Single amino acid substitution and inter-species transmission of MERS-coronavirus from camels to humans: abridged secondary publication

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KEY MESSAGES

1. Middle East respiratory syndrome coronavirus (MERS-CoV) acquires a human-adaptive mutation in the nsp6 gene following inter-species transmission from camels to humans.
2. The nsp6 L232F mutation is associated with increased viral replication competence in the respiratory tracts of humans and mice.
3. The nsp6 L232F mutation affects autophagic machinery within host cells, thereby modifying viral replication competence.
4. MERS-CoV remains a public health threat, and ongoing surveillance in camels and humans is warranted.

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Introduction

Middle East respiratory syndrome coronavirus (MERS-CoV) was first identified as a cause of severe acute respiratory disease in humans in 2012. MERS-CoV remains a potential pandemic threat and a research priority.[1] Inter-species transmission of MERS-CoV from camels to humans is sporadic, possibly through direct or indirect camel contact. Clusters of human cases have been reported, typically in healthcare settings. Inter-species virus transmission from animals to humans is often associated with mutations as the virus attempts to adapt to the new host. A classic example is the adaptative mutation of E627K, which arose in the PB2 gene of avian influenza A virus following transmission to humans.[2] We aimed to identify and characterise host adaptive mutations that emerge when MERS-CoV is transmitted from camels to humans.

Methods

MERS-CoV sequences (full genome and >20 kb) were retrieved. Sequences were aligned using MAFFT and processed for phylogenetic analysis. A bacterial artificial chromosome plasmid construct of the infectious MERS-CoV/China01(GD01) strain was provided by Guangzhou Medical University.

An independent risk assessment of the genetic modification of MERS-CoV was implemented in the Safety Office, the University of Hong Kong. The experiment envisaged was a loss-of-function mutation as we started from a human virus possessing the putative 'human adaptive mutation' to mutate nsp232 to the camel genotype. Thus, the genetic modification procedure did not increase the risk for humans. All handling of infectious virus was carried out at bio-safety level 3 containment.

The GD01 human strain of MERS-CoV exhibits the L232F mutation in the nsp6 gene. We rescued a recombinant GD01 with and without the nsp6 L232F mutation using our previously described methods.[3] Rescued viruses were plaque-purified in Vero cells and subcultured in Huh-7 cells to generate virus stocks, which were aliquoted and stored at -80°C. The genetic identities of the virus stocks were confirmed, and the titre of each virus stock was determined. We characterised the replication kinetics of the two isogenic mutants with the nsp6 L232F mutation using our previously described methods.[3] Cellular RNA was extracted from virus-infected cells. Purified RNA was reverse-transcribed into cDNA using random hexamers and then subject to gene expression. Vero cells were fixed in 10% formalin and processed for transmission electron microscopy. The effect of MERS-CoV infection on autophagic flux was determined by transfection of a tandem-EGFP-mCherry-LC3 reporter plasmid encoding fluorescent LC3, which is detectable as yellow puncta in autophagosomes (APs) and as red puncta in autolysosomes (ALs). Chloroquine and BafA1 were used as controls for the inhibition of autophagic flux. Vero cells were transfected with Lipofectamine 2000 and then infected with MERS-CoV at a multiplicity of infection (MOI) of 0.01.

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Results

We retrieved 502 complete and partial (>20 kb) MERS-CoV genomes from GenBank, which comprised both the Arabian Peninsula clade A/B and Africa clade C sequences. We used these genomes to identify mutations that are more likely to occur in human MERS-CoV. Using the Fisher's exact test adjusted with Bonferroni correction, 11 mutations were significantly more frequent to occur in MERS-CoV sequences from humans than from camels. These mutations were present in nsp3, nsp5, nsp6, nsp13, spike, and nucleocapsid viral proteins. Among these 11 mutations, the top hit (ie, the mutation with the greatest difference in occurrence between human and camel origin sequences) was the leucine (Leu L) to phenylalanine (Phe F) mutation at codon 232 of the nsp6 protein, which occurred in 60 (25.2%) of 238 human sequences but in 1 (0.4%) of 264 camel sequences (from clade B). To visualise the nsp6 mutation in the MERS-CoV phylogeny, we constructed a phylogenetic tree of MERS-CoV sequences exhibiting the nsp6 mutation genotype in human and camel hosts (Fig 1).

