Research Article

A preliminary study on the UL97 gene of cytomegalovirus isolated from patients suspected of ganciclovir resistance in Sri Lanka

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Abstract

Background: Human cytomegalovirus (HCMV) is an opportunistic virus causing infections in immunocompromised patients. The widespread use of ganciclovir to treat CMV infections has led to the development of ganciclovir resistance. To date, no studies have been reported in Sri Lanka on mutations in viral genes detected from CMV identified by PCR. No Sri Lankan studies have been reported to date of ganciclovir resistance associated CMV gene mutations.

Objective: To screen the CMV UL97 gene identified in a cohort of patients who did not respond to ganciclovir for resistance conferring mutations.

Methodology: A retrospective study was carried out on suspected ganciclovir resistant patients who had static or increased levels of CMV viraemia treated with ganciclovir for 2-3 weeks to determine mutations of UL97 gene. DNA of CMV was extracted; Codons 439 – 645 of the UL97 gene was amplified by PCR only from patients who did not respond to ganciclovir and two fragments of 354bp (1317-1671) and 285bp (1650-1935) were sequenced.

Results: Twelve of the 340 patients with CMV infection had static or increased levels of CMV viraemia despite being treated with ganciclovir for 2-3 weeks. No mutation was found in the UL97 gene detected from the 12 patients which could confer ganciclovir resistance. However, there was an incidental finding of a single nucleotide change from thymine to cytosine at the 1368 nucleotide position of the UL97 gene which was detected in all 12 patients.

Conclusion: No mutation was found in the UL97 gene from CMV patients in this preliminary study which could confer ganciclovir resistance. UL54 gene of CMV should be considered in

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addition to UL97 gene in future studies as mutations reported in both genes are known to contribute to ganciclovir resistance.

Keywords: Human Cytomegalovirus; Ganciclovir resistance; UL97 gene

Introduction

Human Cytomegalovirus (HCMV) is an opportunistic pathogen found in increased abundance in immune compromised patients and is still the most significant infectious pathogen in post-transplant patients, despite being under latest treatment regimes and surveillance.[1] HCMV is a lytic virus which causes a cytopathic effect both *in vitro* and *in vivo.*[2] It is reported to cause high morbidity and mortality during the post-transplant period,[1] accelerate progression to AIDS in HIV patients,[3] cause end-organ disease directly and is also associated with increased graft rejection in solid organ transplant patients.[4]

The double stranded DNA genome of HCMV is the largest of all human herpesviruses, with a very high G + C content. It has the E genome architecture which is distinctive for herpesvirus. The gene UL97 encodes the protein UL97 (pUL97), which is a phosphotransferase related to protein kinases. The pUL97 is a serine/threonine kinase, which is found in mature HCMV virions which can perform autophosphorylation. The amino acid sequence of pUL97 contains conserved serine/threonine kinase functional motifs. Protein UL97 has a wide range of both cellular and viral substrates which are vital for the regulation of viral DNA synthesis and in the anabolism of antiviral nucleoside analogs.[5]

Ganciclovir is a deoxyguanosine analogue that phosphorylates to ganciclovir triphosphate which is a competitive inhibitor for HCMV DNA polymerase.[6] Phosphorylated ganciclovir modifies the DNA structure, which moderates and ultimately terminates replication. It was first approved for medical use in 1988.[7] The molecular mechanisms of ganciclovir resistant CMV demonstrate the importance of the two key viral genes, UL97 coding for a viral phosphotransferase and UL54 coding for the viral DNA polymerase.[8] UL97 mutations found in ganciclovir resistant clinical isolates reduce the phosphorylation of ganciclovir while continuing to maintain normal protein kinase functions of pUL97 without impairing viral replication to a great extent. The important role of pUL97 in phosphorylating ganciclovir is an indication that any mutation that alters or weakens its phosphotransferase function will establish resistance to ganciclovir and cross-resistance to any drug that depends on pUL97 for phosphorylation or uses pUL97 as a viral target.[9]

CMV resistance to ganciclovir has been reported throughout the world and is a rising issue pertinent to transplant patients.[10] In Sri Lanka, ganciclovir is used to treat CMV infections and as prophylaxis, especially in immune compromised patients. To date, no cases of ganciclovir resistance have been reported in Sri Lanka. However, there are CMV infected patients who have been reported as non-responders to ganciclovir, both clinically and virologically. Studies related to mutations in viral genes in CMV or resistance to ganciclovir have not been carried out in Sri Lanka to date. This preliminary study was carried out to determine the presence of mutations in CMV UL97 genes isolated from patients who presented to the Medical Research Institute (MRI), to assess the magnitude of CMV resistance to ganciclovir in Sri Lanka.

Methodology

This preliminary study was designed as a retrospective, molecular epidemiological study. The initial target population of the study were patients who were clinically suspected to have CMV infection in 2019 from whom samples were sent to the MRI for determining CMV viral load. Samples from patients who were positive for CMV and who did not respond to ganciclovir were sequenced to find possible mutations in the UL97 gene.

