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Antiviral-resistant cytomegalovirus infections in solid organ transplantation in the Netherlands

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Objectives: Antiviral resistance in cytomegalovirus (CMV) may result from mutations in the molecular targets of antiviral agents. The aim of this study was to investigate both the prevalence of resistance-associated mutations and the factors associated with antiviral resistance in solid organ transplant (SOT) patients with repeated high CMV loads during antiviral treatment.

Methods: SOT patients were selected retrospectively, based on CMV loads of >30000 IU/mL at least twice in a period during which treatment was given. Patient samples were tested for antiviral resistance by Sanger sequencing the UL97 and UL54 genes of CMV, which code for the viral kinase and polymerase. Factors predisposing to and resulting from the development of antiviral resistance mutations were analysed.

Results: Multiple samples from 113 SOT patients were tested, showing resistance-associated mutations in 25 patients (22%). A further 20 (18%) patients showed mutations that were not known to be associated with antiviral resistance. Several factors were associated with development of resistance-associated mutations in UL97 as well as UL54, including human leucocyte antigen (HLA) mismatch, which occurred more frequently in the group of patients with resistance mutations. High-level resistance mutations were most frequently seen in UL97.

Conclusions: This study shows that by selecting patients solely on the basis of virological response to treatment, more patients with antiviral resistance mutations are identified. In this study we confirm findings by other groups that primary infections are associated with resistance development. Moreover, we show that HLA mismatch is associated with the development of antiviral resistance, which suggests a role for host immunity in the development of resistance.

Introduction

Human cytomegalovirus (CMV) infections are important in immunocompromised patients. After solid organ transplantation the virus causes morbidity and mortality, especially if an organ from a CMV-seropositive donor is transplanted into a CMV-seronegative recipient.[1,2] Recipients who are CMV-seropositive prior to transplantation are at risk of reactivations of CMV, due to immunosuppression, but they can also develop superinfections, caused by a different CMV strain present in the transplanted organ.[3] CMV infections following transplantation are associated with end-organ disease such as colitis and pneumonitis, but it has also been shown that graft survival is affected by CMV infections.[4]

First-choice treatment of CMV infections is with either intravenous ganciclovir or its orally administered valine ester, valganciclovir. Because CMV infections are associated with such high morbidity and damage to the transplanted organ, prophylaxis with valganciclovir has been introduced in many protocols, for a duration of several months after transplantation.[5] This tactic is used to prevent or to postpone CMV infections to a time when immunosuppression has been reduced and the patient has a better chance of developing immune control of CMV.[6] Unfortunately, CMV may become resistant to ganciclovir, in which case more toxic second-line treatment has to be used to treat CMV infections, i.e. foscarnet or cidofovir. New antiviral agents with activity against CMV have been developed, such as maribavir and letermovir.

These agents, however, are not universally available yet and have not been included in the most recent consensus guideline for management of CMV infections in solid organ transplant patients.[7] Several factors have been described that are associated with a higher chance of developing antiviral resistance, including type of transplanted organ, suboptimal antiviral dosage, prolonged treatment duration, and sero-discordance with donor seropositivity and recipient seronegativity (D+/R−) transplantation.[1,2,6]

Ganciclovir and valganciclovir require the action of the viral phosphotransferase for their activity, encoded by the UL97 gene. These two agents as well as foscarnet and cidofovir inhibit the viral polymerase, encoded by the UL54 gene. When antiviral resistance is suspected, testing is performed by sequencing these two genes. Several mutations associated with antiviral resistance have been identified.[8–10] The frequencies of resistance development have been studied by different groups and show a range of 5%–10%. However, in most studies patients were selected for resistance testing by clinical suspicion of antiviral resistance, which potentially leads to inclusion predominantly of patients who are relatively ill during the infection. It is less clear whether patients who are in an out-patient setting and who may experience less severe symptoms may also be infected with antiviral-resistant CMV. In this study we show the results of routine sequencing of both UL97 and UL54 of CMV, on the basis of CMV DNA levels, irrespective of the clinical condition of the patient at that time.

