Significance of mutations in the region coding for NS3/4 protease in patients infected with HCV genotype 1b

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Abstract

Background. Fast hepatitis C virus (HCV) replication is one of the reasons for frequent changes in viral genome.

Objectives. The objective of this study was to evaluate the frequency and type of mutation in NS3/4 protease in patients with HCV genotype 1b and to determine the effect of the mutation on viral load, fibrosis stage, alanine aminotransferase (ALT) activity, and alpha-fetoprotein (AFP) level.

Material and methods. The study included 46 treatment-naïve patients, infected with HCV genotype 1b. Mutations were analyzed after isolating HCV RNA, and then evaluating the compliance of the amino acid sequence, using 3500 Genetic Analyzer (Applied Biosystems, Foster City, USA). RNA fragment from nucleotide 1–181 encoding NS3/4 protease was subjected to analysis.

Results. Mutations were demonstrated in 65% of subjects. Changes in the protease region affecting resistance to treatment (T54, Q80, V158, M175, D186) were detected in 10.8% of patients. Substitution mutation at T72 was found most frequently – in 49.9% of cases. In 13% of patients, mutation at G86 was demonstrated, including G86P in 5 patients and G86S in 1 patient. In the group of patients with T72 mutation, viral load was significantly higher (1.3×106 IU/mL vs 1.0×105 IU/mL; $p = 0.01$), AFP level was higher and fibrosis level was lower (1.26 vs 2.17; $p = 0.008$) compared to the patients without the mutation. Cryoglobulinemia was observed in 74% of patients with mutation at position T72.

Conclusions. Natural mutations of the region coding for NS3/4 protease are found frequently in patients infected with genotype 1b, but they may cause resistance to antiviral agents only in 11% of patients. Changes were most frequently found at position T72. Mutations at position T72 are correlated with the cryoglobulinemia occurrence. This is a substitution mutation, accompanied by a high viral load, high ALT activity and AFP level, which may point to a more unfavorable influence of such a modified virus, compared to wild-type virus, onto pathological processes in the liver.

Key words: mutations, cryoglobulinemia, hepatitis C virus genotype 1b, NS3/4 protease

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Introduction

Collectively, 6 hepatitis C virus (HCV) genotypes have been identified, differing in 30–35% of RNA sequence. In Poland, like in most European countries, genotype 1 subtype b (1b) is the most frequent HCV genotype, responsible for chronic infection. Hepatitis C virus replication is characterized by fast rate and frequent occurrence of modifications in the genome. The replication rate is connected with the activity of RNA-dependent RNA polymerase (RdRP). Except normal RNA synthesis, modified RNA strands are also produced during the HCV replication phase. They are characterized by point or multiple amino acid modifications, or even their loss. In the case of HCV, the correction of a modified RNA strand is not possible. Such modifications may be lethal and they may limit further replication, or lead to the appearance of a virus with different biological properties. Modified viral genome usually does not influence the change of biological properties, such as immunogenicity or tropism for hepatocytes; however, resistance to antiviral agents sometimes occurs as the result of such modifications.[1]

NS3 protease, having the properties of a helicase, is responsible for the initiation and unwinding of an RNA strand during the replication of the virus. It is encoded in the C-terminal viral RNA fragment, at positions 1–181. The protease has the properties of an adenosine triphosphatase (ATP) enzyme, nucleotide triphosphatase, necessary for the replication and transcription of RNA. Finding the loci coding for NS3 synthesis allows for designing agents inhibiting viral replication. Such agents are usually aptamers, short DNA or RNA fragments, or peptides binding specifically to a target molecule.[2] The most direct-acting antiviral agents (DDAs) used in HCV infections are aptamers. Replacement or loss of amino acids in NS3 significantly decreases the efficiency of HCV protease. Usually, this happens in the case of mutation at T36, T54, V55, A155, or V170 position. These positions are particularly prone to the resistance against protease ketoamide inhibitors. These compounds form a reversible covalent bond with a catalytic serine of NS3/4A protease. Such situation usually occurs during boceprevir or telaprevir therapy. Linear noncovalent protease inhibitors, such as faldaprevir tripeptide, simeprevir, asunaprevir, and vaniprevir, usually bind to position 168.[3,4] Currently, resistance associated with a single mutation within the HCV genome is not a serious problem, because high doses of antiviral agents or coadministration of several agents with different mechanisms of action guarantee successful therapy (Table 1).