Sera were separated, and DNA extracted using QIAamp® DNA Mini-Kit (QIAGEN, Hilden, Germany). The CMV viral load was determined using the RealStar® CMV PCR 1.0 (Altona, Germany) *in vitro* diagnostic tests, which is based on real-time polymerase chain reaction (PCR) technology. Of the samples positive for CMV, UL97 gene sequencing was done on ganciclovir resistant patients, who were defined as those who did not respond to ganciclovir for more than two weeks and had static or increased CMV viral loads (more than 1000 IU/ml).[11]

A PCR was performed to amplify a length of 354 and 285 nucleotides in the desired region of the UL97 gene. The forward primer 5'-TGGCCGACGCTATCAAATTT-3' and reverse primer 5'-CCCAGCGCCGACAGCTCCGAC-3' was used to amplify nucleotides 1317-1671 and the forward primer 5'-ATGTCGGAGCTGTCGGCG-3' and reverse primer 5'-CGACACGAGGACATCTTG-3' was used to amplify nucleotides 1650-1935 of the UL97 gene. [12] PCR was carried out in a 25μL reaction containing 5 μL of GoTaq® Reaction Buffer, 0.5 μL of GoTaq® DNA Polymerase, 2.5 μL of $MgCl_2$, 1.0 μL of dNTP Mix, 1.25 μL each of both forward and reverse primers, 2.5 μL of DNA and 11 μL of nuclease free water under the following thermal cycling conditions: initial denaturation at 95 °C for 2 minutes and 45 cycles of denaturation at 95 °C for 20 seconds, annealing at 55 °C for 30 seconds, extension at 72 °C for 30 seconds and a final extension at 72 °C for 4 minutes.[12]

Bi-directional sequencing reactions were carried out with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and resolved with Applied Biosystems®3500Dx capillary sequencer (8 capillaries). Both forward and reverse reactions were carried out for each sample with respective primers. The total reaction volume per reaction was 10.0 μL. Thermal cycler parameters were set according to manufacturer's guidelines.

Results

Of the 1333 samples tested for CMV viral load, 340 (25.5%) patients were positive for CMV with a detectable CMV viral load. Amongst these 340 patients, 12 (3.5%) were suspected to have ganciclovir resistance.

Table 1: Demographic details of the patients suspected of ganciclovir resistance

Patient Type	Age (range)	Number of patients	Gender (M:F)
Fetus/infants with congenital infection	1 – 12 months	12	3:1
Cancer patients undergoing chemotherapy	1 - 15 years	12	3:1
Renal transplant patients	46 - 75 years	12	4:0

Sequencing results of the two fragments amplified showed no variations in the UL97 gene in the 12 patients. Compared to the reference sequence of the UL97 gene of CMV Merlin strain reference

(NC_006273.1),[13] no mutations were observed. However, a single nucleotide change from thymine to cytosine was detected at the 1368 nucleotide position of the UL97 gene in all 12 patients.

Discussion

Previous studies indicate that UL97 is the preferred locus of ganciclovir resistant mutations.[9] Around 95% of ganciclovir resistant CMV strains contain one or more mutations in the UL97 gene[14] with 70% of these strains containing mutations in three specific codons (460, 594, and 595) of the UL97 gene.[14] The 2 regions amplified in the present study covered all the known drug resistance mutations in UL97 (between codon 450 – 640).

Though, ganciclovir is used as prophylaxis and to treat CMV infections in Sri Lanka, there are no previous reports available on CMV resistance to ganciclovir or mutations in viral genes in CMV in Sri Lanka. In this study, of 340 patients who were positive for CMV PCR, 12 were suspected as being ganciclovir resistant, indicating that 3.5% of the CMV infected patients may be ganciclovir resistant.

Previous studies have shown that ganciclovir resistance in CMV is caused by mutations in either the UL97 or UL54 gene.[15] Ganciclovir resistance in the absence of mutations in the UL97 gene at codons 460, 594, and 595 could be due to mutations in the UL54 gene which was not examined in this study. The findings of this preliminary study need to be extended to examine mutations in the UL54 gene. Apart from the presence of mutations in CMV genes, other factors such as host immunity, drug compliance and quality of the drug could also be possible causes of ganciclovir unresponsiveness seen among patients.[16]

Interestingly, the single nucleotide change found at the 1368th nucleotide position (466th codon) of the UL97 gene of CMV detected from all 12 patient samples was an incidental finding of this study. This nucleotide change is not significant as it does not result in the change of amino acid as both codons GAU and GAC code for the same amino acid, aspartic acid, which is found in the HCMV strain AD169 (ATCC VR-538). This suggests that the HCMV strain AD169 is present in Sri Lanka, and this was the first time such strain identification was done. The strain AD169 is not common among the Western HCMV strains and is mostly found in the Asian region.[17]

Conclusion

This single nucleotide change in the UL97 gene of CMV could be used as a genetic marker of CMV present in Sri Lanka. Since all tested 12 samples were identified as strain AD169, this may indicate that it could be the most prevalent CMV strain, present in CMV infected patients in Sri Lanka. However, genotypic tests and a larger sample size are needed to accurately identify the prevalent CMV strain(s) in Sri Lanka. Although this optimized PCR assay could help determine ganciclovir resistance, phenotypic assays are also important to corroborate genotypic results. This study was the first in Sri Lanka to sequence the antiviral gene (UL97) of CMV present in suspected ganciclovir resistant CMV infected patients and to detect mutations in the same antiviral gene (UL97).

The authors find that not studying UL54 is a limitation of the study and it is therefore recommended that mutations in both UL97 and UL54 that are responsible for ganciclovir resistance in CMV are examined the in future studies.

Declarations

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Conflicts of Interest: The authors report there are no competing interests.

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