ORIGINAL ARTICLE

Expressions and significances of the *angiotensin-converting enzyme 2* gene, the receptor of SARS-CoV-2 for COVID-19

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Abstract

The *ACE2* gene is a receptor of SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) for COVID-19 (coronavirus disease 2019). To analyze the expression profiles and clinical significances for this gene in humans, RNA-seq data representing 27 different tissues were analyzed using NCBI; total RNA was extracted from different tissues of mouse and semi-quantitative reverse transcriptional-polymerase chain reaction (Q-RT-PCR) was carried out. Immunohistochemistry expression profiles in normal tissues and cancer tissues and TCGA survival analysis in renal and liver cancer were conducted. ACE2 was highly conserved in different species. In normal tissues, *ACE2* expression distributions were organ-specific, mainly in the kidney, male testis and female breast, and cardiovascular and gastrointestinal systems. High level of expression in testis, cardiovascular and gastrointestinal system indicated that SARS-CoV-2 might not only attack the lungs, but also affect other organs, particularly the testes, thus it may severely damage male sexual development for younger male and lead to infertility in an adult male, if he contracted COVID-19. On the other side, high expression of *ACE2* was correlated with increased survival rate in renal and liver cancer, indicating that *ACE2* is a prognostic marker in both renal cancer and liver cancers. Thus, the ACE2 is a functional receptor for SARS-CoV-2 and has a potential anti-tumor role in cancer. Taken together, this study may not only provide potential clues for further medical pathogenesis of COVID-19 and male fertility, but also indicate the clinical significance of the role of the *ACE2* gene in cancer.

Keywords *ACE2* gene · SARS-CoV-2 · COVID-19 · RNA-sequencing · Immunohistochemistry (IHC) · Testis · Cancer

Introduction

The Angiotensin-converting enzyme 2 gene (*ACE2*) (OMIM: 300335), also known as ACEH, is attached to the cell membrane of the outer layer tissues of the heart, arteries,

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lungs, kidney and intestines. It is located on Xp22.2 and encoded an 805-amino-acid-long protein and belongs to the family of angiotensin-converting enzyme of dipeptidyl carboxydipeptidases [1, 2]. ACE2 contains a 17-amino-acid N-terminal signal peptide and a 22-amino acid C-terminal membrane anchor. It also has a conserved motif at the zinc metalloprotease consensus sequence (HEXXH) and a conserved glutamine residue at Glu-402 that served as a third zinc ligand [1]. ACE2 is also has homologous to the human ACE, which plays a role in cleaving angiotensin I into angiotensin 1–9, and vasoconstrictor angiotensin II peptide into the vasodilator angiotensin 1–7 [3, 4]. ACE2 correspondingly acts as the entry point into cells for several coronaviruses. By cleaving angiotensin II and increasing vasodilator angiotensin 1–7, it may act as an important regulator of heart function [1, 5] and plays a protective role in acute lung injury. The expression of *ACE2* in organ- and cell-specific implies its regulatory role of cardiovasculature, kidneys and fertility. Potential anti-tumor effects of ACE2 and future therapeutic perspectives for cancers in ACE2 have been reported [6, 7]. Unfortunately, ACE2 has a high affinity to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Importantly, ACE2 is a key host cell receptor for the spike (peplomer) glycoprotein of the coronavirus HCoV-NL63, also known as severe acute respiratory syndrome coronavirus (SARS-CoV) [8], and the very recently reported coronavirus disease 2019 (COVID-19) in Wuhan called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [9–11]. Diseases associated with the *ACE2* gene include SARS, COVID-19, Middle East respiratory syndrome (MERS) and Hartnup disorder [10, 12, 13]. This might provide a clue that suppressing the expression levels of *ACE2* in cells may help fight the viral infection.

Since its first report in December 2019 [14], the COVID-19 is rapidly spreading worldwide and the number of cases is rising with increasing pace across all countries [15, 16]. Due to its invasive spread, the World Health Organization (WHO) declared COVID-19 as a global pandemic on Mar. 11, 2020 (https://www.who.int/emergencies/diseases/novel-coronavirus-2019/events-as-they-happen) [17]. The onset of COVID-19 causes progressive respiratory failure and even death due to alveolar damage in lungs, because the virus enters host cells through the enzyme ACE2, which is expressed extremely highly in the type II alveolar cells of the lungs. As of the 20th of March 2020, the rate of deaths per number of identified cases is about 4.1% overall, ranging from 0.2 to 15% depending on age and other preliminary health issues. The binding affinity between ACE2 and SARS-CoV-2 is nearly 10- to 20-fold higher than that between ACE2 and SARS-CoV [18, 19]. Hence, in *ACE2*-expressing cells and tissues, *ACE2* gene is the functional receptor of SARS-CoV-2 and plays a critical role in the viral entry into the cells during infection, and it also has

potential anti-tumor roles in cancer. Therefore, in this study, we analyzed the expression profiles of the *ACE2* gene for COVID-19 in different normal tissues and cancer tissues as a prognostic marker in renal and liver cancer.

