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ARAŞTIRMA / RESEARCH

Relationship between disease severity and prognostic indicators and matrix metallaproteinase in patients with stable idiopatic pulmonary fibrosis

Stabil idiyopatik pulmoner fibrozde hastalik şiddeti ve prognostik belirteçler ile matriks metallaproteinaz düzeyleri arasindaki ilişki

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Abstract

Purpose: Blood-derived biomarkers have been extensively considered as possible prognostic indicators in idiopathic pulmonary fibrosis (IPF) recently. In order to assess the value of circulating biomarkers in common IPF clinical practice, the study intends to draw conclusions regarding the link between disease severity, prognostic indicators, and serum matrix metalloproteinase in patients with stable idiopathic pulmonary fibrosis.

Materials and Methods: The study comprised 22 people with an IPF diagnosis that had been verified by a multidisciplinary approach. The sociodemographic details, clinical and radiologic symptoms, pulmonary function tests and the Gender-Age-Physiology (GAP) score were noted. ELISA has been used to research serum MMP concentrations.

Results: There is no statistically significant correlation between the Matrix Metalloproteinase (MMP) 2, MMP 7, MMP 9, and MMP13 and the GAP index and, pulmonary function tests, or disease severity. GAP score was found to be higher in stage 3 in patients with severe disease, in stage 2 in patients with moderate disease, and in stage 1 in patients with mild disease.

Conclusion: There are consistent findings in the literature, despite the fact that the association between MMP and IPF prognostic markers, pulmonary function tests, and disease severity could not be seen in this investigation. However, because they could open the door to a cutting-edge treatment strategy, these indicators should be investigated prospectively in larger series.

Keywords: Idiopathic pulmonary fibrosis, matrix metalloproteianses, GAP, severity, biomarkers

Öz

Amaç: Biyobelirteçler, son zamanlarda idiyopatik pulmoner fibrozis (İPF) olası tanısında prognostik göstergeler olarak kapsamlı bir şekilde değerlendirilmektedir. Yaygın IPF klinik uygulamasında dolaşımdaki biyobelirteçlerin değerini belirleyebilmek için bu çalışma, stabil idiyopatik pulmoner fibrozlu hastalarda hastalık şiddeti, prognostik göstergeler ve serum matriks metalloproteinaz arasındaki bağlantıya ilişkin sonuçlar çıkarmayı amaçlamaktadır.

Gereç ve Yöntem: Çalışma, multidisipliner bir yaklaşımla doğrulanmış İPF tanısı olan 22 kişiden oluşuyordu. Sosyodemografik detaylar, klinik ve radyolojik semptomlar, solunum fonksiyon testleri ve GAP skoru kaydedildi. Serum MMP konsantrasyonları ELISA yöntemiyle ölçülmüştür.

Bulgular: Matriks Metalloproteinaz (MMP) 2, MMP 7, MMP 9 ve MMP13 ile GAP indeksi ve solunum fonksiyon testleri veya hastalık şiddeti arasında istatistiksel olarak anlamlı bir ilişki yoktur. GAP skoru ağır hastalığı olanlarda evre 3'te, orta hastalığı olanlarda evre 2'de, hafif hastalığı olanlarda evre 1'de daha yüksek bulundu.

Sonuç: MMP ile İPF prognostik belirteçleri, solunum fonksiyon testleri ve hastalık şiddeti arasındaki ilişki bu incelemede görülmemesine rağmen literatürde tutarlı bulgular mevcuttur. Ancak, son teknoloji bir tedavi stratejisine kapı açabilecekleri için, bu göstergeler daha geniş serilerde ileriye dönük olarak araştırılmalıdır.

