Infection, Genetics and Evolution 11 (2011) 442–445

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Occult hepatitis C virus infection and associated predictive factors: The Pakistan experience

Muhammad Idrees [a,*], Amreek Lal [b], Fayyaz Ahmed Malik [c], Abrar Hussain [a], Irshad ur Rehman [a], Haji Akbar [a], Sadia Butt [a], Muhammad Ali [a], Liaqat Ali [a], Fayyaz Ahmed Malik [c]

[a] Division of Molecular Virology & Molecular Diagnostics, National Centre of Excellence in Molecular Biology, University of the Punjab, Lahore 53700, Pakistan
[b] Department of Pathology, Sidu Medical College Sidu Sharif Swat, NWFP, Pakistan
[c] Department of Pathology, Independent Medical College, Faisalabad, Pakistan

ARTICLE INFO

Article history:
Received 28 September 2010
Received in revised form 25 November 2010
Accepted 5 December 2010
Available online 22 December 2010

Keywords:
Occult hepatitis C
Etiology
Liver biopsy
Abnormal liver enzymes
Predictive factor

ABSTRACT

The aim of the present study was to determine the presence of HCV RNA in the liver biopsies of patients with abnormal liver tests but without detectable serum HCV RNA and anti-HCV antibodies in sera. Liver biopsies and whole blood of total 31 patients who were negative for anti-HCV antibodies with elevated liver function tests were received at Division of Molecular Diagnostics, University of the Punjab Pakistan from January 2002 to June 2009 for the detection of HCV RNA. HCV RNA status of the subjects was tested by reverse-transcription PCR and quantified using SmartCycler II real-time PCR. HCV genotyping was carried out in HCV RNA positive samples using molecular genotyping method. HCV RNA was found in liver-biopsy specimens from 23 (74.2%) of the total 31 patients negative for anti-HCV antibodies and undetectable serum HCV RNA. HCV RNA of both negative and positive polarity was found in the livers of 8 (25.8%) patients. Genotyping analysis showed that 65% patients were infected with HCV 3a, 17% with 3b, 13% with 1a and 4% patients were found with untypable genotype. In a multivariate logistic regression model, patients having previous history surgeries, male sex and age above 30 years were significantly associated with the presence of occult HCV infection ($p < 0.05$). In conclusion, patients with elevated liver enzymes and negative HCV antibodies and negative serum RNA may have occult HCV infection and its chance increases with previous history of surgeries, in male sex and above 30 years of age.

1. Introduction

The etiology of the liver damage cannot be established in about 10% of patients with elevated levels of liver-function tests (LFTs) after exclusion of all known causes of hepatitis (Carreno, 2006). Occult hepatitis B virus (HBV) has been reported to be the cause of a small proportion of these cryptogenic chronic hepatitis cases (Diodati et al., 1988; Chemin et al., 2001; Castillo et al., 2007). On the other hand occult hepatitis C infection (OHCI) has also been described in patients with abnormal liver enzymes of unknown origin (Carreno, 2006; Halfon et al., 2008; Pardo et al., 2007; Carreno et al., 2008). Subjects with Occult hepatitis C infection have detectable HCV-RNA in the liver or peripheral blood mononuclear cells in the absence of both serum HCV-RNA and anti-HCV antibodies and may display abnormal values of LFTs (DeMarco et al., 2009). OHCI has been reported under two different clinical situations (Carreno et al., 2008). Firstly in anti-HCV negative and serum HCV-RNA-negative patients with abnormal liver function tests and secondly in anti-HCV-positive patients who have no detectable serum HCV-RNA with normal liver enzymes. OHCI may occur in apparently normal individuals and remains a potential risk for the development of liver cirrhosis and hepatocellular carcinoma if not diagnosed well in time.

