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High homologious nucleotide to GBV-C was amplified from DNA of MT2 and HeLa cells and PBMC of human and chimpanzee[1]

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KEY WORDS GB hepatitis agents; monkey diseases; nucleic acid sequence homology; fluorescence microscopy; DNA; human; monocytes; polymerase chain reaction

ABSTRACT

AIM: To determine whether the nucleotide sequences homologous to GBV-C genome exist in the DNA from cell lines of human origin and from peripheral blood mononuclear cells (PBMC) of human and chimpanzee. **METHODS**: DNA of MT2 cell, HeLa cell, and PBMC from six human and one chimpanzee were prepared by using PCR Temperate Preparation Kit. All of the DNA preparations were digested with DNase-free RNase and then were extracted by phenol-chloroform method. By using these DNA as templates, direct nested-PCR (dPCR) respectively amplified with GBV-C 5′-NCR and NS3 primers were carried out. Nucleotide sequences of the dPCR products were analyzed and positions in chromosomes of the amplification fragments were detected by using primed *in situ* (PRINS) sequence-specific labeling with fluorescein. **RESULTS**: DNA fragments amplified with GBV-C 5′- NCR primers were obtained from the DNA of MT2 and HeLa cells and the DNA in 4 of 6 human PBMC samples. DNA fragments amplified with GBV-C NS3 primers were obtained from MT2 and HeLa cells and the DNA in 5 of 6 human PBMC samples and one chimpanzee PBMC sample. The homology of the nucleotide sequences from these amplification products compared with the reported GBV-C genome sequence was from 73.80 % to 79.15 %. Fluorescence staining spots by using PRINS were shown in the PBMC and their chromosomes with positive dPCR results. **CONCLUSION**: The nucleotide sequences with high homology to GBV-C genome at the 5′-NCR and/or NS3 region exist in the DNA of MT2 and HeLa cells and the PBMC DNA of human and chimpanzee. These sequences locate in the chromosomes of PBMC with positive dPCR results.

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INTRODUCTION

GB virus C (GBV-C) and hepatitis G virus (HGV) were recently identified as a novel member of *Flaviviridae* family associated with human hepatitis. The two viral agents are generally considered as two different isolates of the same virus because the homology of their nucleotide sequences and putative amino acid sequences are as high as 85 % and 100 % respectively[1,2]. GBV-C/HGV is a single strand RNA virus but the viral particle has not been discovered yet. GBV-C/HGV genomes do not contain the core-like sequence[3] and the capsid protein may be defective or missing[2]. However, approximate 15 % of GBV-C/HGV infection rate in non A-E hepatitis patients strongly indicate that the virus seems not to be a defective virus[4,5]. HGV sequence in chimpanzees infected from human plasma containing the virus was found to differ from the prototype of the viral genome by 5 nt (2 aa) and 27 nt (2 aa) 77 weeks after inoculation[6]. Sequence comparisons revealed that GBV-C found in chimpanzees was more diverse than variants of GBV-C/HGV found in humans[7]. Therefore, the controversy is presented whether GBV-C/HGV is a true virus[8].

For understanding the origin of the viral genomic fragments, DNA were extracted from MT2 and HeLa cells and from PBMC of human and chimpanzee, and then directly amplified by PCR with no reverse transcription (dPCR) in the present study. The primers used in the PCR were derived from GBV-C 5′-NCR and NS3 region respectively. The nucleotide and amino acid sequences of dPCR products were compared with the report-

ed GBV-C sequence[1]. Primed *in situ* (PRINS) sequence-specific labeling with fluorescein was used to determine the positions of these nucleotide sequences in the dPCR positive PBMC and their chromosomes.

MATERIALS AND METHODS

Source of PBMC PBMC and serum samples were isolated from the peripheral venous blood of three Chinese (No: C1, C2, C3) and three Germans (No: G1, G2, G3). All of the 6 individuals had no hepatitis history and the level of ALT was normal. No HBV DNA or HCV RNA was detectable in the sera from 6 cases by PCR or RT-PCR. A PBMC sample from one healthy chimpanzee with no HBV and HCV infection was offered by University of Munich, Germany.

DNA preparation DNA of PBMC, MT2 cell, and HeLa cells were prepared by PCR Temperate Preparation Kit (Boehringer Mannheim) according to the manufacturer's instruction. Plasmid puc18 DNA was isolated from *E coli* JM109 by Sambrook's method[9]. All of the DNA preparations were digested with DNase-free RNase (Boehringer Mannheim) at 37 ℃ for 2 h. Final concentrations of the RNase and DNA were 10 mg/L and 100 mg/L respectively. DNA in these digestion solutions were extracted by phenol ($pH \geqslant 7.8$, Merck)-chloroform method[9].

