Human Cytomegalovirus (HCMV) Genetic Diversity and Drug Resistance

Subjects: Infectious Diseases

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Human cytomegalovirus (HCMV) is a pathogen with high prevalence in the general population that is responsible for high morbidity and mortality in immunocompromised individuals and newborns, while remaining mainly asymptomatic in healthy individuals. The HCMV genome is 236,000 nucleotides long and encodes approximately 200 genes in more than 170 open reading frames, with the highest rate of genetic polymorphisms occurring in the envelope glycoproteins. HCMV infection is treated with antiviral drugs such as ganciclovir, valganciclovir, cidofovir, foscarnet, letermovir and maribavir targeting viral enzymes, DNA polymerase, kinase and the terminase complex. One of the obstacles to successful therapy is the emergence of drug resistance, which can be tested phenotypically or by genotyping using Sanger sequencing, which is a widely available but less sensitive method, or next-generation sequencing performed in samples with a lower viral load to detect minority variants, those representing approximately 1% of the population. The prevalence of drug resistance depends on the population tested, as well as the drug, and ranges from no mutations detected to up to almost 50%. A high prevalence of resistance emphasizes the importance of testing the patient whenever resistance is suspected, which requires the development of more sensitive and rapid tests while also highlighting the need for alternative therapeutic targets, strategies and the development of an effective vaccine.

Keywords: human cytomegalovirus ; drug resistance ; drug-resistance testing

1. The HCMV Genome and Genetic Diversity

The HCMV genome is a 236 kb long linear double-stranded DNA (dsDNA), which is longer than all other human herpesviruses and one of the longest genomes of all human viruses in general. The genome contains more than 170 open reading frames and encodes approximately 200 genes, including nine gene families, a large number of glycoprotein genes, and homologues of the human HLA class I and G protein-coupled receptor genes [1][2][3][4]. The genome has the highest level of genetic variability of all the known human herpesviruses. The virus is known to readily undergo recombination, and coinfection is frequently observed, especially in individuals with weakened immune systems [5][6][7]. HCMV clinical isolates display genetic polymorphisms in multiple genes, mainly envelope glycoproteins such as UL55 (gB), UL73 (gN) and UL75 (gH) [7]. Glycoprotein B (gB), encoded by the UL55 gene and classified into 5 genotypes (gB1, gB2, gB3, gB4 and gB5), is an abundant and the most highly conserved glycoprotein of CMV. The glycoprotein H (gH), divided into two major genotypes (gH1 and gH2), is an 86 -kDa protein and encoded by the UL75 gene. The highly polymorphic gene UL73 encodes the viral glycoprotein N (gN), which is divided into seven genotypes: gN1, gN2, gN3a, gN3b, gN4a, gN4b and gN4c [8][9][10][11]. Past research has attempted to tie certain polymorphisms to the higher viral fitness of the strain, and, as a consequence, to different clinical manifestations of HCMV disease, and the ability to establish persistent or latent infections, but every attempt to correlate individual alleles with transmission and pathogenesis have so far been unclear or contradictory [12][13][14][15][16].

2. HCMV Antiviral Therapy

Antiviral agents specifically targeting HCMV-like ganciclovir (GCV), valganciclovir (VGV), foscarnet (FOS) and cidofovir (CDV) interfere with the synthesis of viral DNA by binding to the active site of the viral DNA polymerase (UL54) [17].

2.1. GCV and VGV

The frontline drugs for the treatment of HCMV infection and prophylaxis, GCV and its oral prodrug VGV, exhibit only modest antiviral activity that is often insufficient to completely suppress viral replication and drives the selection of drug-resistant variants to continue to replicate and contribute to disease. To obtain to its active form, ganciclovir-5′-triphosphate, a nucleoside analog that targets DNA polymerase, GCV undergoes phosphorylation by both cell and viral kinase (UL97). GCV is most frequently administered as an intravenous formulation due to its low oral bioavailability. VGV is a GCV ester

that is well absorbed after oral administration and rapidly metabolizes to ganciclovir. Both drugs are routinely used for treating HCMV infection after solid organ transplantation, but due to possibility of myelosuppression, it is advised to avoid administration after hematopoietic cell transplantation [18][19].

