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Down regulation of defensin genes during SARS-CoV-2 infection

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Summary. – Defensins, crucial components of the innate immune system, play a vital role against infection as part of frontline immunity. Association of SARS-CoV-2 infection with defensins has not been investigated. In this study, we have investigated the expression of defensin genes in the buccal cavity from patients with COVID-19 infection along with negative control samples. Nasopharyngeal/oropharyngeal swab samples collected for screening SARS-CoV-2 infection in early 2020 from Hyderabad, India, were analyzed for the expression of major defensin genes by the quantitative real-time reverse transcription polymerase chain reaction, qRT-PCR. Forty SARS-CoV-2 infected positive and 40 negative swab samples were selected for this study. Based on the qRT-PCR analysis involving gene specific primers for defensin genes, 9 defensin genes were found to be expressed in the nasopharyngeal/oropharyngeal cavity. Four defensin genes were found to be significantly down regulated in SARS-CoV-2 infected patients in comparison with the control samples based on differential expression analysis. The significantly down regulated genes were defensin beta 4A/B, 106B, 107B, and 103A. Down regulation of human beta defensin 2, 3, 6 and 7 suggests that antiviral innate immune response provided by defensins may be compromised in SARS-CoV-2 infection resulting in progression of the disease. Correction of the down regulation process through appropriate defensin peptide-based therapy could be an attractive method of treatment.

Keywords: host defense; defensins; COVID-19; gene regulation; SARS-CoV-2

Introduction

The small cysteine rich cationic antibacterial proteins, defensins are crucial components of the innate immune system (Oppenheim *et al*. 2003; Ganz 2003; Selsted and Ouellette, 2005; Schneider *et al.,* 2005; Pazgier *et al.,* 2006; Xu and Lu, 2020). They are the first line of defense against bacterial, fungal and viral infections. Based on their pattern of disulfide bonds; mammalian defensins are classified into α, β, and θ subfamilies (Oppenheim *et al*. 2003; Ganz 2003; Selsted and Ouellette, 2005; Schneider *et al.,* 2005; Pazgier *et al.,* 2006; Xu and Lu, 2020). The α-defensins HNP1-4 are predominantly produced by neutrophils, stored in neutrophil azurophilic granules and released in large quantities upon neutrophil activation (Selsted and Ouellette, 2005). The neutrophil defensin genes DEFA1 and DEFA3 enable gene copy number variation, which could potentially relate to their level of expression and regulation of immune responses (Mayumi *et al*., 2017). The β-defensins are expressed in a variety of epithelia especially in the airways (Pazgier *et al.,* 2006). Expression of the HBD1 and HBD4 genes is essentially constitutive, whereas expression of HBD2 and 3 genes is inducible in response to various stimuli (Pazgier *et al.,* 2006; Xu and Lu, 2020). Viruses, bacteria, microbial products, and pro-inflammatory cytokines, such as interleukin 1β (IL-1β) and tumor-necrosis factor (TNF), induce the expression of HBD2 and HBD3 in various cells

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Abbreviations: SARS-CoV-2 = severe acute respiratory syndrome-coronavirus 2; COVID-19 = Coronavirus Disease 2019; qRT-PCR = quantitative real time PCR; HBD = human beta defensin; VTM = viral transport medium

(Ding *et al.,* 2009; Klotman and Chang, 2006; Wilson *et al.,* 2016; Reddick and Alto, 2014; Semple and Dorin, 2012). In addition to its antimicrobial properties, DEFB4 (HBD2) is expressed in leukocytes and acts as a chemokine for cells of the adaptive immune response (Yang *et al.,* 1999). Defensins HD5 and HD6 are human enteric defensins and play important roles in gut immunity (Clevers and Bevins, 2013). Defensin antiviral effects on enveloped viruses (Ding *et al.,* 2009) suggest that defensins might have an active role in COVID-19. It is of interest to examine the association of defensin gene expression during SARS-CoV-2 infection in humans. SARS-CoV-2 has a heterogeneous pattern in exhibiting the symptoms associated with infection (Cao, 2020). The varied pattern of symptoms might be associated with innate immune response of defensins. A detailed expression analysis of human defensin genes could shed light on their role in modulating innate immune response, infection pattern and their association with the COVID-19. In this study, we have examined the expression of defensin genes in cells from nasopharyngeal/oropharyngeal swab samples from normal subjects and patients tested positive for the COVID-19, in early 2020 from Hyderabad, India, by the real-time reverse transcription PCR (qRT-PCR) method. We observed that several defensin genes were down regulated in patients.

