Resistance-Associated Substitutions (RAS) and Clinical Factors as Determinants of Sofosbuvir-Daclatasvir Treatment Outcomes in Chronic Hepatitis C Patients

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ABSTRACT

Background: *Direct acting antivirals (DAAs) have demonstrated remarkable efficacy, in achieving hepatitis C viral (HCV) elimination rates higher than 90%. One particular concern associated with treatment failure is the emergence of resistance associated substitutions (RASs) in the genome. The occurrence of RASs highlights the adaptability and resilience of the HCV. This highlights the importance of RASs mutation, enabling the development of new therapeutic strategies to combat these resistant strains. This study aims to determine the presence of early HCV mutations in chronic hepatitis C in Indonesia and the association between this mutation to the efficacy of sofosbuvir-daclatasvir.* ***Methods****: We conducted a prospective longitudinal study in naïve hepatitis C patient population. The virus was examined for RAS by RNA sequencing before starting treatment. RAS mutations were determined through the cut-off value of RAS mutations that contributed in the successful therapy. All patients were followed up until 12 weeks after completion of treatment to determine the sustained virological response 12 (SVR12).* ***Results:*** *Out of the 58 patients, 9 patients (15.51%) did not achieve SVR. Only 14 patients was further analyzed to provide the association between the mutation and SVR-12 failure after sofosbuvir-daclatasvir therapy. Specifically, 2 patients with L31 mutation and one patient with L31/Y93 mutation achieved SVR. Only one patient with failure to achieve SVR and the mutation was found in Y93H region.* ***Conclusion:*** *The mutation of Y93H may contribute to treatment failure while L31A may increase the susceptibility to DAAs treatment.*

Keywords: *hepatitis C, direct acting antiviral, resistance associated substitutions, mutation, Y93H region.*

INTRODUCTION

Hepatitis C virus (HCV) is a RNA virus encoded structural and non-structural proteins. These non-structural proteins (NS5A and NS5B) are the targets of direct acting antivirals (DAA), which inhibit viral RNA replication and assembly.[1-3] The combination of sofosbuvir 400 mg and daclatasvir 60 mg is recommended as the first line therapy in chronic hepatitis C due to its pan-genotypic nature.[1,4] There are currently 8 major genotypes of HCV that have been identified.[1,4] DAAs have demonstrated remarkable efficacy, achieving HCV elimination rates exceeding 90%. However, antiviral administration based on its genotype is expected to increase the rate of sustained virological response (SVR).[1]

One particular concern associated with

treatment failure is the emergence of resistance associated substitutions (RASs) in the genome. Initial RAS testing is not recommended to be routinely performed before DAA. Even without knowing those mutation, the SVR that can be achieved is still above 90%. This opinion is in line with the recommendation of the European Association for the Study of the Liver (EASL).[5,6] New genotypic resistance testing is recommended to be performed before re-treatment in suspected of resistance or failure. The results will be taken into consideration in treatment modification such as adding, substituting agents, or extending the duration.[1,] Contrary with the other guidelines, the American Association for the study of liver disease (AASLD) and the infectious disease society of America (IDSA) published recommendations regarding RAS screening both prior to and following therapy. In the case of treatment-naïve patients planning to receive sofosbuvir-daclatasvir therapy, it is considered to screen for RAS in the NS5A region for HCV genotype 3.[7,8] RAS mostly occurs in individuals that failed Hepatitis C first line treatment. RAS identification is essential to prevent re-treatment failure and to be checked in patients that failed with first line treatment. Untreated RAS may contribute to the emergence of drug resistant HCV strains.[9] Early identification and introduction of RAS management, may prevent the spread of drug-resistant HCV and preserve effectiveness of current treatments. Secondly, considering the global prevalence of HCV, there are still a significant number of patients who require retreatment.[10] This is also in line with World Health Organization's ambitious goal to eliminate Hepatitis C as public threat by 2030 and Indonesia goal to eliminate Hepatitis C by 2040.[10-12]

