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AMINO ACID SEQUENCE VARIATIONS WITHIN ENVELOPED PROTEIN 2 (E2) OF HEPATITIS C VIRUS SUBTYPE 3a AND RESPONSE TO INTERFERON ALPHA PLUS RIBAVIRIN COMBINATION THERAPY

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Abstract

Hepatitis C virus subtype 3a (HCV-3a) is a highly prevalent type in Pakistan. Although, the response rate of this type is higher to Interferon (IFN) therapy than other types, yet a significant percentage of HCV-3a patients fail to respond to IFN therapy. The variation in the genetic makeup of HCV is regarded as one of the important factors that influenced the response rate of HCV to IFN therapy. This study was conducted to determine the genetic variation in HCV-3a and its association with IFN therapy response. One hundred and fifty samples of HCV-3a patients were collected at the Pathology Department of Shalamar Hospital, Lahore. Out of those, 50 were initial samples of responders (IR) taken at the start of the therapy and 100 of non responders including 50 initial (INR) and 50 last samples (LNR). To determine the association of amino acid sequence variation in E2 protein of HCV-3a with therapy outcome, two regions $E2_{506-564}$ and $E2_{622-714}$ from the E2 protein were amplified and sequenced. In the first region ($E2_{506-564}$) a hyper-variable sequence of 33 amino acids ($E2_{522-554}$) was found where the mutation rate was lower in IR than INR and LNR. In contrast, in the PePHD domain ($E2_{665-676}$) of the second selected region ($E2_{622-714}$) mutation in IR isolates was observed higher than INR and LNR. In conclusion, the treatment response was associated with amino acid (aa) sequence variation in the both selected regions of E2 protein of HCV-3a ($E2_{506-564}$ and $E2_{622-714}$).

Keywords: HCV, subtype 3a, amino acid sequence variation, treatment response.

Introduction

The mechanism of resistance to Interferon therapy in HCV infected patients is not comprehensively understood so far. It has been speculated that many factors related to the host (i.e., Age, gender, and race) and virus (i.e., genotype, viral load, and genetic variation) may be linked with Interferon (IFN) therapy response (1-5).

A lot of work has been done to investigate how HCV escapes the host's immune response and antiviral therapy and how it damages the hepatocytes. One belief is that ineffective response of HCV to antiviral therapy is because of quick mutations in the hyper-variable regions of the viral genome, which cause rapid changes in its enveloped proteins and enables the virus to evade the IFN therapy (6-7). Controversial findings regarding the association of mutation in different regions of the HCV genome (i.e. E2-PePHD, NS5A-PKRBD, NS5A-ISDR and NS5A-V3) with Interferon therapy response have been reported previously (8-16).

To see the correlation of genetic variation in the HCV genome with Interferon therapy most of the previous studies were focused on the Interferon sensitive determining region (ISDR) from non structure protein 5A (NS5A) and RNA-dependent protein kinase eukaryotic transcription factor-2 alpha (PKR/eIF-2α) phosphorylation homology domain (PePHD). Regarding ISDR in most of the studies reported internationally or locally, the researchers agreed on the point that the molecular changes in this region of HCV subtype 3a have no effect on IFN therapy response. However, the variation in ISDR region of HCV subtype 1a was found highly associated with IFN therapy response (14, 17-19). Although some of the researchers believe that the variations in the PePHD domain within the E2 protein of HCV-3a influence the IFN therapy outcome (8-14), yet the results of other studies are conflicting because this region is highly conserved (20-25).

Most of the local studies were based upon small sample size and the samples were taken either at the start or end of therapy and no correlation of viral genomic variation in initial and last samples were observed. Furthermore, most of the studies were focused either ISDR within NS5A or PePHD within E2 protein of the HCV-3a to see the influence of these variations on the success rate of treatment. The correlation of ISDR with treatment response in HCV-3a has not been proven in most of the previous studies. Regarding PePHD domain, the results of previous studies were contradictory. So, there was a need to explore the variation in new regions of the HCV genome and their effect on IFN plus Ribavirin (RBV) combination therapy.

In this study, we examined the amino acid variation within two selected regions $E2_{506-564}$ and $E2_{622-714}$ of E2 protein of HCV-3a to see their association with response to IFNα-2b plus RBV combination therapy in Pakistani patients chronically infected with HCV-3a. The first selected region was first time taken under consideration while the second region that includes PePHD domain was examined before in few previous studies done in Pakistan.

