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Letter

Mutational landscapes of NITD008-resistant EV71 variants revealed through population sequencing

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Dear Editor,

Enterovirus 71 (EV71) is the main pathogen of hand, foot, and mouth disease (HFMD), which is a serious public health threat, especially in the Asia-Pacific region (Wu et al., 2013). Due to the lack of effective antivirals for treatment, supportive therapy remains to be the primary measure for severe infections of EV71. EV71 belongs to the genus *Enterovirus* in the family of *Picornavirridae*. EV71 encodes a polyprotein that is proteolytically cleaved into four structural proteins and seven nonstructural proteins, i.e., VP1 to VP4, 2A to 2C, and 3A to 3D. Moreover, an alternative encoding strategy of harboring a novel open reading frame encoding a short peptide has been recently reported in gut epithelial cells infected with some enteroviruses (Lulla et al., 2019). Structural proteins play a key role in the packaging and maturation of virus particles, while non-structural proteins are mainly involved in the replication process of virus. Among them, the 3D polymerase ($3D^{pol}$) protein functions as an RNA-dependent RNA polymerase (RdRP) that is essential for viral RNA synthesis (Wu et al., 2010).

Nucleoside or nucleotide analogues have been well accepted as therapeutic agents and play a dominant role in the treatment of a number of viral infections (Lou et al., 2014). NITD008 is an adenosine nucleoside analog that has been reported to selectively inhibit viruses in the family *Flaviviridae* and EV71 (Deng et al., 2014; Yin et al., 2009). It is well known that the triphosphate form of NITD008 competes with natural adenosine triphosphate substrates to incorporate into the growing RNA chain and terminate RNA elongation (Yin et al., 2009). However, the role of NITD008 as a mutagen in EV71 infection remains unclear. To clarify this, we evaluated the effect of NITD008 on virus complexity by analyzing the mutational landscape of wild type (WT) and NITD008-resistant EV71 populations in treated or untreated NITD008.

In our previous study, two NITD008-resistant isolates were selected by passaging WT EV71 with increasing concentrations of NITD008, and resistance mutations of 3A and/or 3D were identified (Deng et al., 2014). To test the mutational landscape of NITD008-resistant EV71 viruses induced by NITD008, we selected several mutants ($3A_{V75A}$, $3D_{V63A+M393L}$, $3A_{V75A}$-$3D_{V63A+M393L}$) for deep sequencing. First of all, referring to our previous methods (Deng et al., 2014), the drug concentration of NITD008 was selected as 1 μM, which showed significant inhibition effect for WT virus and no cytotoxicity to Vero cells. Using the same concentration of DMSO as control, Vero cells were infected with WT, $3A_{V75A}$, $3D_{V63A+M393L}$, and $3A_{V75A}$-$3D_{V63A+M393L}$ at a multiplicity of infection (MOI) of 0.1. At 72 hours post-infection (hpi), the three NITD008-resistant viruses ($3A_{V75A}$, $3D_{V63A+M393L}$, $3A_{V75A}$-$3D_{V63A+M393L}$) showed significant resistance relative to WT virus in cytopathic effect (CPE) (Fig. 1A and Supplementary Fig. S1), viral titer and RNA copy number (Fig. 1B), as previously reported (Deng et al., 2014). Subsequently, the RNA samples were used for DNBSEQ™ T7 sequencing platform across the full genome and analyzed by the ViVan pipeline (Isakov et al., 2015). We mined the deep sequence data for minority variants above 1% frequency to calculate the number of minority variants, which can be used as metrics of viral population diversity (Stapleford et al., 2015). Each sample achieved mean depth of more than 100,000 and 100% genome sequence coverage (Supplementary Table S1).

Firstly, the mutation spectra of proteins in WT population and NITD008-resistant populations were compared. Across the whole genome, WT maintained the highest number of mutations regardless of NITD008 induction (73 or 159 mutations), while NITD008-resistant populations had lower levels of mutations (51/49/45 or 123/94/59 mutations) (Fig. 1C and D). Notably, the mutation number of *3D* gene in WT population increased remarkably from 1 to 51 (51-fold) upon NITD008 treatment, providing direct evidence that NITD008 targets viral polymerase during EV71 replication, which is consistent with *in vitro* experiments (Yin et al., 2009). Additionally, in WT population following NITD008 treatment, there was a 17-fold increase in mutation number of *2C* gene. 2C is a highly conserved and multifunctional membrane protein of picornaviruses, which exhibits ATPase and helicase activity *in vitro* (Supplementary Fig. S2, boxes A, B and C) (Wang et al., 2020). Besides, EV71 2C has also been found to contain ATP-independent RNA chaperoning activity that can destabilize RNA duplexes (Xia et al., 2015). Further analysis showed that these mutations were located in the vicinity of motifs A, B, and C (Supplementary Fig. S2), thus we speculated that

