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Research article

Tracing the SARS-CoV-2 infection on the ocular surface: Overview and preliminary corneoscleral transcriptome sequencing

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\begin{abstract}
COVID-19’s impact on the ocular surface has already been recognized, however the molecular mechanisms induced by the infection on the ocular surface are still unclear. The aim of this paper is to provide a first overview of the transcriptional perturbations caused by SARS-CoV-2 on the ocular surface by analyzing gene expression profile of corneoscleral ring samples from post-mortem SARS-CoV-2 positive donors (PD). The presence of SARS-CoV-2 on the ocular surface, in tears and corneal tissues has rarely been detected in infected individuals in both the presence and the absence of ocular manifestations. In this preliminary study, 6 human corneoscleral tissues of 3 PD and two tissues from a negative donor (CTRL) were obtained at the local eye bank. The presence of genomic and sub-genomic SARS-CoV-2 RNAs was assessed by qRT-PCR, while transcriptome analysis (RNA-sequencing) was performed by Illumina. Principal Component Analysis (PCA), search for differentially expressed genes (DEGs) and Gene Ontology (GO)-enrichment analysis were performed. Three samples from PD were found positive for SARS-CoV-2 genomic RNA, although the absence of sub-genomic RNAs indicated an inactive virus. PCA analysis grouped 3 different clusters, one including CTRL, and the other two including, respectively, PD with undetected SARS-CoV-2 (PD-SARS-neg) and PD with detected SARS-CoV-2 (PD-SARS-pos). The DEGs in common with the 2 PD clusters included several genes associable to the interferon pathway, such as ADAMTS4, RSAD2, MMP1, IL6, ISG15 and proinflammatory cytokines. Among the down-regulated genes we found AQP5. GO analysis revealed 77 GO terms over-represented in PD-SARS-neg vs. CTRL, and 17 GO terms in PD-SARS-pos vs. CTRL.

The presence of SARS-CoV-2 RNA and RNA-sequencing reads in ocular surface tissues supports the possibility that the eye acts as an entry route. The modulation of early responsive genes, together with several ISGs suggests a potential protective responsiveness of the ocular tissues to SARS-CoV-2.
\end{abstract}

\section{Introduction}

The random combination of morbidity, high infectivity and the ability of Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) to target several human organs and subvert antiviral defenses contributed to the diffusion of COVID-19 worldwide (Lam et al., 2020). The 12 Open Reading Frames (ORFs) encoded along the positive-sense RNA genome of SARS-CoV-2 codified for structural and non-structural proteins (Fernandes et al., 2020), the latter responsible for the interactions with host receptors and antiviral mediators, to promote viral entry and the overall infection process (Blanco-Melo et al., 2020). \textit{In-vivo} studies have revealed a hyper-activation of Interferon Stimulated Genes (ISGs) as the molecular footprint of SARS-CoV-2 infection in lungs, known as “cytokine storm” (Song et al., 2020). Mechanistically, how SARS-CoV-2 may interfere with immune responses is not completely known, although the reported modulation of long non-coding RNAs (lncRNAs) can dysregulate the fine-tuning mechanisms controlling the immune system (Turjya et al., 2020).

The presence of SARS-CoV-2 on the ocular surface and in tears has rarely been detected in infected individuals in both the presence and the

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absence of ocular manifestations (Zhang et al., 2020; Wu et al., 2020). In fact, the reported prevalence of viral RNA detection in tears varies from 0% to 24%, with higher positivity rates in patients with severe COVID-19, suggesting that a significantly higher possibility of viral transmission exists through tears in patients with moderate to severe COVID-19 (Arora et al., 2021).

In addition, low RNA loads and sub-genomic RNA of SARS-CoV-2 were detected post-mortem in the cornea of patients with COVID-19 viremia in the absence of SARS-CoV-2 infectious particles in any of the samples analyzed, suggesting that the risk of COVID-19 infection via corneal transplant is low even in donors with SARS-CoV-2 viremia (Casagrande et al., 2021). However, COVID-19 infection should remain a contraindication for donation for both penetrating keratoplasty and endothelial keratoplasty (Wan et al., 2021).

