DIAGNOSTIC AND TREATMENT METHODS

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DOI 10.25789/YMJ.2023.84.06

УДК: 616.24-004

SERUM BIOMARKERS IN DIFFERENT TYPES OF PULMONARY FIBROSIS

Idiopathic pulmonary fibrosis (IPF) is a chronic progressive interstitial lung disease of unknown origin with an average life expectancy of 3-5 years after diagnosis. The disease is accompanied by progressive pulmonary fibrosis, decreased lung function, poor response to therapy and early mortality. Various biomarkers, including serum biomarkers, are used for timely and differential diagnosis of idiopathic pulmonary fibrosis (IPF) and COVID-19-associated pulmonary fibrosis (PF), predicting the course of the disease and assessing the effectiveness of specific therapy. Target was to investigate the features of pulmonary fibrosis based on serum biomarkers in patients with ILF and COVID-19-associated fibrosis. Methods. Changes in serum concentrations of biomarkers CA15-3, LOXL2, TGFBR3 and periostin in patients with ILF (n=10), COVID-19-associated pulmonary fibrosis and controls were investigated. Results. Significant differences were found between LOXL2 concentrations in the control and ILF groups (p=0.003), ILF and COVID-19-associated fibrosis groups (p=0.036) and between periostin concentrations in the control and ILF groups (p=0.042). ROC analysis for LOXL2 revealed: in the ILF and control groups AUC=0.854 (95% CI 0.693-1.0; p<0.0001), with a sensitivity of 80.0% and specificity of 76.9%; in the ILF and COVID-19-associated LF groups AUC=0.773 (95% CI 0.556-0.989; p=0.014) with a sensitivity of 99.0% and specificity of 63.6%. For periostin: AUC=0.692 (95% CI 0.469-0.916; p=0.092) with a sensitivity of 50.0% and specificity of 84.6%. Correlation analysis in the pooled group showed a significant correlation for CA15-3 and periostin (rs=0.383; 95% CI 0.042-0.645; p=0.025), LOXL2 and periostin (rs=0.509; 95% CI 0.196-0.727; p=0.002), TGFBR3 and CA15-3 (rs=0.347; 95% CI 0.0-0.62; p=0.044). Conclusions. We found significant differences between serum levels of LOXL2 in ILF group and CG, ILF group and COVID-19-associated LF. ROC analysis yielded the values of the optimal points of group separation by serum LOXL2 and periostin levels. This allows differential diagnosis of different pulmonary fibrosis.

Keywords: pulmonary fibrosis, IPF, COVID-19, CA15-3, periostin, TGFBR3, LOXL2.

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Introduction. Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive interstitial pulmonary disease of unknown origin with an average life expectancy of 3-5 years after diagnosis set up. The disease is accompanied by the development of progressive pulmonary fibrosis (PF), decreased respiratory lung function, poor response to therapy, and premature mortality [7]. In total, there are about 3 million patients with IPF in the world [9]. The estimated primary incidence rate in Europe is from 0.009 to 0.049 / 100,000 in population, in North America - 0.075-0.093, and the overall incidence rate is 0.033-0.251 in Europe and 0.24-0.298 – in North America [8].

When exposed to damaging environmental factors (cigarette smoke, viruses, dust) or as a result of an autoimmune process in IPF, the microdamage of the alveolar epithelium is initiated. Although the triggering mechanisms of the disease may vary, progression of PF is associated with growth factors activation, changes in the concentration of cytokines and chemokines, as well as epigenetic reprogramming of fibroblasts and vascular remodeling [5].

One of the most unfavorable complications of COVID-19 is the development of PF [15], which significantly decreases the quality of life and can subsequently lead to the death. In COVID-19-induced PF, a number of serum biomarkers, predictors of its unfavorable consequences, are being under investigation [1-3].

For the timely identification of patients with PF, as well as the determination of disease phenotypes, the use of serum biomarkers seems to be very promising. Given the common pathogenetic mechanisms of fibrosis, it is suggested that biomarkers of disease may be effective in identifying both IPF and COVID-19-associated PF. As a potential PF biomarkers and response to the antifibrotic therapy SP-D (surfactant protein D), MMP-1, MMP-8, KL-6, CRPM-1, CRPM-8, C3M, C1M, 5mC, mH2A1, TOLLIP and MUC5B [1, 10, 19], as well as CA15-3, TGFBR3, LOXL2 [16], and periostin are under consideration.

The objective was to study the diagnostic value of serum biomarkers LOXL2, periostin, TGFBR3 and CA15-3 in patients with IPF and COVID-19-associated fibrosis.

