Hepatitis c virus (HCV) genotyping detection methods and its clinical significance

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ABSTRACT

In recent years, the incidence rate of Hepatitis c virus (HCV) infection was showing an increasing trend unceasingly. However, HCV typing was more, and clinical symptoms, clinic treatment effects and sensibility to interferon responses of patients with different genotypings had differences. Therefore, effective detection and distinction of HCV genotyping was one of the basic prerequisites for treatment. The detection effects of PCR amplification sequencing and evolutionary tree analysis, which were regarded as effective detection methods of HCV infection, were still needed to be improved in a larger degree. Therefore, researches on HCV genotyping detection methods have become the hotspot and focus of scholars at home and abroad. In recent years, the correct diagnostic rate of HCV were increasing continually, at the same time, relevant researches were also increasing accordingly, but the discussion of which was still needed to be improved in a larger degree. Scholars at home and abroad were all performing studies on more simple and sensitive HCV genotyping methods, and if HCV genotypes could be confirmed by accurate and sensitive detection methods before treatment, it would had important significance for clinical treatment. In this study, we have conducted discussion on HCV genotyping detection methods and its clinical significance.

1. Introduction

Hepatitis C virus, HCV was widely popular in the world, at the last count, about 170 million people worldwide were infected with HCV, and its infection rate was about 3%, in addition, new HCV infection cases annually were about 3-4 million[1]. In China, HCV infection rate was higher than the world average, about 3.2%, that was to say, approximately 40 million, so China was one of the countries with the largest number of HCV infection in the world[2-3]. HCV infection was often characterized by onset hidden, and 50% and above infection cases would develop into hepatitis c, among whom, 1/10 of cases could further develop into liver cirrhosis even liver cancer, moreover, studies showed that among such patients, up to 1%-3% of patients could develop into liver cancer, therefore, we attached importance to its clinical prevention and early diagnosis and treatment[4]. Besides, studies showed that HCV heterogeneity also had outstanding performance, therefore we should pay attention to difference researches on HCV nucleotide and amino acid sequence[5]. HCV genotyping studies helped to further look into HCV genetic features, to analyze relevant distribution of HCV evolution and epidemics, and to confirm phylogenetic development and transmission mode of virus; besides, HCV genotyping studies also helped to realize risk factors of hepatitis c and progress of hepatic diseases, which could provide the basis for the individual establishment of disease therapeutic schedules[6] and meanwhile the basis for diagnosis reagent and vaccine researches.

1. HCV Genotyping Detection Methods

1.1. HCV Genomic Structures and Characteristics

HCV was belong to hepatitis c virus member of flaviviridae family and was a kind of single-stranded positive sense RNA virus, with total length of about 9.6 kB. HCV genome consisted of 341 bp of
5’ untranslated region, 5’ UTR and 27 bp of 3’ untranslated region, 3’ UTR. In 5’ UTR, there had an open reading frame, ORF, and its gene researches showed that it could conduct encoding on polymer precursor protein, with about 3,000 species. After various effects, it could be split and then three kinds of structural protein including E1, E2 glycoprotein, core protein and non-structural protein came into being, which respectively played an important effect on virus particles coding and virus replication and synthesis.

In addition, due to that influence factors were more during the HCV reproduction process and RNA polymerase correction was not obvious, therefore it had outstanding specificity performance and its genetic structure differences also had great effects on itself[7], therefore we thought that we should conduct typing analysis of HCV based on the variation of different genetic structure regions.

Researches on such cases for genotyping were common, among which, researches on technical aspects were more, but researches suggested that genotyping species were more, however in the later period, a unified naming system was established according to the existence of such cases, that was to say, Simmonds system[8], which was more suitable for HCV genotyping studies. And more researches in recent years showed that it could be divided into 7 types and more than 80 subtypes for researches’ satisfaction according to its variation researches[9].

1.2. HCV Genotyping Regions Selection

At present, available regions were including 5’ UTR, NS5, Core and core-envelope 1 region (E1). Which region could be selected as the genotyping basis has become the key to the problem.