Materials and Methods

Sample collection. Human nasopharyngeal/oropharyngeal swab samples in viral transport medium (VTM), received at CSIR-CCMB during early 2020 from Government Hospitals, Hyderabad, India at 4°C for COVID-19 diagnosis were selected for the study with ethical approvals (Institutional Ethics Committee of CCMB -82/2020). A total of 40 SARS-CoV-2 positive samples from the sample pool, having average Ct value of 23.2 for E gene and ORF gene of SARS-CoV-2 (Chu *et al.,* 2020) were selected for the study. Similarly, 40 negative samples as controls for differential expression were selected from the same pool of samples negative to SARS-CoV-2 E and ORF gene. The study has approval of CCMB Institutional Biosafety Committee.

SARS-CoV-2 diagnostics. Total RNA was extracted from the viral transport medium (VTM) swab samples using KingFisher™ Flex system (Thermofisher Scientific Inc., USA). The extracted RNA was analyzed for SARS-CoV-2 presence using single tube reverse transcription-polymerase chain reaction (qRT-PCR) analysis using the Novel Coronavirus (2019-nCOV) qRT-PCR detection kit (Shanghai Fosun Long March Medical Science CO. Ltd, China) for E gene and ORF gene of SARS-CoV-2 (Chu *et al.,* 2020) according to manufacturer's protocol. Quantstudio™ 5 (Thermofisher Scientific Inc., USA) was used to perform qRT-PCR. All the qRT-PCR work was carried out in a BSL-2 facility of CSIR-CCMB, Hyderabad, India.

Table 1. List of defensin genes selected for the study and their primer sequences with annealing temperatures

S. No	Gene	Symbol	Forward primer	Reverse primer	Annealing temperature (°C)
D2	Homo sapiens defensin beta 1	*DEFB1*	atgagaacttcctaccttctgctg	ctctgtaacaggtgccttgaattt	55
D4	Homo sapiens defensin beta 104A	*DEFB104A*	gtgctgctattagccatttctctt	gattcagtaagctctcatcccatt	55
D7	Homo sapiens defensin beta 107A	*DEFB107A*	ccttgataaaagagcttgattcca	tcacagtgaccttccattctctta	55
D8	Homo sapiens defensin beta 105A	*DEFB105A*	ttctcaaggaaatcccaatctcta	actcacagacagcaaactcacct	60
D9	Homo sapiens defensin beta 107B	*DEFB107B*	agagaatggaaggtcactgtgaag	gagatggtccagagagatttatcc	60
D10	Homo sapiens defensin beta 106B	*DEFB106B*	tttgatgagaaatgcaacaaactt	agcagaggcaagagtaaaaagaag	60
D11	Homo sapiens defensin beta 114	*DEFB114*	acatgtaccttggtgaatgctgat	ggtgcacatgtaacttctttgttc	55
D12	Homo sapiens defensin beta 105B	*DEFB105B*	gacttgatttttcccaaccattt	ctgcttctaaggccagaagtgt	55
D13	Homo sapiens defensin beta 108B	*DEFB108B*	tcaaccaagaattgagagcactac	tggttggttttggtttattctttt	55
D14	Homo sapiens defensin beta 112	*DEFB112*	tgttttgctttgtttcatgttttt	cacataaatcctgggtatgacaaa	55
D15	Homo sapiens defensin alpha 1	*DEFA1*	acatcccagaagtggttgtttc	agctcatttttctttttctgcaag	55
D16	Homo sapiens defensin alpha 5	*DEFA5*	aggaaatggactctctgctcttag	tttcaggaccttgaactgaatctt	55
D17	Homo sapiens defensin alpha 3	*DEFA3*	acatggactgctattgcagaatac	aaggaaattgagcagaaggtacag	55
D18	Homo sapiens defensin alpha 6	*DEFA6*	aacagaatattcctatgggacctg	ttgctgaaaggactttatttgaga	60
D19	Homo sapiens defensin beta 4A/B	*DEFB4A/B*	atcctgttacctgccttaagagtg	gtttacatgtcgcacgtctctgat	60
D20	Homo sapiens defensin beta 103A	*DEFB103A*	tcatggaggaatcataaacacatt	gcatttccacactttacaacactc	60
D21	Homo sapiens defensin alpha 1B	*DEFA1B*	ctgagactactcaccccataacct	aaacaaccacttctgggatgtc	55
D23	Homo sapiens GAPDH – housekeeping gene	GAPDH	atgacatcaagaaggtggtgaag	ctgtagccaaattcgttgtcatac	55 and 60