Anahtar kelimeler: İdiopatik pulmoner fibrozis, matrix metalloproteinaz, gap, ciddiyet, biyomarker

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INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) disease is a long illness marked by the thickening and stiffening of lung tissue associated with scar tissue formation. In this condition, the sponge or meaty section of the lung becomes scarred or fibrotic. It is a slow-progressing, highly fatal disease that affects roughly 80% of people within 3–5 years of diagnosis[1]. Idiopathic pulmonary fibrosis (IPF) is the severest form of idiopathic interstitial pneumonia, with a poor prognosis that occurs mainly in elderly adults, suggesting a strong link between the fibrosis process and aging. Chest high-resolution computed tomography (HRCT) is currently considered the main standard diagnostic in all ILD assessments, not only for baseline evaluation, but also for monitoring the disease and prediction of treatment efficacy[2]. HRCT is necessary for the follow-up, usually performed yearly, and in case of deterioration of respiratory function. The disadvantages to its realization are the repeated irradiation, the cost, the accessibility, and sometimes the difficulties of realization related to the supine position. It is worthy of notice to be aware of the radioprotection issue, since the cumulative dose is 7 mSv per examination, which corresponds with 2 years of natural light exposure[3].It is precisely for this reason that the search for new markers to be used or supported in the diagnosis and follow-up of IPF has recently been the focus of attention.

Although the underlying mechanisms linking aging with IPF are not fully understood, it has been hypothesized that IPF patients may have an accelerated process of lung aging, characterized by increased genomic instability, abnormal shortening of telomeres, epithelial cell senescence, mitochondrial dysfunction, and loss of proteostasis, among others[4,5]. Excessive mediators and some processes have been blamed in the pathogenesis. Among the mediators, several matrix metalloproteases (MMPs) which may contribute to modify the lung microenvironment by various mechanisms have specific implications. Thus, these enzymes can not only degrade all the components of the extracellular matrix, but they are also able to release, cleave and activate a wide range of growth factors, cytokines, chemokines and cell surface receptors affecting numerous cell functions including adhesion, proliferation, differentiation, recruiting and transmigration, and apoptosis. Since IPF is a collagenase that degrades the matrix, it makes sense that the amount of various matrix metalloproteinases could be downregulated. However, certain matrix metalloproteinases (MMPs) have a pro-fibrotic role, whilst others play an anti-fibrotic role. These profibrotic MMPs successfully encourage the epithelial to mesenchymal transition, which in turn promotes the growth of fibrosis. These profibrotic groups also cause fibrocyte migration and macrophage polarization. The equilibrium between profibrotic and antifibrotic mediators is ultimately upset by all of these events, leading to an abnormal healing process[6]. Therefore, dysregulated expression of MMPs may have profound impact on the biopathological mechanisms implicated in the development of IPF. There are very contradictory and limited data on the markers studied in serum in IPF. In idiopathic pulmonary fibrosis (IPF), blood-derived biomarkers have been extensively discussed as potential prognostic markers; however, studies have been constrained by analyses using data-dependent thresholds, inconsistent adjustment for confounders, and a variety of endpoints, frequently producing ungeneralizable results.

In the light of the growing evidence, the study aims to conclude about the relationship between disease severity and prognostic markers and serum matrix metalloproteinases in patients with stable idiopathic pulmonary fibrosis in order to evaluate the usefulness of circulating biomarkers in routine IPF clinical practice.

MATERIALS AND METHODS

Study population

Twenty-two individuals with a confirmed diagnosis of IPF by a multidisciplinary perspective were included in the study between 1.1.2020 and 1.5.2020 sequentially in Cukurova University Chest Diseases Department. After each patient signed the informed consent form, they were all enrolled in the research. The Cukurova University Non-Interventional Ethics Committee (96/2020) gave its approval for this cross-sectional study. All procedures performed in the study involving human participants were in accordance with the ethical standards of the hospital, national research committee and the 1964 Helsinki declaration.

At the time of the visit, included patients completed a clinical evaluation that comprised a thoracic ultrasound, pulmonary functional tests, and mMRC

and GAP scores. The most recent thorax CT conducted within three months of enrollment as well as those completed while the patient was enrolled were assessed. A whole blood sample of 10 cc was collected from each patient.