1.2.1. 5’ Untranslated Region (5’ UTR)

5’ UTR was the most conservative region among HCV genomes, and its homology was 92% ~ 100%, besides its phyletic changes and evolution rate were lower. Although this regional gene sequence was highly conservative, because of its well restriction fragment length polymorphism, RFLP and its easier amplification features, the typing values were higher based on it. And it was also the main selected fragment for clinical detection at present. More studies in recent years have shown that HCV genotyping detection method based on 5’ UTR was relatively desirable, which could provide necessary basis for establishment and selection of therapeutic schedules, and its recognition was also higher[10]. But this kind of basis also had its disadvantages, that was to say, it had lower distinction values between type 1 and type 6, which was because similarity of above two types was higher. In view of this point, some type 6 samples could be wrongly divided into 1a or 1b type[11]. Furthermore, it also had relatively poorer distinction effects on genetic subtypes; difference of research results was larger, and meanwhile it had poorer detection values for mixed infection and recombination[12]. Therefore, other regions were necessary for better distinctions.

1.2.2. Non-structural Protein 5B Region, NS5B

From the perspective of bioinformatics analysis, NS5B region could best reflect the HCV evolutionary relations, and research values of variation in this region were higher, and differentiations were also higher, which could reach to 16.5% ~ 20.1%[13], therefore this region had higher distinction values for HCV genotyping, which could better reflect the genovariation, and was often regarded as the main selected region for subtypes distinction.

1.2.3 Core–envelope 1 Region, C–E1

This region was the hypervariable region of HCV genomes and had higher distinction values for species. Its nucleotide variation was relatively larger, and compared with 5’ UTR, it had greater variability. Therefore, its values were relatively higher in genotyping. Studies found that[14] Core region and NS5B region genotyping results were basically the same, and designed primers in Core region were more easier, compared with NS5B, amplification positive rate and accuracy rate in this region were higher. Other studies found that the values of combined studies on Core region based on 5’ UTR were relatively higher, which had higher accuracy for genotyping and had positive significance for genotyping extension, especially had higher distinction values for type 1 and type 6[15].

1.3. HCV Genotyping Methods Analysis

HCV genotyping methods were more, and detection values of various methods had greater differences. Therefore, comparative studies on it had higher significance.

1.3.1. Direct Sequencing Method

HCV genotyping detection methods were more, and direct sequencing method was one kind of method with more studies and was more experienced, which was recognized as “golden standard”[16]. Direct sequencing method: HCV representative gene fragments were effectively amplified, then its nucleotide sequence was detected, finally the sequencing results were compared with known genotype sequences, which was in order to achieve the purpose of HCV typing. But due to its higher cost and higher requirements for laboratory environment and operating personnel experience levels, besides, sequencing method was poorer than other methods in mixed infection detection, so its clinical application was restricted, which was mainly regarded as the reference basis for other detection methods.

1.3.2 Restricted Fragment Length Polymorphic Analysis Method (RFLP)

RFLP method was a kind of traditional HCV genotyping method,
which was sensitive, convenient and practical and didn't need costly equipments, so this method could be carried out in general hospital laboratories. RFLP method: firstly amplification method was adopted for HCV specific regions (5’UTR, C-E1 and NS5B) treatment; then further nucleic acid restriction enzyme (like Haell, Rsa I, Mva I, Hind I, Sscr/Hae III, Rsa I, Mva I, Hind I, Sscr I) was adopted for enzyme digestion; after that it was decomposed into a number of fragments with different lengths for analysis. RFLP results were stable and had good repeatability, which was good for the accurate types of 6 HCV genotypes, and its results were highly consistented with sequencing method. However, the application of this method was restricted due to endonucleases including less species and locus, therefore it had poorer detection values for subtypes and new genotypes.

