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RESEARCH ARTICLE

Immunological Evaluation of TGF-β and Osteopontin of Idiopathic Pulmonary Fibrotic patients in Al-Diwaniyah province

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ARTICLE INFO	ABSTRACT
  *Corresponding Author: Maisam.hilal@gmail.com	Background: Idiopathic pulmonary fibrosis (IPF) is a hetero-geneous sickness considered via an perversely stimulated immunity system. Present study aimed to evaluate of TGF-β and Osteopontin of IPF sick in Al-Diwaniyah province. Methods: the existing training stayed a case-control training comprised 70 sick who were diagnosed (Clinically, and X-ray) with IPF and 70 healthy control groups. The samples collected from individuals visited Al-Diwaniya teaching hospital in Al-Diwaniyah city. ELISA was used in this study for quantitative determination of Osteopontin and TGF-β in serum samples of participants and done it according to company instruction (BT-LAB). Results: The ELISA test showed important rise in concentration mean of TGF-β and Osteopontin in the sick (751.3 ± 118 ng/ml and 1930.5 respectively) compared to the control (548.6 ± 217 ng/ml and 906.26 ± 252 pg/ml respectively) (P< 0.05). The concentration of TGF-β increased in females (999.38 ± 120 ng/ml) while concentration mean of osteopontin increased in males (2125.20 ± 882 pg/ml). Moreover, the concentration mean of TGF-β and osteopontin increased in elderly patients in age group 4 (759.44 ± 107 ng/ml and 2256.12 ± 759 pg/ml respectively).We found a higher mean of Osteopontin concentration in smoking patients (3636.93± 884 0g/ml) compared to non-smokers (1638.31 ± 638 pg/ml), in contrast high serum level of TGF-β determine in non- smokers (568.5 ± 111 ng/ml) compared to smokers (433.74± 127 pg/ml) but without significant association (P>0.05). In conclusion: TGF-β and Osteopontin are clearly associated with IPF and their concentrations may be affected by age and gender. Therefore, these indicators can be relied upon as markers in the analysis of IPF.

INTRODUCTION

Idiopathic pulmonary fibrosis is a long-lasting, developing lung disorder noticeable via ongoing fibrosis in the lungs and histological traits typical of normal interstitial pneumonia. [1]. Symptoms such as heightened coughing and dyspnea contribute to a diminished quality of life. On average, sick diagnosed with IPF have a median persistence of 3–5 years. [1,2]. The immune system is garnering more attention in the context of interstitial lung diseases, as our understanding of its involvement in fibrosis development and response to treatments continues to grow. [3]. Dysregulated immune responses and imbalanced processes of injury, inflammation, and repair are key factors in initiating and advancing idiopathic pulmonary fibrosis. The guiding arm of the immunity system productions vital parts in managing harmful immune reactions, regulating inflammation, and influencing the shift

from inflammation to fibrosis. [4]. It's believed that persistent alveolar-micro injuries result in a sustained and disordered wound healing process, contributing to the progression of IPF. [5]. Fibrogenesis is characterized via a significant buildup of extracellular matrix (ECM) generated via myofibroblasts, which includes substances like collagen, laminin,elastin, fibronectin, hyaluronan, and glycoproteins. This accumulation leads to the irreparable congealing of alveolar walls, impairing the altercation of oxygen and carbon dioxide among the bloodstream and alveolar air. [6,7].

Cytokines and growth features that stimulate fibroblasts and myofibroblasts, leading to additional remodeling of fibrotic tissue, encompass Transforming Growth Factor-β (TGF-β), interleukins for instance IL-1, IL-13, IL-6, and IL-33, platelet-derived growth factor, tumor necrosis factor (TNF)-α, fibroblast growth feature, and leukotrienes. [8]. Once stimulated, fibroblasts generate TGF-β, IL-1β, IL-33, reactive oxygen species, C-X-C motif chemokines (CXC), and C-C motif chemokines (CC). These substances sustain fibrogenesis and attract immune cells, fostering long-lasting inflammation. This sets off a beneficial cycle that bolsters fibrogenesis by prompting the transformation of fibroblasts into myofibroblasts. [9]. TGF-β productions a pivotal character in the progress of pulmonary fibrosis via encouraging the transformation of fibroblasts into myofibroblasts, which then generate an excessive amount of extracellular matrix. This process chiefs to a failure in lung occupation. [7]. TGF-β stands out as a highly influential stimulator of extracellular matrix production, encompassing collagen and various further matrix proteins. Its expression is heightened in both animal models of lung fibrosis and in human lungs affected by fibrosis. In these animal types, the rise in TGF-β appearance occurs prior to the synthesis and deposition of collagen. [8].