Methods

Patient selection and methods

Between January 2010 and June 2015, 3912 solid organ transplantation patients were seen as either in-patients or out-patients, at various points following transplantation. Testing for CMV DNAaemia was performed according to protocol or depending on the clinical condition of the patient. Results were reported in copies/mL, and later translated into IU/mL for this study, based on a standard curve using the WHO international standard (National Institute for Biological Standards and Control, UK). One IU was 0.3 copies (SD 0.028 copies). During the time when the patients had active CMV infections, testing for antiviral resistance was done if the response to antiviral agents was insufficient and the patient had clinical symptoms of CMV disease. For the purpose of this study, samples were selected retrospectively, based on the virological response to treatment. For this, sequencing of the UL97 and UL54 genes was carried out on samples with a CMV load of >30000 IU/mL from patients who had at least two samples above this count, and who were treated for at least 1 week in the period between the two samples. Clinical data were gathered from patients with CMV DNA loads of >30000 IU/mL. CMV viraemia was diagnosed using quantitative real-time PCR in whole blood and plasma, as described previously.[11] In short, the detection was based on a 133 bp fragment of the polymerase gene of CMV, with the addition of a probe to detect all strains (AGT GCA GCC CCG GCC ATC ATT C). The CMV kinase gene UL97 and DNA polymerase gene UL54 were tested using a Sanger sequencing method, by using the HotStarTaq DNA polymerase kit (Qiagen) for amplification. The sequencing reaction was carried out using the amplicons with the BigDye Terminator v3.1 kit (Applied Biosystems) on the T100TM Thermal Cycler (Bio-Rad), after which sequencing was performed using the ABI Prism 3500XL Genetic Analyser (Applied Biosystems). The sequence was subsequently analysed with SeqMan Pro (DNASTAR Lasergene 10) for mutations causing drug resistance for (val-)ganciclovir, cidofovir and or foscarnet.[12] Results were compared with the genome of standard strain AD169 (GenBank 17403) with mutations reported previously.[7] Sequences were classified as WT if there were no differences between the sequence and the comparator. If mutations were previously shown to be associated with antiviral resistance, they were classified as resistance associated (Figure 1). The remaining group, of 'unknown significance', consisted of sequences with mutations that have been previously described as polymorphisms, mutations that have been found before but where there is no conclusive evidence of them being resistance associated, and mutations that have never been reported before.