1.3.3. LiPA Method
Firstly HCV treatment: reverse transcription polymerase chain reaction (RT-PCR) was adopted for amplification and biotin or fluorescein was labelled; then multi-group specific probe designed for 5’ UTR was used for hybridization with amplification products with labelled biotins, afterwards coloration was conducted; finally interpretation of HCV genotypes was performed by scanning. LiPA method was one of typing technology which was universally applicable in clinical laboratories, and its detection results were in consistency with sequencing method. Because early-stage probe was mainly designed for 5’UTR of HCV, and this region was highly conservative and had lower variation rate, therefore this method had poorer detection values for some subtypes of HCV. Recently some researches have conducted improvements for LiPA method, that was to say, combined utilization of Core region design primers and typing probes could effectively solve the deficiency of 5’ UTR.

1.3.4. Type Specific Primers Amplification Method
HCV genotypes were distinguished based on nucleotide sequence differences, then specific primers were designed based on its specificity, afterwards primers and probes were adopted for amplification products treatment, mainly showing amplification once more; finally HCV genotyping was conducted based on amplification results. However, this method needed to make multi-group primers, experimental procedures of which were complicated, and compared with other genotyping methods, its experimental specificity and sensibility were still needed to be improved.

1.3.5. Specific Primers Extension Analysis Method (PSEA)
Primers treatment: when mispairing appeared at the end of primer 3’, tag enzyme was adopted for its treatment, and detection was conducted for mispairing peak, then the frequency was regarded as the basis of typing distinctions. Therefore, the accuracy of this method was relatively higher for typing detection, especially for HCV mixed genotype infection detection, which was particularly used for distinguishing the mixed genotypes with low-proportion (3% and above)[19].

1.3.6. Fluorescence Resonance Energy Transfer Probe Dissociation Curve Analysis Method
Fluorescent quantitation PCR primers and probes were designed for HCV genome specific regions based on fluorescence resonance energy transfer principles, then its amplification products treatment was conducted, that was to say, fluorescence resonance energy transfer probe special enzyme was adopted for its incision treatment, afterwards the processed fluorescence signals were collected, finally dissociation curve treatment was performed based on such structures for achieving the purpose of genotyping. After that, virus-like particles were prepared as positive quality control products. Consequently, complete fluorescence quantitative PCR HCV genotyping detection system was established. At present, MGB probe was applied in many laboratories, which was characterized by easier sequence design, higher sensitivity, faster target sequence identification (typing could be finished within 1h after amplification) and higher specificity. Compared with traditional methods, such kind of detection method had relatively higher sensitivity on HCV RNA.

1.3.7. Genechip Technology
Genechip technology was a kind of newly-developing detection method in recent years, but had higher clinical application values, which was faster and more accurate for typing and mixed infection detection, and compared with "golden standard", accuracy rate of this method was higher, detection effects of which were basically in accordance with sequencing method. Genechip technology method: a large number of specific oligonucleotides fragments were treated effectively, then they were arranged on the support materials, afterwards they were amplified, subsequently, amplification samples and base pairing were hybridized, later, specific fluorescence appeared on the support materials, then chip scanning was performed, finally genotyping results were obtained based on the fluorescence signals after computer analysis. This technical method was characterized by its higher sensitivity, specificity and accuracy, and its easy operation, meanwhile its more prominent performance for mixed infection detection, which had larger research and development space.

1.3.8. Genetic Phylogenetic Tree Analysis Method
Gene sequencing was performed for specific regions (such as 5’UTR, Core, E1 and E2, NS5B) in HCV nucleotide sequences, then sequence results were compared with each other and evolutionary distance of each sequence was analyzed, subsequently, genetic phylogenetic tree was established based on computer software.
Genotypes and subtypes had great differences in different areas. Relevant typing rules which were newly established in 2005 showed that this method had higher reliability, which was basically the most reliable standard for HCV genotyping [23].

1.3.9. HCV Serological Typing Method
This method was mainly designed for polypeptide induced genotype specific antibody detection in NS4 region. This technical method was characterized by its easier detection mode and relatively lower technology pollution, which had higher detection values for large samples and was more suitable for clinical disease screening work. Because antigenicity of HCV gene subtypes was similar, this method had lower detection sensitivity for distinction of gene subtypes and genotype mixed infection, therefore, this method could not take the place of the molecular method of genotyping.