Reverse transcription (RT) Total RNAs from the PBMC samples, one GBV-C RNA positive serum from a non A-E hepatitis patient, and one GBV-C RNA negative serum were prepared using High Pure RNA Isolation Kit (Boehringer Mannheim) according to the manufacturer's instruction. Total RNA preparation 10 μL was added into 10 μL of RT master mixture containing dNTP 0.2 mmol/L, hexanucleotide mix 50 nmol/L, 20 U RNAse inhibitor, 20 U M-MuLV-reverse transcriptase, and 4 μL 5 × RT buffer (Boehringer Mannheim) and was incubated at 37 ℃ for 45 min and then at 95 ℃ for 5 min.

dPCR DNA prepared from the cells and the PBMC were directly amplified by nested PCR (dPCR) with the primers derived from GBV-C 5′-NCR and NS3 region[10,11], and the expected sizes of the two amplification products were 192 bp and 287 bp respectively. 5′-NCR external primers: 5′-ATGACAGGGTTGGTAGGTCGTAAATC-3′ (sense), 5′-CCCCACTGGTCCTTGTCAACTCGCCG-3′ (antisense). 5′-NCR internal primers: 5′-TGGTAGCCACTATAGGTGGGTCTTAA-3′ (sense), 5′-ACATTGAAGGGCGACGTGGACCGTAC-3′ antisense. NS3 region external primers: 5′-GCTCGCCTATGACTCAGCAT-3′ (sense), 5′-GTCACCTCAACGACCTCCTC-3′ (antisense). NS3 region internal primers: 5′-ATCCATAATTGAGACAAAGCTGGA-3′ (sense), 5′-CCACCAACCCACAGTCGGTG-3′ (antisense). DNA 100 ng was added into dPCR master mixture containing dNTP 0.2 mmol/L, external primers 20 pmol/L, 0.01 % (w/v) gelatine, 3 U Taq polymerase [Boehringer Mannheim] and 10 μL 10 × PCR buffer with $MgCl_2$ 25 mmol/L (pH 9.1) for the first round PCR. The product 2 μL from the first round PCR was used as temperate in the second round PCR. dPCR master mixture for the second round PCR contained 30 pmol/L internal primers and the rest agents was the same as in the first round of PCR. Total volume per reaction in the two rounds of PCR was 100 μL. GeneAmp PCR System 2400 (Perkin Elmer) was used as thermal cycler. The reaction parameters were described as the following: 94 ℃ 3 min (×1); 94 ℃ 30 s, 60 ℃ 30 s, (×10); 94 ℃ 30 s, 60 ℃ 30 s (×20, 5 s addition for each of the following cycles); 72 ℃ 7 min (×1). In the dPCR, three controls were co-amplified with the other DNA samples: (1) positive control (cDNA from GBV-C RNA), (2) negative control (puc18 DNA), (3) blank control.

RT-nested PCR RT product 10 μL were used as temperate in the first round of RT-nested PCR. Except of the temperate, the other reagents, concentration of reagents, reaction volumes, and amplification protocol were the same as in dPCR. One GBV-C RNA positive control serum, one GBV-C RNA negative control serum mentioned in RT, and one blank control were set up in the RT-nested PCR.

Agarose gel electrophoresis The products of dPCR and RT-nested PCR were detected by 2 % ethidium bromide stained agarose gel electrophoresis.

Nucleotide sequence analysis DNA fragments from the dPCR products were cloned into PCR2.1 plasmid using T-A Cloning Kit (Invitrogen) according to the the manufacturer's instruction. The recombinant plasmid was amplified *in E coli and then recovered*[9]. The inserted fragments were sequenced by MWG-BIOTECH, Germany.

PRINS Primed *in situ* (PRINS) sequence-specific labeling with fluorescein was used to determine the positions of the amplified sequences in the dPCR positive PBMC and their chromosomes. This assay was performed by the Institute of Human Genetics of Medical U-

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niversity of Lübeck, Germany.