The nsp6 L232F mutation did not alter replication in Calu-3 or Huh-7 cells, but it enhanced replication in Vero cells (Fig 2). The isogenic mutant rGD01-nsp6 232L displayed smaller plaque sizes compared with rGD01-WT, which contains nsp6 232F (1 vs 0.71, P<0.001). These data suggest that the nsp6 L232F mutation increases viral replication. We confirmed this enhanced viral replication competence by a competitive growth assay involving co-culture with different ratios (9:1, 1:1, and 1:9) of rGD01-WT and rGD01-nsp6 232L. We measured the genotype frequencies after various numbers of viral passages in Vero cells (data not shown). After three passages, a distinct nsp6 Phe genotype was observed at all co-culture ratios in Vero cells, implying that rGD01-WT has more robust intrinsic replication kinetics (data not shown). No competitive advantage was observed in Calu-3 cells.

Triplicate cultures were infected with each of the isogenic viruses. In bronchial cultures, rGD01-nsp6 232L exhibited significant reduction of replication at 48 and 72 hours post-infection (hpi) [Fig 2]. Replication did not significantly differ in lung cultures, reflecting a phenotype similar to the results in Calu-3 lung adenocarcinoma cells.

After intranasal inoculation of 10^4 plaque-forming units of virus per mouse, we observed significantly lower titres of rGD01-nsp6 232L virus at 1 to 3 days post-infection (dpi); the greatest difference of 1.71 log10 median tissue culture infectious dose ($TCID_{50}$)/mL was observed on day 1 (Fig 2). We also detected lower amounts of subgenomic viral RNA from rGD01-nsp6 232L at 1 to 3 dpi. These findings suggest that the nsp6 L232F mutation enhanced replication in mouse lung tissue.

Protein levels of pro-inflammatory cytokines and chemokines in the lungs were measured from 4 hpi to 5 dpi. Although both viruses exhibited detectable replication at 1 dpi, there was no significant induction of inflammatory markers compared with mock infection controls (data not shown). These results suggest that effective innate immune antagonism was achieved by both viruses, which is an expected phenotype for MERS-CoV infection. At 2 to 3 dpi, the protein levels of monocyte chemoattractant protein-1, interferon gamma-induced protein-10, and interleukin-6 in mouse lungs were significantly higher upon infection with rGD01-WT than upon infection with rGD01-nsp6 232L. Considering that rGD01-WT had a higher viral load in the lungs compared with rGD01-nsp6 232L at 2 to 3 dpi, the increased innate immune response may reflect the higher viral load in the lungs, rather than the involvement of the nsp6 L232F mutation in immune antagonism.

The nsp6 protein has previously been implicated in AP restriction.[4] Using a tandem fluorescent-tagged LC3 (mRFP-EGFP-LC3) reporter, autophagic flux can be measured based on pH differences between AP and AL environments; the more acidic AL environment quenches the EGFP signal. This reporter can quantify the numbers of autophagic compartments in infected cells. After Vero cells were transfected with mRFP-EGFP-LC3, we infected those cells with each of the two isogenic viruses at an MOI of 0.01 and measured the numbers of autophagic compartments at 24 hpi. There was no difference in AL number between cells infected with either isogenic virus, but the AP number was higher in rGD01-nsp232L-infected cells; thus, the camel Leu residue resulted in lower autophagic flux, indicated by less AP to AL fusion (Fig 3).

