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Original

Investigation of antiviral resistance and escape mutations in children with naive chronic hepatitis B patients and their parents

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In this study, it was aimed to scan the resistance to nucleoside analogs in naive pediatric patients with chronic hepatitis B treatment and their parents and the rate of accompanying possible escape mutations. A total of 34 children who did not receive any treatment regarding chronic hepatitis B and 19 parents who caused vertical transmission or acquired transmission from father were involved in the study. Serological tests concerning hepatitis B virus and transaminases in conjunction with viral load were studied. HBV genotypes, subgenotypes were determined by surface gene sequencings. The gene mutations coding polymerase (*pol*) for resistance against nucleoside analogs and escape mutations in the genes coding surface (*S*) proteins were analyzed with PCR method. All cases were genotype D. Only one pediatric patient was D2; the rest of all pediatric patients and their parents were genotype D1. Resistance was not identified against nucleoside analogs in any children or their parents. HBsAg escape mutations determined in the chronic hepatitis B patients were 18.8% (10 case). It can be speculated with this results that the resistance may not be considered as a problem in the preference of nucleoside analogs in treatment of naive children. Nevertheless, escape mutations were seen as high in both children and parents as well. Since it interests public health on a large scale, advanced studies and evaluation of vaccination escape mutations' rate in broad case series and their follow up are of great importance in the determination of health policies with regard to hepatitis B infection control.

Key words: chronic hepatitis B, children, antiviral resistance, escape mutations.

Chronic hepatitis B virus (HBV) infections cause the death of nearly one million people throughout the world by giving rise to serious complications such as liver cirrhosis and hepatocellular carcinoma.[1] It still continues to be a significant public health problem though its rate decreases day by day with the application of effective vaccination program.[2] Its prevalance shows differences according to countries and regions. Our country is considered as medium endemic area, its HBsAg rate varies between 0.8% and 5.7%.[3]

In the treatment of chronic hepatitis B of pediatric patients, interferon/pegylated interferon (IFN) and nucleoside analogs (NA) (lamivudine, adefovir, entecavir, telbivudine, tenofovir) are used.[4] The treatment with NA is not curative. These drugs suppress HBV replication effectively. While it retards the development of the disease in many patients, new antiviral variants may emerge if antiviral treatment is not repressive sufficiently.[5] Patients carrying resistant strains with NA use can give rise to serious problems for public health. The transmission of resistant HBV strains to individuals not being on medication can increase failure chance in the first-line treatment.[6] The virus can lead to important problems

in the treatment by reducing the antiviral activity against NA in company with mutations in polymerase gene. The determination of appropriate treatment by scanning mutations that can lead to NA resistance in chronic hepatitis B patients and side-effect toxicity reduction in this way and prevention of economic losses and serious complications that can occur in patients are of major importance.[7] However, it has been reported in studies carried out in people infected with HBV that the mutations regarding resistance development during natural replication of the virus can emerge spontaneously even with no NA use.[8] Different results were found in various studies performed on this issue. In a study conducted in adult patient population in Turkey, this rate was reported as 10%.[9]

P area coding polymerase (*pol*) enzyme in HBV genome are divided into 7 sub-zones (A, B, C, D, E, F and G). The mutations regarding NA primarily develop in A, B, C and D sub-groups and two groups are of clinical significance: 1st group: primary resistence mutations (rtI169T, rtA181T/V, rtT184A/C/F/G/I/L/M/S, rtA194T, rtS202C/G/I, rtM204I/V/S, rtI233V, rtN236T, rtM250I/L/V), 2nd group: compensatory (restorative) mutations (rtL80I/V, rtI91L, rtV173L, rtL180M,rtQ215P/S, rtN238D/S/T).[10,11] In the HBV genom organization, overlapping position of polymerase (*pol*) and surface (*S*) genes can lead to amino acid changes in NA drug resistance mutations and HBsAg protein. Thus, HBV strains escaping from antibodies developed with HBV vaccination can appear due to NA treatments. For these mutations that develop concerning HBV *pol/S* gene overlapping, potential vaccination escape mutation associated with antiviral drug (ADAPVEM); Antiviral Drug-Associated Potential Vaccine Escape Mutant concept has started to be used.[12,13] These mutations are of quite importance in terms of public healh.