The prevalence of RAS NS5A ranged from 6%-16% by population sequencing (cut-off point 15%-25%) or deep sequencing (cut-off point 1%).[13] Sharafi, et al[14] reported that of 2,409 isolates of patients with HCV-1 infection showed a prevalence of basal RAS and clinically significant basal RAS (>100x) of 16% and 4.7%, respectively. The prevalence of RAS NS5A was found to be more common in HCV-1b than HCV-1a.[14] Carrasco et al.[15] conducted a study that demonstrated a higher prevalence of two or more baseline RAS in patients who experienced treatment failure with DAA medications (p=0.015). These basal RAS were found to have a significant correlation with therapy failure (p=0.029). Current research indicates that screening for baseline NS5A RAS can serve as a predictive factor for the response to NS5A inhibitor. [16,17]

Gozlan, et al.[18] conducted a study between basal RAS data and failure to achieve SVR12 in 160 patients infected with HCV-1 with DAA-based therapy. Of the 8 patients who received sofosbuvir-daclatasvir, there was no correlation between basal RAS and the achievement of SVR12. Sarrazin, et al[19] also found RAS mutation in patients who treated with sofosbuvir-ledipasvir. Kozuka et al.[20] reported the effect of basal NS5A RAS in 312 HCV-1b-infected NS5A inhibitor-naive patients treated with the sofosbuvir and ledipasvir. The prevalence of multiple basal NS5A RAS was low in NS5A inhibitor naïve patients, but their presence was associated with decreased efficacy of sofosbuvir-ledipasvir. SVR12 was found to be lower in multiple RAS greater than 3 compared to RAS less than 3. A study by Mawatari et al.[17] of 493 HCV-1b infected patients treated with sofosbuvir-ledipasvir showed that 97.6% of patients achieved SVR12 and the remaining 2.2% patients experienced virological failure. Basal RAS NS5A and NS5B were found to correlate with lower SVR12 rate ($p<0.001$). Based on multivariate analysis, the presence of basal RAS was found to be associated with virologic failure.

RAS detection is primarily conducted through population sequencing, also known as direct sequencing, utilizing the Sanger sequencing method.[3,21] However, the lack of commercially standardized formats for population sequencing necessitates the use of in-house methods. A more sensitive alternative is deep sequencing, specifically pyrosequencing, which leverages next-generation sequencing (NGS) technology. This advanced technique can identify viruses with RAS at proportions as low as 1% of the viral species.[21] Both population sequencing and deep sequencing methods typically involve

sequencing the RNA of the HCV and examining mutations in viral amino acids.[7] The cut-off point value used to determine the significance of RAS is 15%. In a small proportion (<15%), RAS has no significant influence on therapeutic response. The proportion of RAS must be at least 15% and above to be clinically associated with failure to achieve SVR.[5,7,21] This study aims to identify possible clinically relevant RASs in HCV NS5A regions contributing to sofosbuvir-daclatasvir treatment resistant in Indonesia. It is hypothesized that RAS posses potential resistance towards sofosbuvir-daclatasvir treatment.

METHODS

Study Population and Design

Blood sample was collected from Hepatobiliary Outpatient Clinic, Internal Medicine Department in Cipto Mangunkusumo Hospital between January 2020 and December 2021. All patients with ≥ 18 years old who received DAA treatment, had tested positive for HCV antibodies for a minimum for 6 months prior and positive for HCV-RNA were involved in this study. Convenience sampling was used for sample selection. The sample size is calculated using a two-proportion test, based on the research literature by Brandão.[22] The SVR (sustained virologic response) rate among patients with NS5A RAS is 22.4%, while the therapy failure rate (non-SVR) among these patients is 66.7%. With a 5% alpha and a 20% beta error, the required sample size is 19 per group, resulting in a total sample size of 38 patients. However, at the time of the study, only 14 patients could underwent further sequencing analysis, leading to a power of study only 35%.

Ethical Statement

The authors declare that all procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by Ethics Committee of Faculty Medicine Universitas Indonesia. Ethical Number KET-1049/UN2.F1/ETIK/PPM.00.02/2019. All participants were signed the informed consent prior of the study. All data collected in this study were kept confidential and can only be accessed by research staff.

Data Collection and Analysis

Prior to therapy, blood samples were analyzed for NS5 mutation of HCV. The duration of treatment was 3 months for non-cirrhotic and 6 months for cirrhotic patients. Three months after completing the treatment, additional blood samples were collected to assess SVR. SVR is determined as undetectable HCV RNA in the serum at least 12 weeks after the end of treatment (12 or 24 weeks treatment depending of Cirrhotic status of patients). Subsequently, bivariate analysis was performed using chi-square or Fisher's exact test to examine the factors influencing the effectiveness of DAA treatment.

