Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com

Detection of cytomegalovirus drug resistance mutations in solid organ transplant recipients with suspected resistance

Rubén López-Aladid[a], Alba Guiu[a], Gemma Sanclemente[b], Francisco López-Medrano[c], Frederic Cofán[d], M. Mar Mosquera[a], Julián Torre-Cisneros[e], Elisa Vidal[e], Asunción Moreno[b], Jose Maria Aguado[c], Elisa Cordero[f], Cecilia Martin-Gandul[f], Pilar Pérez-Romero[f], Jordi Carratalá[g], Nuria Sabé[g], Jordi Niubó[h], Carlos Cervera[i], Anna Cervilla[a], Marta Bodro[b], Patricia Muñoz[j], Carmen Fariñas[k], M. Gemma Codina[l], Maitane Aranzamendi[m], Miguel Montejo[n], Oscar Len[o], M. Angeles Marcos[a,] *Group for Study of Infection in Transplantation of the Spanish Society of Infectious Diseases Clinical Microbiology GESITRA-SEIMC Spanish Network for Research in Infectious

[a] *Department of Clinical Microbiology, Hospital Clinic, Universidad de Barcelona, Barcelona Institute for Global Health, Barcelona, (ISGlobal), Spain*
[b] *Department of Infectious Diseases, Hospital Clinic, Institut d'Investigacions Biomediques August Pi I Sunyer (IDIBAPS), Universidad de Barcelona, Barcelona, Spain*
[c] *Unit of Infectious Diseases, Instituto de Investigación Hospital 12 Octubre (i + 12) University Hospital 12 de Octubre, Universidad Complutense, Madrid, Spain*
[d] *Nephrology and Renal Transplant Department, Hospital Clinic, Universitat de Barcelona, Barcelona, Spain*
[e] *Clinical Unit of Infectious Diseases, Hospital Universitario Reina Sofia-IMIBIC-UCO, Córdoba, Spain*
[f] *Infectious Diseases Department, Hospital Universitario Virgen del Rocío, Sevilla, Instituto de Biomedicina de Sevilla (IBIS), Unit of Infectious Diseases, Microbiology and Preventive Medicine, University Hospital Virgen del Rocío, Spain*
[g] *Department of Infectious Diseases, Bellvitge University Hospital, IDIBELL, Barcelona, Spain*
[h] *Department of Clinical Microbiology, Bellvitge University Hospital, IDIBELL, Barcelona, Spain*
[i] *Department of Medicine, Division of Infectious Diseases, University of Alberto, Edmonton, Canada*
[j] *Department of Clinical Microbiology and Infectious Diseases, Hospital General Universitario Gregorio Marañón, Instituto de Investigación Sanitario Gregorio Marañón, Madrid, Spain*
[k] *Unidad de Enfermedades Infecciosas, Hospital Universitario Marqués de Valdecilla, Universidad de Cantabria, Santander, Spain*
[l] *Microbiology Service, Hospital Vall dHebron, Spain*
[m] *Servicio de Microbiología, Hospital Universitario de Cruces, Bilbao, Spain*
[n] *Unidad de Enfermedades Infecciosas, Hospital Universitario de Cruces, Bilbao, Spain*
[o] *Department of Infectious Diseases, Hospital Universitari Vall d'Hebrón, Universitat Autónoma de Barcelona, Barcelona, Spain*

ARTICLE INFO

Keywords:
Cytomegalovirus
Suspected resistance
Genotypic analysis
Solid organ transplantation

ABSTRACT

Background: Current guidelines recommend that treatment of resistant cytomegalovirus (CMV) in solid organ transplant (SOT) recipients must be based on genotypic analysis. However, this recommendation is not systematically followed.
Objectives: To assess the presence of mutations associated with CMV resistance in SOT recipients with suspected resistance, their associated risk factors and the clinical impact of resistance.
Study design: Using Sanger sequencing we prospectively assessed the presence of resistance mutations in a nation-wide prospective study between September 2013-August 2015.
Results: Of 39 patients studied, 9 (23%) showed resistance mutations. All had one mutation in the UL 97 gene and two also had one mutation in the UL54 gene. Resistance mutations were more frequent in lung transplant recipients (44% p = 0.0068) and in patients receiving prophylaxis ≥6 months (57% vs. 17%, p = 0.0180). The mean time between transplantation and suspicion of resistance was longer in patients with mutations (239 vs. 100 days, respectively, p = 0.0046) as was the median treatment duration before suspicion (45 vs. 16 days, p = 0.0081). There were no significant differences according to the treatment strategies or the mean CMV load at the time of suspicion. Of note, resistance-associated mutations appeared in one patient during CMV pro-

