Research Article

153

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Molecular Analysis of Hepatitis B Virus Reverse Transcriptase Domain for Mutations Associated with Viral Resistance in Pakistani Patients

Pakistanlı Hastalarda Viral Dirençle İlişkili Mutasyonlar İçin Hepatit B Virüsü Ters Transkriptaz Domaininin Moleküler Analizi

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ABSTRACT

Objectives: Current study was designed to screen out the resistant mutations in reverse transcriptase (RT) domain of hepatitis B virus (HBV) genome from non-responder Pakistani patients.

Materials and Methods: A total of 22 patients, receiving different nucleot(s)ide analogues were included in the study. RT domain of the virus from samples of non-responder patients was amplified and sequenced. Sequences were aligned and analyzed for RT domain mutations.

Results: After 18 months, 18 patients were responder and 4 were non-responder. Mean alanine aminotransferase (ALT) and viral load of responder patients decreased significantly as compared to those of non-responder patients. Two of the 4 samples from non-responders were successfully sequenced. Mutations rtY135S, rtI169P, rtV173P, rtL180I, rtA181V, rtT184Y and rtM204V were identified from the sample of patient 1, while rtL80V/rtL80G and rtY135S were identified from the sample of patient 2.

Conclusion: Mutations rtY135S, rtI169P, rtV173P, rtL180I, rtA181V, rtT184Y, rtM204V, rtL80V/rtL80G, and rtY135S are present in genome of HBV circulating in Pakistani patients. These mutations give resistance to virus against lamivudine, telbivudine, adefovir, and partially resistance against entecavir. However, no mutation was found to be associated with the viral resistance against tenofovir.

Keywords: Hepatitis B virus, RT domain, resistant mutations, nucleot(s)ide analogues, HBV genome

ÖZ

Amaç: Mevcut çalışma, yanıtsız Pakistanlı hastalardan hepatit B virüsü (HBV) genomunun ters transkriptaz (RT) domainindeki dirençli mutasyonları taramak için tasarlanmıştır.

Gereç ve Yöntemler: Çalışmaya farklı nükleot(z)id analogları alan toplam 22 hasta dahil edildi. On sekiz ay sonra, 18 hasta yanıt verdi ve 4 hasta yanıt vermedi. Yanıt vermeyen hastaların örneklerinden alınan virüsün RT domaini amplifiye edildi ve dizilendi. Diziler hizalandı ve RT domaini mutasyonları açısından analiz edildi.

Bulgular: Yanıt veren hastaların ortalama alanin aminotransferaz (ALT) ve viral yükü, yanıt vermeyen hastalarla karşılaştırıldığında önemli ölçüde azaldı. Yanıt vermeyen hastalardan alınan 4 örnekten 2'si başarıyla sıralandı. Birinci hastanın örneğinden rtY135S, rtI169P, rtV173P, rtL180I, rtA181V, rtT184Y ve rtM204V mutasyonları belirlenirken ikinci hastanın örneğinden rtL80V/rtL80G ve rtY135S mutasyonları belirlendi.

Sonuç: Pakistanlı hastalarda saptanan HBV genomunda rtY135S, rtI169P, rtV173P, rtL180I, rtA181V, rtT184Y, rtM204V, rtL80V/rtL80G ve rtY135S mutasyonları mevcuttu. Bu mutasyonlar, virüse lamivudin, telbivudin ve adefovire karşı tam, entecavire karşı kısmen direnç sağlamaktaydı. Bununla birlikte, tenofovire karşı viral dirençle ilişkili hiçbir mutasyon bulunmadı.

Anahtar Kelimeler: Hepatit B virüsü, RT alanı, dirençli mutasyonlar, nükleot(z)id analogları, HBV genomu

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Introduction

Hepatitis B virus (HBV), a member of hepadnaviridae family of viruses, is a pathogen of human hepatocytes first recognized in 1960s (1,2). The undesirable effects caused by HBV infection include liver degeneration, liver cirrhosis, hepatocellular carcinoma, and liver failure (3). Approximately 257 million people are chronic carriers of HBV in the world. The annual number of deaths caused by HBV related infections were estimated to be 887000 in 2015 (4). However, the infection rate of HBV has been decreased significantly in developed countries (5,6) but there is no such report from developing and underdeveloped countries, including Pakistan.