Natural HCV mutations and their subsequent selection during the course of antiviral treatment are frequent. Hepatitis C virus mutants may acquire the ability for fast replication as well as competitiveness against wild-type viruses. Moreover, new mutations, unimportant from the point of view of therapy efficacy evaluation, may trigger the mechanisms of broader, unfavorable effect on the organism compared to the activity of a wild-type virus. In-depth analysis of new mutations seems to be inevitable in the monitoring of early oncogenesis, fibrosis or the activation of cryoglobulinemia. This may improve preliminary diagnosis and contribute to the prevention or significant delay of HCC development.[5] The problem is even more important, as patients after effective antiviral therapy still belong to the group with an increased risk for HCC development. Also, it is impossible to exclude geographical differences in the occurrence of mutations, resulting from epidemiological reasons and environmental factors. This justifies undertaking studies on the occurrence of mutations in the HCV genome in patients infected with this virus in different regions of the world.

Objectives

The study was performed in order to evaluate the frequency of mutations in the region coding for NS3/4 protease among people infected with HCV genotype 1b. The type of mutation was established in relation to resistance to antiviral treatment and other mutations, including substitutions. The effect of mutation on viral load as well as inflammation and fibrosis in the liver was studied.

Table 1. Changes in the protease NS3/4 region affecting resistance to protease inhibitor treatment[6–10]

Protease inhibitor	Position; changed amino acid												
	V36	F43	T54	V55	Q80	S122	I132	R155	A156	V158	D168	V170	M175
Boceprevir	M, A		A, S, C, G	A				K, T	S, T, V	I	N	A, T	L
Telaprevir	M, A, L, G, I		A, S				V	K, T, G, M	S, T, V, F, N		N		
Simeprevir					K, R	A, G, R		K, Q			V, E, A, H	T	
Faldaprevir								K, Q	T		V, E, A, N, T		
Paritaprevir			A	A				K, Q	S, T		A, E, H, T, V, Y	A	
Vaniprevir		S						K, G, T			V, Y G, A		
Grazoprevir								Q, K	S, T		A, V		
Danoprevir								R, K, Q			D, E	V, I	
Asunaprevir	M, R							K	T		A		

Material and methods

The study included 46 patients, 18 women and 28 men, aged 49 years on average (from 19 to 72 years), infected with HCV genotype 1b (Table 2). All the patients were treatment-naïve.

Hepatitis C virus infection was documented based on the presence of HCV RNA in the serum. The test was performed with the use of the reverse transcription polymerase chain reaction (RT-PCR) method, with reaction starters specific for viral noncoding 5'-terminal region (5'-UTR). The viral genotype was determined by direct sequencing of the PCR reaction product (Syngen Biotech, Tainan City, Taiwan).

Quantitative evaluation of HCV RNA was performed with the use of COBAS AmpliPrep (Roche, Mannheim, Germany). The procedure was automated, based on the amplification of nucleic acid by PCR. HCV virus RNA was determined quantitatively with the use of quantitative standard HCV QS. The method sensitivity was 11 IU/mL, linearity was 15 IU/mL.

Table 2. The patients' characteristics

Number of patients	46
Women/men	18/28
Age [years] mean range	 49 19–72
HCV-RNA [IU/mL] mean range	 1.1×10^6 4.1×10^4–5.5×10^6
Fibrosis mean range	 1.7 1–4
ALT [IU/mL] mean range	 78 20–360
AFP [ng/mL] mean range	 6.1 1.4–15.0
Cryoglobulinaemia n percentage [%]	 28 61

HCV – hepatitis C virus; ALT – alanine aminotransferase; AFP – alpha-fetoprotein.

