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Genotypic resistance of cytomegalovirus to antivirals in hematopoietic stem cell transplant recipients from Portugal: A retrospective study

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ABSTRACT

The aim of this study was to characterize Human Cytomegalovirus (HCMV) drug resistance mutations in *UL97* and *UL54* genes in allogeneic hematopoietic stem cell transplant (allo-HSCT) recipients in Portugal. We have performed a retrospective study with 22 patients from a cohort of patients with different haematological malignancies submitted to allo-HSCT between 2010 and 2014. Patients were selected according to clinical and laboratory data of HCMV infection and management. HCMV resistance mutations were characterized by sequencing of *UL97* and *UL54* genes. Sequence data were compared with: 1) HCMV genome reference strain AD169; and also 2) *UL97* from Merlin strain (GenBank: AY446894.2), and *UL54* from TB40/E strain (GenBank: ABV71585.1). Resistance mutations were identified in seven patients (32%): five harboured resistance mutations in *UL97*: A594V (n = 2), C592G (n = 1), L595W (n = 1), and C603W (n = 1); and two harboured resistance mutations in *UL54*: P522S and L957F, one in each patient. Several natural polymorphisms and unknown mutations were found in both *UL97* and *UL54*, with the majority of the patients harbouring more than one unknown mutation in *UL97* but only one in *UL54*. No simultaneous mutations were found. This is the first study in Portugal to characterize HCMV *UL97* and *UL54* sequences and to identify HCMV drug-resistance mutations in allo-HSCT patients. The *UL97* resistance mutations found were amongst the most frequent resistant mutations, while *UL54* L957F mutation was here reported for the first time in a clinical specimen. This information provides important information regarding HCMV strains and antiviral resistance in our population.

1. Introduction

Human cytomegalovirus (HCMV) infection is a major cause of morbidity and mortality in patients submitted to hematopoietic stem cell transplant (HSCT) (Ljungman et al., 2011; Sousa et al., 2014). Currently, prophylactic and preemptive therapies are the two major strategies used to prevent infection and/or progression of HCMV disease. Four antiviral drugs are currently used to inhibit effective HCMV DNA synthesis: ganciclovir (GCV) or its oral prodrug valganciclovir (VGCV), cidofovir (CDV), and foscarnet (FOS) (Biron, 2006; Schreiber et al., 2009). The results of HCMV treatment can be influenced by several factors such as high viral load at the start of therapy, prolonged antiviral therapy, subtherapeutic doses of antiviral due to poor compliance or low drug absorption, underlying disease, and intensity of immunosuppression (Baldanti et al., 2004; Eckle et al., 2002). Furthermore, the emergence of

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Abbreviations

allo-HSCT	allogeneic hematopoietic stem cell transplant
CDV	cidofovir
D	donor
D+	day post-transplant
DOI	duration of infection
FOS	foscarnet
GCV	ganciclovir
GVHD	graft-versus-host disease
HCMV	human cytomegalovirus
HLA	human leukocyte antigen
HSCT	hematopoietic stem cell transplant
MRA	Mutation resistance analyser
PCR	polymerase chain reaction
pUL54	viral DNA polymerase
pUL97	viral phosphotransferase
R	recipient
SOT	solid organ transplant
TTI	time to infection
VGCV	valganciclovir

antiviral resistance mutations may contribute to treatment failure and progression of HCMV disease (Chou, 1999, 2015; Deback et al., 2015; Gohring et al., 2015).

HCMV antiviral drug resistance has been found in 1.7%–5.1% of HSCT recipients with an attributable mortality of almost 2% (Choi et al., 2014; Drouot et al., 2014; Hantz et al., 2010; Shmueli et al., 2014). The molecular mechanisms of HCMV resistance to antiviral drugs rely on the presence of mutations within the viral phosphotransferase pUL97 (product of *UL97* gene) that plays a major role in GCV activation, and in the viral DNA polymerase pUL54 (product of *UL54* gene) that constitutes the target of all current drugs (Campos et al., 2016; Chou, 1999; Hakki and Chou, 2011). These mutations confer various levels of resistance, and whereas *UL97* mutations are able to confer resistance only to (V)GCV and are confined to a relatively small genomic region, mutations in *UL54* can confer resistance to all current drugs and are clustered over a much larger area (Campos et al., 2016; Chou, 2010; Deback et al., 2015). Although the majority of *UL54* mutations that confer resistance to GCV also confer resistance to CDV and/or FOS, other mutations confer resistance only to GCV, CDV, or FOS (Campos et al., 2016; Foulongne et al., 2004; Hakki and Chou, 2011; Kleiboeker et al., 2014).

