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SARS-CoV-2 re-infection in a cancer patient with a defective neutralizing humoral response

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To the Editor-in-Chief of Clinical Infectious Diseases
Dr. Robert T. Schooley

Novara, 21st April 2021

Dear Editor,

I am very pleased to submit the enclosed manuscript by Borgogna et al. entitled "SARS-CoV-2 re-infection in a cancer patient with a defective neutralizing humoral response" for consideration for publication as Correspondence in Clinical Infectious Diseases.

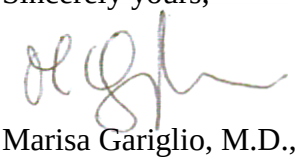
In this letter, we describe an Italian cancer patient who was infected at two separate times with two genetically distant SARS-CoV-2 strains, with reappearance of the first strain four months after the first infection.

This case highlights the concerning risk of re-infection in cancer patients who fail to mount an efficient neutralizing humoral response along with the underlying existence of persistent asymptomatic/undetectable infection.

This letter, including related data, figures, and tables has not been previously published and it is not under consideration elsewhere. I state that all authors have made substantial contributions to the conception and design of the study, or acquisition of data, or analysis and interpretation of data, drafting the article or revising it critically for important intellectual content. All authors have made final approval of the version to be submitted and declare no conflict of interests.

We would like to take this opportunity to thank you in advance for your thoughtful consideration of our work.

Sincerely yours,



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SARS-CoV-2 re-infection in a cancer patient with a defective neutralizing humoral response

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Keywords: SARS-CoV-2, Re-infection, Neutralization humoral response, Cancer patient.

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TO THE EDITOR - Emerging evidence indicates that SARS-CoV-2 re-infection may occur in immunocompromised patients, including cancer patients [1-3]. Here we report a case of a 52-year-old male patient suffering from transitional cell carcinoma of the renal pelvis and ureter who was infected at two separate times with two genetically distant SARS-CoV-2 strains, with reappearance of the first strain four months after the first infection. The patient's past medical history and treatments are summarized in Supplementary Figure 1. On June 23, 2020 (day 0), he had cough and fever and was diagnosed with COVID-19 by SARS-CoV-2 reverse transcriptase-polymerase chain reaction (RT-PCR) assay of a nasopharyngeal swab specimen (Figure 1A). Chest X-ray did not reveal any abnormality, and his clinical conditions improved with resolution of cough and fever within two weeks. On day 35 and 36, two consecutive nasopharyngeal swabs resulted negative for SARS-CoV-2 infection. In the next few months, the patient did not show any respiratory symptoms. However, the deterioration of his cancer condition leading to urinary tract infection and sepsis required further hospitalization. On day 110, an additional SARS-CoV-2 nasopharyngeal swab tested positive. On day 115, the patient died from septic shock and respiratory failure.

Quantitative SARS-CoV-2 viral loads by droplet digital PCR detected 546, 1, and 53 copies/μl on day 0, 110, and 115 nasopharyngeal swabs, respectively (Figure 1A). Whole genome sequencing and phylogenetic analysis of RNA from the first two specimens showed that the viral genome found at day 0 could be grouped in the Nextstrain clade 20B and Pangolin lineage B.1.1, while the strain isolated on day 110 belonged to the Nextstrain clade 20A and Pangolin lineage B.1 (Figure 1B). However, when we sequenced the RNA from the third sample harvested on day 115, we detected again the Nextstrain clade 20B, suggesting that the first infection strain had never been cleared completely. With regard to amino acid changes, by analyzing minority variants in the day 115 specimen, the mutations R203K and G204R, which distinguish B.1 and B.1.1 lineages, were the predominant ones until 65% coverage, but below this cut-off we were also able to detect significant levels of the wild-type virus (Figure 1C). Furthermore, the D614G variant was always present in specimens isolated on day 110 and 115, whereas it was absent, even as a minority variant, in the specimen harvested on day 0. No evidence

of recombination events was observed. Phylogenetic analysis was congruent with both persistent infection with B.1.1 strains (specimens from day 0 and 115) and re-infection with B.1 strain on day 110. Consistent with a scenario of weak immunological response, the neutralizing activity of the day 110 serum against recombinant vesicular stomatitis virus rVSV-SARS-CoV-2-SΔ21 was very low when compared to positive control (Figure 1D and S2) [4, 5].

