Virus Research 238 (2017) 243–252

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Characterization of significant molecular determinants of virulence of Enterovirus 71 sub-genotype B4 in Rhabdomyosarcoma cells

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ARTICLE INFO

Keywords:
Enterovirus 71
Hand foot mouth disease
Virulence
Vaccines
Codon deoptimization
Mutations

ABSTRACT

One of the leading causes of the hand, foot and mouth disease (HFMD) is Enterovirus 71 (EV-A71), displaying symptoms such as fever and ulcers in children but some strains can produce cardiopulmonary oedema which leads to death. There is no FDA-approved vaccine for prevention of severe HFMD. The molecular determinants of virulence for EV-A71 are unclear. It could be a single or a combination of amino acids that determines virulence in different EV-A71 genotype/sub-genotypes. Several EV-A71 strains bearing single nucleotide (nt) mutations were constructed and the contribution of each mutation to virulence was evaluated. The nt(s) that contributed to significant reduction in virulence *in vitro* were selected and each mutation was introduced separately into the genome to construct the multiply mutated EV-A71 strain (MMS) which carried six substitutions of nt(s) at the 5′-NTR (U700C), VP1-145 (E to G), VP1-98E, VP1-244 K and G64R in the vaccine seed strain that had a partial deletion within the 5′-NTR region (nt. 475–485) of Δ11 bp. In comparison to the wild type strain, the MMS showed low virulence as it produced very low RNA copy number, plaque count, VP1 and had 10^5-fold higher $TCID_{50}$, indicative of a promising LAV candidate that should be further evaluated *in vivo*.

1. Introduction

In 2016, there were approximately 2.14 million reported cases of Hand, Foot and Mouth Disease (HFMD), including 204 deaths in China (WHO Western Pacific Region Surveillance Summary 2016). Vaccines against the Hand, Foot and Mouth Disease (HFMD) are highly desirable as HFMD has evolved to become a severe global and life threatening disease, ravaging lives of young children in cyclical epidemics in the Asia Pacific. With rising concern about the virulence of EV-A71, there is an urgent need for a vaccine against EV-A71 to be produced that is endorsed by the United States Food and Drug Administration (FDA). Up to date, several biopharmaceutical companies in China have ended their Phase III Clinical Trials, producing the inactivated vaccine (IV) adjuvanted with alum, against the sub-genotype C4a. Although the efficacy of their IVs was more than 90% against mild HFMD, it only conferred 80% protection against severe HFMD (Chong et al., 2015). The IV induces good humoral immunity but is deficient in the cellular arm of immunity, which is needed for long-term protection. Therefore, there is a need to develop other types of vaccines. The development of live attenuated vaccines (LAVs) is desirable as it is known to induce excellent immunogenicity, can elicit both humoral and cellular immunity and able to confer live-long immunity. A LAV from the BrCr strain (S1-3′) prototype strain carrying mutations in the 3′-NTR, $3D^{pol}$ and 5′-NTR was constructed by Arita et al. (2007). Although there was reduced virulence, mild neurological symptoms were still observed in the 3 cynomolgus monkeys immunized with the EV-A71 (S1-3′) strain. Hence, the plan to use this strain as a LAV was discontinued (Arita et al., 2007).

Before an effective LAV can be developed, there is a need to identify the genetic determinants of virulence. Once the specific determinants of virulence in EV-A71 have been identified, rational design of the LAV can be carried out by site directed mutagenesis (SDM) to target the specific amino acids that are associated with virulence. Classification of genetic determinants of virulence in EV-A71 by analyzing differences in the genome has been published. Sequence comparison between virulent and non-virulent strains showed that 4 amino acids (aa) in VP1 (Gly^{P710}/Gln^{P710}/Arg^{P710}/Glu^{P729}), 1 aa. in the 2A protein (Lys^{P930}) and 4 nucleotides (nt) in the 5′-NTR (G^{P272}, U^{P488} and A^{P700}/U^{P700})

