Protein expression of transmembrane protease serine 4 in the gastrointestinal tract and in healthy, cancer, and SARS-CoV-2 infected lungs

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Abstract. In addition to the angiotensin-converting enzyme 2 (ACE2), a number of host cell entry mediators have been identified for severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), including transmembrane protease serine 4 (TMPRSS4). The authors have recently demonstrated the upregulation of TMPRSS4 in 11 different cancers, as well as its specific expression within the central nervous system using *in silico* tools. The present study aimed to expand the initial observations and, using immunohistochemistry, TMPRSS4 protein expression in the gastrointestinal (GI) tract and lungs was further mapped. Immunohistochemistry was performed on tissue arrays and lung tissues of patients with non-small cell lung cancer with concurrent coronavirus disease 2019 (COVID-19) infection using TMPRSS4 antibody. The results revealed that TMPRSS4 was abundantly expressed in the oesophagus, stomach, small intestine, jejunum, ileum, colon, liver and pancreas. Moreover, the extensive TMPRSS4 protein expression in the lungs of a deceased patient with COVID-19 with chronic obstructive pulmonary disease and bronchial carcinoma, as well in the adjacent normal tissue, was demonstrated for the first time, at least to the best of our knowledge. On the whole, the immunohistochemistry data of the present study suggest that TMPRSS4 may be implicated in the broader (pulmonary and extra-pulmonary) COVID-19 symptomatology; thus, it may be responsible for the tropism of this coronavirus both in the GI tract and lungs.

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Introduction

The entry of the severe acute respiratory syndrome (SARS) coronavirus-2 (SARS-CoV-2) into cells is facilitated by its spike (S) proteins, mainly via binding to key cell entry mediators, such as the angiotensin-converting enzyme 2 (ACE2) (1,2). In addition, the S proteins of SARS-CoV-2 are also primed/activated by the transmembrane protease serine 2 and 4 (TMPRSS2 and TMPRSS4), which appear to play a crucial role in the tropism of this virus and the multi-organ infection in the context of coronavirus disease 2019 (COVID-19) (1,3,4). Indeed, TMPRSS4, along with TMPRSS2, have been found to be capable of activating the viral S proteins, consequently

increasing the SARS-CoV-2 infection of human enterocytes in the small intestine (5). Accordingly, recent data demonstrate the abundant expression of ACE2, TMPRSS2 and TMPRSS4 in enterocytes of the lower gastrointestinal (GI) tract (6). Of note, COVID-19 can also present with a wide spectrum GI symptoms, including diarrhoea, nausea and vomiting (7-9).

Moreover, based on data derived from a systematic review and meta-analysis for the risk and prognosis of patients with COVID-19 (10,11), cancer has been identified as an independent risk factor which holds a positive association with severe COVID-19 infection and related adverse clinical outcomes (12). In accordance with this finding, previous studies, including data from the authors' research group, have documented the differential expression of SARS-CoV-2 infection host cell entry mediators in malignancies (4,13). Indeed, the authors have previously demonstrated that TMPRSS4 is overexpressed in 11 types of cancer, including lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), cervical squamous cell carcinoma, thyroid carcinoma, ovarian cancer, colorectal cancer, pancreatic cancer, adenocarcinoma of the stomach, uterine carcinosarcoma and uterine endometrial carcinoma (14). Furthermore, a recent study on patients with chronic obstructive pulmonary disease (COPD) also demonstrated that TMPRSS4 gene expression was elevated in epithelial brushes and bronchial biopsies of these patients compared to the controls (15).

The present study aimed to expand on previous observations regarding the role of TMPRSS4 in SARS-CoV-2 infection and to provide novel evidence of the abundant protein expression of TMPRSS4 in both the GI tract and in COVID-19-affected lungs.

