The spike glycoprotein of the new coronavirus 2019-nCoV contains a furin-like cleavage site absent in CoV of the same clade

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ABSTRACT

In 2019, a new coronavirus (2019-nCoV) infecting Humans has emerged in Wuhan, China. Its genome has been sequenced and the genomic information promptly released. Despite a high similarity with the genome sequence of SARS-CoV and SARS-like CoVs, we identified a peculiar furin-like cleavage site in the Spike protein of the 2019-nCoV, lacking in the other SARS-like CoVs. In this article, we discuss the possible functional consequences of this cleavage site in the viral cycle, pathogenicity and its potential implication in the development of antivirals.

Human coronaviruses (CoV) are enveloped positive-stranded RNA viruses belonging to the order *Nidovirales*, and are mostly responsible for upper respiratory and digestive tract infections. Among them SARS-CoV and MERS-CoV that spread in 2002 and 2013 respectively, have been associated with severe human illnesses, such as severe pneumonia and bronchiolitis, and even meningitis in more vulnerable populations (de Wit et al., 2016). In December 2019, a new CoV (2019-nCoV) has been detected in the city of Wuhan, and this emerging viral infection was associated with severe human respiratory disease with a ~2–3% fatality rate (Li et al., 2020). The virus that was presumed to have initially been transmitted from an animal reservoir to humans possibly *via* an amplifying host. However human-to-human transmission has been reported, leading to a sustained epidemic spread with > 31,000 confirmed human infections, including > 640 deaths, reported by the WHO in early February 2020. The estimated effective reproductive number (R) value of ~2.90 (95%: 2.32–3.63) at the beginning of the outbreak raises the possibility of a pandemics (Zhao et al., 2020). This prompted WHO to declare it as a Public Health Emergency of International Concern. This is especially relevant because so far there are no specific antiviral treatments available or vaccine. Based on its genome sequence, 2019-nCoV belongs to lineage b of Betacoronavirus (Fig. 1A), which also includes the SARS-CoV and bat CoV ZXC21, the latter and CoV ZC45 being the closest to 2019-nCoV. 2019-nCoV shares ~76% amino acid sequence identity in the Spike (S)-protein sequence with SARS-CoV and 80% with CoV ZXC21 (Chan et al., 2020). In this article,

we focus on a specific furin-like protease recognition pattern present in the vicinity of one of the maturation sites of the S protein (Fig. 1B) that may have significant functional implications for virus entry.

The proprotein convertases (PCs; genes *PCSKs*) constitute a family of nine serine secretory proteases that regulate various biological processes in both healthy and disease states (Seidah and Prat, 2012). By proteolysis, PCs are responsible for the activation of a wide variety of precursor proteins, such as growth factors, hormones, receptors and adhesion molecules, as well as cell surface glycoproteins of infectious viruses (Seidah and Chretien, 1999) (Table 1). Seven PCs cleave precursor proteins at specific single or paired basic amino acids (aa) within the motif $(R/K)-(2X)n-(R/K)\downarrow$, where n = 0, 1, 2, or 3 spacer aa (Seidah and Chretien, 1999). Because of their role in the processing of many critical cell surface proteins PCs, especially furin, have been implicated in viral infections. They have the potential to cleave specifically viral envelope glycoproteins, thereby enhancing viral fusion with host cell membranes (Izaguirre, 2019; Moulard and Decroly, 2000). In the case of human-infecting coronaviruses such as HCoV-OC43 (Le Coupanec et al., 2015), MERS-CoV (Millet and Whittaker, 2014), and HKU1 (Chan et al., 2008) the spike protein has been demonstrated to be cleaved at an S1/S2 cleavage site (Fig. 2) generating the S1 and S2 subunits. The above three viruses display the canonical $(R/K)-(2X)n-(R/K)\downarrow$ motif (Table 1). Additionally, it has been demonstrated that variation around the viral envelope glycoprotein cleavage site plays a role in cellular tropism and pathogenesis. For instance, the pathogenesis of some CoV

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A

B

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651 - T   A   S   I   L                       R ↓ S   T   G   Q   K - 661   CoV-ZXC21
      ACG GCT TCT ATA TTA --- --- --- --- CGT AGT ACA GGC CAG AAA


