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Expression of innate immune response genes in upper airway samples of SARS-CoV-2 infected patients: A preliminary study

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***Background & objectives*: Upper respiratory mucosa is the entryway for SARS-CoV-2, and cells at this site form the first line of resistance against the pathogens. Innate immune response at this point is crucial for managing the replication and early stage symptoms of virus infection. This study was aimed to evaluate the expression of pattern recognition receptors and cytokines in upper airway cells of SARS-CoV-2 infected patients.**

***Methods*: Forty seven nasopharyngeal swab (NPS) specimens from 25 SARS-CoV-2 infected patients and 22 SARS-CoV-2 negative individuals were investigated for expression of toll-like receptors (TLRs), melanoma differentiation-associated protein 5 (MDA5), NOD-like receptors family pyrin domain containing 3 (NLRP3), angiotensin-converting enzyme 2 (ACE2), interleukin (IL) - 6, tumour necrosis factor alpha (TNFα) and type-1 interferons (IFNs) by real time reverse transcription polymerase chain reaction.**

***Results*: Increased expression of TLR2, MDA5 and ACE2 was detected in SARS-CoV-2 infected patients in comparison with controls. MDA5 expression was significantly higher in asymptomatic and mildly symptomatic SARS-CoV-2 positive patients than the patients with severe symptoms. The asymptomatic group showed significant induction of type 1 IFNs than the symptomatic group. Non-specific induction of TLR7 could be observed in nasopharyngeal (NP) cells irrespective of symptoms and SARS-CoV-2 positivity.**

***Interpretation & conclusions*: The findings suggest that increased MDA5 in NP cells of asymptomatic SARS-CoV-2 positive patients may subsequently induce type 1 IFNs to protect the individuals from further clinical severity of SARS-CoV-2 infection. A future prospective study in NPS of larger cohort needs to be performed to confirm our findings.**

Key words Innate immunity - MDA-5 - nasopharyngeal swab - SARS-CoV-2 - toll-like receptors

Patients with severe COVID-19 may develop acute respiratory distress syndrome and acute lung injury, resulting in morbidity and mortality caused by damage to the alveolar lumen by inflammation and pneumonia[1]. However, in 80 per cent of the infected patients the disease remains mild and mostly restricted to the upper and conducting airways[2]. SARS-CoV-2 when inhaled attaches to the epithelial cells of nasal cavity and initiates replication, thereby migrating down the tract along the conducting airways, triggering strong innate immune response. Angiotensin-converting enzyme (ACE2) is the main receptor for both SARS-

CoV and SARS-CoV-2[3,4]. But only some percentage of monocytes/macrophages within the lung express ACE2[5] which indicates that SARS-CoV-2 utilizes other receptors or cellular entry mode in major immune cells[6]. Innate immune cells in the upper airways recognize the viral invasion, by pathogen associated molecular patterns (PAMPs), to mount an antiviral response. Therefore, nasal or nasopharyngeal swabs (NPSs) used for diagnostic purpose may be considered as early markers of the innate immune response, predicting the subsequent clinical course in COVID-19[7].

There are three major classes of cytoplasmic pattern recognition receptors (PRRs) which recognize viral RNA: Toll-like receptors (TLRs), retinoic acid-inducible gene RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs), triggering the expression and activation of interferon (IFN), natural killer (NK) cells, CD8+ T cells and macrophages[8]. For SARS-CoV and Middle East respiratory syndrome (MERS) coronavirus, PAMPs such as viral genomic RNA/dsRNA are recognized either by the endosomal RNA receptors, TLR3 and TLR7 or the cytosolic RNA sensor, RIG-I, melanoma differentiation-associated protein 5 (MDA5), subsequently inducing type I IFN and other pro-inflammatory cytokines comprising the first line of defense at the entry point of viral infection[9]. Although, a number of studies have reported the expression of innate immune response genes by *in silico* studies/bioinformatics analysis of available data or by metagenomic sequencing of upper airway samples, none of these have specifically shown the expression of TLRs in the upper airway cells[10]. It has been reported that compared with other viral acute respiratory infections (ARIs), COVID-19 is characterized by an elevated IFN response but attenuated activation of TLR, interleukin 1 (IL-1) and NLR family pyrin domain containing 3 (NLRP3) inflammasome pathways which are considerably less responsive to SARS-CoV-2[11].

