The Role of Hepatitis C Virus NS5A Region Mutation and SNP IL-28B of Host to Support Successful Pegylated Interferon and Ribavirin Treatment in Patients with HCV-HIV Coinfection: A Prospective Cohort Study

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ABSTRAK

Latar belakang: *infeksi HIV mempercepat progresifitas penyakit pada penderita hepatitis C dan menurunkan angka keberhasilan terapi Peg-IFN/RBV. Mutasi VHC pada regio NS5A-ISDR/PKR-BD meningkatkan hasil luaran yang lebih baik pasien monoinfeksi VHC yang diterapi dengan Peg-IFN/RBV. Polimorfisme SNP IL-28B diprediksi memiliki efek terhadap evolusi quasispecies VHC. Akan tetapi, peran mutasi NS5A dan SNP IL-28B pada koinfeksi VHC-HIV masih belum jelas. Tujuan dari penelitian ini adalah untuk mengetahui peran mutasi VHC NS5A-ISDR/PKR-BD dan polimorfisme SNP IL-28B terhadap keberhasilan terapi Peg-IFN/RBV pada koinfeksi VHC-HIV.* ***Metode:*** *studi ini menggunakan desain studi kohort prospektif. Sampel plasma dikumpulkan dari 30 pasien koinfeksi VHC-HIV dan 8 monoinfeksi VHC. Sekuensing nukleotida PCR dilakukan setelah proses ekstraksi RNA dan sintesis cDNA. Analisis struktur sekunder dan prediksi fungsi mutasi menggunakan program PredictProtein(PP).* ***Hasil:*** *sebanyak 16 pasien koinfeksi VHC-HIV mencapai SVR. Tidak ada pasien monoinfeksi yang mencapai SVR. Mutasi nonnetral ≥1 ditemukan pada 24/30 pasien koinfeksi dan lebih sering ditemukan pada kelompok SVR (14 pasien). Mutasi nonnetral ≥1 ditemukan bermakna secara statistik terhadap status SVR ($p<0,05$), terlepas dari status koinfeksi ataupun monoinfeksi. Dari 27 pasien koinfeksi yang memiliki gen CC, sebanyak 21 pasien memiliki mutasi nonnetral. Struktur yang diduga sebagai binding site didapatkan berbeda dibanding konsensus pada kelompok SVR, sedangkan pada kelompok non-SVR struktur tersebut sama dengan konsensus.* ***Kesimpulan:*** *mutasi nonnetral ≥1 berhubungan dengan tercapainya SVR pada terapi Peg-IFN/RBV, terlepas dari status monoinfeksi atau koinfeksi.*

Kata kunci: *NS5A-ISDR/PKR-BD, SNP IL-28B, koinfeksi VHC-HIV, mutasi nonnetral, SVR.*

ABSTRACT

Background: *HIV infection in HCV-infected patients accelerates disease progression and reduces the success rate of Peg-IFN/RBV treatment. HCV mutation in NS5A-ISDR/PKR-BD region improved the outcome in HCV monoinfection treated with Peg-IFN/RBV. SNP-IL28B polymorphism is predicted to have an effect on HCV quasispecies evolution. However, the role of NS5A mutation and SNP IL-28B in HIV-HCV coinfection is still unclear. The aim of the study is to determine the role of HCV NS5A-ISDR/PKR-BD mutation and SNP IL-28 polymorphism on the successfulness of Peg-IFN/RBV therapy in HCV-HIV coinfection.* ***Methods:*** *prospective cohort was performed in this study. Plasma sample were obtained from 30 and 8 patients with HCV-HIV coinfection and HCV monoinfection, respectively. PCR nucleotide sequencing was performed after RNA virus extraction and cDNA synthesis. Protein secondary structure and prediction of mutation function were analyzed using PredictProtein (PP) program.* ***Results:*** *sixteen HCV-HIV coinfected patients and none from eight HCV patients achieved sustained virological response (SVR). ≥1 non-neutral mutation was found in 24/30 HCV-HIV coinfection and more frequent in SVR group (14 patients). ≥1 non-neutral mutation were found statistically significant for overall SVR achievement ($p<0.05$) in all patients regardless of coinfection or monoinfection status. Of the 27 HCV-HIV coinfected patients with CC-gene, 21 subjects had non-neutral mutation. The structure which was expected as NS5A binding site structure was different from consensus (wild type) in SVR group, while the structure was similar to consensus in non-SVR group.* ***Conclusion:*** *having ≥1 non-neutral mutation was associated with SVR achievement in Peg-IFN/RBV therapy, regardless of monoinfection and coinfection status.*