 Conjunctivitis or keratoconjunctivitis may be a sign of COVID-19 prior the onset of respiratory symptoms (Inomata et al., 2020; Cheema et al., 2020) or be the only sign of the infection (Scalinci and Trovato, 2020; 2019), suggesting that the ocular surface can be both a potential gateway for the SARS-CoV-2 to enter the body and a potential source of infection. On the contrary, the risk for an isolated conjunctival viral activity in patients with a negative nasopharyngeal swab-based RT-PCR seems to be absent or extremely low, suggesting no need to perform additional conjunctival swabs in patients with negative nasopharyngeal swabs (Rokohl et al., 2020; 2021).

Although lungs represent the main SARS-CoV-2 targets, other mucosal tissues can mediate the SARS-CoV-2 entry (Douglas et al., 2020; Collin et al., 2021). We and others reported the expression of SARS-CoV-2 entry receptor ACE2 and associated host protease TMPRSS2 in human cornea and conjunctiva by immunohistochrometry, RNA-sequencing, single-cell RNA-sequencing, Western blot, and assay for transposase-accessible chromatin using sequencing (ATAC-seq) (Leonardi et al., 2020; Mencucci et al., 2021; Zhou et al., 2020; Ma et al., 2021; Grajewski et al., 2021) supporting the possibility of an eye-mediated entry of SARS-CoV-2 (Douglas et al., 2020). Interestingly, the limbal region seems to be particularly prone to infection and may serve as a potential entry route for the virus (Eriksen et al., 2021).

 However, eye antiviral immunity has been rarely investigated and a recent study indicated that SARS-CoV-2 infection is restricted in human corneas, differently from Zika and HSV-1 (Miner et al., 2020).

Animal studies confirmed the ability to detect virus in the nasolacrimal and pulmonary system upon SARS-CoV-2 infection via the conjunctiva in monkeys (Deng et al., 2020) and in golden hamsters (Hoogland et al., 2021). In this last model, the eye transmission route showed evidence of a host respiratory response with elevated levels of interferon –stimulated protein ISG15 and the pro-inflammatory chemokine C-X-C motif chemokine ligand 11 (CXCL11) in the lungs. In the same study, it was suggested that dissemination of virus-derived PAMPs may be responsible for the systemic inflammation observed across tissues, reflecting the inconsistency of the tropism and inflammation in COVID-19 affected patients and the different clinical manifestations.

A recent report showed that SARS-CoV-2 RNA has been isolated from corneal specimens without inducing cytopathic effects and plaque formation in VeroE6 cell cultures (Ferrari et al., 2020). Therefore, the fate of SARS-CoV-2 in the eye is unclear. Based on the availability of ocular samples of SARS-CoV-2-positive donors, we report here preliminary data on the gene expression profile by RNA-sequencing of corneoscleral ring samples.

2. Materials and methods

2.1. Sample collection and description

According to the Italian guidelines set by the National Transplant Center (Rome, Italy), for every tissue donor post-mortem nasopharyngeal swabs are analyzed by means of quantitative Real-Time PCR (qRT-PCR) for identification of the presence of SARS-CoV-2 RNA before the tissue is used for transplantation. Only corneal tissues from donors with a negative post-mortem swab test can be transplanted (Ferrari et al., 2021).

The ocular tissues of 3 donors with positive post-mortem nasopharyngeal swab test for SARS-CoV-2 RNA (positive donor, PD), plus 2 of a negative post-mortem nasopharyngeal swab donor (control donor, CTRL) were obtained at the Fondazione Banca degli Occhi del Veneto (Venice, Italy) (Ferrari et al., 2020). By Italian law, written informed consent from a donor’s next of kin was obtained for the use of tissues for transplantation or, alternatively, for research purposes in agreement with the Declaration of Helsinki. The tissues were used in accordance with the laws of the National Transplant Center (Rome, Italy). Corneas were maintained in storage medium at 31 °C until sample processing (Ferrari et al., 2020).

