Vol. 199, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1994 Pages 37-40

PARTIAL SEQUENCE ANALYSIS OF THE HEPATITIS C VIRAL GENOME IN SINGAPORE PATIENTS

Ann Siew-Gek Lee[1], Keng-Yeen Ng[2], Melissa Kie-Lin Chee[1], Anantharaman Vathsala[3], Hui-Lin Choong[3], Han-Seong Ng[2], and Chong-Jin Oon[1]

[1]Department of Clinical Research, [2]Department of Medicine 2, and [3]Department of Renal Medicine, Singapore General Hospital, Singapore

Received January 10, 1994

SUMMARY: The distribution of hepatitis C viral (HCV) genotypes in Singapore has not been previously determined. We studied the sera of 40 Singapore patients which were PCR-positive for HCV. The HCV genotypes were determined by direct sequencing of amplified sequences of the 5' non-coding region, after reverse transcription. Of the 40 samples, 35/40 (87.5%) were of HCV type 1, 2/40 (5.0%) were of type 2 and 3/40 (7.5%) were of type 3. The most common HCV genotype in this study was the type 1 genotype. Our results confirm the wide geographical distribution of HCV genotypes.

The hepatitis C virus (HCV) is a positive-stranded RNA virus with a large open reading frame that encodes a polyprotein of 3011 aminoacids. It is the major causative agent of post-transfusion non-A non-B hepatitis (1).

The HCV genome consists of the 5' non-coding region, the open reading frame encoding the core protein (C), the glycoproteins envelope 1 (E1) and the nonstructural protein 1/envelope 2 (NS1/E2), the nonstructural proteins 2 to 5 (NS2 to NS5) and the 3' untranslated region. The 5' non-coding region is the most conserved region, while the NS5 region is highly variable (2).

There have been several different terminology used to distinguish the different HCV genotypes. Recently, a new classification has been proposed with sequences classified into four major types (3,4,5). The type 1 group has been subdivided into type 1a (prototype HCV-1) and type 1b (HCV-J, HCV-BK, K1), the type 2 group comprises the type 2a (K2a and HC-J6) and type 2b (K2b and HC-J8) while the type 3 group is subdivided into type 3a (E-b1, HCV-Ta) and 3b (HCV-Tb). HCV type 4 is widespread in Africa but is absent or rare in Europe and the Far East (3, 5).

0006-291X/94 $5.00

Although there have been numerous reports on the distribution of HCV genotypes in the United States, Japan and Europe, little is known about the HCV nucleotide sequence present in South East Asia. We report here the HCV genotypes in 40 Singapore patients.

MATERIALS AND METHODS

Serum samples
Serum samples were obtained from Singapore patients tested positive for anti-HCV antibodies using a second-generation HCV enzyme immunoassy (EIA, Abbott, Germany).
HCV RNA isolation
HCV RNA was extracted from 0.2 ml of serum using the guanidinium thiocyanate/phenol-chloroform method (6), and dissolved in 20µl of DEPC treated water.
Synthetic oligonucleotides
Oligonucleotide primers for cDNA synthesis and PCR were synthesised with a 7500 Milligen/Biosearch DNA synthesiser (Millipore, USA). The primers 939 and 209 for first round PCR and primers 940 and 211 for second round PCR are described elsewhere (4).
Reverse transcription and nested polymerase chain reaction (PCR)
Reverse transcription was carried out at 42°C for 60min using 1.5µM of the antisense primer 209, 10units of avian myeloblastosis virus reverse transcriptase (Promega), 40 units of RNasin (Promega, USA), 600µM each of dATP, dCTP, dGTP, and dTTP and 3µl of purified RNA in a 20µl reaction volume.
Nested PCR was as described previously (4). The reaction mixture contained 0.5µM of each of the outer nested primers, 939 and 209, 1 unit of *Taq* DNA polymerase, 1.5mM of magnesium chloride, 50µM each of dATP, dCTP, dGTP and dTTP. The first PCR was carried out for 25 cycles of 94°C for 1min, 50°C for 1min and 68°C for 1min. This was followed by a final 7min elongation at 72°C. The second round PCR was done using 4% of the first PCR product under the same conditions as before, but using the primers 940 and 211. The amplified products were electrophoresed on a 2% agarose gel stained with ethidium bromide.
Precautions were taken to prevent the contamination of samples: aerosol resistant tips were used, the areas for pre-PCR were physically separated from the post -PCR areas, and negative controls (water and serum) were used.
DNA sequencing
PCR products were purified using the DEAE paper method. Primers used for PCR were also used as sequencing primers to sequence both strands. The primers were end-labelled with ^{33}[P](Dupont, USA) using T4 polynucleotide kinase (GIBCO/BRL, USA). Dideoxy chain termination reactions were performed with the DNA cycle sequencing kit (GIBCO/BRL, USA).

