# 行政院國家科學委員會專題研究計畫　成果報告

## B 型肝炎病毒大表面抗原轉殖小鼠在形成肝癌過程中先天性免疫反應之變化(3/3)

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**Transcriptome analysis of HBV-LS-transgenic mice:**
**An injury-regeneration model of human hepatocarcinogenesis**

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## 中文摘要

藉由微陣列生物晶片技術，幫助我們在人類肝癌中大量表現的基因能有透徹的瞭解與研究。近來相關研究已證實當小鼠大量表現 HBV large-envelope protein 時，會造成肝細胞長期的傷害受損進而導致肝癌的產生。雖然導致人類肝癌的產生與 HBV-LS-tg 小鼠的起因可能不同，但過程中所發生的肝臟病變及肝再生是二者共同的特徵。因此，我們想進一步來證實，當肝細胞的傷害促使〝高度分化的乾細胞〞面臨突變、染色體變異及 DNA 轉錄層次的失控，最後造成肝癌細胞的產生是有相關連的。而 HBV-LS-tg 轉殖小鼠在不同生長時程中會有基因短暫性的大量表現變化，然而這些重大的影響傷害是否會導致肝癌的發生，仍是現今需進一步研究釐清的地方。

HBV-LS-tg 轉殖鼠的特徵包括了長期的高 ALT 酵素活性、肝門靜脈附近的膽管細胞分裂以及局部發炎反應。這些肝細胞的核大小明顯不一、並且在細胞質裡有明顯的微小液泡產生。我們置備了 2 月、4 月、8 月、10 月、14 月及 18 月不同時間月份轉殖鼠的 RNA，藉由 Affymetrix GeneChip Mouse Experssion 430A 的晶片來分析基因轉錄層次的研究。以兩個月的同窩正常小鼠當作對照組，把老化所造成的表現量改變的基因去掉後還剩下 4465 個表現量改變在 2 倍以上的基因。在這些轉殖鼠的 3311 個已知基因中顯現出了明顯的表現量差異趨勢包括了 725 個表現量上升基因和 1038 個表現量下降基因。除此之外我們還從這些基因轉殖鼠中找出了關鍵性的訊息傳遞路徑和新陳代謝的改變。其中幾個較具有代表性的包括了 TGF-β、EGFR、ER stress response、Wnt-signaling、inflammatory responses、eicosanoid synthesis、matrix metalloproteinases，根據這些資料可以找出腫瘤產生時的調控細胞凋亡和細胞週期的調控機制。此外也已有相關研究發現，細胞發炎會促使細胞纖維化的反應最後形成癌細胞。因此透過這些研究將進一步幫助我們瞭解 HBV-LS-tg 轉殖鼠在不同時期所受的生理改變傷害，並將提供我們更多有關人類肝癌形成的線索。

## Abstract

With the advent of the DNA microarray technology, expression profiling of human liver cancers is now a reality. Chronic liver cell injury was clearly demonstrated to cause liver tumors in mice overexpressing HBV large-envelope protein (HBV-LS-tg). Although the initiating events maybe different but chronic hepatitis and regenerative hyperplasia seem to be common features found in both human HCC patients and HBV-LS-tg mice. It is likely that the downstream processes from liver injury put the "differentiated resting hepatocytes" at risk to mutations, chromosomal instability and/or deregulated transcription control and the eventual tumor development. Temporal changes in gene expression during the lifespan of these mice, which may have great influence over the process of hepatocarcinogenesis, however, remain to be an entirely untouched subject.