Abbreviations: EV-A71, Enterovirus 71; PV, poliovirus; 5′-NTR, 5′- non translated region; aa, amino acid; nt, nucleotide; CPE, cytopathic effects; MOI, multiplicity of infection; RD, Rhabdomyosarcoma; PFU, plaque forming units; $TCID_{50}$, 50% tissue culture infectious dose; VP1, viral protein; LAV, live attenuated vaccine; MMS, multiply mutated strain

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http://dx.doi.org/10.1016/j.virusres.2017.07.010
Received 19 May 2017; Received in revised form 8 July 2017; Accepted 8 July 2017
Available online 10 July 2017
0168-1702/

could be genetic determinants for virulent EV-A71 sub-genotype C4 strains (Li et al., 2011). In addition, it was postulated that the EV-A71 mutant displaying high-fidelity (sub-genotype B4) with a single aa. change, (G64R) in its RNA-dependent RNA polymerase (RdRP) enzyme greatly reduced viral pathogenicity *in vivo* (Meng and Kwang, 2014). Kataoka et al. (2015) found that if the aa. glutamic acid was present at position 145 of VP1 (VP1-145E) in EV-A71 (sub-genotype C1), the virus induced neuro-pathogenesis and viremia more efficiently in cynomolgus monkeys than if glycine (G) was found at residue 145 (VP1-145G) (Kataoka et al., 2015).

The analysis of significant molecular determinants of virulence that were responsible for the attenuated phenotypes of the Sabin Oral Polio Vaccine (OPV) strains was due to the complete sequences of 3 poliovirus (PV) genomes and the development of infectious PV cDNA clones. For example, the Sabin 1 strain differed from its wild type strain with 57 nt. substitutions (Nomoto et al., 1982). It was found that the A480G in the IRES region is the most vital determinant of virulence that contributed to the attenuation in the Sabin 1 strain. It could be possible that nt. 480 affects the formation of a structure in the 5′-NTR responsible for neuro-virulence (Kawamura et al., 1989). For the Sabin 2 strain there were only 2 nt. substitutions (nt. 481 in IRES and nt. 2909 in VP1), whereas a total of 10 nt. substitutions were discovered for the Sabin 3 strain. Amongst the 10, there were 3 significant molecular determinants of virulence (C274U in IRES, C2034U in VP3, and U2493C in VP1) (Chia et al., 2014; Huang et al., 2013; Westrop et al., 1989). Virulent strains of EV-A71 are referred to as the new polio as it is neurotropic. Both the enteroviruses share very high sequence homology, particularly in the 5′-NTR. EV-A71 contains a similar nt. G481 that is a significant molecular determinant of neuro-virulence in the PV wild type. Hence, studies on PV in earlier research could be good references to design attenuated EV-A71 viruses.

With advances in molecular biology, novel approaches to viral attenuation can be further studied such as altered replication fidelity and codon de-optimization. As high mutation rates often hinder the effectiveness of a LAV, increasing the replication fidelity can potentially attenuate whole virus population, culminating to a population collapse with the absence of mutating vital immunogenic epitopes. For all species, codon pair usage is biased and some codon pairs are utilized more often than others (Gutman and Hatfield, 1989). A big part of the genetic code is redundant as contiguous pairs of aa. can be coded for by 36 alternate pairs of synonymous codons (Buchan et al., 2006; Sharp et al., 1986). Through the substitution of alternate but synonymous codons within the genome sequence, this would produce different codon pairs but expressing a similar sequence of amino acids. The proteins expressed from these viruses would elicit the same immune response as the wild type viruses. Burns et al. (2006) replaced half of the total codons in the Sabin type 2 OPV strain (within capsid region) with less frequently utilized synonymous codons. They discovered that processing and manufacturing of viral proteins were unaffected but viral fitness was reduced (Burns et al., 2006). Subsequently, they replaced natural capsid region codons with synonymous codons that had an abundance of CpG and UpA dinucleotides. Codon-deoptimized PVs were produced and these viruses had significantly lower overall fitness as indicated by lower viral plaque number and virus yields (Burns et al., 2009).

