行政院國家科學委員會專題研究計畫　成果報告

B 型肝炎病毒基因體變異在慢性 B 型肝炎急性發作的角色探討：一前瞻性全長基因體研究(III)

計畫類別：個別型計畫
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執行單位：國立臺灣大學醫學院內科

計畫主持人：劉俊人
共同主持人：陳培哲，陳定信

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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

（計畫名稱）

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共同主持人：陳培哲教授、陳定信教授
計畫參與人員：

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：國立臺灣大學醫學院內科

中　華　民　國　94　年　10　月　20　日

研究計畫成果精簡報告：

（一）中文摘要

（二）英文摘要

中文成果摘要　　關鍵詞: B 型肝炎病毒，急性發作，全長，基因体，變異，前瞻性

慢性 B 型肝炎急性發作的免疫致病機轉迄今尚未明瞭。病毒株變異或是宿主的免疫反應改變導致病毒與宿主間的平衡狀態破壞，被認為是引發急性發作最重要的因素，但何者為重仍有爭論。本研究欲以前瞻性研究及全長基因體分析來探討病毒株變異與慢性 B 型肝炎急性發作的相關性。縱使真正致病機轉仍未明，臨床觀察及動物實驗常發現血清 ALT 值升高前會出現病毒量驟升的現象，若能解開此病毒量驟升之謎，應對 B 型肝炎急性發作的致病機轉有進一步的了解。病毒日產量極高，其複製腌又欠缺校正錯誤的能力，因此極有機會出現病毒變異株，藉著逃脫宿主免疫能力攻擊或是具有較好的複製能力，使病毒量驟升，進而引發 B 型肝炎急性發作。之前許多相關研究並無法得到一致的結論，次基因体的研究策略及欠缺適當的研究模式為主要因素；更重要的是其採樣進行病毒核酸序列分析的時間點多在急性發作之中或後，因此無法推測這些病毒核酸變異是否真正引發急性發作，或僅是代表宿主免疫攻擊後的產物。前瞻性研究及全長基因體分析當可回答此一重要問題。

第一年計劃中，我們已更廣泛地收集 14 位自發性或是在醫療介入後發生 B 型肝炎急性發作的病患， 我們首先定期分析血清 ALT 值和 HBV DNA 以釐清血清 HBV DNA 和 ALT 值升高的相對應關係。結果相當一致地，在 13 位(93%)病患 ALT 值升高前都會有病毒量竄升的現象。為探討病毒發生變異是否引發急性發作，我們特別在急性發作過程中的四個點收集血清並進行 HBV 全長基因体分析: 收錄病患時、血清病毒量增加到最高時、血清 ALT 值達最高時、和急性發作之後。每一病患在不同時間點得到的 HBV 全長基因体分別與病患收錄時的 HBV 全長基因体比較並找出變異所在，之後我們與已知病毒免疫反應標的區氨基酸序列相對比和利用體外短暫轉染法，來進一步探討這些變異可能代表的免疫學或病毒生物學方面的意義。結果發現在病毒量竄升到最高點時，遑論是發生在急性發作之前或是與急性發作同時發生，病毒基因體幾乎與病患收錄時的病毒基因體保持一致。在急性發作過程中，體內出現不同主要病毒基因體的病患比例隨發作進展而逐漸增高：在血清 ALT 值升到最高值時其比例為 36%；在急性發作之後，其比例則增高為 50%。基因體出現的變異絕大多數是多重核甘酸點變異或次基因體缺損。我們的結果顯示急性發作與病毒發生變異無關。(Liu CJ et al, Gastroenterology 2004)

就病毒長期演變的觀點來看，為探討急性發作後出現的病毒變異是否真會造成新的急性發作，我們在第二年計劃中收錄連續發生急性發作的病患及其系列血清，增殖定序其 HBV 全長基因体並加以比對，以釐清此一問題。結果，我們同樣發現在每一次急性發作中臨床出現肝炎活性之前，病毒基因體幾乎維持不變；而重複發生急性發作的患者在急性發作後會出現病毒變異株，此病毒變異株與下一次急性發作中竄升的病毒株也不相同。另外，我們利用專一於病毒變異株的 PCR 方法證實下一次急性發作中竄升的病毒株已既存於病人收錄時的病毒池中。我們的結果支持某些病毒株的任意活化是造成急性發作的機轉之一。最後，真正具致病性的是肝細胞內的 HBV，而大部份研究僅探討血清中的病毒基因体。為釐清血清中的 HBV 是否能代表肝細胞內的 HBV，我們也自相同病患在同一時間取得其肝及血清檢體，進一步比較二者檢體中病毒基因体的異同。我們發現兩個來源的主要病毒全長基因體以及病毒物種類似程度大致相同。此一發現意味著血清中的病毒株能代表真正具致病性的肝內病毒株。