Keywords: *NS5A-ISDR/PKR-BD, SNP IL-28B, HCV-HIV coinfection, non-neutral mutation, SVR.*

INTRODUCTION

Hepatitis C virus (HCV) infection is one of the leading causes of death in HIV patients and HIV infection in hepatitis C patients increases the risk of cirrhosis, hepatocellular carcinoma (HCC) and death.[1,2] In the United States, approximately 25% of HIV-infected patients also had HCV coinfection.[3] In Cipto Mangunkusumo National General Hospital, around 67.9% of 3,613 new cases of HIV-infected patients had positive anti-HCV serology.[4] Pegylated interferon and ribavirin (Peg-IFN/RBV) is one of treatment options for chronic hepatitis C in patients with HCV-HIV coinfection. Several studies have been conducted to determine the outcome predictor of hepatitis C patients treated with Peg-IFN/RBV.[5-7]

HCV mutation in NS5A IFN sensitivity determining region (ISDR) and protein kinase R-binding domain (PKR-BD) was suspected to be a predictor of Peg-IFN/RBV treatment response. El-Shamy study[8] showed that 71% patients who had ≥1 mutation in NS55A ISDR region achieved SVR. De Rueda study[9] showed that having ≥4 mutation in NS55A PKR-BD region was associated with SVR ($p = 0.001$) in HCV patients who received Peg-IFN/RBV combination therapy. However, different results were reported from two Western studies. Zeuzem, et al[10] reported that there was no correlation between the number of amino acid mutations in ISDR region and the decreased HCV-RNA levels. Hofgartner[11] also reported that amino acid substitution in ISDR region was not related to interferon resistance in HCV genotype 1a patients.

The prediction of mutation function of a protein is influenced by three factors, i.e. structure, sequence and annotation.[12] Amino acid mutations could not be observed only by looking at the number of mutations, but the role of the mutation to its function also needs to be observed. Non-synonymous mutation is defined as a substitution of nucleotide which causes change in the amino acid sequences, while non-neutral mutation is defined as a mutation which causes change in the function.[13] In additon to NS5A mutation, single nucleotide polymorphism (SNP) IL-28B on human chromosome 19 can also predict the response of therapy on HCV infection, but the mechanism is still not clearly known.[14] Therefore, this study was conducted to determine the role of HCV RNA mutations (particularly non-neutral mutations) in NS5A-

ISDR/PKR-BD region and host SNP IL-28B in the successfulness of hepatitis C therapy using combination of Peg-IFN/RBV in HCV-HIV coinfection.

METHODS

Study Design and Patient Selection

A prospective cohort design was used in this study. HCV-HIV coinfected and HCV monoinfected patients were recruited from HIV Clinic and Hepatology Clinic in Cipto Mangunkusumo National General Hospital, respectively, from July 2015 to July 2017. HCV-HIV coinfected patients with CD4 count bertween 200-350 cells/µL for patients who were on antriretroviral therapy (ART) or CD4 count above 350 cells/mL for patients who were not on ART liver fibrorsis >F1 were included in this study. All patients who were willing to participate in this study were asked to sign informed consent form. Strict inclusion and exclusion criteria were applied in order to avoid bias and to control confounding variables in this study. The minimum sample size was calculated by using hypothetical test of differences between two proportions.