The hallmarks of HBV-LS-transgenic mice included chronic ALT elevation, bile duct proliferation in the portal areas and focal lymphocyte infiltration in lobular components. The hepatocytes showed marked variations in the size of nuclei, prominent microvacuoles in the cytoplasm and the 'ground-glass" appearance in older mice. Transcriptome analysis was performed using Affymetrix GeneChip Mouse Expression 430A arrays with RNA samples of transgenic mice of different ages, 2-month, 4-month, 8-month, 10-month, 14-month, and 18-month. The littermates of 2-month age were used as control samples. After minimizing the aging effect, 2288 genes showed 2-fold changes in their expression levels. The transgenic mice displayed strikingly distinct expression patterns with 714 genes up-regulated and 648 genes down-regulated among the known genes. Furthermore, we identified critical signaling pathways and metabolic alteration in the transgenic mice. The most influential signaling pathways included TGF-β, EGFR, ER stress response, Wnt-signaling, inflammatory responses, eicosanoid synthesis, matrix metalloproteinases with which cell death and distinct cell cycle activity were identified during the tumor progression. Potential fibrotic response was identified throughout the process suggesting a propagation of the inflammatory stimulus to fibrotic activity and the ultimate tumor formation. The obtained information will help understand the physiological changes of HBV-LS transgenic mice at different ages, which will also provide valuable information to the processes of hepatocarcinogenesis in human. (NSC 96-2752-B-010 -002-PAE)

## Introduction

Chronic liver cell injury was clearly demonstrated to cause liver tumors in mice overexpressing HBV large-envelope protein (HBV-LS-tg) (Chisari, et al., 1986). Although the initiating events are different but chronic hepatitis and regenerative hyperplasia seem to be common features found in the human HCC patients and mouse system. It is likely that the downstream processes from liver injury put the "differentiated resting hepatocytes" at risk to mutations, chromosomal instability and/or deregulated transcription control and the eventual tumor development. Temporal changes in gene expression during the lifespan of these mice, which may have great influence over the process of hepatocarcinogenesis in HBV-LS-tg mice, however, remain to be an entirely untouched subject. In this study, we conducted a genome–wide transcriptome profiling modulated by HBV-LS transgene and attempted to delineate the molecular events leading to hepatocarcinogenesis in HBV-LS-tg mice.

## Materials & Methods

*Transgenic mice*

HBV-LS-tg mice (C57BL/6J-Tg[Alb1HBV] 44Bri, JR2226) were originally purchased from the Jackson Laboratory and then bred in our laboratory. Due to the overexpression of LS protein in the hepatocytes, these mice develop chronic hepatitis since 4 months of age and develop HCC after 1 year. The line was maintained by breeding male hemizygous tg mice with wild-type females. Resulting in 50% of the wild-type littermates. The serum ALT was monitored by ALT activity assay. Samples of blood, spleen and liver tissues from HBV-LS-tg mice and their littermates at various ages after birth were collected. The accumulated alteration of gene expression was analyzed using oligonucleotide chips.

*RNA isolation and high-density oligonucleotide microarray analysis*

TRIzol Reagent (Invitrogen Life Technologie, Carsbad, CA) was used to prepare total RNA from the frozen liver samples. RNAs were quantified by spectrophotometry at 260 nm. Biotin-labeled antisense cRNA using *in vitro* transcription reaction (IVT) will be synthesized, purified and fragmented and hybridized to GeneChip Mouse Expression 430A arrays (MOE430A) (Affymetrix, Santa Clara, CA) according to the manufacture protocols. MOE430A array has 22690 probesets representing 13406 UniGenes. The arrays were washed and stained with R-Phycoerythrin streptavidin (Molecular Probes) and then were scanned with an Affimetrix GeneArray Scanner. Expression values were normalized across the sample by global scaling normalization. All hybridization intensities less than 100 were converted to a value of 100. Affymetrix MSA 5.0 software was employed to conduct the normalization, to monitor specific hybridization and gene expression. Microarray hybridization and raw data