In the study, it was attempted to match the sample size (n:13) to the prevalence of the condition. Despite the disease's low incidence and the impact of the pandemic, the objective was nevertheless met. Patients who were experiencing an IPF episode and all patients who declined to take part in the trial were sequentially excluded in the study. The trial excluded patients who had symptoms of aggravation in the previous 4 weeks. All IPF patients who admitted to Cukurva University Department of Chest Diseases with a definite diagnosis of IPF enrolled sequentially.

Procedure

Thorax High Resolution Computerized Tomography (HRCT)

Thoracic HRCT scans were evaluated in all patients in the last three months prior to participation in the radiological evaluation. Radiological images were evaluated and graded by a single specialist physician in Cukurova University Faculty of Medicine, Department of Radiology. In the computed tomography that will be taken with contrast in the early arterial phase, the section thickness is 1 mm.Images were acquired with the patient in the supine position with full inspiration covering the entire chest area. Additional sections were made in the prone decubitus position to exclude changes due to gravity. Intravenous contrast agent was not administered. In the computed tomography that will be taken with contrast in the early arterial phase, the section thickness will be 1 mm. Major HRCT images have been described in international standard terminology defined by the Fleischner Society dictionary and in the peer-reviewed literature on viral pneumonia using terms such as ground glass opasities (GGO), crazy-paving pattern, and consolidation[7].Image analysis was evaluated by expert radiologists in our institution using the institutional digital database system (HBYS Mergentech PACS, version v3.22.03.1-20220314).

Fibrotic changes were scored using a semi-quantative technique. An HRCT fibrotic index was obtained by counting the presence and extension of reticulation and honeycomb for each lobe[8]:

0—no reticulation,
1—reticulation without honeycombing,
2—septal reticulation with honeycomb in <25% of a lobe
3— septal reticulation with honeycomb in 2-49% of a lobe
4— septal reticulation with honeycomb in 50-75% of one lobe
5— septal reticulation with honeycomb in >75% of one lobe
Radiological involvements are divided into three;
Mild: scores ≤6; Medium: scores 7-13; Severe: scores ≥14

Gender-Age-Physiology (GAP) model

The most widely validated clinical prediction model is the GAP model, which incorporates age, sex, FVC, and DLCO into a simple score-score index and staging system that predicts one-, two-, and three-year mortality[9]. The severity of the disease was determined according to this model.

Pulmonary Function Tests (PFTs)

PFTs were performed with a calibrated Sensor Medics V-Max 20 Spirometer (Jaeger MS-PFT Analyzer Unit, Wiasys Healthcare GmbH, Höchberg, Germany) in accordance with the ATS guideline in Cukurova University Department of Chest Diseases. Basal forced expiratory volume for 1 second (FEV1) and forced vital capacity (FVC) were measured 3 times and the best values were recorded.

Total lung capacity was measured with the helium dilution technique (Jaeger MS-PFT Analyzer Unit) and Transfer Factor for Carbon Monoxide (TLCO) was measured with the single breath method. It was measured with a single breath technique in which 10% helium and 0.3% carbon monoxide were rapidly inhaled, held for 10 seconds, and then exhaled by measuring the remaining carbon monoxide[10]. Test results are presented as a percentage of predicted values. The results of pulmonary function tests were interpreted according to the ATS/ERS recommendations[11].

Laboratory tests

Serum MMP Level Measurement

In the study, serum MMP values of patients presenting with stable IPF will be studied with Enzyme-Linked ImmunoSorbent Assay (ELISA), Fine Test, China in Cukurova University, CUMERlab. The samples taken from the patients will be kept at room temperature for 60 minutes and the

blood cells will be expected to collapse. In the follow-up, the remaining serum will be taken with the help of a pipette and transferred to 1.5mL Eppendorf tubes and stored at -80 degrees until the day of the study.