* Corresponding author at: Department of Clinical Microbiology, Hospital Clinic, Universidad de Barcelona, Institute for Global Health, (ISGlobal), Villarroel 170, Barcelona 08036, Spain.
Email address: mmarcos@clinic.ub.es (M.Angeles Marcos)

http://dx.doi.org/10.1016/j.jcv.2017.03.014
Received 27 October 2016; Received in revised form 2 January 2017; Accepted 16 March 2017
Available online xxx
1386-6532/

phylaxis and also in a seropositive organ recipient. Incomplete suppression of CMV was more frequent in patients with confirmed resistance.

Conclusions: Our study confirms the need to assess CMV resistance mutations in any patient with criteria of suspected clinical resistance. Early confirmation of the presence of resistance mutations is essential to optimize the management of these patients.

1. Background

Cytomegalovirus (CMV) is one of the most important pathogens affecting solid organ transplant (SOT) recipients. In these patients, CMV is a significant cause of morbidity and mortality associated with both invasive disease and modulating effects in the host immune system [1,2].

The development of antiviral agents and preventive strategies over the past decades have significantly improved patient outcomes, but they have also promoted the development of antiviral-resistant CMV strains that can significantly contribute to adverse clinical outcomes [3–6].

Antiviral drug resistance should be suspected when CMV viremia or clinical disease persist in spite of prolonged antiviral therapy [7,8]. However, not all such cases are associated with genomic resistance mutations. Genotypic testing is the routine method for detecting drug resistance and the basis for the selection of alternative therapies [9].

Clinical suspicion of the development of resistance should be assessed early for prompt initiation of the most appropriate therapy. However, virologic resistance is likely underdiagnosed since mutation assessment is not systematically performed.

Several risk factors have been associated with CMV resistance thereby conditioning the inclusion criteria of some studies [10]. The present study was prospectively conducted within the context of the Group for the Study of Infection in Transplantation (GESITRA), constituting one of its major lines of clinical research. The nationwide network of Spanish hospitals has allowed the inclusion of a wide variety of solid organ transplant recipients.

2. Objectives

The aim of this study was to assess the presence of mutations associated with CMV resistance in solid organ transplant recipients with suspected resistance in a nation-wide study, as well as to determine the associated risk factors and the clinical impact of resistance.

3. Study design

3.1. Setting and study population

We conducted a prospective observational study in nine hospitals included in the Spanish Network for Research in Infectious Diseases (REIPI). Adult solid organ transplant patients with suspected resistance to antiviral drugs were included in the study from September 2013 to August 2015. Resistance was suspected on the presence of progressive or stable viral loads or if clinical symptoms persisted despite the use of adequate antiviral treatment for at least 2 weeks [7,8].

The study was approved by the local Ethics Committees of the participating hospitals and was endorsed by GESITRA.

The coordinating center was the Hospital Clinic of Barcelona, which performed the genotypic resistance testing as well as the data analysis. Patient treatment and follow-up were conducted as per the protocol of each center. All patients were treated with standard-dose ganciclovir/valganciclovir (GCV/VGCV) (adjusted to renal function). Monitoring was based on locally quantified plasma CMV PCR tests. No standardization was imposed. On suspicion of resistance, plasma samples were frozen at −80 °C in the respective hospital and sent in batches to the Microbiology Laboratory of Hospital Clínic every 4 months.

Data of interest at the time and beyond for each participant were registered in a clinical database at each participating hospital. The resistance mutations detected were also included in the database CMV infection and disease and acute cellular rejection were defined as previously reported [7,8].

3.2. Microbiological studies

In the coordinating laboratory, extraction of DNA was performed in 500 μl of plasma of each sample using QIAsymphony system (Qiagen, Hilden, Germany). CMV viral load was confirmed by PCR CMV Real Time (Nanogen Advanced Diagnostics, Italy) according to the manufactureŕs instructions.

Genotypic antiviral resistance testing was based on PCR amplification of the CMV UL97 protein kinase gene (codons 400–670) in a single fragment and the UL54 DNA polymerase gene (codons 300–1000) in four fragments followed by Sanger nucleotide sequencing (see Supplementary Material).