Hepaptitis C virus mutation

Blood plasma was collected from the patients infected with a HCV genotype 1 virus. The total RNA was extracted from 500 µL of blood plasma using a devise for an automatic extraction of nucleic acids EasyMag (Biomerieux, Lyon, France). The fragment of the HCV genome covering NS3/4A was amplified in a nested-PCR with primers described by Paolucci et al.[11] The sequences of the primers were as follows: for the 1st PCR – *1-forward outer 5'-CGAGACCTTGCGGTGGCAGT-3'*, *1-reverse 5'-CAGCCGTYTCCGCTTGGTCC-3'*; for the 2nd PCR – *1-forward inner 5'-CATCACCTGGGGGGCAGACACC-3'*, *1-reverse inner 5'-GTCAGTTGAGTGGCACTCATCAC-3'*. The 1st PCR, prevented by complementary DNA (cDNA) synthesis, was performed with a OneStep RT-PCR kit (EURex Ltd., Gdańsk, Poland) in 25 µL of reaction mixture, containing 12.5 µL of 2 × Master Buffer Mix, 1 µL of both outer forward and reverse primer 10 µM solutions, 1 µL of Master Enzyme Mix solution, and 50–100 ng of RNA. The reaction conditions were as follows: 10 min denaturation at 94°C, followed by 50 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, with an extension at 72°C for 10 min. The 2nd PCR was carried out in 20 µL of reaction mixture, containing 1 × PCR buffer with $MgCl_2$, 0.8 mL of 20 mM deoxynucleotide (dNTP) solution, 1 µL of both inner forward and reverse primer 10 µM solutions, 0.1 µL of Taq DNA polymerase, and 1 µL of the 1st PCR solution (all reagents from EURex Ltd.). The 2nd PCR was performed with the following conditions: 5 min denaturation at 94°C, and then 30 cycles at 94°C for 1 min, 52°C for 1 min and 72°C for 1 min, with an extension at 72°C for 10 min.

The PCR products were checked and separated using an agarose gel electrophoresis, and then extracted from agarose slides, using a PCR clean-up Gel Extraction Kit (Macherey-Nagel, Duren, Germany). The extraction was performed automatically on a QIACUBE machine (Qiagen, Hilden, Germany).

Direct sequencing of cleaned PCR products was performed using an automatic DNA sequencer 3500 Genetic Analyzer (Applied Biosystems, Foster City, USA) and a BigDye Terminator v. 3.1 Cycle Sequencing kit (Applied Biosystems). Nucleotide sequences were compared with the HCV subtype 1b gene for polyprotein, NS3 protease region, partial cds, isolate:MS100913 (GenBank ID: AS709386.1). Particular sequences of codons 54, 55, 71, 72, 80, 86, 158, 168, and 175 of the gene were analyzed to search for the mutations resulting in HCV protease inhibitor resistance.[11]

In all the patients, tests for hepatitis B virus (HBV), detecting the presence of the antigen HBsAg in the serum, and for human immunodeficiency virus (HIV), looking for anti-HIV antibodies (Abbott Laboratories, Lake Bluff, USA) were performed. Moreover, the presence of alpha-fetoprotein (AFP), alanine aminotransferase (ALT) and cryoglobulinemia in the serum was evaluated.

In 20 patients, a liver biopsy was taken, and in 26, an elastography was performed. Sections from liver bioptates were subjected to hematoxylin and eosin staining in order to establish the intensity of the inflammatory process, and to Syrian red staining, evidencing the presence of connective tissue. The morphological state of the liver was described according to Scheuer's classification. In the case of patients with elastography, the liver fibrosis stage was transposed into the Metavir scale (the Metavir scale is comparable to Scheuer's classification).

After the study was approved by the Bioethical Committee at the Medical University of Bialystok, the patients expressed informed consent to participate in the research.

Statistical analysis

Statistical analysis was performed with the χ^2 and the Mann-Whitney U tests. The significance level was established at $p < 0.05$. Studies were performed using STATISTICA PL v. 10 (StatSoft Polska Sp. z o.o., Kraków, Poland) for Windows 10.

Results

In the group of patients with HCV genotype 1b, mutations were detected in 30 out of 46 (65%) patients (Table 3). Mutations potentially connected with resistance to NS3/4 inhibitors (T54 (1), Q80 (1), V158 (1), D168 (1), and M175 (1)) were detected in 5 (11%) patients. Only single mutations of this type were present in individual patients.

