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CLINICAL STUDY

Impact of TTV and SENV infection in chronic hepatitis B or C on liver histology and therapy outcome

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Abstract: *Aim:* To determine the influence of TTV and SENV on histological findings and viral response in patients with chronic viral hepatitis B and C.
Background: The clinical impact of TTV or SENV coinfections in these patients remains unclear.
Methods: Serum and liver biopsy specimens from chronic hepatitis B and C patients, 107 with liver biopsy and 105 who had finished complete antiviral therapy, were investigated for the presence of TTV and SENV.
Results: The Ishak score determined from 107 liver biopsy samples compared according to TTV or SENV coinfection was similar. Among 39 chronic hepatitis C patients with and 43 without virological response, we have found 9 and 3 SENV positive ($p<0.05$) and 18 and 28 TTV positive patients, respectively (not significant). However 11 of 32 biopsy samples obtained in the responder's group and 19 of 31 in non-responders were TTV positive ($p<0.05$). No similar differences were observed among 23 chronic hepatitis B patients. TTV clearance after interferon therapy exceeded 80 %, clearance of SENV 90 %.
Conclusion: TTV or SENV infections did not negatively influence the severity of histological features or the antiviral response in patients with chronic hepatitis B and C. Both viruses were highly sensitive to interferon therapy (Tab. 5, Ref. 29). Full Text in free PDF *www.bmj.sk.*
Key words: TTV, SENV, chronic hepatitis C, chronic hepatitis B, interferon, liver biopsy.

Viral hepatitis B and C constitute the most common etiologic factors of chronic liver diseases with a high rate of progression to cirrhosis or hepatocellular carcinoma resulting in increased mortality (1, 2).

Current medical options make it possible to eradicate viral infection in a significant number of patients or at least to suppress and to prevent progression of chronic hepatitis B (CHB) or C (CHC) to cirrhosis or hepatocellular carcinoma. The most commonly used medication is interferon or peginterferon (in chronic hepatitis C in combination with ribavirin).

In spite of advances made during past years, the antiviral therapy is still not efficient enough. Major factors that negatively influence response to treatment are viral load and genotype with reference to hepatitis C. Concerns are focused on coinfection with other viruses as well (1, 2).

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Regarding cases of hepatitis of unknown etiology, new hepatotropic viruses – TT virus (TTV) and SEN virus (SENV) - have been taken into account during the last few years (3, 4), none the less clinical relevance remains dubious. In addition to detection of the virus in patient's sera samples by PCR sampling, its presence has been confirmed in the liver and also other tissues, with the liver being the essential site of replication (5). Owing to their similar parenteral route of transmission, prevalence among patients with chronic hepatitis B and C is high (6–11). Most of the literature data suggest that there is no direct impact of TTV or SENV upon clinical course of HBV and HCV infection (10–14). However, other observations admit a possible adverse influence of TTV and SENV infections on the clinical course, liver biopsy results or antiviral treatment outcome in patients with chronic hepatitis B and C (15–18).

The main aims of this study were to determine the impact of TT virus and SEN virus coinfection on histological findings in patients with chronic hepatitis B and C and to clarify the effect of TTV and SENV on viral response in chronic hepatitis B and C patients treated with current antiviral therapy as well as the effect of this therapy on TTV and SENV.

Methods

A total of 137 adult patients with chronic hepatitis B and C (86 males and 51 females, mean age 40.1±14.2 years, 98 with CHC and 39 with CHB) diagnosed or hospitalised at The De-

partment of Infectious Diseases in Košice, Slovakia between January 2004 and December 2006 were enrolled and followed-up in our outpatient hepatology clinic. The impact of TTV and SENV coinfection on histological findings was evaluated in a group of 107 patients (79 with CHC and 28 with CHB) who have undergone a liver biopsy. Another group consisted of 105 patients (82 with CHC and 23 with CHB) who have finished the complete course of antiviral therapy following the current therapeutic guidelines to determine the impact of TTV and SENV on response to antiviral therapy.

