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HIGH LEVEL OF RNA HAS2-AS1 IN THE BUFFY COAT OF A PATIENT BLOOD SAMPLE IS A MORE INFORMATIVE PROGNOSTIC MARKER OF COVID-19 CLINICAL COURSE COMPARED TO THE LEVEL OF HYALURONIC ACID IN PLASMA

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Numerous studies have confirmed the association of COVID-19 clinical course with the blood levels of hyaluronic acid and long non-coding RNA HAS2-AS1 as a positive regulator of hyaluronan synthase. We aimed to estimate both the level of hyaluronic acid in plasma and the level of RNA HAS2-AS1 in leukocytes (buffy coat) from the same blood sample at the time of patient admission to the hospital and to analyze the specificity of these indicators as biomarkers of COVID-19 severity. The study involved 78 patients with confirmed COVID-19 who underwent treatment between 2020 and 2021 at the Kyiv City Clinical Hospital N 4. The patients were divided into three groups based on the severity of the disease and classified as mild (n = 12), moderate (n = 36), and severe (n = 30). The level of hyaluronic acid in plasma was determined using the Hyaluronic Acid ELISA kit "Abcam" (USA). The buffy coat was isolated by centrifugation of the blood stabilized with EDTA-K and further aspiration of the leukocyte "cloud". The expression level of HAS2-AS1 in buffy coat leukocytes was estimated using reverse transcription and real-time PCR. According to the obtained data, the level of hyaluronic acid in the plasma of patients with moderate and severe illness was 1.5 and 2.2 times higher compared to the mild illness group, respectively. Meanwhile, the level of RNA HAS2-AS1 in blood lymphocytes (buffy coat) of patient with moderate and severe illness was increased by 7.7 and 22.6 times compared to patients with mild illness. The results of our study demonstrate that, unlike the level of hyaluronic acid in plasma, the level of HAS2-AS1 in a buffy coat is a more reliable prognostic criterion for severe COVID-19 and allows distinguishing patients with varying clinical severity during hospitalization.

K e y w o r d s: hyaluronic acid, RNA HAS2-AS1, COVID-19, plasma, buffy coat, leukocytes.

The impact of the COVID-19 pandemic on health and the global economy has spurred rapid investigations into the mechanisms underlying COVID-19 development and its complications. In the context of COVID-19, there has been a significant increase in demand for chest radiology examinations. Clinicians have drawn attention to an early visual symptom on computed tomography (CT) scans, termed "ground-glass opacity." This distinctive radiological finding of coronavirus disease has prompted substantial interest in understanding its underlying cause.

W. Hellman et al. from the Department of Health of Sweden and the Laboratory Department, citing autopsy results of deceased COVID-19 patients whose lungs were filled with clear, viscous fluid, concluded that these were macromolecules of hyaluronic acid [1]. Staining confirmed the presence of hyaluronan obstructing alveoli and thickened perialveolar interstitium. These authors were the first to hypothesize that hyaluronic acid is pathologically associated with acute respiratory distress syndrome (ARDS) in patients with severe COVID-19.

Interstitial lung edema is one of the leading symptoms of severe COVID-19, directly associated with acute respiratory distress and the need for oxygen support [2, 3]. Zhao F. et al. from the University of Arizona confirmed that hyaluronic acid (HA) is a potent hydrophilic agent contributing to interstitial and alveolar edema in ARDS, leading to histological findings of "hyaline membranes" observed in COVID-19 fatalities [4]. Due to its high molecular

weight and semi-flexible polymer chain, hyaluronic acid forms a highly viscous gel-like fluid, providing blocking properties and supporting tissue barrier functions [5].

Hyaluronic acid is crucial in various physiological and pathological processes [6]. It is synthesized by different cell types, including fibroblasts, endothelial cells, alveolar cells, and others, underscoring its significant role in diverse contexts. Hyaluronic acid is pivotal in the functioning of the endothelial surface layer, protecting endothelial cells, regulating barrier permeability, and serving as a mechanosensory element necessary for functions such as nitric oxide production, vascular integrity, and vasodilation. Hyaluronic acid exhibits excellent hydrophilic properties, allowing it to bind water at a rate exceeding its weight by 1000 times [7].

