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SHORT COMMUNICATION

Sensitivity detection of Abacavir in human through SNP detection of HLA-B*5701 allele

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Abstract: The main objective of this study was to make sure whether randomly taken 12 samples were sensitive to abacavir. The genomic DNA from 12 blood sample were extracted by phenol chloroform DNA extraction method, extracted genomic DNA were amplified and sequenced, thereafter SNPs were detected. Every sample had shown the presence of normal base at SNP position. This study indicated, those randomly taken 12 patients were sensitive to abacavir, so they can consume abacavir if they get infected with HIV.

Key words: Abacavir; Sensitivity; HIV; Genomic DNA; HLA-B*5701.

Introduction

Abacavir, a powerful reverse transcriptase inhibitor, a nucleoside analogue used in antiretroviral therapy for the treatment of HIV infection, is generally well tolerated drug but its adverse effect can cause hypersensitivity in 5% to 9% of patients during the first 6 weeks of treatment, that limits its use in antiretroviral therapy and require clinical surveillance [1]. Detection of SNP at HLA-B*5701 allele can identify patient's risk of developing hypersensitivity reactions, i.e. rash, fever, gastrointestinal tract problems and lethargy [2].

The prodrug abacavir is converted into active drug carbovir triphosphate by cytosolic enzymes. Carbovir triphosphate competes with dGTP for incorporation into viral DNA by HIV reverse transcriptase. Once incorporated, HIV replication is inhibited because carbovir triphosphate does not contain the necessary hydroxyl group on the ribose sugar for further extension, hence causes DNA chain to terminate and thereby HIV replication is inhibited.

Materials and Methods

Study Site

This study was conducted in geneOmbio Technologies Pvt. Ltd. at Pune.

Sample collection

Blood sample, collected in vacutainers containing EDTA to prevent blood clotting, was used for this study. 12 blood samples were collected.

Genomic DNA extraction & quality check

Genomic DNA was extracted following phenol chloroform method using the reagents 1X PBS, 2X STE, 10% SDS, Proteinase K, Equilibrated Phenol, and Chloroform: Iso-Amyl Alcohol (24:1), Isopropanol, 70% Ethanol, Nuclease free water. DNA sample was run through 0.1% (w/v) agarose gel in 0.5% TE, using agarose gel electrophoresis. DNA band was clearly observed under Trans-Illuminator and images were taken by Gel Documentation System.

Primer designing for specific amplification of extracted genomic DNA

The rs ID of HLA-B 5701 was put on dbSNP page and searched for FASTA sequence. Around 48-52 bp of FASTA sequence was copied and gaps were deleted from the sequence and it was then pasted on Primer3 Input.

FASTA sequence

CCTCACACTTACAATGGGACAAGGGGAAC
CAGGAGGCCCCCAAGGGGATCCCTGGGT
TCCACACGAACTCCTCCTACCCTCATTGTG
TGACAGCAGCCATGCCTCCTCCTGGGGAT
CAGGATCTATTACCTGTGCCTGGAGAGGA
GGGGACTCCTCTTCTCACCCGCTGGTCTC
TGGACACATACTGTCCAATTCCCCTGKGG
CAGCTGTAATGTGTAGTTCAATGGGCACT
CATTTGTCCCCTTTTAAGGGTACCCTCCTT
TAGAATCCAGGACCTTCTACCCTGCAGAG
TGTGGTTTTGGGAGAGAAGTGCAAAATC
CCACGACAGGTGAGTTGAAGGAATGGGA
TATGGAGCCACATCCACTTCCACCCCTTG
GTATC

PCR-product size length was selected (300-400 bp) and clicking Pick Primer, once the forward and reverse primer was seen, these were copied and pasted on Primer Blast software to confirm whether the designed primers were specific for HLA-B gene. Database was selected and Homo

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sapiens was selected as organism. After clicking Get Primers, all information was seen indicating the designed primers were confirmed for the HLA-B gene.

Amplification of the desired genomic DNA by PCR and Sequencing of PCR product

Conventional Gel based PCR was performed using Applied Biosystems 2720 Thermal Cycler. Concentration of each PCR ingredient was taken as per the standard reaction. The amplified products were checked on 0.2% (w/v) agarose gel in 0.5X TE, using agarose gel electrophoresis and images of a stained gel was taken by gel documentation system. 300 bp to 400 bp amplicons were generated among 12 samples, suggesting the amplification of correct size as desired.