Viral RNA Extraction and cDNA Synthesis

Viral RNA was extracted from 200 µL plasma samples using a High Pure viral nucleic acid kit. As many as 3 µL of RNA template underwent reverse transcription-polymerase chain reaction (RT-PCR) to produce cDNA by using Tetro cDNA synthesis kit. PCR for NS5A region of genotype 1b HCV used Taq Polymerase DNA and primer pair of 5A-1b-1 (sense) 5'-ATTCCAGGTCGGGCTCAA-3' and 5A-1-2R (antisense) 5'-ACGGTAGACCAAGA CCCGTC-3' for the first stage PCR. Three microliters from the first stage PCR reaction were used for second stage PCR (nested-PCR) by using primer pair of 5A-1b-3 (sense) 5'-ACTTCCATGCTCACCGACCC-3' and 5A-1-4R primers (antisense) 5'-AGAGGGGG CATGGAGGAGTA -3'.[15] These primer pairs were also used as the sequential primer for genotype 1b HCV.

The primer pairs used in the PCR process and sequencing of NS5A region genotypes 1a, 3 and 4 HCV were primer pairs specifically designed using Primer3Plus program based on 30 strands of HCV whole genome from various countries. Three microliters from the first stage PCR reaction were used for the second stage of PCR (nested-PCR). These primer pairs were also used as the sequential primer for genotypes 1a, 3, 4 and 6 HCV. The amplification results were analyzed by electrophoresis using 1.5% agarose gel (in 1x TAE) with SYBR® Safe DNA Gel Stain dye and documented using ultra violet transilluminator.

Nucleotide Sequencing

The purified PCR product was sequenced by using automatic sequencer ABI Prism 3730 XL genetic analyzer DNA sequencer and dye terminator sequencing kit. The electrophoregram of each sequence was examined and modified by using Macimum Composite Likelihood Method (MEGA) software version 7.0.

Secondary Structure Analysis and Mutation Function Prediction

The analysis of secondary structure of HCV NS5A-ISDR/PKR-BD protein sequence was performed by using Predict Protein (PP) program. Screening for non-acceptable polymorphisms 2 (SNAP2) is a service which is provided by PP to predict the effects of amino acid substitution in the change of protein function. The accuracy of SNAP is 77% in detecting 80% of non-neutral mutation.

Data Analysis

Dichotomous data was presented in proportions, while numerical data was presented in mean with standard deviation or median with the interquartile range. Bivariate analysis of categorical data was carried out using Chi Square and numerical data using unpaired T test or Mann-Whitney analysis. The p value <0.05 was considered statistically significant. Statistical analysis was performed by using SPSS version 23.00.

Research Ethics

This study has been approved by the Medical Research Ethics Committee of Faculty of Medicine, Universitas Indonesia with a reference number 725/UN2.FI/ETIK/2015.

RESULTS

Thirty patients with HCV-HIV coinfection and eight patients with HCV monoinfection were included and analyzed in this study.

Genotype 1a was the most common genotype found in the HCV-HIV coinfection group, followed by genotype 3 and 4. No genotype 1b was found in this group. As many as 90% of HCV-HIV coinfected patients had CC gene, while 75% of HCV monoinfected patients had non-CC gene. HCV NS5A-ISDR/PKR-BD mutation was not significantly different between coinfection and monoinfection group. However, ≥1 non-neutral mutation was statistically significant between HCV-HIV coinfected and HCV monoinfected patients (p=0.049). The demographic, laboratory and HCV NS5A mutation characteristics were shown in **Table 1**.

HCV NS5A-ISDR/PKR-BD Mutation and SVR

In this study, the association between neutral, non-neutral, synonymous, non-synonymous mutation and SVR in HCV-HIV coinfected