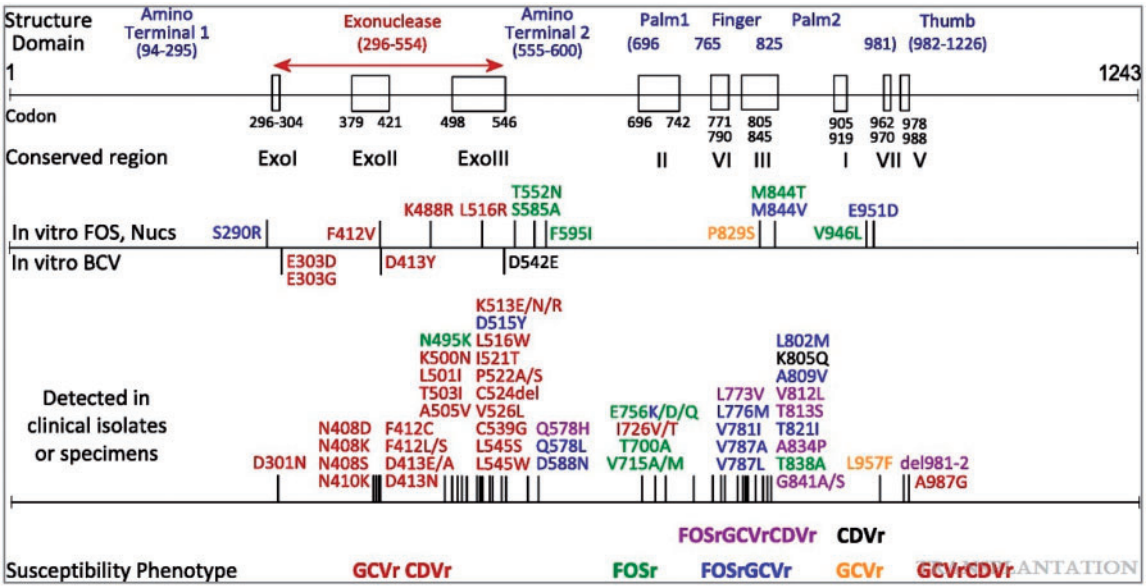

Figure 1. CMV UL54 DNA polymerase gene mutation map. Shown are the structure domains and regions of amino acid sequence conservation in herpesvirus polymerases where resistance mutations are clustered. Corresponding resistance phenotypes are colour coded for the involved drugs. FOS, foscarnet; BCV, brincidofovir; GCV, ganciclovir, CDV, cidofovir; Nucs, nucleoside analogues; r, resistant. Adapted and updated from prior publications. Used with permission of: Kotton C, Kumar D, Caliendo A *et al. Transplantation* 2018; **102**: 900–31.

Statistical analysis

Statistical analysis was performed to establish risk factors causing CMV resistance, including high viral loads, renal function, type of immunosuppression, duration of exposure to antivirals and donor-seropositive and recipient-seronegative (D+/R−) status.[1,13,14] Besides these risk factors, other potential susceptibility factors [e.g. prophylaxis, human leucocyte antigen (HLA) mismatch, age, gender, coinfection with BK virus] were analysed for association with resistance using *t*-tests, χ^2 tests and regression analysis. Linear regression was used to investigate the association between HLA mismatch and maximum CMV load, with log-transformed maximum CMV load as the outcome and number of HLA mismatches as the exposure. All analyses were performed using IBM SPSS Statistics, version 23.

Ethics

All patients in this study participated in the TransplantLines cohort for organ transplantation recipients, and gave written consent to the use of materials and data [Medical Ethical Test Committee (METC) number 2014/077].

Results

A total of 1042 patients had at least one CMV-positive blood sample during the study period. Nearly half of these patients (493; 47%) had only low or moderate CMV loads of <3000 IU/mL. The remaining 549 patients had at least one sample of >3000 IU/mL. Two hundred and eighty patients had at least one blood sample with >30000 IU/mL, of whom 88 patients had at least one sample exceeding 300000 IU/mL.

One hundred and twenty-one patients fulfilled the inclusion criteria of at least two blood samples with >30000 IU/mL taken at least 2 weeks apart, during which treatment was given. In eight patients meeting the inclusion criteria, no sequence was obtained, either owing to insufficient sample or to failed sequencing reaction. These eight patients were excluded from the study. The 113 patients included in the study included 93 recipients of kidney transplants, of whom 1 also received a liver transplant and 1 also received a pancreas. The others were recipients of lungs (n=13), liver (n=4), heart (n=1), combined heart/lung transplant (n=1) and a small bowel transplant (n=1).

In 25 patients (22%) mutations associated with known antiviral resistance were found (Table 1). In 20 patients (18%) mutations compared with WT [standard strain AD169 (GenBank 17403)] sequences were found, but these mutations were not known to be associated with antiviral resistance (Table 2). In 68 patients WT virus was found without mutations. The three groups were regarded as separate groups for analysis.

Factors associated with development of antiviral resistance

Virological factors

Most patients meeting the inclusion criteria had primary CMV infections resulting from CMV sero-discordant transplantations (D+/R−; 79 out of 113 patients, 70%). Thirty-four recipients were CMV seropositive before transplantation; 28 of these recipients had a seropositive donor and 4 received an organ of a CMV-seronegative donor, and in 2 cases the donor serostatus was not known.

In primary CMV infections, antiviral resistance mutations occurred in 28% of cases; 56% of primary infections remained sensitive and 16% showed mutations of unknown significance. CMV infections in seropositive recipients resulted in resistance in only 9% of cases, and 71% remained sensitive. Primary CMV infections were associated with development of antiviral resistance, with an OR of 5.8 (95% CI 1.6–21.2) in comparison with CMV infections in seropositive patients. Viral loads were higher in primary infections than in infections occurring in CMV-seropositive recipients (P=0.012). CMV loads were not significantly higher in patients who developed antiviral resistance compared with the CMV loads in patients who did not develop antiviral resistance (P=0.15). However, antiviral resistance from primary CMV infections developed more frequently in patients with high viral loads compared with CMV infections in seropositive patients (P=0.031).

