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Hepatocellular Carcinoma: Significance of HBV Vertical Transmission

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SUMMARY **In two cases of childhood hepatocellular carcinoma in Thailand, we established vertical transmission of hepatitis B virus infection as the underlying cause. With the first patient, the family history of HBV carriage became evident and a pedigree could be devised which demonstrated the high prevalence among the family members and hence evidence of vertical transmission. In the case of the second patient, we performed PCR and subsequent direct sequencing of HBV DNA isolated from his HBsAg-positive mother's, as well as from his serum, comparing the nucleotide sequences with those of a pregnant woman diagnosed as an asymptomatic HBV carrier, of another asymptomatic HBV carrier and of a reference strain, respectively, all belonging to the same genotype and subtype as the samples tested. Our results clearly indicate the necessity for nation-wide hepatitis B vaccination starting at birth, at least in hyperendemic areas like the Far East, in order to forestall HBV carriage and ensuing cirrhosis and/or HCC by preventing vertical transmission.**

Hepatocellular carcinoma (HCC) constitutes a commonly encountered malignant disease in sub-Saharan Africa, China, and most of the Far East.[1] In Thailand, it represents the most frequent malignant neoplasm among men and the third in importance among women[2] thereby reflecting the considerably higher incidence of HCC in males than in females throughout the world.[3] The etiological factors responsible for contracting HCC have been identified, first and foremost, as chronic carriage of hepatitis B virus (HBV), and furthermore, exposure to aflatoxin, alcohol, oral contraceptives, and, possibly, tobacco smoking. Thus, the epidemiological evidence gathered to date appears to have established HBV as one of the limited number of human viruses to unequivocally cause cancer.[4] In general, the incidence of primary hepatocellular carcinoma (HCC) increases arithmetically with age in both sexes, a finding which correlates with the severity of chronic carriage of HBV ultimately leading to macronodular cirrhosis and/or HCC.[3] Only the oldest age group shows a slight decline, and it should be noted that a significant incidence occurs in childhood. This is predominantly found in countries where HBV is hyperendemic and where the HBsAg carrier state can, therefore, be transmitted vertically from asymptomatic carrier mothers to their offspring. Such cases of vertical transmission of HBV with subsequently ensuing pediatric hepatocellular carcinoma have been reported from Taiwan,[5-8] Japan[9-10] and Senegal.[11]

We report here two cases of pediatric primary hepatocellular carcinoma having occurred in Thailand as a consequence of perinatal infection. The significance of vertical transmission was demonstrated referring to the respective pedigree, on the one hand, as well as by determining the HBs gene sequence of the HBV isolate detected in mother and child.

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MATERIALS AND METHODS

Patient study

Two cases of children histologically diagnosed as HCC admitted to Chulalongkorn University Hospital, Bangkok, were studied with the aim of detecting previous, as well as latent, hepatitis B virus infection in them and also among the various members of their respective families.

Case 1:

A 12-year old boy presented with abdominal mass of a two-month history. He was otherwise healthy. Physical examination revealed no jaundice. On palpation, we detected hepatomegaly with the liver spanning 18 cm, yet firm to the touch. The liver function test showed the following results: TB 2.2 mg/dl; DB 0.3 mg/dl; SGOT 162 U/l; SGPT 32 U/l. The prothrombin time was within the normal range. Ultrasonography showed a solid mass covering the whole right lobe, as well as evidence of the tumor invading the portal vein. Surgical liver biopsy was performed. The histopathological diagnosis was cirrhosis with hepatocellular carcinoma. Three months after diagnosis, the patient passed away.

Case 2:

A 10-year old boy presented with abdominal pain having set in 10 days before admission. He did not display any jaundice. Physical examination revealed hepatomegaly with his liver spanning 8 cm and being firm to the touch. The liver function test showed the following results: TB 0.18 mg/dl; DB 0.03 mg/dl; SGOT 61 U/l; SGPT 32 U/l; albumin 4.8 g/dl; globulin 3.2 g/dl; prothrombin time within the normal range; alpha-fetoprotein 118,000 ng/ml. On the ultrasonogram, an irregular trabecular mass of 7 cm diameter and located in the middle part of the right lobe of the liver became visible. Fine needle aspiration performed for diagnostic purposes showed evidence of hepatocellular carcinoma. The patient underwent hepatic resection of the tumorous mass. Subsequent tissue pathology confirmed the diagnosis of HCC. He was treated by chemotherapy. Three months after the onset of chemotherapy, the patient is still alive.