Based on the earlier reports on coronavirus infection, innate immune response has been shown to play a crucial role in protective/destructive responses thereby, creating opportunity for immune intervention[12]. Individuals with underlying diseases, such as diabetes, hypertension, and cardiovascular disease are susceptible to COVID-19[13]. Additionally, young children with highly effective immune response reported mild symptoms with less severe cases[14]. These reports highlight the importance of innate immune response for disease outcome[12]. The present study was aimed to investigate the expression of host innate immune response genes in upper airway cells as biomarkers in SARS-CoV-2 infected individuals using NPS cells, collected for diagnostic purpose.

Material & Methods

The study was conducted at the Mumbai Unit of the ICMR- National Institute of Virology (NIV) after obtaining approval from the Human Ethics Committee of NIV. Retrospective samples (NPSs, n=47) from the ICMR-NIV, Mumbai Unit repository, received during the months of March to May 2020 from hospitals of Mumbai for SARS-CoV-2 testing, were used for this study based on their available clinical information. The NPS samples previously utilized for testing SARS-CoV-2, were availed from the repository and further categorized into four groups: (*i*) symptomatic positive patients (n=14) with mild/moderate symptoms (fever, cough, congestion, malaise, headache) and severe symptoms (breathlessness, chest pain and comorbidities), (*ii*) asymptomatic positive patients (n=11) [contact cases tested positive by reverse transcription polymerase chain reaction (RT-PCR)], (*iii*) symptomatic negative (n=12) (suspected cases under the case definition criteria and tested negative by RT-PCR), and (*iv*) asymptomatic negatives (n=10) (contact cases tested negative by RT-PCR)[15,16] (Table). Based on RT-PCR results and symptoms with a follow up period, asymptomatic and symptomatic COVID-19 negative samples were taken as controls (Table). Of the total 47 cases enrolled, 46.8 per cent (n=22) were females. The mean age of patients was 39.19±13.64 yr and mean cycle threshold (Ct) value was 26.27±4.547.

Quantification of innate immune response genes by RT-qPCR): NPS aliquots (1 ml collected in viral transport medium) stored at −80°C were thawed and centrifuged in microcentrifuge tube at 200*g* for four minutes. From the cell pellet, total RNA was isolated using the Ambion RNAqueous® Phenol free total RNA Isolation Kit as per manufacturer's instructions (Thermo fisher Scientific, Lithuania, USA)[17]. First-strand cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems by Thermo fisher Scientific, Bedford, MA, USA)[18]. For quantitative real time PCR analysis, TaqMan® Gene Expression Master Mix, (Applied Biosystems by Thermo fisher Scientific, Life Technologies Corporation, CA, USA) and ABI 7500 real-time PCR System (Applied Biosystems, Foster City, CA, USA) were used. Primers and fluorescein amidite-labelled probes were obtained from TaqMan® Gene Expression

Table. Demographic characteristics of the SARS CoV-2 infected and uninfected patients

Variables	SARS-CoV-2 positive (n=25)		SARS-CoV-2 negative (n=22)	
	Symptomatic positive (n=14)	Asymptomatic positive (n=11)	Symptomatic negative (n=12)	Asymptomatic negative (n=10)
Sex				
Female, n (%)	5 (35.71)	6 (54.54)	7 (58.33)	4 (40)
Male, n (%)	9 (64.28)	5 (45.45)	5 (41.66)	6 (60)
Age (yr, mean±SD)	44.21±12.6	43.18±13.82	34.67±14.66	33.2±10.99
Ct value (mean±SD)	26.87±5.141	25.58±4.114	-	-

Ct, cycle threshold

Assays (Applied Biosystems by Thermo fisher Scientific, Life Technologies Corporation, CA, USA). The following probes and primers were used: TLR2 (Hs00152932_ml), TLR3 (Hs01551078_ml), TLR4 (Hs00152939_ml), TLR7 (Hs01933259_Sl), IFN induced with helicase C domain 1 (IFIH-1 or MDA5) (Hs00223420_m1), NLRP3 (Hs00184937_m1), ACE2 (Hs01085333_m1), IL-6 (Hs00985639_m1), tumour necrosis factor alpha (TNFα, Hs01113624_g1), IFNA1 (Hs00855471_g1), IFNB1 (Hs01077958_s1). Expression levels were normalized to the levels of 18s rRNA using Taqman® Reagents Starter kit (Applied Biosystems, Foster City, CA, USA)[19].

Statistical analysis: Comparisons between the symptomatic and asymptomatic COVID-19 cases with control groups were analysed using t test and Mann–Whitney U test to compare two or three variables respectively. Data were analysed using GraphPad Prism 5, Version 5.01 (GraphPad Software, San Diego, CA, USA).