2.2. FOS

FOS, a pyrophosphate analogue, also has increased affinity for UL54 and blocks DNA replication, but does not require phosphorylation; therefore, resistant mutations can only be formed in viral DNA polymerase genes [20]. It is not the drug of choice in first-line preemptive therapy for HCMV due to its considerable nephrotoxicity, and it is administered only if a patient is cytopenic, or resistance to first-line therapy agents has been proven.

2.3. CDV

CDV is a cytidine monophosphate analogue and a competitive inhibitor of the viral DNA polymerase that undergoes phosphorylation using cell kinase so that the resistance mutations can be detected in the UL54 gene. It is also reserved for second-line treatments due to its considerable toxicity [21][22].

2.4. Maribavir

Maribavir (MBV) is an oral benzimidazole nucleoside that effects HCMV DNA synthesis, viral gene expression, encapsidation and viral capsid egress through the inhibition of the UL97 kinase. The United States Food and Drug Administration approved it in November 2021 for the treatment of adult and pediatric refractory/resistant post-transplant HCMV infection [23][24][25]. Resistance mutations to MBV emerge in the UL97 gene, which may result in cross-resistance to GCV and exclude the option of combination therapy [26][27][28]. Mutations in the UL27 region may also attribute to resistance to this drug [29].

2.5. Letermovir

Letermovir (LMV), the HCMV terminase inhibitor, has been recently approved for prophylaxis in stem cell HCMV-seropositive adult hematopoietic cell transplant recipients [30][31]. Resistance mutations are located mainly in UL56, and rarely in UL89 and UL51-terminase subunits, so there is no cross-resistance with other anti-HCMV drugs [32][33]. Clinical experience with LMV as a treatment for active HCMV infection is still limited, but the absence of myelosuppression, oral bioavailability and a good safety profile make LMV an eligible candidate for the treatment of HCMV infections that are resistant to approved agents or as an alternative to poorly tolerated intravenous options [34][35]. The use of novel molecular targets for the inhibition of HCMV replication, such as terminase complex, and exploring new options, like the viral alkaline nuclease, coded by the UL98 gene is the next step toward successful inhibition of HCMV replication. The creation of new therapeutics in order to develop more effective combination therapies would reduce the chance of the emergence of drug resistance [36][37][38].

3. HCMV Drug Resistance

The outcome of HCMV infection in an immunocompromised host, as well as congenital infection, significantly depends on the availability of antiviral therapy. However, there are considerable limitations of the currently registered drugs, such as poor oral bioavailability, associated toxicities and the potential for the development of resistance mutations. Quasispecies carrying resistance mutations to all currently available antiviral therapy have already emerged, partly because of the use of monotherapy in combination with low genetic barrier to resistance and due to the chronic persistence of HCMV infection in immunocompromised patients [39][40]. This emphasizes the need for alternative therapeutic targets, strategies and, of course, the development of an effective vaccine. One possible pathway is studying the distinct stages of the viral replication cycle in order to identify possible new drug targets. Combination therapy would most certainly reduce the probability of the emergence of resistance mutations, all while lowering the required dose, which would increase the tolerability. An alternative approach is to target a host cell protein or pathway that is essential for the completion of viral replication. While there is reasonable concern about the possible toxicity of this mechanism of treatment, it would reduce the possibility of the development of drug resistance [41][42].