2.2. SARS-CoV-2 diagnosis

Total nucleic acids (DNA and RNA) were purified from corneal specimens and storage medium by using a MagNA Pure 96 System (Roche Applied Sciences, Penzberg, Germany). Detection of SARS-CoV-2 RNA and sub-genomic RNA were performed by an in-house qRT–PCR method, which were developed, respectively, according to the protocol designed by CDC that targeted the genes N2 and sequence analysis of E gene sub-genomic mRNA (Wölfel et al., 2019, 2020). RT-qPCR assays were performed in a final volume of 25 μl, containing 5 μl of purified nucleic acids, using One Step Real Time kit (Qiagen) on an ABI 7900HT Fast Sequence Detection System (Thermo Fisher).

2.3. RNA extraction and sequencing

Tissues were homogenized with a manual pestle in liquid nitrogen. Subsequently, 1 ml of Trizol (Life Technologies, Carlsbad, CA, USA) was added to each sample and they were finely homogenized using a T-10 Ultra-Turrax (IKA, Staufen, Germany). Samples were centrifuged at 800 × g to remove the cellular pellets, and further mixed with 200 μl of Chloroform (Sigma-Aldrich, St. Louis, US) and centrifuged at 12,000 × g for 15 min at 4 °C. The water phase was collected and used for RNA extraction with the RNAeasy micro kit (Qiagen, Hilden, Germany) following manufacturer instructions. A DNA removal step was applied using 500 units of RNase-free DNase (Qiagen) at room temperature for 15 min. Total RNA was resuspended in RNase-free water (Thermo Fisher, Carlsbad, US) and the RNA/DNA concentrations in each sample were quantified using the Qubit RNA and DNA high-sensitivity Assay Kit (Thermo Fisher). RNA qualities were assessed with an Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). Due to the low RIN values (below 5), a 3′-end sequencing procedure was adopted using the QuantSeq 3′ mRNA-Sequencing Library Prep Kit FWD for Illumina (Lexogen, Vienna, Austria) for 7 suitable RNA samples out of 8. Libraries were sequenced using an Illumina NextSeq300 (Cribi, UniPD, Padova, Italy) with a 75 single end read layout.

2.4. RNA sequencing data analysis

The raw Illumina reads were trimmed for quality using trimmomatic (Bolger et al., 2014), setting a minimal Phred quality of 25 and removing the sequencing adaptors. FASTQ files were imported in the CLC Genomic Workbench v.21 (Qiagen, Hilden, Denmark) and analyzed as follows. To identify differentially expressed genes (DEGs) the trimmed reads were mapped on the human reference genome (hg19, Ensembl v.99) applying the following parameters: Mismatch cost = 2; Insertion cost = 3; Deletion cost = 3; Length fraction = 0.8; Similarity fraction = 0.8 and adopting a “forward” strand specificity and expression values were counted as Read Per Kilobase of Mapped reads (RPKM). A Baggerley test with false discovery rate (FDR) p-value correction was applied to identify differentially expressed genes (DEGs), setting a cutoff of 2-fold changes (FC) and a 0.01 of FDR p-value. A GO-enrichment analysis
based on Uniprot gene ontology (goa_human_20200423_hg19) was performed, removing hits with mean RPKM below 5.0, absolute fold change below 2 and with an FDR p-value higher than 0.01. Raw RNA-sequencing data were deposited at the NCBI SRA archive with accession ID PRJNA732263.