Since the identification of occult HCV infection in January 2004 by Castillo et al. (2004) and other investigators have found HCV RNA in the PBMC of a high percentage of patients with an occult HCV infection (Carreno, 2006). However, Halfon et al. (2008) had demonstrated that occult HCV infection cannot be found in PBMCs of patients with HCV associated cryptogenic liver diseases therefore are not much reliable in identifying patients with an occult HCV infection. Therefore, the gold standard for the detection of OHCI is the detection of HCV-RNA in hepatocytes as HCV RNA might be present in hepatocytes even in the absence of viral RNA in serum and PBMCs as has already been reported for HBV infecting humans and woodchucks (Halfon et al., 2008; Castillo et al., 2004). No such study on the presence of OHCI is available from South East Asia including Pakistan.

* Corresponding author. Tel.: +92 42 5293141; fax: +92 42 5421316.
E-mail address: idreeskhan@cemb.edu.pk (M. Idrees).

1567-1348/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.meegid.2010.12.004

In the present study we have attempted to determine the etiology of the elevated liver-enzyme levels by investigating occult HCV infection in these patients, utilizing a sensitive nested PCR and real-time PCR assays to detect HCV RNA in liver biopsy specimens from a cohort of patients who were negative for anti-HCV antibodies, negative serum HCV RNA and had abnormal LFTs of unknown origin. Further the role of different demographic, biochemical and virological variables were evaluated for the prediction of OHCI.

2. Methods

2.1. Patients and samples

Our Molecular Diagnostic Laboratory at National Centre of Excellence in Molecular Biology, University of the Punjab is the only ISO Certified facility of the country for the detection and genotyping of hepatitis C virus (HCV). Currently we are receiving on average 4000 sera/plasma/blood samples per month for PCR analysis. In addition to these usual samples, total 31 patient's biopsies along with their 3–5 mL whole blood were also received at this Division from January 2002 to June 2009. All these subjects with unknown etiology had sustained abnormal liver-enzyme levels at least for 2 of the 3 liver enzymes (aspartate aminotransferase, AST; alanine aminotransferase, ALT; alkaline phosphatase, AKP) and bilirubin for 12 months before the liver biopsy was done. All known causes of hepatitis were excluded on the basis of biochemical, analytical, clinical, and epidemiological data such as negative HAV IgM, HBsAg, serum HBV-DNA, anti-HCV antibodies, serum HCV RNA, HEV IgG & IgM, metabolic and genetic disorders, autoimmune diseases, and drug or environmental toxicity. Risk factors including previous history of surgeries, dental surgeries, history of sharing needles, drug abuse, blood transfusions, tattoos, body piercing, and sexual behavior were noted.

2.2. Liver biopsy

The patients underwent a liver biopsy for diagnostic purposes in hospitals by trained doctor. Liver-biopsy specimens were immediately snap frozen in liquid nitrogen, transported to lab and were stored at −70 °C until used for the detection, quantification and genotyping of HCV RNA.

2.3. Biochemistry and serology

Liver function tests such as ALT, AST, ALP and bilirubin levels of all the samples had already been estimated by Auto-analyzer (Hitachi, Tokyo, Japan). All the patients were screened for virological markers using third generation Enzyme-linked Immunosorbant Assay (ELISA) (*DRG Instruments*, Germany) kits for all the samples using the methodology described by the manufactures. The estimated sensitivity and specificity of these tests are 98.9% and 100%, respectively.

2.4. Qualitative and quantitative HCV RNA PCRs

HCV RNA (Sense strand) in serum, and liver was tested by a sensitive reverse transcriptase PCR (RT-PCR) using primers designed from the 5′ noncoding region (5′NCR) of the HCV genome as described earlier (Idrees et al., 2008) with sensitivity of 10 IU/mL. In addition all the samples were also tested by real-time PCR. HCV RNA was quantified in HCV RNA positive samples using SmartCycler II Real-time PCR (Cepheid, USA) using HCV RNA quantitative kits (*Sacace Biotechnologies*, Italy) according to the kit protocol. The lower detection limit of the kit was 250 IU/mL.