Materials and Methods

Patient and sample selection:

In the present study 150 samples of the Hepatitis C patients chronically infected with HCV-3a were collected in Shalamar Hospital Lahore from August 2009 to January 2013. Out of those 150 samples 100 were taken before therapy (50 IR and 50 INR) and 50 at the end of therapy from non-responding patients (LNR). The selection of HCV subtype 3a was based on its high rate of prevalence (60-80%) in Pakistan. The patients were originated from different areas of the province Punjab of Pakistan. All the patients were naive and treated for 6 months with 3MU of recombinant IFNα-2b (thrice a week) and RBV (1000-1200 mg/day according to body weight). The patients co-infected with Hepatitis B virus (HBV) and/or human immunodeficiency viruses (HIV) were excluded from the study. The patients with severe psychiatric problems, uncontrolled diabetes, active TB, less than 50,000/mm^3 platelets, anemia, decompensate liver disease, and the elevated serum creatinine level (>1.5 mg/dL) were also not included in this study.

HCV RNA detection and genotyping: Real-time PCR technique was used for the detection of HCV RNA. To isolate the HCV RNA from the sample, column based Roboscreen RNA isolation kit (Instant virus RNA kit: AJ ROBOSCREEN Germany) was used. The amplification of HCV RNA was done by Real-time amplification kit (Robogene® HCV RNA qualitative kit: AJ ROBOSCREEN Germany). The kit had <100 IU/ml or <200 copies/ml detection limit. HCV genotyping was done by multiplex PCR as explained somewhere else (26).

Amplification of the target sequences $E2_{1800-2032}$ and $E2_{2203-2482}$ **from the E2 protein coding region of HCV-3a:**

Primer designing: Two sets of primers were designed for the amplification of two target sequences $E2_{1800-2032}$ and $E2_{2203-2482}$ (codon 506-564 and 622-714) selected from E2 protein coding region of the HCV subtype 3a (Table-1). The numbering was according to the HCV-3a prototype HCV-NZL1 (Accession number NC_009824). Primer designing was done on Primer3 software (http://bioinformatics.weizmann.ac.il/cgi-bin/primer/primer3.cgi). First set of primers includes two pairs HVE2A, HVE2B and HVE2C, HVE2D for the amplification of $E2_{1800-2032}$ region (codon 506-564). The first pair (HVE2A, HVE2B) of primers was designed to amplify a long DNA fragment in the first PCR round and the second pair (HVE2C and HVE2D) was to amplify the inner portion of the first round amplified sequence. Similarly for the amplification of the second selected region $E2_{2203-2482}$ (codon 622-714), other two pairs of

primers were designed including an outer and an inner pair (PE2A, PE2B and PE2C, PE2D).

Amplification of the first selected region $E2_{1800-2032}$ (codon 506-564)

HCV RNA Isolation: HCV RNA was isolated from 250µL serum sample with RNA isolation kit (TRI REAGENT-LS, TS 120, by the Molecular Research Centre, USA) according to the kit protocol.

Complementary DNA (cDNA) synthesis: The cDNA was generated by RT-PCR using 200 units of M-MLV RT enzyme (Fermentas®) and 0.5µM of outer antisense primer HVE2B. Reverse transcription was done at 40°C for 55 min, followed by heat inactivation of the RT enzyme at 95°C for 5 min.

Amplification of cDNA by nested PCR: For the amplification of cDNA, two PCR rounds (Hemi-nested and Nested) were applied. In the first round, a large fragment of 426 bps from E2 coding sequence of the HCV-3a genome was amplified with outer sense (HVE2A) and outer anti-sense (HVE2B) primers. Other reagents used were: 10X *Taq* Buffer (2µL), dNTPs mix (0.2mM), primers (1µM each), 25mM $MgCl_2$ (2.4 µL), *Taq* DNA Polymerase (1U), nuclease free water up to the final volume of 25 µL. In this round 35 cycles were used, with each cycle consisting of denaturation at 94°C for 45 Sec, annealing at 54°C for 45 Sec, extension at 72°C for 50 Sec, followed by a final extension at 72°C for 10 min.

In the second round inner portion of 233 bps from the first amplified sequence was amplified. All reagents with their concentrations were same as in used in the first round except new pair of primers (inner sense HVE2C and inner anti-sense HVE2D). Thermal conditions for the second round were 94°C for 35 Sec (denaturation), 52°C for 35 Sec (annealing) and 72°C for 45 Sec (extension) for 35 cycles followed by a final extension at 72°C for 10 min.

