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摘要

B 型肝炎病毒(HBV)DNA 嵌入之情形存在於兒童型肝細胞癌(HCC)中。為了研究 HBV 是在什麼時期發生嵌入行為以及其在癌症前期的角色，從 14 名罹患慢性肝炎兒童(含一名 HBV 相關肝硬化病童)得到之 15 個肝組織檢體進行逆向聚合酵素鍊反應(IPCR)法之分析。 此外，本研究中亦針對得自 9 名 HCC 病童之腫瘤及非腫瘤組織進行研究以作為比較。全部 23 名病人中，6 名是處於高度病毒複製期，17 名是低病毒複製期。

14 名慢性 B 型肝炎患者中至今有 3 名被偵測到重組過之 HBV 基因體。在 9 名 HCC 病童中，每人以 IPCR 方法可自 HCC 組織獲得 1 至 2 個宿主病毒交接序列。同時，自非腫瘤部分可獲得多個嵌入型 HBV DNA。除了 2 個 IPCR 產物因病毒 DNA 重組而使得病毒交接點落在 DR1 以外之區域，其他的嵌入型 HBV DNA 之病毒交接點均位於 DR1 附近。

本研究中採用相當敏感之 IPCR 方法來偵測病毒基因體。我們的初步分析發現了在慢性肝炎時期已發生重組之 HBV DNA，這顯示了嵌入宿主基因體之可能性。另外我們也證實在肝癌前組織與肝癌組織中，較慢性肝炎組織容易找到 HBV DNA 嵌入之情況。 HBV DNA 嵌入的時機，或許並非如早先我們所熟知的僅在癌前期或癌發生期，而也可能發生於更早之慢性肝炎時期。當然更進一步的確認將有賴於從慢性肝炎肝組織之基因體中選殖出來病毒-宿主接合點核酸序列來。

關鍵詞：B 型肝炎病毒，兒童肝細胞癌，病毒基因體嵌入

ABSRACT

Hepatitis B virus (HBV) DNA integration is often detected in childhood hepatocellular carcinomas (HCC). To study how early HBV is integrated and its role in pre-cancerous stage, 15 liver tissues obtained from 14 children with chronic hepatitis including one liver tissue from a child with HBV-related liver cirrhosis, and three non-tumorous liver tissues from three children with HCC were analyzed by inverse polymerase chain reaction (IPCR) method. For comparison, 9 tumor tissues from the 9 HCC children were also studied for comparison. IPCR method had the advantage over the conventional southern blot method for it requires only a minimal amount of tissue DNA to detect the integrated viral genomes. Among the 23 patients studied, 6 were in highly replicative phase, 17 were in low replicative phase.

Rearranged HBV DNAs, which were possibly integrated into the host genome, were isolated from the liver of 2 chronic hepatitis and 1 HBV-related cirrhosis children. In the 9 children with HCC, one to two host-viral junctions were amplified from 9 HCC tissues by IPCR. In the meantime, multiple HBV integrants were obtained from their non-tumor counterparts. All the integrated viral junctions were located near DRI, except two IPCR products with viral DNA rearrangement.

In this study, a relatively sensitive strategy, IPCR, was used to detect the integrated viral genome. Our preliminary analysis revealed the possibility that HBV DNA integration into the host genome occurred at least in some children with chronic hepatitis B. In addition, we also proved that integrated HBV DNA and the host-virus junction sequences were more frequently isolated from the precancerous and cancer liver tissues. Therefore, the timing of HBV DNA integration, in contrary to what was generally believed, may appear not only during the precancerous or HCC development stages, but also in earlier stages. Further confirmation of our results will depend on cloning of the viral-host junction sequences from the chronic hepatitis liver tissues.

Key word: hepatitis B virus (HBV) ; childhood hepatocellular carcinoma ; integration of HBV DNA into host genome

INTRODUCTION

Chronic hepatitis B virus (HBV) infection may lead to chronic hepatitis, liver cirrhosis, and even hepatocelluar carcinoma (HCC). In hyperendemic areas such as Taiwan, chronic HBV infection begins very early, since infancy or early childhood. Perinatal transmission of HBV from carrier mothers to their infants is an important route of transmission (1). It accounts for about half of the cases for chronic HBV infection (2). The other half is from horizontal transmission, which occurs mainly during childhood (3).

HBV replicates through reverse transcription of an RNA intermediate, as retroviruses do (4). Insertional mutagenesis has been demonstrated to be an important oncogenesis mechanism of slow transforming viruses (5). The mechanism of hepatocarcinogenesis is unclear, but is considered to be a multi-step process. It is speculated that HBV DNA integration may promote liver carcinogenesis by integration into the sites close to the gene(s) controlling cell growth. It could be achieved either by altering the nature of these gene products through insertional mutagenesis or by changing the gene expression levels via transregulation. Most HBV related hepatocelluar carcinomas contain integrated HBV genomes (6). Two HBV integrants in human were mapped to genes relevants to cell growth (7,8), although most of integration patterns of HBV genome in HCC seem to be non-specific and occur randomly. We have demonstrated integration of HBV genome into tumor and non-tumor liver tissues in children with HCC by southern blot hybridization methods (9). However, how early the event of integration of HBV DNA into host genome occurs in children during the natural course of chronic HBV infection remains unknown. This is an important issue because the antiviral agents should be intervened before integration occurs. It has been showed by Goto et al. (10) the integration of HBV DNA into the liver tissues in children with chronic HBV infection by southern blot hybridization method. Another study showed contradictory results and failed to demonstrate the integrated HBV genome in the liver tissues of childhood chronic hepatitis (11). Yaginuma et al. reported that viral junction at the integration site were restricted to DRI in one childhood HCC and one chronic hepatitis tissues (12). This issue obviously needs to be clarified, particularly by a more sensitive method.

Since the liver tissues obtained by needle biopsies from children with chronic HBV infection are small, it is difficult to adapt the conventional genomic cloning methods to study the viral genome integration. We have successfully established a method of inverse polymerase chain reaction (PCR) to study HBV integration in small pieces of tissue samples with high sensitivity (13). This method can overcome the difficulty in obtaining a relatively large amount of liver for Southern hybridization to study HBV integration into liver genomes. The main procedures of inverse PCR include digestion of small amount of tissue DNA fragments, ligation to circular-form DNA, and then amplification of the circular-form DNA by PCR using primers designed inversely towards the unknown flanking sequences. Although no specific viral junction has been found for HBV integration, about half of the integrants have at

least one of the virus ends located near the 11-base-pair direct repeat sequences (DR1 and DR2) or within the cohesive region (Coh type)(14-17). The Coh type integrants can be grouped into four patterns by their viral end specificity and strand polarity (16-18). They include integrants with one viral end at DRI and the viral DNA extending either downstream through the core gene (type Ⅰ) or upstream through the X gene (type Ⅱ) and the integrants with one viral end at DR2 and the HBV sequence extending through the core gene (type Ⅲ) or the X gene (type Ⅳ) (Fig. 1).

In this study, we have investigated HBV DNA integration in the host. We have studied the integration of HBV DNA into the host genome in children with chronic HBV infection by a more sensitive method, which is inverse PCR. Four groups of HBV primer pairs near DR1 or DR2 were used for amplification of viral-host junction in type Ⅰ -Ⅳ integrants. The flanking sequences of host genome and the sequences of integrated HBV were analyzed when integration of HBV DNA was noted. Through this study, we can generate ample data to better understand the host-virus integration status in children with chronic HBV infection.

SUBJECTS AND METHODS

SUBJECTS

Totally 415 HBsAg carriers children have been long-term followed once every six months. At each visit, physical examination, blood test for live function profiles and HBV markers were tested.

Totally 14 liver tissues from 13 (with one child receiving 2 times of biopsy) of the long-term followed 415 children with chronic HBV infection were studied by inverse PCR to test whether integration of HBV DNA exists or not. Liver biopsy was performed in these children with parental consent. In order to avoid the interference of HBV free form DNA, 10 of these liver tissues were randomly selected from nine anti-HBe seropositive children at biopsy. For comparison, liver tissues from a child with liver cirrhosis and tumor and non-tumor liver tissues from another 9 anti-HBe seropositive children were also obtained through random selection. The clinical details of these 24 children are listed in Table 1.