第三年計劃中，我們已順利完成上述工作，並特別著重分析急性發作前宿主免疫狀態的改變。另一方面，肝內及血清中的病毒株皆源自肝細胞核中的 cccDNA。本計畫同時也證實自肝及血清得到的病毒株等同於 cccDNA。(Liu CJ et al, Hepatology 2004)

我們經由此前瞻性全長病毒基因体研究，成功釐清病毒株發生變異並非造成急性發作的重要因素，此一發現將對慢性 B 型肝炎急性發作的免疫致病機轉有重要貢獻。

ENGLISH ABSTRACT **Key words:** Acute exacerbation, hepatitis B virus, variant, viral load, full-length, genome, prospective

The onset of acute exacerbation (AE) during the course of chronic hepatitis B is likely related to the break of balance between virus and host immune responses. Whether such a break of immune tolerance is triggered major by the changes of host immune status or by an alteration of HBV hepatitis B virus (HBV) genome still remains controversial. To address this issue, a prospective study from silent (asymptomatic) stage to acute exacerbation and a full-length sequencing strategy are needed.

Although the underlying mechanism remains unknown, clinical observations and animal studies clearly show that an upsurge of viral load always precedes or sometimes coincides with AE in chronic hepatitis B. Thereby, a key to the understanding of these AEs seems to unravel the origin and identity of such a viral surge. To clarify these issues, **in the first year**, we have already collected the clinical and serological data from 14 patients who developed acute exacerbation of chronic hepatitis B spontaneously, after discontinuing lamivudine treatment, or during interferon treatment. We first regularly monitored the serum ALT levels and HBV DNA levels during the development of acute exacerbation. Our results consistently showed that serum viral load resurged before the maximal hepatitis activity in 13 (93%). We then performed full-length HBV genome sequencing from the serum samples obtained at 4 points: at enrollment, at the peak of serum viral load, at the peak of serum ALT level, and after acute exacerbation. We found that the viral genome at virologic peak remained almost identical to that at baseline in 12 (86%) of them. On the contrary, the viral genomes obtained after the development of hepatitis exacerbation are different from the corresponding one at baseline in 7 (50%).

Our preliminary results suggested that the development of hepatitis B exacerbation was not related to the changes of HBV genome. Nevertheless, from a viral evolution point of view, we need to consider the origin of the HBV strains at baseline. Some viral strains may exist and remain inactive in the host for a long time before reactivation, as is the case of chemotherapy-induced AE. The other baseline viral strains, especially those from repeated spontaneous AE, may just represent survivors from previous episode of AE. These HBV survivors may either be new viral variants selected out from host immune surveillance in previous exacerbation, or they may remain the same strain as prior to preceding AE and again trigger the current episode of exacerbation.

Our previous results suggested that the development of hepatitis B exacerbation was not related to the changes of HBV genome. Nevertheless, from a viral evolution point of view, we need to consider the origin of the HBV strains at baseline. Some viral strains may exist and remain inactive in the host for a long time before reactivation, as is the case of chemotherapy-induced AE. The other baseline viral strains, especially those from repeated spontaneous AE, may just represent survivors from previous episode of AE. These HBV survivors may either be new viral variants selected out from host immune surveillance in previous exacerbation, or they may remain the same strain as prior to preceding AE and again trigger the current episode of exacerbation. (Liu CJ et al, Gastroenterology 2003)

AE of chronic hepatitis B is usually preceded by re-emergence or increase of HBV in the serum. To investigate their origin, **in the second year,** we compared the identity of the serum viral genome to that in the liver and in previous AE by full-length sequencing. The full-length viral

genome and extent of quasispecies were obtained from serum and liver biopsy specimens at the same time from 9 subjects with hepatitis B exacerbation (group I). Composition of viral quasispecies was compared by the genetic diversity and the average number of nucleotide substitutions within and between different viral sources. Another two patients with repeated AEs (group II) were also enrolled and their serial serum ALT, HBV DNA levels and full-length sequences were determined. In all group I patients, serum viral genome was identical to that in the liver. The genetic diversity and the average number of nucleotide difference were also comparable between serum and liver tissue. In two group II patients, viral variant emerged after previous AE was not identical to that caused subsequent AE. Dominant viral strains for serial AEs in a single patient did not show a sequential evolution, but presented as a horizontal selection of a minor population from the original viral pool. The findings suggested that serum viral strain reflects the intrahepatic one in AE. Random reactivation of the original HBV pool, other than a sequential evolution of one strain, also contributes to the onset of repeated AE.