Defensin qRT-PCR analysis. Gene specific primers were synthesized for a total of 17 various human defensin genes and GAPDH housekeeping gene (Table 1). cDNA was synthesized from 50 ng of total RNA from each COVID positive and negative swab samples using iScript Advanced cDNA synthesis kit for qRT-PCR (BioRad, USA) following manufacturer's protocol. qPCR analysis was performed for all the defensin genes and the housekeeping gene using TB Green Premix Ex Taq 11 (TliRNaseHPlus) kit (Takara, Japan) in Applied Biosystems ViiA™ 7 Real-Time PCR system (USA). The qRT-PCR was performed using the following conditions – initial denaturation at 95°C for 30 s; 40 cycles of 95°C for 15 s, 55 or 60°C for 30 s and 72°C for 15 s; followed by melting curve analysis (Saxena *et al.,* 2012). Amplification of gene specific single qRT-PCR product was confirmed by performing gel electrophoresis of the amplified product in 2% agarose gel and melting curve analysis.

Differential expression analysis. The cycle threshold (Ct) values obtained from the analysis were used for differential expression analysis (Saxena *et al.,* 2012). The mean Ct value of each specific gene and housekeeping gene was calculated and their differential expressions were analyzed based on the 2^ddCt of target and housekeeping genes. The *p* values were calculated using standard t-test for all the observed Ct values. All the statistical analysis was performed using Microsoft Excel statistical software.

Results

Expression of defensin genes

The lists of 17 defensin genes selected for the study are summarized in Table 1. They include both α- and β-defensin genes including isoforms. From these, 9 defensin genes showed expression at detectable limits in the selected nasopharyngeal/oropharyngeal swab samples, which were further selected for the differential expression analysis. The Ct values for the expression of defensin genes were selected in the range of 20.0 to 40.0 for all the genes including housekeeping genes. Undetectable ranges of amplification were excluded from the analysis. The mean Ct values of defensin genes expression of positive and negative SARS-CoV-2 samples were analyzed separately (Table 2).

Based on the expression analysis it was found that SARS-CoV-2 positive samples showed higher Ct values, in comparison to the SARS-CoV-2 negative samples for almost all the defensin genes (Table 2). Differential expression analysis of the Ct value of each of the defensin genes against housekeeping GAPDH gene indicated that four defensin genes, *DEFB4A/B* (HBD-2), *107B* (HBD-7), *106B* (HBD-6), and *103A* (HBD-3) were found significantly down regulated in the SARS-CoV-2 positive swab samples

Table 2. Expression level of defensin genes in COVID-19 positive and negative samples

Gene name	COVID status	Mean	SD	SEM	Differential analysis	*p* value	Significance
COVID status	positive	23.2	7.4	1.2		–	
	negative	-	-	-			
Defensin beta 1	positive	32.5	2.3	0.4	-0.14	0.20	No
	negative	32.1	1.4	0.2			
Defensin beta 105A	positive	31.2	1.6	0.2	-0.13	0.17	No
	negative	30.9	1.8	0.3			
Defensin beta 107B	positive	29.8	2.6	0.4	-1.00	0.01	Yes
	negative	28.5	1.9	0.3			
Defensin beta 106B	positive	29.0	2.5	0.4	-1.33	0.00	Yes
	negative	27.5	1.7	0.3			
Defensin beta 4A/B	positive	29.6	2.7	0.4	-0.95	0.01	Yes
	negative	28.4	2.0	0.3			
Defensin beta 103A	positive	29.1	2.6	0.4	-1.12	0.01	Yes
	negative	27.8	1.9	0.3			
Defensin alpha 3	positive	29.7	3.0	0.5	-0.67	0.07	No
	negative	28.7	2.3	0.4			
Defensin alpha 6	positive	29.9	3.0	0.5	-0.48	0.15	No
	negative	29.2	2.8	0.4			
Defensin alpha 1B	positive	32.8	1.6	0.3	-0.37	0.09	No
	negative	32.2	2.2	0.3			

in comparison to control SARS-CoV-2 negative swab samples (Fig. 1). Non-significant down regulation of expression was observed for the α-defensin genes, *DEFA3, DEFA6* and *DEFA1B* (Fig. 1). *DEFB1* and *DEF105A* did not show any effective changes in their differential expression.