3.3. Data management and statistical analysis

Data were registered using the program OpenClinica 3.1 program [copyright (C) 2005–2014, by LLC GNU Lesser General Public License (GNU LGPL)]. Data were analyzed using Stata version 14.1 (Stata Corp., College Station, TX). We used the *t*-test or Wilcoxon rank-sum test to compare continuous variables and the Fisher test to compare proportions. Following univariate analysis, a logistic regression model was constructed as an exploratory analysis to identify independent factors significantly associated with the presence of a mutation. We did multivariate analyses by logistic regression with a stepwise forward model ($p_{in} < 0.05$, $p_{out} < 0.10$ in the likelihood ratio test). Odds Ratios (OR) and 95% confidence intervals (CI) were calculated for factors associated with the presence of mutation. The threshold of statistical significance was $p < 0.05$ (see Supplementary material).

4. Results

During the study period, we enrolled 43 adults who had undergone solid organ transplantation and in whom CMV antiviral resistance was suspected. Four were excluded from the analysis because sequencing could not be carried out due to low CMV viral load. Finally, 39 patients were included. Table 1 shows the baseline characteristics of the study population.

The kidney was the most frequent type of organ transplant (44%) followed by liver transplant (21%). Donor/recipient CMV serostatus was mainly D+/R- (62%) with fewer R+ (39%). More than 20% of patients received depleting anti-lymphocyte antibodies as induction therapy. The most common maintenance immunosuppressant regimen consisted of tacrolimus, mycophenolate and steroids. Mammalian target of rapamycin inhibitors (mTORi) were used in 4 patients (11%). Half of the patients had received post-transplant prophylaxis and the most common schedule was ≤3 months (68%).

Based on genotype testing, 9 out of 39 (23%) patients tested showed a CMV resistance mutation (Table 2). All of these 9 patients

Table 1
Comparative baseline clinical characteristics of the study population.

Baseline characteristic	Total (39)	Without mutations (30)	With mutations (9)	P-value
Age[a]	52 (13) [39]	54 (12) [30]	46 (15) [9]	0.0914[b]
Sex (male)[c]	30(77%)	23 (77%)	7 (78%)	1.0000[d]
Type of Transplant[c]				
Kidney	17 (44%)	15 (50%)	2 (22%)	0.2512[4]
Heart	6 (15%)	5 (17%)	1 (11%)	1.0000
Lung	5 (13%)	1 (3%)	4 (44%)	0.0068
Liver	8 (21%)	6 (20%)	2 (22%)	1.0000
Kidney-pancreas	3(8%)	3 (10%)	0 (0%)	1.0000
CMV serostatus[c]				
D+/R-	24 (62%)	16 (53%)	8 (89%)	0.1152[d]
D+/R+	14 (36%)	13 (43%)	1 (11%)	0.1189
D-/R+	1(3%)	1 (3%)	0 (0%)	1.0000
Induction therapy[3]				
Basiliximab	19 (49%)	17(57%)	2 (22%)	0.1274[d]
Depleting anti-lymphocyte antibodies	9 (23%)	8 (27%)	1 (11%)	0.6542
Initial maintenance[c]				
Cyclosporine	5 (14%)	3(12%)	2 (22%)	0.5716[d]
Tacrolimus	30 (86%)	23(88%)	7 (78%)	1.0000
Mycophenolate mofetil	31(89%)	22 (85%)	9 (100%)	0.1602
Steroids	27 (77%)	22 (85%)	5 (56%)	0.4161
Use of m-tor inhibitors	4 (11%)	2 (8%)	2 (22%)	0.2226
CMV Prophylaxis schedule[c]				
≤3 months	13 (68%)	10 (83%)	3 (43%)	1.0000[d]
≥6 month	6 (32%)	2 (17%)	4(57%)	0.0180

[a] Arithmetic Mean (SD) [n].
[b] t-test.
[c] n (column percentage).
[d] Fisher's exact test.

Table 2
Resistance mutations in the UL97 and UL54 genes.

Patient	Mutation detected UL97	Ratio[a] GCV	Mutation detected UL54	Ratio[a] GCV/POS/CDV
10	A594V	8.3		
19	M460V	8.3		
29		9.2		
30	C592G	2.9		
34	H520Q	10		
35	M460V	8.3	P522A	3/1/4.1
36	L595S	9.2		
38	M460V	8.3		
42	M460I	5	D413A	6.5/0.8/11

[a] IC_{50} of mutant/IC_{50} of wild type.

had one mutation in the UL 97 gene: 8 with a high-level and one patient with a low-level GCV resistance mutation (C592G) [11]. Two patients also had one mutation in the UL54 gene, D413N and P522A respectively; both mutations are associated with high-grade resistance to GCV and cidofovir (CDV), but neither of the two was to POS [9]. Additionally, 5 mutations of unknown significance were detected, all of which were in the UL54 gene (G604S, V679A, Y708H, S895N, L1018P). One UL97 drug-sensitive mutation (N510S) and several UL54 drug-sensitive mutations (S655L, N685S, A885T, S897L, N898D) were also found [11].

