HBV 感染末期的 HCC 病人組織來確認隨機 DNA 突變
以雷射捕獲顯微技術 (LCM) 分離六位 HCC 病人的肝臟組織，RT-PCR 的結果顯示出表現 pre-SHBSAgs 的細胞中 oggl 的表現量上升，尤其是表現 pre-S₂突變型 HBS 的細胞 oggl 的表現量更是大幅度的上升。此結果意味著在慢性 B 型肝炎感染中，表現 pre-S₂突變型 HBS 的肝臟細胞存有較高量的氧化壓力。

目標五：檢測在慢性 B 型肝炎感染晚期或是 HCC 病人中腫瘤抑制基因／原致癌基因的表現情形
以雷射捕獲顯微技術分離出表現不同突變形式 HBS 的肝臟細胞並分析 p53 突變的情形，我們的結果顯示有一半以上的患者在 p53 密碼子 (exon) 5-8 的位置有突變發生，這表示 pre-SHBSAgs 與腫瘤抑制基因 p53 的突變有關，未來將利用這些雷射捕獲顯微技術分離出的肝臟細胞側測 Rb 和 BRCA1 基因的表現量。

整體而言，我們大致上已完成所有的實驗目標；除此之外，在目標三的部份加入了轉殖基因老鼠模式，此群體計畫原本授予 3 年，但是由於蘇益仁教授任職於 NHRI，因此將在第 2 年中止此群體計畫，我們目前已完成起始所訂立的研究目標”pre-S₂ mutant HBsAg 導致基因體的不穩定性”，我們的結果提供了一模式連接了 pre-S₂ mutant HBsAg 與 HBV-related HCC 的相關性。