Interferon-- and nucleot(s)ide analogues (NAs) are clinically available treatments for HBV. Interferon reduces the hepatitis B surface antigen level from blood alongside immunomodulatory effects but it poses many adverse side effects (7). NAs treatment is easier in use than interferon therapy, though it also has some side effects but fewer (8). Five nucleotide/nucleoside analogues are so far used for treatment of chronic HBV Infection. These are: lamivudine, adefovir dipivoxil, entecavir, telbivudine, and tenofovir disoproxil fumarate. All of these act on the reverse transcriptase (RT) region of the viral genome stopping the production of DNA from pre-genomic RNA (8,9,10).

HBV replication is an error prone process because it has no proofreading activity, leading to high mutation rate in the genome (11,12). Some of these mutations may cause viral resistance against treatment and this antiviral resistance is the greatest stumbling block in HBV treatment (13). Several mutations in the RT domain are considered to be associated with resistance to nucleotide or nucleoside analogues in the treatment of chronic HBV (3,6,14). However, all the mutations occurring in HBV polymerase region are not associated with resistance. A few are well known mutations associated with primary drug resistance to NAs, which are: rtL80G/I, rtI169T/P, rtV173L, rtL180M/I, rtA181T/V/S, rtT184S, rtS202I/G/S, rtM204V/S/I, rtN236T, rtN238D/S/R and rtM250V/I/L.

The objective of current study was to screen out the non-responder patients for resistance mutations in RT domain of HBV from non-responder patients, and to compare some factors of non-responders with responder patients.

Materials and Methods

This was a cross sectional study conducted during August 2020 to March 2021. The surveys were conducted in hospitals to select the patients receiving different nucleotide analogues. A total of 22 chronic HBV patients who completed at least 18 months of NAs treatment were selected with the help of a gastroenterologist. A performa was filled for each patient, which included all the treatment history and other important information. A written informed consent was given to each of the patient and the patients keen to contribute in the study were enrolled. The patients with a positive treatment response were also monitored for breakthrough. Blood samples were collected from all the non-responder patients (Figure 1).

Pre-treatment Factors

Pre-treatment viral factors like genotype, hepatitis B e antigen, and viral load were recorded for all patients before and on every 6 months of treatment. Different host factors like age, gender, body weight, alanine aminotransferase (ALT), dental procedure, previous surgery record, infection age, and previous treatment, history were also recorded.

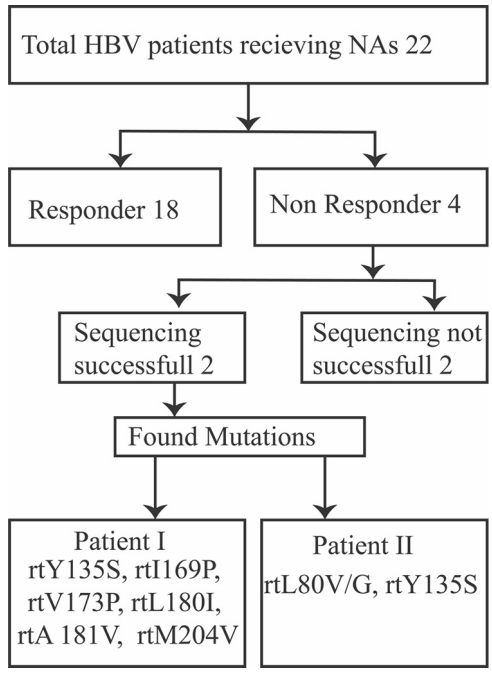

Figure 1. Flow chart of complete study including patients' selection and main findings

HBV: Hepatitis B virus, NAs: Nucleot(s)ide analogues

DNA Extraction and Amplification

Viral DNA was extracted using commercially available kits and a fragment of genome including RT domain was amplified by polymerase chain reaction using previously described primers (15,16). PCR conditions were optimized in a gradient PCR machine and the quantitative measurement of viral load was achieved by a real time PCR machine.