The presence of resistance mutations was significantly higher in lung transplant recipients (44% p = 0.0068) (Table 1). Although D+/R- CMV serostatus was more frequently reported among patients with mutations, the differences did not reach statistical significance. In addition one patient in this group was CMV seropositive. Longer prophylaxis (≥6 months) was more frequent in patients with resistance (57% vs. 17%, p = 0.0180).

Moreover, the presence or not of resistance mutations was comparable regarding at onset and beyond the time of resistance suspicion (Table 3). The mean time between transplantation and time of suspicion was longer in the group with resistance mutations (239 vs. 100 days,respectively, P = 0.0046), as was the median treatment duration before suspicion of resistance (45 vs. 16 days, p = 0.0081). There were no significant differences between the two groups in the treatment strategies at the time of suspicion of resistance, the mean CMV load or the presence of symptoms at suspicion. CMV disease was diagnosed in 49% of patients although biopsy confirmation was achieved in only 3 patients. Viral syndrome was the most frequent clinical presentation. All the patients were receiving standard doses GCV/VGCV doses at the time of suspicion and clinicians empirically modified the treatment accordingly. Patients without mutations mainly received increased doses of GCV or VGCV (73%, p = 0.0153), while in patients with mutations mTORi was added or switched to (67%, p = 0.0039). According to the outcome at 3 months, patients with resistance more frequently had incomplete suppression of CMV replication (67% vs. 30%, p = 0.0631). However, there were no differences in the development of graft rejection (not directly related to CMV infection) or mortality. Of the variables associated with the presence of resistance mutations in the univariate analyses, lung transplantation was the only variable independently associated with increased risk for this mutation (OR 23.20, 95% CI 2.13–252.69) (see Supplementary Material).The area under the ROC curve (AUC) for the multivariate model was 0.71 (95% CI, 0.48–0.93) (see Supplementary material).

Table 4 shows the clinical and virological data of SOT recipients with known CMV resistance mutations.

5. Discussion

The detection of drug resistance in CMV has important implications for patient management, as resistance can have an impact on morbidity and graft survival in transplant recipients [12]. However, clinical (clinical profile) and virologic resistance (laboratory evidence) are not always linked [13].

In our study, only 23% of the patients with suspected resistance showed mutations associated with resistance confirmed by genotypic methods, thereby emphasizing the relevance of antiviral resistance testing whenever resistance is suspected. Unfortunately, it is not widely available and usually cases are presumed to be CMV drug-resistant based only on the lack of clinical and virological response to therapy [14].

In the present study, the presence of mutations was associated with lung transplantation, prophylaxis ≥6 months, a higher mean time between transplantation and suspicion of resistance and longer previous treatment with GCV or VGCV. However, the 9 patients with confirmed

Table 3
Comparison of the data at the onset and beyond the suspicion of resistance.

Characteristic	Total (39)	Without mutations (30)	With mutations (9)	P-value
Transplant and time of suspicion (days)[5]	118 (169)[39]	100 (145) [30]	239 (204) [9]	0.0046[6]
Days of therapy before suspicion[5]	17 (23) [39]	16 (8) [30]	45 (58) [9]	0.0081[6]
Treatment at the time of suspicion[3]				
Prophylaxis	1 (3%)	0	1 (11%)	0.2308[4]
Pre-emptive treatment	26 (67%)	21 (70%)	5(56%)	0.4472
Disease treatment	12 (31%)	9 (30%)	3(33%)	1.0000
CMV viral load at the time of suspicion(c/ml)[1]	147.836 (515.419) [39]	178.870 (585.809) [30]	44.390 (47.105) [9]	0.4997[2]
Symptoms at moment of suspicion[3]				
Viral syndromea	19 (49%)	14 (47%)	5 (56%)	1.000[4]
Gastrointestinal disease	9	8	1	
Pneumonitis	6	4	2	
Hepatitis	2	1	1	
Disseminated (>1 end organ)	1	1	0	
	1	0	1	
Strategy on suspicion[3]				
Increase dose GCV/VGCV alone	24 (62%)	22 (73%)	2 (22%)	0.0153[4]
Switch or add foscanet	8 (21%)	4(13%)	4 (44%)	0.0651
Switch or add mTORi	10 (26%)	4(13%)	6 (67%)	0.0039
Viral Response[3]				
Total suppression of CMV replication	24 (62%)	21(70%)	3 (33%)	0.0631[4]
Incomplete suppressión	15 (38%)	9 (30%)	6 (67%)	
Final outcome[3]				
Graft rejection	5 (13%)	3 (10%)	2 (22%)	0.5716[4]
Dead	3 (8%)	2 (7%)	1 (11%)	0.5558

[a]Presence of fever >38 °C (for at least 2 days in a 4 day period) associated with leucopenia, thrombocytopenia or increase in transaminases.