4. HCMV Drug Resistance Testing

The emergence of drug-resistant forms is a great obstacle to the successful treatment of HCVM infection. Even with a selection of several antiviral drugs, clinical management of the infection is challenging due to its high frequency of drug resistance-associated mutations. The genes encoding the drug targets, UL54 (DNA polymerase) to GCV, CDV and FOS,

UL56 (terminase complex) to LMV, and UL97 (phosphotransferase) to GCV and MBV, are usually the ones carrying resistance mutations [40]. However, for LMV, mutations in the genes that form the terminase complex, like UL89, can have an effect on its susceptibility to the drug [43]. Resistance gene mapping results showed that a single mutation in the UL27 gene is necessary and sufficient for resistance to MBV [29][44]. Drug resistance mutation maps for HCMV are regularly updated with recent information about newly detected polymorphisms that might cause resistance. They provide more detail on cross-resistance properties, and also emphasize the need to expand the regions covered in diagnostic testing. Therapy options for the treatment of HCMV infection are limited, so cross-resistance is a serious obstacle; however, there are few reports about multidrug resistance with mutations in both genes [45][46][47]. Patients should be tested for antiviral resistance whenever resistance is suspected, even after mutations have been identified, as additional resistance mutations can develop. It emphasizes the importance of proving that observed genetic changes confer resistance so that they can be distinguished from polymorphisms [40][48]. Normally, mutations in the UL97 gene occur initially, followed by UL54 mutation after a therapy switch. The appearance of a UL54 mutation alone without any detection of a UL97 mutation is rare. Interestingly, in a number of patients, the UL97 mutation could be detected exclusively in specific compartments, and not in blood. The manifestation of multidrug resistance is mostly associated with combined UL97/UL54 mutations [46][47].

Drug resistance to HCMV can be detected either by genotyping or phenotypically, using a virus grown in cell culture and applying various drug concentrations to it. Cell-associated plaque reduction assay detects drug resistance without the need for genetic information, but it takes time, requires a highly educated staff, is technically demanding and produces results that may vary significantly between different laboratories. Genotypic analysis can be performed without the need for viral isolation, which facilitates and speeds up the process; however, determining the degree of resistance when multiple mutations are detected may be difficult to deduce by genotypic testing alone. Therefore, the phenotypic studies are essential for analysis of the new mutations encountered in clinical isolates [40][48][49].

4.1. Sanger Sequencing

Sanger sequencing-based genotypic analysis is currently the most frequently used method by commercial reference laboratories. Restriction fragment length polymorphism (RFLP) and real time PCR assays can also be used for the detection of drug-resistant mutations. The limitations of the Sanger sequencing method are the strictly prescribed sample requirements, such as viral loads of at least 1000 IU/mL, for successful characterization and the substantial length of the fragment needed for analysis to cover all possible resistance mutation sites is also a challenge. It is necessary to read more than 2000 base pairs for the UL54 gene and about 1000 for the UL97 gene to detect resistance to GCV, VGV, CDV and FOS [40][45][46]. Fragments of 2100 base pairs of the UL56 gene and 800 base pairs of the UL89 gene need to be sequenced to determine resistance to LMV [42]. Resistance mutations to MBV are mostly covered with a nucleotide sequence of the UL97 gene; however, for complete resistance testing, sequencing of the UL27 gene is also required [29][44]. Only variants present in more than 20–30% of the overall viral population can be detected with Sanger sequencing, so the identification of low levels of resistance in a predominantly susceptible population, as well as mixed infections, may fail. Once a nucleotide sequence is read, it is compared to the wild-type referral strain in order to detect any polymorphisms. Only mutations that have a confirmed effect on the susceptibility of the antiviral drugs are reliable for the interpretation of the polymorphisms detected by genotypic methods [40][50]. Genotypic testing based on Sanger sequencing can be conducted in reference laboratories or as an in-house test, which some publications are demonstrating. Hall Sedlak et al. describe a rapid, sequencing-based assay for the UL97 and UL54 genes. This assay is performed in 96-well format with a single master mix and provides clinical results within 2 days. It sequences codons 440 to 645 in the UL97 gene and codons 255 to 1028 in the UL54 gene with a limit of detection of 240 IU/mL [51]. An in-house method for sequencing UL56 gene for detection of resistance mutations to LMV was described in a case report by Bosworth et al. [52]. The interpretation of detected mutations is also challenging but there are free, regularly updated internet algorithms that can be used for the detection of drug resistance mutations obtained by Sanger sequencing, such as Mutation Resistance Analyzer created by University of Ulm (https://dna.informatik.uni-ulm.de/software/mra/app/index.php?plugin=form, assessed on 8 January 2024).

