**The ACE2 Expression in Sertoli cells and Germ cells may cause male
reproductive disorder after SARS-CoV-2 Infection**

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Abstract

COVID-19 spread from Wuhan city to all provinces of China during the Spring Festival. Around tens of thousands of people have been infected. Some studies have demonstrated that the reason for the wider spread of COVID-19 was that SARS-CoV-2 presents a greater affinity for angiotensin-converting enzyme 2 (ACE2) than SARS-CoV. That means the number of ACE2 positive cells is related to the possibility of infection. The expression of ACE2 was mainly localized in the testis, heart, and kidney, but the expression landscape in testis is still unknown. Considering the critical roles of testicular cells for the transmission of genetic information between generations, more and more people are concerned about the effects of COVID-19 on testis. In this study, we analyzed Single-cell RNA-seq datasets of adult human testis. Results showed that ACE2 was expressed in both germ cells and somatic cells, among which Sertoli cells, Spermatogenic Stem cell, and Leydig cells are majority cluster. Moreover, we also found that the positive rate of ACE2 in testes of infertile men was higher than that of normal men, which indicates that SARS-CoV-2 may cause reproductive disorders through pathway activated by ACE2 and the men with reproductive disorder may more likely to be infected with SARS-CoV-2. Our results also showed that the expression level of ACE2 was related to the age of men, and the peak of positive rate was at 30 years old, which indicates young men are more likely to be infected by SARS-CoV-2. Together, this research provides a biological background of the potential route for infection of SARS-CoV-2 and may enable rapid deciphering male-related reproductive disorders induced by COVID-19.

Keywords: COVID-19; ACE2; Testis; Single-cell RNA-seq; Reproductive disorder

1. Introduction

A newly identified coronavirus, SARS-CoV-2, has been posing significant threats to public health [1]. The complete genome sequences revealed that SARS-CoV-2 and severe acute respiratory syndrome (SARS) coronavirus are homologues with a high similarity of nucleotide sequences [2-4]. The receptor of coronavirus determines the virus entry into the host cell and constitute a target for the development of prophylactics and therapeutics [3]. SARS-CoV and SARS-CoV-2 share the main same receptor Angiotensin Converting Enzyme 2(ACE2) [4,5]. Daniel W et al research shows a possible reason for SARS-CoV-2 with rapid global spread was that the SARS-CoV-2 binds ACE2 with higher affinity than SARS-CoV[6]. Therefore, the analysis of ACE2 is essential to obtain the route of infection.

ACE2 was first discovered as an important active enzyme in the renin-angiotensin-aldosterone system and highly expressed in testis, myocardium, and kidney [7]. Through investigating the clinical manifestations after SARS-CoV-2 infection, it was clear that lungs show dramatic changes in histomorphology at onset [8]. SARS-CoV has been known to damage multiple organs, and orchitis is a complication of SARS. [9]. The testis is the key component of the mammalian reproductive system. Therefore, exploring ACE2 expression in divergent testicular cells is pressing.

Although several studies have revealed the expression of ACE2 in testis; the antibody detection might be subjected to non-specificity issue [10]. The cellular characteristics and regulatory networks underlying ACE2 remain to be systematically unveiled.

The recently developed single-cell RNA sequencing (scRNA-Seq) approaches can effectively delineate cell types and uncover heterogeneity that enables us to detect the ACE2 expression in each cell type and give quantitative information [11]. Meanwhile, the damage to some type of cells in the testes by viruses may cause male reproductive disorders [11]. Infertility is the most serious type of reproductive disorder. To thoroughly investigate the connection between ACE2 and reproductive disorders, we performed bioinformatics tools to analyze scRNA-seq data of testis for 5 donors with health and 8 donors with infertility[11, 12].

2. Materials and methods

2.1 Public dataset acquisition and processing

Single-cell datasets (GEO: GSE106487, GSE112013) for human testis were obtained from the Gene Expression Omnibus (GEO; <https://www.ncbi.nlm.nih.gov/>). GSE112013 consists of 6490 single cells and 3002 testicular single cells constitute from 3 normal young males[12]. GSE106487 which include samples from 2 adult normal males, 7 obstructive azoospermia (OA) and 1 nonobstructive azoospermia (NOA) [11]. We analyzed the cell cluster based on published articles and canonical markers[11, 12]. The Seurat (version 3.1.3) was used to read two datasets separately and discriminate different cell types. Firstly, datasets were normalized by NormalizeData method. Then Seurat FindClusters function was used to obtain cell cluster with resolution 0.7 in GSE106487 and 0.4 in GSE112013.

2.2 Identification of cell types and gene expression analysis

To obtain potential marker genes, Seurat function FindAllMarkers was used to perform differential expression analysis with the default parameters[11, 12]. UMAP was used for visualization purposes. In order to find susceptible cell cluster, certain markers such as SOX9 for Sertoli cells, VIT for Leydig cells and DDX4 for Germ cells were separately analyzed using Seurat function VlnPlot. Besides, Canonical markers for cell clusters were selected and plotted using ggplot2 (version 3.2.1).