Amplification of the second selected region $E2_{2203-2482}$ **region** (codon 622-714)

The cDNA from the second region was amplified similarly as the first region using PE2A and PE2B primers in the first round to amplify a 456 bps fragment and PE2C and PE2D primers in the second round for the amplification of the second target sequence $E2_{2203-2482}$ of 280 bps. Other reagents used for the amplification were same. To amplify the second fragment 35 cycles were used in each round (First and second) that contains the thermal conditions 94°C (50 Sec), 54°C (50 Sec) and 72°C (55 Sec) for denaturation, annealing and extension respectively in the first round and 94°C (40 Sec), 54°C (40 Sec) and 72°C (45 Sec) in the second round followed by a final extension at 72°C (10 min) in each round. The second round amplified fragments were visualized by 2% agarose gel electrophoresis stained with Ethidium Bromide.

Sequence Analysis

For the sequencing of amplified regions, the second round PCR positive products were run on 2% agarose gel and purified with the kit (QIAGEN, Valencia, California) according to the kit protocol. The purified DNA was used as a template for the sequencing PCR. The sequencing PCR products were analyzed by the automated sequencer (ABI PRISM 3100 genetic analyzer; Applied Biosystem) using the Big-Dye Terminate cycle sequencing ready to use reaction kit. The product was sequenced to get the nucleotide sequence of the amplified products. The deduced amino acid sequences of the E2 region were compared with the E2 sequences identified in the prototype isolates for HCV-3a (NZL1). Multiple amino acid sequence alignment was carried out using CLUSTAL X (version 1.81) and MEGA (version 2.1) (http://www.megasoftware.net/) to analyze the amino acid sequences (27-28).

Statistical analysis: According to the variable nature, the descriptive statistics was used and all the results were presented in the tabulated form. The quantitative variables were stated as means and standard deviations. Comparisons between groups (IR, INR, LNR) were done by $\chi 2$ or Fisher's exact test for the categorical variables and the Student's *t*-test for the quantitative variables.

Results

Amino acid sequences of the selected regions from the E2 protein of HCV subtype 3a isolates were compared with HCV-3a prototype NZL1 and with each other to see the relation of any sequence change with treatment response. The sequences of responding and non responding isolates were also compared with each other to see any sequence difference between these isolates. Sequences of pre-treatment and end of therapy isolates of non responders were also compared.

In the first selected region ($E2_{506-564}$) the amino acid sequence of carboxyterminal side from 555 to 564 was found highly conserved. On the N-terminal side from 506 to 521 some variations were examined in all the HCV 3a isolates (IR, INR and LNR) as compared to a HCV-3a prototype sequence of NZL1 (Fig-1). In this study a hyper-variable region of 33 amino acid residues from 522-554 ($E2_{522-554}$) was examined in all the IR, INR and LNR isolates with difference in mutation rates. In the isolates of initial samples from responding patients (IR), the differences between the mean number of mutations were observed 5.49 (range, 3-7) including the whole selected region from the amino acid residue 506 to 564 (compared to the NZL1). In the isolates of initial and last samples from non responding patients (INR and LNR) the mean mutation difference in this region was 7.60 (range, 6-11) and 9.71 (range, 8-15) respectively (Table-2). In the hyper-variable region ($E2_{522-554}$) the difference between mean mutation numbers of amino acid sequence of IR, INR and LNR

isolates was 5.06 (range, 2-6), 6.97 (range, 5-9) and 8.57 (range, 7-12) respectively (Table-2).

In the second selected region ($E2_{622-714}$) the amino acid sequence pattern of carboxyterminal side from 677 to 714 was highly conserved in INR and LNR isolates but variable in IR isolates. The N-terminal side of 622-664 was highly variable in all the isolates (IR, INR and LNR) as illustrated in fig-2. In the complete sequence of the selected region from 622 to 714 amino acid residues of IR isolates the difference between mean mutation numbers of amino acid sequence was 5.40 (range, 5-8) compared to the NZL1. In the initial and last samples of non responding patients the mean mutation difference was exhibited 2.8 (range, 0-4) and 2.67 (range, 1-4) respectively (Table-3). In the PePHD domain ($E2_{665-676}$), the mean mutation difference in the numbers of amino acid sequence of IR, INR and LNR isolates was 0.87 (range, 0-2), 0.0 (range, 0-0) and 0.07 (range, 0-1) respectively (Table-3).