Mueller et al. (2006) introduced the biggest number of less frequently utilized synonymous codons in the capsid region of PV type 1 Mahoney. They found a significant decrease in replicative fitness and number of infectious viral progeny. As compared to the wild type, there was also reduction in the viral infectivity up to approximately 1000-fold and decrease in genome translation (Mueller et al., 2006). The codon deoptimized viruses remained attenuated after repeated cell passages and were genetically stable with minimal risk of reversion (Burns et al., 2006; Mueller et al., 2006). As these viruses have sequences that are fairly divergent from circulating wild type viruses, probabilities for further recombination and production of vaccine-derived variants will be reduced. In addition, the codon deoptimized attenuated viruses were genetically stable and showed low risk of reversion.

Previously, we had mutated the EV-A71 virus (sub-genotype B4 virus; 5865/Sin/000009) by substitutions at positions 475, 486, and 487 as these 3 nucleotides corresponded to the significant molecular determinants of neuro-virulence in PV Sabin strains 1, 2 and 3. In EV-A71, we have introduced a partial deletion (PD) (deletion from nt. 475-485 in the 5′-NTR) as it is considered to be genetically more stable than single site mutations. Compared to mutants with specific nt replacements at 475, 486 and 487, the EV-A71 PD mutant carrying the 11 base pair deletion demonstrated the lowest viral RNA copy number, plaque count and VP1 capsid protein. The PD mutant demonstrated low virulence and therefore, could possibly be a good potential seed strain for designing a LAV candidate (Yee et al., 2016).

In this study, we constructed several codon deoptimized EV-A71 viruses by substituting single synonymous codon at positions VP1 (98E), VP1 (244K), VP1 (242K), VP2 (149K) and 2A (930K) to assess the outcome of each codon deoptimization on replication fitness in Rhabdomyosarcoma (RD) cells. If the LAV has single site mutations, there is a strong possibility for reversion to occur. However, if it bears a short deletion and further mutations added to the genome, there is a possibility to reduce reversion and increase the stability of LAVs. In addition, a better attenuated mutant was constructed by introducing multiple mutations into the EV-A71 mutant PD. Hence, we also constructed a multiply mutated EV-A71 strain (MMS) by substituting 6 nucleotides at the 5′-NTR (U700C), VP1-145 (E to G), VP1 (98E), VP1 (242K), VP1 (244K), G64R in the mutant PD (Δ11 bp in 5′-NTR). We evaluated the attenuation of virulence of the MMS in RD cells such as cytopathic effects, viral infectivity by tissue culture infectious dose ($TCID_{50}$), plaque counts, production of VP1 and RNA copy number.

2. Materials and methods

2.1. Tissue culture of RD cell line

Human Rhabdomyosarcoma cells (RD, ATCC # CCL-136) were propagated in Dulbecco's modified Eagle's Minimal Medium/F-12 (DMEM/F-12, Invitrogen, USA), that was supplemented with 1.5% $NaHCO_3$, 10% fetal bovine serum (FBS) (Gibco, USA), 1% non-essential amino acids and 1% penicillin/streptomycin antibiotics. The cells were grown at 37 °C in 5% CO_2 until they reached confluence.

2.2. Virus propagation and storage

A 75 cm^2 tissue culture flask with 100% confluent Rhabdomyosarcoma (RD) cells was infected with 100 µl of virus supernatant. The flask was incubated for 1 h at 37 °C and replaced with fresh DMEM supplemented with 2% FBS. The flask was incubated at 37 °C for 24–48 h and observed for cytopathic effects (CPE). The culture supernatants were harvested and freeze-thawed 3 times. The supernatants were then centrifuged at 10,000 × g for 20 min at 4 °C to remove cell debris. The harvested supernatants were then stored at −80 °C until further use.

2.3. Viral RNA extraction

This process was performed using QIAamp® Viral RNA Mini Kit (Qiagen, Calif., USA). The principle was based on the selective binding properties of a silica-gel based membrane together with micro spin technology to extract viral RNA. The samples were lysed with a buffer which aids in denaturing RNases. The RNA was attached to the membrane which was then washed two times with buffers. The RNA was eluted with DEPC-treated water. An aliquot of 140 µl taken from the samples were processed and the viral RNA was eluted in 50 µl of elution buffer (Qiagen, Calif., USA).