

Title. Nicotinic Cholinergic System and COVID-19: Identification of a potentially crucial snake toxin-like sequence in the SARS-CoV-2 Spike glycoprotein

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Abstract

Smoking is a risk factor for respiratory infections and there is reasonable concern that it may affect COVID-19 susceptibility and severity. Recent studies have focused on the interaction between smoking (and nicotine) and ACE2 expression, suggesting that ACE2 up-regulation could contribute to enhanced viral cell entry. However, case series have shown that there is an unexpectedly low prevalence of smoking among hospitalized COVID-19 cases. Since early April, we were the first to hypothesize that dysfunction of the nicotinic cholinergic system (NCS) may be implicated in the pathophysiology of severe COVID-19. We recently reported that many of the clinical manifestations of severe COVID-19 could be explained by dysregulation of the NCS. In this study, we present an amino acid sequence in the receptor binding domain of the SARS-CoV-2 Spike glycoprotein which is homologous to a sequence of a snake venom toxin. We present the 3D structural location of this “toxin-like” sequence on the Spike Glycoprotein. These findings suggest that SARS-CoV-2 could potentially interact with acetylcholine receptors causing dysregulation of the NCS and the cholinergic anti-inflammatory pathway.

Keywords. COVID-19; SARS-CoV-2; smoking; nicotine; nicotinic cholinergic system; inflammation.

INTRODUCTION

As the global pandemic of Corona Virus Disease 2019 (COVID-19), a disease caused by the acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is evolving, it is imperative to understand the pathophysiology and the risk and potentially favorable factors associated with disease progression and severity in order to offer effective therapies. Smoking is a well-established risk factor for respiratory infection susceptibility and severity [1,2]. Considering the global burden of 1.1 billion smokers, it is crucial to understand the association between COVID-19 and smoking.

During the past few weeks, there is a lot of debate about the interaction between smoking, nicotine and ACE2, the enzyme used by SARS-CoV-2 for cell entry [3]. Recent studies suggest that smoking up-regulates ACE2 [4-6]. Cellular mechanisms have been proposed for the interaction between nicotine and ACE2 expression, [7,8]. The hypothesis is that smoking-induced ACE2 up-regulation will facilitate viral invasion and cell entry contributing to enhanced COVID-19 susceptibility and severity. Similar rationale has been applied to explain the higher risk for adverse outcomes in patients using common medications to treat hypertension, diabetes and cardiovascular disease such as ACE-inhibitors, angiotensin receptor blockers (ARBs) and thiazolidinediones [9]. However, the notion that ACE2 up-regulation contributes to COVID-19 severity has been seriously challenged. Evidence from *in vitro* studies on SARS-CoV suggests that continuous viral infection and replication induces down-regulation of ACE2 resulting in detrimental effects due to unopposed angiotensin II accumulation and activity. [10]. A similar mechanism may apply to SARS-CoV-2 [11]. Case series of COVID-19 patients from the US, Europe and Asia have shown that use of ACE-inhibitors and ARBs was not associated with COVID-19 test positivity and adverse outcome [12,13] while in one study, use of ACE-inhibitors was associated with lower odds for in-hospital death [14]. ACE2 counteracts the adverse effects of angiotensin II by cleaving

angiotensin I and angiotensin II to angiotensin (1-9) and angiotensin (1-7), respectively.

ACE2 deficiency has been observed with ageing, in diabetes mellitus and in heart disease, all of which appear to be risk factors for severe COVID-19 [15-17]. Additionally, children and young women have higher levels of ACE2 than older people, and experience milder COVID-19 [18]. Thus, ACE2 up-regulation is likely to be protective against severe COVID-19 [19].

The association between smoking (and nicotine) and COVID-19 appears to be far more complex than implied by findings on ACE2 expression. A recent meta-analysis of 13 case series of hospitalized COVID-19 patients in China identified an unusually low pooled prevalence of smoking compared to the population smoking rates [20]. While limitations were applicable to this analysis (lack of adjustment for confounding factors, possibility for under-reporting or inaccurate recording of the smoking status etc.), similar observations of low smoking prevalence among hospitalized COVID-19 patients were observed in case series from the US and elsewhere [14,21-23]. To further complicate the interpretation of these findings, some, but not other, studies have found that smoking is a risk factor for adverse outcomes [23,24].