Viral RNA Extraction and Complementary DNA Synthesis (cDNA)

Viral RNA was extracted using QIAamp® Viral RNA Mini Kit. RNA concentration were then measured by BioDrop µLITE spektrofotometer (ISOGEN Life Science, Netherlands). The synthesis of cDNA at the region of NS5A was performed by using Reverse transcription-polymerase chain reaction (RT-PCR) originated from RNA viral template using Tetro cDNA synthesis kit (Bioline, USA). Nested-PCR using Taq DNA Polymerase (Qiagen) was carried out using primers sequences (given in Table 1) for the amplification of cDNA. Primer pair for PCR and sequencing of NS5A HCV genotype 1b were collected from Maria I. Lusida.[23] On the other hand, primer pair for PCR and sequencing of NS5A HCV genotype 1a, 3, 4, and 6, were assembled using Primer3Plus software based on 30 whole genome HCV sequences from around the world. The PCR step is described in **Table 2**. Amplification products were analysed with electrophoresis using agarose gel 1.5% (in TAE 1x) stained with SYBR® Safe DNA Gel Stain (Thermo Fisher Scientific, Inc, CA, USA), documented with transilluminator ultraviolet. All amplicons were purified with ExoSAP-IT™ kit (Thermo Fisher Scientific, Inc, CA, USA) and further sequenced with direct nucleotide sequencing.

Table 1. Primer used for PCR

PCR round	Primer	Genotype	Nucleotide sequence of primers (5'-3')
PCR 1st round	5A-1b-1 (sense)	1b	ATTCCAGGTCGGGCTCAA
	5A-1-2R (antisense)		ACGGTAGACCAAGACCCGTC
Nested PCR	5A-1b-3 (sense)		ACTTCCATGCTCACCGACCC
	5A-1-4R (antisense)		AGAGGGGGCATGGAGGAGTA
PCR 1st round	NS5AF (sense)	1a, 3, 4, 6	TKGTDGRCHTTAAGATCATGRGMGG
	NS5AR (antisense)		TCBAGDGGRGGCATDGASGARYADGA
Nested PCR	SEQa (sense)		GGATGAACMGKCTVATHGCSTTCG
	SEQc (antisense)		AGHGGDGGRTTRTAGTCCGGYCKVGC

Table 2. Thermal Cycler Program

Cycle Step	PCR 1st round		Nested PCR		Cycle
	Temp. (°C)	Time (min)	Temp. (°C)	Time (min)	
Denaturation	94	3	94	3	1
	94	1	94	1	
Amplification	58	1	57	1	35
	72	1	72	1	
Extention	72	10	72	10	1

Nucleotide Sequencing

Purified PCR products were sequenced using ABI Prism 3730 XL genetic analyzer DNA Sequencer (Applied Biosystem, Foster City, CA, USA) and kit dye terminator sequencing (BigDye Terminator v3.1 Cycle Sequencing Kit; Applied Biosystems, Foster City, CA, USA). Each sequence electrogram were measured and edited using Maximum Composite Likelihood Method (MEGA), 7.0 version software. Nucleotide were aligned with BLASTx and reference sequences from various subtypes. Accession number of GenBank for HVC genotype 1b is AB056520, genotipe 1a is AF009606, genotipe 3 is HQ912957, genotipe 4 is AFN53800 dan genotipe 6 is NC009827.

RESULTS

Flowchart of Patient Selection

A total of 101 hepatitis C patients was involved in this study. Among these patients who received sofosbuvir-daclatasvir therapy, 24 patients were unable to complete the treatment for various reasons, including death (3 patients), incomplete treatment (18 patients), and missing medical records (3 patients). Out of the initial group, 77 patients successfully completed the therapy within the recommended duration. Among them, 58 patients had complete data on SVR and the remaining 19 patients lost to follow up. Only 14 patients can undergo further sequencing analysis. The flowchart of the selection of patients could be seen in **Figure 1**.