1.3.10. Other Methods
At this stage, HCV genotyping methods whether molecular biological techniques or serological experiments for clinical laboratories were relatively mature. But both kinds were characterized by their longer time-consuming and complicated procedures in the process of experiments. For better detection methods, higher requirements were needed not only for their distinction of genotyping and subtypes but also for their easier operation, lower cost and sensitivity. Scholar at home and abroad were also researching and developing easier and more sensitive HCV genotyping methods, such as denaturing high performance liquid chromatography (DHPLC), heterogeneous molecular mobility analysis and molecular beacon and gold nanoparticle technology. Although these detection methods were not enough perfect, their easier, faster, sensitive detection mode and good specificity have provided new basis for HCV detection.

2. HCV Genotyping Clinical Significance

2.1. HCV Genotypes Epidemic Distribution
Genotypes and subtypes had great differences in different areas[24], and its distribution had obvious regional and population distribution differences. HCV genotyping was good for analysis of transmission routes of hepatitis c and was good for discovering the possible new epidemic subtypes in the future as soon as possible, which could provide scientific basis for HCV prevention and control.

HCV genotype 1 showed a worldwide distribution, which was the main genotype in America, Europe, Australian and Japan. America and Northern Europe mainly had the most epidemic distribution of subtype 1a; subtype 1b was the most common genotype in Europe and Asia. Type 2 was common genotype in developed countries, South America and Asia. Japan mainly had the common subtype 2a distribution; subtype 2b was widely distributed in Northern Europe and America; subtype 2c was the most common subtype in Western Europe, southern Europe, Pakistan and India. Type 3 especially subtype 3a was epidemic in Europe, America, Australian and South Asia. Type 4 and Type 5 mainly has a distribution in Africa and the Middle East. Type 6 and its subtypes were mainly discovered in Southeast Asia, among which, majority of new cases was subtype 6a in Thailand, Vietnam and Burma. Clinical pathogenicity of Type 7 was relatively weak, which was recently found in Central Africa and Thailand cases[25]. Along with the rapid development of globalization and the increasingly strengthened connection between international communities, movement of population was increasing frequently in modern society, and past geographical barriers were lost, which resulted in the rapid spread of HCV genotypes between countries[26].

HCV epidemic in China was mainly characterized by the existence of lower epidemic in general areas and higher epidemic in parts of areas, which showed an uneven scattered distribution. High morbidity of HCV infection was caused by iatrogenic infection, like hematodialysis, repeated use of medical equipments and non one-off injection equipments and iatrogenic transmission induced population infection[27]. The most epidemic subtypes of HCV in China were lb and 2a, but genotypes in different areas had differences. Genotypes in northern areas were single, mainly showing lb and 2a distribution; genotypes in southern areas were more, mainly showing 1b distribution, besides, 2a, 3a, 3b and 6a each had a larger proportion. In the early stage, HCV genotypes were relatively single, as time goes by, subtype 1b and 2a were reduced gradually, but subtype 3a, 3b and 6a were increased by years, and subtypes were increased by years.

2.2. Relationship between HCV Genotypes and Clinical Treatment

2.2.1. HCV Different Genotypes Prognosis
75%-85% of acute hepatitis c would become chronic, and HCV would bring continuous and chronic damages to liver without timely treatment, then hepatitis c could be developed into liver cirrhosis and liver cancer. Many relevant studies showed that[28] effects of HCV genotyping on liver of patients had great differences, therefore HCV genotypes distinction had higher clinical values for disease prognosis, meanwhile studies showed that prognosis of type 1 patients was relatively poorer, and compared with type 2 and 3 patients, hepatitis c of type 1 patients was easier to be aggravated and developed into liver cirrhosis even liver cancer. Moreover, studies also showed that prognosis of type 1b patients was much more worse, hepatitis c of type 1b patients was easier to be developed into liver cirrhosis and liver cancer, and patients in such stage accounted
also had obvious differences in the therapeutic effects of hepatitis C patients with different genotypes, which was related to the high HCV RNA capacity of genotype 1b leading to persistent infection of patients and immune responses could not be obtained easily. Relevant studies also showed that HCV genotypes had obvious statistical differences with HCV RNA capacity and liver damage indexes.