4.2. Next-Generation Sequencing

Next-generation sequencing (NGS) technology offers a more sensitive, higher resolution view of emerging antiviral resistance capable of detecting minority variants down to as little as 1%, and it may be performed in samples with a lower viral load. However, cost per sample is still substantially high; NGS technology is not available at all laboratories; specialized skills are required for analysis; there is a scarcity of databases which summarize the clinically relevant antiviral resistance mutations for use in a bioinformatics pipeline; and detection of unexpected organisms or commensals of uncertain significance NGS assays is not widely used for the detection of HCMV antiviral resistance. Standardization of

the method and diagnostic utility in comparison with traditional Sanger sequencing remains to be completed, but NGS is a powerful tool with a growing role in managing immunocompromised patients with suspected infection and is recommended for use in clinical trials. NGS can also provide data on the HCMV genotypes circulating in the population, which may facilitate the development of vaccines and immunobiological preparations, enable dynamic monitoring of risk groups (pregnant, newborns, children of the first year of life and patients who underwent solid organ transplantation), predict the epidemiological situation for cytomegalovirus infection, and improve the system of epidemiological surveillance of infections in general [53][54].

Evaluation of NGS as a Diagnostic Tool

There are a few evaluations of NGS as a diagnostic tool for detection of resistance mutations. In 2010, Schindele et al. demonstrated, using pyrosequencing, that the detection of GCV resistance-associated mutations occurring in the HCMV open reading frame of UL97 can be both fast and sensitive, with a minimum level of 6% mutant sequence variants. When compared to conventional dideoxy chain terminator sequencing, the method was more sensitive in detecting minor HCMV-mutant fractions in a wild-type population and, therefore, a useful tool for the early detection of emerging resistant mutations [55]. Nanopore sequencing was used by Li et al. to determine the complete genomes of HCMV in high-viral-load clinical samples without viral DNA enrichment, PCR amplification or prior knowledge of the sequences. Their data prove that improvements have been made and that, compared to Illumina, the final genomes from a urine sample and a lung sample achieved 99.97 and 99.93% identity and that Nanopore sequencing is capable of determining HCMV genomes directly from high-viral-load clinical samples with a high accuracy [56]. Garrigue et al. demonstrated that NGS technology allows a deeper discrimination of the emergence and persistence of a drug resistance mutation, which could be pertinent to the investigation of when routine Sanger sequencing detects only wild-type strains. Moreover, NGS-improved sensitivity helps in studying viral abundance, dynamics and diversity, which are less approachable with Sanger sequencing [57]. A dual-step NGS-based clinical assay that utilizes full-length gene amplification with a long-range PCR followed by shotgun sequencing for mutation analysis was developed by von Bredow et al. Their test achieved satisfactory performance with 96.4% accuracy, 100% precision and an analytical sensitivity of 300 IU/mL with a 20% allele frequency, showing that the implementation of a robust NGS LDT offers greater testing flexibility and sensitivity, accommodating a more diverse patient population [58]. Streck et al. compared NGS to Sanger sequencing and demonstrated two-test agreement for determining antiviral resistance/susceptibility and 88% (22/25) agreement at the level of resistance-associated mutations. The limit of detection of the NGS method was determined to be 500 IU/mL, and the lower threshold for detecting mutations associated with resistance was established at 15% [59]. The feasibility of the ViroKey® SQ FLEX Genotyping Assay was assessed by examining 38 pediatric and 88 adult patient samples. The test proved to be most effective in detecting mutations in samples with a viral load above 1000 IU/mL, and it detected the 10 most important drug-resistant mutations, the most frequent being A594V, found in 5% of all tested samples [60].

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