Discussion

Interferon alpha (IFN-α) activates the double-stranded RNA protein kinase-R (PKR) that hampers the translation of both viral and cellular proteins by phosphorylating the α-subunit of eukaryotic initiation factor (eIF2α) (29). The nonstructural protein 5A (NS5A) of HCV obstructs the function of activated PKR through the PKR-binding domain (PKRBD) and is suggested one of the possible mechanisms of HCV to evade the antiviral effects of IFN-α (30-31).

It has also been described previously that the mutations within the Interferon sensitive determining region (ISDR) of HCV subtype 1a and 1b are associated with IFN-α sensitivity. The ISDR is a part of the PKR-binding domain of HCV protein NS5A (8, 17, 21, 32-37). Other studies especially reported from Western countries examined very rare mutations within this region (18, 38-39). No correlation between the variations within the ISDR of HCV-3a and treatment response was examined previously in Pakistan or other Asian countries where the HCV-3a is the most prominent type (17-19).

Most of the previous studies reported from Pakistan and outside were conducted either on the ISDR domain of NS5A or PePHD domain of E2 and the results of those studies regarding the association of mutations in these regions with treatment response were conflicting. Secondly, most of these studies were based upon a small number of the samples that were taken only at the end of therapy in non responding patients (8-14). However, In the present study, a total of 150 samples were included that were taken both at the start and end of the therapy in responding and non-responder patients infected with HCV subtype 3a and treated with IFN-α 2b plus Ribavirin combination therapy.

To see the effect of mutations in the HCV subtype 3a envelope protein 2 (E2) on the treatment response, two regions from E2 ($E2_{506-564}$ and $E2_{622-714}$) were selected in the present study. The amino acid sequence on the carboxyterminal side of the first region ($E2_{506-564}$) generated in the present study was highly conserved, while the N-terminal side showed variations from the amino acid sequence of the prototype HCV-3a (NZL1). A hyper-variable region of 33 amino acids ($E2_{522-554}$) with different mutation rates was examined in all the isolates (IR, INR and LNR) (Fig-1). The mean mutation difference in the numbers of amino acid sequence in this hyper-variable region was higher in INR and LNR isolates as compared to IR isolates. It was also interesting to see that the mutation rate was higher in the last samples of non responding isolates (LNR) as compared to their initial samples (INR). It indicates that this region in non-responding patients was under positive selection pressure during the therapy. The increase in mutation rate of this region may be helping the virus to survive against antiviral therapy that was also examined previously (40).

In the hyper-variable region ($E2_{522-554}$) two permanent replacements of amino acids as compared to HCV3a-NZL1 prototype were observed. At position 543 the Glutamine (Q) was permanently replaced with Leucine (L) and Lysine (K) was replaced by Threonine (T) at position 535 in most of the cases. At position 554 the amino acid position was unstable where the Serine (S) was replaced by Valine (V), Alanine (A) or Threonine (T). Two other amino acids Arginine (R) and Threonine (T) were replaced by Lysine (K) and Asparagine (N) at position 523 and 529 respectively (Fig-1).

Like in the first region the carboxyterminal side of the second selected region $E2_{622-714}$ was also found conserved in non responding isolates (INR and LNR). The situation in responding isolates (IR) was contrasted where the variation was observed in most of the cases. On the N-terminal side mutation was present in all the isolates either these belong to responding (IR) or non responding (INR and LNR) isolates (Fig-2). Mutation rate in the complete selected region ($E2_{622-714}$) (including PePHD domain and both carboxyterminal and N-terminal regions) was high in IR isolates as compared to INR and LNR (Table-3).

In PePHD domain the mutation was only present in IR isolates while the INR and LNR were highly conserved except one LNR isolate in which Glutamine (Q) was replaced by Leucine (L) at position 668. Although in most of the cases mutation in PePHD domain was associated with treatment response that was also observed in previous studies (8-14), yet in the four cases of IR isolates no mutation was seen in the PePHD domain.