Prophylaxis was given to all non-kidney transplant patients according to organ-specific protocols. Nine kidney transplant patients also received prophylaxis because they participated in a study in which prophylaxis had to be given. In all 27 patients who received prophylaxis, CMV infections were delayed until after discontinuation of valganciclovir. There were no breakthrough infections during prophylaxis. Five patients who had received prior valganciclovir prophylaxis developed antiviral resistance, whereas 16 patients who received prophylaxis did not develop antiviral resistance (not significant, P=0.8).

Many patients in this study had reduced renal function, with a mean estimated glomerular filtration rate (eGFR) of 52.5 (±4.6 SEM). Reduced eGFR with necessity for lower dosing of antivirals was also not associated with resistance development (P=0.49).

Host factors

The average cumulative HLA mismatch of the patients included in the study was 2.6. The cumulative HLA mismatch was significantly higher for patients who developed antiviral resistance than in patients without antiviral resistance (2 versus 3) (P=0.012). Maximum CMV load was not related to HLA mismatch: for every extra HLA mismatch, the geometric mean maximum CMV load was 0.99 times as high (95% CI 0.85–1.15).

Patients with WT virus had a mean age of 53.3 (±1.8 SEM) years versus 46.2 (±2.1 SEM) years for patients who developed resistant CMV (P=0.02). Younger patients were not more likely to have primary infections. The 20 patients who only had infections with CMV with mutations of unknown significance had a mean age of 52.6 (±3.6 SEM) years (Tables 1 and 2).

Effects of antiviral resistance

Overall mortality of this cohort in the study period was 27%. Mortality for patients with antiviral resistance was not significantly different, i.e. 22% (P=0.34). In the group of patients without mutations mortality was highest, at 34%; however, the difference in mortality between these groups was not significant (P=26). Also, the occurrence of leucopenia, as the most common side effect of (val-)ganciclovir treatment, was not associated with resistance development (P=0.36).

Treatment

CMV disease was treated in all 113 patients initially with oral valganciclovir adjusted to renal function. Twenty-nine patients received intravenous ganciclovir because of poor clinical response to oral valganciclovir (17 with WT virus, 10 with resistant CMV and

Table 1. Characteristics of 25 patients with mutations associated with resistance