We aimed to study resistance mutations against NA in naive pediatric chronic HBV cases treatment and vaccination escape mutations in this research. In addition, we analyzed antiviral resistance in parents of pediatric patients that we knew there was a vertical transmission or acquired transmission from father and we intended to study whether this resistance was transmitted vertically or not.

Material and Methods

Patients

Pediatric patients aged between 0-18 not being on any kind of antiviral drugs and followed due to chronic hepatitis B infection at Pediatric Gastroenterology, Hepatology and Nutrition Department were involved in the study. All parents diagnosed with chronic hepatitis B – without considering if they received treatment or not- were involved in the study by taking their consents. During routine controls of patients and their parents with HBV, blood samples containing 2 ml EDTA were separated and they had been kept at -80°C until they were studied. Study protocol was approved by institutional ethical committee. Informed written consent was obtained from the parents of all children and the parents known to have chronic hepatitis B.

The personal information of patients such as their age, sex, routine laboratory data (Aspartate aminotransferase-AST, alanin aminotransferase-ALT, HBsAg, HBeAg, AntiHBe, HBV-DNA, HDV) were obtained from hospital records. Chronic HBV infection was classified under 3 immunological phases: 1- immune tolerance phase, 2-immunoreactive phase, 3- inactive carriers.[14] The data of parents of patients and their treatments were recorded.

Determination of HBV genotype

HBV genotypes of National Center for Biotechnology Information (NCBI, US National Library of Medicine, Bethesda, USA,http://www.ncbi.nih.gov/projects/genotyping/formpage.cgi) were determined by genotyping tool describing genotype dependent on viral nucleotide series. Genotyping tool functions by using BLAST to compare a question series with a reference series cluster for known genotypes.[15]

DNA sequence analysis (Determination of polymerase and surface gene mutations)

The area coding reverse transcriptase between HBV *pol* gene, 80th-250th amino acids were arranged by Sanger dideoxy sequencing technique. For HBV *pol* gene amplification product synthesizing as with lenghth of ~740 bp, forward (F: 5'-tcgtggtggacttctctcaatt-3') and reverse (R: 5'-cgttgacagactttccaatcaat-3') primaries were used. Ten minutes preliminary denaturation at 95°C for PCR, then 35 loops at

Table I. Sociodemographic and Laboratory Data.

	Children (n=34)	Parents (n=19)
Age	11±5.1	31.6±6.9
Gender		
Female	13 (38.2%)	18 (94.7%)
Male	21 (61.8%)	1 (5.3%)
AST	37±21.8	40.2±36.3
ALT	35.4±20.2	51.5±66.3
HDV	1 (2.9%)	0
Classification		
Immune tolerantt	19 (55.9%)	8 (42.1%)
Immuneactive	1 (2.9%)	6 (31.6%)
Inactive carrier	14 (41.2%)	5 (26.3%)
Genotype		
D1	33 (97.1%)	19 (100%)
D2	1 (2.9%)	0
Treatment	0	6 (31.55%)
Resistance against NA	0	0

AST, aspartate aminotransferase, ALT, alanine aminotransferase, HDV: hepatitis D virus, NA: nucleoside analog

95°C for 45 seconds, at 60°C for 45 seconds and at 72°C 45 seconds temperature/time cycle were used.[16] Obtained PCR products were purified. Sequencing was performed by using Big Dye Terminator CycleSequencing Kit (Amersham Pharmacia Biotech Inc., USA) on ABI PRISM 3130 (Applied Biosystems Inc., USA) platform. Electropherograms were evaluated visually and analyzed with Geno2pheno program (Center of Advanced European Studiesand Research, Germany) in fasta format. In the area overlapping with HBV reverse transcriptase and S gene; 121., 135., 137., 139.-149., 151-153., 155-157., 161., 164., 172., 173., 175., 176., 182., 193-196th amino acid positions were analyzed in terms of mutations.[17]

HBsAg amino acid changes were studied under 2 titles as ADAPVEM and typical HBsAg amino acid changes. Typical HBsAg amino acid changes, however, were classified as HBIg-selected escape, vaccine escape, hepatitis B misdiagnosis and immune-selected escape amino acid.[18]

Statistical analysis

SPSS 18 program (SPSS Inc, USA) was used for the analysis of descriptive statistical parameters (average, standard deviation, minimum and maximum).