In humans, hyaluronic acid is synthesized by three transmembrane isoforms known as hyaluronan synthases (HAS1, HAS2, and HAS3) [8]. Among all hyaluronic acid synthases, HAS2 plays a pivotal role in hyaluronic acid synthesis due to its catalytic properties, high expression compared to other HAS isoforms, and its critical role in cardiac development [9].

Recently, it has been demonstrated that a crucial regulator of HAS2 gene expression and hyaluronic acid production is the long non-coding RNA (lncRNA) antisense 1 to hyaluronan synthase 2 (HAS2-AS1), belonging to the class of natural antisense transcripts [10] It has been shown that the production of hyaluronic acid and the expression of HAS2 can be stimulated under hypoxic conditions, as the promoter of HAS2-AS1 contains a hypoxia-responsive element (HRE) responsive to HIF-1α [11]. Hypoxia plays a decisive role in the pathogenesis of COVID-19, and the expression of HAS2-AS1 with subsequent activation of hyaluronic acid production may be considered a leading mechanism determining the severity of the clinical course.

It is not coincidental that CY Lin et al. emphasized the association between elevated hyaluronic acid levels and severe cases of Dengue fever, especially those characterized by "cytokine storm," characterized by elevated levels of inflammatory cytokines such as IL-1β, IL-6, and TNFα [12]. This connection is grounded in the fact that these cytokines are potent hyaluronic acid synthase 2 (HAS2) stimulants in various cell types, including endothelial cells, lung alveolar epithelial cells, and fibroblasts. Papakonstantinou et al., in their studies, demonstrated that hyaluronic acid is associated with the severity and outcome of ARDS and predicts overall survival, proposing to consider hyaluronic acid and its degrading enzyme HYAL-1 as potential targets for controlling airway inflammation and remodeling in ARDS [13].

In 2020, Ding M. et al., for the first time, showed in a small sample of COVID-19 patients that the level of hyaluronic acid and type III procollagen in plasma could be used as indicators of poor prognosis; however, authors consider dynamic observations of lymphocyte counts and CRP levels to be more informative [14].

In our study, it was demonstrated that, unlike plasma hyaluronic acid, the level of HAS2-AS1 in leukocytes is a more reliable prognostic criterion for severe COVID-19 and clearly distinguishes patients with subsequent mild, moderate, or severe courses already at the hospitalization stage.

Materials and Methods

Clinical examination. Patients (78 individuals) were admitted to the infectious diseases department of Kyiv City Clinical Hospital No. 4 after 6-9 days of outpatient treatment since the onset of the illness. COVID-19 was laboratory-confirmed by real-time PCR in all patients. The participant group was homogeneous and comprised 33 men and 45 women with obesity (BMI >30), arterial hypertension, and subcompensated diabetes (mean blood glucose level - 7.57 mmol/l) receiving antidiabetic medications. The average age of the patients was 61.0 ± 15.4 years. Glucocorticosteroids were not prescribed during the pre-hospital treatment stage.

IRB approval. All participants provided written informed consent. The relevant national competent authorities reviewed and approved the study: the Research Ethics Committee (No. 3/20 dated 27.08.2020) and the Hospital Ethics Committee (No. 104/1-284 dated 13.05.2021), which were used to obtain patient and healthy donor samples.

The clinical severity of the disease was determined based on oxygen saturation (pulse oximetry) and the duration of oxygen dependence according to the WHO classification. Disease severity was classified as follows: mild disease: illness without signs of viral pneumonia and hypoxia; Moderate disease: clinical signs of pneumonia (fever, cough, dyspnea, increased respiratory rate) without evidence of severe pneumonia, including $SpO_2 \geq 90\%$ on room air; Severe disease: illness with clinical symptoms

of pneumonia (fever, cough, dyspnea, increased respiratory rate) plus one of the following: respiratory rate >30 breaths/min, severe respiratory distress, or SpO_2 <90% on room air.

According to the specified criteria, all patients were divided into three groups based on the severity of the disease and classified as mild (n = 12), moderate (n = 36), and severe (n = 30) according to the Recommendations of the National Health Commission of Ukraine. The severity of COVID-19 was assessed at the time of discharge from the hospital or the patient's death.

Blood collection and buffy coat preparation. The Buffy coat isolation method involved centrifugation for 10 min at 400 g of stabilized blood previously collected in S-monovettes (Sarstedt, Germany) with EDTA-K as an anticoagulant. Following centrifugation, the upper layer of the leukocyte "cloud" was carefully aspirated using a sampler and utilized for subsequent RNA isolation.