PCR product was further purified by Enzymatic Purification using the reagents exonuclease, SAP, buffer, and water.

Table 2: The thermal cycler program which amplify the DNA

Steps	Temperature (°C)	Time	Number of Cycles	Final Volume (ul)
Initial Denaturation	95°C	5minute		
Denaturation	95°C	30second		
Annealing	55°C	30second	35Cycles	25ul
Extension	72°C	30second		
Final Extension	72°C	10minute		
Cooling	4°C	Until use		

Table 3: Time and temperature for obtaining the optimum temperature in enzymatic reaction by PCR

37°C	95°C	4°C
1 Hour	10 Minutes	Until use

After enzymatic purification, the purified product was gone for cycle sequencing PCR.

If PCR amplicon is 4ul, primer is 1ul, sequencing buffer is 1.8ul and RR is 0.5ul, then amount of water is, {10-(4+1+1.8+0.5)} ul= (10-7.3) ul= 2.7ul for cycle sequencing. Total reaction volume is 10ul.

If PCR amplicon is 3ul, primer is 1ul, sequencing buffer is 1.8ul, RR is 0.5ul, then amount of water is, {10-(3+1+1.8+0.5)} ul= (10-6.3) ul= 3.7ul for cycle sequencing. Total reaction-volume is 10ul.

Temperature required for cycle sequencing:

The next step of sequencing is clean up which was done using absolute alcohol, EDTA, ethanol, hidi. Thereafter sequencing plate was placed into the Applied Biosystem 3130 Genetic Analyzer, the sequencing PC was set properly and started. Once sequencing run was done, data were assimilated by Sequencing PC using the software collection supplied with the DNA Sequencer. The software ABI Sequencing Analysis Software v 5.3 with KB Base caller v1.4 interpreted the four fluorescent dyes, and produced the raw data nucleotide sequence as a display of peaks, i.e. an electropherogram. The highest peak at every specific nucleotide position was allocated as the nucleotide corresponding to the color of that peak. The color allocated for each nucleotide was:

- Red = T (thymine)
- Blue = C (cytosine)
- Green = A (adenine)
- Black = G (guanine)

The sequence can be read from the electropherogram as the definitive nucleotide order. If the software were unable to interpret a particular fluorescent peak a letter *N* was assigned for that base position. This could be caused by two bases being present at one position (heterozygosity) or weak peak height. In addition to the chromatogram trace, automated DNA Sequencers generate a text file of sequence data by calling the bases associated with each peak (base calling). It is important to manually check the electropherogram against the predicted base, as errors are reasonably common. Commonly, errors occur near the beginning and the end of any sequencing run. Chromas Lite, free software was used to displays the electropherogram.

SNP detection

Chromas Lite software was installed in computer. All those 12 DNA sequence's results were copied from Sequencer machine and pasted in computer. Chromatogram file was opened (one by one). Thereafter clicking Edit, Copy Sequence, FASTA Format, and the DNA sequence was copied. The copied sequence was pasted on word. The rs id of HLA-B gene was put on dbSNP and the FASTA sequence of HLA-B gene was shown. The FASTA sequence was copied and pasted in word. K was there in the FASTA sequence. (K represents SNP). 10 to 15 base prior to K, were copied and pasted in Find and Replace window. Thereafter the whole DNA sequence (from Sequencing Machine) was selected. Clicking Find in & Current Selection from Find and Replace window, the portion of sample DNA sequence similar to the FASTA sequence of HLA-B gene, was selected. The base came after the selected portion, was the SNP. In all 12 samples, the base T (Thymine) was present in place of K (single nucleotide polymorphism), which represents normal allele (base T in place of K, represents normal allele and if G is present in place of K, then it represents mutated allele). In all the 12 samples the SNP represented the normal allele (T), and not the mutated allele (G). The IUPAC CODE Table helped to determine

whether the SNP represented normal allele or mutated allele.