[1] Median (IQR)[n].
[2] Wilcoxon Rank Sun test.
[3] n (column percentage).
[4] Fisher's exact test.
[5] Arithmetic Mean (SD) [n].
[6] *t*-test.

resistance presented one or more risk factor to develop resistance described in other studies (10).

One of the most important findings of this study is that the development of GCV resistance is associated with prolonged use of GCV or VGCV. It is well known that drug resistance generally occurs after lengthy drug exposure with incomplete viral suppression, resulting in increasing viral load or disease despite therapy [7,11]. In the present study, patients with confirmed CMV resistance mutations had received longer antiviral treatment than patients without mutations. On the other hand, patients receiving antiviral prophylaxis for ≥6 months more frequently presented confirmed GCV-resistant CMV than those who received GCV less than three months. Although previous studies have argued that extending VGCV prophylaxis from 100 days to 200 days did not significantly affect the incidence of GCV resistance, the results of our study suggest the opposite [15]. During CMV prophylaxis the incidence of GCV resistance is low [4], but it should not be ignored as shown in one of the patients studied. We observed that lung transplant recipients had the highest incidence of GCV resistance in accordance with previous reports [10]. Although it has been proposed that this may be associated with a specific immunosuppression protocol risk, local organ CMV load, or anatomical factors, it is important to note that lung transplant patients receive prophylaxis for a longer period of time. Four out of 5 patients who had undergone lung transplantation in our study presented resistance (80%) and all had received long antiviral prophylaxis of between 6 and 12 months as recommended by clinical guidelines [7,8].

The other group of patients with a higher risk of GCV resistance was that of CMV D+/R- organ recipients [16]. Although not statistically significant, probably due to the low number of patients included in the study, 89% of patients with mutations associated with resistance were D+/R-. However, resistance can also occur in seropositive organ recipients, as observed in one patient in the present study.

Similar to other reports, the use of anti-lymphocyte antibodies as induction therapy was not associated with the emergence of resistance in our study, but this may be due to the low number of patients included [13]. Surprisingly, we did not observe significant differences in CMV viral load at the time of suspicion between the two groups of patients. This result contrasts with previous studies, in which higher viral loads were a risk factor for GCV resistance [4]. Thus, it is important to suspect GCV resistance when viral load increases or does not decrease independently of viral load.

Although clinical risk factors for drug resistance are becoming better defined, resistance should be confirmed by diagnostic laboratory testing in order to support therapeutic decisions related to the switching to therapies with potential adverse effects [7,8]. In many instances, empiric treatment is used until genotypic analysis results are available. Some mutations associated with low levels of resistance can be overcome by increasing doses of GCV [17]. This probably explains why doses of GCV were increased on suspicion of resistance. As plasma GCV levels were not available, it cannot be ruled out that suboptimal drug levels were maintained for prolonged periods. However, the only patient with a low-grade mutation in our study did not respond to high doses of GCV, and thus, other factors likely contributed to treatment failure, as previously suggested [18]. In addition, neither can we rule out the possible accumulation of different mutated strains under antiviral selective pressure not detected by Sanger sequencing [9,19,20].

Increasing evidence is available regarding the antiviral effects of mTORi; these drugs have also been successfully used in the management of SOT recipients with resistance mutations [21]. Interestingly, three of 4 patients with mutations not receiving mTORi (patients 10, 38 and 42) presented bad prognosis.

The consequences of drug-resistant CMV strains may vary broadly from asymptomatic viremia to tissue-invasive disease and unfavorable clinical outcomes [5]. Incomplete suppression was more frequent in patients with virological resistance, which may be a risk factor for the de-

Table 4
Clinical and virological data in solid organ transplant recipients with a cytomegalovirus resistance mutation.

