Autoantibodies against SUMO1-DHX35 in long-COVID

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Abstract

Long COVID is a collection of symptoms as a late sequelae of SARS-CoV-2 infection. It often includes mental symptoms such as cognitive symptoms, persisting loss of smell and taste, in addition to exertional dyspnea. A role of various autoantibodies (autoAbs) has been postulated in long-COVID and is being further investigated. With the goal of identifying potentially unknown autoAbs, we screened plasma of patients with long COVID on in-house post-translationally modified protein macroarrays including citrullinated, SUMOylated and acetylated membranes. SUMOylated isoform DEAD/H (Asp-Glu-Ala-Asp/His) box helicase 35 (SUMO1-DHX35) was identified as only candidate antigen. In adult patients with long COVID, IgG autoAbs against SUMO1-DHX35 of IgG class were found in seven of 71 (9.8%) plasma samples, of IgM and IgG class in one of 69 (1.4%) samples, not in 200 healthy adult controls, not in 442 healthy children, and 146 children after SARS-CoV-2 infection. All autoAb-positive seven patients were female. AutoAb titers ranged between 200 to up to 400 By point mutagenesis and expression of FLAG-tagged mutants of DHX35 in HEK293 cells, and subsequent SUMOylation of purified constructs, lysine 53 was identified as a unique, never yet identified, SUMOylation site. The autoAbs had no reactivity against the non-SUMOylated mutant (K53R) of DHX35. To summarize, autoAbs against SUMO1-DHX35 were identified in adult female patients with long-COVID. Further studies are needed to verify the frequency of occurrence. The function of DHX35 has not yet been determined and there is no available information in relation to disease implication. The molecular mechanism causing the SUMOylation, the potential functional consequences of this post-translation modification on DHX35, and a potential pathogenicity of the autoAbs against SUMO1-DHX35 in COVID-19 and other possible contexts remain to be elucidated.

1. Introduction

Long Corona Virus Disease (Long-COVID) and post-COVID are a collection of symptoms that present as late sequelae of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection. It often includes mental symptoms such as functional cognitive impairments, fatigue, in addition to exertional dyspnea [1–3]. Whereas long-COVID usually describes persistence or onset of new symptoms for a period longer than 4 weeks, post-COVID19 includes cases with symptoms still present after 12 weeks [4]. The symptoms are sometimes diffuse, and the disease burden may be variable [5]. In contrast to the critical acute form of COVID-19 that affects men significantly more often than women, long COVID appears to affect women more often [6]. SARS-CoV2 seems to be an extremely immunogenic virus as many of the disease complications arise from immune phenomena such as cytokine release syndrome. In addition, an enhanced activity of B lymphocytes with over production of autoAbs has also been shown in patients with COVID-19, especially those with severe disease [7–12]. AutoAbs against interferon-ω and α
2. Material and methods

This study was approved by the local Ethical Review Board (42/21 and CORSAA) and conducted according to the Declaration of Helsinki. Blood plasma samples were taken after written informed consent in the outpatient long/post COVID ward of the Department of Internal Medicine V of the Saarland University Hospital (Homburg/Saar, Germany). Blood samples of adult patients with acute COVID-19 were obtained at the department of Internal Medicine II and V of the Saarland University Hospital (Homburg/Saar, Germany), samples of healthy adult controls were obtained from company medical service of the University Hospital of Saarland (Homburg/Germany), and samples of healthy children and children after SARS-CoV-2 infection were provided by the central trial office COKIBA cross sectional trial (Regensburg, Germany).

2.1. Autoantibody screening for post-translationally modified autoantigens

Plasma samples of 70 patients with long COVID were diluted 1:500, pooled together and preadsorbed overnight against an expression clone of SLP2 produced by the same expression system in E. coli. Subsequently these samples were further diluted 1:2 (resulting in a final dilution of 1:1000) and screened on protein macroarrays containing clones of UniPEx 1 and 2 cDNA expression libraries (Bioscience, Dublin, Ireland), as previously described [21,22]. To search for further antigens, this pooled plasma samples were screened against various post-translationally modified UniPex 1 and 2 protein macroarrays, including SUMOylation, ubiquitination, citrullination, and acetylation. SUMOylation of protein macroarrays was performed as previously described [23] and ubiquitination was performed with synchronized HeLa cell extracts [24]. For acetylation, macroarrays were incubated with Tris buffer, 50 mM, pH 8.0 containing 150 mM NaCl, 5% (v/v) glycerol, 0.1 M trichostatin A, 10 mM ethylenediaminetetraacetic acid, 10 mM dithiothreitol, 20 μg recombinant p300 and 20 μM coenzyme A (Sigma #2056) for 30 min at 37 °C. The reaction was stopped by excessive washing. Glutathione S-transferase (GST)-tagged p65 served as control for excess acetylation amino acid residue 310.