Methods. In longitudinal prospective non-randomized study 34 patients were enrolled: 1st group – patients with IPF (n=10), 2nd - COVID-19-associated PF (n=11), and 3d - control (CG) (n= 13). All patients were hospitalized in the Pulmonology or Thoracic Department of Bashkir State Medical University Clinic (Ufa). The diagnosis was established on the basis of a clinical examination, laboratory and instrumental studies, including high-resolution computed tomography, spirometry and video-assisted thoracoscopic lung biopsy. As part of the clinical and instrumental examination, body mass index and functional respiration parameters

(VC, FEV_1, FEV_1/VC) were assessed. In addition, for each group the proportion of smoking patients was determined by means of smoking index, as well as the proportion of patients with various concomitant diseases.

The concentration of biomarkers in blood serum was determined by enzyme immunoassay with further determination of optical density using a photoelectrocolorimeter with a wavelength 450 nm. The following reagent kits were used: RayBio® Human LOXL2 ELISA Kit (USA), RayBio® Human TGF-beta RIII ELISA Kit (USA), CHEMA® CA15.3 (M12)-ELISA (Russia), and Aviscera Bioscience® HUMAN PERIOSTIN/OSF- 2 ELISA KIT (USA).

The study was approved by the Local Ethical Committee of Bashkir State Medical University, protocol No. 3 from 21 September, 2022. All patients signed informed consent to participate in the study.

Statistical analysis was performed by means of STATISTICA program (version 10.0). Nonparametric statistics methods were used: data were presented as median (interquartile range Q1; Q3). To compare all three groups, the Kruskal-Walli's test was used, and Wilcoxon-Mann-Whitney U test - for paired comparisons. When determining the threshold values of biomarkers concentrations to differentiate the groups, ROC analysis was utilized with sensitivity and specificity calculation. A nonparametric correlation analysis was also carried out with the calculation of the Spearman correlation coefficient. A $p<0.05$ level was considered to be statistically significant.

Results and discussion. In the Table 1 the clinical and demographic characteristics of patients in the study groups, and in Table 2 – comparative results of determining the levels of biomarkers in the blood serum of the subjects are presented. The differences between groups were determined in age (less in control, 42.0 (33.0; 51.7) years versus 56.5 (51.8; 63.4) and 59.0 (52.5; 63.7) in IPF and COVID-19 LF groups, respectively), and in IPF group, the respiratory function parameters vital capacity (VC) (63.8% (54.3; 88.9)) and force expiratory volume (FEV_1, 57.0% (48.1; 93.4)), which were less than in other groups. COVID-19 group was more likely to consists males compared to IPF and control groups (30% vs. 54.5 and 42.0%, respectively). There were no significant differences between other parameters.When comparing the concentrations of the biomarkers, the significance levels of the U-test and the Kruskal-Walli's test are presented in Table. 3. Analyzing the results using the Kruskal-Walli's test, the significant differences between all three groups for LOXL2 ($p = 0.015$) were identified. Significant differences in LOXL2 levels in pairwise comparisons of IPF and CG groups ($p=0.003$) and IPF and COVID-19-associated PF groups were also found ($p=0.036$). Differences were also found for periostin in a pairwise comparison of the IPF and CG groups ($p=0.042$).

Further, the ROC analysis was performed for groups with significantly difference in biomarkers level. When dividing the IPF versus control group according to the LOXL2 level (Fig. 1), the AUC was 0.854 (95% CI 0.693-1.0, $p<0.0001$). The optimal LOXL2 group cut-off point was 20.6 pg/ml (sensitivity 80.0% and specificity 76.9%). When comparing IPF

Table 1

Clinical and demographic characteristics of patients

Indicator		IPF	COVID-19-PF	Control
(n)		10	11	13
Age, years		56.5 (51.8; 63.4)	59.0 (52.5; 63.7)	42.0 (33.0; 51.7)
Gender, n (%)	M	3 (30.0)	6 (54.5)	4 (30.8)
	F	7 (70.0)	5 (55.5)	9 (69.2)
BMI, kg/m^2		28.6 (24.9; 29.5)	28.4 (24.6; 34.2)	28.4 (24.6; 34.2)
Smoking, n (%)	Yes	1 (10.0)	6 (54.5)	4 (30.77)
	No	9 (90.0)	5 (55.5)	9 (69.23)
VC, % estimated		63.8 (54.3; 88.9)	85.74 (66.6; 92.6)	81.1 (52.0; 87.6)
FEV1, % estimated		57.0 (48.1; 93.4)	83.2 (73.2; 91.2)	86.97 (66.3; 94.1)
FEV1/VC, % estmated		104.7 (86.9; 114.1)	105.3 (94.5; 115.1)	100.7 (87.9; 108.3)
DM 2 type, n (%)		1 (10.0)	0	0
AH, n (%)		2 (20.0)	3 (27.3)	0

P.s.: BMI – body mass index, FEV – forced expiratory volume, FEV1/TL – Tiffeneau index, DM 2 type – diabetes mellitus of 2nd type, AH – arterial hypertension.