Osteopontin (OPN) is a glycophosphoprotein with high levels of phosphorylation, derived from the concealed phosphoprotein 1 gene. [10]. Osteopontin is widely present in numerous tissues, including the kidney, bone marrow, brain, and lung. [11]. Osteopontin is a solvable extracellular matrix (ECM) particle found abundantly in various tissues, including the kidney, , bone marrow, brain and lung. [12]. Physiologically, it is thought that OPN appearance is elevated in utmost tissues and body liquids complicated in wound curing and altering. [13, 46]. Furthermore, OPN appearance is identified in numerous pathological environments because of its role in changeable cell migration and diversity. [14]. As an illustration, both hepatic s and erum OPN ranks are elevated in individuals with alcohol related liver sickness and non-alcoholic fatty liver sickness. [11]. Concerning lung sickness, OPN is discovered to enable the development of lung tumor via promoting epithelial-to-mesenchymal transitions and cell proliferation. [15, 45].

Regrettably, there is a lack of comprehensive studies on the immune mechanism of lung fibrosis globally. Many immune indicators, whether cellular or mediators, remain inadequately researched. [13-15]. In Iraq, particularly in Diwaniyah province, there is a dearth of studies examining the part of TGF-β and osteopontin in patients with IPF. This prompted the initiation of our current research on this subject..

Patients and samples collection

Study Strategy and Blood Sample Collection: the existing training was a case-control study comprised 70 patients who were diagnosed (Clinically, and X-ray) with IPF, in addition, 70 healthy control groups who had no times past or medical indication of PF or asthma. The patients were seen to Al-Diwaniyah teaching hospital in Al-Diwaniyah city from the dated of January, 2023 until the end of November 2023. All information about recovery patients and control group was noted in questionnaire forma during direct meeting with patients and control individuals, the questionnaire forma, which contain name, age, gender, and smoking or not. Three ml of blood placed in a plane tube which left to clotted at room temperature (20- 25 ºC) for 30 minutes, at 2500 rpm centrifuged for 10 minutes, and formerly separated the serum into four parts in a ependroff tubes, stored at -20ºC for immunological assays.

Immunological assays: ELISA was used in this study for quantitative determination of (Osteopontin and TGF-β) in patient and healthy control serum samples and done according to company instruction (BT-LAB). The optical density (OD rate) stayed measured by means of a microplate reader fixed to 450 nm within 10 minutes after the addition of the stop solution. ELISA results were calculated based on the optical density readings for both standards and samples. Subsequently, a standard curve was generated via scheming the mean OD value for every standard on the X-axis alongside the concentration on the Y-axis, and a best-fit curve stayed drawn concluded the points on the graph.

Ethical Approval: The study stayed conducted following the approval of both the patient and the Iraqi Ministry of Health.

Statistical analysis: The statistical test stood prepared by means of the Statistical Set for the Social Sciences, style 22, with Microsoft Excel 2010. The outcomes stood measured statistically different while the probability (P value) was less than 0.05 [16].

RESULTS

The ages of the patients extended from 30 to 90 years, with an mean age of 59.5 ± 17.92 years, and the mutual of them stayed female (44%), as most of these females were within the age grouping of 30 – 45 years, as in Table (1 and 2). Moreover, we found the majority of sick in the third age grouping (60-75 years), at a rate of 36%. On the other hand, the average age of healthy people was 51.7 ± 13.41 years, and the percentage of females was also more than that of males (42%) as shown in table (3). Statistically, we did not invention important variances (P >0.05) when comparing the genders and ages of patients with the control.

To conclude the character of smoking in the occurrence and progress of the disease, we conducted a questionnaire about the percentage of smoking among patients. We found that the number of smokers were 7 individuals (10%) out of 70 patients (P = 0.002), as in Figure (1).

Table (1): The case-control alteration in mean age.

Age (years)	Healthy controlling	Cases	*P* value
Range	31- 88	30 – 90	
Mean ± SD	51.7 ± 13.41	59.5 ± 17.92	0.114 [NS]
SE	1.60	2.14	
N	70	70	

Table (2) : Dissemination of sick with IPF over age grouping and sex

Age groups	Age range	Men	Women	N (%)	*P* value
Group 1	**30 -45**	1	13	14 (20)	0.001 [S]
Group 2	**45- 60**	1	10	11 (16)	0.046 [S]
Group 3	**60-75**	16	8	24 (34)	0.042 [S]
Group 4	**≥ 75**	8	13	21 (30)	0.057 [NS]
Total	**30 – 90**	26	44	70 (100)	0.039 [S]

Table (3) : The case-control alteration in sex dissemination.