Table 1. Demographic, laboratory and mutation characteristics

Variables	HCV-HIV coinfection (n = 30)	HCV monoinfection (n = 8)
Demographic data		
Gender, n (%)		
- Male	29 (96.7)	2 (25)
- Female	1 (3.3)	6 (75)
Age (years)[a]	36.43 (3.4)	51.13 (10.02)
Cirrhosis, n (%)		
- Yes	15 (50)	0 (0)
- No	15 (50)	8 (100)
Laboratory data		
CD4 (cells/μL)[b]	495 (219-886)	-
Viral load (log IU/mL)[b]	1.47 x 106 (3.49 x 105–2.17 x 107)	2.53x106 (1.73x104–1.39 x 107)
Genotype, n (%)		
1a	23 (76.7)	0 (0)
1b	0 (0)	8 (100)
3	4 (13,3)	0 (0)
4	3 (10)	0 (0)
IL28B gene variation, n (%)		
- CC	27 (90)	2 (25)
- Non-CC	3 (10)	6 (75)
SVR status, n(%)		
- SVR	16 (53.3)	0 (0)
- Non SVR	14 (46.7)	8 (100)
Mutation characteristics[b]		
- Number of neutral mutation	5 (2–9)	4 (2–6)
- Number of non-neutral mutation	1 (0–11)	0 (0–3)
- Number of synonymous mutation	3 (1–8)	2 (0–3)
- Number of non-synonymous mutation	3 (0–12)	3 (2–7)
- Total mutation in all categories	6 (3–20)	5 (3–9)
Amino acid substitution, n (%)		
- neutral ≥ 4/ < 4	24 (80%)/ 6 (20%)	4 (50%)/ 4 (50%)
- non-neutral ≥ 1/ < 1	23 (76.7%)/ 7 (23.3%)	3 (37.5%)/ 5 (62.5%)
- synonymous ≥ 3/ < 3	19 (63.3%)/ 11 (36.7%)	2 (25%)/ 6 (75%)
- non-synonymous ≥ 3/ < 3	22 (73.3%)/ 8 (26.7%)	7 (87.5%)/ 1 (12.5%)

[a]: mean (SD); [b]: median (minimum - maximum)

patients was not statistically significant. However, in the subanalysis in all patients regardless of coinfection or monoinfection status, having ≥1 non-neutral mutation was associated with higher SVR rate (p=0.04). (**Table 2)** The presence of both non-neutral and non-synonynous mutation was not associated with SVR achievement in HCV-HIV coinfected patients. (**Table 3**)

HCV NS5A-ISDR/PKR-BD Mutation and SNP IL-28B

No significant association was found between HCV NS5A-ISDR/PKR-BD mutation and SNP IL-28B in HCV-HIV coinfected patients. However, patients with CC gene have a tendency to acquire non-neutral and non-synonymous mutation in HCV NS5A region. (**Table 4)**

Table 2. HCV NS5A-ISDR/PKR-BD mutation and SVR rate

Variables	Coinfection and Monoinfection (n = 38)			
	Non SVR	SVR	Total	p
NS5A-ISDR/PKR-BD mutation				
- (neutral), ≥ 4	17 (60.7%)	11 (39.3%)	28 (100%)	*0.713
- (neutral), < 4	5 (50%)	5 (50%)	10 (100%)	-
NS5A-ISDR/PKR-BD mutation				
- (non-neutral), ≥ 1	12 (46.2%)	14 (53.8%)	26 (100%)	*0.040
- (non-neutral), < 1	10 (83.3%)	2 (16.7%)	12 (100%)	-
NS5A-ISDR/PKR-BD mutation				
- (synonymous), ≥ 3	10 (47.6%)	11 (52.4%)	21 (100%)	*0.197
- (synonymous), < 3	12 (70.6%)	5 (29.4%)	17 (100%)	-
NS5A-ISDR/PKR-BD mutation				
- (non-synonymous), ≥ 3	19 (65.5%)	10 (34.5%)	29 (100%)	*0.128
- (non-synonymous), < 3	3 (33.3%)	6 (66.7%)	9 (100%)	-

* Chi square

Table 3. HCV NS5A-ISDR/PKR-BD mutation and SVR rate in HCV-HIV coinfected patients