Results & Discussion

NPS samples used in the study showed similar Ct values for SARS-CoV-2 in asymptomatic and symptomatic positive samples indicating less variation in the viral RNA content between both the groups, as reported by others also[20].

Increased expression of TLR2, NLRP3 and ACE2 in SARS-CoV-2 positive patients: COVID-19 is characterized by an elevated IFN response but attenuated activation of TLRs, IL-6 and NLRP3 inflammasome pathways which are considerably less as compared with other acute respiratory illness[11]. In our study, relative expression of TLR2 was observed only in SARS-CoV-2 positive individuals and was negligible in negative individuals (P=0.0126) (Fig. 1A). However, no significant difference in TLR2 expression was observed between the symptomatic and asymptomatic positive groups. Upregulation of TLR2 in NPS of SARS-CoV-2 positive patients suggest viral recognition by TLR2 at the entry point.

TLR3 and TLR4 were minimally expressed in both SARS-CoV-2 positive and negative groups and had no significance within the groups, except in a few positive samples where the TLR4 expression was higher (data not shown). An *in silico* study on relation of SARS-CoV-2 and TLRs has reported TLR4 involvement in recognizing molecular patterns of SARS-CoV-2 for producing inflammatory responses[21]. However, we did not find any association of TLR4 expression with the severity of the disease. Since TLR7 recognizes single-stranded RNA (ssRNA), a non-specific increase in TLR7 expression in nasopharyngeal (NP) cells of COVID-19 positive and negative groups in our study indicated infection with other ssRNA viruses, which needs to be investigated (Fig. 1B). Notably, irrespective of SARS-CoV-2 positivity, TLR7 expression was comparatively higher in symptomatic individuals than the asymptomatic individuals, suggesting its involvement in immunopathogenesis. As per an immunoinformatic approach, the SARS-CoV-2 genome contains more ssRNA fragments which is recognized by TLR7/8 as compared to the SARS-CoV genome, indicating hyperactivation of innate immune response by SARS-CoV-2, thereby inducing an effective proinflammatory response, supporting our observations[22]. In another study with four young male patients with severe COVID-19, a rare loss-of-function variant of X-chromosomal TLR7 was identified and supposed to be responsible for impaired type I/II IFN responses[23]. Altogether, the observations suggest requirement of a balanced TLR7

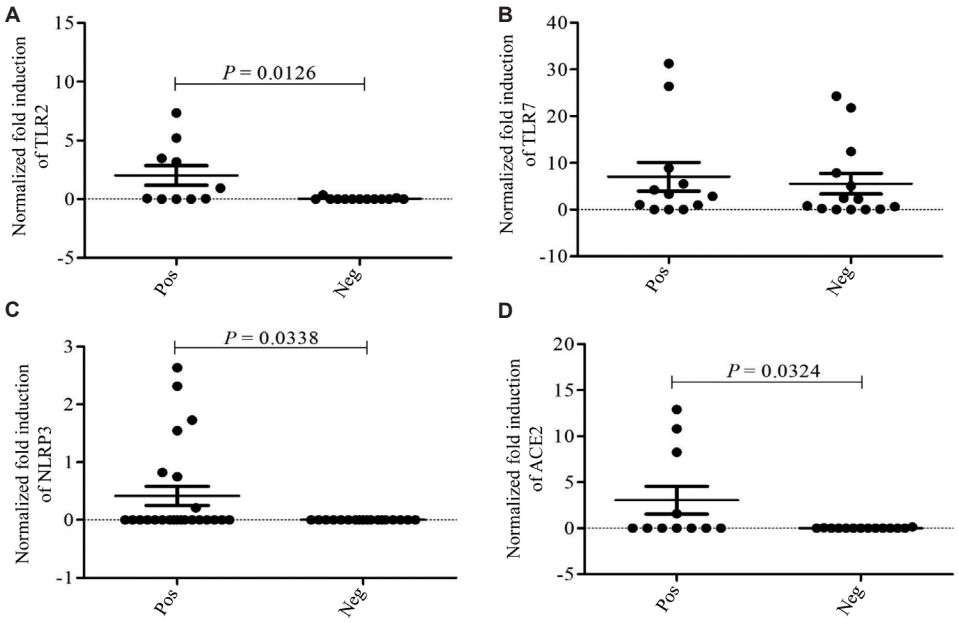

Figure 1. Differential expression of innate immune response genes in nasopharyngeal (NP) cells of SARS-CoV-2 infected and uninfected patients. Comparisons for mRNA expression of (**A**) TLR2: between SARS-CoV-2 positive group (n=10) versus negative control group (n=13) (*P*=0.0126), (**B**) TLR7: between SARS-CoV-2 positive group (n=12) versus negative control group (n=14), (**C**) NLRP3: between SARS-CoV-2 positive group (n=24) versus negative control group (n=18) (*P*=0.0338), (**D**) ACE2: between SARS-CoV-2 positive group (n=11) versus negative control group (n=14) (*P*=0.0324). TLR, toll-like receptors; NLRP3, NLR Family pyrin domain containing 3; ACE2, angiotensin-converting enzyme 2.

mediated antiviral and inflammatory host response for protection against severe COVID-19.