In a parallel approach, we conducted LC3 immunoblotting to measure autophagic flux. LC3 is a well-known autophagic marker that exists in two forms: LC3-I, found in the cytoplasm, and LC3-II, bound to the AP membrane. Changes in the LC3 immunoblotting band intensity ratio of LC3-II/actin (reflecting cellular AP level) in response to BafA1 (an inhibitor of AP to AL fusion) can provide information about autophagic flux. Among Vero cells infected at an MOI of 0.01, only rGD01-WT-infected cells exhibited an increase in the LC3-II/actin ratio upon treatment with BafA1 at 24 hpi, suggesting that rGD01-WT-infected cells had higher autophagic flux than rGD01-nsp6 232L-infected cells (Fig 3). At 48 hpi, both viruses blocked AP to AL fusion, indicated by the absence of BafA1-mediated changes in the LC3-II/actin ratio. We regarded LC3-I as a proxy for double-membrane vesicle (DMV) levels; rGD01-WT-infected cells showed higher levels of LC3-I compared with rGD01-nsp6 232L-infected

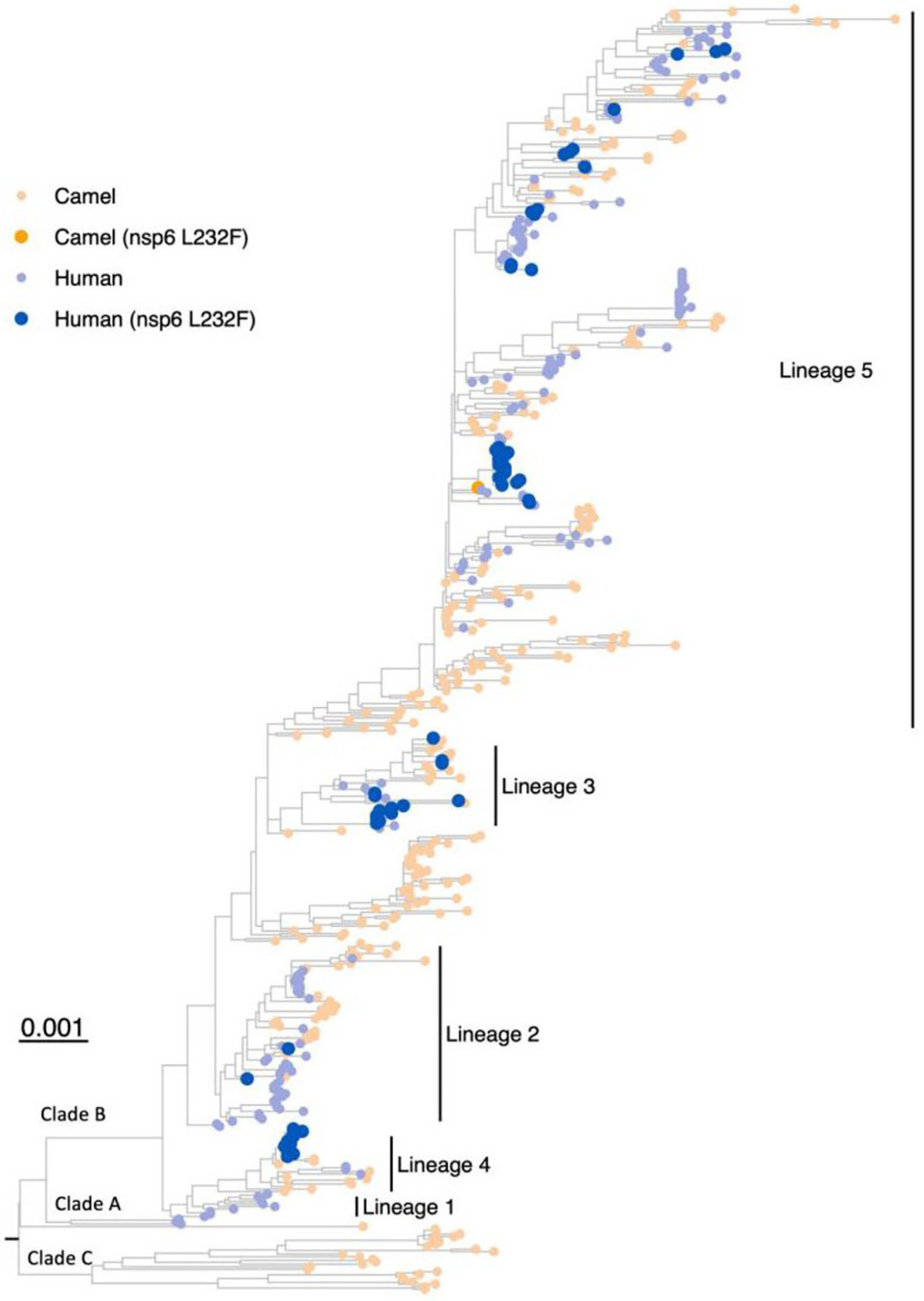

FIG 1. The Middle East respiratory syndrome coronavirus (MERS-CoV) nsp6 L232F mutation preferentially occurs in humans, compared with camels, in 502 complete and partial MERS-CoV genomes