Determination of total protein by BCA method

The amount of protein obtained will be determined by the temperature-dependent color change principle and the BCA (Bicinchoninicacidassay) method, which is used to determine the total protein amount. BCA solution kit will be used in the experiment. Standards of 2000ug, 1000ug, 500ug, 250ug, 125ug, 62.5ug, 31.25ug and 0ug were prepared with BSA (bovine serum albumin). After taking 5 μL of the proteins in Eppendorf and diluting them with 1/5 distilled water, both standards and proteins will be transferred to 96-well cell culture dishes as 10 microliters and in pairs. After adding 200uL of BCA working solution, the plates will be shaken in the plate shaker, and then incubated at 37°C for 30 minutes and read at 562nM wavelength in the plate reader.

ELISA experiments

The serum obtained and stored at -86 will be thawed and studied simultaneously. Experiments will be carried out in accordance with the company protocol of elisa kits designed for MMP subtypes. BD FACS calibur branded 4-channel flow cytometry device will be used for the experiments.

Research infrastructure

Flow **cytometry** experiments in the project, as well as Eliza and Western Blot experiments will be carried out in the Pulmonary Diseases Molecular Research Laboratory located in CUMERLAB. In the laboratory, there are plate reader spectrophotometer, western tanks and electrophoresis systems, centrifuge devices for centrifugation and storage of samples, BD Facscalibure brand 4-channel flow cytometry device and -20 and -86 deep freezers for Eliza.

Statistical analysis

SPSS 22 program was used in the analysis of the data. The minimum required sample size was calculated as 13 based on literature with a precision of 5% and 95% confidence. Descriptive statistics for continuous variables are given as mean±standard deviation while for categorical variables were given as frequency and percentages. Shapiro Wilk test was used as the normal distribution test. Spearman correlation analysis was used in the analysis of quantitative data that did not fit the normal distribution, the Kruskal Wallis test was used in the comparison of 3 or more groups that did not fit the normal distribution, and the Pearson Chi-square test was used in the comparison of categorical data. A value of $p<0.05$ was considered statistically significant.

Table 1. Sociodemographic characteristics of the patients

Gender	**n**	**%**
Male	21	95.5
Female	1	4.5
HRCT patern		
UIP	15	68.2
Probable	4	18.2
Indeterminate	3	13.6
Diagnositc method		
Upon HRCT* patern	17	77.3
Histopathologic	5	22.7
Cigarette		
Never	5	22.7
Ex-smoker	16	72.7
Smoker	1	4.5
Treatment regimen		
None	5	22.7
Pirfenidone	13	59.1
Nintedanib	4	18.2
Total	22	100.0

*HRCT: high reolution computerized tomography.

RESULTS

The mean age of 22 IPF patients included in our study was 68.95±7.58 (min:58-max:81). Sociodemographic characteristics of the patients are given in Table 1..

When the correlations between GAP index and Matrix metalloproteinases (MMP), MMP 2, MMP 7, MMP 9 and MMP13 were examined, it was found that there was no statistically significant correlation. A moderately strong negative correlation was found between MMP 2 and MMP 13 (Table 2)

When the correlations between pulmonary function tests (PFTs) and Matrix metalloproteinases (MMP), MMP 2, MMP 7, MMP 9 and MMP13 were examined, it was found that there was no statistically significant correlation (Table 3).

When the relationship between disease severity (according to FVC) and GAP stage was examined, it was found that there was a statistically significant difference; it was found to be higher in stage 3 in patients with severe disease, in stage 2 in patients with moderate disease, and in stage 1 in patients with mild disease (Table 4). When MMP levels were compared according to disease severity, it was found that there was no statistically significant difference between the groups (Table 5)

Table 2. Correlations between GAP index and Matrix metalloproteinases

		GAP index	**MMP 2**	**MMP 7**	**MMP 9**	**MMP 13**
GAP* index	r	1.000	0.363	-0.184	0.256	-0.150
	p	.	0.097	0.412	0.262	0.506
MMP** 2	r		1.000	0.363	0.075	-0.617
	p		.	0.097	0.748	**0.002**
MMP 7	r			1.000	0.025	-0.119
	p			.	0.913	0.597
MMP 9	r				1.000	0.401
	p				.	0.072
MMP 13	r					1.000
	p					.