Table 2

Biomarker levels in the study groups

Indicator	IPF	COVID-19 PF	Control
Periostin, ng/ml	10.9 (6.6; 18.3)	6.9 (5.1; 13.1)	6.9 (4.1; 9.9)
CA 15-3, U/ml	3.6 (2.7; 6.1)	4.1 (3.1; 5.5)	3.3 (1.9; 5.0)
LOXL2, pg/ml	49.9 (23.4; 84.7)	13.8 (13.1; 62.2)	15.2 (12.6; 23.4)
TGFBR3, ng/ml	389.3 (330.5; 682.4)	472.1 (291.0; 859.6)	379.6 (223.9; 675.7)

Table 3

Significance levels of the Kruskal-Wallis p-test and the Wilcoxon-Mann-Whitney U-test when comparing biomarker levels in the studied groups

Indicator	Kruskal-Walli's test	U-criteria of IPF versus control	U-кcriteria of COVID-19 PF and control	U-criteria of IPF and COVID-19 PF
LOXL2	**0.015**	**0.003**	0.955	**0.036**
Periostin	0.145	**0.042**	0.631	0.512
TGFbR3	0.638	0.557	0.303	0.756
CA15-3	0.443	0.468	0.228	0.605

group versus COVID-19-associated PF group (Fig. 2), the AUC was 0.773 (95% CI 0.556-0.989, p=0.014). The optimal point for dividing the groups by LOXL2 level was 14.0 pg/ml (99.0% and 63.6%). When splitting the IPF group versus CG based on the periostin level (Fig. 3) the AUC was 0.692 (95% CI 0.469-0.916, p=0.092) and cut-off point between groups for periostin was 11.1 ng/ml (sensitivity 50.0% and specificity 84.6%).

Spearman's rank correlation coefficients were also calculated for biomarkers levels in the combined group of subjects. A significant positive correlation was found between the concentrations of LOXL2 and periostin (rs=0.51; 95% CI 0.20-0.73, p=0.002), TGFBR3 and CA15-3 (rs=0.35; 95% CI 0. 0-0.62, p=0.044), and CA15-3 and periostin (rs=0.38; 95% CI 0.04-0.65, p=0.025).

A special feature of our study was a comparative analysis of the diagnostic value of four current biomarkers of PF - LOXL2, periostin, TGFB3, CA15-3 in two groups of patients with different fibrosis diseases - IPF and COVID-19-associated PF. Despite the relatively small number of groups, significant intergroup differences and correlations in the levels of the studied biomarkers were found. The biomarker LOXL2 showed the biggest diagnostic significance, and seems to have a prominent role in the formation of fibrotic tissue. LOXL2 (lysyl oxidase-like protein 2) cross-links collagen fibers activates fibroblasts [11], promotes the synthesis and accumulation of collagen, and strengthens the intercellular matrix. Previously, the increased expression of LOXL2 gene was detected in patients with IPF compared to the control group [12]. An increase in the level of LOXL2 was also recorded in IPF [3, 13] and in fibrosis of other localizations – liver and cardiac fibrosis [11]. Serum LOXL2 concentrations greater than 700 pg/mL have been associated with higher risks of IPF progression [3]. The results of our study, where has shown the highest levels of LOXL2 found in patients with IPF and characterized by aggressive development of PF are consistent with the data from other studies. At the same time, significantly lower levels of LOXL2 in COVID-19-associated LF probably reflect the less malignant nature of the fibrotic process in this group of patients.