Patient	Type of Transplant (CMV serostatus)	Induction therapy	Prophylaxis received (months)	Treatment at the time of suspicion[a] (days of therapy)	CMV mutation (days post-TX)	CMV viral load at the time of suspicion (copies/ml)	Symptomatic at the time of suspicion	Therapeutic adaptation at the time of suspicion	Outcome[b]
10	Liver (D+/R−)	No	0	Preemptive therapy. VGCV 450 mg/12 h (15d)	UL97: A594 V (63 d)	9.300 c/ml	No	Increased GCV	Total suppression Cell-mediated rejection
19	Kidney (D+/R+)	Basiliximab	1M	Disease VGCV 450 mg/12 h (60d)	UL97: M460 V (235 d)	11.234 c/ml	Viral syndrome[c] Suspected gastritis	Increased GCV/ Everolimus/POS	Incomplete suppression, successive reactivations
29	Heart (D+/R−)	No	3M	Preemptive therapy GCV 2.5 mg/Kg/12 h (84d)	UL97: L595S (350 d)	154.486 c/ml	No	increased GCV	Incomplete suppression, successive reactivations
30	Kidney (D+/R−)	D.A.L	3M	Disease VGCV 450 mg/12 h (45)	UL97: C592G (102 d)	11.605 c/ml	Suspected colitis	Increased GCV/Sirolimus	Incomplete suppression, successive reactivations
34	Lung (D+/R−)	No	6 M	Prophylaxis VGCV 900 mg/24 h (180d)	UL97: H520Q (210 d)	33.772 c/ml	Confirmed pneumonitis	POS/Sirolimus	Total resolution
35	Lung (D+/R−)	No	7M	Preemptive therapy VGCV 900 mg/12 h (20d)	UL97:M460 V UL54: P522A (235 d)	68.494 c/ml	No	POS/Sirolimus	Total resolution
36	Lung (D+/R−)	No	6M	Preemptive therapy GCV 10 mg/Kg/12 h (26)	UL97: L595S (210 d)	38.700 c/ml	No	Increased GCV/ Everolimus	Total resolution
38	Liver (D+/R−)	No	0	Disease VGCV 900 mg/12 h (45d)	UL97: M460 V (340 d)	11.375 c/ml	Confirmed pneumonitis and retinitis	VGCV/POS	Incomplete suppression Death
42	Lung (D+/R−)	Basiliximab	12M	Preemptive therapy GCV 7.5 mg/Kg/12 h (200d)	UL97: M460I UL54: D413N (400 d)	2.800 c/ml	No	Increased GCV/ Leflunomide	Incomplete suppression Cell-mediated rejection

CMV, cytomegalovirus; D, donor; R, recipient; TX, transplantation; GCV, ganciclovir. VGCV, valganciclovir; POS, foscarnet; D.A.L. depleting anti-lymphocyte antibodies.

[a] Drug/doses.

[b] Outcomes (CMV replication, viral response, rejection and mortality) at 3 months.

[c] Presence of fever >38 °C (for at least 2 days in a 4 day period) associates with leucopenia, thrombocytopenia or increase in transaminases.

velopment of multidrug resistance or a worse evolution. Nonetheless, we were only able to analyze patient data up to 3 months after the suspicion of resistance.

In our study, genotypic testing showed that the 9 patients with resistance mutations presented one of the "canonical" mutations in the viral UL97 kinase gene [11]. In addition, mutations in the UL54 gene were detected in 2 lung transplant patients. While UL97 mutations generally anticipate UL54 mutations and confer only GCV resistance, CMV strains with mutations in both UL97 and UL54 have higher levels of phenotypic resistance *in vitro*, indicating that they might have an additive effect on the GCV susceptibility of an isolate, with significant clinical repercussions. In our study, of the 2 patients with mutations in the UL54 gene, one presented cellular rejection but the other showed total resolution. Furthermore, mutations in UL54 induced by therapy with one antiviral agent can confer cross-resistance to other antiviral agents used to treat CMV even without previous exposure [22], as has been demonstrated in our study. An ongoing difficulty with interpretation of UL54 mutations is the relatively frequent occurrence of natural sequence polymorphisms of unknown significance. In our study we found 5 of these polymorphisms, making the characterization of the resistance phenotype of previously unrecognized mutations essential for interpretation of genotypic data [23].

In the cohort of patients studied, 77% had no evidence of resistance mutations. We used Sanger sequencing with which a mutation can only be detected if it is present in more than 20% of the virus population. Hence, it is possible that mutations in minority virus variants at the time of sampling remained undetected, and only deep sequencing techniques can fully describe the kinetics of the emergence of resistant mutations [24,25].