Measurement of hyaluronic acid (HA) in blood plasma. For the measurement of hyaluronic acid levels, we used the enzyme-linked immunosorbent assay (ELISA) method (ab287799 – Hyaluronic Acid ELISA, Abcam, USA). This assay principle involves the detection of hyaluronic acid utilizing Biotin-Detection Antibody and HRP-Streptavidin Conjugate Read, followed by result detection at 450 nm within 20 min using the Immunochem-2100 reader (HTI, USA).

RNA isolation, reverse transcription, and real-time PCR. Total RNA was extracted from the buffy coat using the phenol-chloroform extraction method. The RNA concentration was determined using a NanoDrop Spectrophotometer ND1000 (NanoDrop Technologies Inc., USA). Reverse transcription was performed using a RevertAid TM H Minus First Strand cDNA Synthesis Kit (Fermentas, Germany) on 6 µg of the total RNA with a random hexamer primer. The obtained single-stranded DNA was used for the PCR.

Real-time PCR detection was performed using the Applied Biosystems 7500 Real-Time PCR Systems (Applied Biosystems, USA) with the Universal PCR Master Mix (Applied Biosystems, USA) reagent mixture. The following temperature profile was used for the PCR reaction: activation of AmpliTaq Gold DNA polymerase at 50°C for 2 min, initial denaturation at 95°C for 10 min, denaturation for 15 sec, annealing at 60°C for 60 sec, extension and fluorescence detection. The last two steps were repeated for 40 cycles. The expression level of HAS2-AS1 was determined relative to the expression level of beta-actin. mRNA levels were quantitatively calculated using the delta-Ct method.

Human ACTB (Beta Actin) Endogenous Control (VIC™/MGB probe, primer limited)Green features. Catalog number: 4326315E. TaqMan Assay ID: Hs99999903_m1.

Statistical analysis. Statistical analyses were provided using licensed software Origin (https://www.originlab.com/). When two groups were present, normally distributed data was analyzed using the two-sided t-test, and skewed data was analyzed using the Mann–Whitney test. Correlations were tested using Pearson's correlation coefficient in normally distributed data and Spearman's correlation coefficient in skewed data. The data will be presented as Mean ± SEM. SEM is the standard deviation of the mean of random samples drawn from the original population.

Results

Hyaluronic acid levels in the plasma of COVID-19 patients. The level of hyaluronic acid in the plasma of patients varied significantly within a wide range, from 8.3 to 344.7 ng/ml. In the group of patients with mild symptoms, the level was 68.4 ± 44.98 ng/ml (Fig. 1). In the group of patients with moderate symptoms, the level of hyaluronic acid was 96.8 ± 51.63 ng/ml. The corresponding data were 140.4 ± 67.43 ng/ml in patients with severe symptoms. There was no statistically significant difference between the groups of patients with mild and moderate symptoms, which the absence of signs of respiratory distress in these groups can easily explain. However, the statistical difference between the mild/moderate group and the group of patients with severe symptoms was significant, P = 0.006.

lncRNA HAS2-AS1 expression. In patients with mild COVID-19 symptoms, a relatively high level of this long non-coding RNA is already observed (4.8 ± 1.77 arbitrary units). In patients with moderate severity of the disease, it increases by 7.7 times - 37.0 ± 13.27 arbitrary units (P < 0.05), and in severe cases, it increases by 22.6 times - 108.2 ± 25.28 arbitrary units (P < 0.05) (Fig. 2) compared to patients with mild symptoms. The level of expression is presented in arbitrary units. The reference gene is β-actin. Statistically significant results: *P < 0.05, **P < 0.01.