Control cases

The sequences of HB s gene, subtype adr, derived from two patients, one an asymptomatic pregnant woman diagnosed as an HBV carrier (C1) and the second one another asymptomatic HBV carrier (C2), were selected to serve as controls.

Laboratory tests

Serological test

The test for HBsAg was performed using the commercially available kit produced by Abbott Laboratories, North Chicago, Illinois.

HBV DNA detection

The selection of primer sets for HBV DNA amplification was based on sequence data provided by Dr. M. Yano at the WHO Collaboration Center, Nagasaki, Japan.

HBV DNA was amplified by nested PCR in an automated thermocycler (Perkin Elmer Cetus) as described elsewhere.[12] In order to cover the HBV S gene completely, four separate amplification reactions were performed employing four different sets of primer pairs (Bio-Synthesis, Lewisville, Texas). The primer sequences and their respective nucleotide numbers were as follows:

Outer primers:
F1, 5'-GGA GCG GGA GCA TTC GGG CCA-3' (nt. 3022-3042);
R2, 5'-CCA GGA CAA ATT GGA GGA C-3' (nt. 366-348);
Inner primers:
F2, 5'-CAT CCT CAG GCC ATG CAG TGG A-3' (nt. 3193-3214);
R1, 5'-TGT AAC ACG AGC AGG GGT CCT A-3' (nt. 201-180);
Outer primers:
F2, 5'-CAT CCT CAG GCC ATG CAG TGG A-3' (nt. 3193-3214);
R4, 5'-ATG GCA CTA GTA AAC TGA GCC-3' (nt. 689-668);
Inner primers:
F3, 5'-CTC GTG TTA CAG GCG GGG T-3' (nt. 191-209);
R3, 5'-ACA AAC GGG CAA CAT ACC TTG-3' (nt.475-455);
Outer primers:
F4, 5'-GTC CTC CAA TTT GTC CTG G-3' (nt. 348-366);
R5, 5'-AGC CCA AAA GAC CCA CAA TTC-3' (nt. 1015-995);
Inner primers:
S2-1, 5'-CAA GGT ATG TTG CCC GTT-3' (nt. 455-474);
S1-2, 5'-CGA ACC ACT GAA CAA ATG GC-3' (nt. 704-684);
Outer primers:
R6, 5'-GGC GAG AAA GTG AAA GCC TG-3' (nt. 1103-1084);
S2-1, 5'-CAA GGT ATG TTG CCC GTT-3' (nt. 455-474);
Inner primers:
F5, 5'-TGC CAT TTG TTC AGT GGT TCG-3' (nt. 684-704);
R5, 5'-AGC CCA AAA GAC CCA CAA TTC-3' (nt. 1015-995).

Regarding the reactions performed, 5 µl of the respective DNA sample were added to a reaction mixture containing 1 U of *Taq*

DNA polymerase (Perkin Elmer Cetus), each of four deoxynucleotide triphosphates (Promega) at a concentration of 200 μM, the primer pair required for the first amplification round, and the primer pair required for the second amplification round, respectively, each individual primer at a 1 μM concentration, 10 mM Tris buffer and 1.5 mM $MgCl_2$ at a final volume of 50 μl. The first amplification round consisted of one cycle at 94°C, 55°C and 72°C for 1 minute each, followed by 30 cycles comprising a 30 second denaturation step at 94°C, a 30 second annealing step at 55°C, and a second extension step at 72°C, each. Amplification was concluded by one cycle at 94°C for 1 minute, 55°C for 2 minutes and 72°C for 10 minutes. For the second amplification round 5 μl of the first PCR product were added to the reaction mixture and amplification was performed in a manner identical to the first round.

Ten microliters of each amplified DNA sample were loaded on a 2% Nusieve agarose gel containing ethidium bromide. Electrophoresis was performed at 120 V for 45 minutes and the product band specific for each individual test, that is, 224, 286, 250, and 332 base pairs, respectively, was visualized on a UV-light box.

DNA purification and sequencing

The PCR product was purified for sequencing using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's specifications and subsequently subjected to 2% agarose gel electrophoresis in order to ascertain its purity.