The limitations of the present study include the modest sample size and the groups are not well balanced. Thus our results should be confirmed in a large and well balanced cohort. Another of the main limitations of this study is that the analysis of GCV resistance of the mutations was performed only when suspected. We performed a multicenter study to cover the diversity of clinical and virological practices across Spanish centers without exclusion criteria. We included only patients not responding to therapy despite adequate antiviral treatment since these patients represent the group with the highest risk of antiviral-resistant CMV, avoiding unnecessary testing of other patients.

In conclusion, our study confirms the need to assess CMV resistance mutations in any patient with criteria of suspected clinical resistance. Early confirmation of the presence of resistance mutations is essential to optimize the management of these patients.

Funding

This study was supported by Health Research Fund (FIS), Department of Health, Spain; Agency for Health Technology Assessment and Research Supported by Ministerio de Economía y Competitividad, Instituto de Salud Carlos III (PS12/02131).

Competing interests

None declared.

Ethical approval

The study was approved by the local Ethics Committees of participating hospitals (2012/7598) and was endorsed by Group for the tudy of Infection in Trasplantation (GESITRA).

Acknowledgments

We thank Sergi Sanz and Susana Mendez(Head Biostatistics & Data Management Unit. Barcelona Institute for Global Health) and Albert Gabarrus (Statistician in Applied Research in Respiratory Diseases, IDIBAPS, UB, CIBERES) for their statistical analysis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcv.2017.03.014.

References

[1] J.A. Fishman, Infection in solid-organ transplant recipients, N. Engl. J. Med. 357 (2007) 2601–2614.
[2] V.C. Emery, Investigation of CMV disease in immunocompromised patients, J. Clin. Pathol. 54 (2001) 84–88.
[3] A.P. Limaye, L. Corey, D.M. Koelle, C.L. Davis, M. Boeckh, Emergence of ganciclovir-resistant cytomegalovirus disease among recipients of solid-organ transplant, Lancet 356 (2000) 645–649.
[4] L. Couzi, S. Helou, T. Bachelet, K. Moreau, S. Martin, D. Morei, M.E. Lafon, B. Boyer, S. Alain, I. Garrigue, P. Merville, High incidence of anticytomegalovirus drug resistance amongD + /R − kidney transplant recipients receiving preemptive therapy, Am. J. Transplant. 12 (2012) 202–209.
[5] G. Bouvin, N. Goyette, H. Rollag, A.G. Jardine, M.D. Pescovitz, A. Asberg, J. Ives, A. Hartmann, A. Humar, Cytomegalovirus resistance in solid organ transplant recipients treatd with intravenous gangiclovir or oral vangalciclovir, Antiv Ther. 14 (2009) 697–704.
[6] A.J. Eid, S.K. Arthus, P.J. Deziel, M.P. Wiilhelm, R.R. Razonable, Emergence of drug-resistant cytomegalovirus in the era of vanganciclovir prophylaxis:therapeutic implications and outcome, Clin. Transpl. 22 (2008) 162–170.
[7] C.N. Kotton, D. Kumar, A.M. Caliendo, A. Asberg, S. Chou, L. Danziger-Isakov, A. Humar, Updated international consensus guidelines on the management of cytomegalovirus in solid-organ transplantation, Transplantation 96 (2013) 333–360.
[8] J. Torre-Cisneros, J.M. Aguado, J.J. Caston, L. Almenar, A. Alonso, S. Cantisán, J. Carratalá, C. Cervera, E. Cordero, M.C. Fariñas, M. Fernández-Ruiz, J. Fortún, E. Frauca, J. Gavaldá, D. Hernández, I. Herrero, O. Len, F. Lopez-Medrano, N. Manito, M.A. Marcos, P. Martín-Dávila, V. Monforte, M. Montejo, A. Moreno, P. Muñoz, D. Navarro, P. Pérez-Romero, A. Rodriguez-Bernot, J. Rumbao, R. San Juan, J.M. Vaquero, E. Vidal, Spanish Society of Transplantation (SET), Spanish Society of Transplantation (SET), Group for Study of Infection in Transplantation of the Spanish Society of Infectious Diseases and Clinical Microbiology (GESITRA-SEIMC), Spanish Network for Research in Infectious Diseases (REIPI), et al., Management of cytomegalovirus infection in solid organ transplant recipients: SET/GESITRA-SEIMC/REIPI recommendations, Transpl. Rev. 30 (2016) 119–143.
[9] M. Hakki, S. Chou, The biology of cytomegalovirus drug resistance, Curr. Opin. Infect. Dis. 24 (2011) 605–611.
[10] A.K. Le Page, M.M. Jager, J.M. Iwasenko, G.M. Scott, S. Alain, W.D. Rawlinson, Clinical aspects of cytomegalovitus antiviral resistance in solid organ transplant recipients, Clin. Infect. Dis. 56 (2013) 1018–1029.
[11] N.S. Lurain, S. Chou, Antiviral drug resistance of human cytomegalovirus, Clin. Microbiol. Rev. 23 (2010) 689–712.
[12] A.P. Limaye, L. Corey, D.M. Koelle, C.L. Davis, M. Boeckh, Emergence of ganciclovir-resistant cytomegalovirus disease among recipients of solid-organ transplants, Lancet 356 (2000) 645–649.
[13] S. Hantz, F. Garnier-Geoffroy, M.C. Mazeron, I. Garrique, P. Merville, C. Mengele, L. Rostaing, F. Saint Marcoux, M. Essig, J.P. Rerolle, S. Cotin, R. Germi, S. Pillet, Y. Lebranchu, P. Turlure, S. Alain, French CMV Resistance Survey Study Group, Drug-resistant cytomegalovirus in transplant recipients: a French cohort study, J. Antimicrob. Chemother. 65 (2010) 2628–2640.
[14] R. Vaz, F. Barros, I. Tavares, M. Bustorff, I. Ferreira, M. Pestana, Ganciclovir-resistant cytomegalovirus infection in renal transplantation, Clin. Kidney 7 (2014) 210–213.
[15] G. Bouvin, N. Goyette, M. Farhan, J. Ives, R. Elston, Incidence of cytomegalovirus UL97 and UL54 amino acid substitutions detected after 100 or 2000 days of vanganciclovir prophylaxis, J. Clin. Virol. 53 (2012) 208–213.
[16] H.A. Myhre, D.H. Dorenberg, K.I. Kristiansen, et al., Incidence and outcomes of ganciclovir-resistant cytomegalovirus infections in 1244 kidney transplant recipients, Transplantation 92 (2011) 217–223.
[17] I. Gracia-Ahufinger, J. Gutiérrez-Aroca, E. Cordero, E. Vidal, S. Cantisán, D. del Castillo, C. Martin-Gandul, A. Rivero, J. Torre-Cisneros, Use of high-dose ganciclovir for the treatment of cytomegalovirus replication in solid organ transplant patients with ganciclovir resistance inducing mutations, Transplantation 95 (2013) 1015–1020.
[18] A.P. Limaye, Ganciclovir resistant cytomegalovirus in organ transplant recipients, Clin. Infect. Dis. 35 (2002) 866–872.