Patients with CHC were treated with a combination of peginterferon alfa-2a or alfa-2b and ribavirin for 48 or 24 weeks, respectively, according to HCV genotype. Patients with CHB were treated with peginterferon alpha-2a or standard interferon alfa-2a or alfa-2b for 48 or 24 weeks, respectively, according to the type of interferon and HBV pre-core mutation. There were 90 out of 105 patients who finished a complete course of therapy and had a control serum for TTV and SENV evaluation at the end of treatment available. This group served for determination of the antiviral treatment efficacy on TTV or SENV clearence.

The virological response in patients with CHC was defined as clearance of HCV RNA at the end of treatment or follow up (end of treatment response or sustaind viral response), the group of patients without response included all non-responders and those who have relapsed. Similarly, the virological response in patients with CHB was defined as decrease of HBV DNA under 10^5 copies per mL after the end of treatment or follow up, and non-responders and relapsers were considered as patients without response.

The presence of TTV after viral DNA isolation from patient's sera samples and liver biopsy specimens was determined by using two different methods with N22 or UTR primers, respectively, and SENV by establishing at least one of its strains, SENV-D or SENV-H. Detection of TTV or SENV in serum or biopsy sample or both was considered as TTV or SENV positivity. All biopsy samples were classified by Ishak score (grading and staging). The fibrosis in patients with Ishak stage 4 to 6 was considered as severe, in patients with Ishak stage 3 and less as mild or moderate. We used the Student t-test and Chi-square test for statistical evaluation. Significance was discriminated by a p-value less than 0.05.

Detection of TTV

DNA extracted from blood serum or biopsy specimen by QIAamp DNA Mini Kit (QIAGEN) was used as a template for PCR reaction. Each DNA sample was resuspended in 100 µl of elution buffer. PCR was performed using two different sets of primers:

1. TTV DNA based on primers from N22 region was identified by semi-nested PCR as described previously by Okamoto, 1998 (19). A total volume of PCR reaction mixture was 50 µl. Each tube contained 5 µl of template DNA, 1.5 U polymerase (RecTac Taq polymerase, Invitrogen), concentration 0.2 mM of each dNTP (dATP, dTTP, dGTP, dCTP), 1.5 mM MgCl2, and amount 50pM of each of primers. First PCR utilized NG 059 sense primer (5´-ACAGACAGAGGAGAAGGCAACATG-3´) and NG 063 antisense primer (5´-CTGGCATTTTACCATTTCCAA-AGTT-3´). PCR protocol consisted of initial denaturation (96 °C/ /6 min) followed by 35 cycles (94 oC/30 s; 60 oC/45 s and 72 °C/ /45 s), and final extension (72 °C/2 min). Second PCR was carried out with NG 061 sense primer (5´-GGCAACATGTTGTG-GATAGACTGG-3´) and the same NG 063 antisense primer for 25 cycles under the same PCR conditions.

2. TTV DNA based on primers from UTR region was identified by PCR protocol as described previously by Takahashi (20). A total volume of PCR reaction mixture was 25 µl. Each tube contained 5 µl of template DNA, 1.5 U polymerase (HotStar Taq polymerase, Quiagen), concentration 0.2 mM of each dNTP (dATP, dTTP, dGTP, dCTP), 2.5 mM MgCl2, and amount 50 pM of each of primers: T801-sense primer (5´-GCTACGTCACT-AACCACGTG-3´) and T935-antisense primer (5´-CTBCGGTG-TGTAAACTCACC-3´, B=G,C alebo T). PCR protocol consisted of initial denaturation (96 °C/15 min) followed by 55 cycles (95 °C/20 s; 65 °C/20 s and 72 °C/20 s), and final extension (72 °C/2 min).

PCR products (271 bp for TTV N22 and 199 bp for TTV UTR region) were detected by agarose gel electrophoresis.