METHODS

HBV Markers:

Hepatitis B surface antigen and its antibody (HBsAg and anti-HBs), antibody to hepatitis B core antigen (anti-HBc), hepatitis B e antigen and its antibody (HBeAg and anti-HBe) were tested by radioimmunoassay using the commercially available kits manufactured by Abbott Company (Dianansbott, North Chicago, U.S.A.).

Liver Histologic Examinations:

Liver tissues were obtained through needle biopsy or wedge biopsy during operation. The liver tissues were snap-frozen in liquid nitrogen immediately for storage until use, or fixed in formalin, sectioned and stained with H & E, Masson- Trichrome stain, and

immunocytochemical stains for HBsAg and HBcAg.

DNA Extraction:

Genomic DNA of Liver tissues from children with chronic HBV infection were extracted by a commercial kit (QIAamp DNA Mini Kit, QIAGEN). The extracted DNA was diluted to the concentration of 1 ng/ul and stored at -70℃ and subjected to inverse PCR.

Inverse PCR:

One μg of liver tissue DNA was digested with restriction enzyme either *Sau3*AI, *Aci*I, or *Bfa*I (New Endgland Biolabs, U.S.A.) at 37℃ for 4 hrs., then the enzyme was inactivated at 68℃ for 15 min. DNA fragments was self-ligated at a concentration of 0.5 ng/ul, at 16℃ for 18 hrs, then the ligase was inactivated at 94℃ for 15 min. Four groups of HBV primers, designed as H, B, R, and D (internal primers H2 and P5, B2 and B1, R2 and H5, D2 and D1; external primers H4 and P11, B4 and B3, R4 and P11, D4 and D3), were synthesized for type Ⅰ, Ⅱ, Ⅲ, and Ⅳ integrants, respectively (Fig. 1).

To perform the first round of PCR , 2.5, 5, 10 and 15 ng of circular form of tissue DNA were added to each reaction tube which contains 1X PCR buffer (Protech, Taiwan) 15 pmol of internal primer pairs, 100uM of deoxy-nucleoside triphosphate (dNTPs), a 1 unit of DNA polymerase (Protech, Taiwan) in a total volume of 50 ul. The PCR was set 94℃ for 1 min, primer annealing at 50℃ for 1 min, and extension at 72℃ for 2 min. In the second round PCR, 4ul of the first inverse PCR product was then added to a new reaction tube containing 15 pmol of external primer pairs and the same mixture was as used in the first round of PCR. Forty cycles were performed with the same program.

The inverse PCR product was digested with *Sau3*AI (for type Ⅰ and type Ⅱ products) or *Aci*I (or *Bfa*I; for type Ⅲ and type Ⅳ products) and subjected to gel electrophoresis with 3% Nusieve agarose gel (Sigma, St. Louis, MO USA) to confirm the authenticity of the products. The sizes of expected IPCR products derived from free HBV DNA are about 270-bp from type Ⅰ products, 300-bp from type Ⅱ , 265-bp from type Ⅲ , and 490-bp from type Ⅳ products. The products of the above-mentioned sizes were not further analyzed in this study. Other bands with expected size-shift during electrophoresis were picked for cloning and sequencing. That is, loss of 53-bp after enzyme digestion in type Ⅰ products, loss of 76-bp in type Ⅱ products, loss of 63-bp in type Ⅲ products, and loss of 58-bp in type Ⅳ products.

Nested PCR:

To confirm whether the IPCR products were from rearranged HBV, direct PCR was performed on undigested liver genomic DNA template using two pairs of primers designed for each IPCR clone. For clone HgS11, the first pair of primers is primer P3: 5' tctgctctgtatcgggagg 3' (HBV sequence 2001-2019) and primer H2 (described in previous paragraph). The second pair of primers is primer P4: 5' ttagagtctccggaacatt 3' (HBV sequence 2022-2040) and primer H4 (described in previous paragraph). For clone 189S38H, the first

pair of primers is primer P5: 5' gacaccgcttcagctttgt 3' (HBV sequence 1992-2010) and primer H2. The second pair of primers is primer P6: 5' gaatccttagagtctcctg 3' (HBV sequence 2016-2034) and primer H4.The PCR was performed with the same condition as described in our previous report (28). Again, the PCR products were subjected to gel electrophoresis with 3% Nusieve agarose gel (Sigma, St. Louis, MO, USA) to confirm the authenticity of the products.

Cloning and Sequencing:

The IPCR DNA products were precipitated by alcohol and was inserted to the cloning vector (pGEM-T Easy Vector System, Promega, Madison, Wisconsin, USA). Through transformation into JM 109 competent cells and maxipreparation, a large amount of plasmids containing the IPCR products were obtained and were subjected for sequencing. DNA sequencing was performed using AmpliCycle Sequencing Kit (PE Applied Biosystems, Greenwich, CT, U.S.A.). Radioactive $^{\alpha}$33P-dATP-labelled T7 and SP6 sequences were used as primers for sequencing reaction. The obtained sequences were then subjected to computer comparison with HBV and human sequences in GeneBank.

RESULTS

Clinical Course and Liver Histology.

The data of the 23 children were shown in Table 1.

Analysis of IPCR Products from Liver Samples of Children with Chronic Hepatitis

Fourteen liver samples from 13 children with chronic hepatitis and one liver tissue from one child with liver cirrhosis were analyzed by IPCR method. According to the results of enzyme digestion and electrophoresis, analysis of the PCR products revealed a major band of 270-bp in samples amplified by type I –specific primers. Minor bands of various sizes lost expected size after *Sau*3AI digestion, which were subsequently cloned and sequenced.

Products of free form HBV DNA also were amplified by other three types of primers in eight samples. However, other minor products did not show expected shift patterns after enzyme digestion. These results indicated that free form HBV DNA was the template for amplification. The integrants might not exist since they were undetectable by this method.

The inverse PCR products in six children had larger sizes than presumed self-ligated HBV *Sau*3AI, *Aci*I, or *Bfa*I cut fragments, and showed expected shift after enzyme digestion. They were considered as candidate products containing possible integrated HBV DNA and its flanking cellular sequences, thus were subjected for further analysis. However, cloning and sequencing of these products showed that they were products caused by mutation of the *Sau*3AI cutting site of HBV at nucleotide 1983 from GATC to CATC in three children, at nucleotide 1984 from GATC to GGTC in one child, at nucleotide 1633 from GATC to GGTC,

and by mutation of the *Aci*I cutting site at nuleotide 1471 from CCGC to CCGG in one child (Table 2). The results showed that a point mutation at viral *Sau3*AI or *Aci*I sites was present in these products and no cellular sequences were obtained. We suggest that these major bands may derive from free viral DNA. (Table 2). Interestingly, in addition to these clones, we also isolated 3 unique HBV DNA-containing IPCR products (Table 2 and Fig. 2) from three of the same group of children (case12, 13, and 14; 2 with chronic hepatitis and 1 with HBV-related cirrhosis). These sequences are unique since they do not contain mutations or polymorphism of *Sau*3AI or *Aci*I sites. Instead, they seemed to be amplified from rearranged HBV DNA templates. A possible mechanism to explain the result was shown in Fig. 3 using clone HgS11H as an example. However, these 3 IPCR products did not contain host genome sequences. This could be partially attributed to the repetition nature of the rearranged HBV DNAs, which make two *Sau3*AI sites (or other enzyme sites used in IPCR) so close that the viral sequences between them can be readily amplified in IPCR. To confirm these products were not derived from DNA recombination caused by enzyme digestion and religation procedures during IPCR, we designed two pairs of primers which are adjacent in the sequence of HBV genome and performed direct PCR on undigested liver genomic DNA from these patients (Fig. 2A and 2B). The sizes of nested PCR products from HgS11H and 189S38H were the same as what we expected to see in the rearranged HBV DNA (Fig.2 and data not shown). Therefore, it is highly suggestive that at least these 2 IPCR products were amplified from originally rearranged HBV genomic DNA template. As for clone 121S22H (Fig. 2C), we are now determining whether it also arose through a similar mechanism. In addition, since some very minor products may not be successfully cloned in the experiments, we cannot conclude definitely that these children have no HBV integration in the cellular sequences.