Patients and methods

Patient selection, autopsy and sample acquisition. The lung tissue of 1 patient (77-year-old male with advanced poorly differentiated bronchial carcinoma under radiation therapy) with non-small cell lung carcinoma (NSCLC) with concurrent COVID-19 infection was retrieved from a clinical autopsy (the specimen was obtained at the Institute of Pathology 'Pathologie Grünstrasse', Düsseldorf, Germany). Appropriate written consent of the patient/next of kin and ethical approval were granted by the Ethics Committee of Hannover Medical School (ethics reference no. 9621_BO_K_2021). The autopsy [77-year-old male with the following comorbidities: COPD, heart-hypertrophy, bronchial carcinoma (NSCLC) after radiation treatment] was performed in a room with adequate airflow (>6 air changes per hour of total room volume) at conditions similar to recommendations for autopsies of suspected Creutzfeldt-Jakob disease (i.e., hazmat suits, boots, goggles and FFP2/3 masks) with an *in corpore* technique analogous to that used in forensic institutions. The thoracic organs were eviscerated, and the heart was separately dissected in the direction of blood flow. The lungs, trachea and larynx were wholly exenterated and perfused via the trachea with phosphate-buffered formalin. The trachea was then closed with a clamp and the specimens were left in formalin at room temperature for 72 h prior to further dissection. The lungs were subsequently cut into 0.5-1-cm-thick parasagittal slices and examined macroscopically. The areas of interest (tumour tissue, lung tissue adjacent to the tumour and peripheral lung tissue) for histology were identified and fitting tissue samples embedded in paraffin and cut into 5-μm-thick slices on coated glass slides (SuperfrostPlus, Gerhard Menzel B.V. & Co. KG) for immunohistochemical staining. The included COVID-19 specimen was positively PCR-tested on a nasal swab, as well as lung tissue, and was found to be positive from the reverse transcription-PCR (RT-PCR) of formalin-fixed paraffin embedded tissue.

Paraffin-embedded tissue microarray slides, each containing 48 cores, were also purchased from US Biomax (US Biomax, Inc.; cat. nos. BN114c32 and LC241L; Tables SI and SII). All tissue samples were collected under the highest ethical standards with the donors giving informed consent [under Health Insurance Portability and Accountability Act (HIPAA)-approved protocols]. Additional control samples of lung tissue were retrieved from archived routine samples from Hannover Medical School (ethics reference no. 1741-2013).

Immunohistochemistry. The slides were deparaffinised and rehydrated, followed by antigen retrieval. Briefly, 100 ml sodium citrate (Thermo Fisher Scientific, Inc.) was heated in the microwave for 2 min, followed by the addition of slides and further heating at 1-min intervals for 10 min (keeping the slides just below boiling temperature, at 90˚C). Blocking was performed using 5% BSA (Thermo Fisher Scientific, Inc.) in PBS for 40 min at room temperature. This was followed by overnight incubation at 4˚C with primary rabbit monoclonal antibodies for TMPRSS4 (cat. no. ab188816, Abcam; 1:500 dilution 5% BSA in PBS). Following three washes at room temperature with PBS (10 min each), the slides were incubated with secondary antibody (1:200 in 1% rabbit serum; ZytoChem Plus HRP-DAB kit, rabbit, cat. no. HRP008DAB-RB, Zytomed Systems GmbH) for 60 min at room temperature. After washing with 0.025% Triton X-100, to remove any unbound secondary antibody, streptavidin-HRP conjugate from the same kit was added to the slides and left to incubate at room temperature for 30 min in a humidity chamber. At room temperature, the slides were then washed and subjected to 3,3'-diaminobenzidine (DAB) (Vector Laboratories, Inc.) staining for 5 min, counterstained with haematoxylin (Merck KGaA) for ~10 sec and washed with 0.1% sodium bicarbonate at room temperature. The slides were then analysed for the immunoreactivity of TMPRSS4 protein using a light microscope (Carl Zeiss AG). A pheochromocytoma (adrenal gland) core was used as a positive control (Fig. S1A; part of the array #BN114c32), and an ovarian cancer core (part of the array #BC11115d045) where the primary antibody was omitted was used as a negative control (Fig. S1B).