Products Purification

The amplified DNA fragments were purified for sequencing using ethanol precipitation kit protocol (Beckman Coulter, USA).

Sequencing

Sequencing of the purified DNA fragments of RT domain was obtained commercially by sending the DNA to commercial service providers where the sequencing was performed by chain termination method. The sequencing instrument used was "CEQ 8000 XL" analysis system for the sequencing reaction.

Statistical Analysis

The sequences were aligned with wild type HBV sequences and analyzed for resistant mutations in the RT domain. Manual analyses of sequences were also carried out. The mutations were also confirmed by "geno to pheno HBV", the online data base for HBV genome analysis.

Ethical Approval

The study was started after the approval from "humans and animals ethics committee", University of Poonch Rawalakot. An

informed consent was given to each of the patient for reading and signing before his/her enrolment to the study.

Results

Patients and Treatment Details

During the study period, 22 hepatitis B patients receiving treatment were enrolled at Pakistan Atomic Energy Commission General Hospital Islamabad. Out of the 22 enrolled patients, 18 (81.8%) showed response during treatment while 4 (18.2%) did not show any response to treatment and considered non-responder after 18 months of treatment (Figure 1). The mean age of responder patients was calculated to be 36.45±14.89 years while the mean age of non-responder patients was calculated to be 38.50±13.63 years. No significant difference (p=0.660) of mean age between responder and non-responder patients was found (Table 1).

In total, 12 patients were male and 10 were female. Out of the four non-responder patients, 2 were male and 2 were female while 10 of the 18 responder patients were male and eight were female (Table 1).

Viral Load Comparison

The mean pre-treatment viral load of responder patients was found to be 7.24E7±3.56E5 while the mean pre-treatment viral load of non-responder patients was 7.13E7±2.32E3. There was no significant difference (p=0.183) of mean viral load between responder and non-responder patients before the treatment (Table 1).

After 6 months of treatment, the viral load of responder patients was significantly lower as compared to the viral load of non-responder patients (p=0.009). At this stage, the mean viral load of responder patients was calculated to be 9.74E4±3.46E5 while the mean viral load of non-responder patients after 6 months of treatment was 9.02E7±2.42E3 (Table 1).

The mean viral load of responder patients after 12 months of treatment was 7.57E3±3.27E4 while the mean viral load of non-responder patients after 12 months was 3.96E5±1.48E3 (Table 1) and the difference was again significant statistically (p=0.021).

After 18 months of treatment, the responder patients had undetectable or very low viral load in serum but the non-responder patients still had a mean viral load of 4.63E5±6441 copies/mL (Table 1).

ALT

The mean pre-treatment ALT of responder patients was found to be 53.90±31.16 while mean ALT of non-responder was 55.11±14.51. There was no significant difference (p=0.697) of mean ALT between responder and non-responder patients before treatment (Table 1).

The mean ALT of responder patients after six months of treatment was 41.35±20.51 while mean ALT of non-responder patients after six months was 47.61±9.27. There was no significant difference (p=0.705) of mean ALT between responder and non-responders (Table 1).

After 12 months of treatment, the mean ALT of responder patients was 30.55±8.34 while mean ALT of non-responder after twelve months was 44.61±8.13. There was significant difference (p=0.001) of mean ALT between responder and non-responder after 12 months of treatment (Table 1).

The mean ALT of responder patients after 18 months of treatment was 26.50±4.12. While mean ALT of non-responder after eighteen months was 43.83±8.06. There was significant difference (p=0.000) of mean ALT between responder and non-responder patients after 18 months of treatment (Table 1).

Mutational Analysis

The blood samples of all 4 non-responder patients were sent for sequencing but unfortunately, DNA of two samples was not successfully sequenced while the remaining two samples were sequenced successfully. RT mutations, well known for their role in resistance, were found in both of these samples.