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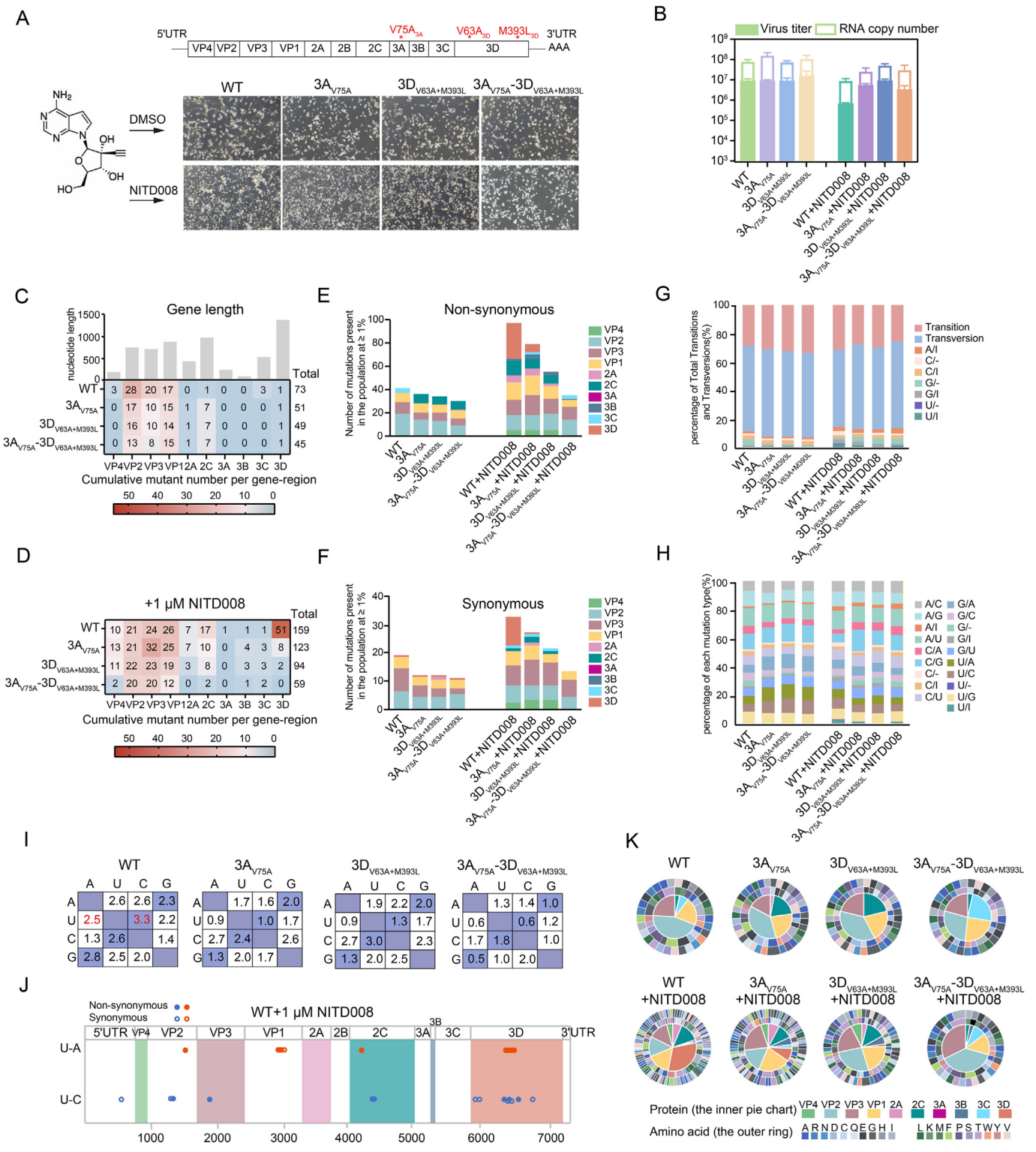

Fig. 1. Determination of mutation spectrum in population during EV71 replication base on deep sequencing. **A** Cytopathic effect (CPE) of wild type (WT) and NITD008-resistant populations on Vero cells following 0 or 1 μM NITD008 treatment at 72 hpi. **B** Viral titers and RNA copy numbers of deep-sequencing samples were measured by plaque assay and qRT-PCR, respectively. **C, D** The number of mutations present in each structural and non-structural protein without or with NITD008. **E, F** The number of (non)synonymous mutations of each protein gene in population. **G** The percentages of transversions (A↔T, A↔C, C↔G, G↔T) and transitions (A↔G and U↔C) are shown. **H** The percentages of specific nucleotide mutations are shown following 0 or 1 μM NITD008 treatment. **I** The fold changes in the number of nucleotide mutation types among populations treated with 1 μM and 0 μM NITD008 were compared (mutation number of virus treated by 1 μM NITD008 divided by that of 0 μM NITD008). **J** NITD008-mediated U-A and U–C mutations are distributed across the EV71 genome. **K** The conversion types of amino acid mutations in population. The inner pie chart represents the protein in which the amino acid mutation is located, the outer ring represents the amino acid of the reference sequence, and the outermost ring represents the amino acid of the mutant sequence. All values represent the number of unique statistically significant minority variants.