Overall, this case highlights the concerning risk of re-infection in cancer patients who fail to mount an efficient neutralizing humoral response along with the underlying existence of persistent asymptomatic/undetectable infection.

Acknowledgement: We thank Marcello Arsura for critically reviewing the manuscript. The study protocol was approved by the Institutional Review Board of the University-Hospital Maggiore della Carità-Novara (Italy) and the patient provided written inform consent.

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Potential conflict of interest: The authors declare no conflict of interest.

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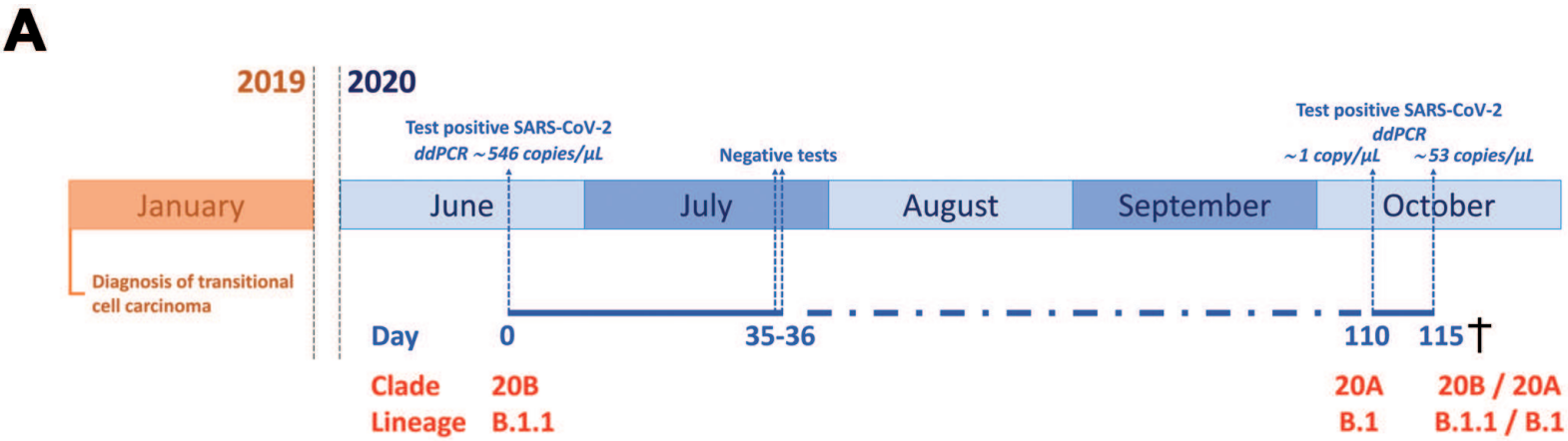
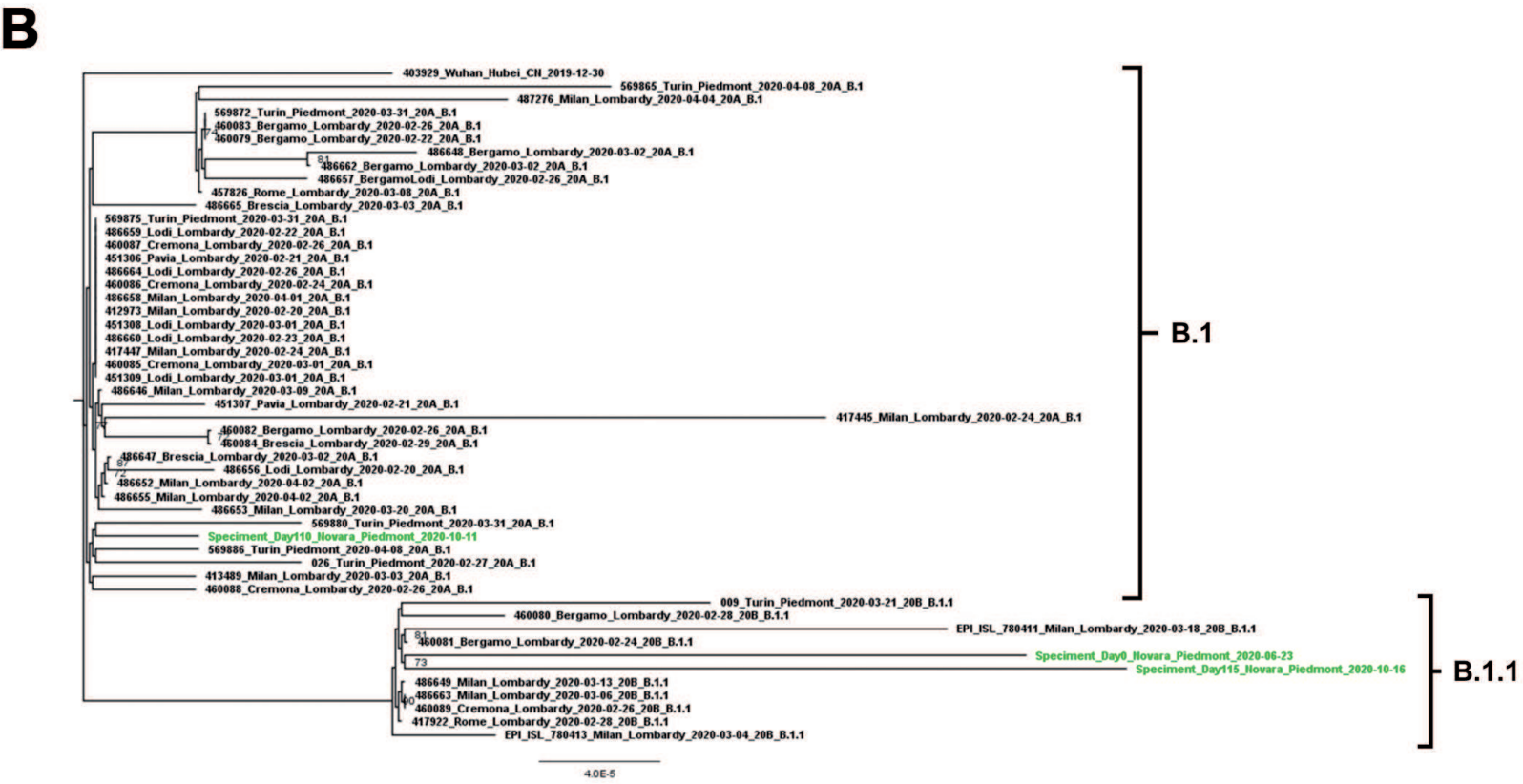
Figure legend