Demographic Characteristics

Only 14 patients had complete data regarding the mutation analysis of HCV and SVR-12 status after treatment. The gender distribution was predominantly male, with the mean age of 52 years old. Among the 14 patients, 8 had cirrhosis prior to the initiation of DAA. A 12-week follow up post-treatment, a sum of 13 patients achieved SVR-12. These patients were further analysed to provide the association between the mutation and SVR-12 failure after sofosbuvir-daclatasvir therapy. Detailed characteristics of the patients is presented in **Table 3.**

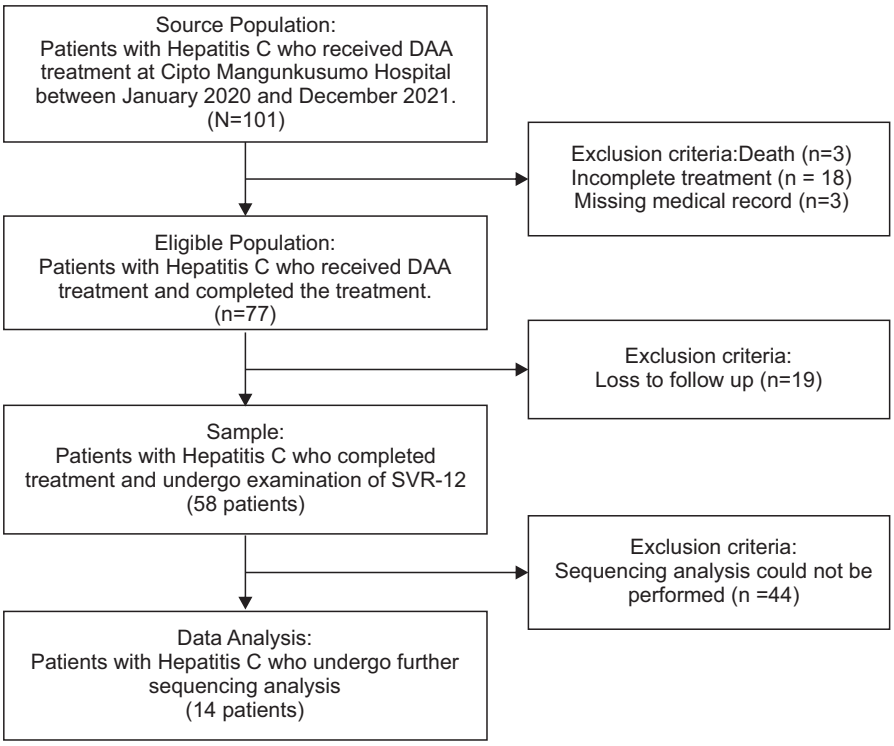

Figure 1. Flowchart of The Selection of Patients.

Table 3. Demographic Characteristics

Characteristics	Total (n = 14)	Non Mutation (n = 11)	Mutation (n = 3)	p Value
Demographic Data				
Gender, n				
Men / Female	9/5	7/4	2/1	1.000
Age, Mean (Standard deviation)	52 (15.73)	48 (13.27)	65 (20.26)	0.111
Cirrhosis				
Decompensated/Compensated/No Cirrhosis, n	4/4/6	2/3/6	2/1/0	0.165
Liver stiffness measurement, kPa Mean (Standard deviation)	18.11 (15.09)	14.6 (15.72)	28.8 (6.14)	0.168
Data Laboratorium				
Hemoglobin, g/dl, (Standard deviation)	12.91 (2.74)	13.07 (2.91)	12.33 (2.45)	0.696
Leucocytes, $10^3/mm^3$, median (minimum–maximum)	5.06 (1.05 – 9.70)	5.33 (4.45 – 6.72)	4.66 (4.21 – 4.93)	0.368
Platelet, x $10^3/mm^3$, Mean, (Standard deviation)	161 (85.9)	166 (95.2)	142 (44.8)	0.682
Albumin, Mean, (Standard deviation)	3.61 (0.76)	3.60 (0.88)	3.64 (0.38)	0.948
AST, U/L, median (minimum–maximum)	81 (18 – 350)	49 (30 – 77)	34 (34 – 192)	0.937
ALT, U/L, median (minimum–maximum)	51 (22 – 86)	64 (29 – 82)	31 (28 – 36)	0.287
RAS Characteristics				
Mutation in L31 (M/A)	1	0	1	-
Mutation in Y93 (F/H)	1	0	1	-
Mutation in L31 and Y93 (M/F)	1	0	1	-
Treatment Outcome				
Hepatitis C treatment, n				
Non SVR/ SVR	1/13	0/11	1/2	0.214

The detail about mutation analysis is described in **Table 4.** Among the mutation in the NS5A region, RAS could be found in L31, Y93, or both L31 and Y93 region. Two patients who had RAS in L31 achieved SVR and each patient had different point mutation (M point and A point). Out of 2 patients with RAS in Y93, one patient achieved SVR while the other patient did not. In patient who did not achieve SVR, the point mutation was found in H point. There was one patient with RAS at both L31 (site M) and Y93 (site F). This patient achieved SVR.