Most frequently, in 50% of cases, substitution at T72 not affecting the efficacy of antiviral therapy was detected. In 6 out of 46 (13%) patients, mutation at G86 was found, including 5 cases of G86P mutation; there was also 1 case of G86S. Other mutations were demonstrated sporadically (Table 4).

In the group of patients with a mutation at position T72, viral load was significantly higher than in patients without this mutation (1.3×10^6 IU/mL vs 1.0×10^5 IU/mL; $p = 0.01$) (Fig. 1).

Among the patients with T72 mutation, the average fibrosis stage in the hepatic tissue was significantly lower than in patients without this mutation (1.26 vs 2.17; $p = 0.008$) (Fig. 2).

Cryoglobulinemia was observed in 28 out of 46 (61%) patients. Among patients with a mutation at position

Table 3. Substitution mutations and cryoglobulinemia found in patients

No.	Patient	Position								Cryoglobulinemia
		T54	I71	T72	Q80	Q86	V158	D168	M175	
1	CA			X						X
2	WI			X		X				X
3	BM			X						
4	OW		X	X						X
5	JW						X			X
6	BB			X						
7	SR				X	X				X
8	ND			X						X
9	LM			X						X
10	PJ			X						X
11	KS			X						
12	BB								X	
13	GA					X				X
14	NA			X						X
15	WM					X				
16	KP					X				X
17	RJ			X						X
18	OM			X						X
19	AW			X						X
20	ZA			X						
21	SM			X						X
22	RL			X						X
23	WA			X						X
24	SS			X						X
25	KG					X				X
26	RW			X						X
27	GM			X				X		X
28	DW	X		X						
29	GH			X						
30	TR			X						X

Table 4. The substitution mutations (type and amount)

Position	n (%)	Type
T54	1 (2.17%)	Thr54Ser
I71	1 (2.17%)	Ile71Val
T72	23 (49.91%)	Thr72Ile
Q80	1 (2.17%)	Gln80Leu
Q86	5 (10.85%) 1 (2.17%)	Gln86Pro Gln86Ser
V158	1 (2.17%)	Val158Gly
D168	1 (2.17%)	Asp168Glu
M175	1 (2.17%)	Met175Asn

T72 (17/23), the incidence was significantly higher compared to those without the mutation (6/16), (74% vs 38%; p = 0.023).

Among the patients with T72 mutation, AFP level was significantly higher than in patients without this mutation (Fig. 3). In the same group of patients, higher ALT activities were documented compared to patients without this mutation (82 IU/mL vs 72 IU/mL); however, the difference was not statistically significant.

No patient was detected with HBs and anti-HIV.

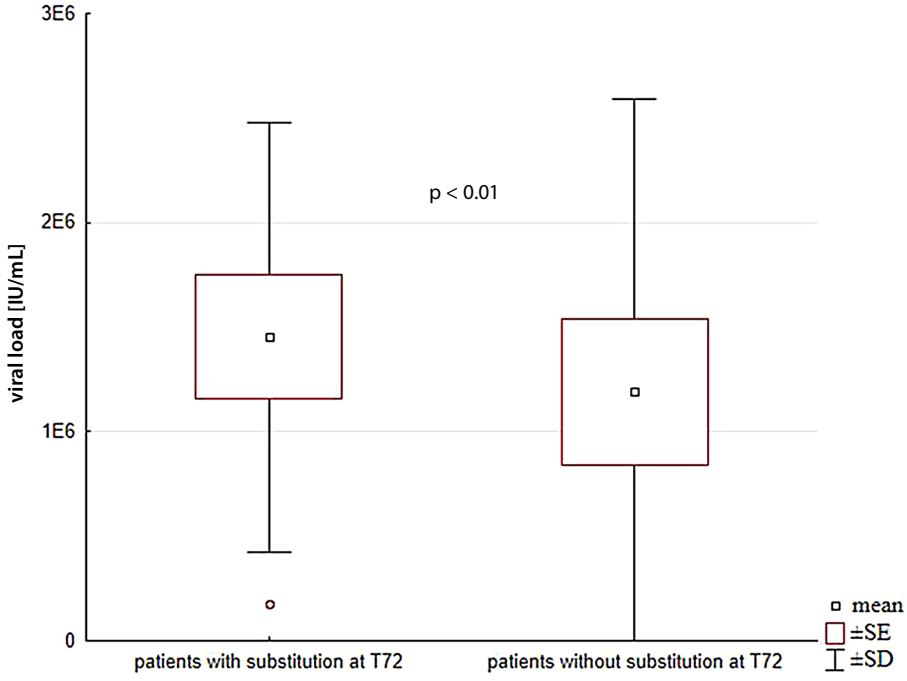

Fig. 1. Viral load in patients with and without substitution at T72 gene of HCV RNA

HCV – hepatitis C virus.