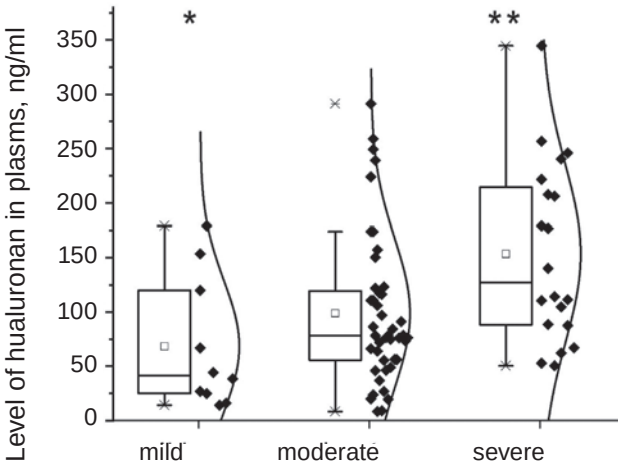

Fig. 1. Hyaluronic acid levels in the plasma of COVID-19 patients

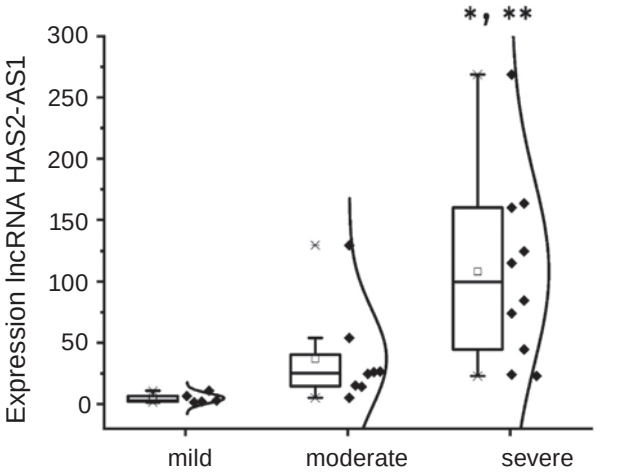

Fig. 2. HAS2-AS1 expression in COVID-19 patients' buffy coat

In the discussion, please ask the researcher to mention what are the reasons that led to a difference in hyaluronic acid between plasma and the buffy coat, which led him to point out the importance of the increase in hyaluronic acid in the buffy coat for the diagnosis of COVID-19, which is more suitable than plasma. It is also preferable to enhance its results with other studies of hyaluronic acid for Covid-19 patients.

Discussion

The obtained experimental data demonstrate the relationship between the level of hyaluronic acid in the plasma of patients even before the onset of clinical complications and the risk of developing respiratory failure. These results are consistent with other studies, such as Ding M. et al., on hyaluronic acid as an early warning indicator of adverse prognosis for critical patients with COVID-19 [14], and confirm the hypothesis that HA is a significant prognostic marker for assessing the severity of COVID-19. The level of hyaluronic acid in the plasma also reflects the course of the disease, as well as the increased level of HA in the respiratory secretion in individuals with a severe course of COVID-19 compared to healthy ones [15, 16].

Thus, in his work, Ti Yang considers hyaluronic acid as a marker of fibrosis by measuring its level on a chemiluminescent immunoassay and claims that changes in serum HA were significantly associated with clinical outcomes and suggests considering HA as a marker for patients with COVID-19 [17]. Another research group led by Yanyan Li from Beijing Ditan Hospital also measured HA in patients with COVID-19 and proposed the use of HA as a simple alternative biomarker to predict the progression of SARS-CoV-2 infection. [18].

Considering HA not only as a pathogenic factor or an indicator of disease severity but also as a promising target for COVID-19 treatment opens therapeutic intervention possibilities [19]. In the context of the association between hyaluronic acid and the development of pulmonary hypertension (PH), compelling results have been shown in studies, indicating that treatment with 4-methylumbelliferone (4MU), an inhibitor of hyaluronan synthesis that also affects mRNA expression of HA synthases, has the potential to prevent the development of PH and treat established PH, especially when associated with lung fibrosis [20]. Other researchers also confirm that under experimental conditions, both *in vitro* and *in vivo*, 4-MU suppresses the synthesis of HA and may become a new therapeutic agent against inflammation, autoimmune processes, and cancer [22-23].

Recently, Nagi and colleagues evaluated the effectiveness of 4-methylumbelliferyl glucuronide (4-MUG), a metabolite of 4-MU, which was thought to limit the utility of 4-MU for systemic inhibition of HA synthesis. Their paper demonstrated that 4-MUG can suppress HA synthesis independently of its conversion to 4-MU and without depleting the precursor of HA, UDP- GlcUA [24]. However, this molecule is not specific to any HAS isoforms, and there are significant limitations to its therapeutic use. The prolonged exposure to 4-MU has side effects,

such as inducing the development of atherosclerosis [25] and hepatocellular carcinoma [26].