RESULTS

RT-nested PCR No DNA fragments were shown in the RT-nested PCR products of 7 PBMC samples, negative control and blank. The RT-nested PCR products of positive control showed two target DNA fragments with expected sizes. One of the two fragments was amplified from GBV-C 5'-NCR and the other from GBV-C NS3 region.

dPCR No DNA fragments were found to exist in the dPCR products with 5'-NCR primers or NS3 region primers in puc18 DNA sample and blank. A single DNA fragment with expected size (about 192 bp) was shown in each of the dPCR products amplified with 5'-NCR primers from DNA samples of MT2 and HeLa cells, 2 of the 3 Germans (G1 and G2) and 2 of the 3 Chinese (C1 and C2). A single DNA fragment with expected size (about 287 bp) was shown in each of the dPCR products amplified with NS3 region primers from DNA samples of MT2 and HeLa cells, all of the 3 Germans (G1, G2 and G3), 2 of the 3 Chinese (C1 and C2) and chimpanzee. The results of dPCR are displayed in Fig 1.

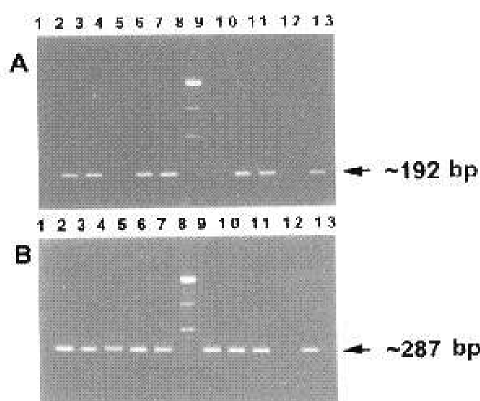

Fig 1. Amplification results by dPCR with GBV-C 5'-NCR primers (A) and GBV-C NS3 region primers (B) of the DNA from MT2 and HeLa cells and the PBMCs of human and chimpanzee. Lane 1, 12, and 13: negative control [puc18 DNA], positive control (cDNA from GBV-C RNA), and blank respectively; Lane 2 – 6: MT2 DNA, HeLa DNA and PBMC DNA from chimpanzee, G1, G2 respectively; Lane 7: marker; Lane 8 – 11: PBMC DNA from G3, C1, C2, and C3 respectively.

Nucleotide sequence analysis The homology of the nucleotide sequences from dPCR products amplified with 5'-NCR primers from the DNA samples of HeLa, G1, G2, C1, and C2 compared to the reported GBV-C sequence[1] are 76.19 %, 77.14 %, 76.19 %, 76.19 %, and 74.29 % respectively. The homology between the 5 sequences range from 90.54 % – 99.05 %. The homology of the nucleotide sequences from dPCR products amplified with NS3 region primers from the DNA samples of HeLa, G1, G2, C1, C2, and chimpanzee compared to the reported GBV-C sequence[1] are 73.80 %, 79.06 %, 78.60 %, 79.15 %, 78.60 %, and 74.76 % respectively. The homology between the 6 sequences range from 79.15 % – 99.45 %. The homology comparison mentioned above did not contain the primer sequences. These nucleotide sequences are shown in Fig 2 and 3.

PRINS Positive fluorescein staining spots were found to exist in each of the PBMC samples and their chromosomes with positive dPCR results (shown in Fig 4), indicating positions of the amplified sequences in the PBMC and chromosomes.

DISCUSSION

GBV C is a single strand RNA virus with one open reading frame related to non A-E human hepatitis. Small core region in GBV-C genome indicates that the virus has no sequence responsible for encoding a core protein. This differs from hepatitis C virus (HCV) which belongs to the same family of GBV-C[5]. Furthermore, the deduced amino acid sequences upstream of the putative E1 gene from different GBV-C RNA isolates varied in length and composition, indicating the capsid protein may be defective or absent[2,12]. It is possibile that the capsid protein is provided by another virus[13]. However, 10 % – 15 % of GBV-C infection rates in non A-E hepatitis patients suggested that it is not a defective virus. It is considered that GBV-C might be a subvirus but this is contradictory to the genomic structure of the virus. Since the biological characteristics of GBV-C are peculiar, GBV-C is suspected not to be a virus.