According to Taylor *et al*. (1999) (41), the PePHD domain within the E2 protein of HCV interacts with PKR in vitro and suppressed its activity, that may help the HCV to evade the antiviral effects of IFN. Any variation in the sequence of this region is believed to affect the IFN therapy response outcome. However, conflicting results have also been reported in other studies where this region was found highly conserved in responding cases (20, 22-23, 25, 42-45). It was also seen in the present study where 4 out of 15 responding cases (IR) had no mutation and only one of the non responding cases (LNR) out of 15 had mutation at one place. It shows that we could not predict the treatment response accurately only relying on the mutation of PePHD domain. So the flanking regions of both sides of the PePHD domain should also be included to predict the therapy response precisely, as done in the present study where the mutation rate in amino acids sequence of the complete selected region (codon 622-714) was noted high in responding cases than in non responding cases.

On the carboxyterminal side of this second region the Aspartate (D) at position 629 and 657 was seen replaced by Asparagine (N) in most of the IR, INR and LNR isolates. The Threonine (T) at position 645 was unstable (replaced with different amino acids in different isolates). In PePHD domain at position 668 Glutamine (Q) was replaced by Leucine (L) in most of the IR isolates but no change at this position was found in INR isolates and in LNR isolates only in one isolate the Q was replaced by L.

In summary, the findings of the present study show that the isolates with <7 variations in aa sequence of the first selected region ($E2_{506-564}$) or <6 variations in its hyper-variable region ($E2_{522-554}$) of the initial samples show better response to IFN plus RBV treatment. The isolates with >4 variations in aa sequence of pretreatment samples of the second selected region ($E2_{622-714}$) or >1 variations in its PePHD domain ($E2_{665-676}$) also revealed better response. Therefore, aa sequences of these regions could be used as therapy response predictor at the start of treatment in HCV-3a infected patients.

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Table-1 Primer sequences used to amplify the selected regions from the E2 coding sequence of the HCV-3a NZL1 (Accession number NC_009824)

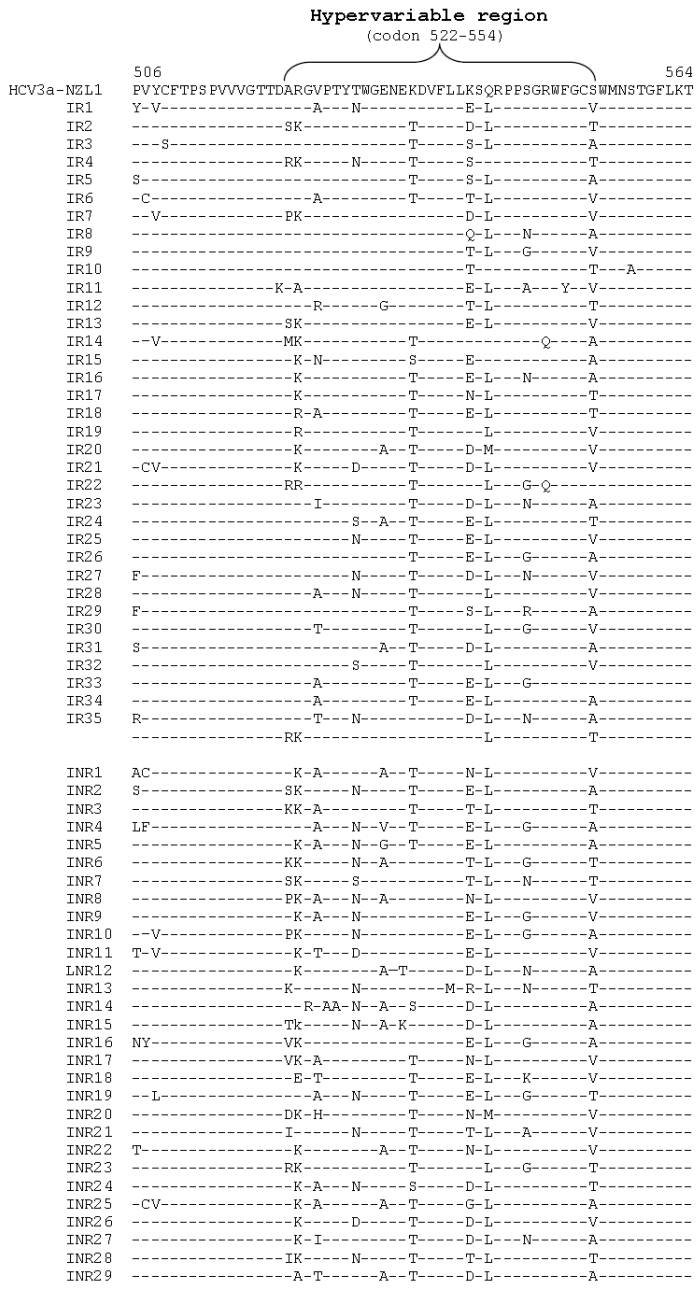
Primer Number	Primer Name	Primer Sequence (5'-3')	Specification	Nucleotide Position
Primers for $E2_{1800-2032}$ region (codon 506-564)				
1	HVE2A	5' gct gca agc cca tca ctt tc 3'	Outer sense	1712-1731
2	HVE2B	5' acg tgg tct cgg gat gtt tc 3'	Outer anti-sense	2137-2118
3	HVE2C	5' ctg gca cta cgc acc tag ac 3'	Inner sense	1800-1819
4	HVE2D	5' acg tct tga gaa acc ccg tg 3'	Inner anti-sense	2032-2013
Primers for $E2_{2203-2482}$ region (codon 622-714)				
1	PE2A	5' cat ccc gag acc acg tac ag 3'	Outer sense	2122-2141
2	PE2B	5' cat cag cat cag cca aag gg 3'	Outer anti-sense	2577-2558
3	PE2C	5' tgg cat tac cca tgt aca gtc 3'	Inner sense	2203-2223
4	PE2D	5' tgc cag atc caa cgc cat aa 3'	Inner anti-sense	2482-2463