Mutational Profile of Patient 1

Patient 1 was male of 49 years who received lamivudine and entecavir treatments. The RT domain of the virus isolated from this patient was detected with rtY135S, rtI169P, rtV173P, rtL180I, rtA181V, rtT184Y and rtM204V mutations. These mutations are known to be associated with viral resistance against lamivudine telbivudine adefovir and entecavir. The patient was non-responder against lamivudine due to compensatory mutations rtL180I,

Table 1. Comparison of responder and non-responder patients in the study

Factor		Responder	Non-responder	Sig.
Age		36.45±14.89	38.50±13.63	0.662
Gender	Male	10 (83.3%)	2 (16.7%)	0.377
	Female	8 (80%)	2 (20%)	
Viral load	Pre treatment	7.24E7±3.56E5	7.13E7±2.32E3	0.183
	After 6 months	9.74E4±3.46E5	9.02E7±2.42E3	**0.009**
	After 12 month	7.57E3±3.27E4	3.96E5±1.48E3	**0.021**
	After 18 month	Undetectable or very low	4.63E5±6441	**0.001**
ALT	Pre treatment	53.90±31.16	55.11±14.51	0.697
	After 6 months	41.35±20.51	47.61±9.27	0.705
	After 12 month	30.55±8.34	44.61±8.13	**0.001**
	After 18 month	26.50±4.12	43.83±8.06	**0.000**

ALT: Alanine aminotransferase, Sig: Signature

rtV173P, rtL180I and rtM204V, while it was non-responder against entecavir due to rtM204V, rtI169P, rtT184Y and rtL180V (Table 2).

Mutational Profile of Patient 2

Patient 2 was a female of 41 years who was treated with lamivudine for 18 months. According to resistance profile, the patient was resistant against lamivudine due to compensatory mutations rtL80V/rtL80G and rtY135S. This mutational profile shows that the patient is not resistant against adefovir, entecavir, and tenofovir (Table 2).

Discussion

The quantitative factors like viral load and ALT significantly decreased during treatment in responder patients while not in non-responders. However, the sample size of the study was low and not enough for comparative analysis of quantitative factors. So, the study was designed to detect the RT mutations responsible for resistance instead of quantitative comparison.

In this study the resistant mutations rtY135S, rtV173P, rtL180I, rtM204V, rtA181V, rtI169P, and rtT184Y were detected which made the patients non-responder against lamivudine, telbivudine, adefovir and entecavir. However, no mutation was found in association with tenofovir.

In a similar type of previous study from Pakistan, almost same mutations were detected from multiple drug resistant patients (16). The mutations reported in that study were: rtL80G, rtY135S, rtI169P, rtV173L, rtL180M, rtA181V, rtT184Y, rtM204V and rtN248H, which were reported to be associated with lamivudine, telbivudine, adefovir and entecavir. Mutation rtN248H was not found in current study, however it was reported in the only previous study from Pakistan. Mutation rtY135S was found in current study which was only reported in the other study from Pakistan (16). It was reported previously from Pakistan that resistance mutations are found frequently on the positions rtL80V/G, rtY135S, rt169P and rt248H while present in low proportion on positions rt184Y and rtL80G.

Another recent study from Iraq reported the mutations rtL80I/V, rtV173L, rtL180M, rtA181S, rtA194T, rtS202I, rtM204V/I, rtN236T and rtM250L/V associated with resistance against lamivudine, telbivudine, adefovir, entecavir and tenofovir (17). However, the mutations rtA194T, rtN236T, and rtM250L/V were not found in our study. Mutation on position rtA194T was generally considered as associated with tenofovir resistance.