Results

A total of 34 children aged between 1-18 years and 19 parents known to have chronic hepatitis B (only one of them acquired from father, the others were vertical transmission) were involved in the study. The average age of children was 11±5.1 years. Of the cases, 21 of them were male and 13 were female. Average AST and ALT levels of the cases were 37±21.8 and 35.4±20.2 IU/L. Of the pediatric patients, 14 were inactive carrier, 19 were in the immune tolerance phase and 1 was in immune reactive form. HBV infection was accompanying to hepatitis D coinfection in only one patient. The average age of parents was 31.6±6.9 years. NA use was identified in 6 mothers. All cases were identified to have received tenofovir. Genotype D was determined in all children and parents. D2 subgroup was established in a 10 year-old girl and D1 subgroup was determined in the rest of all children and their parents. Resistance against NA was not observed in any scanned pediatric cases or their parents (Table I).

In 10 cases (18.8%), escape mutation was determined with regard to HBV surface protein. The mutations were seen detected in 6 children and 4 mothers. There were more than one escape mutations in 2 cases. In 5 patients (3 parents, 2 children) HBIg selected escape (M133L, T123A, Y134N, Q129R), in 5 cases (3 children, 2 parents) immune selected escape (I110L, Y100S, P120T, Q101H), and in 2 cases vaccine escape mutation (M133L, S143L) were determined. The same mutation

was established in only one mother and her child (Q101H-immune selected escape). The remaining mutations were seen detected in only mother or child (Table II).

Discussion

Even when no treatment was received in individuals with HBV infection, spontaneous resistence was reported against NA's.[7] This can be dependent on both factors related to the virus and host factors. Primarily, these mutations may emerge naturally; because HBV has a high replication and mutational rate. Moreover, HBV reverse transcriptase does not have a correction function to repair misplaced nucleotides. Secondly, a patient not requiring any treatment can be infected with a mutant virus derived from a patient who was treated with NA. Thirdly, the patient may have some treatments with direct anti-HBV activity, however, s/he may be unaware of it.[19]

There is no recommendation regarding antiviral resistance scan in naive chronic HBV treatment. In addition, there is insufficient information concerning the clinical importance of all scanned mutations. Still, it is reported that there can be clinical benefits of frequently used and previously known NA resistance and there will be positive effects on side-effect, cost-effectivity and loss of time in treatment.[4,8,20]

In the studies carried out in different patient groups, it was revealed that various mutations decreasing drug sensitivity could be determined in the treatment of naive cases. Nevertheless, there is no specific research concerning NA resistance in the treatment of naive chronic HBV cases in pediatric age groups. In several studies, resistance rate against NA was reported between 0% and 57% in the treatment of naive patients.[21,22] In 2 studies performed in our country, the rate was reported as 3.8% and 10% [9,21]. There can be many reasons of difference in the results such as the study design, race, geographical differences, study method, age distribution and number of patients involved in the study. We did not determine a resistance against NA in parents that led to transmission vertically in the treatment of naive children with chronic HBV infection in our study. With these results, in cases that we were or were not aware of their vertical transmission, in antiviral choice we did not determine negative data associated with resistance against the treatment and we could speculate that resistance should not be a problem when beginning treatment. Nonetheless, the number of pediatric children involved in the study and parents identified to have vertical transmission may not be adequate to fully evauate this situation. A broad array of case series are needed in this issue.

In our study we did not determine a clinical important drug resistance mutation between parents and their child. This might have been

Table II. Hepatitis B Surface (S) Protein Escape Mutations in Our Patients.