2.5. Detection of HCV negative strand

HCV RNA of negative polarity was detected in liver biopsies using nested RT-PCR as well as real-time. Total RNA was isolated from 2 to 5 mg of liver biopsy utilizing the Gentra Total RNA Isolation Kit (Life Technologies USA); and, after precipitation, the RNA pellet was dissolved in 20 μL of diethylpyrocarbonate (DEPC) treated water. Complimentary DNA (cDNA) was synthesized using RT-PCR kit (Invitrogen, USA), with 0.5 μg of total RNA from liver according to the procedure given in the kit protocol. The cDNA was then subjected to PCR amplification as described previously in detail (Idrees et al., 2008). Qualitative real-time PCR was done by SmartCyclerII (Cepheid, USA). All PCR assays were performed according to the recommendations of Kwok and Higuchi (1989). Genotyping of HCV positive samples were done as described previously (Idrees, 2008).

2.6. Statistical analysis

The statistical analyses of the data were performed using SPSS package release 9.0 (SPSS). Comparisons between groups were made by Student's t test (for continuous variables) and by Fisher's exact test (for categorical data). The Odd ratio together with its 95% CI and the corresponding p values were calculated to assess the relative risks/predictors to have the HCV RNA in the patients liver cells using logistic regression. Epidemiological and clinical data of the patients (gender, age, previous history of surgeries, history of sharing needles/syringes, previous blood transfusions, levels of AST, ALT, GGTP, ALP, presence or absence of steatosis and liver fibrosis stage were included in a logistic regression analysis to identify independent factors associated with occult HCV infection. A two-tailed p-value $< .05$ was considered to denote statistical significance.

3. Results

Table 1 shows the main characteristics of the study population. Liver biopsies of total 31 patients with sustained elevated LFTs (at

Table 1
Demographic characteristics and main results of the studied subjects ($N = 31$).

Characteristics	Value
Age (years) (Mean ± SD)	32 ± 11
Gender – male	54.8%
Estimated duration of infection (Mean ± SD)[a]	12.4 ± 10
ALT (Mean ± SD)	84.29 ± 11
AST (Mean ± SD)	69.35 ± 9
Liver fibrosis stage	
F0	64.5%
F1	35.5%
Tissue PCR for HCV positive strand	
Positive	74.2%
Negative	25.8%
Tissue PCR for negative HCV strand	
Positive	25.8%
Negative	74.2%
HCV genotypes	
3a	65.2%
3b	17.4%
1a	13.0%
Indetermined	4.3%
Viral load	
>0.2 MIU/mL	65.2%
≤0.2 MIU/mL	34.8%

SD, standard deviation; ALT, alanine amino transferases; AST, aspartate amino transferases; F1, fibrosis stage 1; F0, fibrosis stage 0.

[a] Duration of occult HCV infection was estimated from risk factors such as previous history of surgeries, dental surgeries, history of sharing needles, drug abuse, blood transfusions, tattoos, body piercing, and sexual behavior were noted.

least ALT & AST) for 1 year and with unknown etiology of liver disease were referred to us for definitive diagnosis. All subjects were negative for all serological and viral markers in their serum samples.

To investigate occult hepatitis infection using strand-specific nested PCR and Real-time-PCR, it was found that 23 (75%) out of the 31 subjects found negative for anti-HCV antibodies and viremia had HCV-RNA in their livers. Patients with or without occult HCV infection had no significance in ALT and AST levels. Patients with occult HCV were significantly older than those lacking HCV RNA in their livers and most likely had a longer duration of elevated LFTs. It was also found that HCV was replicating in the livers of 8 (34%) of the subjects having occult HCV infection using PCR assays for negative HCV RNA strands. In 25% of our subjects the etiology of their elevated liver enzymes cannot be established as they were negative for all serological and viral markers in serum and liver biopsy specimen. The viral load was detected in these positive tissue samples that were not very high (0.1–1.2 MIU/mL). Genotyping results of the HCV RNA positive showed that 15 (65%) patients with occult HCV infection had genotype 3a, 4 (17%) had genotype 3b, 3 (13%) had 1a and in 1 (4%) genotype was not determined. The histological diagnosis of the 31 liver-biopsy specimens from the patients with abnormal LFTs ranged from no change to minimal nonspecific changes (F0–F1). Of the 23 patients with occult HCV infection, the number of cases with fibrosis level F1 (according to METAVIR) was higher in patients with occult HCV infection (10/12; 83.3%) than in patients without intrahepatic HCV RNA (13/19; 68.4%).