3. Results

Three post-mortem nasopharyngeal swabs of corneal donors were detected positive for genomic SARS-CoV-2 RNA (Ferrari et al., 2020). The presence of SARS-CoV-2 RNA was tested in the explanted corneas and two tissues were found positive (Donor 1 and 3, Table 1), whereas SARS-CoV-2 RNA was found in the conservative media of one cornea only (Donor 3, right cornea). We exploited the remaining parts of the explanted corneas, mostly referring to the corneoscleral ring, to perform RNA extraction and transcriptome analysis (RNA-sequencing), to identify differentially expressed genes in comparison to two control samples of a non-infected individual processed similarly (CTRL) (Table 1). The hardness of the corneoscleral tissue required a strong homogenization step and this probably contributed to the overall low RNA quality. We obtained a total of 0.26–2.1 μg of RNA per sample with RNA integrity values (RIN) below 5, whereas for one sample (D3_L) the RNA quality was not sufficient to proceed with library preparation. The absence of contaminating DNA was achieved through a DNase step and verified using DNA- and RNA-specific quantifications (Table 1). qRT-PCR revealed the presence of SARS-CoV-2 RNA only in two of the extracted RNAs (D3_R and D1_L) (Table 1), whereas sub-genomic SARS-CoV-2 RNAs was undetectable in all the samples. Illumina sequencing yielded 29.7 million (M) of high-quality reads (3.4–5.5 M reads per sample). As much as 1,850 reads of sample D3_R mapped to the 3′-end of the SARS-CoV-2 genome, whereas SARS-CoV-2 reads were not present in the other samples.

3.1. Corneoscleral gene expression profile by RNA-sequencing

Principal Component Analysis (PCA) based on the whole gene expression profiles grouped the 7 samples in 3 clusters according to the presence of SARS-CoV-2 RNAs in cornea or corneoscleral tissues, with the positive donors Donor 1, 2 and 3 clustered together on the PC1 axis (explaining 26.5% of the variation) and the control samples being more distributed on the PC2 axis (18.9% of the variation, Fig. 1). The first cluster included the samples of Donor 2 (positive donor with undetected SARS-CoV-2, PD-SARS-neg), the second cluster included samples of positive donors with detected SARS-CoV-2 in the eye (PD-SARS-pos), whereas the third cluster included CTRL samples. Using a hypergeometric test performed on the Gene Ontology (GO) Biological processes annotations, we reported 77 GO terms over-represented comparing PD-SARS-neg vs. CTRL, and 17 GO terms comparing PD-SARS-pos vs. CTRL, whereas 5 GO terms differentiated PD-SARS-pos vs. PD-SARS-neg samples (Supplementary Table 2). A total of 12 DEGs were common to all the three comparisons (ADAMTS4, CSRP1, NFKB1, TM4SF1, CXCL2, IRF1, Ccdc71L, NINJ1, TNFAIP2, SOCS3 and PLAUR) (Figs. 2 and 3 and Supplementary Table 1) and are characterized by incremental expression values, starting from CTRL, PD-SARS-pos and PD-SARS-neg samples. Among the down-regulated genes we found AQP5 (−4.8x), ANK3 (−2.2x) and KRT19 (−3.6x). Only in the comparison between PD-SARS-neg samples vs. CTRL, intracellular dsRNA receptors appeared upregulated, such as MDA5 (3x), RIG-I (3x) and ZNF31 (2.9x).

Using a hypergeometric test performed on the Gene Ontology (GO) Biological processes annotations, we reported 77 GO terms over-represented comparing PD-SARS-neg vs. CTRL, and 17 GO terms comparing PD-SARS-pos vs. CTRL, whereas 5 GO terms differentiated PD-SARS-pos vs. PD-SARS-neg samples (Supplementary Table 2). A total of 8 common GO terms, including 159 non-redundant genes, are detectable when comparing PD vs. CTRL samples, and included response

![Fig. 1. Principal Component Analysis based on whole transcriptome expression data. The samples labeled in blue are negative controls, whereas the ones labeled in red refer to positive donors. Triangles indicate samples negative or not-detected for SARS-CoV-2, whereas dots are samples with SARS-CoV-2 detected in the cornea and in the corneoscleral tissue (D1-D3). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)](image)

**Table 1**

Sample description and sequencing results. The donor code, cause of death, post-mortem sampling time and the results of the nasopharyngeal swab, detection of SARS-CoV-2 RNA in the cornea, conservative medium and corneoscleral ring were reported. The presence of SARS-CoV-2 was evaluated in the cornea and in the corneoscleral ring (Table 1). Total obtained RNA in micrograms, the sequencing library ID and number of high-quality reads were also indicated per sample.