Results and Discussion

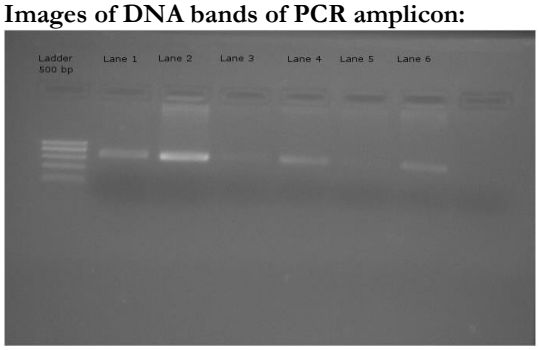
All 12 samples had shown a DNA band of 300-400bp in gel (this is the PCR amplicon's band).

Below are the FASTA sequence of 12 sample's HLA-B*5701 allele:

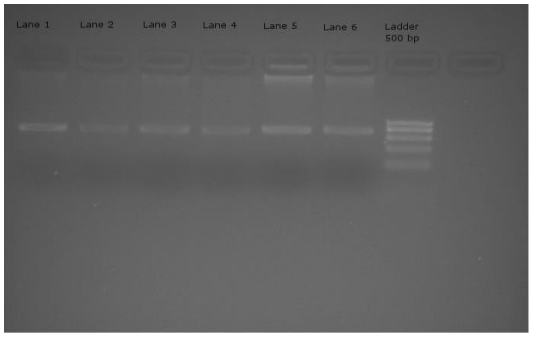
1. TAAGTGGTTCCTGGGCGCATCATAGTCGT TCGTCGGATCTATTACCTGTGCTGGAGAG GAGGGGATCCTCTTCTCACCCGCTGGTCT CTGGACACATACTGTCCAATTCCCCTGTGG CAGCTGTAATGTGTAGTTCAATGGGCACT CATTTGTCCCCTTTTAAGGGTACCCTCCTT TAGAATCCAGGACCTTCTA
2. GCTGGTTCCAGGCCTCCTTCTGGGACAGG ACTATTACCTGTGCCTGGAGAGGAGGGG ACTCCTCTTCTCACCCGCTGGTCTCTGGGC ACATACTGTCCAATTCCCCTGTGGCAGCTG TAATGTGTAGTTCAATGGGCACTCATTTG TCCCCTTTTAAGGGTACCCTCCTTTAGAAT CCAGGACCTTCTACCCTGC
3. CCTATGTCAAATTTTTCTTTTTGGGGATCA GGATCTATTACCTGTGCCTGGAGAGGGG GGATCCTCTTTCTCACCCGCTGGGCTCTG GACACATACTGTCCAATTCCCCTGTGCAGC TGTAATGTGTAGTTCAATGGGCACTCATT TGTCCCCTTTTAAGGTACCCTCCTTTAGAA TCCAGGACCTTCTACCCTGCA
4. GAAGGCCGGGGTTTTCTTTCTTAGTCAGG ACTATTACCTGTGCCTGGAGAGAGGGGAT CCTCTTCTCACCCGCTGTCTCTGGACACAT ACTGTCCAATTCCCCTGTGCAGCTGTAATG TGTAGTTCAATGGGCACTCATTTGTCCCCT TTTAAGGGTACCCTCCTTTAGAATCCAGG ACCTTCTACCCTGCAGAG
5. GGGGTGGTGTTGCTTCTTGGTTTGGCTCC TTAGACAGGATCTATTACCTGTGCCTGGA GAGAGGGGATCCTCTTCTCACCCGCTGGT CTCTGGACACATACTGTCCAATTCCCCTGT GCAGCTGTAATGTGTAGTTCAATGGGCAC TCATTTGTCCCCTTTTAAGGGTACCCTCCT TTAGAATCCAGGACCTTC
6. GGGGCGTCAAGTTTTTCTTCCGAAACAGG ATCTATTACCTGTGCCTGGAGAGGAGGG GATCCTCTTCTCACCCGCTGGTCTCTGGAC ACATACTGTCCAATTCCCCTGTGGCAGCTG TAATGTGTAGTTCAATGGGCACTCATTTG TCCCCTTTTAAGG
7. GGGAGCAGGATGGTCCTCCCGAACGTTCC CGATCTATTACCTGTGCCTGGAGAGGAGG GGACTCCTCTTCTCACCCGCTGGTCTCTGG ACACATACTGTCCAATTCCCCTGTGGCAGC TGTAATGTGTAGTTCAATGGGCACTCATT TGTCCCCTTTTA
8. TGGGGGGGGGGGACAGGATGAATGGAA TGGCACTATCCGATTCTTCATGTGCCGGG AGAGAGGGGACTCCTCTTCTCCCCGCTGG TCTCTGGACACATACTGTCCAATTCCCCTG TGGCAGCTGTAATGTGTAATTCAATGGGC ACTCATTTGTCCCC
9. CGCCTTAGCGTAGAAGTGGTATGTGTCTA CGTATCCGATTCCTTACCTGTGCCTGGAG AGGAGGGGACTCCTCTTCTCACCCGCTGG TCTCTGGGCACATACTGTCCAATTCCCCTG TGGCAGCTGTAATGTGTAGTTCAATGGGC ACTCATTTGTCCC
10. GAAATTACGTGCGGGTGGTAAGTGTGTA CGACCCGATCTATTACCTGTGCCTGGAGA GGAGGGGACTCCTCTTCTCACCCGCTGGT CTCTGGACACATACTGTCCAATTCCCCTGT GGCAGCTGTAATGTGTAGTTCAATGGGCA CTCATTTGTCCCCTTTTAAGGGTACCCTCC TTTAGAATCCAGGACCT
11. CACCAAAAGTCCTGTCTTCTCCTGTCGGTT CCCGATCTATTACCTGTGCCTGGAGAGGA GGGGACTCCTCTTCTCACCCGCTGGTCTCT GGGCACATACTGTCCAATTCCCCTGTGGC AGCTGTAATGTGTAGTTCAATGGGCACTC ATTTGTCCCCTT
12. CCAGTGGGGGGCCCGGTGGTATCTCCTCT ACGTACCCGATCTATTACCTGTGCCTGTGA GAGGAGGGGACTCCTCTTCTCACCCGCTG GTCTCTGGACACATACTGTCCAATTCCCCT GTGGCAGCTGTAATGTGTAGTTCAATGGG CACTCATTTGTCCCCTTTTAAGGGTACCCT CCTTTAGAATCCAGGACCTTCTACCCTGCA GAGT