The ACE2 expression distribution across different cell types in human testis was evaluated. According to the previous research articles, SARS-CoV-2 tends to attack cells through receptor ACE2, therefore, we defined that ACE2 positive ratio is the number of ACE2 positive cells which expression value is larger than 0 within a cluster divided by a cluster total cell number. To clarify the relationship between ACE2 and regulatory protein, we used RNA and protein network data of ACE2 through GeneCards (<https://www.genecards.org/>). All data are available online. The relationship network data was imported into Cytoscape (version 3.7.2), and ACE2 was highlighted in yellow.

3. Results

3.1 Single-cell analysis of the health & reproductive disorder testicular cells

In this study, 12 different cell types with 4 types of somatic cells and 8 germ cell types were identified (FIG.1A, S1)[11]. The different donors were labeled for more accurate data analysis with cells colored based on their donors of origin, as indicated on the figure key. Nomal (N), Obstructive Azoospermia (OA), Nonobstructive Azoospermia (NOA) (FIG.1B)..

3.2 The mRNA expression of ACE2 in adult human testicular cells.

Then we analyzed the expression of ACE2 positive cells in different types (FIG.1C), and the ACE2 positive cells were expressed in both somatic and germ cells (FIG.2A, B). The Sertoli cells were significant enrichment of ACE2 expression (90% of cells) (FIG.1D). About 9% cells with ACE2 positive in SSCs, and also highly expressed in later spermatogenic cells (FIG.1C, D). Somatic cells are a key component of the testicular microenvironment, which plays a significant role in the maintenance of SSCs and spermatogenesis. ACE2 was widely expressed in somatic cells and SSC, which may be involved in the regulation of early spermatogenesis (FIG.2A,B).

3.3 The analysis of ACE2 expression in normal and infertility donors.

To further determine the characteristic expression of ACE2, we analyzed the proportion of ACE2 positive cells in different donors, and found that the positive rate of the normal male was lower than that of the donor with reproductive dysfunction (FIG.3A,B,C). This indicated that the over activation of ACE2 might affect normal spermatogenesis. Considering the homology of the SARS-CoV and SARS-CoV-2, we performed to analyze the published pathological sections of the testis of SARS patients in 2003(FIG.3C) [9]. The testicular cells of the infected SARS were seriously damaged, IgG was highly expressed in the Sertoli cells and germ cells of the patients, and there was also expressed in the Leydig cells, which was similar to the distribution of ACE2 in the testis (FIG.1C, 3C, 5C) [9].

3.4 Single-cell analysis of ACE2 in young adult human testicular cells.

In different ages, SARS-CoV-2 had different infection rates[13]. Then we analyzed some published young samples for comparison[12]. After initial quality controls, 12 different cell types were identified of in testis (FIG.4A, S2). For accurate data analysis, we marked donors with a different color (FIG.4B). ACE2 was still

identified in different cell types (FIG.5A). Especially, Sertoli cells, SSCs, and Leydig cells were the high proportion of the cell types in young samples, with similar characteristics of previously data (FIG.5A,B,C).

3.5 The mRNA expression of ACE2 in testicular cells at different age.

Then we analyzed the positive rate of normal individuals in different age groups. 30-year-old individuals were the highest positive rate with 2.84%, followed by 20 years of age (1.37%), while 60-year-old men were about 0.87% (FIG.6A,B,C). Moreover, we analyzed the online data of GeneCards to find out potential regulatory mechanism of ACE2 in testis. The results show that ACE2 has related regulatory effect on 5-Hydroxytryptamine Receptor 3A (HTR3A) and Cholinergic Receptor, Nicotinic Alpha 1 (CHRNA1). HTR3A mediated apoptosis indirectly via TNF- α production by enhancing serotonin release[14]. CHRNA1 interacts with inflammation related Leukocyte immunoglobulin-like receptor subfamily B member 3 (LILRB). ACE2 presents associated with Meprin A Subunit Alpha (MEP1A), the tumorigenesis-related factor[15]. This data suggest the potential mechanism of abnormal function or even damage in patients infected with SARS-CoV-2 (FIG.6D).

4. Discussion

SARS-CoV-2 and SARS-CoV have very similar spike protein 3-D structure which was considered to have strong binding affinity to human cell receptor ACE2[6]. Analysis of ACE2 is an important reference for studying the COVID-19 infection process and an important basis for rapid diagnosis of diseases[6,16]. At present, most cases of COVID-19 show symptoms of respiratory infections, but some patients also present symptoms of gastrointestinal infections, such as diarrhea[17]. A patient with pain in the testes appeared in Fujian, China on February 10, 2020. This means that SARS-CoV-2 can invade the reproductive system, but the infection mechanism still is not clear.