Studies have demonstrated that in allogeneic HSCT (allo-HSCT) patients, HCMV antiviral drug resistance is frequently associated with mutations in *UL97* (Drouot et al., 2014; Hamprecht et al., 2003; Marfori et al., 2007) while rare in *UL54* (Gohring et al., 2013; Gregg et al., 2014). Furthermore, a large number of new mutations of unknown clinical significance have been identified in either clinical or laboratory isolates (Komatsu et al., 2014). Despite several studies have described the prevalence and characterized HCMV resistance mutations in allo-HSCT, it is still necessary to conduct more studies especially in centres with high number of transplantations. Hence, with this study, we aimed to characterize HCMV *UL97* and *UL54* resistance mutations in allo-HSCT recipients from our hospital in Portugal.

2. Patients and methods

2.1. Study population

We performed a retrospective study in a cohort of patients with different haematological malignancies submitted to allo-HSCT between 2010 and 2014 at the *Bone Marrow Transplant Service* from the *Portuguese Oncology Institute of Porto (IPO Porto)*.

All patients were monitored for HCMV infection as previously described (Sousa et al., 2014). Preemptive therapy with (V)GCV was chosen as first-line treatment: oral VGCV (900 mg/12 h) or intravenous GCV (5 mg/kg/12 h), in the context of gastrointestinal intolerance. Patients with (V)GCV-associated complications (neutropenia, nausea/vomiting) or with no change in viral loads for at least 2 weeks were treated with FOS (60 mg/kg/8 h) as a second-line treatment. Preemptive treatment was given for 2–3 weeks or until HCMV negativity was reached. Maintenance therapy was initiated after two consecutive negative results for HCMV with VGCV (450 mg/d) or GCV (5 mg/kg/d) or FOS (90 mg/kg/d) during 5 days per week, at least until D+100 and even longer in high-risk patients. Antiviral doses were adapted to patient's renal function.

Patients were selected for HCMV resistance mutation analysis if at least one of the following criteria was satisfied: 1) HCMV reactivation prior to 30 days after transplant; 2) viral load at first positive detection >5 antigen-positive cells per 5.0×10^4 polymorphonuclear leukocytes by pp65 antigenemia or $>10^4$ copies/mL in whole blood by quantitative real-time polymerase chain reaction (PCR); 3) HCMV positivity lasting at least one month; and 4) no significant decrease of HCMV load after 2 weeks of antiviral treatment.

The study was approved by the local ethical committee (CES IPO: 73/2015) and did not interfere with routine procedures decided by clinicians.

2.2. Data collection

Patients demographic characteristics (gender, age), clinicopathological data (underlying diseases, stem cell source, Human Leucocyte Antigen (HLA) match, conditioning regimen, and donor and recipient HCMV serostatus), and treatment information (type of antiviral: GCV, VGCV, FOS or CDV; dose and duration) were collected from individual clinical records by a clinician. HCMV infection data (time to infection and duration of infection) were collected from clinical records as previously described (Sousa et al., 2014).

2.3. HCMV antiviral drug resistance genotyping

Nucleic acid extraction was performed from peripheral blood samples using *Magna Pure Compact Nucleic Acid Kit* (Roche, Indianapolis, USA). HCMV genotypic antiviral resistance was performed with nested PCR protocols for *UL97* and *UL54* adapted from literature (Boutolleau et al., 2009, 2011) using *KAPA Hifi HotStart DNA Polymerase* (Kapabiosystems, Boston MA, USA). The PCR products resulting from the nested PCR were purified with the *Qiagen QIAquick Gel Extraction Kit* (Qiagen, Hilden, Germany) and used for sequencing. Sequencing was performed with overlapping primer pairs (Boutolleau et al., 2009, 2011) using the *Prism Big Dye® Terminator Cycle Sequencing Ready Reaction kit* (Life Technologies, Foster City CA, USA) and the automated sequencer *ABI Prism® 3730 Genetic Analyzer* (Life Technologies, Foster City CA, USA).

2.4. HCMV sequence analysis

The sequence data were analysed using the *4Peaks for Mac OS X 10.3* version 1.7.2 freeware program available at http://nucleobytes.com/index.php/4peaks. Sequence data were compared with the HCMV genome reference strain AD169 (GenBank accession No: BK000394) using SeqScape v2.5 software (Applied Biosystems, Foster City CA, USA). Mutations were reported as resistant