RESULTS

The HCV genome was extracted from the sera of 40 Singapore patients, reverse transcribed and amplified by nested-PCR using primers to the 5' non-coding region. Six of these patients were on haemodialysis and 15 were post renal transplant patients previously on haemodialysis.

The HCV genotypes of the 40 patients studied were determined by comparison of the sequences obtained with known nucleotide sequences of HCV genotypes (7-12).

Thirty-five of 40 samples (87.5%) were of the type 1 genotype, 2/40 (5.0%) was of the type 2 genotype and 3/40 (7.5%) were of the type 3 genotype. The most prevalent HCV genotype

observed was the type 1. HCV while type 4 was not observed in any of the Singapore patients.

A single base insertion of adenosine between nucleotides -138 and -137 was observed in one patient. Insertions at this position have also been observed in other type 1 isolates (3, 5).

All 6 haemodialysis patients and 15 renal transplant patients were infected with the type 1 genotype.

DISCUSSION

The hepatitis C virus may be genotyped by using type-specific primers for PCR (13, 14), by restriction fragment length polymorphism (15-16), by reverse-hybridisation of amplified sequences with oligonucleotides specific for the different genotypes (3) or by DNA sequencing of the amplified region.By using DNA sequencing, one is able to detect small differences in the nucleotide sequence which may be undetectable by the other methods mentioned.

When using the polymerase chain reaction to amplify the viral sequence, it is important that the primer sequence is selected in a highly conserved region. Mismatches between the primer and the viral sequence would prevent amplification. The 5' non-coding region was selected for analysis since it has been reported to be highly conserved (17). However, within this region, there are sequence variations which allow discrimination between HCV types (3, 4, 16, 17, 18, 19).

In Singapore, the HCV seroprevalence in patients with liver disease (3.6%-14.9%) and in the random population (0.08%) is lower compared to figures reported in developed countries (20, unpublished observations).

The most common genotype in Singapore is type 1 (87.5% of our cases). The HCV type 1b genotype is the most prevalent genotype in Japan, accounting for approximately 70% of cases (14, 21). In Japan, the next most prevalent type is type 2 (K2) comprising 31.6% (21) and 24.2% (14) of cases. Although blood transfusion has been strongly associated with HCV transmission, many of our patients have had a history of dialysis of transfusion locally and had not been treated overseas. This would suggest that this HCV type 1 is of a local origin and not due to overseas exposure.

In Thailand, 4 of 10 samples studied were of type 3 (11). This is in contrast to the low incidence of type 3 observed in Singapore (7.5%). Since the distribution of HCV genotype in other countries in South East Asia has not been characterised, the geographical distribution of these subtypes and their disease association remain unknown.

Furthermore, recent studies have also shown that better responses to alpha-interferon therapy is associated with HCV genotype 2 than other types, hence emphasising the importance of HCV genotyping.

ACKNOWLEDGMENTS: This study was supported by grants from the Singapore Totalisator Board, Shaw Foundation and the Department of Clinical Research, Ministry of Health, Singapore.