Table 2 shows the results of multivariate analysis. Each variable was simultaneously adjusted to the others. The independent risk factors that remained significantly associated with the presence of intrahepatic HCV RNA (occult HCV infection) were history of previous surgeries/dental procedures (OR, 1.3; 95% CI, 0.5–4.2, $p < 0.05$), age >30 years (OR, 1.28; 95% CI, 0.5–3.8, $p < 0.05$) and male sex (OR, 1.28; 95% CI, 0.5–3.8, $p < 0.05$).

Table 2
Results of multivariate logistic regression analysis of variables associated with prediction of occult HCV infection.

Variable	No. of HCV RNA positive participants/total no. of participants	Adjusted OR	95% CI
Age (years)			
>30	14/17	1.28	0.5–3.8
≤30	9/14		
Sex			
Male	14/17	1.28	0.5–3.8
Female	9/14		
History of previous surgeries/dental procedure			
Yes	10/11	1.3	0.6–4.2
No	13/20		
History of sharing needles			
Yes	22/25	0.2	0.1–0.6
No	1/6		
ALT levels			
Yes	16/16	0.5	0.2–0.9
No	7/15		
AST levels			
Yes	15/15	0.5	0.3–1.0
No	8/16		
Liver fibrosis stage			
F1	10/12	0.8	0.6–1.3
F0	13/19		
Steatosis			
Present	15/16	0.4	0.2–0.8
Absent	6/15		

OR, odd ratio; CI, confidence interval; ALT, alanine amino transferases; AST, aspartate amino transferases; F1, fibrosis stage 1; F0, fibrosis stage 0.

4. Discussion

Abnormal results on liver-function tests may have various causes, and the etiology of the liver damage can be established in most cases. However, there are patients in whom the etiology of long-standing abnormal results on liver-function tests is of unknown origin and for these patients occult hepatitis C infection should be considered. The existence of occult HCV infection has been reported by various researchers from different parts of the world (Carreno, 2006; Castillo et al., 2007; Pardo et al., 2007; Carreno et al., 2008) excluding South East Asia.

In the current study we were able to establish occult HCV infection in patients with chronic liver disease. To the best of our knowledge, this is the first study that has analyzed the presence of HCV RNA in liver samples from patients with chronic hepatitis in the absence of anti-HCV antibodies and serum HCV RNA, from this region of the world. For all of our patients the etiology of their elevated liver enzymes could not be established as they were negative for all serological viral markers in serum. It was found in the current study that 75% of the patients with unknown etiology had HCV-RNA in their livers. The results of the current study are in consistence with the results reported by Stapleton et al. (2004) who had reported anti-HCV negative HCV infection in patients with cryptogenic liver disease and persistently abnormal results of liver tests.

Absence of serum HCV RNA in patients with occult HCV infection may have no HCV replication; however, we were able to detect HCV RNA of negative polarity in the liver biopsy specimens of 34% subjects who had occult HCV infection, suggesting an ongoing viral replication in patients liver cells. It has already been reported that the patients with occult HCV infection even do not have detectable circulating RNA could still be potentially infectious (Castillo et al., 2005; Carreno, 2006). Here the question arise that why the HCV RNA and anti-HCV antibodies is not detectable in the sera of patients in whom the HCV even replicate in their hepatocytes? The possible reasons for the absence of anti-HCV antibodies and HCV RNA in serum may be due mutations affecting the virus translation capacity, its encapsidation capacity, or the formation and release of virions into blood circulation causing extremely low viremia and low production of anti-HCV antibodies that may be below the sensitivity limits of the currently available HCV RNA detection and anti-HCV ELISAs methods (Carreno, 2006). Further this detection of negative HCV RNA strands in the livers of about on third of the subjects with occult HCV infection is very imperative finding as it translates viral replication in the livers, which involves synthesis of a negative RNA intermediary.

