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Note

High genetic stability in MDCK-SIAT1 passaged human influenza viruses

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ABSTRACT

MDCK-induced amino acid (AA) mutation, such as D151G/N in the neuraminidase (NA) of influenza A/H3N2 viruses, is of concern. MDCK-SIAT1 cells, modified derivatives with an increased expression of α2,6-linked sialic acid receptors are increasingly used due to their superiority in a viral recovery. However, MDCK-SIAT1 induced AA mutations have not been fully examined. In this study, we compared NA and hemagglutinin (HA) genes of recent circulating influenza viruses isolated after an MDCK-SIAT1 passage with those directly obtained from the original samples. A total of 22 samples collected during the 2016-17 seasons included 9 A/H3N2, 5 H1N1pdm, and 8 B viruses. None of the deduced AA mutations in the NA or HA segments were detected after an MDCK-SIAT1 passage, except for one AA mutation in the NA of an influenza B virus sample. NA D151G/N changes were not seen in any of the MDCK-SIAT1 passaged A/H3N2 viruses, even in the small variants analysis conducted using deep sequencing. AA mutations induced by an MDCK-SIAT1 passage are currently rare, although careful observation is needed in the future.

MDCK (Madin-Darby canine kidney) cells have been widely used for the isolation of human influenza viruses because of their high proliferative and economic efficiency. However, poor isolation and propagation of recent epidemic viruses, especially A/H3N2, in MDCK cells are major concerns. We also experienced a significant decrease in the isolation rate of recent A/H3N2 viruses in MDCK cells, resulting in difficulties in the subsequent analysis, such as the hemagglutination inhibition (HI) test. In the early 2000s, MDCK-SIAT1 cells were developed as a modified derivative with an increased expression of α2,6-linked sialic acid receptors, abundantly expressed on human respiratory cells [1]. They have been increasingly used due to their superiority in a viral recovery [2]. A stable production of influenza viruses has been obtained even in our institution, using MDCK-SIAT1 cells instead of MDCK cells.

A culture-derived induction of amino acid (AA) mutations might have a negative impact on data interpretation in the subsequent analysis. The propagation of recent influenza A/H3N2 viruses in MDCK cells was shown to induce the D151G/N substitution within neuraminidase (NA) catalytic sites. This concurrently results in a reduction in NA catalytic activity and an increase in its binding activity to α2,3-linked sialic acid receptors, predominantly expressed on MDCK cells [3]. This additional NA function caused difficulties in accurately interpreting antigenicity based on the HI test. A genetic comparison between A/H3N2 viruses directly obtained from clinical samples and isolated by an MDCK cell passage showed significant MDCK-induced D151G/N substitutions (~30%) [4]. Moreover, definite AA mutations in even hemagglutinin (HA) were found after an MDCK passage in these reports [4]. Multiple HA AA mutations were also induced by an MDCK passage in other viral types, A/H1N1 and B [5]. In contrast, despite an increasing utilization opportunity, there is little information on MDCK-SIAT1-induced mutations in the influenza genome, particularly regarding a direct comparison between clinical and cultivated samples. In this study, we sequenced the full length of NA and HA genes of recent circulating A/H3N2 viruses directly obtained from clinical samples and isolated after an

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MDCK-SIAT1 passage of the same sample, in order to examine culture-derived mutations. In addition, this comparison was also conducted in the other human viral types, A/H1N1pdm and B. We also tried to detect the emergence of small AA variants induced on passaging in MDCK-SIAT1 cells, using a deep sequencing method.