*GAP: Gender-Age-Physiology, **MMP: matrix metallo proteinase

Table 3. Correlations between Pulmonary function tests and MMP levels

		FEVL	**FEV1%**	**FEV1/FVC**	**FVCL**	**FVC%**	**MMP 2**	**MMP 7**	**MMP 9**	**MMP13**
FEV1(L)	r	1.000	0.627	-0.156	0.913	0.564	-0.204	0.033	-0.034	0.075
	p	.	0.002	0.488	<0.001	0.006	0.361	0.883	0.882	0.740
FEV1(%)	r		1.000	-0.182	0.571	0.901	-0.242	0.165	-0.290	-0.045
	p		.	0.419	0.006	0.000	0.278	0.464	0.201	0.841
FEV1/FVC	r			1.000	-0.473	-0.506	0.020	0.142	0.225	-0.077
	p			.	0.026	0.016	0.930	0.529	0.327	0.732
FVC(L)	r				1.000	0.648	-0.213	-0.028	-0.153	0.051
	p				.	0.001	0.342	0.903	0.509	0.820
FVC(%)	r					1.000	-0.118	0.133	-0.350	-0.127
	p					.	0.602	0.556	0.120	0.573
MMP2	r						1.000	0.363	0.075	-0.617
	p						.	0.097	0.748	0.002
MMP7	r							1.000	0.025	-0.119
	p							.	0.913	0.597
MMP9	r								1.000	0.401
	p								.	0.072
MMP13	r									1.000
	p									.

*FEV1:Forced expiratory in the first second, **FVC:Forced vital capacity, ***MMP: matrix metallo proteinase, Spearman correlation analysis

Table 4. The relationship between disease severity and Gender Age Physiology index

			GAP* stage			p
			1	2	3	
Severity of the disease according to PFT**s	Severe	n	0[a]	1[a, b]	1[b]	0.022
		Column %	0.0	10.0	50.0	
	Moderate	n	0[a]	4[b]	0[a, b]	
		Column %	0.0	40.0	0.0	
	Mild	n	10[a]	5[b]	1[b]	
		Column %	100.0	50.0	50.0	

Symbols a and b indicate the statistical difference between cells; *GAP: Gender-Age-Physiology ; **Pulmonary function test; Pearson Chi-square

Table 5. Comparison of MMP levels according to disease severity

	Severity of the disease according to pulmonary function tests			
	Severe	Moderate	Mild	p
	X̄±S.D.	X̄±S.D.	X̄±S.D.	
MMP 2	2.44±0.25	2.35±0.16	2.18±0.56	0.501
MMP 7	1.34±0.78	0.96±0.33	1.41±1.25	0.645
MMP 9	13.89±8.65	11.45±2.82	10.79±3.88	0.714
MMP 13	0.31±0.09	0.34±0.05	0.38±0.15	0.801

***MMP: matrix metallo proteinase; Kruskal Wallis Test

DISCUSSION

IPF is an epithelial-driven illness that has recently gained attention. When type II alveolar epithelial cells are microinjured in healthy lungs, the body may heal the cells. TGF-b, PDGF, TNF-a, angiotensin, MMPs, and several chemokines are secreted in large quantities when lung microinjuries persist due to the activation of alveolar epithelial cells by aberrant wound healing. These mediators encourage fibroblast proliferation, migration, and differentiation into myofibroblasts, which are immune to cell death and release collagens and other elements of the extracellular matrix (ECM). MMPs have been linked to the breakdown of ECM, which is controlled by a delicate balance between MMPs and tissue inhibitors of MMPs (TIMPs) according to earlier research[12-18]. There are very few studies comparing the amounts of these markers in relation to the severity of the illness, despite the fact that MMPs are present in IPF patients at various levels. What is basically underlined in this study is that there is no significant relationship between serum MMP levels and disease severity and pulmonary function tests in stable IPF cases.