Patient no.	Age (years)	M/F	Trans-planted organ	Prophylaxis	Donor/ recipient serostatus	Treatment duration (months)	Treatment	Maximum CMV load (IU/mL)	Mutations of unknown significance (affected gene)	Resistance-associated mutations found	Affected gene	Affected antiviral	Treatment duration before mutation (days)	Infection duration (months)	Cumulative HLA mismatch
1	58	F	kidney	no	+/−	49.5	VGC	1.2×10^6		L595F	UL97	GCV (HL)	64	45.5	2
2[a]	65	F	kidney	no	+/−	8	GCV, FOS	1.1×10^6		C603WL595S	UL97UL97	GCV (HL) GCV (HL)	216	18.5	3
3	71	M	kidney	no	+/−	7	VGC	4.7×10^5	A590V (UL97)	A594P	UL97	GCV (UL)	235	8.5	6
4	52	M	kidney	no	+/−	10	FOS	5.1×10^5		A594VL501F	UL97UL54	GCV (HL) GCV, CDV	148	69.5	3
5[a]	40	F	kidney	no	+/−	5	FOS	8.0×10^5		A594VL501F	UL97UL54	GCV (HL) GCV, CDV	132	7	0
6	52	M	kidney	no	+/−	11.5	VGC	6.5×10^5		A594V	UL97	GCV (HL)	332	19.5	4
7	27	F	kidney	no	+/−	24	GCV	1.5×10^6		M460VL516R	UL97UL54	GCV (HL) GCV, CDV	394	19	3
8[a]	24	F	kidney	no	+/−	4	FOS	1.7×10^6		L595S	UL97	GCV (HL)	138	7	3
9	25	F	kidney	no	+/−	3	VGC	7.6×10^5	D1129N (UL54)	A594V	UL97	GCV (HL)	161	8	3
10	24	M	kidney	no	+/−	12	VGC	1.6×10^6		Del595-597	UL97	GCV (HL)	108	33	3
11	64	M	kidney	no	+/−	16	GCV	9.0×10^5	V321I (UL54)	Del595	UL97	GCV (HL)	124	16	4
12	57	M	kidney	no	+/−	6	VGC	2.0×10^5		M460V A595TA505T	UL97UL97 UL54	GCV (HL) GCV (IL) GCV, CDV	115	12.5	3
13	64	F	kidney	no	+/−	6	VGC	1.8×10^5	L609F (UL97)	A594VT503I	UL97UL54	GCV (HL) GCV, CDV	167	8.5	3
14	55	M	kidney	no	+/−	3.5	GCV	8.3×10^4	R1030C (UL54)G1031A (UL54)	L595S	UL97	GCV (HL)	129	6	3
15	42	F	kidney	no	+/−	7.5	GCV, FOS	2.0×10^5		Del595A987G	UL97UL54	GCV (HL) GCV, CDV	88	7.5	0
16[a]	52	F	kidney	yes	+/−	6	FOS	7.4×10^4		A594VC603RM460I	UL97UL97UL97	GCV (HL) GCV (HL) GCV (HL)	128	4	1
17[a]	24	F	kidney	no	+/−	11.5	FOS	3.4×10^5	L394F (UL54)E877K (UL54)	L595S	UL97	GCV (HL)	115	79	6
18	25	M	kidney	no	+/−	2	GCV	1.4×10^6		L595S	UL97	GCV (HL)	129	16	6
19	48	F	kidney	no	+/−	8	FOS	3.2×10^6		L595F, H469Y	UL97	GCV (HL)	240	11	6
20[a]	54	F	kidney	yes	−/+	6	VGC	1.1×10^6	L609F (UL97)	C603WP522S	UL97UL54	GCV (HL) GCV, CDV	452	3.5	4
21	40	M	kidney	no	+/+	9.5	VGC	5.0×10^5		del597-599P522S	UL97UL54	GCV (HL) GCV, CDV	97	10	2
22	58	M	lung	yes	+/−	8	GCV	1.1×10^5		L595S	UL97	GCV (HL)	244	7.5	NA
23	52	F	lung	yes	+/−	6	GCV	3.2×10^5	T892A (UL54)	L595S	UL97	GCV (HL)	156	38	NA
24	49	F	lung	yes	+/+	8	GCV, FOS	1.6×10^5		L595S	UL97	GCV (HL)	195	4.5	5
25	36	M	heart	no	+/−	9.5	GCV, FOS	7.6×10^5		Del599-603	UL97	GCV (HL)	160	10.5	0

All patients were initially treated with oral valganciclovir (VGC), and were continued on VGC or treatment was switched to ganciclovir (GCV) or foscarnet (FOS). F, female; M, male; HL, high-level resistance; IL, intermediate resistance; LL, low-level resistance; NA, not available.
[a]Patients whose samples were checked for resistance mutations during the clinical illness.