The virulence and pathogenicity of COVID-19 are reportedly associated with viral activation of cytoplasmic NLRP3 inflammasome[24]. NLRP3 expression was upregulated in NPS of a few patients with significant difference between positive and negative groups (*P*=0.0338) (Fig. 1C) but the expression was not associated with clinical symptoms. A study on upper airway host transcriptional response to SARS-CoV-2 by metagenomic sequencing showed suppressed innate immune response and less responsive NLRP3 inflammasome pathways in airway epithelial cells in comparison to other viral ARIs[25]. Further, studies in large cohort are required to confirm the findings.

An induction of ACE2 in NP cells of SARS-CoV-2 positive cases was observed (Fig. 1D), confirming the presence of receptor in nasal epithelial cells (*P*=0.0324) as shown by others[26,27]. The exact role of ACE2 in SARS-CoV-2 infection is questionable due to the evidences of low ACE2 expression in human respiratory system, raising the hypothesis of alternate receptors required for the SARS-CoV-2 entry[28].

Increased expression of MDA5 in SARS-CoV-2 positive asymptomatic patients: MDA5 is known to mediate activation of interferon stimulating genes (ISGs) essential for antiviral IFN responses. It is speculated that SARS-CoV-2 ssRNA, recognized by MDA5 and RIG-I triggers increased expression of IFNs and induces pro-inflammatory cytokines such as IL-6, TNF-α, IL-1 through NFκB[9,12]. In concordance with the speculations, our study showed significantly higher MDA5 expression Fig. 2A (*P*=0.002) in SARS-CoV-2 positive as compared to the negative group indicating its possible role in type1 IFN induction and protection against disease severity. Furthermore, within symptomatic positive group, higher MDA5 expression in mild symptomatic group was observed as compared to the severe symptomatic individuals (*P*=0.0472) (Fig. 2B). Using HEK293 MDA5 KO cells, Liu *et al*[29] have demonstrated a significant role for ISG15 in MDA5-mediated antiviral response, supporting our result.

Expression of interferons and inflammatory cytokines: Type 1 IFN and other pro-inflammatory cytokine expression form the primary defence system at the entry point of viral infection, generating direct inhibitory effects on viral propagation. In our study, NP cells