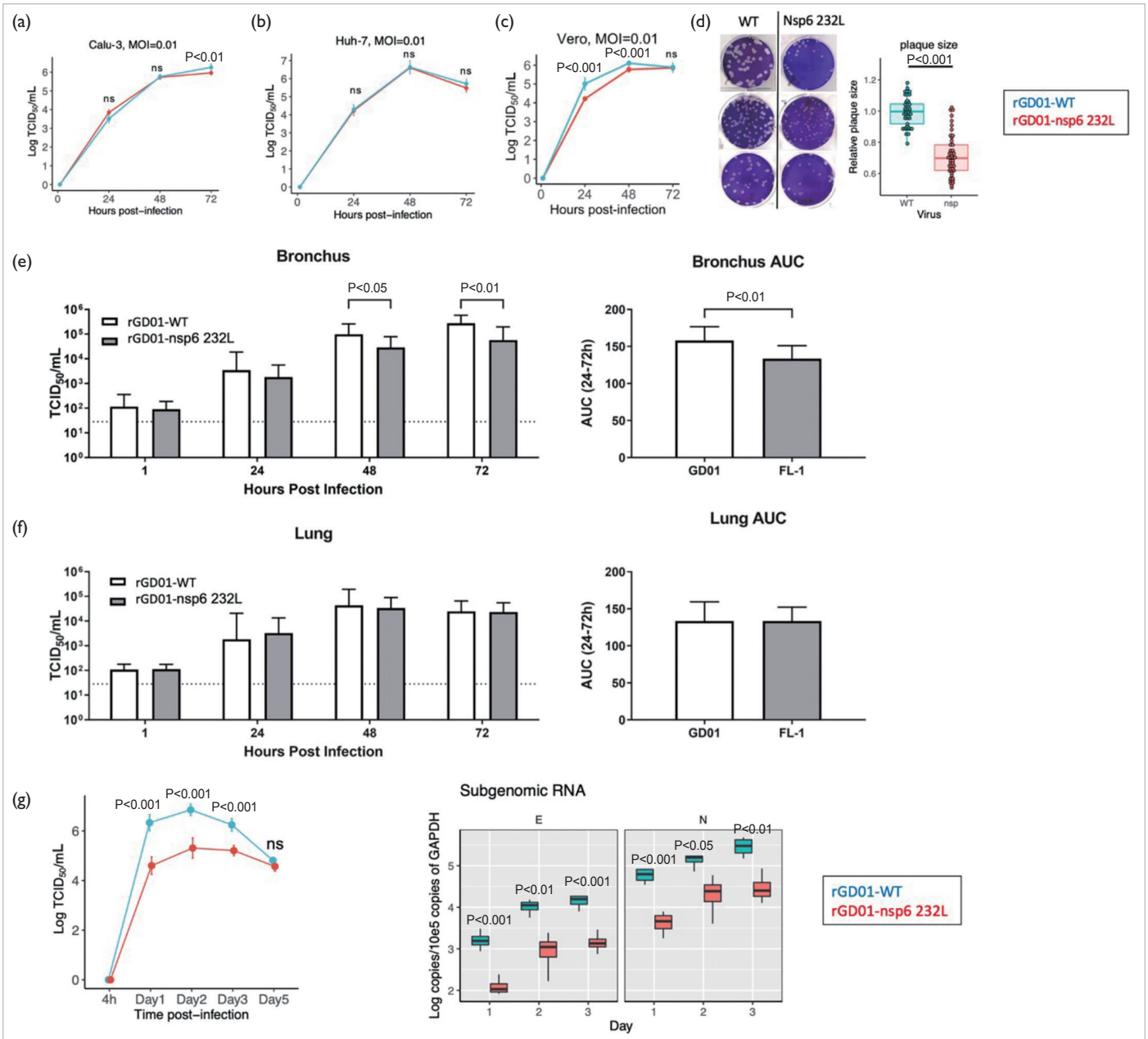

FIG 2. Viral replication kinetics of rGD01-WT and rGD01-nsp6 232L: (a) Calu-3 cells, (b) Huh-7 cells, and (c) Vero cells were infected at a multiplicity of infection (MOI) of 0.01. (d) Relative plaque sizes of rGD01-WT and rGD01-nsp6 232L viruses were determined in Vero cells. Viral replication kinetics in ex vivo cultures of human (e) bronchial and (f) lung tissues. The horizontal dotted line denotes the limit of detection in median tissue culture infectious dose ($TCID_{50}$) assays. (g) Viral replication kinetics in the lungs of human DPP4-knockin mice.

cells at both 24 and 48 hpi, indirectly suggesting that rGD01-WT infection led to higher numbers of DMV structures for viral RNA synthesis.

Nsp3, 4, and 6 reportedly induce the formation of DMV structures in an overexpression system. We used electron microscopy to measure the formation of DMV structures induced by each of the isogenic viruses. In Vero cells, single-membrane virus compartment vesicles containing virions were observed for both viruses at 24 hpi, whereas distinct DMV structures were not evident (data not shown). Therefore, we could not quantify the difference in DMV formation induced by the two isogenic viruses. However, we observed a larger vesicle diameter and higher virion number in each vesicle in rGD01-WT-infected cells than in rGD01-nsp6 232L-infected cells, confirming that rGD01-WT induces greater viral production.