Table 2. Characteristics of 20 patients with mutations of unknown significance

Patient No.	Age (years)	M/F	Transplanted organ	Prophylaxis	D/R serostatus	Treatment duration (months)	Treatment	Maximum CMV load (IU/mL)	Mutations found	Affected gene	Infection duration (months)	Cumulative HLA mismatch
1[a]	75	F	kidney	no	+/−	4	FOS	7.7×10^5	H469Y N510S	(UL 97) (UL 97)	11	5
2	40	M	kidney	no	+/−	35	VGC	1.8×10^5	G374D	(UL54)	54.5	0
3	45	M	kidney	no	+/−	6	VGC	3.1×10^5	T610M	(UL54)	10	NA
4[a]	36	F	kidney	no	+/−	2.5	VGC	2.3×10^5	G1141S	(UL54)	7	3
5	52	F	kidney	no	+/−	7.5	VGC	9.8×10^4	I562T	(UL54)	13	3
6	71	M	kidney	no	+/−	11	VGC	1.1×10^6	A786V	(UL54)	34	5
7	36	M	kidney	no	+/−	1.5	VGC	4.7×10^5	S1126A	(UL54)	7	1
8	80	M	kidney	no	+/−	1	VGC	8.0×10^4	S1114F	(UL54)	7	0
9	68	M	kidney	no	+/−	6	VGC	9.2×10^5	V655A	(UL97)	12	4
10	37	F	kidney/liver	yes	+/−	9	VGC	8.5×10^4	G354D	(UL54)	10.5	5
11	62	M	kidney	yes	+/+	6	VGC	9.5×10^5	Ins686-TS894LP656S	(UL54) (UL54)	43	NA
12	70	F	kidney	no	+/+	2	VGC	2.0×10^5	S612G	(UL54)	7	3
13	63	F	kidney	no	+/+	6.5	FOS	9.9×10^5	H686Y	(UL54)	55	0
14	45	F	kidney	no	+/+	5	VGC	3.1×10^5	G503SG340R	(UL97) (UL54)	8	0
15	39	M	kidney	no	+/+	9	VGC	1.6×10^5	A247TT885M	(UL97) (UL54)	13	4
16	61	M	kidney	no	−/+	6	VGC	1.0×10^6	M526TG895E	(UL97) (UL54)	21	3
17	59	F	lung	yes	+/−	3	GCV	6.1×10^5	G874E	(UL54)	29	NA
18	22	M	lung	yes	+/+	1.5	GCV	4.0×10^5	G687S	(UL54)	1	4
19	39	M	heart	yes	+/−	11	GCV	5.7×10^5	P931S	(UL54)	2.5	4
20	52	F	intestine	yes	+/−	14	VGC	4.8×10^4	P1029L	(UL54)	6.5	5

All patients were initially treated with oral valganciclovir (VGC), and were continued on VGC or treatment was switched to ganciclovir (GCV) or foscarnet (FOS). F, female; M, male; NA, not available.
[a]Patients whose samples were checked for resistance mutations during the clinical illness.

2 in the unknown mutations group). Thirteen patients received foscarnet (10 in the group with resistance, 2 in the group with WT virus and 1 in the group with unknown mutations). Five of the 13 patients had been treated with intravenous ganciclovir before this switch. Fifteen patients with (val-)ganciclovir-resistant CMV never received foscarnet, of whom 3 died during the study period. One of these deaths was attributable to CMV infection. The other patients died of malignancy and *Pneumocystis jirovecii* pneumonia, and in these patients CMV loads were negative at time of death.

Patients who had infections with resistant CMV were treated for a longer period of time than patients with WT virus [mean 9.9 ($\pm$1.8 SEM) months versus mean 6.5 ($\pm$0.8 SEM) months, P=0.02]. Treatment duration was measured as the total period during which antiviral agents were given as treatment, excluding treatment interruptions and prophylaxis. The group of patients with mutations of unknown significance had a mean treatment duration of 7.6 ($\pm$1.6 SEM) months. The infections lasted even longer than the treatment period, because treatment was frequently stopped when clinical response was observed, but while CMV was still detectable. Patients with antiviral resistance had infections with mean duration of 18.8 ($\pm$3.9 SEM) months versus 13.2 ($\pm$1.8 SEM) months for patients with WT CMV infections (P<0.02). Patients with mutations of unknown significance had infections with a mean duration of 12.0 ($\pm$3.8 SEM) months.

The 20 patients with mutations of unknown significance were treated for a shorter duration, and only 3 patients had a clinical disease course, prompting switch of antiviral therapy to something other than valganciclovir. None of these patients died during the follow-up period.

Antiviral resistance was detected on day 179 (mean $\pm$20 SEM) after start of the antiviral treatment.