Samples for influenza virus isolation were collected through our clinical study network of general practitioners throughout Japan, with informed consent, from patients who showed a positive result with a rapid influenza diagnostic tests kit. All viral samples were collected before the initiation of neuraminidase inhibitors (NAIs) treatment. A total of 22 clinical samples isolated during the 2016-17 seasons were used, including 9 A/H3N2, 5 A/H1N1pdm, 5 B/Victoria, and 3 B/Yamagata viruses. MDCK-SIAT1 cells were purchased from DS Pharma Biomedical Co. (Osaka, Japan). Monolayer-cultured MDCK-SIAT1 cells were inoculated with viruses directly collected from the clinical samples, and the cells were incubated at 34 °C and 5% CO_2. The wells were monitored daily until seven days for virus growth by cytopathic effects, and the supernatant of the wells that exhibited sufficient cytopathic effects was collected. Viral RNA was extracted directly from the clinical samples and from a single MDCK-SIAT1 cell passaged supernatants using the Maxwell 16 LEV simply RNA Cells Kit (Promega, Madison, WI). RT-PCR was performed using the A/H3N2 and A/H1N1pdm RNA samples. PCR primers, synthesized based on the 3′ and 5′ terminal nucleotides that are common to all human influenza A virus segments [6] were as follows: forward primer (Uni-12), 5′-ACGCGTGATCAGCAAAAGCAGG-3′ and reverse primer (Uni-13), 5′-ACGCGTGATCAGTAGAAACAAGG-3′. The PCR consisted of 31 cycles of a denaturing step at 94 °C for 30s, an annealing step at 57 °C for 30s, and an extension step at 72 °C for 2 min. RT-PCR was also conducted using the influenza B virus RNA samples, based on the method reported by Zhou et al. [7]. After amplicon preparation, deep sequencing was conducted using the Illumina MiSeq sequencing system (Illumina, San Diego, CA) [8]. Data processing was then performed using the pipeline prepared by Amelieff Co. (Tokyo, Japan). Finally, genome sequences were constructed using the reference sequence and filtered variants. The NA and HA AA sequence was deduced from the obtained nucleotide sequence [8]. The presence of single-nucleotide variants (SNV) at particular base positions was examined based on the BAM files obtained and the SNV criteria reported by McCrone et al. [9].

The comparison of NA and HA genes of A/H3N2, A/H1N1pdm, and B viruses isolated after an MDCK-SIAT1 passage to those directly obtained from the original samples is shown in Table 1. No nucleotide or AA substitutions were detected from the MDCK-SIAT1 passaged viruses, irrespective of viral type, except for the substitution of C with T at 737 bp, leading to the S246L AA change in the NA segment of a B/Yamagata sample. The AA site 246 in the NA segment of B/Yamagata was not within the catalytic sites. Sanger sequencing confirmed the S246 and L246 in the clinical and MDCK-SIAT1 passaged sample, respectively (data not shown). Deep sequencing revealed no evidence of SNV at the 737 bp in the clinical sample, suggesting the emergence of a nucleotide substitution induced by an MDCK-SIAT1 passage (data not shown).

Nucleotide and AA variations at AA site 151 in the NA segment of A/H3N2 viruses isolated from MDCK-SIAT1 passaged samples and those directly obtained from the same clinical samples are shown in Table 2. All the clinical samples exhibited D151, not including G151 or N151. In addition, the low-frequency SNV satisfied by the defined SNV criteria of GGT(G) and/or AAT(N) at site 151 were not detected within a single clinical sample. Thus, the G151 and N151 variants did not coexist together with D151 in the unpassaged A/H3N2 viruses. The MDCK-SIAT1 cultivated A/H3N2 viruses were all occupied by NA D151. No small SNV of GGT(G) or AAT(N) at site 151 in a single sample were detected from the MDCK-SIAT1 passaged viruses.

Oh et al. reported a direct HA comparison between clinical samples and their MDCK-SIAT1 passaged influenza viruses in all human viral types [5]; in their study, a few AA mutations were induced via an MDCK-SIAT1 passage for A/H3N2 and A/H1N1 viruses (one AA site in A/H3N2 and none in A/H1N1). Additionally, in influenza B viruses, multiple AA mutations induced by an MDCK-SIAT1 passage were detected from multiple samples. The effect of an MDCK-SIAT1 passage on AA mutations in NA genes was not examined in their study. It has been presumed that AA mutations in the NA and HA genes of A/H3N2 viruses might be induced less efficiently in MDCK-SIAT1 cells than in MDCK cells [2,10]. Our study confirmed little evidence of MDCK-SIAT1-derived AA mutations in NA or HA genes of not only A/H3N2, but also of A/H1N1pdm and B viruses, by the direct comparison between clinical and cultivated samples, although it is not still convincing about culture-induced NA AA mutations in A/H1N1pdm and B viruses between MDCK and MDCK-SIAT1 cells.