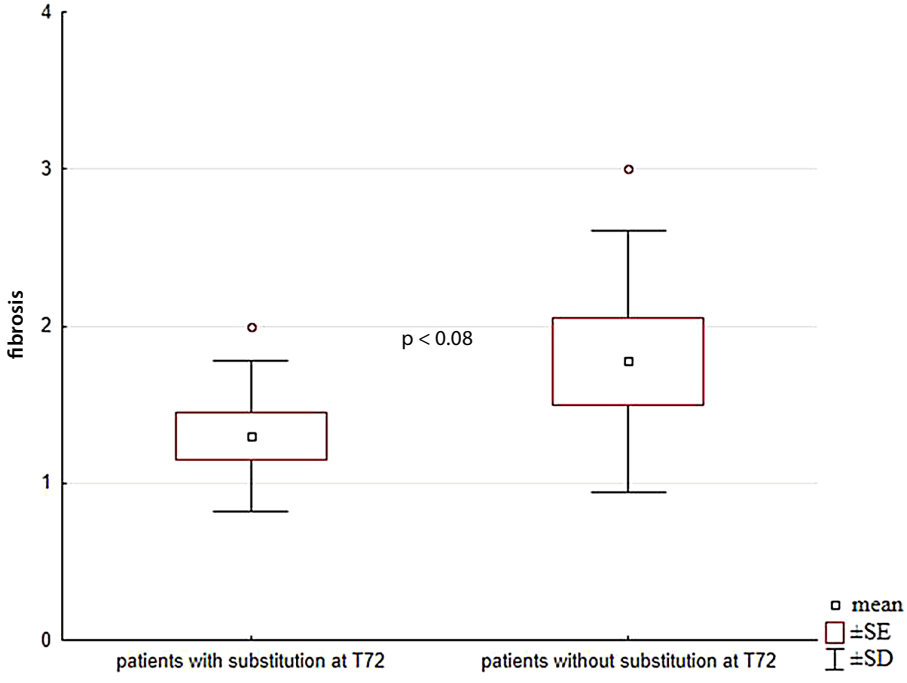

Fig. 2. Liver fibrosis in patients with and without substitution at T72 gene of HCV RNA

HCV – hepatitis C virus.