UALCAN database and statistical analysis. UALCAN (http://ualcan.path.uab.edu/), an online transcriptomic database, was used to investigate the mRNA expression levels of TMPRSS4 for comparisons between LUAD and LUSC and normal tissues, as well as stratifying for sex as a clinicopathological parameter. The method used for differential analysis in the present study was one-way ANOVA, using disease state (tumour or normal) as variables for calculating differential expression: Gene expression-disease state. A P-value <0.05 was considered to indicate a statistically significant difference.

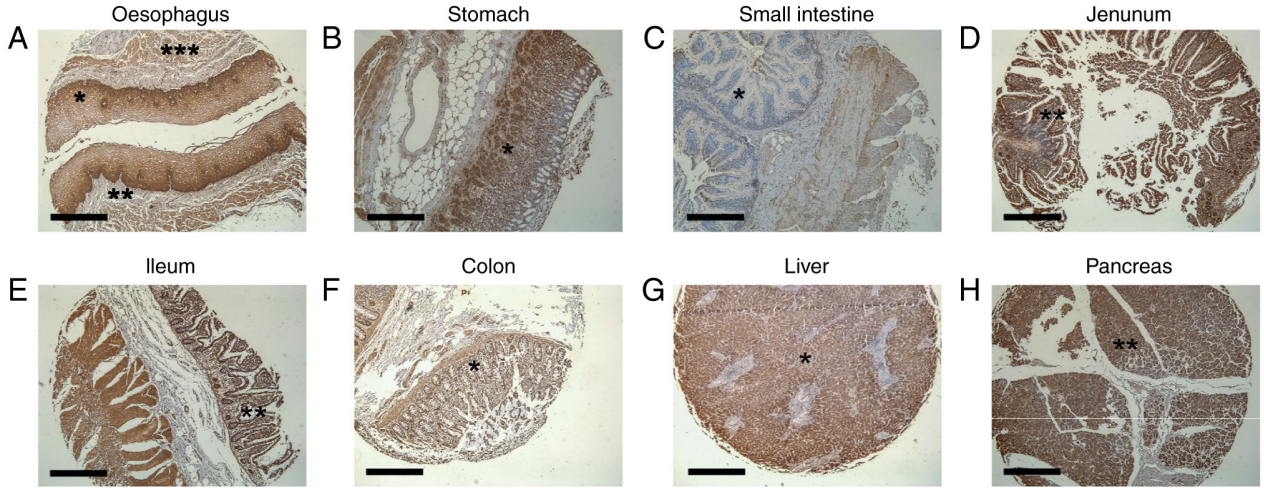

Figure 1. Immunohistochemical staining for transmembrane protease serine 4 in the upper and lower gastrointestinal tract, liver and pancreas at x20 magnification. (A) Mid-oesophagus exhibiting cytoplasmic staining in the oesophageal epithelial cells (*), submucosal glands (**) and lower muscularis mucosae (***). (B) Gastric fundic mucosa exhibiting positivity in the surface epithelial cells and the mucinous parietal cells (*). The zymogenic and endocrine cells in the basal zone also exhibited diffuse cytoplasmic staining. (C) In the small intestine, there was weak staining of the cytoplasm of goblet cells and enterocytes (*). In the (D) jejunum and (E) ileum, the cytoplasmic expression was more pronounced compared to the large intestine (**). (F) Colonic mucosa exhibiting simple columnar surface epithelium and mucosal crypts with cytoplasmic and weak nuclear membrane staining of goblet cells and adjacent enterocytes (*). (G) The liver exhibited cytoplasmic staining of hepatocytes together with bile ducts and ductules (*). (H) In the pancreas, there was pronounced cytoplasmic staining of acinar cells (**). Scale bar, 500 nm.