      ACT CAG ACT AAT TCT CCT CGG CGG GCA CGT AGT GTA GCT AGT CAA
676 - T   Q   T   N   S   P   R   R   A   R ↓ S   V   A   S   Q - 690   2019-nCoV
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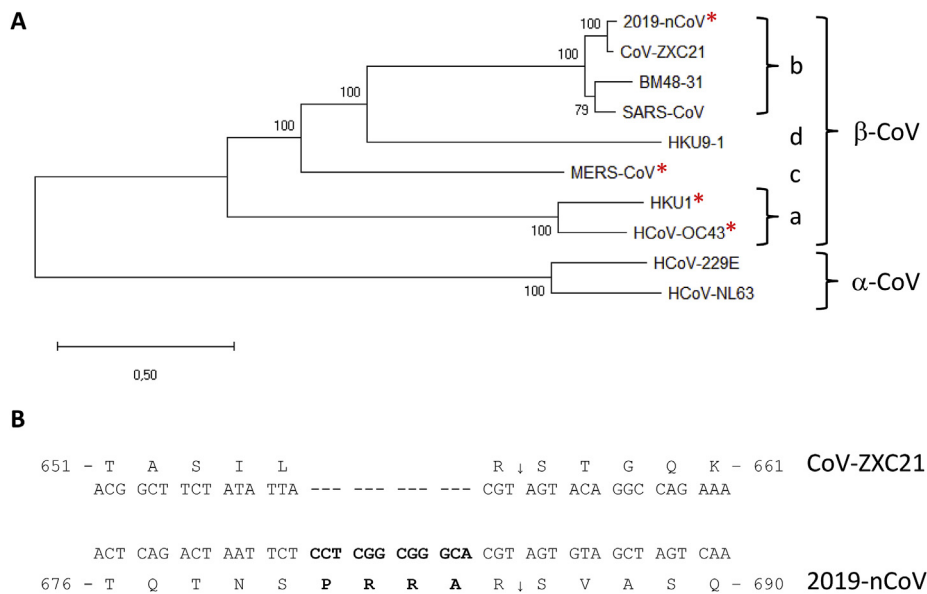
Fig. 1. Characterization of an nCoV-peculiar sequence at the S1/S2 cleavage site in the S-protein sequence, compared SARS-like CoV. (A) Phylogenetic tree of selected coronaviruses from genera alphacoronavirus (α-Cov) and betacoronavirus (β-CoV), lineages a, b, c and d: 2019-nCoV (NC_045512.2), CoV-ZXC21 (MG772934), SARS-CoV (NC_004718.3), SARS-like BM4821 (MG772934), HCoV-OC43 (AY391777), HKU9-1 (EF065513), HCoV-NL63 (KF530114.1), HCoV229E (KF514433.1), MERS-CoV (NC019843.3), HKU1 (NC_006577.2). The phylogenetic tree was obtained on the Orf1ab amino acid sequence using the Maximum Likelihood method by Mega X software. Red asterisks indicate the presence of a canonical furin-like cleavage motif at site 1; (B) Alignment of the coding and amino acid sequences of the S-protein from CoV-ZXC21 and 2019-nCoV at the S1/S2 site. The 2019-nCoV-specific sequence is in bold. The sequence of CoV-ZXC21 S-protein at this position is representative of the sequence of the other betacoronaviruses belonging to lineage b, except the one of 2019-nCoV. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Comparative sequences of envelope protein cleavage site(s) in coronaviruses (above) and in other RNA viruses (below). Empty boxes: no consensus motif detected..

Coronavirus	S1/S2, site 1	S1/S2, site 2	S2'
2019-nCoV	SP**RR**AR↓S**V**AS	IAY↓TMS	SKPS**KR**↓SF
CoV-ZX21	TASIL**R**↓STGQ	IAY↓TMS	SKPS**KR**↓SF
Bat-AC45	TASIL**R**↓STGQ	IAY↓TMS	SKPS**KR**↓SF
SARS-CoV	TVSLL**R**↓STGQ	IAY↓TMS	LKPT**KR**↓SF
BM48-31	SSTLV**R**↓SGGH	LAY↓TMS	LKPT**KR**↓SF
HKU9-1	ADSLP**R**↓LQLV	VNY↓DPL	GATTY**R**↓SA
MERS-CoV	TP**R**SC**R**↓SVPG		GSRSA**R**↓SA
HKU1	SR**R**K**RR**↓SISA		CGSSS**R**↓SF
HCoV-OC43	KN**RR**S**R**↓GAITT		SKASS**R**↓SA
HCoV-229E	IAVQP**R**↓NVSYD		SRVAG**R**↓SA
HCoV-NL63	IPVRP**R**↓NSSDN		SRIAG**R**↓SA