Table-2 Amino acid sequence variation of HCV E2 regions (codon 506-564) in comparison with HCV-3a NZL1 (Accession number NC_009824)

E2 Region	$IR_{(n=35)}$ Mean(Range)	$INR_{(n=35)}$ Mean(Range)	$LNR_{(n=35)}$ Mean(Range)
$E2_{506-564}$	5.49(3-7)	7.60(6-11)	9.71(8-15)
$E2_{522-554}$ (Hypervariable region)	5.06(2-6)	6.97(5-9)	8.57(7-12)

Table-3 Amino acids sequence variation of HCV E2 regions (codon 622-714) in comparison with HCV-3a NZL1 (Accession number NC_009824)

E2 Region	$IR_{(n=15)}$ Mean(Range)	$INR_{(n=15)}$ Mean(Range)	$LNR_{(n=15)}$ Mean(Range)
$E2_{622-714}$	5.40(5-8)	2.8(0-4)	2.67(1-4)
$E2_{665-676}$ (PePHD)	0.87(0-2)	0.0(0-0)	0.07(0-1)


Hypervariable region
(codon 522-554)
506
564
HCV3a-NZL1 PVYCFTPSPVVVGTTDARGVPTYTWGENEKDVFLLKSQRPPSGRWFGCSWMNSTGFLKT
IR1 Y-V----------A---N----------E-L----------V----------
IR2 ----------SK----------T-----D-L----------T----------
IR3 ---S----------T-----S-L----------A----------
IR4 ----------RK-----N-----T-----S----------T----------
IR5 S----------T-----S-L----------A----------
IR6 -C----------A---------T-----T-L----------V----------
IR7 --V----------PK----------D-L----------V----------
IR8 ----------Q-L---N------A----------
IR9 ----------T-L---G------V----------
IR10 ----------T----------T---A------
IR11 ----------K-A----------E-L---A---Y--V----------
IR12 ----------R------G--------T-L----------T----------
IR13 ----------SK----------E-L----------V----------
IR14 --V----------MK----------T----------Q----A----------
IR15 ----------K-N---------S-----E----------A----------
IR16 ----------K----------T-----E-L---N------A----------
IR17 ----------K----------T-----N-L----------T----------
IR18 ----------R-A---------T-----E-L----------T----------
IR19 ----------R----------T-------L----------V----------
IR20 ----------K--------A--T-----D-M----------V----------
IR21 -CV----------K-----D-----T-----D-L----------V----------
IR22 ----------RR----------T-------L---G-Q----------
IR23 ----------I---------T-----D-L---N------A----------
IR24 ----------S--A--T-----E-L----------T----------
IR25 ----------N-----T-----E-L----------V----------
IR26 ----------T-----E-L---G------A----------
IR27 F----------N-----T-----D-L---N------V----------
IR28 ----------A---N-----T-------L----------V----------
IR29 F----------T-----S-L---R------A----------
IR30 ----------T---------T-------L---G------V----------
IR31 S----------A--T-----D-L----------A----------
IR32 ----------S-----T-------L----------V----------
IR33 ----------A---------T-----E-L---G----------
IR34 ----------A---------T-----E-L----------A----------
IR35 R----------T---N----------D-L---N------A----------
----------RK----------L----------T----------
INR1 AC----------K-A------A--T-----N-L----------V----------
INR2 S----------SK-----N-----T-----E-L----------A----------
INR3 ----------KK-A---------T-----T-L----------T----------
INR4 LF----------A---N--V--T-----E-L---G------A----------
INR5 ----------K-A---N--G--T-----E-L----------A----------
INR6 ----------KK-----N--A--------T-L---G------T----------
INR7 ----------SK-----S----------T-L---N------T----------
INR8 ----------PK-A---N--A--------N-L----------V----------
INR9 ----------K-A---N----------E-L---G------V----------
INR10 --V----------PK-----N----------E-L---G------A----------
INR11 T-V----------K-T---D----------E-L----------V----------
LNR12 ----------K--------A-T------D-L---N------A----------
INR13 ----------K------N---------M-R-L---N------T----------
INR14 ----------R-AA-N--A--S-----D-L----------A----------
INR15 ----------Tk-----N--A-K------D-L----------A----------