Unfortunately, we were unable to test HCV RNA in the PBMCs of the subjects. However, it has been reported in several previous studies that patients who have abnormal liver function tests and are anti-HCV and serum HCV-RNA negative may have an occult HCV infection in liver but not necessarily in PBMC (Castillo et al., 2004; Halfon et al., 2008). Though the liver biopsies appear useful for the diagnosis of occult HCV infection, however, they are nonetheless delicate surgeries in low-middle income countries. Recently Castillo et al. (2010) has described that the use of patient's serum after ultracentrifugation in non-invasive assays may be useful for the diagnosis of occult HCV infection and should be further investigated. The findings of the current study further suggest that occult HCV infection shall play a role in the pathogenic process in a number of patients with abnormal LFTs. However, in general, occult HCV infection seems to induce a mild liver disease, because 56.5% of our patients with occult HCV infection had no changes and 43.5% had minimal nonspecific changes. This observation of the current study is consistence with the findings of Pardo et al. (2007) who had demonstrated that occult HCV infection is a milder disease with liver damage than damage caused

by chronic HCV infection. Most fascinatingly, liver steatosis was found high in patients with intrahepatic HCV RNA than those with unknown etiology. It has already been reported that steatosis is a common liver feature in chronic hepatitis C and HCV genotype 3 is directly associated with this fat accumulation, supporting our finding as our 65% patients with occult HCV were infected with the HCV 3a genotype that is associated with fat accumulation in liver cells. Recently a changing pattern of HCV genotypes prevalence was observed in Pakistan overtime, with an increase in the relative proportion of genotype 3a (Butt et al., 2010) that may have the capability to increase fat accumulation in HCV infected patients overtime in this region of the world resulting in large number of patients with steatosis.

Most importantly, the results of our study further demonstrated that occult HCV infection was associated with previous history of surgeries (general & dental), an age >30 years old, and a male sex. Importantly our results confirm that surgeries were the most important risk factor associated with occult HCV infection. Contrary to our expectation no association was found between occult HCV infection and history of injections with used needles, high LFTs levels and liver fibrosis stage.

Our study had few limitations. First, the information about risk factors was collected from a questionnaire that may vary in completeness and be more vulnerable to bias. Second, the sample size is very small as few patients were referred to us for definitive diagnosis even many patients are seen in clinical practice with abnormal liver enzymes of no known etiology as most of the patients are unwilling to undergo liver biopsy due to painful liver biopsy procedure and cost resulting in depriving a significant proportion of patients from definitive diagnosis. Third, the patients PBMC were not tested for HCV RNA. Fourth the levels of Zinc, iron and were not tested in liver biopsies as the biopsies were only sufficient for nucleic acid extraction only. Even in the presence of these limitations still we believe that this is a very important study because it addresses the issue of occult hepatitis C infection and associated factors for the first time in this region. We think that the findings of the current study may help determine the cause of many cases of previously unexplained abnormal liver enzyme elevations if confirm by other researchers.

5. Conclusion

In conclusion, patients with negative anti-HCV antibodies and negative serum HCV-RNA may have HCV RNA in their livers. The current study further adds important information regarding variables such as age, sex and history of previous surgeries/dental procedures that are independently related with the presence of occult HCV infection.

Ethical approval

Ethics Committee of National Centre of Excellence in Molecular Biology, University of the Punjab approved the study.

Conflict of interest

None of the authors who participated in this study have commercial or other associations that might pose conflict of interest.

Acknowledgements

We thank all the Gastroenterologists, Hepatologists, clinicians and subjects for their cooperation in the current study. This project was partially supported by Ministry of Science & Technology, Government of Pakistan.

References

Butt, S., Idrees, M., Akba, H., Rehman, I.U., Awan, Z., Afzal, S., Hussain, A., Shahid, M., Manzoor, S., Rafique, S., 2010. The changing epidemiology pattern and frequency distribution of hepatitis C virus in Pakistan. Inf. Gen. Evol. 10, 595–600.