2.2. Expression of candidate antigens

The expression clone of DHX35 was recombinantly expressed with C-terminal FLAG (DYKDDDDK) tag by pSFI vector in HEK293 cells. Additionally, C-terminally FLAG-tagged full-length and FLAG-tagged fragments of different lengths of DHX35 were expressed. Secondary modification, 500 μL of cell lysates were incubated for 10 min at room temperature with 10 μL anti-FLAG-affinity matrix (Sigma #A2220) and washed afterwards. SUMOylation of DHX35 was performed as previously described. RAN-GST served as control for successful SUMOylation [23,24]. Post-translationally modified proteins were washed, eluted by administration of FLAG peptide (100 μg/mL) and brought into solution in phosphate-buffered NaCl (PBS). SUMOylation was verified by sumo-specific Abs (Biozol #BZL08843). For site-directed mutagenesis of DHX35, the QuickChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used. cDNA coding full length DHX35 and mutants, each C-terminally FLAG-tagged, were constructed. Mutants produced to identify the SUMO1-site were designed according to analysis software SUMOplot™ They contained lysine to arginine exchanges at positions predicted with the highest probability to be subjected to SUMOylation, namely K53R, K198R, K336R, K413R, K646R, K694R and K696R.

2.3. ELISA for antibodies against DHX35 and against SUMO1-DHX35

Enzyme-linked immunosorbent assay (ELISA) for autoAbs was performed as previously described [25]. In short, SUMOylated DHX35 was coated onto Nunc MaxiSorp plates (eBioscience, Frankfurt, Germany) pre-coated with murine anti-FLAG monoclonal Abs at a dilution of 1:2500 (s/v; Sigma-Aldrich, Munich, Germany) at 4 °C overnight. After blocking with 1.5% (w/v) gelatin in Tris-buffered saline (TBS) and washing steps with TBS with Triton X-100, the individual plasma samples were diluted 1:100. ELISA was performed according to standard protocols with the following Abs: biotinylated goat antihuman heavy and light chain immunoglobulin G (IgG) at a dilution of 1:2500 (Dianova, Hamburg, Germany); subclass-specific sheep anti-human IgG1, IgG2, IgG3 and IgG4 (Binding Site Group, Birmingham, UK) at dilutions of 1:5000; goat anti-human IgM (Dianova) at a dilution of 1:2500; or goat anti-human IgA (Dianova) at a dilution of 1:2500. Following this step, corresponding biotinylated secondary Abs were used for immunoassays carried out to detect IgG subclasses and IgM. Peroxidase-labelled streptavidin (Roche Applied Science, Indianapolis, IN) was used at a dilution of 1:50,000.

2.4. Western blot and IEF of SUMO1-DHX35

Isoelectric focusing (IEF) and western blotting (including native western blotting with non-reducing sample pretreatment and gradient gels without sodium dodecyl sulfate) was performed on whole blood cell lysates of patients with anti-SUMO-DHX35 Abs and autoantibody-negative controls.

3. Results

3.1. Screening of post-translationally-modified protein arrays and ELISA for anti-SUMO-DHX35 antibodies

Plasma samples of a series of 71 patients with long-COVID-19 (Supplemental Table 1), 69 adult patients hospitalized with acute COVID-19, 200 healthy adult controls (collected before 2019), 442 healthy children and 146 children with SARS-CoV-2 infection were included in this study. Screening of post-translationally modified protein arrays with these plasma samples identified SUMO1-DHX35 as potential antigen. In 7 of 71 adult patients with long-COVID autoAbs against SUMO1-DHX35 were detected (Fig. 1 A) with titers ranging between 200 and 400 (Fig. 1 B). AutoAbs did exclusively bind SUMOylated DHX35, but not non-SUMOylated DHX35. SUMO1-DHX35-autoAbs belonged predominantly to IgG1 class in patients with long-COVID (Fig. 1 C), the SUMO1-DHX35-Ab-seropositive patients with acute COVID-19 presented IgM class and different IgG subclass Abs (Fig. 1 C). In contrast, SUMO1-DHX35-Abs were not found in 442 healthy children, and in none of 146 children tested after SARS-CoV-2 infection. The sensitivity and specificity of positivity for SUMO-DHX35 in long-COVID, were 9.8% and 99.5%, respectively, whereas the positive predictive value and negative predictive value were 87.5% and 77%, respectively.
Of note, only patients with past COVID-19 exposition were included in the control group (n = 215).