processing was performed in the National Microarray & Gene Expression Analysis Core Facility of the National Research Program for Genomic Medicine at Yang-Ming University. Microarray data was analyzed using GeneSpring software (Silicon Genetics, Redwood City, CA). $Log_2$ ratio of hybridization intensity between LS-tg and 2-month littermate was used to calculate the relative gene expression level. Gene-wise hierarchical clustering of the affected genes was established to define genes uniquely up- or down-regulated by 2-fold at a single or multiple time points. Total of 2288 probesets showed 2-fold changes. Clustering using K-means generated specific gene groups with distinct expression patterns. Total of 1362 probesets, 714 up-regulated and 648 down-regulated, were identified in fourteen clusters (Fig. 2, Left). The genes showed uniquely induced/elevated expression patterns at 2-month (cluster 1), 4-month (cluster2), 8-month (cluster 3), 10-month (cluster 4), 14-month (cluster 5) and 18-month (cluster 6) were selected and subjected to further analysis. Expression of selected genes was verified with real-time RT-PCR using the TaqMan protocol (Applied Biosystems, Inc.). Network analysis of gene activities was performed using the Ingenuity Network Analysis (Ingenuity Systems, Mountain View, CA).

*Real-time quantitative PCR*

Complementary DNA (cDNA) was prepared from the total RNA with SuperScript RNaseH⁻Reverse Transcriptase (Invitrogen, Carlsbad, CA). Expression of distinct genes was examined using ABI17700 System (Applied Biosystems, Inc.). These quantitative PCRs defined a threshold cycle ($C_t$) of detection for the target or for beta-2-microglobulin ($\beta_2$m) in each cDNA sample. Sequence-specific primers and probes, as specified by the ABI Assay ID, were purchased from Applied Biosystems. PCR reactions were carried out in a total volume of 5 μl with the TaqMan universal PCR Master Mix according to the manufacture's protocol (PerkinElmer Life Sciences).

*Histochemistry*

Paraformaldehyde fixed (4%), paraffin-embedded liver section of Ls-tg mice at different ages were studied. Tissue sections of 5-μM-thick were deparaffinized in xylenes, rehydrated in graded alcohol, and equilibrated in PBS. To inactivate the endogenous peroxidase activity, the sections were treated with 0.1% hydrogen peroxide in PBS for 10 min prior to HE staining.

**Results and Discussions**

*Pathophysiology of HBV-LS transgenic mice*

The transcript of HBV-LS in the transgenic mice increased progressively in the first 10 months but the level of transcripts seems to be reduced later in life as shown in

Fig. 1A. Elevated expression of alpha-fetoprotein (AFP) was only detected in the liver of 14-month-old mice (Fig. 1A). These transgenic mice developed an age-dependent liver damage as reflected by the appearance of abnormal liver nodules (Fig. 1B, a, arrows), necrotic lesions as small white spots on the livers and increasing level of serum ALT (Fig. 1C). “Ground-glass hepatocytes” (GGH) reflecting overproduction of HBV large envelope polypeptide in endoplasmic reticulum (ER) was seen scattered throughout the livers of 18-month-old mice (Fig. 1D, d, arrows). The histological sections of the livers showed bile duct proliferation in the portal areas (Fig. 1D, a), focal lymphocyte infiltration in lobular components (Fig. 1D, b), marked variations in the size of nuclei and prominent microvacuoles in the cytoplasm which were not found in the liver of the age-matched littermates (Fig. 1D, c). Despite the observation of the several hallmarks of liver injury induced by overproduction of LS antigen in this strain of mice, the low incidence of typical hepatocellular hyperplasia or liver tumors is different than that reported by Chisari, et al. (1987). It needs to be confirmed whether the difference in tumor incidence is related to a partial loss of the transgene copies.

## Transcriptome analysis of HBV-LS transgenic mice

The pathophysiological results indicate that LS-tg mice overproduce HBV-LS protein within the hepatocytes develop severe, prolonged hepatocellular injury that initiates a programmed response, characterized by inflammation, chronic hepatitis and regenerative hyperplasia. Temporal changes in gene expression during the lifespan of these mice, which may have great influence over the process of hepatocarcinogenesis remain uncharacterized. A comprehensive transcriptome analysis of livers of HBV-LS transgenic mice was performed using GeneChip Mouse Expression 430A array (MOE430A), which has 22690 probesets representing 13406 UniGenes.

