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Genetic diversity of the human adenovirus species C DNA polymerase

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ABSTRACT

Background: Human Adenovirus (HAdV) are responsible for severe infections in hematopoietic stem cells transplant (HSCT) recipient, species C viruses being the most commonly observed in this population. There is no approved antiviral treatment yet. Cidofovir (CDV), a cytidine analog, is the most frequently used and its lipophilic conjugate, brincidofovir (BCV), is under clinical development. These drugs target the viral DNA polymerase (DNA pol). Little is known about the natural polymorphism of HAdV DNA pol in clinical strains.

Methods: We assessed the inter- and intra-species variability of the whole gene coding for HAdV DNA pol of HAdV clinical strains of species C. The study included 60 species C HAdV (21 C1, 27 C2 and 12 C5) strains isolated from patients with symptomatic infections who had never experienced CDV or BCV treatments and 20 reference strains. We also evaluated the emergence of mutations in thrirteen patients with persistent HAdV infection despite antiviral treatment.

Results: We identified 356 polymorphic nucleotide positions (9.9% of the whole gene), including 102 positions with nonsynonymous mutations (28.0%) representing 8.7% of all amino acids. The mean numbers of nucleotide and amino acid mutations per strain were 23.1 (± 6.2) and 5.2 (± 2.4) respectively. Most of amino acid substitutions (60.6%) were observed in one instance only. A minority (13.8%) were observed in more than 10% of all strains. The most variable region was the NH2 terminal domain (44.2% of amino acid mutations). Mutations in the exonuclease domain accounted for 27.8%. The binding domains for the terminal protein (TPR), TPR1 and TPR2, presented a limited number of mutations, which were nonetheless frequently observed (62.5% and 58.8% of strains for TPR1 and TPR2, respectively). None of the mutations associated with CDV or BCV resistance were detected. In patients receieving antiviral drugs with persistent HAdV replication, we identified a new mutation in the NH2 terminal region.

Conclusions: Our study shows a high diversity in HAdV DNA pol sequences in clinical species C HAdV and provides a comprehensive mapping of its natural polymorphism. These data will contribute to the interpretation of HAdV DNA pol mutations selected in patients receiving antiviral treatments.

1. Introduction

Human adenoviruses (HAdV) are ubiquitous DNA non-enveloped viruses belonging to the family *Adenoviridae* and the Mastadenovirus genera. More than sixty types divided into seven species from A to G have been described (Seto et al., 2011). HAdV infections cause various symptoms that usually remain limited in immunocompetent adults. In contrast, in immunocompromised patients, especially in pediatric hematopoietic stem cell transplant HSCT recipients, HAdV are an important cause of morbidity. They can lead to disseminated diseases with a high fatality rate (Feghoul et al., 2015a,b; Lion et al., 2003, 2010; Mynarek et al., 2014). Endogenous reactivation of persistent HAdV appears to be the main cause of HAdV infections in those patients (Feghoul et al., 2015a,b; Kosulin et al., 2016a,b; Markel et al., 2014; Veltrop-Duits et al., 2011). HAdV species A (types 12 and 31) and C (types 1, 2, 5 and 6) have the capacity to establish persistent infection in intestinal T lymphocytes of the digestive tract (Kosulin et al., 2016a,b) and are the most commonly observed in HSCT patients (Echavarría, 2008; Feghoul et al., 2015a,b; Kosulin et al., 2016a,b; Lion, 2014; Matthes-Martin et al., 2012).

There is currently no formally approved antiviral drug for the treatment of HAdV infections. Cidofovir (CDV) is the most frequently used although it has not been validated with randomized trials (Lindemans et al., 2010). CDV is a cytidine analog that inhibits the viral

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DNA polymerase (DNA pol). After intracellular phosphorylation, cidofovir diphosphate competitively inhibits the incorporation of deoxycytidine triphosphate into viral DNA by viral DNA pol. Brincidofovir (BCV), a lipid conjugate of CDV that offers an increased tissue distribution and intracellular uptake, is currently under clinical investigation (Painter et al., 2012; Paolino et al., 2011). In clinical trials for CMV prophylaxis and HAdV infection treatment, oral administration of BCV has been associated with gastrointestinal adverse events. To limit the digestive toxicity, shorter courses of BCV treatment of oral administration and intravenous formulation are in clinical development. Like most direct-acting antivirals, resistance is due to mutation in the target gene emerges under selective pressure. Despite a limited intracellular concentration and thus a low selective pressure, some mutations in HAdV DNA pol have been reported in a laboratory strain ATCC HAdV 5 after twenty passages in A549 cells with increasing CDV concentration (Kinchington et al., 2002). With a similar experimental design, some mutations have also been selected using BCV in the laboratory strain ATCC HAdV type 5. The combination of T87I and V303I mutations was associated with a fivefold increase in IC50. BCV with its better pharmacokinetics characteristics might select mutations in the viral DNA pol at a higher rate than CDV (Sethna et al., 2014). Ongoing clinical trials should unveil phenotypic and genotypic profiles of HAdV strains occurring in therapeutic failures.

Little is known about the natural polymorphism of HAdV DNA pol, and more particularly in clinical strains. A better characterization of polymorphism is needed for better understanding and interpretation of mutations detected in patients receiving antiviral treatment. Indeed, deciphering the variability in each functional domain would allow prediction of the likely impact of any novel mutation reported in HAdV DNA pol. (Hoeben and Uil, 2013a,b).