3.2. SUMOylated isoform of DHX35

Expression of point-mutated and C-terminally FLAG-tagged DHX35 constructs resulted in K53 as site of SUMOylation. K53R mutant could not be SUMOylated (Fig. 2A) and autoAbs of patients’ sera did not bind to this mutant anymore (Fig. 2B). Altering the other potential arginine sites and thus impairing their potential SUMOylation, had no influence for antibody binding. Western blots of lysates prepared from peripheral cells collected from long-COVID-19 patients with or without autoAbs did not reveal a different, eventually atypic post-translational modification of SUMO1-DHX35 (Fig. 2C). The SUMO1-isoform was detectable in SUMO1-DHX35 seropositive and seronegative patients.

3.3. Clinical characteristics of patients presenting autoAbs against SUMO1-DHX35

A summary of patients’ characteristics with or without autoAbs against SUMO1-DHX35 is presented in Table 1. Remarkably all patients presenting autoAbs were female, compared to 56% in the cohort without autoAbs. Patients with autoAbs had no history of allergies, compared with 25% of patients in the group without autoAbs. In addition, 28% vs. 3.5% of patients had a history of autoimmune diseases and 28% vs. 5% of patients had a history of smoking in the cohort with and without autoAbs respectively. Apart from age (p = 0.0295) there were no statistically significant differences regarding any of these clinical characteristics.

4. Discussion

Exact mechanisms of long-COVID are still largely unclear. Pathogenic autoAbs have been proposed to contribute and several have been described/associated. Here we report about the detection of autoAbs against SUMO1-DHX35 in a subset of patients with long/post COVID. This study demonstrates that screening samples for Ab reactivity on post-translationally modified protein arrays can reveal unexpected antigenic targets, which are totally invisible when usual techniques based on non-modified proteins are exploited.

The specificity of SUMO1-DHX35-reactive Abs newly identified in this study seems to be relatively high for adult patients with long/post COVID, especially when IgG Abs are considered. The presence of this autoAb should be further investigated in other infectious context and also in autoimmune and rheumatologic diseases. So far SUMO1-DHX35 was not known as an autoantigen, and neither was DHX35. DHX35 is described as an RNA helicase (the human genome codes for 95 non-redundant helicases). SUMO1-DHX35, also known as Probable ATP-dependent RNA helicase DHX35, is involved in altering RNA secondary structure and pre-mRNA splicing as a part of the DEAD box protein family. However, no information regarding its specific biological function have been described thus far.

We have observed that SUMOylation of DHX35 is a prerequisite for autoantibody binding. We further show that a subset of patients possess SUMO1-DHX35-reactive Abs. We found however that the SUMO1 post-translational modification was equally present in cell protein extracts from SUMO1-DHX35 sero-positive or -negative long COVID patients and healthy controls. This finding led us to conclude that the level of protein SUMOylation does not seem to be a triggering factor for
Fig. 2. A) Western blots of different point-mutated, full-length and C-terminally FLAG-tagged constructs of DHX-35, which were either treated post-translationally to be SUMO1ylated or not. Using anti-FLAG or anti-DHX35 as primary antibodies demonstrates gain of molecular weight due to added SUMO1-group in all mutants but K53R. SUMO1-specific primary antibody confirms K53 as SUMO1-site. B) ELISA using different point-mutated, full-length and C-terminally FLAG-tagged constructs of DHX-35 as antigens. These were either used directly (above) or treated post translationally by SUMO1ylation (below). Anti-FLAG, anti-SUMO1, or plasma of long-COVID patients were used. C) Western blot of DHX35 of whole blood cell lysate using either anti-SUMO1- or commercially available anti-DHX35 antibodies. Blood cell lysates were derived of anti-SUMO1-DHX35-antibody positive and negative patients.
autoAb formation in a specific way as it is known for other autoantigens in clinical immunology and also hematology [19, 20, 26–33] The reasons why COVID-19 patients develop SUMO1-DHX35 reactive Abs remain unknown.

AutoAbs against SUMO1-DHX35 occurred in a subgroup of patients with long-COVId. In our cohort, it was specific, since non-modified DHX35 was not recognized by plasma IgG Abs, and positivity was mostly revealed in women. In addition, patients with SUMO1-DHX35 reactive Abs were older. The reason for their occurrence in this context and whether these Abs play a pathogenetic role should be investigated in future studies. Beside this, other limitations of this study include the relatively small number of patients included, which makes difficult to establish associations with clinical characteristics, and the missing knowledge on HLA-haplotypes.

In conclusion, this work has uncovered a novel post-translational modification never identified so far: a SUMOylation that takes place at residue 53 of DHX35. This finding may have significant functional importance since this residue also bears a site for ubiquitination.

Author contribution
L. Thurner, N. Fuchs, K. Dobet, K. Deppen, M. Knieps, and B. Thurner planned the study. L. Thurner, N. Fuchs, K. Dobet, K. Deppen, M. Knieps, and B. Thurner revised the manuscript. M. Charron, N. Fuchs, L. Thurner, and E. Röhrlein performed the experiments. R. Briel and C. H. obtained and managed clinical samples and data.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtauto.2022.100171.

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