There are several MMP subtypes that have been found and are reportedly effective in IPF. MMP-2, MMP-7, MMP-9, and MMP-13 stood out among them. Lung fibroblasts, endothelial cells, and alveolar epithelial cells all express MMP-2. Chemokines and growth factors are under control of it, and it also promotes extracellular matrix via regulating the Wnt/b-catenin signal network[19]. MMP-7, which is expressed in fibroblasts and lung epithelial cells, is the most extensively studied MMP in IPF. MMP-7 has a broad affinity for ECM components and is capable of degrading a variety of proteins. In the early and middle phases of pulmonary fibrosis, MMP-7 contributes to the occurrence and progression of fibrosis[20]. Patients with IPF have higher MMP-9 levels in their broncho alveolar lavage fluid, which is broadly expressed in all leukocytes, fibroblasts, epithelial cells, and endothelial cells. It is almost certain that blocking MMP-9 will prevent macrophage-induced fibroblast migration[21,22]. Alveolar and bronchiolar epithelial cells express MMP-13. MMP-13 is markedly elevated in pulmonary fibrosis and regulates ECM deposition in lung fibrosis. It is the first article analyzing several MMP subtypes in IPF, to the best of our knowledge and according to the literature search.

In a cohort study with 28 IPF patients, surfactant protein-D, MMP-7 and Krebs von den Lungen-6 have been shown to be correlated with pulmonary function tests and a supporter in clinical practice of IPF[23]. A recent systematic review including nine studies and a large number of participants indicated higher basal MMP-7 level as a poor prognostic factor for disease progression and mortality[24]. Another study revealed that MMP-7 concentrations could be used to accurately predict outcomes[25].

bronchoalveolar lavage fluid MMP-7 were positively correlated with HRCT interstitial scores in IPF[26].

Serum MMP-7 and bronchoalveolar lavage fluid MMP-2 and MMP-9 levels potentially has been shown to be useful diagnostic markers but not prognostic markers in a dog IPF model[27]. Some studies blamed MMP-12 as a potential biomarker in asthma, COPD and pulmonary fibrosis[28]. With scarce data in literature, several pathophysiological aspects still await elucidation.

IPF has developed into a category of disorders where positive outcomes are attained and the anticipated survival is increased with current anti-fibrotic therapies. In addition to these advancements, it has been hypothesized that biomarkers may influence the pathogenesis of the illness, and anti-MMP therapies have even been tested. The use of biomarkers in diagnosis or therapy, as well as their predictive capabilities, are still largely unknown. Serum MMP levels were examined in this study in various clinical manifestations of IPF in an effort to identify whether they would be a feasible indicator of severity. There was no connection between serum MMP levels and disease severity and prognosis scores.

Despite the fact that there was no link between IPF and MMP in the study that was presented, it must be remembered that it was a cross-sectional, single-center study with scant patient data. By following the recommendations' standards, invasive procedures like taking bronchoalveolar lavage samples were avoided, and only the patients' blood levels were measured. These samples were evaluated in the same lab at the same time. It would be useful to show how the levels of these samples have changed over the disease's follow-up. The specific function that MMPs play in the onset, progression, prognosis, and mortality of IPF is unclear, as far as we are aware. These markers should be looked into prospectively in bigger series, though, since they could lead to a ground-breaking therapeutic approach. Further research is required to confirm early results about the most efficient diagnostic and prognostic signs that may soon be incorporated into customary IPF clinical practice.

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