Detection of SENV

SENV-D and SENV-H DNA was identified by PCR protocol as described previously by Kao, 2002 (21). A total volume of PCR reaction mixture was 25 µl. Each tube contained 5 µl of template DNA, 1U polymerase (HotStar Taq polymerase, Quiagen), concentration 0.2 mM of each dNTP (dATP, dTTP, dGTP, dCTP), 2.0 mM MgCl2, and amount 100 pM of each of primers: SENV-D sense primer (5´-GTAACTTTGCGGTCAACTGCC-3´) or SENV-H sense primer (5´-GGTGCCCCTWGTYAGTTGGC-3´), respectively and universal antisense primer same for both strains (5´-CCTCGGTTKSAAAKGTYTGATAGT-3´) (K=G or T, S= C or G, Y=C or T). PCR protocol for both SENV strains consisted of initial denaturation (96 °C/14 min) followed by 31 cycles (95 °C/1 min; 57.1 °C/1 min;72 °C/1 min), 11 cycles (95 °C/1 min; 55 °C/1 min; a 72 °C/1 min), and final extension (72 °C/1 min). PCR products (231bp for SENV-D, and 230bp for SENV-H) were detected by agarose gel electrophoresis.

Results

Liver biopsy samples from 107 patients (79 with CHC and 28 with CHB) who have undergone liver biopsy were investigated to determine the impact of TT virus and SEN virus coinfection on their histological findings. According to the degree of fibrosis by Ishak score 23 patients(21.5 %) had severe fibrosis and 84 (78.5 %) had mild to moderate fibrosis.

In the group of patients with severe fibrosis 1 TTV-N22, 12 TTV-UTR and 3 SENV positive liver biopsy samples were found. Among patients with mild or moderate fibrosis 2 positive cases of TTV-N22, 34 cases of TTV-UTR and 11 positive cases of SENV were confirmed. There were no significant differences between both groups (Tab.1). There was also no significant dif-

Tab. 1. Evidence of TTV and SENV in liver biopsy specimens according to degree of fibrosis.

Fibrosis	TTV-N22		TTV-UTR		SENV	
	positive	negative	positive	negative	positive	negative
Mild or moderate	2	82	34	50	11	73
Severe	1	22	12	11	3	20
Overall	3	104	46	61	14	93

Chi-square test was used for statistical evaluation. No significant differences were found.

Tab. 2. Mean values of Ishak score according to TTV or SENV infection.

	TTV-N22			TTV-UTR			SENV		
	positive n=3	negative n=104	p value	positive n=46	negative n=61	p value	positive n=14	negative n=93	p value
Staging±SD	2.33±1.25	2.49±1.49	NA	2.45±1.56	2.52±1.43	NS	2.50±1.55	2.49±1.48	NS
Grading±SD	5.67±0.47	5.07±1.91	NA	4.71±1.84	5.36±1.86	NS	5.08±2.87	5.09±1.70	NS

Student t-test was used for statistical evaluation. NA = not applicable. NS = non-significant.

Tab. 3. Coinfection of TTV and SENV with HCV according to treatment response.

	With response		Without response		Statistical
	serum n=39	biopsy n=32	serum n=43	biopsy n=31	significance
TTV-N22	1(2.6 %)	0 (0 %)	5 (11.6 %)	1 (3.2 %)	NS
TTV-UTR	18 (46.2 %)	11* (34.4 %)	28 (65.1 %)	19* (61.3 %)	*$p<0.05$
SENV	9† (23.1 %)	5 (15.6 %)	3† (7.0 %)	2 (6.5 %)	†$p<0.05$

Chi-square test was used for statistical evaluation. NS = non-significant.

ference comparing mean values of Ishak score from all liver biopsy samples according to TTV or SENV coinfection (Tab. 2).