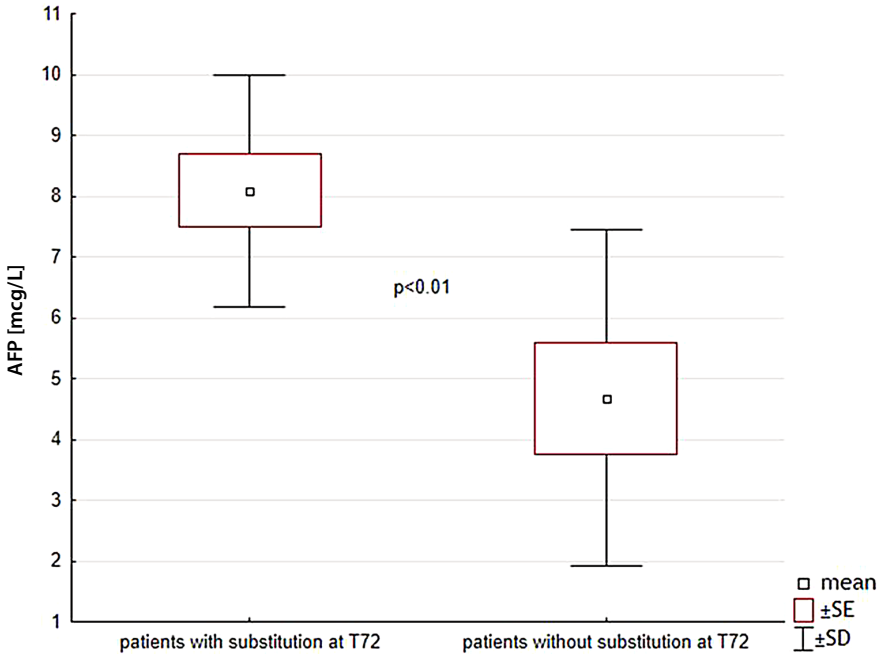

Fig. 3. The activity of AFP in the serum in patients with and without substitution at T72 gene of HCV RNA

AFP – alpha-fetoprotein; HCV – hepatitis C virus.

Discussion

Mutations in HCV genotype 1 are frequent. Jaspe et al. reported the possibility of such mutations in even 79% of patients infected with HCV genotype 1, before the start of therapy. Moreover, there are considerable differences in the prevalence of individual mutations, depending on the geographical region (Table 5).[12] A study by Miura et al., performed on patients infected with HCV genotype 1b, in 25 chronically infected patients without cirrhosis, 29 with cirrhosis and 25 with hepatocellular carcinoma (HCC) demonstrated the highest frequency of mutations in the part of the virus encoded in the region from position 61 to 100 (usually at positions 70, 75 and 91), in patients with liver cirrhosis and carcinogenesis.[13]

Equally frequently such mutations appear in patients treated with antiviral agents; however, these are different mutations. Danish Hepatological Group documented the occurrence of mutations in the HCV genome in 71%

Table 5. Frequency of amino acid substitution R7 0Q and L/C91M in patients with HCV genotype 1a and 1b infection with respect to different geographic regions[10]

Genotypes	n	Geographic regions	Substitution [%]	
			R70Q	L/C91M
1a	27	Venezuela	3.7	0
	27	USA	3.7	0
	0	Japan	0	0
1b	38	Venezuela	79	68
	34	USA	65	82
	80	Japan	40	31
	70	China	10	61

of patients unsuccessfully treated with telaprevir; however, many of these mutations do not affect the resistance to antiviral therapy.[14] In our research, the frequency of all mutations was 49%, but mutations in the region of NS3/4 protease, underlying the resistance to DAAs, were demonstrated in 11% of patients, which is in accord with the observations of other authors. Ferraro et al. detected such mutations in 14% patients infected with HCV genotype 1b at positions V36, F43, T54, I153, R1 55, and D168.[15] In similar studies, Paolucci et al., on the basis of a group of 39 treatment-naïve people infected with HCV genotype 1b, demonstrated the presence of mutations at positions V55, Q80 and M175 in 10% of the patients.[11] In the context of antiviral therapy, mutations decreasing the efficacy of boceprevir and telaprevir are most often detected, which, considering the frequent adverse reactions of these agents, justifies the recommendation to avoid their use in the treatment.[16,17] Most authors evaluating HCV mutations focus on determining their effect on the resistance to antiviral agents. This is certainly important, although the presence of other mutations not influencing the efficacy of antiviral therapies may play an important role in the biology of the virus and pathophysiological implications of the infection.

Hepatitis C virus RNA does not integrate with nucleic acids of humans. It seems that one of the most important pathways stimulating HCC development in the course of HCV infection is the influence of specific viral proteins synthesized on the basis of viral genome. Many reports suggest that mutations in the viral RNA may influence the synthesis of such proteins. Viral NS3 protein exerts a specific influence on the early stage of carcinogenesis in hepatocytes. It inhibits the activity of cellular p21 (WAF1) protein, while p21 remains in close cooperation

with p53, one of the main factors controlling the neoplastic process. Although the deficiency of p21 protein does not directly cause HCC development, the influence of this protein on a higher growth rate and proliferation of newly formed neoplastic cells has been confirmed.[18]