Figure 1. A) Timeline of clinical presentations and SARS-CoV-2 testing, including viral loads (copies/µl) and the strains found in the study patient. Timing of relevant clinical events, such as outcome of diagnostic tests, is shown; **B)** Phylogenomic analyses of described SARS-CoV-2 strains in the study patient. The tree was constructed by the maximum likelihood method. Clade information as inferred by Nextstrain and Pangolin nomenclatures is shown; **C)** Viral genome classification and amino acid mutations identified according to Nextclade and Pangolin among the three specimens harvested on day 0, 110, and 115; **D)** Serum neutralizing assay against rVSV-SARS-CoV-2-SΔ21 with a sample harvested at day 110. Data are representative of two independent experiments performed in duplicate. Error bars represent the standard deviation. Patient (blue dot), normal human serum (Neg) (black triangle), positive serum = COVID-19 convalescent serum (Pos) (red square).

Figure 1

Click here to access/download;Figure (.tif and .eps files only);Figure 1_case_CMYK.eps





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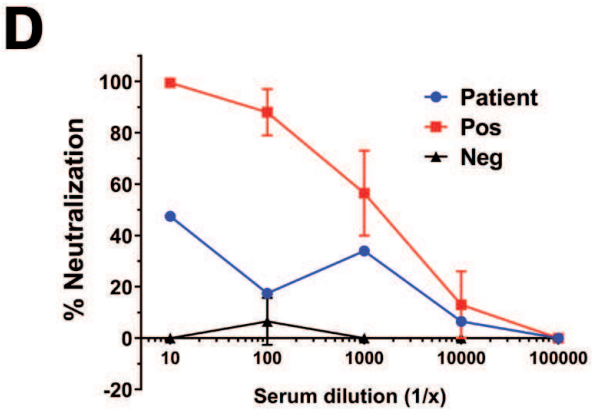
Amino acid mutations among the strains found in the study patient							
Specimen	Clade	Lineage	Gene				
			ORF1b	S	ORF3a	N	ORF10
Day 0	20B	B.1.1	P314L	A846V	R30S, R30L	R203K, G204R	
Day 110	20A	B.1	P314L	D614G			
Day 115	20B	B.1.1	P314L	D614G	Q17L	R203K, G204R	V30L