For determining the concentration of the amplified DNA, we measured the absorption at 260 nm of every sample in a UV spectrophotometer (Shimadzu UV 160 A). The concentration was calculated according to the formula that 1 OD_{260} = 50 μg double-stranded DNA. Between 10 and 30 ng/μl (3-6 μl) of every respective DNA were subjected to cycle sequencing using dye-labeled terminators (8 μl and 3.2 pmole of specific primer at a final reaction volume of 20 μl) which represents a rapid and convenient method for performing enzymatic extension reactions for subsequent DNA sequencing on the ABI Prism 310 Genetic Analyzer (Perkin Elmer Cetus). This round of amplifications was performed according to the manufacturer's specifications using the inner forward primer employed in each individual test in order to amplify the particular DNA strand of interest for further sequencing. Cycle sequencing consisted of 25 cycles at 96°C for 10 seconds (denaturation), 50°C for 5 seconds (annealing), and 60°C for 4 minutes (extension). The reaction was concluded by cooling the thermal ramp to 4°C. The extension products were subsequently purified of excess un-incorporated dye terminators by ethanol precipitation according to the manufacturer's specifications (Perkin Elmer Cetus) and subsequently prepared for loading on the ABI Prism 310 Genetic Analyzer.

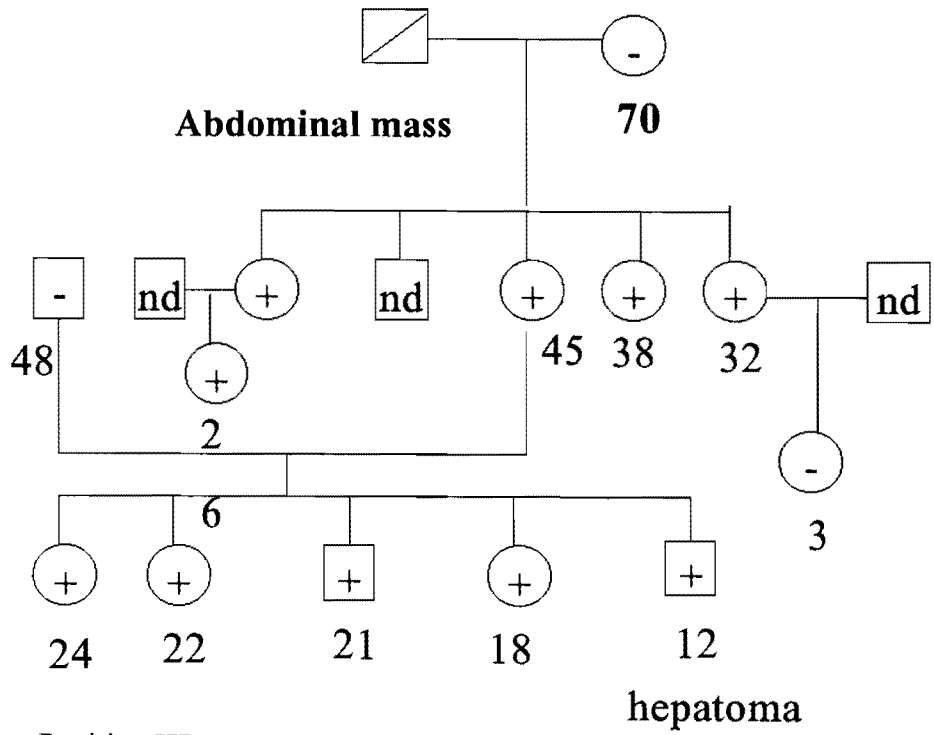
For all the subsequent steps we referred to the ABI Prism 310 Genetic Analyzer user's manual (Perkin Elmer Cetus). All sequences thus obtained were confirmed by performing the cycle sequencing once more with the inner reverse primers.

RESULTS

In the first case, the patient's pedigree shows a high incidence of HBV infection in the family, including his mother and siblings, as depicted in Fig. 1.

In the second case, the patient's mother had been diagnosed positive for HBsAg, his father HBsAg-negative. Since he is the only child and the parents' siblings are untraceable we could not establish his pedigree beyond his first-degree relatives. On the HBV DNA samples isolated from mother (M) and son (S), direct sequencing of the S gene was performed. (GenBank nucleotide sequence database, accession nos. AF072465, AF072466).