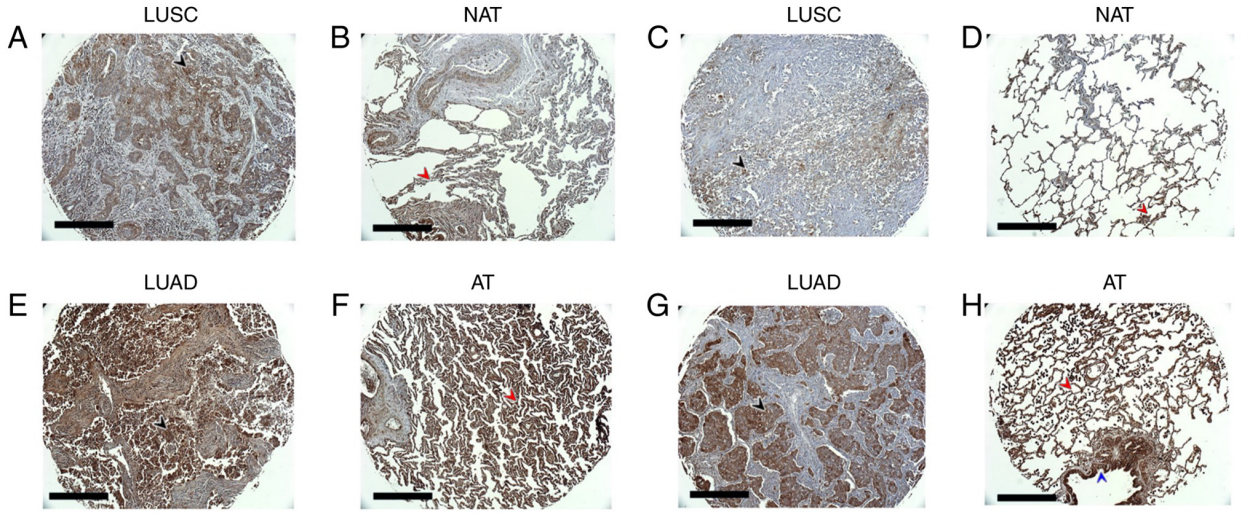

Figure 2. Immunohistochemical staining for TMPRSS4 in human non-small cell lung cancer at x20 magnification. High expression of TMPRSS4 in LUSC cells (black arrowhead) with a lesser expression in (A) the adjacent peritumoral tissue, and a high expression in alveolar epithelial cells (red arrowhead) in (B) the NAT of a male patient (53 years of age, T2N0M0; grade 2, stage IB). (C) Lesser, but detectable staining of TMPRSS4 in lung squamous cell carcinoma cells (black arrowhead) with minor staining in the adjacent peritumoral tissue, and (D) a high expression on alveolar epithelial cells (red arrowhead) in the NAT of a female patient (56 years of age, T2N0M0; grade 3, stage IIIA). Protein expression in lung adenocarcinoma cells (black arrowhead) with (E) a lesser expression in the adjacent peritumoral tissue (LUAD) and (F) a high expression on alveolar epithelial cells (red arrowhead) in cancer adjacent lung tissue (AT) of a male patient (65 years of age, T2N2M0, grade 3, stage IIIA). (G) Expression in LUAD (black arrowhead) and a (H) high expression on both bronchial (blue arrowhead) and alveolar epithelial cells (red arrowhead) in cancer adjacent lung tissue of a female patient (35 years of age, T4N1M0, grade 3, stage IIIA). Scale bar, 500 nm. TMPRSS4, transmembrane protease serine 4; LUSC, lung squamous cell carcinoma; LUAD, lung adenocarcinoma; NAT, normal adjacent tissue; AT, adjacent tissue.

Results

TMPRSS4 expression in the GI tract tissue microarray. In a recent study, SARS-CoV-2 virions were identified in the intestines and colon tissue of 2 patients using transmission electron microscopy, suggesting a causal role of this virus in bowel damage (16). Using immunohistochemistry, the present study demonstrated that TMPRSS4 was abundantly expressed at the protein level in the oesophagus, stomach, small intestine, jejunum, ileum, colon, liver and pancreas (Fig. 1). In the oesophagus, staining was evident in epithelial cells, submucosal glands and the lower muscularis mucosae. This is of increasing importance given oesophageal mucosal lesions caused by SARS-CoV-2 have been shown to result in upper GI bleeding in a 77-year-old man (17). Another study also presented the case of a patient with COVID-19 with acute oesophageal necrosis (18). In