Materials and methods

Materials and machines

Materials used in this study were RNAsimple Total RNA Kit (TIANGEN, Beijing, China), ReverTra Ace qPCR RT Master Mix (Cat No. FSQ-201, TOYOBO, China) [20]. Antibody for ACE2 was come from Sigma-Aldrich (HPA000288) or R&D Systems (CAB026174) with dilution of 1:500. And the following machines used: Microscissor, tweezers, Benchtop centrifuge (Thermo Scientific), UV spectrophotometer, Veriti 96 well thermal cycler PCR (Applied Biosystems, USA).

RNA-sequencing profiles

In order to determine tissue-specificity, the *ACE2* gene expression profiles were analyzed from RNA-sequencing (RNA-seq) data, performed on tissue samples from 95 human individuals representing 27 different tissues, using the NCBI program (https://www.ncbi.nlm.nih.gov/gene/59272) [21]. These data were collected from RNA-seq normal tissues (BioProject: PRJEB4337; Publication: PMID 24309898).

Homology analysis

Homologs of the *ACE2* gene were performed by the NCBI program (https://www.ncbi.nlm.nih.gov/homologene?LinkName=gene_homologene&from_uid=59272) [20, 22, 23]. Multiple sequence alignment was performed by Clustal Omega program.

Total RNA extraction

RNA extraction from mice tissues was executed according to our previously reported standard protocols [22, 24–26]. Tissues from 2 month old adult mice included retina, lung, uterus, ovary, breast, testis, spleen, kidney, liver, brain, small intestine, skeletal muscle, and whole blood. After extraction, 1 µL of RNA sample was used to determine and record the concentration value using the UV spectrophotometer, before stored in a − 20 °C refrigerator [20].

Reverse transcription (RT)

Total RNA was transcribed into cDNA according to our previously described methods [24, 27]. The reverse transcription Polymerase Chain Reaction (RT-PCR) amplification reaction system was 20 µL, including 5×RT Master Mix 4 µL, RNA template 2 µg, and ddH$_2$O was added to make the final volume 20 µL. The RT-PCR cycle was programmed as follows: 37 °C for 15 min, 50 °C for 5 min, then at 98 °C for 5 min, and finally hold at 4 °C. Then, the PCR product was then diluted six fold and stored at a − 20 °C refrigerator, or used it directly in the next experiment.

Semi-quantitative RT-polymerase chain reaction (Q-RT-PCR)

Q-RT-PCR was conducted according to our previously reported protocols [22, 24]. Primers for the *Ace2* gene (NM_001130513.1) in mouse were (RT-ACE2-mF: TCC ACGATCCCATGCCTATG; RT-ACE2-mR: TGCTGA AGACCCACTTTGCT). Mouse *ß-actin* gene was used as an internal control [20, 22]. Primers for RT-PCR spanned at least one intron to avoid amplification of genomic DNA. The

reaction system used for PCR amplification was 10 µL, consisting of Ace2 primers 1 µL, 2×Taq PCR Master mix 5 µL, cDNA 1 µL, ddH$_2$O 3 µL. PCR program was as follows: pre-denaturation at 95 °C for 90 s, denaturation at 95 °C for 40 s, annealing at 60 °C for 30 s, extension at 72 °C for 30 s for 31 cycles; final extension at 72 °C for 5 min, and storage at 4 °C. Each assay was performed as a triplicate. PCR products and 2 kb DNA molecular weight size ladder were loaded in a 1.8% agarose gel to be confirmed by electrophoresis [22].