Virus	Envelope Protein	Cleavage site
HIV	Gp160	VQRE**KR**↓AV
Influenza Virus H5	HA	**RKRKKR**↓GL
Avian H5N1 A/HK/98	HA	**RERKRKKR**↓GL
Avian H5N1 TKY/ENG	HA	**NTPQR**K**KR**↓GL
Human CMV	gB	**HKRTKR**↓ST
Human RSV	F protein	**KKRKRR**↓FL
Yellow Fever Virus	PrM	SR**RSRR**↓AI
Zika Virus	PrM	AR**RSRR**↓AV
Ebola virus	GP	GR**RTRR**↓EA
2019-nCoV (S1/S2) site	Spike Protein	TNSP**RR**AR↓SV
2019-nCoV (S2') site	Spike Protein	SKPS**KR**↓SF

has been previously related to the presence of a furin-like cleavage site in the S-protein sequence. For example, the insertion of a similar cleavage site in the infectious bronchitis virus (IBV) S-protein results in higher pathogenicity, pronounced neural symptoms and neurotropism in infected chickens (Cheng et al., 2019).

Similarly, in the case of influenza virus, low-pathogenicity forms of influenza virus contain a single basic residue at the cleavage site, which is cleaved by trypsin-like proteases and the tissue distribution of the activating protease(s) typically restricts infections to the respiratory and/or intestinal organs (Sun et al., 2010). Conversely, the highly pathogenic forms of influenza have a furin-like cleavage site cleaved by different cellular proteases, including furin, which are expressed in a wide variety of cell types allowing a widening of the cell tropism of the virus (Kido et al., 2012). Furthermore the insertion of a multibasic motif

RER**RR**K**KR**↓GL at the H5N1 hemagglutinin HA cleavage site was likely associated with the hyper-virulence of the virus during the Hong Kong 1997 outbreak (Claas et al., 1998). This motif exhibits the critical Arg at P1 and basic residues at P2 and P4, as well as P6 and P8 and an aliphatic Leu at P2' positions (Table 1) (Schechter and Berger nomenclature (Schechter and Berger, 1968)), typical of a furin-like cleavage specificity (Braun and Sauter, 2019; Izaguirre, 2019; Seidah and Prat, 2012).