The aim of the present work was to sequence the entire gene coding for the DNA pol of clinical HAdV strains of species C, the most frequent species observed in HSCT patients, to determine inter- and intraspecies variability and to map the different domains involved. The study included 60 species C HAdV strains isolated from pediatric and adult patients with symptomatic infections and who had never experienced CDV or BCV treatments.

2. Materials and methods

2.1. Patients and viral strains

Between 2010 and 2015, a total of 60 HAdV clinical strains including 21 HAdV C1, 27 HAdV C2 and 12 HAdV C5 were obtained from respiratory and stool samples of 60 patients (26 females and 34 males) with respiratory digestive or disseminated infections. The age of the patients ranged between 3 months and 66 years (mean ± SD age 21.5 ± 22.0 years). The main clinical characteristics are summarized in Supplementary Table 1. Forty patients were HSCT recipients (66.7%) (21 children and 19 adults), 11 patients were hospitalized in the infection disease department, seven patients in the general pediatric department and two in the clinical immunology department. Viral strains were obtained from 41 stool and 19 respiratory samples. No patients had received CDV, BCV or ribavirin before or at the time of sample collection.

HAdV species A and species B are also associated with severe infections in HSCT patients. In our center, we identified less samples of these species than HAdV C. Fifteen samples of HAdV A and four of HAdV B could be tested. Clinical characteristics of patients with HAdV species A and species B are given in Supplementary Table 2.

In order to identify wheter specific mutations in the DNA pol could be selected under treatement with cidofovir or brincidofovir, we analyzed stool or plasma samples in patients who had a persistent detection of HAdV with at least 3 $\log_{10}$ copies/ml during treatment or within 3 weeks after discontinuation. From January 2014 to September 2017, thrirteen patients with those criteria and with available samples were analyzed.

HAdV were isolated in A549 cells in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) (Invitrogen, Carlsbad, CA), 100 U/ml penicillin (Gibco, ThermoFisher Scientifics, Auckland, NZ), and 100 µg/ml streptomycin sulfate (Gibco, ThermoFisher Scientifics, Auckland, NZ). Cells were incubated at 5% CO_2 and 37 °C. For viral propagation, 300 µl of specimen and 700 µl of media were inoculated in a 25 cm^2 corning cell culture flask. Twenty-four hours later, the inoculum was removed and replaced with 7 ml of DMEM with 2% FCS. Cell suspension was harvested when a cytopathic effect was observed.

2.2. Ethics statement

The study was carried out in accordance with the Declaration of Helsinki. This study was a non-interventional study with no addition to usual procedures. Biological material and clinical data were obtained only for standard viral diagnostic following physicians' prescriptions (no specific sampling, no modification of the sampling protocol). Data analyses were carried out using an anonymized database. According to the French Health Public Law (CSP Art L 1121–1.1), such protocol was exempted from informed consent application. The two parents or guardians of pediatric recipients of HSCT gave written informed consent to all aspects of the transplantation procedure and to the use of medical records for research.

2.3. Sample pretreatment and extraction

Before inoculation in A549 cells or nucleic acid extraction, stool and respiratory samples were pre-treated. Stool specimens were prepared by dilution of 1 g or 1 ml of stool in 9 ml of phosphate buffered saline (PBS). The resulting suspension was subjected to three −20 °C freeze-thaw cycles followed by a centrifugation step. The supernatant was passed through a 0.45 µm filter (Minisart Plus syringe filters; Sartorius Stedim Biotech GmBH, Goettingen, Germany). The respiratory samples were fluidized by the use of the digest-EUR® (EuroBio, Courtaboeuf, France) and filtered through a 0.2 µm filter (Minisart Plus syringe filters; Sartorius Stedim Biotech GmBH, Goettingen, Germany).

Nucleic acids were purified from 200 µl pre-treated samples or viral isolate and eluted in 100 µl using the QIAsymphony system with the Qiasymphony Virus/DSP pathogen kit (Qiagen, Courtaboeuf, France).

2.4. Adenovirus real time PCR and typing

Adenoviruses were detected and quantified with the Adenovirus Rgene kit (bioMérieux/Argene, Varhilles, France) according to the manufacturer's instructions (Feghoul et al., 2016). Identification of HAdV species A to F was performed using six individual real-time PCR assays as previously described (Feghoul et al., 2015a,b). These assays were carried out on an ABI 7500 thermocycler (Life Technologies, Carlsbad, CA). Adenovirus type identification was performed by sequencing hypervariable region 7 (HVR7) of the *hexon* gene as previously described (Sarantis et al., 2004).

2.5. HAdV DNA polymerase gene amplification and sequencing

HAdV DNA pol of species C HAdV was amplified in two fragments (F1 and F2) of 2393 base pairs (bp) and 1810 bp, respectively, using two pairs of primers (F1F, F1R, F2F, F2R) (Table 1). The PCR mix for amplification of both fragments consisted of AmpliTaq Gold 360 Mastermix (Applied Biosystems, Life Technologies, Carlsbad, CA, USA), 10 µM of forward and reverse primers and 5 µl of template DNA. Amplification conditions included an initial denaturation step of 5 min at 95 °C followed by 35 cycles of 30 s at 95 °C, 30 s at 57 °C for F1 and 64 °C for F2, 2 min at 72 °C and a final extension step for 5 min at 72 °C. After purification using the ExoSAPIT® kit (Affymetrix, Inc., Santa