Supplemental information regarding clinical course, SARS-CoV-2 genome detection, and immunological assays

A 52-year-old male patient attending the oncology department at the University-Hospital Maggiore della Carità of Novara, Italy, was diagnosed with transitional cell carcinoma of the renal pelvis and ureter at the beginning of 2019. No other relevant comorbidities were reported. Computed tomography (CT) scan showed mediastinal and retroperitoneal lymphadenopathy with pulmonary and bone metastases. As depicted in Supplementary Figure 1, the patient underwent six chemotherapy courses of cisplatin plus gemcitabine—one every 21 days—obtaining a partial response. In January 2020, as the patient experienced nodal and bone tumor progression with thrombosis of the inferior vena cava, a second-line treatment with the checkpoint inhibitor pembrolizumab was commenced along with enoxaparin. After the fourth administration of pembrolizumab, the anticancer treatment was discontinued as the patient had developed obstructive uropathy with renal failure and urinary tract infection (UTI), which required bilateral nephrostomy and antibiotic administration. On June 23, 2020 (day 0), he had cough and fever, and was diagnosed of COVID-19 by SARS-CoV-2 reverse transcriptase-polymerase chain reaction (RT-PCR) assay of a nasopharyngeal swab specimen (cycle threshold, Ct, values for SARS-CoV-2 E, RdRp, and N genes ranged from 25 to 26). Chest X-ray did not show any abnormality, and the patient was again treated with antibiotics for UTI recurrence. Within two weeks, his clinical conditions improved with resolution of cough and fever. On day 35 and 36, two consecutive nasopharyngeal swabs were negative for SARS-CoV-2 infection by RT-PCR assay. In the next few months, the patient did not show any respiratory symptoms. The CT scan performed on day 91 showed nodal, bone, and local tumor progression, with bilateral thrombosis of femoral veins, treated with enoxaparin. Due to this condition, on September 22, he was started on a third-line therapy with vinflunine but, after a single administration, had to be hospitalized due to oliguria and pyuria. On day 110, the patient had fever caused by an ongoing *Escherichia coli*-induced sepsis. RT-PCR assay of a nasopharyngeal swab resulted positive again, causing concern for a recurrence of COVID-19 (Ct values of 34 and 36 for E and N genes, and over 40 for the RdRp gene). An abdominal CT scan performed on

day 113 showed thrombosis of the inferior vena cava, of the right iliac vein, and of both femoral veins. On day 115, he died from septic shock and respiratory failure.

The patient's blood was only available on day 110. Rapid immunochromatographic test on blood resulted positive for IgG anti-SARS-CoV-2 N protein. Very low levels of IgG anti-SARS-CoV-2 spike protein were found in this sample (1200 AU/ml with the low threshold < 2.544 AU/ml). In addition, anti-receptor binding domain (RBD) antibodies were determined by a different ELISA, which confirmed the presence of a very low reactivity. Consistently, the neutralizing activity performed using the replication-competent chimeric VSV expressing the SARS-CoV-2 spike protein (rVSV-SARS-CoV-2-SΔ21) was very low when compared to convalescent positive control (Figures 1D and S2) [4]. The fact that VSV harbors the D614 form of the spike protein—the same found in the strain isolated on day 0—and that the G614 form is reportedly unable to interfere with the neutralizing titre [5] rules out any detection bias of our approach, indicating that the patient did fail to mount an appropriate neutralizing humoral response.

Materials and Methods

Ethical approval and informed consent

The study protocol was approved by the Institutional Review Board of the University-Hospital Maggiore della Carità-Novara (Italy) and the patient provided written inform consent.

SARS-CoV-2 RNA detection and quantification in nasopharyngeal swabs

Our clinical microbiology laboratory utilized the Allplex 2019-nCoV Assay (Seegene) for molecular detection of SARS-CoV-2 in COVID-19 patients. Allplex 2019-nCoV Assay is a multiplex real time PCR (RT-PCR) assay for simultaneous detection of 3 target genes of SARS-CoV-2 (RdRP, N, and E, respectively). A specimen is considered positive if the gene target has a cycle threshold (Ct) of < 40.