INR16 NY----------VK----------E-L---G------A----------
INR17 ----------VK-A---------T-----N-L----------V----------
INR18 ----------E-T---------T-----E-L---K------V----------
INR19 --L----------A---N-----T-----E-L---G------T----------
INR20 ----------DK-H---------T-----N-M----------V----------
INR21 ----------I------N-----T-----T-L---A------V----------
INR22 T----------K--------A--T-----N-L----------V----------
INR23 ----------RK----------T-------L---G------T----------
INR24 ----------K-A---N-----S-----D-L----------T----------
INR25 -CV----------K-A------A--T-----G-L----------A----------
INR26 ----------K-----D-----T-----D-L----------V----------
INR27 ----------K-I---------T-----D-L---N------A----------
INR28 ----------IK-----N-----T-----T-L----------T----------
INR29 ----------A-T------A--T-----D-L----------A----------

```
INR30  -CV-------------V--A---------T-----N-L---N------T----------
INR31  -------------------A---N-----T-----E-L---N------T----------
INR32  -------------------A---N-----T-----E-L---G------A----------
INR33  ----------------R------------T------ESQ---------T----------
INR34  YFLLLP----------------------T-----N-L---N------A----------
INR35  ----------------I------N-----T-----T-L---A------V----------

LNR1   ----------------PK-A--F---A--T-----D-L---G------A----------
LNR2   VSAL-S-T---------K-A------A-VT-----E-L---G------A----------
LNR3   --HMLP----------SK-----N-----T-----T-L---A------T----------
LNR4   -----------------K-A---N-----T-----E-L---G------A---T------
LNR5   S---------------VK-A---N--A--S-----G-L---G------A----------
LNR6   SDI-------------KK-----N-----T-----E-L---G------T----------
LNR7   S---------------SK-A---N-----T-----N-L----------T----------
LNR8   ----------------PK-A--F---A--T-----D-L---G------A----------
LNR9   ----------------VK-A---Q--A--T-----E-L---G------T---A------
LNR10  -CV-------------T-TA------T--S-------L---G------A----------
LNR11  --V---------------K-A---N-----T-----E-L----------V----------
LNR12  ----------------SK-A---R---A-T-----D-L---N------T----------
LNR13  ----------------KQ-A---N--A--T-----E-L----------T----------
INR14  -----------------RTAA-N--A-K------D-L----------A----------
LNR15  ----------------K-A---N-----GT-----E-L---G------A----------
LNR16  -C--------------VK-A---K-----T-----E-L----------V----------
LNR17  G---------------V--A---------S-----E-L---G------V----------
LNR18  ----------------EE-T--------KT-----E-L----------A----------
LNR19  HEY-------------K-----NF----T-----S-L----------A----------
LNR20  -----------------K-----S-----SY----N-M---N------A----------
LNR21  ----------------IK-----N--G--T-----T-L----------T----------
LNR22  TEY-------------K-----N-----T-----S-L---N------A----------
LNR23  -----------------K-A---N--A--T-------L---G------A----------
LNR24  ----------------EK-AA------T-T-----N-L---A------T----------
LNR25  ----------------RK-A---N--A--T-------L---G------A----------
LNR26  -----------------V-N---N-----T-M---D-L---K------V----------
LNR27  F---------------K-I--------GT-----T-L---N------A----------
LNR28  ----------------IK-----N--G--T-----T-L----------T----------
LNR29  ----------------PQ-T---KF----T-----G-L----------A----------
LNR30  --V-------------VA-A---------T-----N-L---G------T----------
LNR31  ----------I-----K--A---N-A---T-----D-L----------T----------
LNR32  ----------------P--A---N--S--T-----E-L----------A--D-------
LNR33  -S--------------KH-A------A--T-----H-LQ---------T----------
LNR34  -LCLLP----------A---N-----T-----T-----N-Q----A----------
LNR35  ---------I-----KHE-A--FN--G-KT-----D-L---N------V----------
```