NITD008 may interfere with the ATPase and/or helicase activity of 2C protein. According to the reported nucleic acid helix unwinding assays (Fang et al., 2021), we found that NITD008 had a weak inhibitory effect on helicase activity (Supplementary Fig. S3), but since 2C is a multifunctional protein, more precise *in vitro* experiments are needed to confirm this conclusion. However, most of the 7 mutations increased in 2A protein of WT population following NITD008 treatment were synonymous mutations, indicating that NITD008 had little effect on 2A protein. Furthermore, in all NITD008-mutagenized populations, the proportion of non-synonymous mutations was 3 times greater than that of synonymous mutations, indicating that all populations were undergoing purification selection, in which case the viruses eliminated deleterious mutations to ensure normal gene function (Fig. 1E and F). In addition, we found that most synonymous and non-synonymous mutations of all populations were located in VP2 to VP1 regions and WT population was more pronounced. These data showed a trend for flexible codon usage in the structural protein, particularly for WT virus, suggesting that it possesses a high degree of variant diversity.

Nucleotide/amino acid-specific mutation reflects the molecular mechanism of viral resistance to drugs, which may provide reference for the development of antiviral drug. To determine if NITD008 was being incorporated at higher levels in WT population, the numbers and types of transitions and transversions occurring in each population were analyzed (Fig. 1G). Treatment with NITD008 increased the number of transitions in WT population relative to the NITD008-resistant populations, which may reflect a strategy of "fine-tuning" of the virus under environmental stress to balance variation with functional stability (Smith et al., 2013). Furthermore, treatment with NITD008 resulted in about 2.5-fold and 3.3-fold increases in the number of U-to-A and U-to-C nucleotide mutations for WT population (mutation number of virus treated by 1 μM NITD008 divided by that of 0 μM NITD008), respectively, much higher than that of the NITD008-resistant populations ($3A_{V75A}$, $3D_{V63A\ +\ M393L}$ and $3A_{V75A}$-$3D_{V63A\ +\ M393L}$) (Fig. 1H and I). Further analysis showed that the NITD008-associated mutations (U-to-A and U-to-C) were mostly concentrated in the 3D polymerase region (12 of 23 mutations), and the proportion of non-synonymous mutations was larger than that of synonymous mutations in the whole genome (65% vs. 35%) (Fig. 1J and Supplementary Table S2). The mutation bias of virus induced by nucleoside analogues may accelerate the generation of drug-resistance sites. Interestingly, both $3A_{V75A}$ and $3D_{V63A}$ mutations (GUG-GCG) just resulted from the U-to-C nucleotide substitution (Deng et al., 2014). Further analysis of the protein/amino acid mutation spectrum showed that subsequent to the treatment of NITD008, the mutation types of protein and amino acid both were increased in WT population, while the NITD008-resistant populations ($3A_{V75A}$, $3D_{V63A\ +\ M393L}$ and $3A_{V75A}$-$3D_{V63A\ +\ M393L}$) exhibited resistance to these increases (Fig. 1K). In terms of major amino-acid mutation pattern, WT population changed from "VP2+VP1+VP3" to "3D + VP2+VP1" upon NITD008 treatment, but other NITD008-resistant populations remained "VP2+VP1+VP3", indicating that WT virus may fine-tune the amino-acid mutation pattern to counter the selection pressure of drug. Finally, the measurement of specific infectivity has been useful for determining lethal mutagenesis for mouse hepatitis virus (MHV) and other RNA viruses (Smith et al., 2013; Rozen-Gagnon et al., 2014). After treatment with NITD008, the specific infectivity of WT EV71 was lower than that before treatment. (Supplementary Table S3), which was similar to the mutagenesis of ribavirin on poliovirus (Graci et al., 2012), indicating that NITD008 has a potential mutagenic effect on EV71.

In summary, deep sequencing technology was used to systematically analyze the mutation characteristics of the whole-genome EV71, aiming to clarify the antiviral mechanism of NITD008 against EV71 by comparing the mutational landscapes between WT population and NITD008-resistant populations during replication. Through deep-sequencing data mining, we found a possible target 2C of NITD008 against WT EV71 in addition to the previously reported 3D polymerase, indicating that NITD008 may play an antiviral role by interfering with multiple proteins. Considering that 2C is the most conserved nonstructural protein of enteroviruses and rhinoviruses, NITD008 show the potential to be further developed as broad-spectrum antiviral drugs against all enteroviruses, rhinoviruses, and even other picornaviruses. Furthermore, our results show a correlation between the antiviral effect of NITD008 and the induction of predominantly U-to-A, as well as U-to-C transitions, supporting mutagenesis as one mechanism of action for NITD008 in the control of EV71 infection. Mutagenesis should be explored as an antiviral strategy to develop new broadly antiviral drugs, which are urgently needed in the modern world with increased risk of emerging or re-emerging diseases that require rapid intervention.

FOOTNOTES

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Other detailed materials and methods and supplemented data were attached in supplementary files.

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