| CODE | DONOR | CAUSE OF DEATH | POST-MORTEM TIME | RESULT OF NS | SARS-CoV-2 in cornea | SARS-CoV-2 in conservative medium | SARS-CoV-2 in CTRL | RNA (μg) | HQ reads |
|------|-------|----------------|------------------|--------------|----------------------|----------------------------------|-------------------|----------|----------|
| D1_L | Donor 1 | Lung cancer | 4h 25m | positive | Detected | n.d. | Detected (30) | 2.17 | 6,641,526 |
| D1_R | Donor 2 | Prostate cancer | 15h 55m | positive | Detected | n.d. | n.d. | 0.52 | 6,158,830 |
| D2_L | Donor 3 | Glioblastoma | 8h 40m | positive | Detected | n.d. | n.d. | 0.67 | 6,336,917 |
| D3_L | Donor 4 | Control | 3h 30m | negative | Detected | n.d. | Detected (29) | 0.26 | 9,731,146 |

D = donor; C = control; CR = corneoscleral ring; NS = nasopharyngeal swab; n.d. = not detected.
to chemical, response to organic substance, response to stress, cytokine-mediated signaling pathway, cellular response to chemical stimulus, response to stimulus, response to biotic stimulus (Supplementary Table 2). If considered alone, these 159 genes clustered the seven RNA-sequencing datasets according to the SARS-CoV-2 infection status and not according to the donor origin, indicating that they displayed incremental expression levels from control to PD-SARS-pos and with the highest expression levels in PD-SARS-neg (Supplementary Fig. 1).

3.2. RNA editing enzymes are poorly regulated by SARS-CoV-2 infection

Since in our previous study we found high basal expression levels of editing enzymes in healthy corneal and conjunctival tissues (Leonardi et al., 2020), here we investigated if the expression levels of the double-stranded RNA-specific adenosine deaminase (ADAR-1) and apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) genes in PD vs. CTRL samples are modified. We retrieved detectable expression levels (>3 TPMs) for ADAR1, ADARB2 and ADARB1 and for APOBEC3A, C and G. ADAR1, ADARB2 and APOBEC3A reached considerable expression levels in some samples, often exceeding 100 TPMs, although only ADAR1 and APOBEC3A appeared somewhat upregulated in SARS-CoV-2 infected samples (Table 2).

4. Discussion

The host response to SARS-CoV-2 extends beyond the typical

| Name | Description | D1_L | D1_R | D3_R | D2_L | D2_R | C1_R | C1_L |
|------|-------------|------|------|------|------|------|------|------|
| ADAR | dsRNA-specific adenosine deaminase | 90.7 | 66.8 | 141.6 | 77.6 | 98.5 | 67.9 | 54.4 |
| ADARE2 | dsRNA-specific editase B2 | 47.8 | 93.0 | 84.2 | 108.6 | 102.4 | 397.4 | 301.4 |
| ADARB1 | dsRNA-specific editase 1 | 31.1 | 26.3 | 67.9 | 43.0 | 32.6 | 46.5 | 46.5 |
| APOBEC3A | DNA dC > dU-editing enzyme APOBEC3A | 57.9 | 50.1 | 39.8 | 144.3 | 260.1 | 41.5 | 17.3 |
| APOBEC3C | DNA dC > dU-editing enzyme APOBEC3C | 25.8 | 21.5 | 35.6 | 16.3 | 6.0 | 42.2 | 42.5 |
| APOBEC3G | DNA dC > dU-editing enzyme APOBEC3G | 3.2 | 30.4 | 25.5 | 9.4 | 0.8 | 8.6 | 32.9 |
respiratory tract in several organs distal to the site of infection. As observed in cell culture and animal models, it is clear that the host response to SARS-CoV-2 results in an early aberrant IFN response juxtaposed with an overproduction of chemokines (Blanco-Melo et al., 2020). This response is defined by low levels of type I and III interferons with a moderate IFN-stimulated genes (ISGs), contrasted by elevated expression levels of chemokines and IL-6.