All FASTA sequence has one base colored with Red, and that denoted the SNP which was originally written as K. Each sample had shown T (Thymine base) in SNP position, which signified that, all HLA-B*5701 allele (of the 12 samples) had never undergone mutation. Therefore, it can be concluded that, any samples are not resistant to abacavir and all samples are sensitive to abacavir, so they can consume abacavir if get infected with HIV.

Images of DNA bands of PCR amplicon:



First lane indicates ladders of different size (from 100bp which is at the bottom position, to 500bp which is at the top position), 2nd lane indicates PCR amplicon's band of sample S_1, 3rd lane indicates PCR amplicon's band of sample S_2, 4th lane indicates PCR amplicon's band of sample S_3, 5th lane indicates PCR amplicon's band of sample S_4, 6th lane indicates PCR amplicon's band of sample S_5, 7th lane indicates PCR amplicon's band of sample S_6.

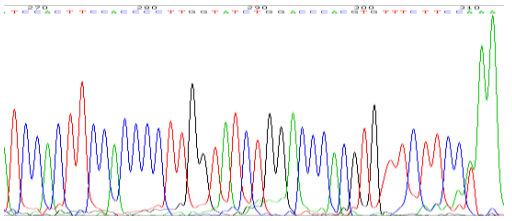


Last lane indicates ladders of different size (from 100bp which is at the bottom position, to 500bp which is at the top position), 1st lane indicates PCR amplicon's band of sample S_7, 2nd lane indicates PCR amplicon's band of sample S_8, 3rd lane indicates PCR amplicon's band of sample S_9, 4th lane indicates PCR amplicon's band of sample S_{10}, 5th lane indicates PCR amplicon's band of sample S_{11}, 6th lane indicates PCR amplicon's band of sample S_{12}.

The output of DNA sequence in Chromas Software had shown excellent chromatogram.

1.

2.