The nucleotide sequences obtained were translated into the corresponding amino acid sequences according to the universally valid genetic code and compared with the S gene of the *adr* strain sequence[13] (Fig. 3). The DNA isolates used as control sequences were selected from a variety of previously sequenced HBV DNAs, subtype adr, derived from various asymptomatic HBsAg-positive women attending the outpatient clinic at Chulalongkorn Hospital, as well as healthy blood donors (C1, C2, accession nos. AF072464, AF074449) (Fig. 2, Table 1). The amino acid sequences coded for by HB S gene deduced from the nucleotide sequences are shown in Fig. 3. The subtype and genotype were determined according to the classification arrived at by Magnius and Norder.[14]



\+ = Positive HBsAg

\- = Negative HBsAg

nd = not tested

Fig. 1 Pedigree of the first patient showing a high incidence of HBV infection among his family members.

Table 1 Quantitative analysis of the nucleotide substitution rates observed upon sequencing of DNA isolates derived from controls C1 and C2, mother (M), son (S) (Genbank accession nos. AF072464, AF074449, AF 072465, AF 072466) in relation to reference strain,[13] as well as to each other.

Comparing nucleotide sequence	Base pair Difference	Base pair Homology
Ref *vs.* C1	29	95.97
C1 *vs.* M	17	97.64
C1 *vs.* S	16	97.78
M *vs.* S	9	98.75
C2 *vs.* C1	14	98.06
C2 *vs.* M	19	97.36
C2 *vs.* S	22	96.94

Ref	atggagaaca	caacatcagg	attcctagga	cccctgctcg	tgttacaggc	ggggtttttc
C1	_____g__	_c_____	_____	_____	_____	_____
C2	_____	tcg_____	_____	_____	_____	_____
M	_____	tcg_____	_c_____	_____	_____	_____
S	_____g__	tc_____	_____	_____	_____	_____

Ref	ttgttgacaa	gaatcctcac	aataccacag	agtctagact	cgtggtggac	ttctctcaat
C1	____a_g	_____	_____	_____	_____	_____
C2	____a_g	____tct__	_____	_____	_____	_____
M	____a_g	_____	_____	_____	_____	_____
S	____a_g	__g_____	_____	_____	___t____	_____

Ref	tttctagggg	gagcacccac	gtgccctggc	caaaattcgc	agtccccaac	ctccaatcac
C1	_____	_____a__	___ t_____	_____ t__	_____a___	_____
C2	_____	_____a__	___ t_____	_____ t__	_____a___	_____
M	_____	_____	___ t_____	_____	_____a__	_____
S	_____	_____	___ t_____	_____	_____a__	_____

Ref	tcaccaacct	cttgtcctcc	aatttgtcct	ggctatcgct	ggatgtgtct	gcggcgtttt
C1	_____	_____	_____	__ t_____	_____	_____
C2	_____	g_____	_____	__ t_____	_____	_____
M	_____	_c_____	_____	__ t_____	_____	_____
S	_____	_____	_____	__ t_____	_____	_____

Fig. 2 Nucleotide sequences of the HBs gene region of HBV DNA isolated from mother of son with HCC (M) (Genbank accession no. AF 072465), her son with HCC (S) (AF 072466), and asymptomatic carriers (C1) (AF 072464), C2 (AF074449) as controls, compared to a reference strain for HBV, subtype adr.[13]

Ref	atcatattcc	tcttcatcct	gctgctatgc	ctcatcttct	tgttggttct	tctggactac
C1	____c____	_____	_____	_____ c___	_____g___	_____ t__t
C2	____c____	_____	_____	_____ c___	_____g___	_____ t__t
M	____c____	_____	_____	_____ c___	_____	_____ t__t
S	____c____	_____	_____	_____ c___	_____	_____ t__t

Ref	caaggtatgt	tgcccgtttg	tcctctactt	ccaggaacat	caactaccag	cacgggacca
C1	_____	_____	_____a___	_____ t__a	____a____	t_____
C2	_____	_____	_____a__	_____a	___ga____	t_____
M	_____	_____	_____ c	_____ t__a	____a____	t_____
S	_____	_____	_____ c	_____ t__a	____a_____	t_____

Ref	tgcaagacct	gcacgattcc	tgctcaagga	acctctatgt	ttccctcttg	ttgctgtaca
C1	_____a____	_____	_____	_____	_____	_____a_
C2	_____a____	_____	_____	_____	_____	_____
M	_____a____	_____	_____	_____	_____	_____
S	_____a____	_____	_____	_____	_____	_____

