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TECHNICAL ADVANCE

Development and Validation of a Template-Independent Next-Generation Sequencing Assay for Detecting Low-Level Resistance-Associated Variants of Hepatitis C Virus

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To develop hepatitis C virus (HCV) direct-acting antiviral (DAA) drugs that can treat most HCV genotypes and offer higher barriers for treatment-resistant mutations, it is important to study resistance-associated variants (RAVs). Current commercially available RAV detection assays rely on genotype- or subtype-specific template-dependent PCR amplification. These assays are limited to genotypes and subtypes that are often prevalent in developed countries because of availability of public sequence databases. To support global clinical trials of DAAs, we developed and validated a template-independent (TI) next-generation sequencing (NGS) assay for HCV whole genome sequencing that can perform HCV subtyping, detect HCV mixed genotype or subtype infection, and identify low-level RAVs at a 5% fraction of the viral population with sensitivity and positive predictive value $\geq$ 0.9. We compared TI-NGS with commercial genotype- or subtype-specific Sanger sequencing assays, and found that TI-NGS both confirmed most of variants called by Sanger sequencing and avoided biases likely caused by PCR primers used in Sanger sequencing. To confirm TI-NGS assay's variant calls at the discrepant positions with Sanger sequencing, we custom designed template-dependent NGS assays and obtained 100% concordance with the TI-NGS assay. The ability to reliably detect low-level RAVs in HCV samples of any subtype without PCR primer-related bias makes this TI-NGS assay an important tool in studying HCV DAA drug resistance. *(J Mol Diagn 2016, 18: 643–656; http://dx.doi.org/10.1016/j.jmoldx.2016.04.001)*

Hepatitis C is a liver infection caused by the blood-borne virus hepatitis C virus (HCV). According to the Centers for Disease Control and Prevention, an estimated 170 million individuals worldwide are chronically infected with HCV, and approximately 10% to 20% of them will develop severe liver diseases.[1] HCV is a single-stranded RNA virus with a high degree of genetic variability and nucleotide sequence diversity, caused by the lack of a proofreading 3′ to 5′ exonuclease activity in its RNA-dependent RNA polymerase.[2] The complex genetic variability of HCV has been classified into four hierarchical strata: genotypes, subtypes, isolates, and quasispecies.[3] A consensus proposal for HCV classification was initially presented in 2005[4] and updated in 2014,[5] which classified HCV isolates into seven genotypes (GTs) and 67 confirmed subtypes, with 24 confirmed subtypes belonging to GT 6. There are geographical variations in genotype prevalence, with GT 1 and 3 more prevalent globally, whereas GT4 is more prevalent in North Africa and the Middle East, and GT2 and 6 are found most frequently in East Asia.[6] The most diverse HCV genotype GT6 is prevalent in China and Southeast Asian countries.[7]

For both traditional treatment with pegylated interferon α/ribavirin and the currently available HCV direct-acting antiviral (DAA) drugs, different genotypes and subtypes of HCV have different antiviral treatment responses.[8,9]

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Therefore, accurate genotyping and subtyping of HCV is needed for the selection of proper treatment regimens. In the developed world, most cases of HCV infection occur among people who inject drugs (PWID).[10] In clinical trials of DAA drugs with PWID subjects, mixed HCV genotype/subtype infections observed in PWID[11] can affect treatment success. A sensitive assay that can detect low levels of genotypes and/or subtypes not targeted by DAA drugs in mixed infected PWID is required for the accurate evaluation of DAA treatment success. In chronically infected patients, HCV exists as a complex mixture of genetically distinct but closely related genomes described as a quasispecies,[12] caused by continually high viral production combined with error-prone RNA replication. The quasispecies provide a large pool of genetic variants that can adapt to new selection pressures such as host cell defense and antiviral treatments, resulting in chronic infection and HCV drug resistance.[13] For the development of interferon-free DAA drugs that are pan-genotypic and have a higher barrier for resistance variants that either exist before treatment or emerge during treatment, it is important to have a sensitive sequencing assay that can detect viral genetic changes and potential DAA treatment resistance—associated variants (RAVs) for all HCV genotypes and subtypes.