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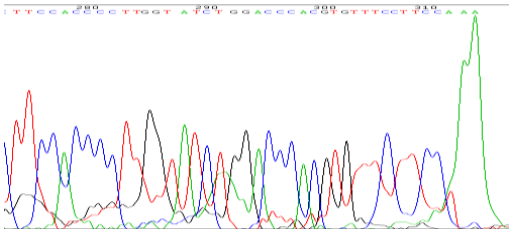

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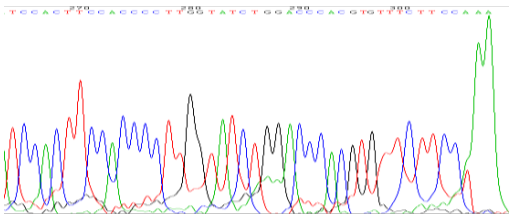

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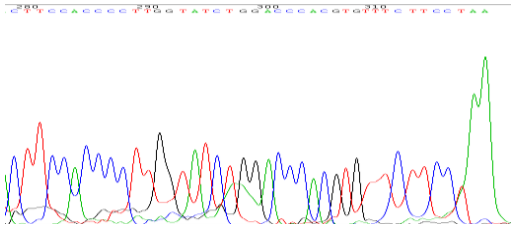

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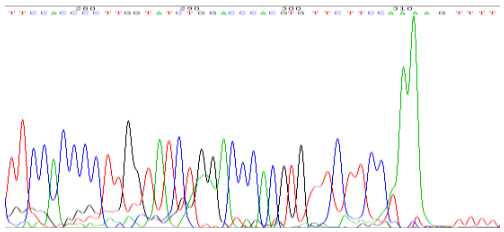

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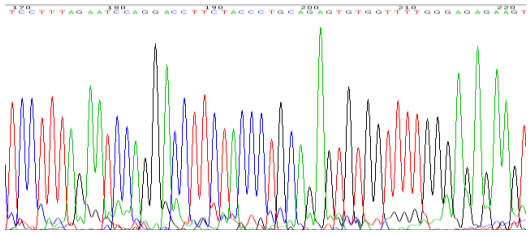

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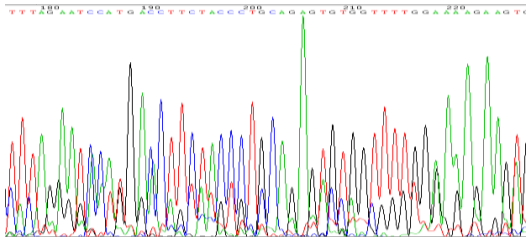

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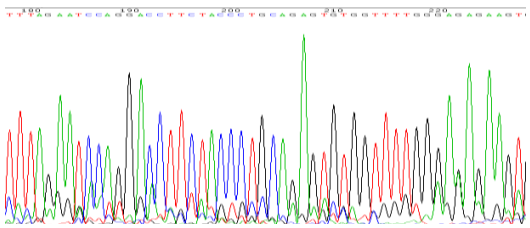

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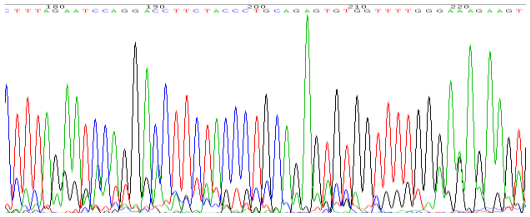

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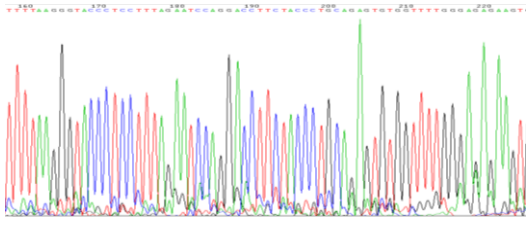
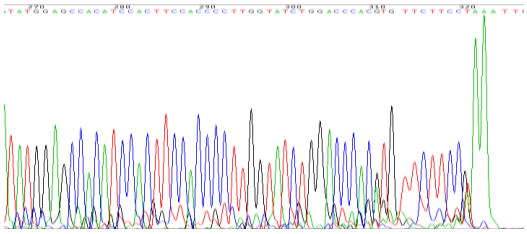


12. 

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