For droplet digital PCR (ddPCR), total RNA was extracted from 200 μl of nasopharyngeal swabs using QIAamp viral RNA mini kit (Qiagen) following the manufacturer's instruction. SARS-CoV-2 genomic RNA was quantified by means of the QX200 Droplet Digital PCR System (ddPCR, Biorad) using

SARS-CoV-2 Droplet Digital PCR Kit (Biorad, CA, USA). SARS-CoV-2 quantification was expressed in copy number/µl of swab.

Whole-genome sequencing and phylogenetic analysis

Whole genome sequencing was performed from the nasopharyngeal swabs harvested on day 0, 110, and 115 by next generation sequencing using the CleanPlex SARS-CoV-2 Panel (Paragon Genomics Inc, CA, USA) on the Illumina MiSeq platform using the 2 × 150 cycle paired-end sequencing protocol. The resulted reads were mapped and aligned to the SARS-CoV-2 reference genome (GenBank accession number: NC_045512.2) using Geneious software, v. 9.1.5 (http://www.geneious.com). SARS-CoV-2 sequences were assigned to clade and lineage using Nextclade (https://clades.nextstrain.org/) and Pangolin applications (https://pangolin.cog-uk.io/). Alignment was performed using MAFFT and manually cropped to a final length of 29,400. The three sequences were included in the Italian dataset of all published strains from northern Italy, obtaining a final dataset of 51 strains. Maximum likelihood tree was built using IQTree V.2 with bootstrapping of 1,000 and selected GTR+I+G model (general time-reversible+ invariant sites+ gamma distribution). The RDP5 software was used to investigate the presence of potential recombination.

Quantitative SARS-CoV-2-specific antibodies

For quantitation of serum IgG reactive to SARS-CoV-2 spike protein, the COVID-SeroIndex Kantaro Quantitative SARS-CoV-2 IgG Antibody RUO Kit (R&D Systems, Bio-Techne, Minneapolis, USA) was used following the manufacturer's instruction. Additionally, WANTAI SARS-CoV-2 antibody ELISA (Beijing Wantai Biological, China) was performed to evaluate anti-RBD antibodies. The presence of anti-N protein antibodies was tested by Immunochromatographic COVID-19 IgG/IgM Rapid Test (PRIMA Lab SA, Balerna, Swiss).

SARS-CoV-2-specific neutralizing antibody assay

Vero E6 and Vero E6-TMPRSS2 (kindly provided by John Hiscott, Pasteur Institute Rome) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich, Milan, Italy). The replication-competent vesicular stomatitis virus r(VSV)-