Immunohistochemistry expression profiles from normal and cancer tissues

Immunohistochemistry (IHC) expression profiles of ACE2 for normal tissues of testis and lung were analyzed through the Human Protein Atlas (https://www.proteinatlas.org/ENSG00000130234-ACE2/tissue). Pathological IHC, TCGA RNA samples expression profiles and survival analysis of ACE2 for the lung, renal and liver cancer tissues were analyzed in the Human Protein Atlas from renal cancer patient data (https://www.proteinatlas.org/ENSG00000130234-ACE2/pathology/renal+cancer) and liver
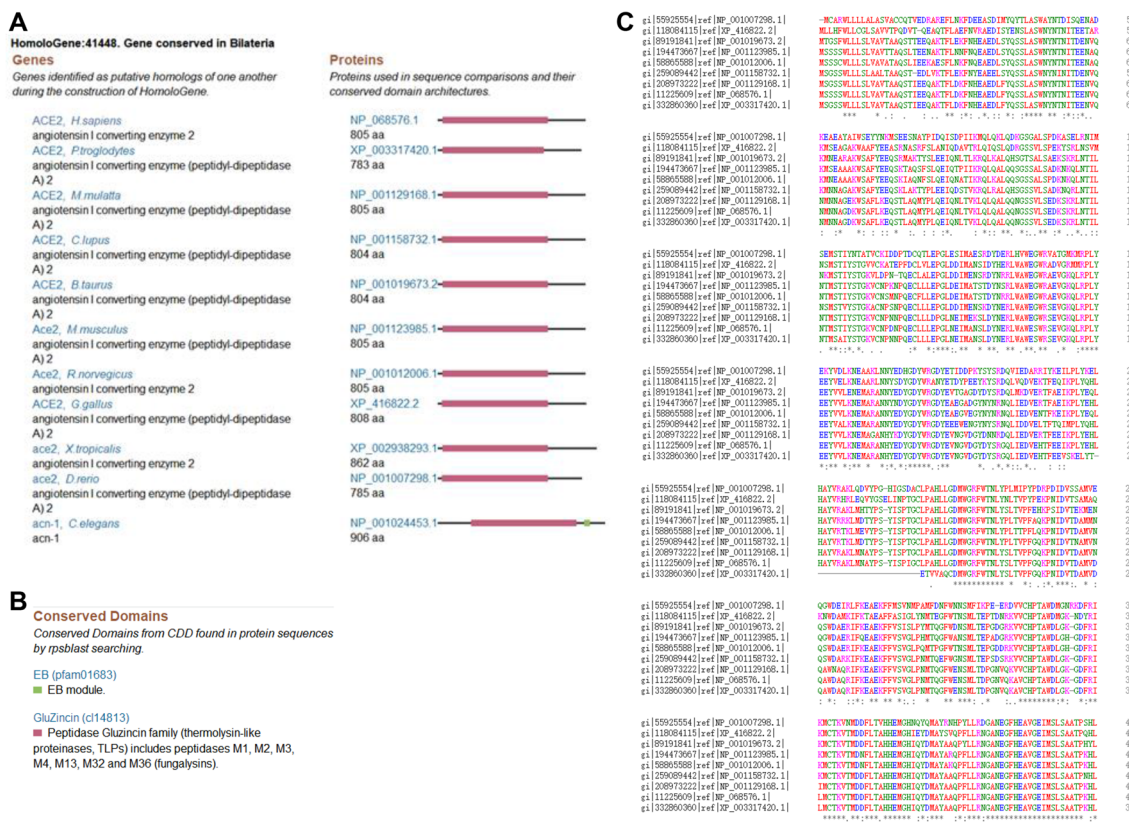

Fig. 1 Homologs of the ACE2 proteins. **a** Conservation for ACE2 in 11 different species. **b** The indicated two conserved domains. **c** Multiple sequence alignment results of the N-terminus of ACE2 (RBD located terminal) among indicated different species. "*" indicates the identity whereas "." and ":" show the similarity. Amino acids from 1 to 418 of human ACE2 are presented

cancer patient data (https://www.proteinatlas.org/ENSG0 0000130234-ACE2/pathology/liver+cancer), respectively. The standard immunostaining protocol for IHC can be found at protocols.io (https://www.protocols.io/view/hpa-cell-atlas -standard-immunostaining-protocol-x2dfqa6). Normal tissues were represented by samples from three individuals each, using one core per individual; cancer tissues were sampled from two cores of each individual and protein expression was annotated in tumor cells. Specimens containing normal and cancer tissue were collected from anonymized paraffin-embedded material of surgical specimens. Note that samples used for IHC did not correspond to the samples in the TCGA dataset.

Survival analysis in TCGA

Patients were classified into two expression groups based on the value of FPKM (fragments per kilobase of exon model per million reads mapped) in each gene and the correlation between expression level and patient survival was examined. The Kaplan–Meier survival estimators were used to examine the prognosis of each group of patients, and the patient survival outcomes were compared by log-rank tests. The genes with log-rank $P < 0.001$ by Kaplan–Meier analysis were defined as a prognostic. If the group of patients with high expression of the prognostic gene had a higher observed event than expected, it was an unfavorable prognostic gene;

otherwise, the results showed a favorable prognosis. Genes with a median expression less than FPKM value of 1 were minimally expressed and classified as unprognostic, even if they showed significant prognosis by survival analysis.

Results

Determination of ACE2 conservation

Homologs of the ACE2 protein demonstrated that it is highly conserved in different species, such as, chimpanzee, Rhesus monkey, cow, dog, mouse, rat, chicken, zebrafish, *C. elegans*, and frog (Fig. 1a, b). This suggested that ACE2 from these animals of different species could potentially bind the receptor binding domain (RBD) of the spike glycoprotein [11, 28], making them probable natural hosts for SARS-CoV-2. Multiple sequence alignment of the N-terminus of ACE2 (RBD located terminal) among different species showed very high levels of identity and similarity in the amino acids (Fig. 1c).