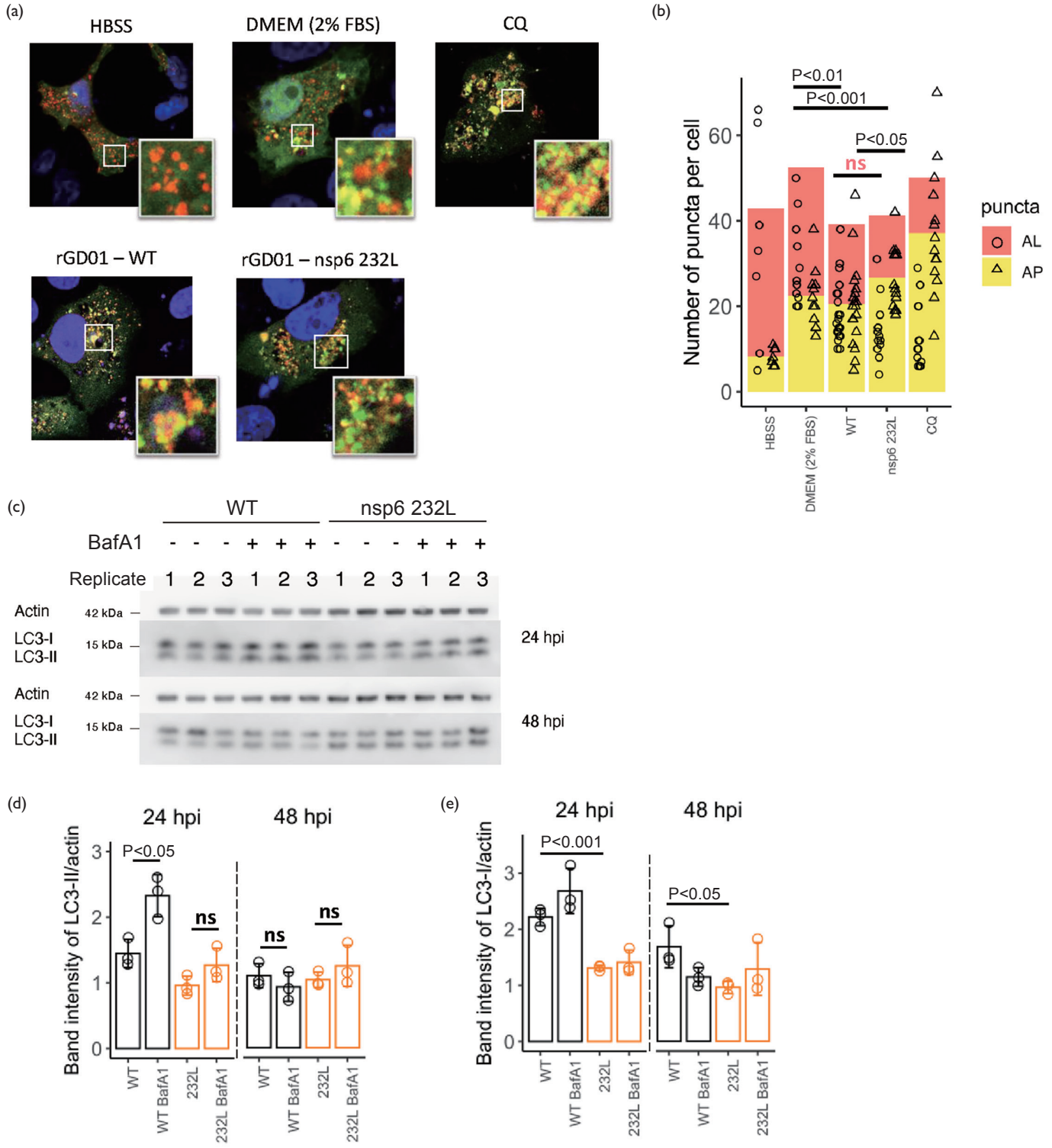

FIG 3. The nsp6 L232F mutation modulates autophagy and double-membrane vesicle quantity. (a) Vero cells transfected with mCherry and EGFP-tagged LC3 were infected with each of the two viruses at a multiplicity of infection (MOI) of 0.01 and subjected to immunofluorescence measurements. Hanks' Balanced Salt Solution (HBSS)-mediated induction of cell starvation served as the high autophagic flux control, Dulbecco's modified Eagle medium (DMEM) supplemented with 2% fetal bovine serum (FBS) served as the basal autophagic flux control, and chloroquine (CQ, 100 mM)-mediated inhibition of autophagosome (AP) to autolysosome (AL) fusion served as the low autophagic flux control. (b) Summary statistics of the numbers of AP and AL puncta counted. (c) Immunoblotting of LC3-I/II in Vero cells infected at an MOI of 0.01. To block AP to AL fusion, cells were incubated with BafA1 (0.1 μM) for 2 hours before collection of protein lysates. Band intensity of (d) LC3-II/actin ratio (representing AP level) and (e) LC3-I/actin ratio (representing a proxy measurement of double-membrane vesicle level).