RAS in Y93 at site F and H showed a different SVR-12 outcome which may be associated to sofosbuvir daclatasvir treatment resistant pattern. For the purpose to assess phenotypic outcomes of these mutation sites, the clinical characteristics between these two point mutation sites were further examined and compared. Patient with Y93(H) had a higher viral load compared to patient with Y93(F). Additionally, liver injury (particularly alanine transamine) and cirrhotic stage were also higher in patient with Y93(H). **Table 5.** shows comparison of characteristics of patient who had mutation in Y93(H) and did not achieve SVR to patient's with mutation in L31(M)/Y93(F) who achieved SVR.

DISCUSSION

The present study examined various RASs in NS5A region which may affect SVR outcome post sofosbuvir daclatasvir treatment. The RASs identified in this population were Y93, L31, and both combined. It was previously known that RAS at these site were responsible for resistance to direct-acting antiviral (DAA) treatment.[24]

Patients in this study with NS5A region mutation at L31M and L31A achieved SVR. This finding is in line with a previous study, although specially performed on patient with chronic hepatitis C and inherited blood disorders, reported that L31M mutation decreased the potency of daclatasvir but remained sensitive to sofosbuvir.[25] Moreover. L31M had a lower impact on ombitasvir in HCV genotype 1 patient.[26] To date there is no research that described the susceptibility of L31A mutation towards DAA treatment as found in this study.

In this study, the presence of RASs at both Y93(F) and L31(M) did not affect the outcome of SVR. A research reported that a combined mutation of L31M/V and Y93(H) was found to be more resistant towards DAA treatment. However, the mutation in the other study was Y93(H) contrary to the mutation found in our study which

Table 4. NS5A region RAS characteristics

RAS Characteristics	Total	Non SVR (n = 1)	SVR (n = 13)	P
L31				
Mutation/ Non Mutation	2/12	0/1	2/11	1.000
Mutation in L31 (M/A)	1/1	0/0	1/1	
Y93				
Mutation / Non Mutation	2/12	1/0	1/12	0.290
Mutation in Y93 (F/H)	1/1	0/1	1/0	
L31 and Y93				
Mutation / Non Mutation	1/13	0/1	1/12	1.000
Mutation in L31 (M) and Y93 (F)	1	0	1	

Table 5. Patient Characteristics

Patient 1. **Y93 (H)** **Non SVR**	**Patient 2.** **Y93 (F) and L31 (M)** **Achieve SVR**
Viral load 9.42x 10^5 IU/mL	Viral load 1.22x 10 IU/mL
AST/ALT 26/350	AST/ALT 31/41
Decompensated cirrhosis with variceal bleeding	Decompensated cirrhosis with variceal bleeding
Albumin 3.4	Albumin 3.4
Total Bil 3.4	Total bilirubin 0.99
INR 0.9	INR 1.06
Fibrosis F4 35.8 kPa	Fibrosis F4 24.5 kPa
Child Pugh B	Child Pugh A

was Y93(F), this is different kind of mutation. For Y93(H) mutation in this study is in line with other study where H mutation in Y93 was present in patient that did not reach SVR. It is well known that Y93H mutation conferred potency reduction of daclatasvir, particularly in HCV genotype 1b and genotype 3.[27,28] Additionally, Y93F mutation was also associated to confer resistance towards ledipasvir and ombitasvir in HCV genotype 1a.[29] It is yet known whether Y93(F) has contributed to susceptibility towards successful DAA treatment in our patient. Another explanation could be HCV genotype carrying Y93(F) mutation has made it susceptible to DAA treatment. This finding highlights the importance of determining HCV genotype in patient to determine the resistance pattern in each genotype.

The patient who did not achieve SVR had a higher viral load, high level liver injury tests and high CTP score. Aside from NS5A region mutation, these outcomes are usually associated with treatment failure and ongoing disease progression. As comparison were made between patients, the outcomes might also be affected by each individual's disease pathophysiology and not associated with the mutation itself. Therefore, more research is required to describe whether these clinical characteristics were associated with phenotypic appearance of Y93(H), Y93(F) and L31(M).