The mutations rtM204I and rtL180M, detected in our study, were most frequently found in previous studies from different areas of the world (2,3,16,17,18,19,20,21,22,23,24,25,26,27,28,29,30,31,32,33,34,35,36,37,38,39,40,41,42). In many of the studies (7,9,16,17,20,22,24,25,26,27,28,29,32,39,40,41), these two mutations were reported to have an association with lamivudine while in some other studies (7,9,16,17,31,32,35,37), these were found to be associated with telbivudine resistance as well. These reports confirm that the mutations rtM204I and rtL180M have association with lamivudine and telbivudine resistance. Besides telbivudine and lamivudine resistance, these mutations were also reported to have some association with other NAs like adefovir, entecavir and tenofovir (2,3,18,19,23,30,33,38,42).

The mutations rtV173P and rtL80G, detected in our study, were second most frequent mutations reported in previous studies (2,9,16,17,18,19,26,29,37,43,44). In some studies (9,16,17,26,29,37,43,44), rtV173P was reported to have an association with lamivudine while in some other studies (9,16,17,37,43), it was found to be associated with telbivudine. In some studies (16,17,26,37,43), the mutation rtL80G was reported to have an association with lamivudine and telbivudine resistance. These reports confirm that the mutations rtV173P and rtL180M have association with lamivudine and telbivudine resistance. In some studies (2,18,19), rtV173P was reported to have association to other NAs too.

The mutations rtA181V detected in our study was third most frequent mutation reported in previous studies (7,9,16,17,33,34,37,38,40,41). In all studies, rtA181V was reported to have an association with adefovir resistance while it was reported to cause multiple drug resistance in some studies too. This mutation was also detected in our study from a non-responder patient.

The mutations rtI169P and rtT184Y detected in our study were fourth most frequent mutations reported previously (16,19,29,33,35,39,40). In some studies (16,19,29,39,40), rtT184Y was found to be associated with entecavir resistance while in some other studies, these mutations were shown to have an association with adefovir and lamivudine resistance too (33,35). In three studies, mutation rtI169P was reported to be associated with entecavir resistance too (16,19,29).

In Pakistan, the mutational analysis is not performed before the start of therapy which increases the risk of treatment failure in chronic HBV patients. It is mainly due to lack of facility and lack of awareness. Another fact behind the unavailability of mutation testing is the unavailability of experts who can carry out the mutational screening. Present study confirms that the resistance

Table 2. Resistance mutations profile of the non-responder patients in the study

	Patient 1		Patient 2	
	Detected mutations	**Resistance prediction**	**Detected mutations**	**Resistance prediction**
Lamivudine associated	rtY135S, rtL180I, rtV173P, rtL180I, rtM204V	Resistant	rtI80V, rtL80G, rtLY135S	Resistant
Adefovir associated	rtA181V	Resistant	None	Susceptible
Telbivudine associated	rtI169P, rtT184Y	Resistant	rtI80V, rtL80G	Resistant
Entecavir associated	rtM204V, rtV173P, rtL180I	Partly	None	Susceptible
Tenofovir associated	None	Susceptible	None	Susceptible

mutations are present in the genomes of viruses circulating in the country. So, it is necessary to analyze the RT domain of virus before start of therapy in the patients.

Study Limitations

The study has a small number of non-responder patients that is a limitation of current study.

Conclusion

Mutations rtY135S, rtI169P, rtV173P, rtL180I, rtA181V, rtT184Y, rtM204V, rtL80V/rtL80G, and rtY135S are present in genome of HBV circulating in Pakistani patients. These mutations give resistance to virus against lamivudine, telbivudine, adefovir, and partially resistance against entecavir. However, no mutation was found to be associated with viral resistance against tenofovir

Ethics

Ethics Committee Approval: The study was started after the approval from "humans and animals ethics committee", University of Poonch Rawalakot.

Informed Consent: An informed consent was given to each of the patient for reading and signing before his/her enrolment to the study.

Peer-review: Externally peer-reviewed.

Authorship Contributions:

Concept: M.M. Design: M.M., M.A.A., Data Collection or Processing: S.J., Z.U.R., M.A.A., Analysis or Interpretation: M.M., S.J. Literature Search: S.J., Z.U.R. Writing: M.M., S.J., Z.U.R.

Conflict of Interest: No conflict of interest was declared by the authors.

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