The coronavirus S-protein is the structural protein responsible for the crown-like shape of the CoV viral particles, from which the original name "coronavirus" was coined. The ~1200 aa long S-protein belongs to class-I viral fusion proteins and contributes to the cell receptor binding, tissue tropism and pathogenesis (Lu et al., 2015; Millet and Whittaker, 2014). It contains several conserved domains and motifs
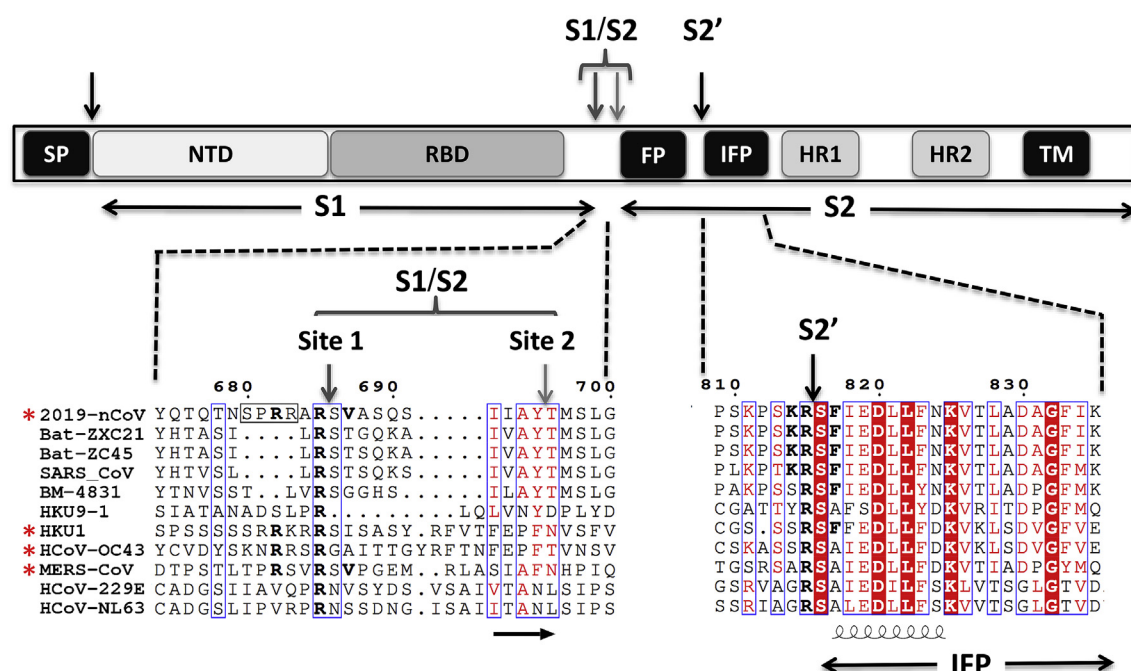
Fig. 2. Schematic representation of the human 2019-nCoV S-protein with a focus on the putative maturation sites. The domains were previously characterized in SARS-CoV and MERS-CoV: Signal peptide (SP), N-terminal domain (NTD), receptor-binding domain (RBD), fusion peptide (FP), internal fusion peptide (IFP), heptad repeat 1/2 (HR1/2), and the transmembrane domain (TM). The SP, S1↓S2 and S2′ cleavage sites are indicated by arrows. The sequence of different CoV S1/S2 and S2′ cleavage sites were aligned using Multalin webserver (http://multalin.toulouse.inra.fr/multalin/) with manual adjustments and the figure prepared using ESPript 3 (http://espript.ibcp.fr/ESPript/ESPript/) presenting the secondary structure of SARS-CoV S-protein at the bottom of the alignment (PDB 5X58) (Yuan et al., 2017). Insertion of furin like cleavage site is surrounded by a black frame. Red asterisks indicate the presence of a canonical furin-like cleavage motif at the S1/S2 site. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(Fig. 2). The trimeric S-protein is processed at the S1/S2 cleavage site by host cell proteases, during infection. Following cleavage, also known as priming, the protein is divided into an N-terminal S1-ectodomain that recognises a cognate cell surface receptor and a C-terminal S2-membrane-anchored protein involved in viral entry. The SARS-CoV S1-protein contains a conserved Receptor Binding Domain (RBD), which recognises the angiotensin-converting enzyme 2 (ACE2) (Li et al., 2003). The SARS-CoV binds to both bat and human cells, and the virus can infect both organisms (Ge et al., 2013; Kuhn et al., 2004). The RBD surface of S1/ACE2 implicates 14 aa in the S1 of SARS-CoV (Li et al., 2005). Among them, 8 residues are strictly conserved in 2019-nCoV, supporting the hypothesis that ACE2 is also the receptor of the newly emerged nCoV (Wan et al., 2020). The S2-protein contains the fusion peptide (FP), a second proteolytic site (S2′), followed by an internal fusion peptide (IFP) and two heptad-repeat domains preceding the transmembrane domain (TM) (Fig. 2). Notably, the IFPs of the 2019-nCoV and SARS-CoV are identical, displaying characteristics of viral fusion peptides (Fig. 2). While the molecular mechanism involved in cell entry is not yet fully understood, it is likely that both FP and IFP participate in the viral entry process (Lu et al., 2015) and thus the S-protein must likely be cleaved at both S1/S2 and S2′ cleavage sites for virus entry. The furin-like S2′ cleavage site at **KR↓SF** with P1 and P2 basic residues and a P2′ hydrophobic Phe (Seidah and Prat, 2012), downstream of the IFP is identical between the 2019-nCoV and SARS-CoV (Fig. 2). In the MERS-CoV and HCoV-OC43 the S1/S2 site is replaced by **R**XX**R**↓SA, with P1 and P4 basic residues, and an Ala (not aliphatic) at P2′, suggesting a somewhat less favourable cleavage by furin. However, in the other less pathogenic circulating human CoV, the S2′ cleavage site only exhibits a monobasic **R**↓S sequence (Fig. 2) with no basic residues at either P2 and/or P4 needed to allow furin cleavage, suggesting a less efficient cleavage or higher restriction at the entry step depending on the cognate proteases expressed by target cells. Even though processing at S2′ in 2019-nCoV is expected to be a key event for