This study has its own limitation. During this study, genotype testing was not mandatory for patients, as sofosbuvir-daclatasvir is a pan-DAA medication with wide-ranging activity against multiple hepatitis C virus genotypes. Furthermore, the sample size in this study was quite small. With only 14 subjects undergoing sequencing analysis, it's doubtful that this smaller sample size can reliably meet the research objectives, as it results in lower statistical power and increased risk of errors, suggesting that conclusions should be drawn with caution. However, despite these limitations, our study provides valuable insights into the specific RAS mutations in the NS5A region observed in Indonesian hepatitis C patients. This adds to the existing scientific knowledge and contributes to understanding the mutation profiles of HCV in this population.

CONCLUSION

In conclusion, this research highlights the occurrence of RAS in the NS5A region, particularly Y93 and L31. The NS5A-L31A mutation may increase HCV susceptibility to DAA treatment, while the occurrence of Y93H reduces the effectiveness of daclatasvir. Beyond these mutations, other factors such as HCV RNA viral load, the level of liver injury, and inflammation may also play a significant role.

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CONFLICT OF INTEREST

The authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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AUTHOR'S CONTRIBUTION

JK, DRB and IH proposed and conducted the study. ADH collected the sample and GA analyzed the data. All authors contributed in designing the study, interpreting results, and writing the final manuscript. JK is the guarantor.

AVAILABILITY OF DATA AND MATERIALS

The dataset is available from the corresponding author on reasonable request.

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APPENDIX

A. Mutation L31M, Y93F

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3     SWLRDIWDWICEVLSDFKTWLQAKLMPQLPGIPFISCQRGYKGVWRGDGIMQTRCHCGAQ  62
      ...WE....V.T.........K...L.LM.....L........E.....V.H.K.P...E

63    IAGHVKNGSMRIVGPRTCSNMWSGTFPINAYTTGPCTPSPAPNYSRALWRVAAEEYVEIR  122
      L...IR......I..K....T.H........F....GV.I.....KF.....S......V.

123   QVGDSHYVTGMTTDNIKCPCQVPAPEFFTEIDGVRLHRYAPACKPLLREEVSFRVGLHQY  182
      R.........V.Q.......*.........VY.I....H..K......D....S...PS.

183   PVGSQLPCEPEPDVAVITSMLTDPSHITAE  212
      V..........T..V.M........D.M..
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Mutasi L31M, Y93F

B, Mutation L31A

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1     SGSWLRDIWDWICEVLSDFKTWLQAKLMPQLPGIPFISCQRGYKGVWRGDGIMQTRCHCG  60
       ..H..CT....V.S.........S..V..A...L......T..........V.S...P..

61    AQIAGHVKNGSMRIVGPRTCSNMWSGTFPINAYTTGPCTPSPAPNYSRALWRVAAEEYVE  120
      .SVT.........LA.....A...H......E.....S..C.P...T........NS...

121   IRQVGDSHYVTGMTTDNIKCPCQVPAPEFFTEIDGVRLHRYAPACKPLLREEVSFRVGLH  180
      V.R...F..I..A.E.QL.......G......V.....Q....P.E....Y.IT.TAW.N

181   QYPVGSQLPCEPEPDVAVITSMLTDPSHITAE  212
      S.AI............S.L....RESF...D.
```

Mutasi L31A, Y93 negatif

C. Mutation Y93H

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1    SGSWLRDIWDWICEVLSDFKTWLQAKLMPQLPGIPFISCQRGYKGVWRGDGIMQTRCHCG  60
     .......V.....T..I.......S..L.R...V..F..................T.P..

61   AQIAGHVKNGSMRIVGPRTCSNMWSGTFPINAYTTGPCTPSPAPNYSRALWRVAAEEYVE  120
     ...T.............K......H.......H.............K..........M.

121  IRQVGDSHYVTGMTTDNIKCPCQVPAPEFFTEIDGVRLHRYAPACKPLLREEVSFRVGLH  180
     VTR...F.........................L..........P...........Q...N

181  QYPVGSQLPCEPEPDVA  197
     ..L.R...........T


Mutasi L31 negatif, Y93H
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