Ref	aaaccttcgg	acggaaactg	cacttgtatt	cccatcccat	catcctgggc	tttcgcaaga
C1	_____ a___	_____ t__	_____	_____	____t____	_____
C2	_____ a___	_____ t__	_____	_____	____t____	_____
M	_____	_____ t__	___c____	_____	____t____	_____
S	_____	_____ t__	___c____	_____	____t____	_____

Fig. 2 (continued)

Ref	ttcctatggg	agtgggcctc	agtccgtttc	tcctggctca	gtttactagt	gccatttgtt
C1	_____	___t____	_____	__t_____	_____	_____
C2	_____	_____	_____	__t_____	_____	_____
M	_____	_____	_____	_____	_____	_____
S	___a____	_____	_____	_____	_____a_	_____

Ref	cagtggttcg	cggggctttc	ccccactgtt	tggctttcag	ttatatggat	gatgtggtat
C1	_____	ta__t_____	_____	_____	_____	_____
C2	_____	ta_____	_____	_____	___t____	_____
M	_____	ta_____	_____	_____	_____	_____
S	_____	ta_____	_____	_____	_____	_____

Ref	tgggggccaa	gtctgtacaa	catcttgaat	ccctttttac	ctctattacc	aattttcttt
C1	_____	_____	_____	_____	_____	_____
C2	_____	_____	_____	_____	_____	_____
M	_____c	_____	_____	_____	_____	_____
S	_____	_____	_____	_____	_____	_____

Ref	tgtctttggg	tatacattta	aaccctaata	aaaccaaacg	ttggggctac	tcccttaact
C1	_____	_____	_____	_____	a_____	_____
C2	_____	_____	_____	_____	a_____	_____
M	_____	_____	_____	_____	_____	_____
S	_____	_____	_____	_____	_____	_____

Fig. 2 (continued)

Ref.	MENTTSGFLG	PLLVLQAGFF	LLTRILTIPQ	SLDSWWTSLN	FLGGAPTCPG	QNSQSPTSNH
C1	___S_____	_____	__KG____	_____	_____	__L__Q____
C2	____IA_____	_____	__KG.S___	_____	_____	__L__Q____
M	____A__L___	_____	__KG____	_____	_____	_____Q____
S	___SI_____	_____	__KG____	_____	_____	_____Q____

Ref.	SPTSCPPICP	GYRWMCLRRF	IIFLFILLLC	LIFLLVLLDY	QGMLPVCPLL	PGTSTTSTGP
C1	_____	_____	_____	__L__G_____	_____I	___ST_____
C2	___C_____	_____	_____	__L__G_____	_____	____TR_____
M	_____	_____	_____	__L_____	_____	___ST_____
S	_____	_____	_____	_____	_____	___ST__L____

Ref.	CKTCTIPAQG	TSMFPSCCCT	KPSDGNCTCI	PIPSSWAFAR	FLWEWASVRF	SWLSLLVPFV
C1	_____	_____	_____	_____	_____	_____
C2	_____	_____	__T_____	_____	_____	_____
M	_____	_____	_____	_____	_____	_____
S	_____	_____	_____	_____	_____	_____

Ref.	QWFVGLSPTV	WLSVIWMMWY	WGPSLYNILS	PFLPLLPIFF	CLWVYI
C1	____V_____	_____	_____N	_____	_____
C2	_____	_____	_____N	_____	_____
M	____V_____	_____	_____N	_____	_____
S	_____	_____	_____N	_____	_____

Fig. 3 Amino acid sequences of HBsAg deduced from the nucleotide sequences of an asymptomatic HBsAg-positive mother (M), her child with HCC (S) and two additional asymptomatic carriers (C1 and C2) as controls, compared to a reference strain for HBV subtype adr.[13]