Virus-Host Junction in Children HCCs and Their Non-Tumor Counterparts

To study HBV integration in livers in the pre-cancer status, the non-tumor liver tissues from 9 HCC children were analyzed by IPCR method. After sequencing and computer comparison, nine DNA fragments containing HBV-host junctions were obtained from ten HCC tissues, which were designated as 669Ha, 669Hb, 1144H, 1743Ha, 1743Hb, 29615H, 10285H, 19031H, and 17439B. We also found that multiple viral-host junctions were present in the non-tumor counterpart from one patient. Indeed, twelve IPCR products contained flanking cellular sequences of integrated HBV were obtained from the non-tumor parts and designated as 663N, 1132Na to 1132Ng, 1741 Na, 1741Nb, 90114H, and 121132H (Table 3 and Fig.4). The sizes of the inserted cellular DNAs in each IPCR product ranged from 30 to 500 bp. All of these obtained viral-host junctions were located near DR1, except 1743Hb, which was located at nucleotide position 3219 of HBV, and 17439B, which was located at nucleotide position 1789. Both were probably due to HBV DNA rearrangement. In addition, we also isolated 6 (2 from HCC tissues and 4 from non-tumor parts) clones (Table 3, 19059H, 90113D, 90110A, 21855H, 21752H, and 10281H) which may arise from the rearranged and integrated HBV DNA templates. However, the viral-host junctions could not be identified in

these clones either.

DISCUSSION

This study has investigated liver tissues obtained mainly in the low viral replication phase, as represented by seronegative HBeAg. This is because (1) this phase was regarded to be a later stage of HBV infection and more likely to detect viral integration into the host genome; (2) to prevent the interference of large amount of free form HBV DNA. Yet the results of electrophoresis revealed that there was still some free form HBV DNA in the liver of the anti-HBe seropositive children. Sequencing of these PCR products which are putative candidate products of integration, turned out to be free form HBV DNA with mutation at the restriction enzyme cutting sites. Therefore, it is very important to know this caveat and to exclude these products in further analysis.

It is of great interest that from livers of 2 chronic hepatitis and 1 HBV-related cirrhosis patient we isolated 3 IPCR products (clone HgS11H, 189S38H, and 121S22H) which may derive from rearranged HBV DNA templates. The results of direct nested PCR using undigested cellular genomic DNA from 2 of the 3 patients as the template further indicated that the rearrangement of HBV DNA was not caused by enzyme digestion and religation occurring in the IPCR procedure. It is understandable that viral-host junctions were not identified in these chronic hepatitis clones, since the rearrangement created repeated HBV sequences which spanned the sites of primers and restriction enzymes (e.g. *Sau3*AI site) we chose in IPCR and may thus interfere the cloning of putative host sequences. However, currently we are designing new experiments to circumvent this obstacle and to obtain direct evidence supporting the existence of integrated HBV DNAs in host genome of these children. To our knowledge, no such large-scale DNA rearrangement has ever been identified in free-form HBV genomes. In contrast, it is well known that both the cellular and the integrated HBV DNAs undergo frequent and various DNA rearrangements, at least in HCC tissues or cell lines (20). Therefore, it is likely that these IPCR clones were amplified from the integrated and rearranged HBV DNA in children with chronic hepatitis. Indeed, two previous reports (10, 12) have showed that integrated HBV DNA could be found in the host genome from patients with chronic hepatitis. Our results is the third report to describe this finding and may help shape the hypothesis that HBV integration and rearrangement may occur in earlier stage during chronic hepatitis infection. Further clarification of this point may shed some light into the understanding of the role played by HBV DNA integration in HCC development.

In addition, we have successfully cloned HBV-host junction sequences from childhood HCC tissues by IPCR method (Fig. 4). The respective non-tumor tissues of HCC children are good materials for studying the status and the effect of HBV integration in the pre-cancerous stage of the liver. Nine HCC tissues and their non-tumor counterparts were analyzed in this study. The electrophoresis and sequencing results showed that at least in some HCC patients (Table 3, e.g. case 16 and clone 1132Na-Ng), more integrated forms of viral DNA seemed to be present in non-tumor parts of the livers than the tumor parts. This finding is consistent with

the monoclonal nature of tumor cells and the polyclonal nature of the non-tumor tissues. All viral junctions obtained were located near DR1, except two products located at HBV nucleotide position 3219 and position 1789 due to viral DNA rearrangement. Although IPCR products amplified by type II-IV primers were also present in eight tissue samples, they were finally demonstrated to be free form or rearranged HBV DNAs. Interestingly, as we found in chronic hepatitis children, several IPCR products isolated from HCC or its non-tumorous portion (Table 3) were also presumed to derive from rearranged HBV DNA templates. This is also consistent with previous knowledge (9, 12, 20) that rearrangements of integrated HBV DNA are frequently encountered in host genomes of HCC or pre-cancerous livers. Moreover, the rearrangement of HBV DNA also seemed to interfere the isolation of viral-host junctions in these clones.

Few previous reports could demonstrate HBV viral genome integration into the host genome (10-13). According to our results, we agree with the generally accepted conception that compared to HCC and its precancerous liver, integration of HBV genome into the host DNA may not occur frequently or extensively in the liver cells of children with chronic hepatitis, since the integrated sequences were not easily identified even using the very sensitive method like IPCR. Alternatively, the integration may have occurred in some chronic hepatitis children with a different way (e.g. complex rearrangement) which escaped our detection due to the intrinsic limit of the IPCR method. In contrast, it is more common to identify the integrated HBV DNAs in the tumor and non-tumorous liver tissues of children with HCC. Once it occurs, HBV DR1 is a preferred viral junction to be integrated. However, we would like to emphasize again the exciting possibility suggested by our results that at least in some children with chronic hepatitis B, rearranged HBV DNA existed in liver cells and it is likely that these rearranged DNAs may have integrated into the host genome. The possible biological significance behind it can not be overlooked, since early integration of HBV may have impacts on the expression of viral proteins, on the growth and death of host hepatocytes, and more importantly, on the development of HCC. To confirm our observation, further experiments are now underway in our lab, aiming to isolate the putative viral-host junction(s) in livers of chronic hepatitis B.

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FIGURE LEGENDS

Figure 1. Schematic representation of the location of HVB primers relative to four types of HBV integrants. Each type of integrants has one viral end located near viral direct repeat (DR) 1 or 2 as previously described by Robinson (18). The thick and thin vertical arrows indicate restriction enzymes *Sau*3AI and *Aci*I cutting sites, respectively. White bar denotes HBV; hatched bar denotes cellular sequences. Horizontal arrows represent HBV primers designed for amplification of each type of integrants: H set primers for type I integrants, B set for type II, R set for type III, and D set for type IV.

Figure 2. Chronic hepatitis livers have IPCR sequences possibly arising from rearranged HBV DNA templates. The 3 IPCR sequences are from the livers of 2 chronic hepatitis patients (189S38H and 121S22H) and 1 cirrhosis child (HgS11H), respectively. A) showed detailed information of clone 189S38H. Its corresponding locations in HBV genome are shown in the map of A1), which also indicates the relative locations of primers used in IPCR (H4 and P12) and primers for nested PCR (H2 and P5, and H4 and P6). The arrows show the directions (5' to 3') of primers. It is clearly shown that without recombination, the nested PCR on the non-digested original HBV genome can not generate small-sized (e.g. within several hundred base pairs) PCR products. In contrast, the HBV DNA rearranged in a manner as shown in A2) can generate two PCR products of specific sizes (218 bp and 182 bp) in the nested PCR. Similar information of clone HgS11H and the design of primers (H2 and P3; H4 and P4) used to determine possible rearrangement of template HBV DNA are shown in B). Again, if the rearrangement occurs as shown in B2), the two PCR products will be 173 bp and 150 bp in size. No PCR product can be isolated in B1), in which no rearrangement occurs. Finally, some basic information of cone 121S22H is shown in C). For this clone, nested PCR has not been performed to evaluate the possibility of HBV DNA rearrangement.