Another group of 105 patients (82 with CHC and 23 with CHB) who have finished the complete course of therapy was followed-up to clarify the effect of TTV and SENV on viral response in chronic hepatitis B and C treated with current antiviral therapy based on interferon as well as the effect of this therapy on TTV and SENV clearance. The end of treatment response or sustained virological response was achieved in 39 of CHC patients (37 also with biochemical response), while 43 patients were either non-responders or have relapsed. Similarly, 14 of CHB patients had a virological response (8 patients with wild type virus infection had a complete response i.e. normalisation of ALT and HBeAg seroconversion, from 6 patients with precore mutation – 4 also had normalisation of ALT and 2 had only virological response) while 9 were without response.

Among CHC patients with virological response we have confirmed 9 SENV, 1 TTV-N22 and 18 TTV-UTR positive cases. Examining 32 liver biopsy samples in this group we have confirmed 11 positive cases of TTV-UTR, 5 positive cases of SENV but no TTV-N22 positivity. In the group of CHC patients without virological response we have observed 3 SENV, 5 TTV-N22 and 28 TTV-UTR positive cases, with 2 SENV, 1 TTV-N22 and 19 TTV-UTR positive biopsy samples out of 31 performed biopsies. The number of SENV infected patients was statistically higher in the group of responders than in patients without response ($p<0.05$). The rate of TTV infected persons using both PCR detection methods was higher in patients without response than in responders, but not significantly. The only significant difference was in the number of TTV-UTR positive biopsy samples between responder's group and the group without response (11 of 32 vs 19 of 31). No other notable differences between both groups were found (Tab. 3).

In patients with CHB who have had a virological response we have confirmed 2 SENV, 1 TTV-N22 and 6 TTV-UTR positive cases. Liver biopsy samples were also examined in 10 of them and we confirmed only 2 positive cases of TTV-UTR, 1 positive case of SENV but no TTV-N22 positivity. Among CHB patients without virological response there was only 1 SENV and 1 TTV-UTR positive patient, however no positive biopsy sample (only 2 biopsies performed in this group). Differences between both groups were not significant (Tab. 4).

Out of 105 patients who finished a complete course of therapy there were 90 cases (69 CHC and 21 CHB patients) who had a

Tab. 4. Coinfection of TTV and SENV with HBV according to treatment response.

	With response		Without response		Statistical
	serum n=14	biopsy n=10	serum n=9	biopsy n=2	significance
TTV-N22	1 (7.1 %)	0 (0 %)	0 (0 %)	0 (0 %)	NS
TTV-UTR	6 (42.9 %)	2 (20.0 %)	1 (11.1)%	0 (0 %)	NS
SENV	2 (14.3 %)	1 (10.0 %)	1 (11.1 %)	0 (0 %)	NS

Chi-square test was used for statistical evaluation. NS = non-significant.

Tab. 5. Clearance of and novel infection by TTV and SENV after antiviral treatment (evaluated in 90 patients).

	Positive before treatment	After treatment		Negative before treatment	After treatment	
		positive	negative		positive	negative
TTV-N22	5	1	4 (80.0 %)	85	0 (0 %)	85
TTV-UTR	41	7	34 (82.9 %)	49	0 (0 %)	49
SENV	14	1	13 (92.9 %)	76	5 (6.6 %)	71

control serum sample for TTV and SENV evaluation available after the end of the treatment. Comparing sera before and after treatment, in these 90 patients, we found clearance of SENV, TTV-N22 and TTV-UTR, in 13 out of 14 cases, 4 out of 5 cases and 34 out of 41 cases, respectively. The rate of TTV clearance using both PCR detection methods exceeded 80 %, clearance of SENV reached 92.9 %. On the other hand 5 of 76 SENV negative patients before treatment became positive during the follow up, but no newly TTV infected persons were noticed during the same time period (Tab. 5).

The clearence of at least one of both viruses occurred in 43 patients (86.0 % of positive cases before treatment). Six patients out of 7 with TTV and SENV coinfection cleared both TTV and SENV, but only one of them cleared all viruses including HCV and another one including HBV. We have found no association of statistical relevance between clearance of TTV and SENV and clearance of HCV.