It has been suggested that NA D151 is detected almost exclusively in unpassaged clinical samples of A/H3N2 viruses and that even low-frequency variants of G151 or N151 within a sample are not seen in their samples [11]. These findings provide evidence of MDCK-induced D151G/N substitutions in A/H3N2 viruses. The emergence of small populations of G151 and/or N151 variants together with D151 after an MDCK passage has also been reported [12]. In the egg cultivation of human influenza viruses, culture-induced AA mutations of A/H3N2 viruses have been focused on HA genes rather than NA genes. These mutations concentrated on HA receptor-binding sites (RBS) and/or HA antigenic sites adjacent to RBS, are explained as an adaptation to enhance multiplicity of infection [13]. Recently, multiple NA AA mutations, particularly T148I, in an egg passage were found without AA mutations at HA antigenic sites [14]. In our study, none of the NA catalytic sites, HA RBS, or RBS-surrounding antigenic sites obtained AA mutations after an MDCK-SIAT1 passage. MDCK-SIAT1 cells overexpress α2,6-linked sialic acid receptors, exclusively expressed on human respiratory cells when compared with MDCK and egg cells [1]. Thus, our results suggest that, in an MDCK-SIAT1 passage, no AA mutations are necessary to adapt to α2,3-linked sialic acid receptors, predominantly expressed on MDCK or egg cells. Recently, the NA antigen as a target of broadly protective antibodies has been re-evaluated for the development of more efficient influenza vaccines [15]. Thus, it is

Table 1
MDCK-SIAT1 passaged mutations in influenza A/H3N2, A/H1N1pdm, and B viruses in Japan during the 2016-17 season.

Segment	A/H3N2 (n = 9)		A/H1N1pdm (n = 5)		B (n = 8)	
	Nucleotide	Amino acid	Nucleotide	Amino acid	Nucleotide	Amino acid
NA	None	None	None	None	C737T	S246L
HA	None	None	None	None	None	None

The full length of NA and HA genes in influenza viruses isolated after MDCK-SIAT1 passage was compared with those directly obtained from the original clinical samples.
MDCK-SIAT1 induced nucleotide and amino acid substitutions are shown in 9 A/H3N2, 5 A/H1N1pdm, and 8 B isolates.
The NA C737T substitution was detected in a single sample of B isolates.
NA, neuraminidase; HA, hemagglutinin.

Table 2
MDCK-SIAT1 passaged amino acid variations at NA site 151 in influenza A/H3N2 viruses.

Sample	Origin	Read frequency (%) of the codons at NA AA site 151			SNV (G151 and/or N151)
		GAT (D)	GGT (G)	AAT (N)	
17-20028-2	Clinical	99.81	0.19	0.00	None
17-20028	MDCK-SIAT1	100.00	0.00	0.00	None
17-20029-2	Clinical	100.00	0.00	0.00	None
17-20029	MDCK-SIAT1	99.85	0.15	0.00	None
17-20032-2	Clinical	99.85	0.15	0.00	None
17-20032	MDCK-SIAT1	100.00	0.00	0.00	None
17-20033-2	Clinical	99.81	0.19	0.00	None
17-20033	MDCK-SIAT1	99.04	0.96	0.00	None
17-20035-2	Clinical	100.00	0.00	0.00	None
17-20035	MDCK-SIAT1	99.49	0.22	0.29	None
17-20036-2	Clinical	99.70	0.30	0.00	None
17-20036	MDCK-SIAT1	100.00	0.00	0.00	None
17-20037-2	Clinical	100.00	0.00	0.00	None
17-20037	MDCK-SIAT1	99.89	0.11	0.00	None
17-20041-2	Clinical	99.53	0.24	0.23	None
17-20041	MDCK-SIAT1	99.85	0.15	0.00	None
17-20044-2	Clinical	99.26	0.74	0.00	None
17-20044	MDCK-SIAT1	98.97	0.38	0.65	None

Read frequency means the ratio of read numbers sequenced for each nucleotide to a total of read numbers sequenced at specific nucleotide positions. Read numbers are obtained from the BAM files.
The evaluation of SNV is based on the BAM file data and the SNV criteria reported by McCrone et al. [9].
NA, neuraminidase; AA, amino acid; SNV, single-nucleotide variants.

desirable that artificial NA AA mutations, which were frequently found in MDCK and egg passages, should be limited to an extremely lower extent for ensuring vaccine efficacy. MDCK-SIAT1 cells are consistently efficient at isolating and propagating recent circulating human influenza viruses. AA mutations induced by MDCK-SIAT1 cultivation are currently rare, regardless of viral types, and need to be carefully observed in the future.

Conflicts of interest

The authors declare no competing financial interests.

Statement

All authors meet the ICMJE authorship criteria including that all authors should have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, and (3) final approval of the version to be submitted.

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