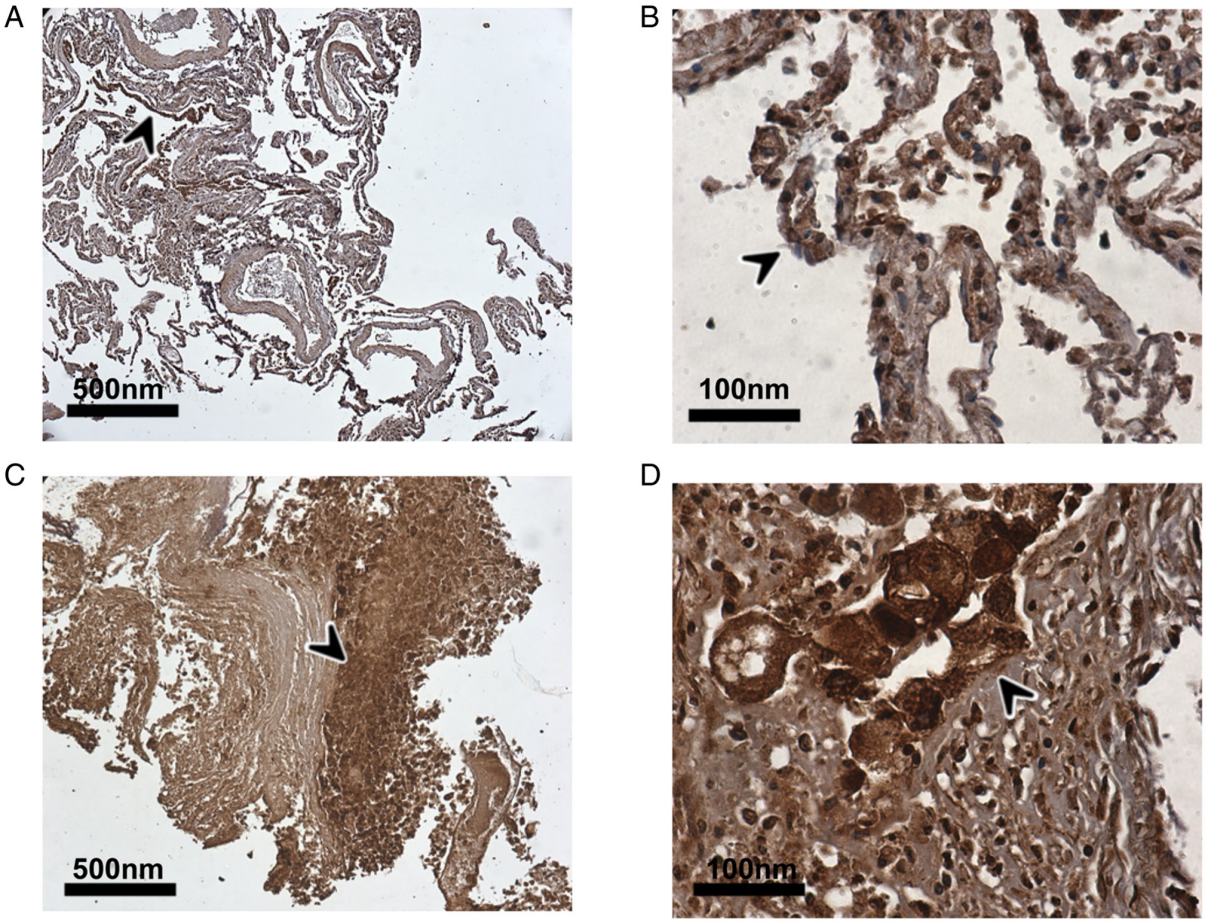

Figure 3. Immunohistochemical staining for transmembrane protease serine 4 in (A and B) human normal lung tissue (A) x20 magnification; (B) x40 magnification, and in (C and D) human lung tumour tissue [(C) x20 magnification; bronchial carcinoma non-small cell lung cancer following radiation treatment; (D) x40 magnification; male, 77 years old with chronic obstructive pulmonary disease]. Of note is the prominent expression on bronchial epithelial cells (panel A, arrowhead) and alveolar epithelial cells (panel B, arrowhead) in (A and B) normal lung tissue, as well as the prominent expression on bronchial carcinoma cells (panels C and D, arrowhead) with peritumoral stroma showing less staining.