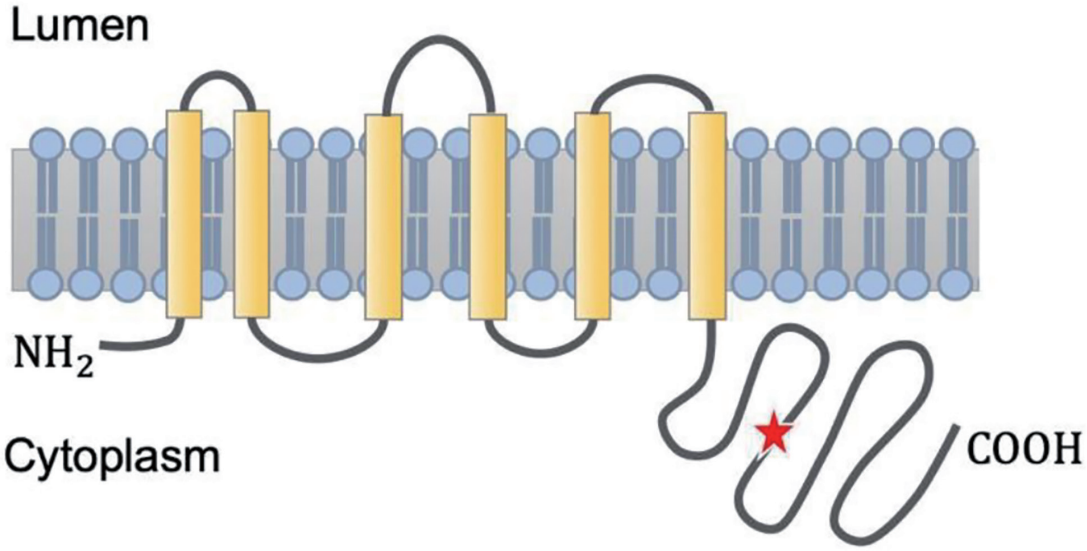

FIG 4. Membrane topology of MERS-CoV nsp6 and the location of the L232F mutation.

Discussion

We compared MERS-CoV mutations in camels and humans, which revealed a potential adaptive mutation, nsp6 L232F, that preferentially occurred in humans. MERS-CoV nsp6 is a highly conserved non-structural protein among Betacoronaviruses that consists of six transmembrane domains and a trailing C-terminal domain in the cytoplasm. The L232F mutation identified is located at the C terminus of nsp6, which potentially alters the intrinsic function of nsp6 (Fig 4).

We used reverse genetics to explore the phenotypic effects of this mutation in recombinant isogenic viruses. Compared with a Leu residue that is present in most camel MERS-CoVs, the recurrent Phe residue mutation in human MERS-CoVs led to enhanced replication in Vero cells, ex vivo cultures of human bronchial tissue, and the human DPP4 mouse model. The underlying mutation did not cause significant changes in interferon antagonism in vitro.

We explored the mechanistic basis for the effects of the nsp6 L232F mutation on viral replication competence in human cells. We investigated its effects on cellular autophagy, as CoV nsp6 has been suggested to hijack autophagy machinery for viral replication and generation of DMV structures. Both isogenic viruses showed autophagic inhibition with fewer ALs. Furthermore, LC3-II immunoblotting showed that the nsp6 L232F mutation resulted in less AP accumulation and a higher autophagic flux. However, the physiologic consequences of this phenotype are unclear because CoV manipulation of autophagy is not fully understood. Additionally, LC3-I is reportedly associated with DMVs. Our data showed that rGD01-WT-infected cells exhibited higher levels of LC3-I compared with rGD01-nsp6 232L-infected cells, possibly reflecting the higher number of DMVs in rGD01-WT-infected cells and corresponding increase in viral production. Further experiments that involve immunofluorescence staining of dsRNA(+) LC3 (+) puncta could validate this hypothesis. Electron microscopy did not show distinct DMV structures at 24 hpi. However, virion-containing vesicles were more visible.

Conclusion

The amino acid substitution nsp6 L232F is associated with transmission of MERS-CoV to humans. This mutation confers a replication advantage to the virus in Vero cells, ex vivo cultures of human bronchial tissue, and experimentally infected mice. This mutation affects AP function, but its effects on viral replication competence or host adaptation remain unclear. Multiple studies have detected the nsp6 L232F mutation during the generation of mouse-adapted MERS-CoV strains through passage in mice.[5]

We identified a potential human-adaptive mutation in the nsp6 protein of human MERS-CoV, which increased viral replication competence in some models. Our findings illustrate the potential

for MERS-CoV to achieve greater viral fitness through repeated inter-species transmission from camels to humans. We recommend systematic serial sampling of specimens from MERS-infected humans to determine whether this nsp6 mutation becomes increasingly prevalent during the course of infection in humans.

Funding

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Disclosure

The results of this research have been previously published in:

1. So RTY, Chu DKW, Hui KPY, et al. Amino acid substitution L232F in non-structural protein 6 identified as a possible human-adaptive mutation in clade B MERS coronaviruses. J Virol 2023;97:e0136923.

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