Figure 3. An illustration of how IPCR product (using HgS11H as an example) was generated from rearranged HBV DNA template. The open line and the solid line, which are joined at the position marked as nucleotide 2088 and 1773, represent two arms of rearranged HBV DNA sequences, respectively. The nucleotides of the left arm are partially repeated sequences of that of the right arm. However, the exact length of repeated sequence and its upstream frontier in the left arm has not been characterized yet. Since the repeated sequence has spanned through the GATC (Sau 3A1 site), the rearrangement will cause enzyme digestion at both ends, religation, and the IPCR product as shown in the lowest panel of the figure.

Figure 4. Summary of the virus-host junction sequences obtained from three non-tumorous tissues. Lowercase letters represent cellular sequences adjacent to HBV. The HBV

primers for IPCR are boxed. The nucleotide number of each viral junction is shown according to Valenzuela et al. (19).

Table1. The clinical details of the 24 children with chronic hepatitis B virus infection, cirrhosis, or hepatocellular carcinoma

Case	Sex	(Initial/Final age)	Peak ALT	Maternal HBsAg	At biopsy or operation			
					Age	ALT	HBe/Anti-HBe	Histology
1	M	8/12	215	P	11	36	P(at@)/N	CH-miAnoF
2	M	3/22	262	P	16	262	P(4Mo before@)/N	CH-miAnoF
3	F	4/17	470	P	12	404	P(3Mo before@)/N	CH-miAnoF
4	M	11/17	481	P	12	304	P(14Mo before@)/N	CH-moAmiF
					13	416	N/P(at @)	CH-miAnoF
5	F	0/15	145	P	1	141	N/P(9Mo after@)	CH-miAnoF
6	M	2/19	65	P	8	12	N/P(6Y after@)	CH-MC
7	F	6/16	68	P	9	8	N/P(6Mo after@)	CH-modAnoF
8	M	4/17	101	N	8	13	N/P(7Mo after@)	CH-MC
9	M	0/23	201	P	7	6	N/P(5Y after@)	CH-MC
10	M	7/26	300	N	18	16	N/P(8Y after@)	*CH-miAnoF
11	F	0/20	160	P	15	102	N/P(3Y after@)	CH-miAmiF
12	M	13/30	1074	N	15	31	N/P(1Y after@)	Ma A mod F
13	F	1/24	830	N	10	16	N/P(1Y after@)	*CH-miAmodF
14	M	11/18	29	?	11	20	N/P	Cirrhosis
15	M	12/12	66	P	12	39	N/P	HCC and non-tumor tissue (cirrhosis)
16	M	10/19	392	P	10	211	N/P	HCC and non-tumor tissue (CH-MC)
17	M	8/8	23	P	8	23	N/P	HCC and non-tumor tissue (cirrhosis)
18	M	1.5/4	201	P	1	201	P/P	HCC and non-tumor tissue (cirrhosis)
19	M	1.5/ 11	620	P	11	40	N/P	HCC and non-tumor tissue (cirrhosis)
20	M	7/8	69	P	8	69	P/P	HCC and non-tumor tissue (cirrhosis)
21	M	14/15	118	P	14	31	N/P	HCC and non-tumor tissue (cirrhosis)
22	M	9/12	31	?	9	26	N/P	HCC and non-tumor tissue (cirrhosis)
23	M	7/8	522	P	7	114	N/P	HCC and non-tumor tissue (cirrhosis)

M=male, F= female, P=positive, N=negative, Mo=month(s), Y=year(s), CH=chronic hepatitis, A=activity, F=fibrosis, mi=mild, mod=moderate, no=no, MC=minimal change, * =with previous histologic change of chronic active hepatitis two to nine years ago, @=HBe seroconversion, ? = unknown or not performed

Table 2. Results of IPCR in 14 children with chronic hepatitis B virus infection (including cirrhosis patient)

Case No.	IPCR clone and the possible explanation	Integration of HB into host genome
1	No	No
2	No	No
3	No	No
4-1	HBV Aci I site mutation (1468-1471 CCGC->CCGG)	No
4-2	No	No
5	HBV Sau3 AI site mutation (1983-1986 GATC->CATC)	No
6	HBV Sau3 AI site mutation (1983-1986 GATC->CATC)	No
7	No	No
8	No	No
9	HBV Sau3 AI site mutation (1983-1986 GATC->CATC)	No
10	No	No
11	No	No
12-1	HBV Sau3 AI site mutation (1983-1986 GATC->CATC)	No
12-2	**121S22H**, possible HBV rearrangement	Possible
13-1	HBV self ligation	No
13-2	HBV Bfa I site polymorphism	No
13-3	**189S38H**, possible HBV rearrangement	Possible
14-1	HBV Sau3 AI site mutation (1632-1635 CATC->GGTC)	No
14-2	HBV Bfa I site polymorphism	No
14-3	**HgS11H**, possible HBV rearranegement	possible

Table 3. Results of IPCR in 10 children with hepatocellular carcinoma

Case No.	IPCR clone, size, and the possible explanation	Integration of HBV into host genome
15-T-1	669Ha	Yes
15-T-2	669Hb	Yes
15-NT	663N, 548 bp cellular seq.	Yes
16-T	1144H	Yes
16-NT1-7	1132Na-1132Ng	Yes
17-T-1	1743Ha	Yes
17-T-2	1743Hb	Yes
17-NT-1	1741Na	Yes
17-NT-2	1741Nb	Yes
17-T-3	17439B, 482 bp with 135 bp cellular seq.	
17-NT-3	No	
18-T-1	19031H, 286 bp with 196 bp cellular seq.	Yes
18-T-2	422 bp, HBV Sau3 AI artifacts	No
18-NT-1	19059H, 256bp, possible HBV rearrangement	Possible
18-NT-2	743 bp, HBV Sau3 AI artifacts	No
19-T	No	No
19-NT-1	90114H, 446 bp with 347 bp cellular seq.	Yes
19-NT-2	90113D, 485 bp, possible HBV rearrangement	Possible
19-NT-3	90110A, 613bp, possible HBV rearrangement	Possible
19-NT-4	156 bp, HBV Sau3 AI mutation and self-ligation	No
19-NT-5	370 bp, HBV self-ligation	No
20-T-1	21855H, 238 bp, possible HBV rearrangement	Possible
20-T-2	363 bp, Sau3 AI site mutaion	No
20-NT	21752H, 226 bp, possible HBV rearrangement	Possible
21-T-1	10285H, 538bp with 463 bp cellular seq.	Yes
21-T-2	102811H, 201 bp, possible HBV rearrangement	Possible
22-T	No	No
22-NT-1	121132H, 356 bp with 264 bp cellular seq.	Yes
22-NT-2	306 bp, Bfa I site mutation	No
22-NT-3	353 bp, HBV self-ligation	No
22-NT-4	508 bp, HBV self-ligation	No
23-T-1	296125H, 797 bp with 712 bp cellular seq.	Yes
23-T-2	1231 bp, HBV self-ligation	No
23-NT-1	273 bp, HBV self-ligation	No
23-NT-2	280 bp, Sau3 AI artifacts	No
23-NT-3	769 bp, HBV self-ligation	No

Figure 1.