Current commercially available RAV detection assays are template dependent, which means that PCR primers were designed on the basis of conserved nucleotide sequences shared among isolates of particular HCV genotypes or subtypes to amplify specific genes targeted by DAA drugs. This approach requires that the particular genotypes and subtypes are sufficiently represented in public nucleotide sequence databases. It also requires a robust and accurate subtyping assay that can analyze all encountered subtypes before performing the subtype-specific RAV assays. Although there are several commercial RAV assays validated for the well-known genotypes or subtypes such as 1a and 1b, samples with known genotype but unknown subtypes or subtypes with limited nucleotide sequence information pose challenges to such a template-dependent approach. That is especially true for samples of GT6, with its large number of diverse subtypes, many of which are poorly represented in sequence databases.

Herein, we describe a pan-genotypic template-independent (TI) next-generation sequencing (NGS) assay for HCV whole genome sequencing that can perform HCV subtyping, detect mixed genotype or subtype infection, and identify RAVs. The assay is built on an RNA-seq method using random primed cDNA synthesis, combined with a simple RNA extraction method, and a human rRNA depletion procedure that eliminates the majority of non-HCV NGS reads observed in previous methods.[14] The assay can reliably detect RAVs at a 5% fraction of the viral population with viral load of 10^6 IU/mL with both high sensitivity and positive predictive value. The ability to reliably detect low-level RAVs in HCV samples of any subtype with a robust experimental procedure makes our TI-NGS assay a promising tool in studying the dynamics of viral genetic changes with HCV DAA treatments to enable better clinical outcomes.

Materials and Methods

HCV Specimens

For assay development, three high viral load HCV plasma samples were purchased from Discovery Life Sciences (Los Osos, CA), and two HCV serum samples were purchased from SeraCare Life Sciences (Milford, MA). These five commercial samples represented five subtypes and four genotypes: GT1a, 1b, 2b, 4a, and 6a. Plasma samples collected from normal healthy volunteers (NHVs) by BioreclamationIVT (New York, NY) were pooled and confirmed to be HCV negative and used for serial titrations of the high viral load commercial HCV samples. To mimic HCV mixed genotype/subtype infections, the commercial GT1a and 1b samples were also mixed at 5%, 10%, 25%, 50%, 75%, 90%, and 95% of 1a in a 1b background at a total viral load of 10^6 IU/mL, using the viral load information provided by the vendors.

Seventeen clinical plasma samples described in this article were collected from a clinical trial with HCV DAA drugs (*ClinicalTrials.gov*, identifier: NCT01932762). They were from 10 HCV patients whose samples were initially genotyped as GT6 by the Siemens VERSANT HCV Genotype INNO-LiPA 2.0 Assay (Malvern, PA). More accurate HCV subtyping assay using NS5B small amplicon Sanger sequencing based phylogenetic analysis[15] showed that two samples from two patients actually belong to GT1b. Among the 10 patients, eight were treated successfully with HCV DAA drugs and thus only their baseline screening samples were collected and analyzed with the sequencing assays. However, there were two GT6 patients who failed HCV DAA treatment. For each of them, multiple samples were collected on different follow-up dates after treatment. Please note that all patients enrolled in the clinical trial provided informed consent in writing, and the study protocol was in accordance with the global standards of the International Conference on Harmonization—Good Clinical Practices, the Council for International Organizations of Medical Public Policy Statement: Clinical Trial Ethics Sciences International Ethical Guidelines for Biomedical Research Involving Human Subjects (CIOMS, 2002), the Pharmaceutical Research and Manufacturers of America (PhRMA, 2009) Principles on Conduct of Clinical Trials, applicable local regulatory requirements, and following the ethical principles that have their origin in the Declaration of Helsinki.

In Vitro—Transcribed HCV RNA Sample

HCV GT1a (H77) replicon cell lines were treated with various concentrations of the HCV NS3/4A protease inhibitor grazoprevir and the NS5A replication inhibitor