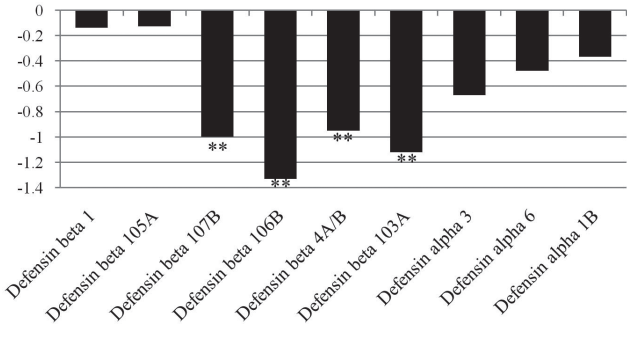

Fig. 1

Differential expression level of defensin genes
Differential expression level of defensin genes in COVID-19 positive samples in comparison to COVID-19 negative samples. (**p <0.01).

Discussion

The β-defensin genes *DEFB4A/B*, *107B*, *106B*, and *103A* were found significantly down regulated based on differential analysis against control for SARS-CoV-2 infection (Fig. 1, Table 2). Genes corresponding to HD-5 and HD-6 i.e., *DEFA5* and *DEFA6* were not expressed in nasopharyngeal/oropharyngeal samples, as would be expected. HD-5 and HD-6 are expressed and secreted in Paneth cells (Clevers and Bevins, 2013). It is of particular interest to observe the down regulation of the HBD-2 (*DEFB4A/B*) gene. HBD-2 represents the human defensin that is produced by epithelial cells following contact with bacteria, viruses, or cytokines such as IL-1 and TNF-α (O'Neil 2003). HBD-2 appears to activate the primary antiviral innate immune response (Kim *et al.,* 2018). Although the nature of the regulation is not completely characterized, both the MAPK signal transduction pathway and the NF-κB transcription factor have been suggested to be involved (Pace *et al.,* 2017). Also, the less investigated *DEFB106* and *DEFB107* were also down regulated.

The antiviral activities of human defensins have been the subject of extensive investigations (Kim *et al.,* 2018; Pace *et al.,* 2017; Park *et al.,* 2018; Ahmed *et al.,* 2019; Wang *et al.,* 2020). Antiviral activity of defensins against HIV and other viruses suggest that defensins have a role in host-defense not only against bacteria. Their expression in several cell types and tissues (Pace *et al.,* 2017) suggest that they can act as the first line of defense against viruses targeting various cell types. HD-5 has been shown to inhibit SARS-CoV-2 *in vitro* by binding to angiotensin-converting enzyme 2 (ACE2), the cellular receptor for the virus (Wang *et al.*, 2020). Hence, the interest in exploring defensins for potential application as anti-viral therapeutics (Park *et al.*, 2018; Ahmed *et al.*, 2019).

Gilbert *et al.* (2021) have reported that infection by SARS-CoV-2 resulted in increase in the levels of *DEFB1* (HBD-1), *DEFB4A* (HBD-2) and *DEFB103* (HBD-3) transcripts in adults (15–65 years) but not in children ≤ 15 years, from nasopharyngeal swab samples. We have observed down regulation in the expression levels of *DEFB4* and *DEFB103* in addition to other β-defensin genes. We are of the opinion that the differences in β-defensin gene expression in the two studies reflect variations in mucosal anti-viral innate immunity of different populations in addition to differences in expression due to age. Modulation of defensin genes is complex and is dependent on various factors (Ryan and Diamond, 2017). Notwithstanding the complexity, we observe down regulation of several β-defensin genes on SARS-CoV-2 infection in the nasopharyngeal region.

Conclusion

The down regulation of several defensin genes suggests that innate immunity provided by defensins is compromised in SARS-CoV-2 infection resulting in progression of the disease caused by the virus. Also, virus-mediated down regulation of defensin expression could result in augmented colonization of the uppermost airway by bacteria resulting in lung infection. Association of defensin genes with SARS-CoV-2 infection suggests that up regulating defensin gene expression could be an attractive therapeutic intervention. Defensin peptides that are part of the full-length human defensins HBD-1-3 that exhibit antimicrobial activities could also conceivably act as effective antiviral agents for therapy against SARS-CoV-2 (Hoover *et al.,* 2003; Krishnakumari *et al.,* 2006, 2018).

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