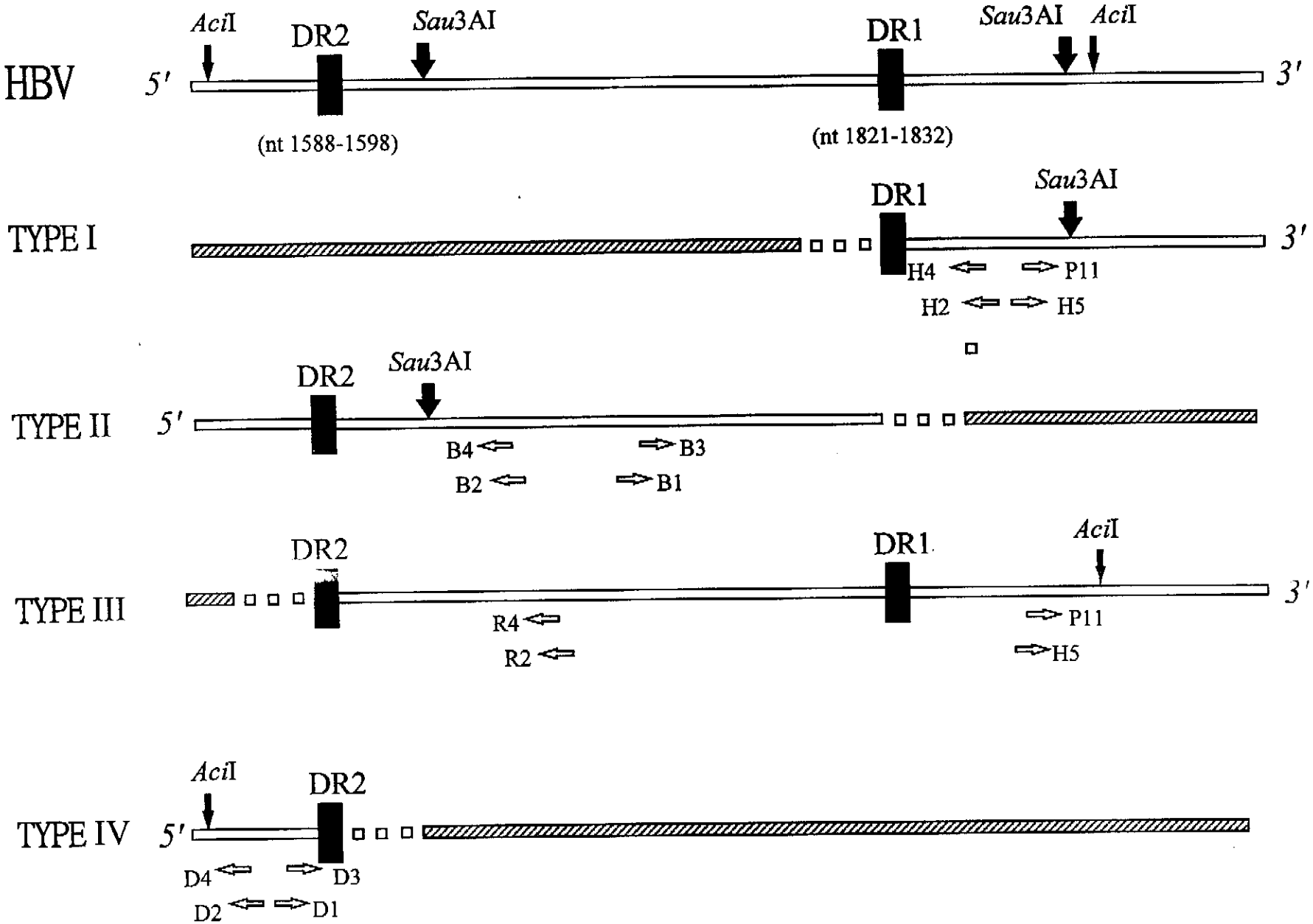
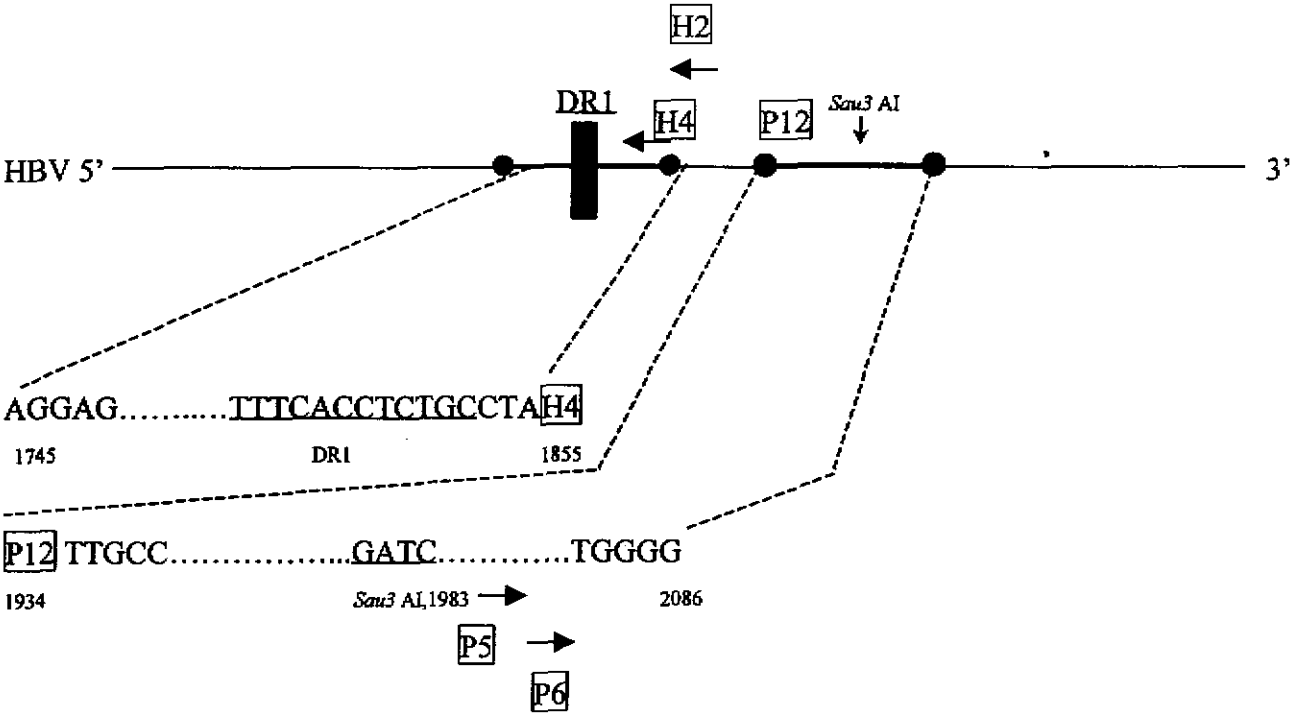

HBV
5′
AciI
DR2
Sau3AI
DR1
Sau3AI
AciI
3′
(nt 1588-1598)
(nt 1821-1832)
TYPE I
DR1
Sau3AI
3′
H4
P11
H2
H5
TYPE II
5′
DR2
Sau3AI
B4
B3
B2
B1
TYPE III
DR2
DR1
AciI
3′
R4
P11
R2
H5
TYPE IV
5′
AciI
DR2
D4
D3
D2
D1

Figure 2.

A)

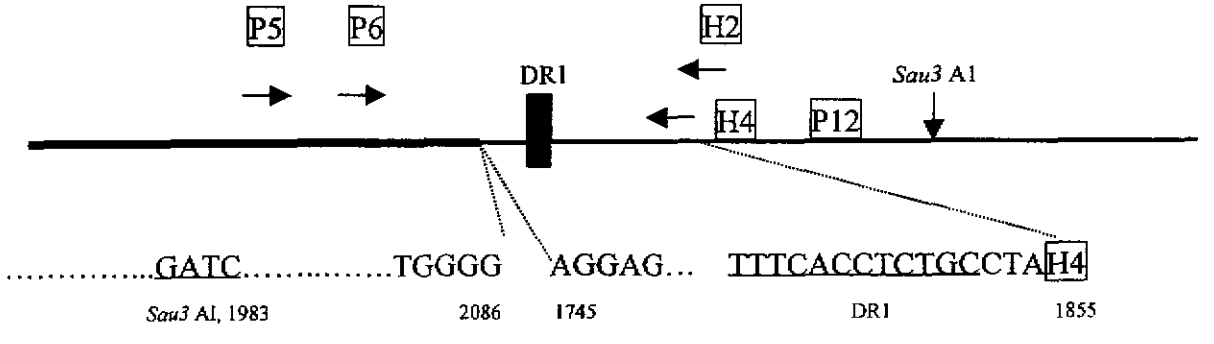
Case No.	Clinical Feature	Clone No.	Size of IPCR product	(IPCR seq.) = Corresponding HBV sequemce	Note
13	Chronic Hepatitis	189S38H	265 bp	(1-153) = 1934-2086 (155-265) =1745-1855	Possible HBV Rearrangement

A1) Original HBV genome



If no rearrangement → no nested PCR product

A2) Rearranged HBV DNA:



If rearrangement occurred: presence of PCR products and the sizes will be:

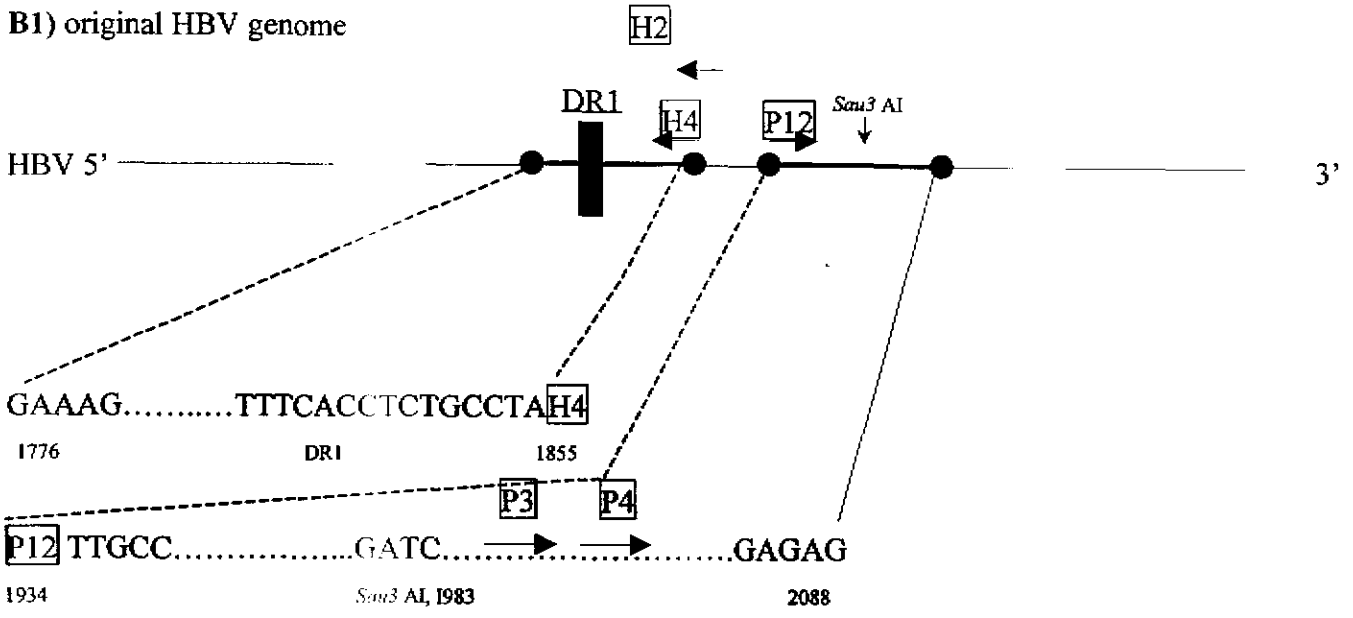
1) H2 + P5: 218 bp

2) H4 + P6: 182 bp

B)

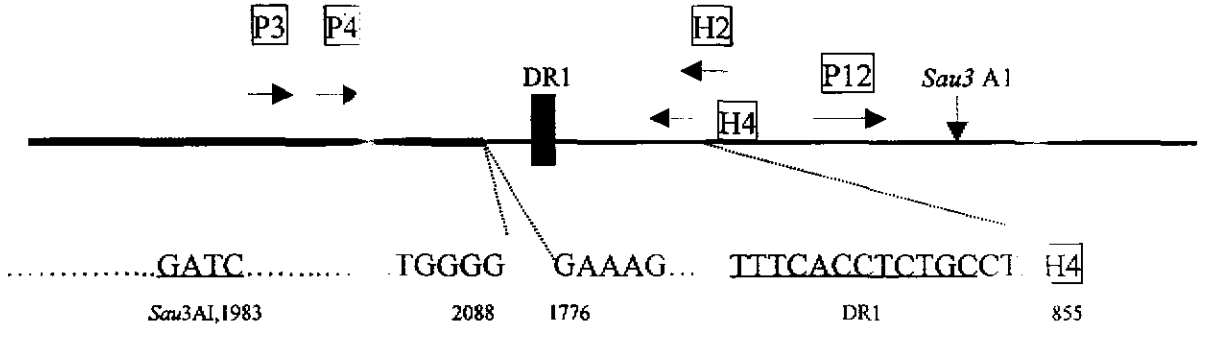
Case No.	Clinical Feature	Clone No.	Size of IPCR product	(IPCR seq.) = Corresponding HBV sequemce	Note
15	HBV-related cirrhosis	HgS11H	238 bp	(1-155) = 1934-2088 (156-238) =1776-1855	Possible HBV Rearrangement

B1) original HBV genome



If no rearrangement → no nested PCR product

B2) Rearranged HBV DNA:

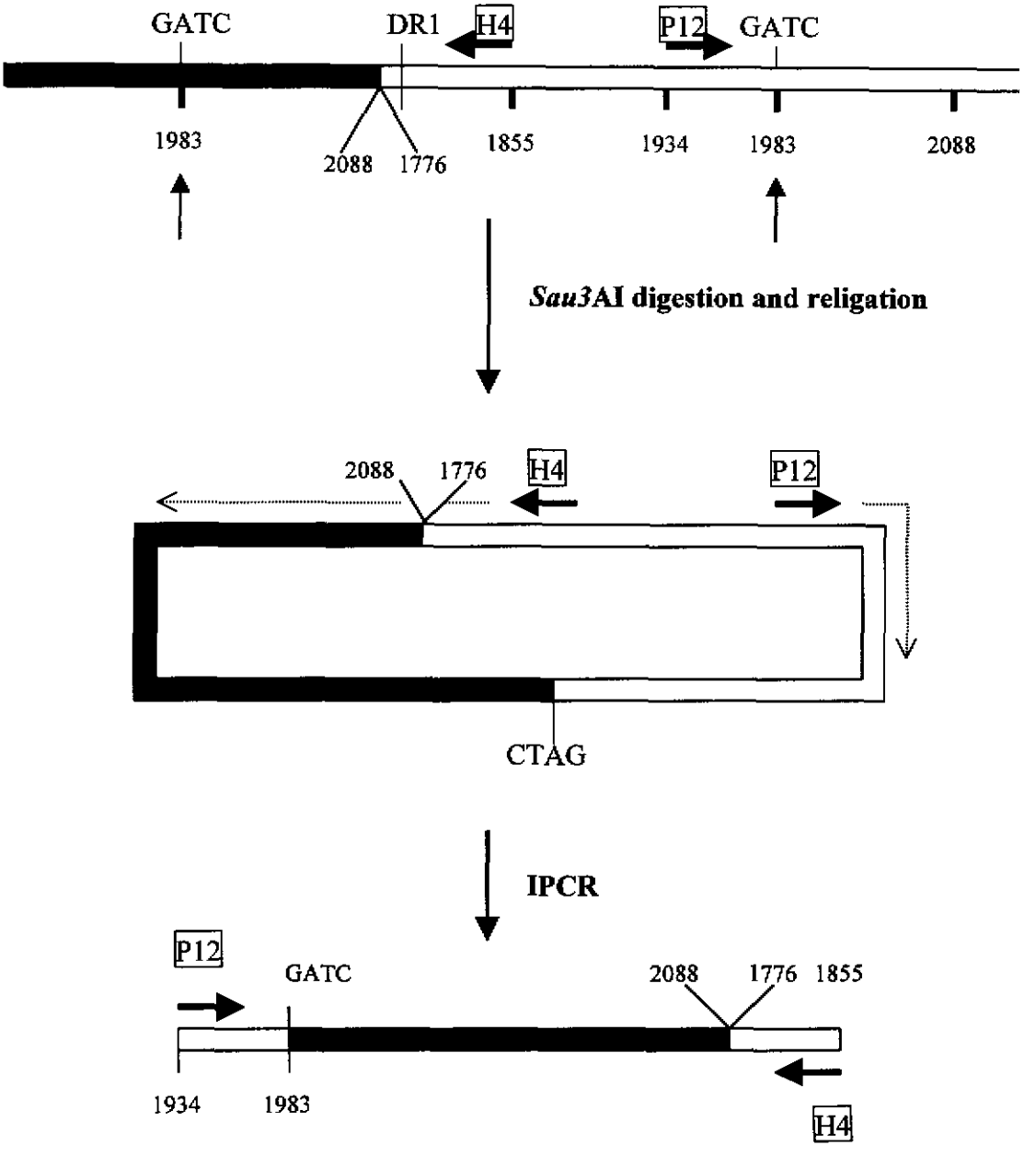


If rearrangement occurred: presence of nested PCR products and the size will be:

1) H2 + P3: 173 bp

2) H4 + P4: 150 bp

Figure 3.