REFERENCES

1. Choo, Q.-L., Kuo, G., Weiner, A.J., Overby, L.R., Bradley, D.W., and Houghton, M. (1989) Science 244, 359-362.
2. Choo, Q.-L., Kuo, G., Weiner, A., Wang, K.-S., Overby, L., Bradley, D., and Houghton, M. (1992) Sem. Liver Disease 12, 279-288.
3. Stuyver, L., Rossau, R., Wyseur, A., Duhanel, M., Vanderborght, B., Van Heuverswyn, H., and Maertens, G. (1993) J. Gen. Virol. 74, 1093-1102.
4. Chan, S.-W., McOmish, F., Holmes, E.C., Dow, B., Peutherer, J.F., Follett, E., Yap, P.L. and Simmonds, P. (1992) J. Gen. Virol. 73, 1131-1141.
5. Simmonds, P., McOmish, F., Yao, P.L., Chan, S.-W., Lin, C.K., Dusheiko, G., Saeed, A.A., and Holmes, E.C. (1993) J. Gen. Virol. 74, 661-668.
6. Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156-159.
7. Choo, Q.-L., Richman K.H., Han, J.H., Berger K., Lee, C., Dong, C., Gallegos, C., Coit, D., Medina-Selby, A., Barr, P.J., Weiner, A.J., Bradley, D.W., Kuo, G., and Houghton, M. (1991) Proc. Natl. Acad. Sci. USA 88, 2451-2455.
8. Kato, N., Hijikata, M., Ootsuyama, Y., Nakagawa, M., Ohkoshi, S., Sugimura, T., and Shimotohnok (1990) Proc. Natl. Acad. Sci. USA 87,9524-9528.
9. Okamoto, H., Machida, A., Miyakawa, Y., and Mayumi, M. (1991) J. Gen. Virol. 72, 2697-2704.
10. Okamoto, H., Kurai, K., Okada, S.I., Yamamoto, K., Lizuka, H., Tanaka, T., Fukuda, S., Tsuda, F., and Mishiro, S. (1992) Virology 188, 331-341.
11. Mori, S., Kato, N., Yagyu, A., Tanaka, T., Ikeda, Y., Petchclai, B., Chiewsilp, P., Kurimura, T., and Shimotohno, K. (1992) Biochem. Biophys. Res. Commun. 183, 334-342.
12. Cha, T.A., Beall, E., Irvine, B., Kolberg, J., Chien, D., Kuo, G., and Urdea, M.S. (1992) Proc. Natl. Acad. Sci. USA 89, 7144-7148.
13. Okamoto, H., Sugiyama, Y., Okada, S., Kurai, K., Akahane, Y., Sugai, Y., Tanaka, T., Tsuda, F., Miyakawa, Y. and Mayumi, M. (1992) J. Gen. Virol. 73, 673-679.
14. Kato, N., Ootsuyama, Y., Ohkoshi, S., Nakazawa, T., Mori, S., Hijikata, M. and Shimotohno, K. (1991) Biochem. Biophys. Res. Commun. 181, 279-285.
15. Nakao, T., Enomoto, N., Takada, N., Takada, A. and Date, T. (1991) J. Gen. Virol. 72, 2105-2112.
16. McOmish, F., Chan, S.-W., Dow, B.C., Gillon, J., Frame, W.D., Crawford, R.J., Yap, P.-L., Follett, E.A.C. and Simmonds, P. (1993) Transfusion 33, 7-13.
17. Bukh, J., Purcell, R.H., and Miller, R.H. (1992) Proc. Natl. Acad. Sci. USA 89, 4942-4946.
18. Burk, J., Purcell, R.H., and Miller, R.H. (1993) Proc. Natl. Acad. Sci. USA 90, 8234-8238.
19. Simmonds, P., Holmes, E.C., Cha, T.-A., Chan, S.-W., McOmish, F., Irvine, B., Beall, E., Yap, P.L., Kolberg, J., and Urdea, M.S. (1993) J. Gen. Virol. 74, 2391-2399.
20. Chia, S.C., Fock, K.M., Oon, C.J., and Chua, K.L. (1991) Annals Acad. Med. Singapore 20, 728-731.
21. Enomoto, N., Takada, A., Nakao, T., and Date, T. (1990) Biochem. Biophys. Res. Commun. 170, 1021-1025.