the present study, in the small intestine, there was weak staining of the cytoplasm in goblet cells and enterocytes. In the jejunum and ileum, cytoplasmic expression was more pronounced compared to the large intestine. The colonic mucosa exhibited cytoplasmic and weak nuclear membrane staining of goblet cells and the adjacent enterocytes (Fig. S1C for higher magnification). In the stomach, the gastric fundic mucosa exhibited positivity in the surface epithelial cells and the mucinous parietal cells. Finally, the observations of the expression of TMPRSS4 were expanded to the liver and pancreas. The liver exhibited the staining of hepatocytes together with bile ducts and ductules, whereas in the pancreas, pronounced cytoplasmic staining of the acinar cells was evident.

TMPRSS4 expression in lungs of patients with NSCLC and in a patient with COVID-19 with COPD. It is currently known that COVID-19 severity increases in patients with lung cancer (19). Moreover, previous meta-analyses have revealed a higher mortality rate in patients with COVID-19 and cancer (20-22). In a previous study, the authors demonstrated that the TMPRSS4 gene was significantly upregulated in LUAD and in LUSC (14). The present study determined the protein expression of TMPRSS4 in 4 patients with NSCLC; 2 patients diagnosed with LUAD and 2 patients with LUSC (Fig. 2). The expression of TMPRSS4 was evident in the tumour cells of patients with LUSC (Fig. 2A and C) and LUAD (Fig. 2E and G) and to a varying degree in the peritumoral stroma. Of note, TMPRSS4 was also highly expressed in tumour adjacent morphological normal lung tissue, particularly on bronchial and alveolar epithelial cells (Fig. 2B,D,F and H). This finding corroborates mRNA data demonstrating a similar gene expression of TMPRSS4 in males and females in LUAD and LUSC (Fig. S1D and E).

A recent systematic review and meta-analysis demonstrated that patients with COVID-19 with COPD have a significantly increased risk of poor clinical outcomes (23). The present study demonstrates for the first time, to the best of our knowledge, the extensive protein expression of TMPRSS4 in tumour cells in the lungs of a deceased patient with COVID-19 with COPD and bronchial carcinoma (Fig. 3C and D), as well as in alveolar epithelial cells of the adjacent non-tumour tissue (Fig. 3A and B), comparable to the results obtained for the LUAD and LUSC samples without COVID-19 infection.

Discussion

The present study provides novel (to the best of our knowledge), comprehensive evidence regarding the protein distribution of

TMPRSS4 in the GI tract and lungs. Recently, two different groups described the involvement of TMPRSS4 as a SARS-CoV-2 entry mediator in the GI tract (5,6), suggesting that a leaky gut may allow SARS-CoV-2 to spread to other organs (e.g., the liver) (5). Based on the findings of the present study using a tissue microarray, evidence of TMPRSS4 protein expression across the GI tract (oesophagus, stomach, small intestine and colon), as well as in the pancreas and liver is provided. In relation to the latter, it is noteworthy that up to 70% of patients with COVID-19 exhibit abnormal liver function tests upon admission to hospital, a finding which is transient in the majority of cases (24). As such, this high hepatic TMPRSS4 protein expression suggests that this mediator may be involved in the SARS-CoV-2 infectivity of the liver, explaining, at least in part, why immunocompromised patients with liver cirrhosis or hepatic cancer are more susceptible to COVID-19 infection (25,26). In addition to the liver, recent research interests have also focused on the involvement of the pancreas in the context of COVID-19, particularly since it has been suggested that pancreatic ACE2 expression can cause damage of this vital exocrine and endocrine organ following SARS-CoV-2 infection (27). Indeed, an association between pancreatitis and COVID-19 has been reported (28). In accordance with such findings, the present study provides novel data (at least to the best of our knowledge) on widespread TMPRSS4 protein expression in the pancreas. Given that the main cell entry mediator (ACE2) is present in the pancreas, it is plausible that the co-expression of these two SARS-CoV-2 cell entry mediators may facilitate increased local viral infectivity, which can lead to pancreatic damage. Of note, previous research on SARS-CoV has demonstrated that this coronavirus can bind to ACE2 and damage the pancreatic islets (endocrine portion of the pancreas), thus causing acute diabetes (29).