It is unclear if non-respiratory organs, such as the ocular surface, can contrast SARS-CoV-2 infection because of a potential heightened immunity of these tissues. This tissue, which expressed SARS-CoV-2 receptors such as ACE-2 and associated protease TMPRSS2, often showed undetectable levels of SARS-CoV-2 and mild possible associable symptoms, even in severe COVID-19 cases (Rokohl et al., 2020).

To investigate the ocular surface involvement in COVID-19 and to further investigate potential implications in corneal transplantation, we produced RNA sequencing data of the corneoscleral ring tissue, based on five SARS-CoV-2 positive and two negative samples. RNA-sequencing expression profiles clustered these seven samples firstly according to the presence of SARS-CoV-2 RNAs either in the cornea or in the corneoscleral ring. We can suppose that these corneoscleral ring expression profiles are stimulated by punctual intakes of SARS-CoV-2 mediated by hand touching or viral particles in aerosols (Dawood, 2021). An alternative hypothesis is that the secretion or spillover of sub genomic material, forming a protected ribonucleoprotein complex, may be a prevalent PAMP that is disseminated from the primary site of replication (Hoagland et al., 2021). This will indicate that the systemic response promoted by SARS-CoV-2 infection is strong enough to consistently modulate gene expression profiles in distal tissues, such as the eye, making it a responsive organ more than a secret place to hide the virus (Rokohl et al., 2020, 2021).

The presence of different expression profiles between SARS positive donors with RNA-positive cornea and SARS positive donors with RNA-negative cornea may also suggest different infection stages. The higher number of DEGs in PD-SARS-neg and the upregulation of dsRNA sensors exclusively in these samples suggested that an antiviral response is mounting, possibly after a recent viral uptake. Notably, the zinc-finger helicase ZNFX1, which was recently reported as an early dsRNA sensor able to boost the antiviral response by stimulating ISGs (Wang et al., 2019), resulted highly expressed and induced in PD-SARS-neg samples. ZNFX1 was reported as negatively correlated with monocytes by SARS-CoV-2 infection of the donors and subsequently by the presence of SARS-CoV-2 RNAs and along SARS-CoV-2 evolution (Wang et al., 2020). We previously reported a high basal expression levels of RNA editing enzymes on the ocular surface (Leonardi et al., 2020, 2021). Abundant C-to-U variations traced on SARS-CoV-2 RNAs and along SARS-CoV-2 evolution (Wang et al., 2020) are indicative of the activity of apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC), whereas less frequent A-to-G variations marked the activity of double-stranded RNA-specific adenosine deaminase (ADAR-1) (Di Giorgio et al., 2020). Although these RNA editing enzymes appeared poorly modulated in our samples, the determination of presence and possible modulation of post-transcriptional modifications during infection will require dedicated studies, since our 3′-RNA-sequencing are not suitable to detect RNA editing events.

5. Conclusions

We reported gene expression profiles of corneoscleral samples from SARS-CoV-2-positive individuals. The modulation of Interferon responsive genes suggested a good responsiveness of the ocular tissues to SARS-CoV-2, either mediated by a continuous input of viral particles from the micro-environment of infected individuals or by a systemic response. The detection of early response genes, such as ZNFX1 and other dsRNA receptors in PD-SARS-neg samples suggested a temporal diversification of the analyzed samples with possible outcomes in the restriction of infection. The presence of SARS-CoV-2 reads in one corneoscleral sample supported the possibility that the eye acts as an entry route, although additional studies will be necessary to understand the resilience of ocular tissues to the viral infection. The use of Nanopore direct RNA-sequencing aimed to detect gene expression levels together with RNA modifications at single base resolution (Harel et al., 2019) might disentangle the role of RNA editing enzymes during SARS-CoV-2 infection.

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Declaration of competing interest

Authors have no conflict of interest.

Appendix A. Supplementary data

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