In our study, periostin has also confirmed its diagnostic value as a biomarker of PF. The increase in its expression level is observed during inflammation, resulting in remodeling and fibrosis of lung tissue in IPF, chronic obstructive pulmonary disease, bronchial asthma

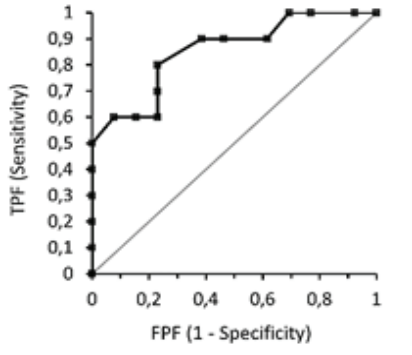

Fig. 1. ROC-analysis of LOXL2 on cut-off point between IPF and control group.

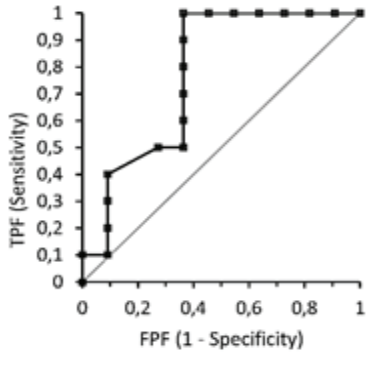

Fig. 2. ROC-analysis of LOXL2 on cut-off point between IPF and COVID-19-associated pulmonary fibrosis.

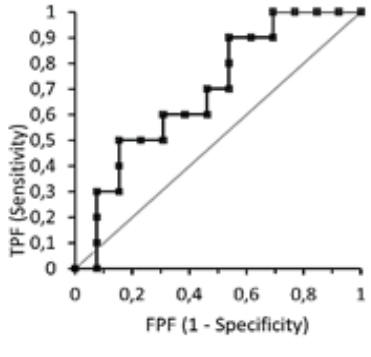

Fig. 3. ROC-analysis of periostin on cutt-off point between IPF and control group.

and lung cancer [14]. Monomeric periostin is one of the most sensitive and specific markers of IPF (AUC=0.958) [2]. At the concentration of 11.2 ng/ml, the sensitivity and specificity were 90.0% and 91.2%, respectively. In the total periostin the cut-off point was 77 ng/ml with AUC 0.843, sensitivity - 73.3% and specificity - 79.6% [2], which was superior to similar indicators of other important biomarkers of pulmonary fibrosis SP-D and KL-6 [20]. The increase in serum periostin concentrations greater than 117 μg/ml was associated with progression of IPF [3], deterioration of VC and DLCO diffusion capacity over six months [6].

Despite the fact that in our study the other two biomarkers TGFBR3 and CA15-3 did not reveal diagnostic significance, for a number of reasons they remain promising tool for diagnosing of PF and assessing other accompanying mechanism such as apoptosis. TGFBR3 is a type 3 transforming growth factor receptor TGF-β. Inhibition of TGFBR3 aggravates the development of pulmonary fibrosis [10]. Under certain conditions, TGFBR binds TGF-β [19], which leads to a decrease in the synthesis of smooth muscle actin-α (SMA-α), fibronectin, type I collagen due to inhibition of SMAD2/3, PI3K/Akt and MAPK signaling pathways TGF-β [1].

CA15-3 is a malignant antigen that is most actively expressed in breast location of cancer [4]. However, the increased concentration of CA15-3 was also observed in IPF [6]. According to d'Alessandro M. et al., the plasma concentration of CA15-3 in IPF was more than 5 times higher than in the control level [18]. In another study, estimation of CA15-3 levels enables to differentiate patients with different types of PF [17].

The discovered correlations between the studied biomarkers reflect their direct and indirect interaction as the links in the complex signaling pathways involved in PF [5]. On this basis, biomarkers of PF are considered not only as an important diagnostic and prognostic criteria, but also as the markers for highly effective targeted therapy in the future [12-14].

Conclusions. Significantly elevated levels of the biomarkers LOXL2 and periostin were found in IPF compared with both control and COVID-19-associated PF groups. The cut-off points to differentiate IPF versus COVID-19-associated LF groups, and IPF versus CG groups based on the LOXL2 level of 20.6 pg/ml and 14.0 pg/ml, respectively, were established. Similarly, for periostin, the cut-off point for IPF versus CG was 11.1 ng/ml. In the combined group of patients, the concentrations of periostin and CA15-3, periostin and LOXL2, TGFBR3 and CA15-3 were positively correlated with each other. Further studies of the plasma/serum concentrations of these biomarkers in patients with IPF and COVID-19-associated LF are needed, which in the future will increase the efficiency of diagnosis and prognosis of pulmonary fibrosis of various etiologies in order to determine the optimal treatment tactics.

The study was carried out with the support of a grant of Russian Science Foundation, agreement No. 22-25-00019 from 16 December 16, 2021.

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