The mRNA expression profiles for *ACE2* in human and *Ace2* in mouse

The mRNA expression profiles for the *ACE2* gene in humans were analyzed from RNA-sequencing data in the indicated

Fig. 2 The mRNA expression profiles in human and mouse. **a** The *ACE2* expression profiles in the indicated 27 different human tissues. RPKM stands for number reads per kilobase (kB) of transcript per million mapped reads. In RNA-Sequencing, the relative expression of a transcript was proportional to the number of cDNA fragments that originate from it. **b** The *Ace2* gene mRNA expression profile analysis in the different tissues of organisms in the mouse. *NC* negative control, *muscle* skeletal muscle
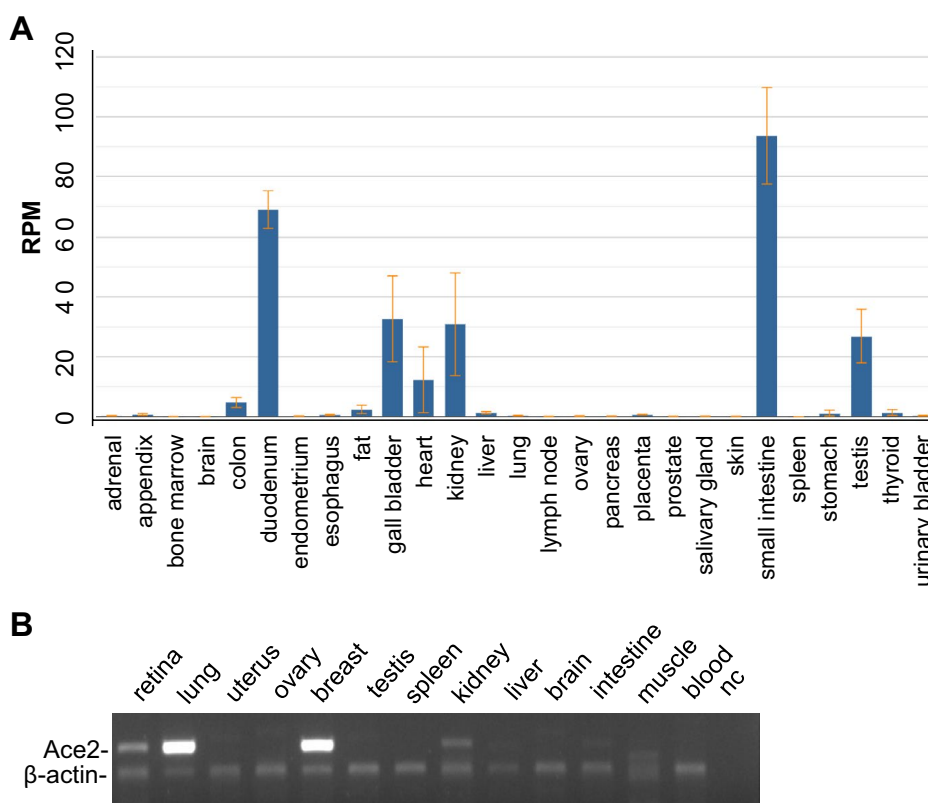
27 different human tissues. The RPKM (Reads Per Kilobase of transcript per Million mapped reads) values of *ACE2* expression in the small intestine was found to be highest, with approximately 93.7, followed by the duodenum (69), gall bladder (32.6), kidney (30.8), testis (26.89), and heart (12.3), but it was found to be lowest in the spleen with approximately 0.007 (Fig. 2a, Table 1). Thus, these results showed the biased expression profile of *ACE2* mRNA in the small intestine, duodenum and 5 other tissues.

Analysis of the expression profile of *Ace2* mRNA in the 13 indicated different tissues from mice was conducted by Q-RT-PCR (Fig. 2b). Surprisingly, the results showed that *Ace2* transcripts are highly expressed in lung and breast, lower in expression in the retina, kidney and small intestine, but very weakly expressed in the testis; where were no detectable expressions in other tissues we tested (Fig. 2b). Comprehensively, *ACE2* expression distribution is organ-specific, and it is mainly localized in the kidney, the cardiovascular and gastrointestinal systems, male testis and female breasts. The expression of *ACE2* in different tissues suggests multiple routes of SARS-COV2 transmission.

IHC analysis results in testis and lung tissues

IHC analysis using the ACE2 antibody found selective membranous expression in renal tubules, intestinal tract, gallbladder and testis (Fig. 3, data not shown). In normal testis tissues, ACE2 presented high staining in cells of seminiferous ducts and leydig, located in both cytoplasmic and membranous region of the cells (Fig. 3a, b). In other male tissues, including ductus deferens, epididymis, seminal vesicle, and prostate, it was not detected or its expression is very low (data not are shown).