2.2.2. HCV Genotyping Guidance for Clinical Treatment

Nucleotide sequence of patients with different genotypes and patients with same genotypes but different subtypes had a certain difference, and however difference was less than 30% and between 20% and 25% respectively, which was also the important cause of HCV infection clinical symptoms differences. Genotyping detection for HCV infection patients could play important guidance effects on clinical treatment and could predict the curative effects of patients, and then appropriate measures were taken for reducing the adverse effects of drugs and reducing the economic burden of patients.

In recent years, clinical treatment for HCV infection patients were more inclined to targeted treatment, that was to say, targeted treatment was based on infected genotypes and subtypes and the virology responses during treatment. For such kind of patients in the clinical treatment, interferon had higher recognition, but in view of HCV genotyping differences, especially differences in interferon responses, therefore a better knowledge of genotyping of patients was necessary before treatment. And clinical demands for such aspect were constantly improved, and relevant requirements have been included in HCV infection prevention guidelines, which was in order to achieve the purpose of more accurate and targeted therapeutic effects.

2.2.3 Genotyping Significance and its Effects on Therapeutic Effects

For the treatment of hepatitis C in the clinic, Peg-IFNα and ribavirin combined treatment was regarded as the standard, and removing or inhibiting intracorporeal HCV continuously for better therapeutic effect was the main target of this treatment, that was to say, 24 weeks after treatment, HCV RNA could not be detected in serum, and its final target was to improve or relieve liver damages, and to inhibit its development into liver cirrhosis and liver cancer. Interferon therapeutic effects of hepatitis C patients with different genotypes also had obvious differences, type 1 and 4 had poorer responses, which was belong to "refractory hepatitis C", while type 2, 3 and 6 were easier to obtain persistent virus responses.

Studies found that SVR rate of hepatitis C patients with HCV genotype 1 to interferon and ribavirin combined treatment was about 50%, while SVR rate of patients with HCV genotype 2 and 3 was over 80%[30], which was possibly because that HCV infection of genotype 2 and 3 was easier to induce immune responses. Other studies found that patients with type 1b and high HCV RNA capacity infection were not easy to obtain persistent responses after treatment[37], therefore, significance of the above factors was high, which also indicated the treatment characteristics of type 1 especially type 1b patients, so we should attach more importance to the genotyping detection and distinction of such patients and provided targeted intervention. Large doses of interferon combined with ribavirin treatment could effectively promote the clinical therapeutic effects of such patients, but intervention effects of which on other types were not obvious relatively[38].

2.3 HCV Genotypes and Vaccine Research

Researchers in various countries found in the process of long-term researches that protein derived from HCV gene hypervariable regions could induce protective immunity, and immune evasion occurred, which brought great difficulties for vaccine development. What's more, due to HCV genotypes and subtypes were more, when one kind of vaccine was successfully developed, HCV gene have mutated, so this kind of vaccine would lose its clinical values, which make HCV vaccine development do not get breakthrough progress.

3. Conclusion and Prospect

Studies show that in view of that effects of different HCV genotypes on hepatitis C patients were great, especially for prognosis and therapeutic effects, therefore, we should especially attach importance to distinction of genotypes and subtypes for such patients before treatment, which was in order to provide basis for establishment and selection of therapeutic schedules and then to achieve effective therapeutic effects; besides, it could promote the therapeutic safety in a larger degree and reduce the economical burden of patients, and then could achieve better treatment purposes. In addition, combined with the current situations that HCV infection rate was high in China and primary hospital laboratories lacked HCV genotyping techniques, we should develop faster, easier, high automatic and low-cost detection methods, which was in order to provide necessary prerequisites for hepatitis C prevention in our country.

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