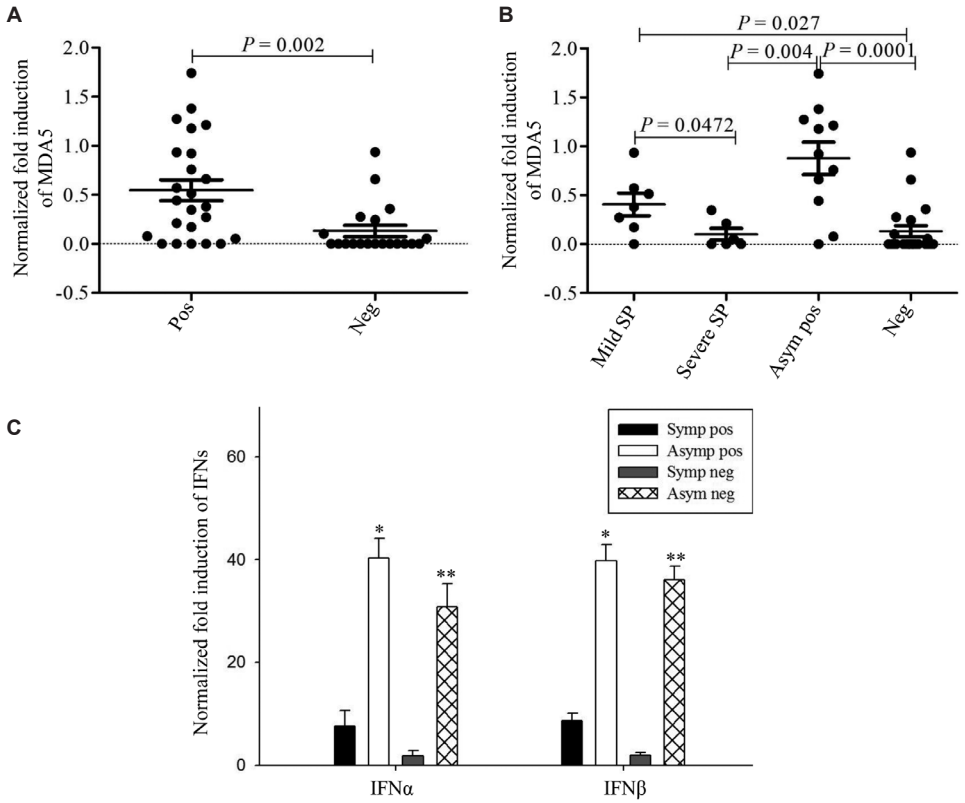

Figure 2. Differential expression of innate immune response genes in nasopharyngeal (NP) cells of SARS-CoV-2 infected and uninfected patients. Comparisons for mRNA expression of MDA5: (**A**) between SARS-CoV-2 positive group (n=24) versus negative control group (n=20) (*P*=0.002), (**B**) between SARS-CoV-2 mild symptomatic positive (n=7), severe symptomatic positive (n=6), asymptomatic positive (n=11), negative (n=20) (mild vs. severe, *P*=0.0472), (severe sym pos vs. asym pos, *P*=0.004), (mild vs. neg, *P*=0.027), (asym pos vs. neg, *P*=0.0001), (**C**) Comparisons for mRNA expression of IFNs (IFNα and IFNβ between SARS-CoV-2 symptomatic positive (pooled, n=5), asymptomatic positive (pooled, n=5), symptomatic negative (pooled, n=5), asymptomatic negative (pooled, n=5) (IFNα sym pos vs. asym pos *P*=0.0218, sym pos vs. asym neg *P*=0.0512, asym pos vs. sym neg *P*=0.0106, sym neg vs. asym neg *P*=0.0248), (IFNβ sym pos vs. asym pos *P*=0.0124, sym pos vs. asym neg *P*=0.0122, asym pos vs. sym neg *P*=0.0007, sym neg vs. asym neg *P*=0.0006). *P* *<0.05 compared to symptomatic positive group, **<0.01 compared to symptomatic negative group. TLR, toll-like receptors; MDA5, melanoma differentiation associated protein 5; IFN, interferon; SP, symptomatic positive

of asymptomatic individuals showed significantly higher expression of type 1 IFNs (IFN α and β) than the symptomatic group, irrespective of SARS-CoV-2 positivity (IFNα, *P*=0.0218; IFNβ, *P*=0.0124) (Fig. 2C). Since there was a significantly increased expression of MDA5 in NP cells of asymptomatic COVID-19 patients than symptomatic, we suggest the role of MDA5 in induction of type 1 IFN in asymptomatic group. However, another study has identified patients with high titres of neutralizing autoantibodies against type I IFN-α2 and IFN-ω in 10 per cent of cases with severe COVID-19 pneumonia which was not found in asymptomatic or mildly symptomatic SARS-CoV-2 positive cases[30]. The neutralizing auto-Abs against type I IFNs may counteract the small amount of IFN released during the SARS-CoV-2 infection thus leading to the disease. Since our study is only based on gene expression in the NP cells, a further study analysing these patients for auto-antibodies to IFN would confirm the findings.

SARS-CoV-2 positive individuals expression of both IL-6 and TNFα as compared to the negative individuals, but there was no significant statistical difference (data not shown).

Small sample size and unavailability of detailed information regarding interval between symptom onset and sample collection was the major limitations of our study. Collection of mixed NPS and oropharyngeal swabs for COVID-19 diagnosis as per the ICMR guideline in India[16], restricted availability of separate NPSs for the study, therefore, only the NPSs collected during the early outbreak period could be utilized retrospectively. In addition, low cell count in NPS further restricted the analysis of other PRRs.

To summarize, NP cells of 47 individuals including COVID-19 positive and negative cases were studied, for upper respiratory tract gene expression. Our data revealed unique characteristics of the host transcriptional response to SARS-CoV-2 infection, providing insights regarding the involvement of innate immune receptors in progression of disease at an early stage.

In conclusion, the upper respiratory epithelium of asymptomatic SARS-CoV-2 positive patients could induce MDA5 expression in response to viral infection leading to subsequent induction of type 1 IFN pathway, which may protect the individuals from further disease severity. Future prospective studies in a larger cohort of patients are required to validate our findings.

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***Conflicts of Interest*:** None.

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