IHC analysis of normal lung tissue indicated that ACE2 expression in alveolar macrophages is moderate and is mainly located in both the cytoplasm and membrane (Fig. 3c, blue arrows). A few positive staining of ACE2 in type I alveolar epithelial cells (Fig. 3c, black arrow) and type II alveolar epithelial cells (Fig. 3c, red arrows) was also revealed; but most of type II alveolar epithelial cells were ACE2 negative stained (Fig. 3c, dashed red arrows).

Expression analysis results in cancer tissues

IHC analysis using the ACE2 antibody (CAB026174) indicated that renal cancers and a few cases of colorectal and liver cancers exhibited moderate to strong immunoreactivity in the membrane (Fig. 4a). The remaining cancer tissues generally did not exhibit expression (Fig. 4a).

Table 1 Expression of *ACE2* mRNA in human different tissues

Sample	Numbers	RPKM values	Counts
Adrenal	3	0.287 ± 0.08	15,093
Appendix	3	0.751 ± 0.291	37,467
Bone marrow	4	0.049 ± 0.029	6316
Brain	3	0.045 ± 0.023	2583
Colon	5	4.695 ± 1.662	583,961
Duodenum	2	69.049 ± 6.292	2,262,846
Endometrium	3	0.2 ± 0.055	11,145
Esophagus	3	0.589 ± 0.17	51,888
Fat	3	2.389 ± 1.378	130,013
Gall bladder	3	32.606 ± 14.37	2,936,794
heart	4	12.309 ± 10.958	1,505,105
Kidney	4	30.81 ± 17.148	2,168,804
Liver	3	1.294 ± 0.368	87,261
Lung	5	0.345 ± 0.102	31,059
Lymph node	5	0.063 ± 0.071	7576
Ovary	2	0.194 ± 0.106	13,770
Pancreas	2	0.199 ± 0.029	13,240
Placenta	4	0.675 ± 0.102	83,518
Prostate	4	0.099 ± 0.037	7124
salivary gland	3	0.147 ± 0.009	16,539
Skin	3	0.089 ± 0.053	8127
Small intestine	4	93.724 ± 16.1	7,099,334
Spleen	4	0.007 ± 0.003	702
Stomach	3	1.177 ± 0.937	96,543
Testis	7	26.895 ± 8.944	6,060,081
Thyroid	4	1.392 ± 0.928	187,069
Urinary bladder	2	0.33 ± 0.107	19,218

TCGA dataset of the RNA-sequencing data from RNA cancer patients indicated that mRNA levels of *ACE2* increased in renal cancer of 877 patients (Fig. 4b, average FPKM of 26.9 in renal cancer, and average FPKM of 2.3 in liver cancer).

Survival analysis in cancer patients

Given that our studies focused on *ACE2* expression and showed that *ACE2* expressions are higher in both normal and cancer tissues of kidney and liver than that of lung tissues, further clinical relationships between the *ACE2* expression and survival outcomes were also investigated. From the available data of renal and liver cancer patients, survival analysis was performed, and the renal and liver survival results are shown in Fig. 5. High expression of *ACE2* was correlated with long survival in renal cancer
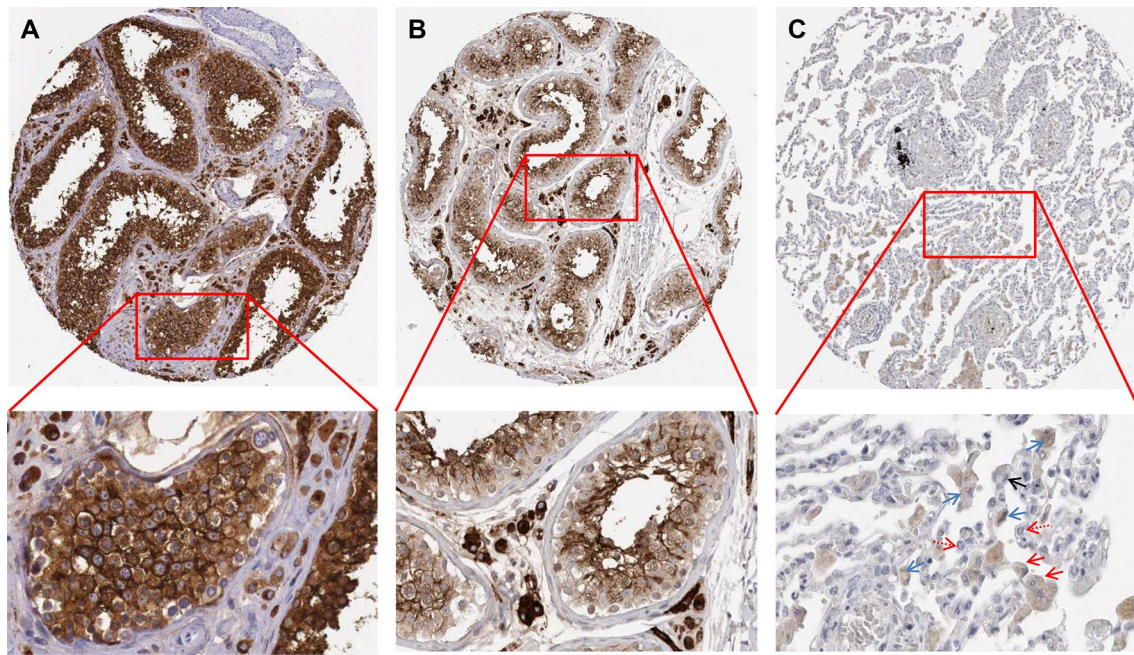
Fig. 3 Immunohistochemistry (IHC) images of normal tissues (testis and lung) for ACE2. **a** IHC image of normal testis tissue from a male of age 38 (Patient id: 305). **b** IHC image of normal testis tissue from a male of age 26 (Patient id: 2254). **c** IHC image of normal lung tissue from a female of age 49 (Patient id: 2268). Bottom panels, enlarged pictures from top panels. Blue arrows indicate the representative positive results in macrophages, black arrow indicates the representative positive results in type I alveolar epithelial cells, red arrows indicate the representative positive results in type II alveolar epithelial cells (**c**, red arrow), but dashed red arrows indicate the representative negative staining of type II alveolar epithelial cells. (Color figure online)