Using dPCR and the two sets of primers derived from GBV-C genome respectively produced the DNA fragments with expected sizes amplified from DNA temperates of MT2 cell and Hela cell and the PBMC of human and chimpanzee. These dPCR assays at least repeated for three times and the same results were obtained. The dPCR products amplified with GBV-C 5'-NCR primers and amplified with GBV-C NS3 region primers

```
(1) 1     TGGTAGCCACTATAGGTGGTCTTAAGGGGAGGCTACGGTCCCTCTTGCG
(2) 1     ..........................A.A...T..A.A.T.......T.
(3) 1     ..........................AC....T..A.A.T.......T.
(4) 1     ..........................A.A...T..A.A.T.......T.
(5) 1     ..........................AC....T..A.A.T.......T.
(6) 1     ..........................A.A...T..A.A.T.......T.

(1) 51    CATATGGAGGAAAAGCGCACGGTCCACAGGTGTTGGTCCTACCGGTGT A
(2) 51    .C.GC.AC.AG.CC......................C...........G.
(3) 51    .C.GC..C.AG.CC......................C...........G.
(4) 51    .C.GC..C.AG.CC.........T............C...........G.
(5) 51    .C.GC..C.AG.CC......................C.....A.....G.
(6) 51    .C.GCA.C.AG.CC......................C...........T.

(1)100    ATAAGGACCCGGCGCTAGGCACGCCGTTAAACCGAGCCCGTTACTCCCCT
(2)101    ......G....A..TC....T..T....................C.A...
(3)101    ......G....A..TC....T..T....................C.A...
(4)101    ......G....A..TC....T..T....................C.A...
(5)101    ......G....A..TC....T..T....................C.A...
(6)101    ......G....A..TC.....T.T.................A..C.G...

(1)150    GGGCAAACGACGCCCACGTACGGTCCACGTCGCCCTTCAATGT
(2)151    ...........................................
(3)151    ...........................................
(4)151    ...........................................
(5)151    ...........................................
(6)151    ...........................................
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Fig 2. Nucleotide sequences from the dPCR products of DNA samples amplified with GBV-C 5′-NCR primers. (1) the reported GBV-C 5′-NCR sequence[1], (2) – (6) sequences of the dPCR products from HeLa DNA and the PBMC DNA of G1, G2, C1, and C2. Underlined areas indicate the primer positions.

compared with the reported GBV-C genome sequence[1] were 74.29 % – 77.14 % and 73.80 % – 79.15 % respectively. This higher homology indicated that the nucleotide sequences in the amplification products had a close correlation to GBV-C genome. PRINS is a sensitive method to detect specific sequences in cell or in chromosome[14]. The PRINS detection results of this study showed that the temperate of amplification fragments located in the PBMC's chromosomes, which was consistent with the results of dPCR. The results of dPCR and PRINS in this study come to a conclusion: the nucleotide sequences with high homology to GBV-C 5′-NCR and NS3 region may generally exist in human PBMC.

In the present study, no DNA fragments could be seen in the dPCR products amplified with GBV-C 5′-NCR primers from puc18 DNA and the PBMC DNA of D3, C3, and chimpanzee, and the dPCR results amplified with GBV-C NS3 region primers from puc18 DNA and C3 PBMC DNA were also negative. Although the dPCR and PRINS performed in different institutes, their results were conformed to each other. These indicated that the possibility of contamination in the study could be eliminated and the results of this study were reliable.

Since GBV-C genome has no gene encoding transcriptase, the results mentioned above is quite significant but is difficult to explain. At least, the following two possibilities should be considered: (1) GBV-C is coinfected with a reverse transcription virus and rely on reverse transcriptase of the latter to integrate GBV-C genomic fragments into chromosomes of host cells, (2) some of sequences in GBV-C genome are derived from the mRNA transcribed from host chromogene.

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(1) 1     ATCCATAATTGAGACAAAGCTGGACGTTGGTGAGATCCCCTTTTATGGGC
(2) 1     ........................T..G..A..A........C.......
(3) 1     ........................T..G......................
(4) 1     ........................T.....A...................
(5) 1     ........................T..G......................
(6) 1     ........................T..G......................
(7) 1     ........................T..G..A...........C.......

(1) 51    ATGGTATCCCCCTCGAGCGTATGAGGACTGGTCGCCACCTTGTATTCTGC
(2) 51    .C..C..A..T..G...A.G...C....C..GA.G.....C..G.....T
(3) 51    ....C..A.....G.....G...C....C..AA.G..T..C..G......
(4) 51    ....A......T.G...A.G...C.......GA.G.....C..G......
(5) 51    ....C..A.....G.....G...C....C..AA.G..T..C..G......
(6) 51    ....C..A.....G.....G...C....C..AA.G..T..C..G......
(7) 51    ....C..A.....T.....G...C.A..C..GA.G..T..CA........

(1)101    CATTCCAAGGCGGAGTGC GAGAGATTGGCCGGCCAGTTCTCCGCGCGGG
(2)101    .....A..A..A...... ...C.CC.C..G.....A.....TT.CA...
(3)101    ..C..A...........TA...C.CC.......T.....T..T. CA...
(4)101    ..C..A.....T...... ...C.CC.T..G.....A......T.TA...
(5)101    ..C..A...........T ...C.CC.......T.....T..T..CA...
(6)101    ..C..A...........T ...C.CC.......T.....T..T..CA...
(7)101    ...........T.....T ...C.TC.T..............T..TA...

(1)150    GGGTTAATGCCATCGCCTATTATAGGGGTAAGGACAGTTCCATCATCAAA
(2)150    ....C........T........C........A........T.........
(3)151    .T..A..C.....T..........................A.....T..G
(4)150    ....C..C....................A..A........A.........
(5)150    ....A..C.....T..........................A.....T..G
(6)150    ....A..C.....T..........................A.....T..G
(7)150    ....G.....T..T........C.....C..A..............TGTG

(1)200    GACGGAGACCTGGTGGTTTGTGCGACAGACGCGCTCTCTACCGGGTACAC
(2)200    ..T..G...........G..C..T...........A..C..T........
(3)201    .................G.....T...........A..C..T........
(4)200    ..............C..G..C..............A..C..T........
(5)200    .................G.....T...........A..C...........
(6)200    .....G...........G.....T...........A..C...........
(7)200    ...........A.....G.....T...........A..C..T.....T..

(1)250    AGGAAACTTCGATTCTGTCACCGACTGTGGGTTGGTGG
(2)250    T.....................................
(3)251    T.....T.....C.........................
(4)250    T..GG.................................
(5)250    T.....T.....C.........................
(6)250    T.....T.....C.........................
(7)250    T..G..T...............................
```