the final activation of the S-protein, the protease(s) involved in this process have not yet been conclusively identified. Based on the 2019-nCoV S2′ sequence and the above arguments, we propose that one or more furin-like enzymes would cleave the S2′ site at **KR↓SF**. In contrast to the S2′, the first cleavage between the RBD and the FP (S1/S2 cleavage site, Fig. 2) has been extensively studied for many CoVs (Lu et al., 2015). Interestingly the S1/S2 processing site exhibits different motifs among coronaviruses (Fig. 2, site 1 & site 2), with many of them displaying cleavage after a basic residue. It is thus likely that the priming process is ensured by different host cell proteases depending on the sequence of the S1/S2 cleavage site. Accordingly the MERS-CoV S-protein, which contains a **R**SV**R**↓S**V** motif is cleaved during virus egress, probably by furin (Mille and Whittaker, 2014). Conversely the S-protein of SARS-CoV remains largely uncleaved after biosynthesis, possibly due to the lack of a favourable furin-like cleavage site (SLL**R**-ST). In this case, it was reported that following receptor binding the S-protein is cleaved at a conserved sequence AYT↓M (located 10 aa downstream of SLL**R**-ST) by target cells' proteases such as elastase, cathepsin L or TMPRSS2 (Bosch et al., 2008; Matsuyama et al., 2010, 2005; Millet and Whittaker, 2015). As the priming event is essential for virus entry, the efficacy and extent of this activation step by the proteases of the target cells should regulate cellular tropism and viral pathogenesis. In the case of the 2019-nCoV S-protein, the conserved site 2 sequence AYT↓M may still be cleaved, possibly after the preferred furin-cleavage at the site 1 (Fig. 2).

Since furin is highly expressed in lungs, an enveloped virus that infects the respiratory tract may successfully exploit this convertase to activate its surface glycoprotein (Bassi et al., 2017; Mbikay et al., 1997). Before the emergence of the 2019-nCoV, this important feature was not observed in the lineage b of betacoronaviruses. However, it is shared by other CoV (HCoV-OC43, MERS-CoV, MHV-A59) harbouring furin-like cleavage sites in their S-protein (Fig. 2; Table 1), which were shown to be processed by furin experimentally (Le Coupanec et al.,

2015; Mille and Whittaker, 2014). Strikingly, the 2019-nCoV S-protein sequence contains 12 additional nucleotides upstream of the single Arg↓ cleavage site 1 (Figs. 1B and 2) leading to a predictively solvent-exposed P**R**RA**R**↓**SV** sequence, which corresponds to a canonical furin-like cleavage site (Braun and Sauter, 2019; Izaguirre, 2019; Seidah and Prat, 2012). This furin-like cleavage site, is supposed to be cleaved during virus egress (Mille and Whittaker, 2014) for S-protein "priming" and may provide a gain-of-function to the 2019-nCoV for efficient spreading in the human population compared to other lineage b betacoronaviruses. This possibly illustrates a convergent evolution pathway between unrelated CoVs. Interestingly, if this site is not processed, the S-protein is expected to be cleaved at site 2 during virus endocytosis, as observed for the SARS-CoV.

Obviously much more work is needed to demonstrate experimentally our assertion, but the inhibition of such processing enzyme(s) may represent a potential antiviral strategy. Indeed, it was recently shown that in an effort to limit viral infections, host cells that are infected by a number of viruses provoke an interferon response to inhibit the enzymatic activity of furin-like enzymes. It was also demonstrated that HIV infection induces the expression of either the protease activated receptor 1 (PAR1) (Kim et al., 2015) or guanylate binding proteins 2 and 5 (GBP2,5) (Braun and Sauter, 2019) that restrict the trafficking of furin to the *trans* Golgi network (PAR1) or to early Golgi compartments (GBP2,5) where the proprotein convertase remains inactive. Altogether, these observations suggest that inhibitors of furin-like enzymes may contribute to inhibiting virus propagation.

A variety of approaches have been proposed to inhibit furin activity to limit tumour growth, viral and bacterial infection. Thus, a variant of the naturally occurring serine protease inhibitor α-1 antitrypsin harbouring a consensus furin cleavage, called α-1 antitrypsin Portland (α1-PDX), inhibits furin and prevents the processing of HIV-1 Env (Anderson et al., 1993). The addition of a chloromethylketone (CMK) moiety to the C-terminus of a polybasic cleavage motif and a decanoyl group at the N-terminus to favour cell penetration (dec-RVKR-cmk) irreversibly blocked the enzymatic activity of furin, PC7, PC5, PACE4 and PC7 (Decroly et al., 1996; Garten et al., 1994). Finally, the elucidation of the crystal structure of furin resulted in the design of a 2,5-dideoxystreptamine-derived inhibitor, where two molecules of the inhibitor form a complex with furin (Dahms et al., 2017). As furin-like enzymes are involved in a multitude of cellular processes, one important issue would be to avoid systemic inhibition that may result in some toxicity. Accordingly, it is likely that such small molecule inhibitors, or other more potent orally active ones, possibly delivered by inhalation and exhibiting a slow dissociation rate from furin to allow for sustained inhibition, deserve to be rapidly tested to assess their antiviral effect against 2019-nCoV.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2020.104742.

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