[19] S. Sijmons, M. Van Ranst, P. Maes, Genomic and functional characteristics of human cytomegalovirus revealed by next-generation sequencing, Viruses 6 (2014) 1049–1072.
[20] J.M. Iwasenko, G.M. Scott, A.R. Naing, A.R. Glanville, W.D. Rawlinson, Diversity of antiviral-resistant human cytomegalovirus in heart and lung transplant recipients, Transpl. Infect. Dis. 13 (2011) 145–153.
[21] N. Sabé, J. González-Costello, I. Rama, J. Niubó, M. Bodro, J. Roca, J.M. Cruzado, N. Manito, J. Carratalá, Successful outcome of ganciclovir-resistant cytomegalovirus infection in organ transplant recipients after conversión to mTOR inhibitors, Transpl. Int. 25 (2012) e78–82.
[22] S. Chou, N.S. Lurain, K.D. Thompson, R.C. Miner, W.L. Drew, Viral DNA polymerase mutations associated with drug resistance in human cytomegalovirus, J. Infect. Dis. 188 (2003) 32–39.
[23] S. Chou, G. Boivin, J. Ives, R. Elston, Phenotypic evaluation of previously uncharacterized cytomegalovirus DNA polymerase sequence variants detected in a valganciclovir treatment trial, J. Infect. Dis. 209 (2014) 1219–1226.
[24] B. Schindele, I. Apelt, J. Hofmann, A. Nistsche, D. Michel, S. Voigt, Improved detection of mutated human cytomegalovirus UL97 by pyrosequencing, Antimicrob. Agents Chemother. 54 (2010) 5234–5241.
[25] S. Chou, R.J. Ercolani, M.K. Sahoo, M.I. Lefterova, L.M. Strasfeld, B. A: Pinsky, Improved detection of emerging drug-resistant mutant cytomegalovirus subpopulations by deep sequencing, Antimicrob. Agents Chemother. 58 (2014) 4697–4702.