Furthermore, the authors, as well as other researchers have demonstrated that TMPRSS4 is overexpressed in LUAD and LUSC, which are both conditions that predispose to cases of severe COVID-19 infection (30-33). Recently, the cellular distribution of ACE2, TMPRSS2 and TMPRSS4 was examined in normal and LUAD samples using single-cell RNA-sequencing (33). Han *et al* (33) demonstrated that TMPRSS4 expression was highest and most frequently detected (75%) in malignant pulmonary cells, primarily of epithelial origin. Notably, ACE2 appears to be co-expressed in the same cells in LUAD, alluding to potential higher infectivity, but also to implications for the management of patients with lung cancer and severe COVID-19. Moreover, as aforementioned, patients with COPD are also at higher risk of poor COVID-19-related outcomes. The exact mechanisms contributing to this higher risk are still under investigation, with data demonstrating that both ACE2 and TMPRSS4 expression levels are upregulated in the epithelial brushing and bronchial biopsy samples of patients with COPD (15). The present study demonstrated the abundant expression of TMPRSS4 in the lung tissue (both adenocarcinoma and normal adjacent lung tissue) of a patient with COVID-19 with COPD and cardiac hypertrophy, who succumbed to the disease.

However, it should be acknowledged that there are a number of limitations to the present study. The present study did not use an *in vitro* model to further study the interactions between TMPRSS4 and other SARS-CoV-2 cell entry mediators. In addition, a quantitative analysis of the protein expression on tissue microarrays was not performed. Finally, there was a limited number of patients used in the present study.

In conclusion, the findings of the present study demonstrate the widespread protein expression of TMPRSS4 in the GI tract, liver and pancreas, and provide further evidence regarding the expression of TMPRSS4 in the lungs. Indeed, collectively, the data of the present data study suggest that TMPRSS4 may be implicated in both the pulmonary and extra-pulmonary COVID-19 symptomatology/manifestations, functioning as another host cell SARS-CoV-2 entry mediator, contributing to the tropism of this new coronavirus. The pharmacological inhibition of viral entry via TMPRSS4 may thus present an interesting additional target for the development of antiviral agents; however, additional studies using larger cohorts of patients with COVID-19 and lung carcinoma patients are required.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HSR, IK, EK, DJ, DAS and CW were involved in the conceptualization of the study. RK, PK, CW, PJ and JLR were involved in the study methodology (immunohistochemistry and statistical analyses). RK, PK, DJ, PJ, CW and EK were involved in formal analysis. RK, IK, JLR and EK were involved in the writing and preparation of the original draft. RK, HSR, DJ, CW, JLR, PK, PJ, IK, DAS and EK were involved in the writing, reviewing and editing of the manuscript. DJ and EK supervised the study. EK was involved in project administration. HSR and DJ was involved in funding acquisition. EK and IK are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. EK and IK confirm the authenticity of all the raw data. All authors have read and agreed to the published version of the manuscript.

Ethics approval and consent to participate

The present study was conducted according to the guidelines of the Declaration of Helsinki. Appropriate patient/next of kin written consent and ethical approval were ensued by the Ethics Committee of Hannover Medical School (OE 9515; ethics reference no. 9621_BO_K_2021); Institute for Pathology, Hannover Medical School, Hannover, Germany. All tissue samples were collected under the highest ethical standards with the donors giving informed consent (under Health Insurance Portability and Accountability Act (HIPAA) approved protocols). Additional

control samples of lung tissue were retrieved from archived routine samples from Hannover Medical School (ethics reference no. 1741-2013).

Patient consent for publication

Not applicable.

Competing interests

DAS is the Editor-in-Chief for the journal, but had no personal involvement in the reviewing process, or any influence in terms of adjudicating on the final decision, for this article. The other authors declare that they have no competing interests.

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