Abbrevations:

IR =Initial sample of the responding patient

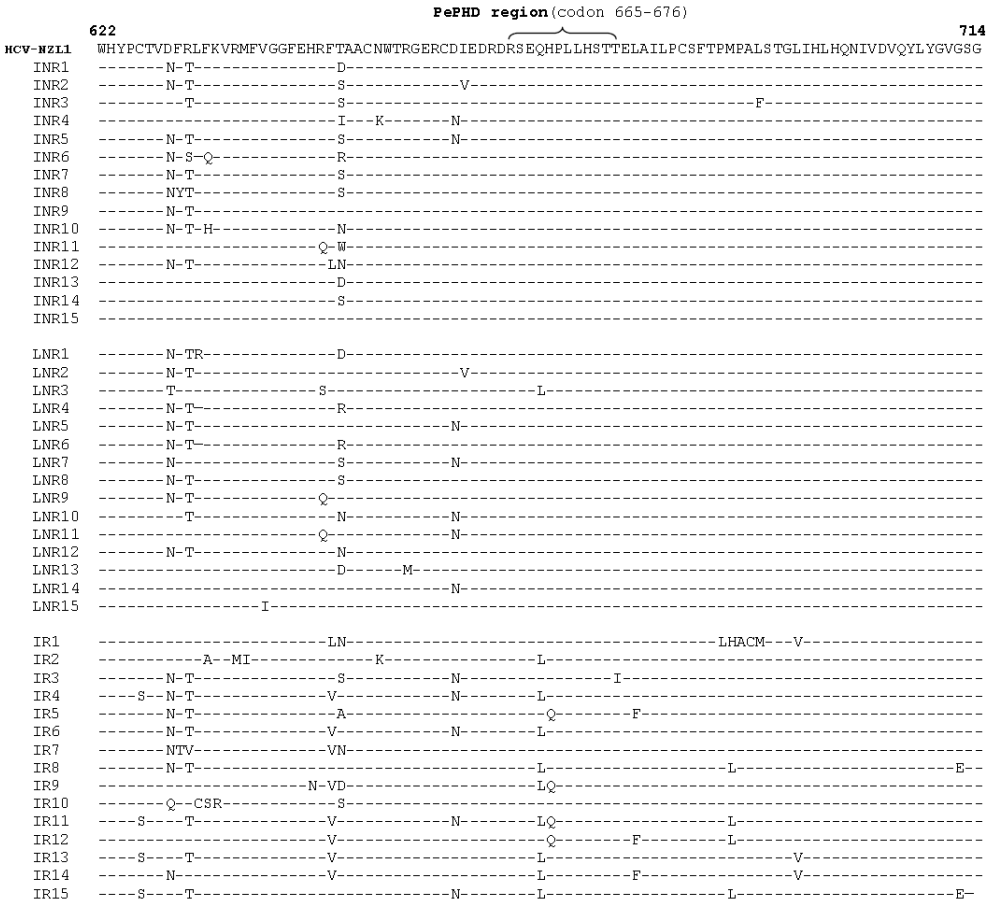
INR =Initial sample of the non-responding patient

LNR =last sample of the non-responding patient

Amino acid residues are indicated by standard single code

Dashes indicate the identical amino acid residues to the reference sequence (HCV3a NZL1, ACCESSION NC_009824)

Fig-1 Sequence alignment of amino acid residues 506 to 564 according to HCV-3a NZL1 (Accession number NC_009824)



Abbrevations:

IR =Initial sample of the responding patient

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Amino acid residues are indicated by standard single code, Dashes indicate the identical amino acid residues to the reference sequence (HCV3a NZL1, ACCESSION NC_009824)

Fig-2 Sequence alignment of amino acid residues 622 to 714 according to HCV3a-NZL1 (ACCESSION NC_009824)