Resistance mutations found

Twenty-five patients had infections with CMV with decreased susceptibility to antiviral agents (Table 1). Most patients had infections with a virus that was only resistant to ganciclovir, with mutations only in the UL97 gene. Seven were resistant to both ganciclovir and cidofovir. One patient was infected with a virus that only had a mutation in UL54 (Patient 3). The other seven patients had mutations in both UL97 and UL54. Resistance-associated mutations in UL97 most frequently occurred at amino acid positions 595 (n=9) and 594 (n=7). Deletions around amino acid positions 595 and 597 were also frequently found (n=4), and mutations at positions 603 and 460 were detected three times each. Most mutations at these loci result in high-level resistance to ganciclovir, with the IC_{50} increased 5- to 15-fold. The A595T mutation found in Patient 16 results in intermediate-level resistance to ganciclovir, with IC_{50} increased 2- to 5-fold. Patient 5 had the A594P mutation, which has not been previously described. However other amino acid changes at this locus have been shown to lead to either high-level or intermediate-level resistance to ganciclovir.[7]

Although eight patients had mutations in the UL54 gene that are known to be associated with reduced susceptibility to one or

more antiviral agents, only two mutations in this gene were seen in more than one patient. These mutations occurred at amino acid positions 501 and 522, in the exonuclease III domain of the polymerase, resulting in resistance to both ganciclovir and cidofovir.[9]

Twenty patients only had mutations that were not known to be associated with antiviral resistance (Table 2). Nine patients who had resistance-associated mutations also had mutations that were not previously shown to be associated with antiviral resistance. Most of these mutations (n=27) were in the UL54 gene; three were in the UL97 gene. Only one of these mutations of unknown significance occurred more than once. This mutation, which was found in Patients 4 and 19, is at amino acid position 609 of the UL97 gene, in a part of the gene where mutations have been identified that have been associated with antiviral resistance. Both these patients also had resistance-associated mutations in UL97.

Discussion

This study shows the prevalence of antiviral resistance in CMV infections in solid organ transplant patients. The study was carried out in patients who were selected on the basis of repeated high CMV loads (>30000 IU/mL in two samples), which helps explain the relatively high prevalence of resistance-associated mutations (22%). Unfavourable clinical response to valganciclovir and ganciclovir prompted resistance testing in only 6 out of 27 patients and switch to foscarnet in only 10 patients. The systematic UL97 and UL54 sequencing of all patients with repetitive high CMV loads clearly led to the inclusion of more patients than would have been the case if only patients with clinical suspicion of ganciclovir resistance had been tested, and more antiviral resistance was detected as a result.[15] This means that in our study possibly more relatively healthy patients were included, which might explain the finding that antiviral resistance in our study was not associated with higher mortality, contradicting what other researchers have found.[16] Also, in our cohort the patients with antiviral resistance were significantly younger than the patients with WT virus, which could also explain the relatively good outcome in the group with antiviral resistance. The mortality in this group was 22%, which was not significantly different from the mortality in the group of patients without mutations, which was 34% (P=0.26).