In the third year, the above full-length genomic sequencing and comparison work has been finished. In addition, the serial serum samples have been collected and cytokines/chemokines including Th1 and Th2 cytokines immediately before the onset of hepatitis B exacerbation will be determined to see if certain antiviral host factors become defective before AE. Furthermore, since all serum and intrahepatic HBVs originate from the covalently closed circular DNA (cccDNA) within the nucleus of the infected hepatocyte. We have already compared the nucleotide sequences and genetic complexity among serum viral genome, intra-hepatic one and the cccDNA. (Liu CJ et al, Hepatology 2004)

We have provided evidence to clarify whether alteration of viral genome or changes in host cytokine profiles is related to the trigger of AE. Future studies should focus on the host factors dysfunctional before AE and allow active replication of HBV.

成果報告：

The main goal of the three-year project had been achieved and relevant results have been published in Gastroenterology year 2003 and Hepatology year 2004. Attached please find our original articles disclosing the relevant sections of the project final report.

We have already collected serial serum samples for cytokines and chemokines assays right before, during and after the process of acute exacerbations. We will performed these assays soon and hope to identify the critical host factors relevant the development of exacerbations in these patients.

已發表文獻：

1. **Liu CJ**, Chen PJ, Lai MY, Kao JH, Chang CF, Wu HL, Shau WY, Chen DS. A prospective study characterizing full-length hepatitis B virus genomes during acute exacerbation. Gastroenterology 2003;124:80-90.
2. **Liu CJ**, Kao JH, Wang HY, Lai MY, Chen TC, Chen PJ, Chen DS. Origin of serum hepatitis B virus in acute exacerbation: Comparison with HBV in the liver and from other exacerbation. Hepatology 2004;40:310-317.

SELF EVALUATION:

EXPECTED RESULTS:

In the 3-year project, certain important results have been achieved as expected and published, as described in the following.

(1). Correlation between serum surge and hepatitis activity, and temporal relationship between emergence of viral variant and hepatitis B exacerbation We documented that viral load increased in all patients developing acute exacerbation, and further clarified the relationship between the increase of viral load and the peak of hepatitis activity. Most importantly, we identified that reactivation rather than variation of pre-existing HBV strain is more likely responsible for hepatitis exacerbation in chronic hepatitis B. **(achieved)**

(2). Impact of viral evolution on the trigger of acute exacerbation (Years 1-3). We collected patients who developed consecutive episodes of acute exacerbation. By serially determining the full-length viral genomes in the same individuals, we elucidated viral variants emerged during and after the development of acute exacerbation; and the emerged viral variant was not related to the next episode of exacerbation in the Years 2-3 project. **(achieved)**

(3). Identity of serum and liver dominant viral genomes (Year 1-2). By determining the serum and liver dominant viral genomes obtained at the same time, we clarified the serum HBV strain can represent the pathogenic viral strains which are responsible for the liver damage. **(achieved)**

(4). Correlation of serum cytokine profiles and the development of exacerbation (Year 3). By determining the serum cytokines serially before and during the development of hepatitis B exacerbation, we hope to identify if such noncyolytic antiviral mechanisms are disturbed before exacerbation. **(ongoing)**

(5). *In vitro* phonotype assays of HBV variants (Years 2-3). From subcloning experiments and *in vitro* phenotype assays, we documented the evolution of viral quasispecies, and clarify the emerged viral strain occurring during acute exacerbation did not possess unique replication advantages or secretion behavior. **(achieved)**

ANTICIPATED TRAINING FOR RESEARCH ASSISTANT:

As expected, the research assistant has learned how to collect samples, how to extract serum and liver viral DNA, how to perform PCR, direct sequencing, cloning experiment, *in vitro* transformation and transient transfection assays, etc. These techniques will be of much help to the assistant, for future studying or work.