GATC
DR1
H4
P12
GATC
1983
2088
1776
1855
1934
1983
2088
*Sau3*AI digestion and religation
2088
1776
H4
P12
CTAG
IPCR
P12
GATC
2088
1776
1855
1934
1983
H4

1132Nd

.....actaaatattTCG//H4

1830

1132Ne

.....cttgtagaacAA H4

1834

1132Nf

.....caggcatgtgCCTCTGC//H4

1826

1132Ng

DR1

.....catatctcttTTTCACCTCTGC//H4

1820

1743Ha

.....atttctgtgaTCA//H4

1823

1743Hb

DR1

.....agtggtggagTTC//GGTGAACTTTTTCACCTCTGC//H4

3219 3052 1816

1741Na

DR1

.....accctcccacCTAAG//TTTCACCTCTGC//H4

1744 1820

台大醫學院小兒科　張美惠教授

心得報告

會議名稱：第一屆世界小兒胃肝膽營養學大會

開會地點：美國波士頓

會議期間：89年8月5日至8月9日

一、參加會議經過：

大會由8月5日上午8:30開始至8月9日晚上閉幕典禮共五天。本人於8月3日晚上自台北出發，於8月4日抵達波士頓。大會行程緊湊，內容豐富，本人又因擔任亞太小兒胃腸肝膽營養學會會長，乃8名國際執行委員之一，故持別忙碌。

第一天(8月5日)：再教育學程(I)

主題：A Global View of Pediatric Gastroenterology 小兒胃腸學之世界觀。

子題I：肝膽及營養疾病之篩檢

其內容包含篩檢之基本原則，以及Celiac病，炎性腸症，囊狀纖維化及遺傳性胰臟炎，血色素沈積症及威爾森病，病毒性肝炎等疾病之篩檢。其重點在於認識疾病及各地發生率；討論何時是最適當可行之篩檢時機；各種篩檢方法之敏感度，特異性及準確性；如何判讀個別病人之篩檢結果；在不同發生率之地區如何判讀結果；瞭解各種檢查之好處及限制。認識組織transglutaminase抗體測驗在Celiac病篩檢的角色；pANCA及ASCA血清篩檢在炎性腸症之角色，以糞便標記及杜普勒超音波篩檢疾活性之準確性等。

子題II ：消化道疾病之機轉：

其內容涵括：

a. 脂肪酸先天代謝異常與肝病：瞭解脂肪酸之運送及在粒線體氧化之疾病的機轉及臨床與生化表現；其在兒童猛暴性肝炎重要角色；如何作正確診斷等。
b. 腸神經系統與消化道蠕動疾病，瞭解中樞神經系統與腸神經系統之雙向溝通，此對治療功能性腸病新治療之發展有助益。

c. 胃腸黏膜之癒合轉：瞭解調節胜肽對促進癒合之效果及機轉，及未具治療潛力之新方法。

d. 肥胖與體重調節及食慾的關係，及對成功治療之啟示，

e. 食物過敏的免疫生物學之機轉，及對診斷及治療之進一步瞭解。

第二天(8月6日)

子題III：小兒消化學疾病之治療：進入下一世紀之展望

其內容涵蓋：

a. 肝炎之預防與治療，由本人演講。

b. 基因治療在胃腸肝膽疾病之應用。

c. 下一世紀炎性腸症之治療

d. 門脈高壓之新治療法

e. 小腸移植結果的進步。

第三天(8月7日)

8月7日至9日為大會節目，很高興看到在plenary lecture及symposium中本人、陳慧玲醫師、倪衍玄醫師等人多篇論文被引用。尤其是NIH Hoofnagle醫師引用吾等在全民預防注射後兒童肝癌下降之數據，提到台灣在B型肝炎防治上的成就，可能是本世紀最重要的醫療貢獻之一，此台灣B型肝炎防治大家共同付出之努力與成果令人感到格外的驕傲。

除了我們台灣代表發表二篇口頭及多篇壁報自由論文之外，本人亦擔任meet-the-professor之professor，本人、吳子聰主任與陳偉德教授、亦主持大會自由論文口頭報告。整個大會對於胃腸、肝膽、營養學各領域研究之新進展均有討論，尤其一些新基因的發現，及其功能特性之研究，肝細胞移植，代謝性肝疾病之藥物或酵素治療，炎性腸症之新治療，胚胎發展學之新進展等等，均有新的知識與收獲。

此次參加世界大會在學術及國際交流方面均頗有收穫，是一個令人難忘的回憶。

Hepatitis B Vaccination and Hepatocellular Carcinoma Rates in Boys and Girls

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Tzee-Chung Wu, MD
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for the Taiwan Childhood Hepatoma Study Group

Context Hepatocellular carcinoma (HCC) has a male predominance and is clos related to hepatitis B virus (HBV) infection. Hepatitis B virus vaccination was launcl in 1984 in Taiwan for neonates of mothers carrying hepatitis B e antigen, resultin a decreased incidence of HCC in children. The effect on boys vs girls is not knowi

Objective To evaluate the association between a HBV vaccination program with cidence of childhood HCC by sex.

Design and Setting Analysis of data collected from Taiwan's National Cancer Reg try System and the Taiwan Childhood Hepatoma Study Group between 1981 and 19

Participants Children aged 6 to 14 years who were diagnosed as having HCC (2 boys and 70 girls).

Main Outcome Measure Incidence of HCC in boys and girls before and after imp mentation of the vaccination program.

Results The boy-girl incidence ratio decreased steadily from 4.5 in 1981-1984 (bef the program's introduction) to 1.9 in 1990-1996 (6-12 years after the vaccination pr gram was launched). The incidence of HCC in boys born after 1984 was significantly duced in comparison with those born before 1978 (relative risk [RR], 0.72; $P=.002$). I significant decrease in HCC incidence was observed in girls born in the same periods (R 0.77; $P=.20$). The incidence of HCC in boys remained stable with increasing age, wh an increase of HCC incidence with age in girls was observed. These age and sex effe remained the same regardless of birth before or after the vaccination program.

Conclusion Our results suggest that boys may benefit more from HBV vaccinati than girls in the prevention of HCC.

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HEPATOCELLULAR CARCINOMA (HCC) occurs mainly in adults 40 to 60 years of age.[1] However, in areas hyperendemic for hepatitis B virus (HBV) infection, HCC may develop in children.[2] We have found a nearly 100% hepatitis B surface antigen seropositivity rate in Taiwanese children with HCC, indicating an intimate relationship between HBV and childhood HCC.[2] In 1984, a hepatitis B vaccination program was launched and has effectively reduced the prevalence of HBV infection, chronic HBV infection rate,[3] and incidence of HCC in children in Taiwan.[4]

A male predominance of HCC has long been observed.[5,6] The mechanism is unknown, but a tumorigenic effect of androgens has been suggested.[7] We found a male-female ratio of 3-4:1 in children with HCC,[8] which is similar to that in adults. Since the influence of hormones in children is much less than that in adults, investigating HCC in children may facilitate understanding of the mechanism of HCC. We therefore studied children with HCC by sex before and after the implementation of the HBV vaccination program.

METHODS

Subjects

A vaccination program was implemented in Taiwan in July 1984.[9] Hepatitis B immunoglobulin was given to neonates of highly infectious mothers carrying hepatitis B e antigen. All infants received 3 or 4 doses of HBV vaccine.

According to our previous observation, HCC in children was diagnosed mainly in those older than 6 years,[2] while hepatoblastoma was diagnosed in younger children.[10] In this study, we included children 6 to 14 years of age with liver cancer to preclude the inclusion of hepatoblastoma.

Two independent childhood hep toma registry systems were used in th study to ensure accuracy. Data from t following 2 systems, including the nam identification number, birth date, se

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Members of the study group are listed at the e of this article.

Corresponding Author and Reprints: Mei-Hw Chang, MD, Department of Pediatrics, National T. wan University Hospital, No. 7, Chung-Shan S. Roa Taipei, Taiwan, Republic of China (e-mail: mhcha @ha.mc.ntu.edu.tw).