In this study, we found Sertoli cells, SSC and Leydig cells have a high ACE2 positive proportion in the expression profiles of human testis scRNA-Seq. ACE2 is associated with inflammation-related HTR3A and tumorigenesis-related CHRNA1. The testicular cell type with inflammation in SARS patients is consistent with the Sertoli cell cluster in our scRNA-Seq analysis, where ACE2 expression is

abundant.[9].Moreover, ACE2-positive cells had a more pronounced distribution in adults with reproductive disorders in scRNA-Seq results (FIG. 3A, B, C). These data indicate that the pathogenesis of SARS-CoV-2 is closely associated with ACE2 expression in human testis.

Another major point in this study, we found that the proportion of ACE2 positive cells in testis is more than 1%, which is higher than that in lung, indicated that the testis might have a high-risk potential infection organ [18].SARS-CoV had a lower lethality rate for male mice at about 2 months of age than for 8-month-old male mice, and relatively higher mortality in middle-aged mice [19].In our study, the 30-year-old individual was one with the highest ACE2 positive rate, while about 20 years individual was relatively low, and the lowest ratio was the 60-year-old.These data indicate that ACE2 expression may be correlated with age.

5. Conclusions

Therefore, our findings suggest that clinicians should take care of the male reproductive health of COVID-19 patients. At present, there is less research for the research of regulation male reproductive system of ACE2, and further experimental verifications are needed to help us better reveal the process of disease and determine more accurate treatment options.

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Conflict of interests

All authors read and approved the final manuscript and declare no competing financial interests.

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A

UMAP_2

UMAP_1

Legend:

- Elongated S'tid
- Differentiated S'gonia
- Early primary S'cytes
- Sertoli cells
- Round S'tid
- Sperm
- Macrophages
- Differentiating S'gonia
- SSCs
- Late primary S'cytes
- Leydig cells
- Mononuclear cells

B

UMAP_2

UMAP_1

Legend:

- N1
- N2
- Q1
- Q2
- Q3
- Q4
- Q5
- Q6
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Notes: **A:** UMAP plot of 3002 testicular cells to visualized cell-type clusters. **B:** UMAP plot of single-cell transcription data with cells colored based on their donors of origin, as indicated on the figure key. Nomal (N), Obstructive Azoospermia (OA), Nonobstructive Azoospermia (NOA). **C:** Expression patterns (violin plot) showing the expression of ACE2 in the 12 clusters. The Sertoli cells are the highest positive cluster. **D:** The percentage of ACE2-positive cells in different populations.

FIG.2

Fig2. The mRNA expression of ACE2 in human testicular cells

Notes: **A:** Box plot showing the expression of ACE2 cells in the 12 clusters. The Sertoli cells are the highest positive cluster. **B:** UMAP plot showing the expression of ACE2-positive cells across the DDX4 -positive cells (Germ cells) clusters, SOX9-positive cells (Sertoli cells) and VIT-positive cells(Leydig cells).

FIG.3

Fig3. The analysis of ACE2 expression in normal spermatogenesis and male infertility donors

Notes:A-C: UMAP plots showing the characteristics of N,OA and NOA spermatogenesis donors. D: IHC stain with IgG. A) Testis from the control C01, showing IgG signals in the blood vessel (arrow). B) Testis from SARS patient S08, showing extensive IgG signals in testis. C) Negative control with is type control antibody. Bar ¼ 50 μm[6].

FIG.4

Fig 4. Single-cell analysis of young man testis

Notes: A: UMAP plot of 6490 testicular cells to visualize cell-type clusters.B: Cell colors based on the origins (donors) were shown in the UMAP. The labeled mark individual donors.

FIG.5

Fig 5. The mRNA expression of ACE2 in young man testicular cells

Notes: **A:** UMAP plot showing the expression ACE2 across clusters. The Sertoli cells (SOX9) are the highest percentage of ACE2-positive cells, SSCs are the types with the highest ACE2 positive rate in the Germ cells (DDX4), and some Leydig cells (VIT) are positive. **B:** The violin plot of ACE2 gene expression across all cell types. **C:** The percentage of ACE2-positive cells in different type cells.

A

B

C

Type	Age	Cell number	Cell number(ACE2+)	Percentage
Normal	17/24/25	6490	89	1.37%
Normal	30	528	15	2.84%
Normal	60	575	5	0.87%

D

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292 **Fig 6. The mRNA expression of ACE2 in testicular cells of different ages**

Notes: A: tSNE plot of testicular cells to visualize cell-type clusters (60 years old). The violin plot of ACE2 gene expression across all cell types in testis.**B:** tSNE plot of testicular cells to visualize cell-type clusters (30 years old), and violin plot of ACE2 gene expression across all cell types in testis.**C:** The percentage of ACE2-positive cells of different ages.**D:** Interacting proteins for ACE2.

FIG.S1

Fig S1.

BOX plot patterns of Coordinated Gene Expression and Selected Cell-Type-Specific Marker

FIG.S2

FigS2.

BOX plot Patterns of Coordinated Gene Expression and Selected Cell-Type-Specific Marker.