Variables	Therapeutic response		P value
	Non-SVR (n = 14)	SVR (n = 16)	
The association between mutations in non-synonymous and non-neutral			
Mutation (+) in Non-synonymous with Mutation (+) in Non-neutral	8 (47.1%)	9 (52.9%)	*0.220
Mutation (+) in Non-synonymous with Mutation (-) in Non-neutral	4 (80%)	1 (20%)	
Mutation (-) in Non-synonymous with Mutation (+) in Non-neutral	1 (16.7%)	5 (83.3%)	
Mutation (-) in Non-synonymous with Mutation (-) in Non-neutral	1 (50%)	1 (50%)	

* Chi square test

Table 4. HCV NS5A-ISDR/PKR-BD mutation and SNP IL-28B polymorphism

	ISDR/PKR-BD mutation (neutral)		P	ISDR/PKR-BD mutation (non-neutral)		P
	Mutation (-)	Mutation (+)		Mutation (-)	Mutation (+)	
CC gene	6 (22.2%)	21 (77.8%)	*1.000	6 (22.2%)	21 (77.8%)	*1.000
Non CC gene	0 (0%)	3 (100%)		1 (33.3%)	2 (66.7%)	
	Synonymous		p	non-synonymous		P
	Mutation (-)	Mutation (+)		Mutation (-)	Mutation (+)	
CC gene	10 (37%)	17 (63%)	*1.000	7 (25.9%)	20 (74.1%)	*1.000
Non CC gene	1 (33.3%)	2 (66.7%)		1 (33.3%)	2 (66.7%)	

* Chi square test

Protein Secondary Structure and SVR

There is no significant association between HCV NS5A mutation and the change of protein secondary structure in HCV-HIV coinfected patients. In a separate analysis, the change of protein secondary structure was not associated with SVR achievement (data is not shown).

Binding Site Structure in SVR and Non-SVR Groups

In the in-depth analysis, the structure which was expected as HCV NS5A binding site structure was not different between the wild type (consensus) and non-SVR group. Meanwhile, this structure was different between SVR group and consensus, as shown in **Figure 1**.

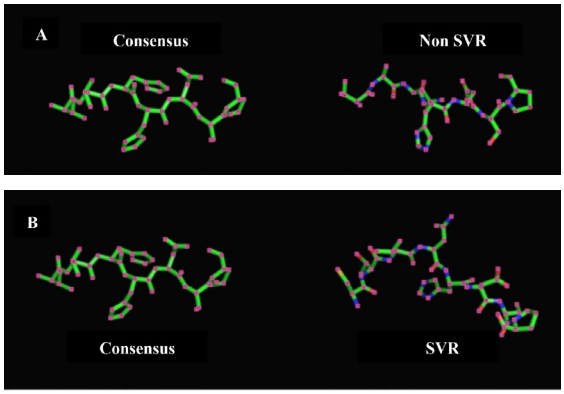

Figure 1. Binding site structure of HCV NS5A.

Figure 1A) The comparison between binding site structure of HCV NS5A wild type (consensus) and non-SVR group. No significant difference between wild type and non-SVR group. Figure 1B) The comparison between binding site structure of HCV NS5A consensus and SVR group. The binding site structures were completely different between the two.

DISCUSSION

The most common HCV genotype in HCV-HIV coinfected patients was 1a (76.7%), followed by genotype 3 (13.3%) and 4 (10%). All HCV monoinfection patients had genotype 1b. Geographical factors had a major role in the spread of HCV genotypes. Genotype 1b is the most prevalent genotype found in Indonesia.[16] The change of genotypes distribution in the HCV-HIV group in this study might be due to HIV route of transmission.[17]

As many as 53.3% of HCV-HIV coinfected patients achieved SVR. None of monoinfected patients achieved SVR. Several factors have been known to play major roles in poor treatment response, which include old age, genotype 1 HCV, high viral load of HCV, advanced fibrosis and non-CC gene.[7,18] Old age, non-CC gene and genotype 1 HCV might be the cause of no SVR in HCV monoinfection group in this study.