Although these preliminary observations should be interpreted with caution, they raise the possibility that nicotine might be protecting patients from developing severe COVID-19 that would require hospitalization. This could be achieved through its action on the nicotinic cholinergic system (NCS). While most researchers have focused on the association between smoking (and nicotine) and ACE2 regulation and expression, none has examined the possibility of a direct interaction between SARS-CoV-2 and the NCS. Since early April we hypothesized for the first time that the NCS may be implicated in the pathophysiology of severe COVID-19, and we recently expanded on this hypothesis [25,26]. The NCS is an important pathway that regulates the inflammatory response. The effects are mediated mainly by the vagus nerve and by alpha7 nicotinic acetylcholine receptors (nAChRs) on

macrophages and other immune cells [27]. This so-called “cholinergic anti-inflammatory pathway” has been implicated in preventing sepsis and Acute Respiratory Distress Syndrome in animal models [28,29]. Alpha7 nAChRs are also expressed in human bronchial epithelial and endothelial cells [30], which are targeted by SARS-CoV-2 or are indirectly affected by COVID-19. Thus, it is possible that the uncontrolled inflammatory response in COVID-19 is caused by dysregulation of the NCS. Dysfunction of the NCS could also explain other clinical manifestations of COVID-19 such as anosmia and thromboembolic complications [26]. We therefore hypothesized that there may be a direct interaction between SARS-CoV-2 and the NCS. To examine this hypothesis, we compared amino acid sequences between SARS-CoV-2 and snake venom neurotoxins. The latter are well-established inhibitors of the NCS [31,32].

METHODS

Sequence retrieval of the protein sequences of both virus related spike proteins and “three finger” neurotoxins from various species was performed using the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) databases. BLAST searches were performed using Mega BLAST [33] with the UNIPROT protein database [34] and by using BLASTP (protein–protein BLAST) with default parameters. Multiple sequence alignments were performed using and Clustal-O) [35,36] and the Clustal Omega and Muscle methods through the UGENE program [37] that was also used to depict the sequence alignments and to incorporate the available conservation or diversity information. Default parameters were used for the alignment. Molecular modelling and superposition were performed by using PyMOL [38].

RESULTS

Figure 1 presents the sequence of the SARS-CoV-2 spike glycoprotein which is homologous in sequence to an equivalent of the Neurotoxin homolog NL1, part of the toxin's "three-finger" interacting motif, and the amino acids within this sequence which are identical (red) or functionally equivalent (yellow) to Neurotoxin homolog NL1 toxin. This peptide fragment (aa 375-390) is part of the Receptor Binding Domain (aa 319-541) of the SARS-COV-2 Spike protein (the domain through which the Spike protein recognizes the ACE2 on the host's cell surface) neighboring to the ACE2 Receptor Binding Motif (aa 437-508) [34].

***Figure 1.** Sequence of the SARS-CoV-2 Spike glycoprotein which is homologous to Neurotoxin homolog NL1 and amino acids within this sequence which are identical (red) or functionally equivalent (yellow) to Neurotoxin homolog NL1 toxin.*

SARS-CoV-2 Spike Glycoprotein	375	STFKCYGVSPTKLDL	390
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Figure 2 presents the alignment of the region aa 350-400 of the spike protein of SARS-CoV-2 glycoprotein with a number of spike proteins of various animal coronaviruses. The region aa 375-392 is contains cysteine-tyrosine amino acids which are characteristic of snake toxin sequences and are highly conserved among different coronavirus species.

Figure 3. Alignment of region aa 373-390 of the SARS-CoV-2 Spike glycoprotein with a number of other coronaviruses Spike proteins and snake toxins, emphasizing the Cysteine-Tyrosine motif. Alignment of a number of snake toxins indicating regions with identity (A), and the amino acid hydrophobic/hydrophilic profile (purple=polar, red=hydrophobic, blue=charged) of this region (B).