Carreno, V., Bartolome, J., Castillo, I., Quiroga, J.A., 2008. Occult hepatitis B virus and hepatitis C virus infection. Rev. Med. Virol. 18 (3), 139–157.

Castillo, I., Bartolomé, J., Quiroga, J.A., Barril, G., Carreño, V., 2010. Diagnosis of occult hepatitis C without the need for a liver biopsy. J. Med. Virol. 82 (9), 1554–1559.

Carreno, V., 2006. Occult hepatitis C virus infection: a new form of hepatitis C. World J. Gastroenterol. 12, 6922–6925.

Castillo, I., Pardo, M., Bartolome, J., Ortiz-Movilla, N., Rodriguez-Inigo, E., deLucas, S., Salas, C., Jimenez-Heffernan, J.A., Perez-Mota, A., Graus, J., Lopez-Alcorocho, J.M., Carreno, V., 2004. Occult hepatitis C virus infection in patients in whom the etiology of persistently abnormal results of liver-function tests is unknown. J. Infect. Dis. 189, 7–14.

Castillo, I., Rodriguez-Inigo, E., Bartolome, J., deLucas, S., Ortiz-Movilla, N., Lopez-Alcorocho, J.M., Pardo, M., Carreno, V., 2005. Hepatitis C virus replicates in peripheral blood mononuclear cells of patients with occult hepatitis C virus infection. Gut 54, 682–685.

Castillo, I., Rodriguez-Inigo, E., Lopez-Alcorocho, J.M., Bartolome, J., Pardo, M., Carreno, V., 2007. Comparative study on the clinical and virological characteristics among patients with single occult hepatitis B virus (HBV), single occult hepatitis C virus (HCV) and occult HBV and HCV dual infection. J. Med. Virol. 79, 236–241.

Chemin, I., Zoulim, F., Merle, P., Arkhis, A., Chevallier, M., Kay, A., Cova, L., Chevallier, P., Mandrand, B., Trepo, C., 2001. High incidence of hepatitis B infections among chronic hepatitis cases of unknown aetiology. J. Hepatol. 34, 447–454.

DeMarco, L., Gillio-Tos, A., Fiano, V., Ronco, G., Krogh, V., Palli, D., Panico, S., Tumino, R., Vineis, P., Merletti, F., Richiardi, L., Sacerdote, C., 2009. Occult HCV infection: an unexpected finding in a population unselected for hepatitis disease. PLoS ONE 4 (12) doi:10.1371/journal.pone.0008128.

Diodati, G, Pontisso, P., Bonetti, P., Stenico, D., Noventa, F., Alberti, A., Realdi, G., 1988. Cryptogenic chronic liver disease and serum or liver hepatitis B virus markers. Their possible correlations and etiologic significance. Digestion 39, 251–256.

Halfon, P., Bourlière, M., Ouzan, D., Sène, D., Saadoun, D., Khiri, H., Pénaranda, G., Martineau, A., Oulès, V., Cacoub, P., 2008. Occult HCV infection revisited with ultra-sensitive real-time PCR assay. J. Clin. Microbiol. 46 (6), 2106–2108 doi:10.1128/JCM.00345-08.

Idrees, M., 2008. Development of a new HCV genotyping assay and its suitability for the detection of common genotypes in Hepatitis C patients in Pakistan. J. Virol. Methods 50, 50–56.

Idrees, M., Lal, A., Naseem, M., Khalid, M., 2008. High prevalence of hepatitis C virus in the largest province of Pakistan. J. Dig. Dis. 9, 95–103.

Kwok, S., Higuchi, R., 1989. Avoiding false positives with PCR. Nature 339, 237–238.

Pardo, M., Lopez-Alcorocho, J.M., Rodriguez-Inigo, E., Castillo, I., Carreno, V., 2007. Comparative study between occult hepatitis C virus infection and chronic hepatitis C. J. Viral. Hepat. 14, 36–40.

Stapleton, J.T., Schmidt, W.N., Katz, L., 2004. Seronegative hepatitis C virus infection, not just RNA detection. J. Infect. Dis. 190, 651–652.