patients, with 75% high survival rate and 58% low survival rate respectively (Fig. 5a, P-value 0.000021); high expression of *ACE2* was correlated with longer survival in 365 liver cancer patients, with 64% high survival rate and 37% low survival rate respectively (Fig. 5b, P-value 0.00042), indicating that *ACE2* is a prognostic marker in renal and liver cancers. Thus, high expression of *ACE2* is favorable for a higher 5-year survival rate of cancer patients in renal and liver cancer.

Discussion

In this study, we performed data analysis of RNA-sequencing from NCBI and quantitative RT-PCR from mouse, and found that *ACE2* expression distribution is organ-specific and mainly found in the kidney, male testis, female breast, and cardiovascular and gastrointestinal system. This finding is consistent to Harmer et al. reports, showing its highest expression is in the testis, renal tissues and cardiovascular tissues, and all portions of the gastrointestinal tract [29]. IHC analysis using ACE2 antibody in normal testis tissues showed that ACE2 is presented high staining in cells of seminiferous ducts and leydig. High level of ACE2 expression in the testis, cardiovascular and gastrointestinal

system indicated that SARS-CoV-2 might not only attack the lung, but also other organs namely the testis, and may affect sexual development for younger boys and cause infertility in adult males if they had been infected by the SARS-CoV-2. Surprisingly, the results showed high expression of *Ace*2 transcript in the lungs of the mouse but low in humans. However, Itoyama et al. [2] identified an alternative 5-prime untranslated exon and found expression of the alternative 5-prime exon in various major organs including in the lungs, demonstrating that different *Ace2* transcripts existed. However, this does not discount the possibility that the ACE2 expression in the lungs of humans may truly be lower. With this regard, we looked at the expression levels by western blotting in human normal tissues, and we found that the ACE expression in the lungs is truly very low, comparing to the testis tissues (link here: https://www.abcam.com/ace2-antibody-ab15348.html). Taken together, even though lung cells may be the main target cells of SARS-CoV-2, other organs may also be vulnerable [30], particularly in the testis which may lead to male infertility if he had COVID-19.