DISCUSSION

Among the family members of the first case, all of whom are sharing the family home in Chachoengsao province, that is, are living in the same village as an extended family, a high incidence of HBV carriers was prevalent, in that all the siblings proved infected with HBV. Their infection might convincingly have originated from their mother during the perinatal period especially as the risk for infants born to carrier mothers, in particular those positive for HbeAg, is estimated to amount to between 65 and 93%, unless the newborns are subjected to immunoprophylaxis at birth.[15-20] Along those lines, the significance of vertical transmission of HCC in children has been reported from Taiwan in that serum HBsAg was found positive in 94% of the mothers of altogether 33 HCC children, a rate much higher than that obtained (50%) for the mothers of

control HBsAg carrier children. In contrast to that, the serum HBsAg positive rate of the fathers of HCC children was not different from that of the control group. Hence, maternal transmission of hepatitis B virus during the perinatal period or early childhood has been demonstrated to be the most important mode of hepatitis B virus infection in HCC children in Taiwan.[21] The results arrived at in the second case, which had been provided by direct sequencing of the S gene of hepatitis B virus, unequivocally demonstrate a particular mode of transmission of HBV to be an important and possibly necessary factor in the development of HCC. In these patients, infection by their mothers who are chronic carriers of HBV undoubtedly occurred either at birth or early in life. This has become obvious not merely by the presence of HBV DNA in the children's sera, but even more so by the viral DNA isolated from the patient belonging to the same genotype (genotype C) and subtype (adr) as that obtained from his mother. Thus, our results are consistent with those obtained in Taiwan,[5] that when the mother is an HBsAg carrier, a large proportion of infants are infected in the perinatal period, thereby becoming carriers themselves which might well predispose them to develop HCC. Moreover, while the results arrived at by the researchers in Taiwan were exclusively based on serological data which leaves some space to speculation, our data were arrived at by means of direct sequencing, which enabled us to characterize the HBV sequences detected as far as the specific viral strain. Progress towards malignant neoplasm might be favored by yet another condition worth considering, in that HBsAg has also been detected in the sera of some patients with Down's syndrome, hematological malignancies, lepromatous leprosy, and in some patients maintained on chronic hemodialysis.[22-25] These findings have pointed to the possibility of an impaired immunological state representing a permissive factor, thus allowing the hepatitis-associated antigen to be chronically positive in these patients. Moreover, according to a group of researchers who have summarized genetic, as well as immunological factors in relation to HBsAg, susceptibility to chronic infection with this agent might be an inherited trait.[26]

Along those lines, it is remarkable that by direct sequencing performed in the second family we were able to detect HBV DNA belonging to the same genotype and subtype in the mother and her son diagnosed with HCC. Although both genotype C, as well as subtype adr are those most commonly encountered in Thailand, comparison of the child's nucleotide sequence with that of his mother on the one hand, and with that of two asymptomatic carriers (C1, C2) used as controls on the other, revealed a much higher similarity between the sequences of mother and son, respectively. This finding would suggest the HBV strain isolated from the child with HCC having originated from and having been vertically been passed on by his mother. The reason for the observed similarity between mother and child not to be complete identity without question lies in the fact that hepatitis B virus which in the course of its replication passes through an intermediary RNA stage is hence subjected to a far higher amount of random mutations than any other DNA virus. This natural mutation rate also accounts for the rather high difference in base pairs observed upon comparing between the controls (C1, C2). As our group have performed the sequencing reaction using both forward and reverse primers, internal control of the results thus obtained was automatically provided.

In any event, the strong relationship between chronic HBV infection and development of cirrhosis and/or primary hepatocellular carcinoma renders primary prevention by vaccination against hepatitis B virus infection a crucial necessity in order to reduce the incidence of the chronic HBsAg carrier state, particularly in countries of high endemicity. The efficacy of the vaccine in preventing HBV infection in adults has been well established.[27-28] In infants born to HBsAg-positive, HBeAg-positive mothers, administration of hepatitis B vaccine, either alone or combined with HBIg at birth has demonstrated a protection rate amounting to more than 90%.[29-31] and has furthermore proven to provide long-term protective immunity for a duration of more than 7 years, irrespective of having been followed by a booster dose at month 60.[32] In order to prevent HBV infection during childhood years, vaccination of HBsAg-negative children ought to be performed, as well. Prevention of HBV infection by vaccination will eventually lead to the eradication of the chronic HBsAg-positive carrier state, thereby significantly reducing the incidence of HCC.[33] It might even be speculated that a combination of gene therapy, which is fast progressing, and medicinal prophylaxis will achieve a reduction not only in the vast number of chronic

HBV carriers, but equally decrease progression to primary hepatocellular carcinoma in the not too distant future.

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