Fig 3. Nucleotide sequences from the dPCR products of DNA samples amplified with GBV-C NS3 region primers. (1) the reported GBV-C NS3 region sequence[1], (2) – (7) sequences of the dPCR products from HeLa DNA and the PBMC DNA of G1, G2, C1, C2, and chimpanzee. Underlined area indicate the primer positions.

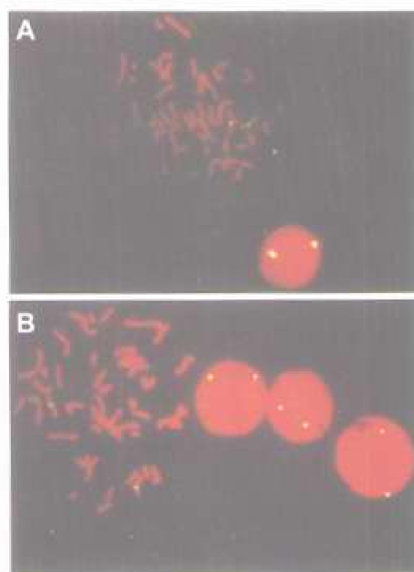

Fig 4. Results of PRINS detecting the positions of DNA fragments amplified with GBV-C 5′-NCR primers (A) and GBV-C NS3 region primers (B) from the PBMC samples. Yellow spots indicated the positive results of PRINS detection. ×1000.

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从 MT2 和 HeLa 细胞及人和黑猩猩 PBMC 的 DNA 扩增出与 GBV-C 高同源性的核苷酸[1]

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关键词　GB 肝炎剂；猴疾病；核酸序列同源性；荧光显微镜检查；DNA；人类；单核细胞；聚合酶链反应

目的：检测人源性细胞株 DNA 及人和黑猩猩外周血单核细胞(PBMC) DNA 中是否存在与 GBV-C 基因组同源的核苷酸序列. **方法：**用 PCR 模板制备试剂盒提取 MT2 和 HeLa 细胞 DNA 及 6 例人和 1 例黑猩猩 PBMC DNA, DNA 经 DNase 的 RNase 消化后再用苯酚-氯仿提取. 直接以上述 DNA 为模板，用 GBV-C 5′-NCR 和 NS3 区引物进行套式 PCR(dPCR)扩增, dPCR 产物进行核苷酸序列分析，并用引物介导原位扩增(PRINS) DNA 序列特异荧光标记技术确定扩增片段在染色体上的位置. **结果：**从 MT2 和 HeLa 细胞 DNA 及 4 例人 PBMC DNA 标本中获得 GBV-C 5′-

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NCR 引物扩增片段. 从 MT2 和 HeLa 细胞 DNA 及 5 例人和 1 例黑猩猩 PBMC DNA 标本中获得 NS3 区引物扩增片段. 这些扩增产物的核苷酸序列与 GBV-C 基因组同源性为 73.80 % - 79.15 %. PRINS 检测结果显示 dPCR 阳性的 PBMC 及其染色体上有荧光着色. **结论**: MT2 和 HeLa 细胞 DNA 及人和黑猩猩 PBMC DNA 中存在与 GBV-C 5′-PCR 和/或 NS3 区同源性较高的核苷酸序列, 这些序列位于 dPCR 阳性的 PBMC 染色体上.

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