However, in the context of SARS-CoV-2 infection and cancer, is ACE2 friend or foe? Since ACE2 might play potential anti-tumor effects of and future therapy for cancer [6, 7], we analyzed *ACE2* expression and its clinical significance associated with survival rates of cancer patients.
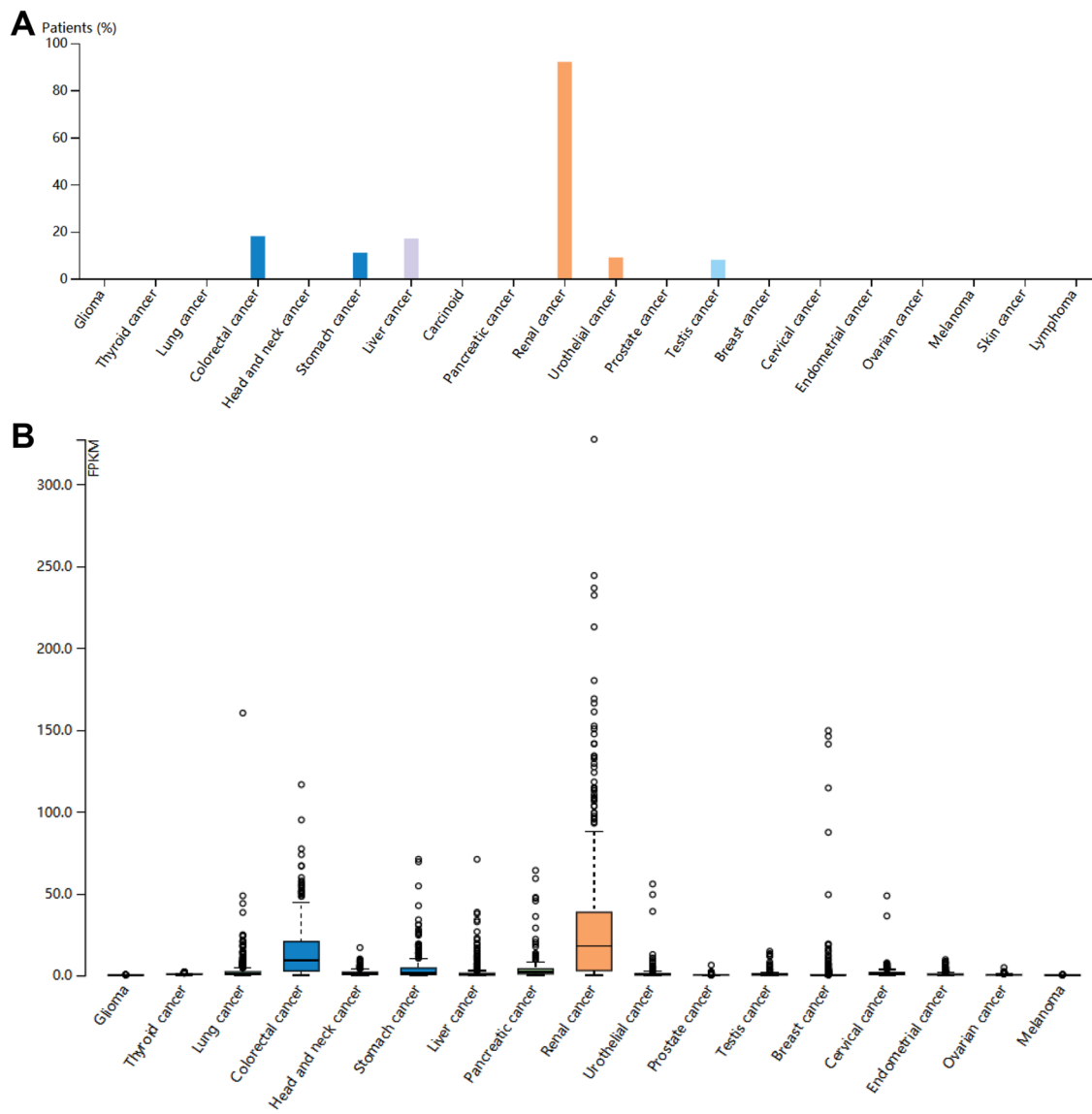
Fig. 4 The protein and mRNA expression overviews for ACE2 in humans. **a** The ACE2 protein expression overviews. Tissue microarrays are used to show antibody staining for IHC in 20 different types of cancer with total samples of 216 cancer patients. Color-coded bars indicate the percentage of patients with high and medium protein expression levels in each cancer. The types of cancer are color-coded according to which type of normal organ cancer originates from. ACE2 protein expression with low or un-detectable will results in a white bar. **b** The *ACE2* mRNA expression overviews. RNA expression overview shows RNA-seq data from The Cancer Genome Atlas (TCGA) from 17 cancer types representing 21 cancer subtypes with a corresponding major cancer type in the Human Pathology Atlas for comparisons. The FPKMs for each gene were subsequently used for quantification of expression with a detection threshold of the FPKM value of 1. Genes were categorized using the same classification as described above. RPKM stands for number fragments per kilobase of exon per million reads

In cancer tissues, renal cancer and a few cases of colorectal and liver cancer exhibited moderate to strong membranous immunoreactivity to ACE2; the remaining cancer tissues were all generally negative. RNA-sequencing indicated that mRNA levels of *ACE2* were enhanced in renal and liver cancer. Further survival analyses showed that high expression of *ACE2* is correlated with longer survival in renal and liver cancer, indicating that ACE2 is a prognostic marker in both renal cancer and liver cancer.