Since the duration of disease progression in LS-tg mice is protracted and mice tend to develop abnormal liver nodules at older age, we studied the temporal changes of gene expression from mouse of different ages. To identify genes differentially expressed at specific phases of the disease process, liver RNA samples were obtained from mice of the following age groups, 2 months, 4 months, 8 months, 10 months, 14 months and 18 months. Gene profiles of the littermates of 2-month and 18-month were also included in the analysis. To avoid the aging-related alteration of gene expression, we excluded those genes, which were highly induced in18-month-old littermates, i.e. 2-fold higher expression level than the 2-month littermates. Within the

remaining 6053 probesets, total of 2288 probesets showed 2-fold changes at any age from the littermates of 2-month-old. Clustering using K-means generated distinct expression patterns. Total of 1362 probesets, 714 up-regulated and 648 down-regulated, were identified in fourteen clusters (Fig. 2, A). The genes showed uniquely induced/or elevated expression patterns at 2-month (cluster 1, 29 genes), 4-month (cluster 2, 21 genes), 8-month (cluster 3, 26 genes), 10-month (cluster 4, 27 genes), 14-month (cluster 5, 429 genes) and 18-month (cluster 6, 63 genes) were selected and subjected to further analysis. The relative expression level of various genes was compared using $Log_2$ ratio of hybridization intensity between genes expressed in LS-tg and 2-month littermates. Gene-wise hierarchical clustering of the affected genes was established (Fig. 2, B, C). A widespread alteration of gene expression, total of 429 genes up-regulated and 280 genes down-regulated, was observed in mice of 14-month-old suggesting that translation of these altered genes may have great bearing on the development of liver disease.

A survey of the functions of the altered genes was conducted (Fig. 3). Percentage of differentially regulated genes, both up- and down-regulated, for the enzymes, the nucleic acid binding proteins, the structural proteins and proteins for transport are 35.8%, 12.1%, 12% and 11.5% respectively.

*Differential expression profile in LS-tg mice*

To better understand the effects of these differentially induced genes in disease development we ranked the genes by fold induction from 6 age groups as shown in Table 1 to Table 6. Most of the genes were upregulated 2~4 fold but the scale of transcription activation during 14-month is striking.

In the 14-month-old mouse livers, expression level of 59 genes (59/425=14%) was elevated 4-fold or more. Level of expression of the remaining 366 genes was elevated 2-3 fold (Table 5-1). Drastic changes of transcription activity in mice of 14-month-old suggested an ongoing large-scale modification of cell activity. Most importantly, many of the highly activated genes in 14-month-old tg mice are cell-cycle regulated genes (*MCM2, 3, 6; CDC2A, Ki67, topoisomerase II, PCNA, CCNB1, CDC2*, etc) or structural/cytoskeletal genes (tubulins, vimentin, deratin, kinesin, etc) reflecting an ongoing cell proliferation. To verify the microarray results, the expression level of 6 cell cycle genes was examined with the real-time RT-PCR. As shown in Fig. 4, *Ccna2, Ccnd1, Pcna, Ccnb2, Cdc20* and *Cdkn1a* (p21) were uniquely induced in mice of 14-month-old. Unexpectedly, the transcription activity of these 6 cell cycle genes dropped precipitously from 14-month to 18-month in both microarray and real-time PCR analysis.

To explore the signaling and network dictating the transcription changes in 14-month-old mice, we subjected this group of 425 genes to Ingenuity Pathway Analysis (Ingenuity Systems). This network analysis software identifies a network of cell cycle genes regulated in the liver of 14-month-old tg mice *in vivo*. The network is

displayed graphically as nodes (genes/gene products) and edges (the biological relationships between the nodes). Nodes are displayed using various shapes that represent the functional class of the gene product. The intensity of the node color indicates the degree of up-regulation (red). Under each node, the fold change in expression is marked by $Log_2$ ratio. As shown in Fig. 5, a total of 35 and 27 differentially expressed genes were brought into the network 1 and network 2, respectively. In each network, several nodes were identified by the pathway analysis as part of the network. These two networks involve the activities of cell cycle, DNA replication, recombination and repair with non-overlapping gene sets. The network analysis further strengthens the possibility that LS-tg mice of 14-month-old experienced transcription reprogramming for cell proliferation