Nevertheless, the obtained results clearly demonstrate that although the level of HA as a prognostic marker reflects the course of the disease, hyaluronic acid does not have high specificity and is not sufficiently indicative. The difference between the mild and moderate patient groups is practically absent, while it is twice as significant between the mild and severe groups. In contrast to hyaluronic acid, HAS2-AS1 is more informative and shows a significant difference between the groups at the pre-hospital stage, 22 times greater. This result allows HAS2-AS1 to be considered a better biomarker for predicting the course of COVID-19 than HA. Since long non-coding RNAs function in the cytoplasm and nucleus of cells, i.e. intracellularly, it is most informative, in our opinion, to determine their level in leukocytes.

The contemporary progress in studying long non-coding RNAs (lncRNAs) is difficult to overstate. In the context of COVID-19, the direct involvement of at least four lncRNAs – MALAT1, NEAT1, TUG1, and GAS5 – has already been established, which are considered potential therapeutic targets in COVID-19 [27, 28]. Furthermore, in the pathogenesis of acute respiratory distress syndrome (ARDS), compelling evidence suggests that modulation of lncRNAs NORAD, RAD51-AS1, lnrCXCR4, and other lncRNAs may reduce the expression of target cytokines, enhance anti-inflammatory immunity, and mitigate cytokine storms [29].

Conclusion. Our study has demonstrated that, unlike hyaluronic acid (HA) levels in plasma, the level of HAS2-AS1 in leukocytes serves as a more reliable prognostic criterion for severe COVID-19 and clearly distinguishes patients with subsequent mild, moderate, or severe courses already at the hospitalization stage.

Conflict of interest. The authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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ВИСОКИЙ РІВЕНЬ HAS2-AS1 В ЛЕЙКОЦИТАХ Є БІЛЬШ ІНФОРМАТИВНИМ ПРОГНОСТИЧНИМ МАРКЕРОМ КЛІНІЧНОГО ПЕРЕБІГУ COVID-19 У ПАЦІЄНТІВ ВИСОКОГО РИЗИКУ ПОРІВНЯНО З ГІАЛУРОНОВОЮ КИСЛОТОЮ В ПЛАЗМІ КРОВІ

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Багато наукових досліджень підтверджують зв’язок клінічного перебігу COVID-19 з рівнями гіалуронової кислоти та довгої некодуючої РНК HAS2-AS1, яка є позитивним регулятором синтази гіалуронової кислоти. Наша мета полягала в оцінці рівнів гіалуронової кислоти у плазмі і РНК HAS2-AS1 у лейкоцитах зі зразка крові, взятого під час прийому пацієнта в лікарню, а також у аналізі специфічності цих показників як біомаркерів важкості перебігу COVID-19. У дослідженні брали участь 78 пацієнтів із підтвердженим COVID-19, які отримували лікування у 2020-2021 роках у Київській міській клінічній лікарні № 4. Пацієнтів розділили на три групи за важкістю захворювання: легкий перебіг (n = 12), середній (n = 36) та важкий (n = 30). Рівень гіалуронової кислоти у плазмі визначали за допомогою ELISA-набору Hyaluronic Acid від Abcam (США). Лейкоцити ізолювали центрифугуванням крові з додаванням EDTA-K та подальшою аспірацією лейкоцитарної «хмаринки». Рівень HAS2-AS1 у лейкоцитах оцінювали методами зворотньої транскрипції та PCR у реальному часі. За отриманими даними рівень гіалуронової кислоти у плазмі в пацієнтів із середнім та важким перебігом був в 1,5 і 2,2 раза вище відповідно до групи з легким перебігом. У той же час рівень РНК HAS2-AS1 у лейкоцитах пацієнтів із середнім та важким перебігом збільшувався відповідно на 7,7 та 22,6 разів у порівнянні з пацієнтами із легким перебігом. Результати нашого дослідження свідчать, що, на відміну від рівня гіалуронової кислоти у плазмі, рівень

HAS2-AS1 у лейкоцитах є більш надійним прогностичним критерієм важкого перебігу COVID-19 і дозволяє відрізняти пацієнтів із різним прогностичним клінічним перебігом ще на етапі госпіталізації.

Ключові слова: гіалуронова кислота, РНК HAS2-AS1, COVID-19, плазма, лейкоцитарна плівка, лейкоцити.

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