GENETIC EVOLUTION OF HUMAN CORONAVIRUS OC43 IN NEURAL CELL CULTURE

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1. INTRODUCTION

Human coronaviruses (HCoV) are ubiquitous in the environment and are responsible for up to one-third of common colds. HCoV-OC43 possesses a genome that comprises genes encoding various structural and nonstructural proteins. Amongst these proteins, the S protein is biologically very important because it could be involved in determination of viral tropism. Indeed, it could for instance be associated with the capacity of the virus to reach the central nervous system (CNS) and possibly trigger neurological disorders. It could also confer the host species specificity observed with coronaviruses. In past years, we have shown that HCoV-OC43 is neurotropic and neuroinvasive, as it persistently infects neural cell cultures[1] and human brains.[2] Although we have suggested that OC43 could remain genetically surprisingly stable in the environment,[3] it is known that coronaviruses can adapt in cell culture or under selection pressure, for instance related to immune system evasion.

2. MATERIALS AND METHODS

2.1. Viruses, Cell Lines, and Persistent Infections

The ATCC HCoV-OC43 strain (VR-759) was grown on the HRT-18 rectal tumor cell line. Persistent infection were carried out in those HRT-18 cells, as well as in the MO3.13 oligodendrocytic,[4] H4 neuroglial, U-87 MG astrocytic, and TE-671 rhabdomyosarcoma cell lines (ATCC). Other cell lines used for virus susceptibility are described in Table 2. Four infections were performed in the H4 cell line, whereas the HRT-18, MO3.13, and H4 cell lines were acutely infected as controls.

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2.2. Virus Purification

Virus from persistent infections was purified at different passages. Prior to purification, virus was clarified and precipitated with polyethylene glycol (PEG) 8000 (Sigma). Accudenz (Accurate Chemicals) was used to perform gradient purification.

2.3. RT-PCR and Sequencing

Viral RNA was extracted using the GenElute Direct mRNA Miniprep Kit (Sigma) and reverse transcribed with MMuLVreverse transcriptase (Invitrogen). The Expand High-Fidelity *Taq* polymerase (Roche) was used to perform PCR. Primers specific to the HE, S and N genes were used to amplify target regions.[3] PCR amplicons were purified using the Qiaex II gel extraction kit (Qiagen) prior to sequencing, which was carried out by Bio S&T (Montréal, Québec, Canada).

2.4. Assays for Viral Susceptibillity and Modulation of Tropism and Infectivity

Prior to performing assays for modulation of tropism and infectivity, susceptibility of different cell lines to HCoV-OC43, ATCC strain, was determined (Table 2). The same cell lines were then infected with virus isolated from different purifications (HRT-18 P33, P54, P110, and P155; H4 P47 and P90; H4 P56.1, P56.2, P56.3, P116.1, P116.2, and P116.3; TE-671 P38 and P79; U-87 MG P35, and MO3.13 P5, P6, and P22) in order to correlate the observed mutations with a modulation of tropism or infectivity. Supernatants were titrated using an indirect immunoperoxidase assay (IPA), as previously described.[5]

3. RESULTS

Persistent infections of neural cell lines were initially performed to determine whether virus carrying mutations in genes encoding the surface protein S originated as a consequence of viral persistence. The HE protein gene and the nucleocapsid protein gene N were also sequenced in order to determine if these genes contributed to adaptation in cell culture. Viral particles released from persistently infected neural cell lines were isolated and purified by gradient centrifugation, and genomic RNA was sequenced. Results showed various mutations in the S gene but very few in HE and N genes, suggesting that the S gene is responsible for adaptation to the cellular environment, which could be associated with neurotropism, neuroinvasion, and presumably neuropathogenesis (Table 1). Almost every acquired mutation (Table 1) was conserved at subsequent passages, suggesting that they could confer an adaptive advantage and a stable phenotype to the virus. Five mutations were predominant and were found in almost all persistent infections (D24Y, S83T, H183R, Y241H, and N489H). The first four mutations are located in the putative receptor binding site, whereas the fifth one is located within the hypervariable region.

To correlate the observed mutations in the S gene with viral replication and tropism, assays for modulation of tropism and infectivity[6] were performed using cell lines originating from various human tissues as well as from various animal species, for which

Table 1. Location of S mutations at various passages of persistently infected cells.