In univariate analysis, mutation of HCV NS5A-ISDR/PKR-BD region was not statistically significant between monoinfection and coinfection groups. Meanwhile, there was a tendency of increased mutation incidence in the coinfection group. To the best of our knowledge, no study has reported this finding in HCV-HIV coinfected patients. A study reported that increased mutation rate of NS5A resistance-associated substitutions (RAS) in domain I is more common in HCV-HIV coinfected patients than in HCV monoinfected patients, however this was associated with therapeutic response to daclatasvir.[19]

It is suspected that the high number of mutations in HCV-HIV coinfection was the result of increased rate of HCV replication. TGF-β1 expression in hepatocytes and circulation were increased due to HIV infection. TGF-β1 is a stimulator for HCV replication, thus increasing HCV replication in hepatocytes by 2-3 folds.[20,21] Another mechanism is HIV infection acting as inducer of HCV replication in human macrophages.[22] Elevated rate of HCV replication increases the probability of mutation on this RNA virus that lack of proofreading ability.[23,24]

The presence of HCV NS5A-ISDR/PKR-BD mutation was not statistically significant towards the achievement of SVR in this study. Of the 30 HCV-HIV coinfected patients, non-neutral mutation and non-synonymous mutation were found in 23 and 22 patients, respectively. As many as 17 patients had both mutations. To explore the contribution of non-neutral mutation in SVR, having ≥1 non-neutral mutations was found statistically significant towards the achievement of SVR ($p<0.05$), regardless of the coinfection or monoinfection status. There was no significant association between non-neutral mutation and SVR rate in both groups, however HCV-HIV coinfected patients who had non-neutral mutation tended to have higher SVR rate. Therefore, the role of non-synonymous mutation is assumed to have less impact in SVR rate if

compared to non-neutral mutation.

NS5A structure in HCV acts as interferon antiviral inhibitor.[25] Therefore, non-neutral mutation of HCV NS5A-ISDR/PKR-BD can become advantageous mutation as it is related to better outcome in Peg-IFN/RBV therapy.

Non-neutral mutation is known to affect the protein structure.[14] In this study, there was no significant association between the changes of HCV NS5A secondary structure in NS5A-ISDR/PKR-BD region and SVR achievement in the coinfection group. This is probably because the changes in secondary structure of all NS5A did not have any major roles in SVR achievement. In the in-depth analysis of NS5A secondary structure which was focused on the structure which was predicted as binding site, it was found that the HCV NS5A binding site structure in HCV-HIV coinfected patients who did not achieve SVR was not too different from the binding site structure according to HCV consensus (the wild type). HCV-HIV coinfected patients who achieved SVR had a different binding site structure compared to the wild type (**Figure 1**). Therefore, the changes of binding site structure associated with ≥1 non-neutral mutation is expected to play an important role in SVR achievement in HCV-HIV coinfected patients treated with Peg-IFN/RBV.

The association between SNP IL-28B polymorphism and HCV NS5A mutation was not statistically significant. However, there was a tendency for patients with CC gene to have a higher rate of non-neutral and non-synonymous mutations. Similar findings were reported by Halfon[26], i.e. CC gene was associated with SVR achievement, particularly in genotype 1 with OR of 3.3 (1.58-6.9) and p = 0.03. Khubaib et al[27] also reported similar result, i.e. CC gene was an independent predictor of SVR in patients with chronic HCV infection.

The antiviral effect of interferon is achieved through the activation of IFN cascade. The main molecule in IFN pathway is signal transducer and activator of transcription 1 (STAT1). This