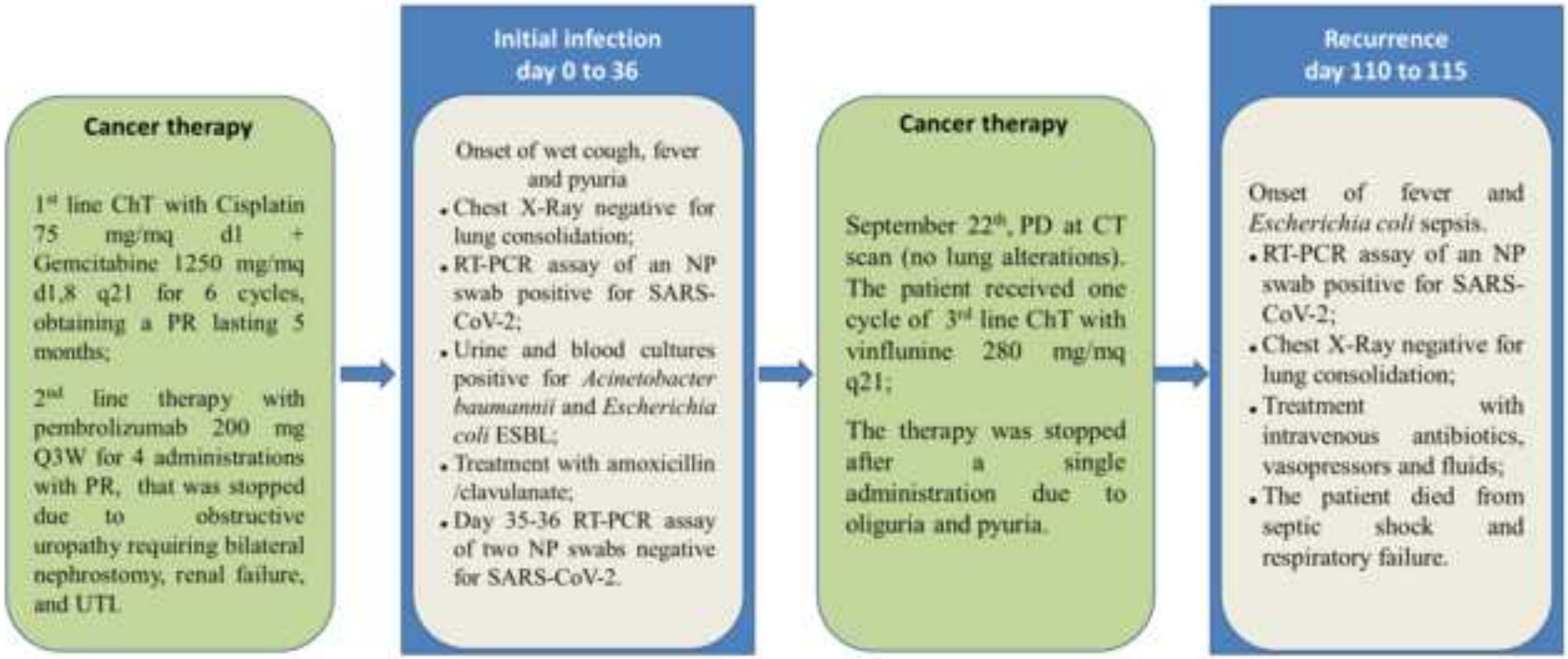
eGFP-SARS-CoV-2-SΔ21 was kindly provided by Sean P.J. Whelan (Washington University School of Medicine, USA) (Case et al., 2020). To grow the virus, Vero E6 cells were infected with a low MOI (0.01) and maintained at 34°C from then on. Cell supernatants were harvested upon visualization of extensive cytopathic effect and detaching of cells at approximately 24 hpi. The spike gene was sequenced after each time the virus had grown. Viral RNA was extracted and an RT-PCR performed to sequence the spike gene. The virus was titrated by flow cytometry. Serum samples were heat-inactivated at 56°C for 30 min. Indicated dilutions of sera were incubated with rVSV-SARS-CoV-2-SΔ21 at an MOI of 0.05 for 1 h at 37°C. Antibody-virus complexes were added to Vero E6-TMPRSS2 cells in 96-well plates and incubated at 37°C for 24 h. Subsequently, cells were fixed in 4% formaldehyde (Millipore Sigma) containing DAPI for 15 min on ice, when fixative was replaced with PBS. Images were acquired with the Leica THUNDER imager (Leica Microsystems, Wetzlar, Germany) in both the DAPI and FITC channels to visualize nuclei and infected cells (*i.e.*, eGFP-positive cells), respectively (5X objective, 9 fields per well, covering the entire well). Images were processed using the Leica Application Suite X (LAS X). A background number of GFP^+ cells were subtracted from each well using an average value determined from at least 2 uninfected wells. Data were processed using Prism software (GraphPad Prism 6.0).

Supplementary Figures

Figure 1. *Clinical course and therapy.*

Figure 2. *Representative images of the neutralization assay for each serum (Patient, Positive and Negative controls) at the dilution 10^{-1}.* Vero E6-$TMPRSS2^+$ cells were infected with rVSV-SARS-CoV-2-S-Δ21 at an MOI of 0.05 after pre-incubation with each serum. At 24 h post infection (hpi), the cells were fixed and nuclei stained with DAPI. Images were acquired using the fluorescence microscope and merged: nuclei are depicted in blue (DAPI), infected cells in green (green fluorescent protein-GFP) (9 fields per well, covering the entire well).

Figure S1: Clinical course and cancer therapy



ChT: chemotherapy; Q3W: every three weeks; PR: partial response; UTI: urinary tract infection; RT-PCR: reverse-transcriptase–polymerase-chain-reaction; NP: nasopharyngeal; ESBL: extended spectrum beta-lactamase; PD: progressive disease; CT: computed tomography

Figure S2