In mice of 18-month-old, mixed activity of cell proliferation and cell-death was observed. Cell death-inducing DNA fragmentation factor A (*CIDEA*) and cell death-inducing DFF A-like effector c (*CIDEC*) were induced to high level. CIDE, a novel family of cell death activators with homology to the 45 kDa subunit of the DNA fragmentation factor (DFF45) that promotes cell death and DNA fragmentation. Expression of CIDE-A induced DNA fragmentation in 293T cells (Inohara, et al., 1998). CIDEC (Liang, et al., 2003) is another member of CIDE family. Expression of AATK, another gene associated with apoptosis was also increased. AATK is a novel gene which is induced during apoptosis. It encodes a protein with a tyrosine kinase domain at the N-terminal end and a proline-rich domain at the C-terminal end. AATK expression may be a necessary pre-requisite for the induction of growth arrest and/or apoptosis of myeloid precursor cells (Gaozza, 1997). It is tempting to speculate that abundant mRNAs of these apoptosis-inducing genes in tg mice of 18-month of age is associated with injury-induced cell death/necrosis. On the contrary, genes may assist cell proliferation in this age of mice include G0S2, FGFR1, STAT3, etc. G0S2 expression is required to commit cells to enter the G1 phase of the cell cycle (Cristillo, et al., 1997). In 18-month livers, we found co-expression of TFF3 (3.4X)and STAT3 (2.2X). STAT3 signaling was implicated in human colonic cancer cells during intestinal trefoil factor 3 (TFF3) and vascular endothelial growth factor-mediated cellular invasion and tumor growth (Christine, et al., 2005). It is unclear yet the biological roles of the mixed activities of cell proliferation and cell-death in LS-elicited liver disease.

*Comparative microarray analysis*

Manifestation of chronic hepatitis and injury-induced regeneration in LS-tg mice is reminiscent of human hepatocarcinogenesis (Chisari, 1995). The link between injury and transformation is established in the HBV transgenic model and ER-overload as revealed as “ground-glass hepatocytes” has also been found in human HCC/cirrhosis patients (Wang, et al., 2003). Shared transcription profiles between these two disease entities should reveal the common elements of hepatocarcinogenesis. We conducted a

comparative microarray analysis between LS-tg arrays and human HCC microarray. The HCC microarray was performed using Affymetrix oligonucleotide gene chip U133 Plus 2.0 with 10 HCC liver samples and 2 normal liver tissues. Relative gene expression was calculated as $Log_2$ ration of HCC/normal liver. An in-house program was used to combine gene array results from the mouse and human arrays. Genes listed from Table 1 to Table 6 were used to construct the following array comparison tables.

Globally, the expression profiles of LS-tg mice and human HCC are not identical. One reason being that HCC specimens assayed in the microarrays were not the "early tumors or hyperplasia". On the other hand, the case of LS-tg mouse represents a gradual evolving hyperplasia. It is, thus, conceivable that most of the genes activated in younger LS-tg mice were not active in human HCC. Nonetheless, human HCC samples shared certain degree of gene expression features with that of the 14-month-old tg-mice because high cell proliferation activity is common to hyperplasia and most of the tumor types (Whitfield, et al., 2000). Our preliminary results on transcriptome and comparative microarray analysis of HBV-LS-tg mice and human HCC demonstrated that LS-tg mice, like human HCC, requires a complex interplay of groups of genes to succeed cellular transformation.

Expression of ER-overload induced genes, CHOP, ATF3 and ATF6 (Pahl, 1999; Shuda, et al., 2003), were peaked prior to 10-month in LS-tg mice (data not shown). Combining the temporal appearance of EOR (ER-overload response), the "ground-glass hepatocytes" phenotype and the drastically altered transcriptome in 14-month-old LS-tg mice, we suggest that EOR may elicit injury/regeneration response and is a prerequisite in steering cell cycle progression. Potential candidate genes for hepatocarcinogenesis deduced from the microarray analysis are listed in the context. This is the first time that a genome-based expression profiling was conducted on the HBV-LS-transgenic mouse system.

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