HRT-18 P155**	H4 P90	H4 P116.1	H4 P116.2	H4 P116.3	TE-671 P79	U-87 MG P35	MO3.13 P22
D30H*	D24Y	D24Y	D24Y	N25Y	N27Y	D24Y	D115H
S83T	V161V	P35S	P35S	P35S	P34S	S83T	T148I
L85Q	H183R	S83T	S147Y (D)	S83T	L85R	H183R	Y241H
D115H	V240V	E170K	H183R	Y119H	S258R	Y241H	M670T
T148I	Y241H	H183R	Y241H	S147P	A373V		P973S
H183Q	N441K	Y241H	N441K	H183R	R757S		A1090V
S258R	Q541L	A469V	E460D	Y241H	G785D		V1213A
S366G	R570P	R570R	H482Y	N489H	P972L		
N413T	N639N	T855I	F683Y	K506T	P973S		
F420S	T855I	N880K (I)	L693F	T641S	A978S		
N489H	D875H	L893H	A759E	N768T	T1086N		
K506N	L893R	S959C	S898S (I)	E896K	D1170A		
T536N	A965V	W974L	V980A	S901F			
Q541L	T975A	T975P	N1203 (D)	W974L			
R757H	I1227T	V980A	I1227T	F982L			
E896D	T1245I	S1093S		V986I			
C897G		G1169G		G1169D			
E933G		M1222K		E1236A			
F982L		D1232Y					
S1192R		P1249L					
T1225I		I1304I					
P1228S							

* D, deletion; I, insertion. ** Passages (and purification numbers) are indicated below the cell line.

susceptibility to HCoV-OC43 infection was previously determined (Table 2). These analyses revealed that mutations found throughout the S gene could affect the latter viral properties in certain cell lines. Amongst the virus variants obtained following persistent infections and virus purifications, five showed extended cellular tropism and increased replication titers *in vitro*: U87-MG P35, H4 P47, H4 P56.3, H4 P116.1, and H4 P116.2 (data not shown). Furthermore, some variants isolated from persistent infections were more virulent in mice and could form plaques, in opposition to the ATCC HCoV-OC43 reference strain VR759 (data not shown).

4. DISCUSSION

We have identified several mutations in the S gene of the HCoV-OC43 genome following persistent infections in different cell lines. These mutations will help us to further characterize viral adaptation during persistence and to understand mechanisms that are implicated in viral tropism and infectivity. Future studies will be carried out using an infectious cDNA clone of the OC43 strain assembled in a BAC vector.[7,8] The construction of this clone was performed in collaboration with F. Almazán and L. Enjuanes and will provide an invaluable tool to further understanding the underlying mechanisms for viral replication and tropism. In combination with the experiments described above, the clone will be useful in elucidating the molecular basis of human coronavirus neuropathogenesis.

Table 2. Susceptibility of various cell lines to the HCoV-OC43 ATCC strain.

Cell line	Origin	Tissue	Type	IPA [1]	Susceptibility [2]
HeLa	Human	Uterus	Epithelial	3.75	Low
MT4	Human	Bone marrow	T lymphocyte	2.0	No
U937	Human	Bone marrow	Monocyte	≤0.5	No
Jurkat E6.1	Human	Bone marrow	T lymphocyte	≤1.5	No
Raji	Human	Bone marrow	B lymphocyte	4.5	Yes
HL-60	Human	Bone marrow	Monocyte	3.25	Low
WI-38	Human	Lung	Fibroblast	4.0	Yes
L132	Human	Lung	Epithelial	3.25	Low
Caki-2	Human	Kidney	Epithelial	≤1,5	No
SW 156	Human	Kidney	Epithelial	≤1.5	No
NCI-N87	Human	Stomach	Epithelial	≤1.75	No
Arpe-19	Human	Eye	Epithelial	≤1.5	No
FHs 74 Int	Human	Intestine	Epithelial	3.5	Low
TK6	Human	Spleen	T lymphocyte	≤1.75	No
17 Cl-1	Mouse	Embryo	Fibroblast	≤1.75	No
L929	Mouse	Subcutaneous	Fibroblast	≤1.5	No
N-11	Mouse	Brain	Microglial	≤1.5	No
DBT	Mouse	Brain	Glial	≤2.25	No
J774 A.1	Mouse	Bone marrow	Macrophage	4.5	Yes
A20	Mouse	Bone marrow	B lymphocyte	3.25	Low
S.END.1	Mouse	Skin	Endothelial	≤1.5	No
Cos-7	Monkey	Kidney	Fibroblast	≤1.5	No
Vero	Monkey	Kidney	Epithelial	≤1.75	No
Vero E.6	Monkey	Kidney	Epithelial	≤1.5	No
B104	Rat	Brain	Fibroblast	3.25	Low
BHK-21	Hamster	Kidney	Fibroblast	4.0	Yes

[1] Indirect immunoperoxidase assay (infectious titers in $TCID_{50}$/mL).
[2] Titers from 0 to 3, not susceptible; titers over 3 and under 4, low susceptibility; titers of 4 and over, susceptible.

5. REFERENCES

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