A

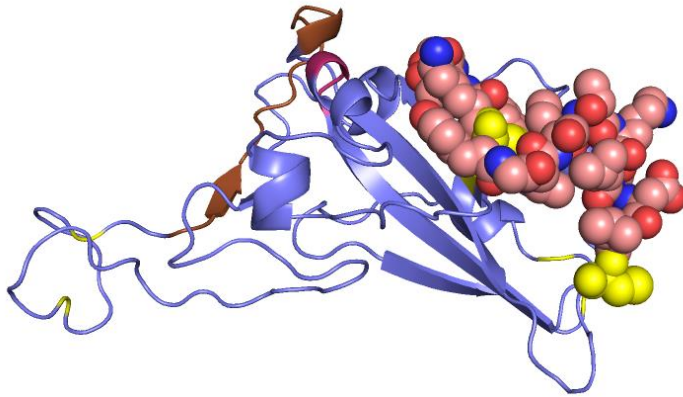
Q9DEQ3.1 Neurotoxin homolog NL1 [Naja atra]	30	T	P	E	T	T	E	I	C	P	D	S	W	Y	F	C	Y	K	I	S	L	A	D	-	-	-	G	I	55
ADF50014.1 short-chain 3 finger toxin [Bungarus flaviceps]	30	N	P	K	T	T	E	L	C	G	H	S	M	Y	F	C	Y	K	N	S	W	I	Y	-	-	-	R	C	55
Q8AY52.1 Candiduxin-2 [Bungarus candidus]	30	N	P	K	T	T	E	L	C	R	H	S	M	Y	F	C	F	K	N	S	W	I	A	-	-	-	G	C	55
ETE57194.1 L345 [Ophiophagus hannah]	27	-	V	H	G	L	C	T	C	E	P	D	E	K	F	C	F	R	K	T	T	M	F	-	-	-	F	P	51
CAA90966.1 cardiotoxin N [Naja naja]	29	-	P	P	F	Y	K	T	C	A	A	G	K	N	L	C	Y	K	M	F	M	V	A	-	-	-	A	I	52
AAR33036.1 atratoxin b [Naja atra]	21	Q	T	P	T	T	K	T	C	S	-	G	E	T	N	C	Y	K	K	W	W	S	D	-	-	-	H	F	45
Q7T215.1 Erabutoxin c [Laticauda semifasciata]	30	Q	P	Q	T	T	K	T	C	S	P	G	E	S	S	C	Y	H	K	Q	W	S	D	-	-	-	F	F	55
P60770.1 Cobrotoxin; [Naja atra]	30	Q	T	P	T	T	G	C	S	G	G	E	T	N	C	Y	K	K	R	W	R	D	-	-	-	H	F	55	
AAF75223.1 alpha neurotoxin [Pseudonaja textilis]	28	-	Y	F	D	T	V	C	K	P	Q	E	T	I	C	Y	R	Y	I	I	P	A	-	-	-	T	H	C	52
CSILC5.1 Alpha-elapitoxin-Oh2a [Ophiophagus hannah]	30	-	P	I	N	A	E	T	C	P	P	G	E	N	L	C	Y	T	K	M	W	C	D	A	W	C	S	S	57
AHZ08823.1 Ionaq neurotoxin [Micropechis ikaheca]	29	-	D	V	K	S	K	T	C	P	P	G	E	N	L	C	Y	T	K	T	W	C	D	A	F	C	G	S	56
P60615.1 Alpha-bungarotoxin [Bungarus multicinctus]	30	-	P	I	S	A	V	T	C	P	P	G	E	N	L	C	Y	R	K	M	W	C	D	A	F	C	S	S	58
P15817.1 Kappa-3-bungarotoxin [Bungarus multicinctus]	30	T	P	-	-	-	C	T	C	P	N	G	O	D	I	C	F	R	K	A	Q	C	D	N	F	C	H	S	56

B

6VSB A:373-390 Chain A, SARS-CoV-2 spike glycoprotein	S	F	S	T	F	-	-	K	C	Y	G	V	S	P	T	K	L	N	D	L	-	-
Q1Q54048.1:373-390 spike protein [Panqolin coronavirus]	S	F	S	T	F	-	-	K	C	Y	G	V	S	P	T	K	L	N	D	L	-	-
AID16716.1:364-381 spike glycoprotein [Bat SARS-like coronavirus]	S	F	S	T	F	-	-	K	C	Y	G	V	S	P	T	K	L	I	D	L	-	-
NL1 sp Q9DEQ3.1 3S08 NAJAT	P	D	S	W	Y	-	-	F	C	Y	K	I	S	L	A	D	G	N	D	V	-	-
4UY2 ALPHA-BUNGAROTOXIN	P	G	E	N	-	-	-	L	C	Y	R	K	M	W	C	D	V	F	C	S	-	-

This fragment, containing an amphipathic sequence of alternating polar and hydrophobic amino acid residues with selectively charged amino acids in a conserved order, probably lies on the Spike protein surface and is not buried in the domain core (**Figure 4**).

Figure 4. Graphical representation of the SARS-CoV-2 Spike Glycoprotein (one subunit) with the sequence aa375-390 shown in ball and sticks.



DISCUSSION-CONCLUSIONS

Animal venoms and especially snake venoms have evolved to contain a wide diversity of proteins that induce inflammatory and toxic effects [40]. Their pharmacological properties have been well-studied, revealing a complex mode of action. Many of these toxins exert their action by binding to the muscle or the neuronal type nAChRs [41-43].

The consistent observations of a low rate of smoking among hospitalized COVID-19 patients (despite the limitations and perplexities), the potential links between dysfunction of the NCS and clinical manifestations of COVID-19 and the identification of regions with increased homology between SARS-CoV-2 spike protein and neurotoxins in the receptor binding domain, generates the hypothesis of an interaction between SARS-CoV-2 and nAChRs leading to NCS dysregulation. If SARS-CoV-2 infection is not successfully controlled by the initial immune response, continuous viral replication could eventually cause considerable dysregulation of the NCS and the cholinergic anti-inflammatory pathway, leading to a hyper-

immune response and cytokine storm. The latter has been implicated as an important mechanism in severe COVID-19 and adverse outcomes [44]. Nicotine is a nicotinic cholinergic agonist that could protect nAChRs. The apparently “contradictory” findings by some investigators of a positive association between smoking and adverse outcomes among hospitalized COVID-19 patients may in fact be supportive to this hypothesis. Smokers experience abrupt nicotine cessation once hospitalized, and therefore, nicotine levels are expected to drop to non-detectable levels within a few hours after hospital admission. This hypothesis needs to be further explored through binding and functional experiments which are currently ongoing. In any case, it should be emphasized that smoking has detrimental health effects and cannot be recommended as a protective measure. However, the potential benefits of nicotine intake through the use of approved pharmaceutical products need to be examined.

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