The occurrence of compensatory mutations in the course of HCV infection is a poorly studied process. Such mutations usually occur as a response to the loss of efficiency of nucleic acid, connected with the primary, unfavorable mutation. The new mutation may change the biological properties of the virus.[19] During the replication of HCV RNA, numerous mutations take place, resulting in the synthesis of new viral proteins, possibly showing different biological properties compared to normally synthesized proteins. Compensatory mutations occur at the same time as mutations conferring resistance to antiviral agents, but may also appear as independent mutations. This is particularly important in the case of the gene encoding protease NS3/4, responsible for the initiation and replication of the virus. At the moment, it is thought that mutations I71, T72 and Q86 are compensatory mutations in the HCV infection. Mutations I71 and T72 are compensatory for mutations V55 and M175, and compensatory mutation Q86 is present in the case of mutation at position Q80.[15] In our research, compensatory mutation T72 was observed in 1 patient in relation to substitution at position T54. Also, mutation Q86 was detected in a single patient, who also had a mutation at Q80.

In the group of patients infected with HCV genotype 1b, Paolucci et al. demonstrated the presence of a mutation at T72 in 28% and at I71 in 13% of patients.[11] In our studies, mutation at position T72 was found in 49.9% (23/46) of patients and at I71 in only 1 patient.

Our results point to a significantly higher viral load and AFP level among the patients with a mutation at position T72 compared to patients without this mutation. This may suggest a stimulatory effect of this mutation on viral replication, which is reflected in higher ALT activity and less advanced fibrosis (more inflammation) in the group of patients with observed mutations. Lee et al. demonstrated that the infection with genotype 1b in itself is a risk factor for HCC compared to other genotypes of the virus. Moreover, a significantly higher HCV viral load was detected in patients with genotype 1b in whom HCC was diagnosed (397 patients with the cancer, 410 without).[20]

The study by Hu et al. performed on 63 patients infected with HCV genotype 1b with coexisting HCC showed more frequent occurrence of mutations at positions A028C, G209A, C219U/A, U264C, A271C/U/A, C378U, G435A/C, and G481A (mutations affecting the resistance to antiviral agents), and compensatory mutations at positions K10Q, R70Q, M91L, and G161S in comparison to 188 patients infected with HCV, but without HCC.[21]

Vallet et al. demonstrated a high frequency of mutations at positions Y56F, I71F, T72I, Q86P, P89S, S101G/D, R117H, S122G/T/N, V132I, and V170I in the group of treatment-naïve patients infected with HCV genotype 1. These mutations may significantly affect the whole codon of NS3 protease. Authors postulate that these mutations may lead to more frequent HCC development and more advanced fibrosis stage.[22] It seems that this compensatory mutation at position T72, unimportant from the point of view of resistance to drugs, may lead to an increased rate of HCV replication. Increased HCV replication is one of the prediction factors for HCC development. Yoshimi et al. demonstrated that in the case of mutation at position Y93H in the fragment encoding NS3/4 protease of HCV genotype 1b, higher HCV replication, higher ALT activity and more frequent HCC development may be expected. However, the mutation itself does not influence the efficacy of antiviral treatment.[23] Verga-Gérard et al. pointed to unfavorable significance of transforming growth factor beta (TGF-β) activation in the stimulation of HCC development. Their studies on NS3/4 protease showed that modification at position S139A was accompanied by the stimulation of TGF-β synthesis, upregulating HCC development.[24] Mutations at position R70 and L/C91 are present in genotype 1b only, and are substitution mutations. Many studies show that they may result in higher frequency of HCC, liver steatosis and higher resistance to interferon (IFN). The results of the study by Jaspe et al. demonstrate big differences in mutation frequency at positions R70 and L/C91, depending on geographical distribution of the population (Venezuela vs United States R70: 79% vs 69%; L/C91: 68% vs 75%).[4] It seems that the studies evaluating mutations within the genome of HCV are not currently very important from the point of view of resistance to new antiviral agents. Such studies may contribute new data concerning HCC development and stimulation of histopathological changes in the liver.

Conclusions

Mutations in the region encoding NS3/4 protease were present in 65% of treatment-naïve patients infected with genotype 1b, but they might affect the resistance to antiviral agents only in 11% of cases. Compensatory mutation at position T72 was present in 49.9% of patients, who showed a higher viral load and AFP concentration than patients without this mutation. A mutation at position T72 was correlated with the cryoglobulinemia occurrence. This may point to an unfavorable role of this mutation in the stimulation of pathological processes in the liver, connected with the infection.

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