Besides the finding that only six patients who met the inclusion criteria had been tested because antiviral resistance was suspected clinically, the most striking result of this study was that 15 patients with resistance mutations did not receive second-line treatment. Although one of these patients died as a result of CMV infection, the infections did not cause life-threatening illness in the majority of patients. The main reason why resistant CMV may not always become a clinical problem can be inferred from the time between start of the CMV infection and the moment when the antiviral resistance was detected, which was 179 (mean ±20 SEM) days after start of treatment. This suggests that even if antiviral resistance is bound to occur, valganciclovir is sufficient to delay clinically relevant infections until months after transplantation, when anti-rejection treatment has been reduced and host immunity against the virus is developing.[17] Other studies also found that ganciclovir resistance occurs quite late in the course of disease.[16] Moreover, some of the mutations found in this study have been previously shown to result in reduced replication fitness.[18,19] Continuing ganciclovir in infections caused by these viruses may therefore still have conferred partial antiviral activity in some of the cases. Reduced replication fitness nevertheless has not been found for all mutations identified in this study, suggesting that host immunity factors determined to a large extent the outcome of the infections in the 15 patients who did not receive antiviral treatment that was effective against the infection.[20] The importance of host immunity in development of CMV antiviral resistance is also suggested by the finding that patients with higher HLA mismatches are more likely to develop resistance. We did not find an association between the highest measured viral loads and resistance development. The reasons for this could be that the patients included in the study all had very high CMV loads, resulting in the selection of a cohort in which all patients have viral loads in the same general range. It is therefore not surprising that patients with higher HLA mismatches did not have significantly higher CMV loads. In our transplantation centre, anti-rejection drugs are adjusted to HLA mismatch only in renal transplantation. The subsequent greater degree of immunodeficiency in these patients could result in the emergence of resistant viruses, because suppression of the virus is more dependent on the antiviral agent than in patients with lower levels of anti-rejection drugs and better immune responses. A link between HLA mismatch and development of antiviral resistance has never been reported before. It is possible that our finding, although statistically significant, is caused by minor confounding factors. A limitation of the study is that we do not have data on related kidney donors and unrelated donors. We believe that related donors could have lower HLA mismatches as well as being infected with the same CMV genotype, which leads to fewer CMV infections after transplantation. This, however, would only be true for seropositive donor/recipient pairs, of which only 28 were included in our study.

Primary CMV infections constituted the majority of the infections in this series of severe CMV infections. In all three groups (WT, resistance mutations and unknown mutations) primary infections made up more than half of the cases. Primary infections, however, were more likely to result in antiviral resistance, which has been shown before by other groups.[7,14]

Undeniably, CMV infections were severe and lengthy in patients with and without antiviral resistance in this study. The infections were usually active over months after transplantation, at a mean of 18.8 (±3.9 SEM) months in patients with resistance-associated mutations and 13.2 (mean ±1.8 SEM) in patients with WT infections (P=0.02). Exposure to antiviral agents was protracted as a consequence: 9.9 (mean ±1.8 SEM) months in the group with resistance and 6.5 (mean ±0.8 SEM) months in the group with WT virus (P<0.02).

In our study, antiviral prophylaxis was not found to be a risk factor for the development of resistance, but because the use of prophylactic valganciclovir was dependent on the transplanted organ we are not able to draw any conclusions from our data. Other studies show conflicting results about prophylaxis as a risk factor.[15,21] Due to the fact that in our study the number of kidney transplant patients was greater than the number receiving other organs, we were not able to attribute higher risk of resistance development to particular organ transplantations.

Most resistance-associated mutations occurred in the UL97 gene, but 30% of resistant CMV had mutations in UL54. These UL54 mutations, which all emerged during valganciclovir treatment, resulted in resistance to ganciclovir and cidofovir in eight

cases. Other authors also found that UL54 mutations occur during valganciclovir treatment.[15,20,22]

The most frequently described UL97 mutations cluster at codons 460, 520 and 590–607. These 'canonical' mutations account for 80% of all cases in previous studies, similar to our result (Figure 1). In our study all 25 patients had at least one of these canonical UL97 mutations.[9,10]

Although the number of UL54 mutations was high at 31, only 8 were known to cause antiviral resistance. These eight all induce resistance to ganciclovir and cidofovir. The vast majority of the UL54 mutations of unknown significance are most likely polymorphisms not causing resistance to antivirals. The data of the patients with these mutations show that their infection and treatment did not differ significantly from those of patients with WT CMV infections. Patient 10 was the only patient in this group in whom antiviral resistance was suspected for clinical reasons. She was tested for antiviral resistance, and treated with foscarnet for reasons of clinical failure on ganciclovir.

In conclusion, this study shows 22% antiviral resistance in a cohort of patients with repetitive high CMV loads while undergoing treatment. Most patients (19 out of 25) with resistance mutations had not been tested for antiviral resistance during their course of disease, and as a result 15 out of 25 were not treated with a definite active antiviral agent. Development of resistance is associated with factors that impact host immunity, including serostatus of the recipient, and HLA mismatch. More research is needed into how host immunity and HLA mismatches contribute to development of antiviral resistance.

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Transparency declarations

None to declare.

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