Discussion

The presence of TTV has been confirmed in the liver and also other tissues, with liver being probably the essential site of replication (5). Possible impairment of the liver by TTV could not be ruled out in some studies (16, 22). Likewise a higher fibrosis score in TTV positive patients with CHC than in TTV negative was described (11). On the other hand the liver biopsy results described by other authors did not differ between CHC patients with or without TTV infection (9, 23). Similarly available data sugests that there is no relationship of histopathologic findings and SENV infection in CHC patients (14, 24). However different studies correlate the rate of progression of fibrosis and SENV positivity (25). Our data support the statement that both, TTV and SENV infections are not associated with a worsening of histological findings in patients with CHB and CHC.

Coinfection by TTV or SENV among HBV and HCV patients is quite frequent. According to literature data, TTV coinfection of HBV infected patients ranges between 18 to 35 % (6–10). Figures regarding HCV patients are analogous and range from 8 to 42 % (7-9, 11, 26). Furthermore, authors of different studies have found the SEN virus prevalence among CHC patients is about 20 % (14, 24, 27). The rate of TTV and SENV coinfection observed in both groups of chronic hepatitis in our study corresponds with this data.

However, there was no direct impact of TTV upon clinical course and treatment outcome of CHB and CHC observed in most studies (8, 10–13). On the contrary, in another study a significantly lower occurrence of TTV was noticed in the group of patients with chronic hepatitis C who had complete response at the end of interferon treatment compared to patients who had incomplete response or did not respond at all (15). Our results using both PCR methods have shown a slightly but not significantly higher number of TTV infected persons among CHC patients without response than responders. However, we noticed a significant difference in the number of TTV-UTR positive biopsy samples taken from CHC patients between the responder group and the group without response, so we cannot exclude a possible negative influence of TTV infection on antiviral treatment in CHC altogether. On the other hand, there was also described an antagonistic correlation between HCV and TTV showing that TTV coinfection resulted in an increased sustained response rate to interferon treatment (28). In patients with CHB we did not observe any influence of TTV infection on interferon treatment.

Similarly as in case of TTV, most authors did not confirm negative impact of SENV infection on clinical course and efficacy of interferon treatment in HCV infected patients, although SEN virus prevalence among chronic hepatitis C patients was relatively high (14, 24, 27, 29). On the contrary, some studies

have shown decreased treatment response rates in SENV infected chronic hepatitis C patients treated with interferon and ribavirin (17) and also decreased treatment response rates in SENV infected chronic hepatitis B patients treated with lamivudin (18). According to our results SENV infection did not negatively affect the efficacy of antiviral treatment in CHC patients. Conversely, the number of SENV infected CHC patiens was higher in the group of responders to interferon therapy. In CHB patients the SENV coinfection had no influence on the treatment in our study.

The clearance rate of both TTV and SENV after interferon treatment observed in most of studies was rather high. From the TTV positive patients about 45-55 % lost TTV DNA after treatment (11, 13, 26). In the case of SENV infection the virus remained undetectable after therapy in 69-78 % of patients who were initially positive (14, 24, 27). Our results confirmed these observations with documented clearance of over 80 % TTV and over 90 % SENV infections. The more pronounced effect of interferon on TTV and SENV in our study could be explained by using mainly peginterferon compared to standard interferon used mostly in former TTV studies. The clearance of TTV and SENV was not associated with clearance of HBV or HCV.

We conclude, that TTV or SENV infections had no apparent influence on the severity of histological features or the antiviral response in patients with chronic hepatitis B and C. Further studies are needed to evaluate the possible adverse effect of TTV positive findings in liver biopsy samples on the outcome of interferon treatment in chronic hepatitis C patients. Both TTV and SENV were highly sensitive to interferon therapy but the clearance of TTV and SENV was not associated with clearance of HBV or HCV.

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