In conclusion, *ACE2* expression distributions were organ-specific, mainly in kidney, male testis, female breast, and the cardiovascular and gastrointestinal systems. Thus, SARS-CoV-2 infection may most likely affect male infertility. On the other hand, high expression of *ACE2* is correlated with
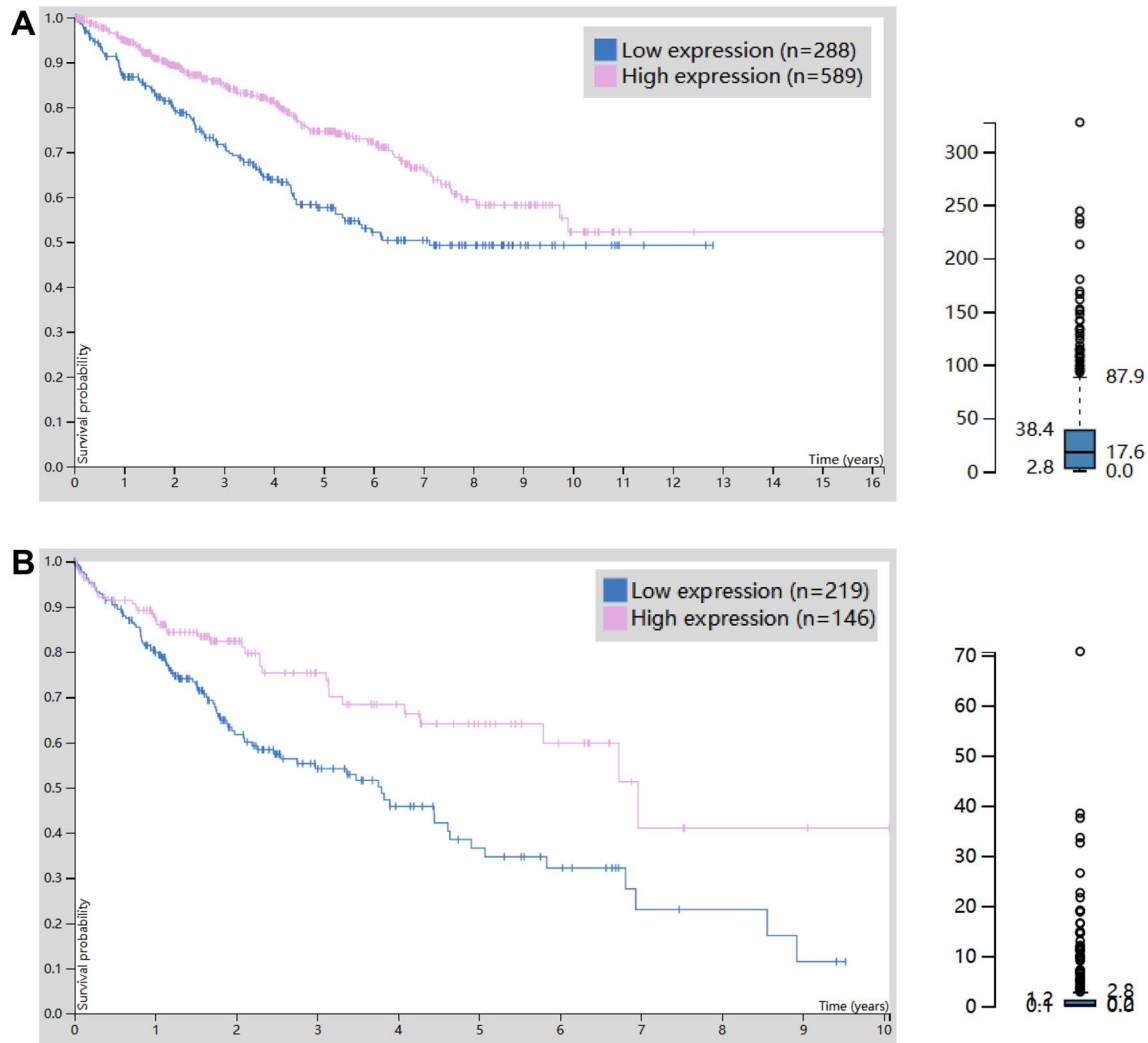
Fig. 5 Kaplan–Meier plots for all cancers where high expression of the *ACE2* gene has a significant (P < 0.001) association with patient survival are shown in this summary. Prognostic marker is indicated in renal cancer (favorable) and liver cancer (favorable). Whether the prognosis is favorable or unfavorable is indicated in brackets. **a** Kaplan–Meier plot includes *ACE2* expression and survival data for patients with renal cancer. **b** Kaplan–Meier plot includes *ACE2* expression and survival data for patients with liver cancer. Right panels indicate the TCGA RNA-seq dataset for renal and liver cancer respectively

long survival in renal and liver cancer, indicating that *ACE2* is a prognostic marker in both renal cancer and liver cancer. The binding affinity between ACE2 and SARS-CoV-2 is nearly 10- to 20-fold higher than that between ACE2 and SARS-CoV [18, 19]. Hence, *ACE2*-expressing cells and tissues can potentially act as targets of the novel coronavirus. With all this combined, ACE2 is a functional receptor for SARS-CoV-2 and has potential anti-tumor role in cancer and may pose conflicting medical benefits and concerns. Altogether, this study may not only provide potential clues for further clinical management of COVID-19 pathogenesis and possible male fertility, but it might also indicate the clinical significances of the *ACE2* gene in mortality rates of certain cancers.

Compliance with ethical standards

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