Table 1. Incidence of Hepatocellular Carcinoma in Boys and Girls Aged 6 to 14 Years in Taiwan, 1981-1996

	Boys			Girls				
Year of Diagnosis*	No. of Cases	Population	Incidence†	No. of Cases	Population	Incidence†	Total Incidence	Male-Female Ratio
1981-1984	56	5 182 103	1.08	12	4 897 529	0.24	0.67	4.5
1984-1990	94	10 753 818	0.87	33	10 143 281	0.32	0.61	2.7
1990-1996	51	10 396 566	0.49	25	9 769 501	0.26	0.38	1.9

*1981-1984, July 1981 to June 1984; 1984-1990, July 1984 to June 1990; and 1990-1996, July 1990 to June 1996.
†Number per 100 000 population.

address, etc, were checked and merged, and any repetition was deleted. The case information was confirmed by the reporting hospitals. The capture-recapture method was used to estimate the total number of cases of childhood HCC (Epi Info, version 6.04; Centers for Disease Control and Prevention and the World Health Organization). The number of cases identified by systems 1 and 2 was estimated to be 86% (95% confidence interval [CI], 80%-92%) of the actual total number of children with HCC.

System 1: National Cancer Registry System

Cases of hepatoma diagnosed between July 1981 and June 1996 were analyzed from the data bank of the National Cancer Registry System at the National Department of Health. This registry was established in 1979. Cases are reported by the department of medical records in each of the 167 hospitals with more than 50 beds in Taiwan.

System 2: Multicenter Childhood Hepatoma Study Group

To ensure the accuracy of the data from the National Cancer Registry, we formed a multicenter Childhood Hepatoma Study Group to register children with hepatoma during the same study period. Pediatric gastroenterologists or oncologists from 17 major hospitals, including all 12 tertiary referral centers in Taiwan, participated.

Statistical Analysis

The study population was stratified both by age at diagnosis and the year of birth. Children with HCC who were older than 6 years on July 1, 1984, when the HBV vaccination program was launched, were born before July 1978. Children born before 1978 and after 1984 were the respective cohorts without and with the effect of HBV vaccination. Children born between 1978 and 1984 were born during the transition to full implementation of the HBV vaccination program. They might have received HBV vaccination beyond infancy.

Age-specific and birth-year–specific incidences of HCC were calculated for boys and girls. Relative incidences of HCC among children divided into groups by age, birth cohort, and sex were analyzed using Poisson regression.[11] The modification of age effect on HCC incidence by sex was statistically tested by cross production of age and sex variables (the interaction term) and expressed in separate models when the age trends were significantly different between female and male.

Table 2. Effect of Birth Year on the Development of Childhood Hepatocellular Carcinoma by Sex*

	Boys		Girls	
Birth Year	RR (95% CI)	*P* Value	RR (95% CI)	*P* Value
1966-1977	1.00 (Referent)		1.00 (Referent)	
1978-1983	0.83 (0.71-0.96)	.02	1.02 (0.79-1.30)	.90
1984-1989	0.72 (0.59-0.89)	.002	0.77 (0.52-1.15)	.20

*RR indicates relative risk; CI, confidence interval.

RESULTS

A consistent predominance of HCC in boys was found throughout the observation period. The incidence of childhood HCC declined gradually in boys during 1981-1996, while the incidence in girls remained stable (TABLE 1). Although the trend of the predominance in boys remained, the boy/girl ratio of the incidence of HCC declined gradually with time from 4.5 for years of diagnosis 1981-1984 to 1.9 for years of diagnosis 1990-1996.

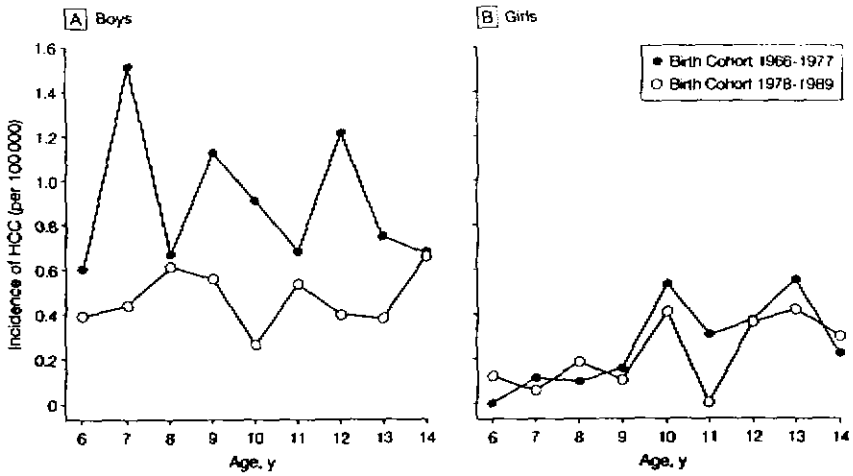
The relative risk (RR) of HCC in boys born between 1978 and 1984 declined significantly in comparison with those born before 1978 (RR, 0.83; $P=.02$); the trend of decrease in the RR of HCC was even more evident in those born after 1984 when compared with those born before 1978 (RR, 0.72; $P=.002$) (TABLE 2). However, the trend of decline in girls was not significant for the birth cohort born between 1978 and 1983 vs those born before 1978 (RR, 1.02; $P=.90$) or for those born after 1984 vs those born before 1978 (RR, 0.77; $P=.20$).

The age trend of HCC risk was significantly modified by sex. The risk of HCC in boys remained constant from age 6 to 14 years, while the risk of HCC in girls, though lower than in boys, increased significantly with age. In separate models, there was no significant age trend for boys (RR, 0.97; 95% CI, 0.92-1.03; $P=.33$), while the incidence of HCC in girls increased significantly by 1.15 times for each year increment of age (RR, 1.15; 95% CI, 1.04-1.28; $P=.007$). The age effect in boys and girls was the same before and after vaccination (FIGURE).

COMMENT

In the present study, we observed a predominance of HCC in boys both before and after the HBV vaccination pro-

Figure. Incidence of Hepatocellular Carcinoma (HCC) in Boys and Girls Aged 6 to 14 Years



A, The incidence of HCC in boys born after July 1978 was significantly lower than in those born before July 1978 ($P=.001$). The incidence of HCC in boys from 6 to 14 years did not change regardless of the birth year, suggesting that the age effect on the incidence of HCC was not prominent in boys. B, The incidence of HCC in girls aged 6 to 14 years increased with age, regardless of the birth year. The incidence of HCC in girls did not change in different birth cohorts.

gram. This predominance cannot be explained by the effect of sex hormones, as in adults. Tumor suppression gene regulation, the metabolism of carcinogens, or genetic alterations have been proposed to differ between men and women and need further study.[5]

This predominance decreased after the vaccination program because the incidence of HCC decreased significantly in boys but not in girls. Why the vaccination program seems to have had more of an effect on boys remains unclear. The low incidence of HCC in girls may render the statistical comparison of the incidences difficult. It is possible that HCC in girls is less intimately related to HBV infection than in boys, but seems unlikely given our previous observations[2] and evidence of HBV infection in girls with HCC born after implementation of the program (unpublished data by authors). The possibility that intrauterine infection with HBV, which would not be affected by vaccination, occurs more frequently in female infants also is unlikely, as there was no female predominance in infants who were seropositive for the hepatitis B surface antigen at birth.[12] Additionally, there was no difference in the vaccination coverage rate between male and female infants in Taiwan. (National Taiwan University Hospital's coverage rate is 100% for all mature neonates. The number of delivery of neonates is approximately 3000 per year. The national coverage rate for neonates was between 84% and 94% for 1986 to 1994 [M. H. Chang, unpublished data]). It also seems unlikely that case finding for such a serious disease would differ between boys and girls or change over time.

Seroepidemiologic studies in Taipei conducted in both 1984 and 1994 in children showed no or a slight predominance in boys in the incidence of HBV infection.[3,13] In contrast, the remarkable predominance of HCC in boys suggests that factor(s) in addition to chronic HBV infection may contribute to hepatocarcinogenesis in males, particularly the early occurrence in prepubertal males.

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