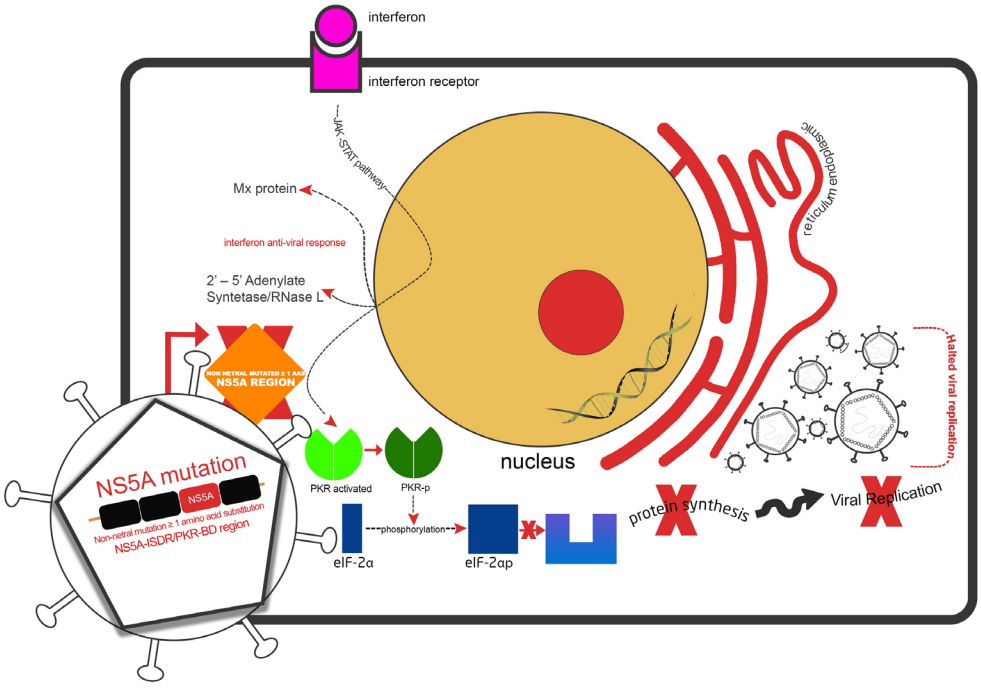

Figure 2. The mechanism of non-neutral mutation of HCV NS5A-ISDR/PKR-BD in patiets who were treated with Peg-IFN/RBV therapy. The replication process of HCV continues because eukaryotic initiation factor-2 α (eIF-2α) is not phosphorylated. When a patient is treated with Peg-IFN/RBV regimen, interferon binds to interferon receptor which then activates protein kinase-R (PKR). Activation of PKR leads to phosphorylation of eIF-2α which will inhibit protein synthesis for translation process, so that HCV replication stops. Through its NS5A structure, HCV suppresses PKR activation, thus inhibiting phosphorylation of eIF-2α and the HCV replication process continues. The non-neutral mutation of NSA-inteferon sensitivity determining region/protein kinase R – binding domain (NS5A-ISDR/PKR-BD) ≥ 1 amino acid, particularly in the structure which is considered as binding site, reactivates PKR and stops the HCV replication process. Therefore, non-neutral mutation of HCV NS5A-ISDR/PKR-BD is associated with better outcome in Peg-IFN/RBV therapy.

molecule will undergo phosphorylation and translocation into nucleus, which then will induce the interferon-stimulating gene (ISG).[25] The activity of ISG plays an important role in the mechanism where CC gene affects SVR. Patients with CC gene are known to have lower ISG activity, which indicated that IFN activity is not optimal yet, therefore combination therapy with Peg-IFN/RBV will increase the response of IFN. High ISG levels in patients with non-CC gene indicates that IFN activity is optimal and therefore provision of interferom-based therapy will result in low SVR.[28,29]

Figure 2 explains the effect of non-neutral mutation of HCV NS5A- ISDR/PKR-BD to the outcome of Peg-IFN/RBV therapy.

The recommendation for chronic hepatitis C therapy has shifted towards Direct Acting Antiviral (DAA). The use of DAA in Indonesia for hepatitis C therapy is currently facilitated by government program and the supply is sometimes not sustainable. Therefore, the use of interferon-based therapy is still one of the options for chronic hepatitis C therapy in Indonesia and other countries with low DAA availability.

CONCLUSION

HCV-HIV coinfected patient with CC gene and ≥ 1 non-neutral mutation in NS5A-ISDR/PKR-BD region tends to have better outcome with Peg-IFN/RBV therapy.

RECOMMENDATION

A study with larger sample size is recommended. This study is also expected to become a basis for other researchers to study the HCV NS5A mutation as initial predictor for response of of DAA therapy .

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