Case	Age	Gender	Classification	Genotype	Escape mutation
1	1	Male	Immune tolerant	D1	M133L (HBIg selected escape+Vaccine escape)
2	27	Female	Immunoreactive	D1	I110L (immuneselected escape), T123A (HBIg selected escape+immune selected escape)
3	39	Male	Inactive carrier	D1	Y134N (HBIg selected)
4	17	Male	Immune tolerant	D1	Y100S (immune selected escape), G130R (immune selected escape)
5	10	Female	Immune tolerant	D1	S143L (vaccine escape+HBsAg misdiagnosed escape)
6	15	Male	Immune tolerant	D1	Q129R (HBIg selected escape)
7	17	Male	Inactive carrier	D1	P120T (immune selected escape)
8	20	Female	Immunoreactive	D1	Q101H (immune selected escape)
9	6	Male	Immunoreactive	D1	Q101H (immune selected escape)
10	19	Female	Immunoreactive	D1	Q129R (HBIg selected escape)

due to the low level of these mutations in the HBV pool, undetectable by direct sequencing. The monitoring of drug resistance mutations in chronic hepatitis B patients is mainly based on the direct sequencing, line probe assay and clonal analysis techniques. The utility of these assays and the ability to detect resistance variants' sensitivity are different. Direct sequence analysis is considered the gold standard for characterizing HBV DNA isolates. The ability to detect resistance variants by direct sequencing is increased for variants representing more than 10% of the viral population[23]. Line-probe assay, detecting only for known mutations, is specific and reproducible and significantly more sensitive than direct sequencing. This technique is a rapid and accurate detection of mutants, which make up as little as 5% of the HBV population, with a sensitivity of 990 copies/ml or 1.7E+2 IU/ml at a 95% confidence interval. Minor HBV populations can be identified by clone analysis; however, this is beyond the capacity of routine laboratories. However, it is more sensitive (<1% of viral population) for the detection of resistant mutants that are present in low concentrations than other techniques.[24]

In chronic HBV patients, genotype has an important place in response to the treatment. It is reported that genotype A and B are related to a better response to treatment compared to genotype D and C.[25] When genotype distribution of patients were taken into account, we saw that all cases had genotype D. When subgroups were examined, only one of the cases had D2, the rest had D1. These results are similar to previous studies conducted in our country. HBV genotype D is determined as predominant in our country; subgenotypes are informed according to their rate as the following; D1 (70-100%); D2 (2-22%); D3 (2-10%) and D4 (0.5-2%).[21,26,27]

In NA treatments of patients with chronic B hepatitis, RT loop of HBV genome were analyzed in terms of drug resistance mutations, on the other hand, it is possible to analyze together with *S* gene mutations overlapping this area. In the hepatitis B virus genome organization; being in the overlapping position of polymerase (*pol*) and surface (*S*) genes can lead to amino acid changes in HBsAg protein and nucleoside analog drug resistance mutations. Thus, HBV strains escaping from antibodies developed with HBV vaccination emerge. In these strains described as immune escape strains, mutations occuring as major neutralization loop known as "a" determinant of *S* gene and around 124-149th amino acid position and taking place around them are held responsible.[17,28]

Clinically significant escape mutation in HBV cases are: vaccination, HBIg, misdiagnosis and immune escape mutations.[18] In the present study, escape mutation was determined in 18.8% cases (n=10). In 2 pediatric cases, vaccination escape (3.8%), immune escape in 5 cases and HBIg escape mutations were identified in 5 cases. In a study carried out by Sayan et al.[16], this rate was found as 8.3% in all HBV patients whether they received treatment or not. In studies performed in Gambia, Spain and Korea, however, this rate was determined at higher rates (38%, 40%, 33%).[17,29] These differences in results were thought to arise from focusing on sub-typology of all amino acids of the so-called protein due to methodology difference in DNA sequencing. The clinical importance of escape mutations has been increasing day by day. These mutations can cause response of hepatitis B vaccination to reduce, untruthful negativities in HBsAg diagnosis tests and insufficiency in preventing hepatitis B immunoglobulin (HBIg).[17,28]

In conclusion genotype D was the determined genotype in all children and their parents in line with general data of our country. Resistance while initiating NA in naive children's treatment was not considered as a major problem. The mutation transmission from parent to child by vertical transmission could not be shown. The rate of escape mutations defined in recent years and the importance of which has been increasing with each passing day was 18.8%. While this rate was lower compared to that of different countries, increasing incidence of these forms can affect public health in a negative way due to resistance of these mutant viruses' to treatment, vaccination and HBIg application. A broad array of case series and the follow-up of their incidence are of great importance in the determination of health politics.

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