Gender	Healthy controlling		Cases		P value
	N.	%	N.	%	
Female	42	60	44	63	0.537 [NS]
Male	28	40	26	37	0.536 [NS]
Total	70	100	70	100	

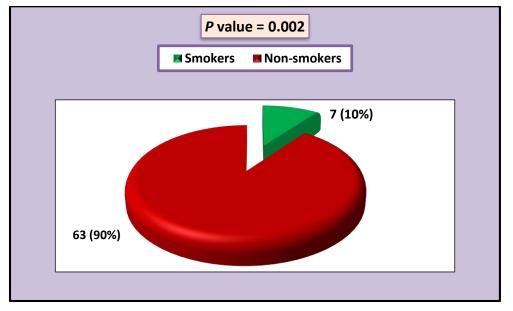*** NS= No Significant Association (*P*>0.05)**

Figure (1): Smoking rate among sick with idiopathic pulmonary fibrosis

The outcomes of the ELISA test showed significant increase in concentration mean of TGF-β and Osteopontin in the sick serum (751.3 ± 118 ng/ml and 1930.5 respectively) compared to the control (548.6 ± 217 ng/ml and 906.26 ± 252 pg/ml respectively)(P< 0.05), as in Table (4). When distributing the concentration of these indicators according to the genders of the patients, we found that the concentration of TGF-β increased in females (999.38 ± 120 ng/ml) while concentration mean of Osteopontin increased in males (2125.20 ± 882 pg/ml), as in the table (5). On the additional hand, we found that the mean absorption of TGF-β and Osteopontin increased in elderly sick in age group 4 (759.44 ± 107 ng/ml and 2256.12 ± 759 pg/ml respectively) as in Table (6).

In Table (7) we found a higher mean of Osteopontin concentration in smoking patients (3636.93± 884 0g/ml) compared to non-smokers (1638.31 ± 638 pg/ml), in contrast high serum level of TGF-β determine in non- smokers (568.5 ± 111 ng/ml) compared to smokers (433.74± 127 pg/ml) without important relationship (P>0.05).

Table (4): Appraisal of some immune indicators between patients and controls

Immune markers	Cases	Control	T test	95 %CI	P value
	Mean ± SD	Mean ± SD			
TGF-β (ng/ml)	751.3 ± 118	548.6 ± 217	6.94	145.339 to 261.261	0.038 [S]
Osteopontin (pg/ml)	1930.5 ± 750	906.26 ± 252	10.94	847.16 to 1220.84	0.009 [S]

Table (5): Distribution studied immunologic markers according to patients' gender

Immune markers	Females	Males	T test	95 %CI	P value
	Mean ± SD	Mean ± SD			
TGF-β (ng/ml)	999.38 ± 120	467.28 ± 95.4	29.09	495.83 to 568.17	0.013 [S]
Osteopontin (pg/ml)	1825.71± 699	2125.20 ± 882	3.562	-466.50 to -133.50	0.019 [S]

Table (6): Distribution of TGF-β and Osteopontin according to patients' age groups

Immune markers	Grouping 1	Grouping 2	Grouping 3	Grouping 4	P value
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	
TGF-β (ng/ml)	602.29 ± 125	515.91± 89	508.19 ± 120	759.44 ± 107	0.037 [S]
Osteopontin (pg/ml)	1798.55 ±688	1611.34± 705	1929.04 ± 812	2256.12 ± 759	0.023 [S]

Table (7): Distribution studied immunologic markers according to cigarettes smoking

Immune markers	Smokers	Non-smokers	T test	95 %CI	P value
	Mean ± SD	Mean ± SD			
TGF-β (ng/ml)	433.74± 127	568.5 ± 111	6.685	-174.623 to -94.897	0.021 [S]
Osteopontin (pg/ml)	3636.93± 884	1638.31 ± 638	15.133	1737.47 to 2259.77	0.005 [S]

DISCUSSION

In our results, we found that IPF increases with age (over 60 years) especially in males while it appears less commonly under 60 years especially in females. However this result agreed with number of results of pervious studies [17,18]. According to Leuschner *et al.* study, Out of the total 1,009 patients, 350 (34.7%) were aged 75 years or older. Compared to younger patients, elderly IPF patients exhibited a greater number of co-morbidities (3.6 ± 2.5 vs. 2.8 ± 2.3; p < 0.001). [18]. The reason of increasing IPF with age discussed by Raghu *et al.*, (2018) who showed that IPF predominantly affects individuals aged over 60 years, attributed to biological changes that render aging lungs more susceptible to the disease. [19]. Lately, genetic changes have been pinpointed that correlate with a heightened risk of developing IPF. However, these alterations may also elevate the likelihood of other age-related lung conditions like long-lasting obstructive pulmonary disease (COPD) or lung tumor [20]. While the precise link among aging and the onset of IPF remains indistinct, various processes associated with the disease have been identified, such as telomerase limitation, mitochondrial dysfunction, cellular aging, and dysregulation of the extracellular matrix. [18]. A recent study from a US registry exposed a negative association among age and the utilization of antifibrotic therapy (pirfenidone and nintedanib). [21]. Conversely, Leuschner et al.'s study revealed that more than half of both ageing and nonelderly sick stood prescribed anti-fibrotic treatment when they joined the study, indicating no discrepancies based on age. [18] Recent findings indicate that patients aged 75 years and above with IPF are more prone to discontinuing pirfenidone over a one-year follow-up period and experiencing a greater occurrence of gastrointestinal disorders. [22].

In recent times, there has been a growing recognition of the gender dimension in diseases like COPD or lung cancer. While it's established that IPF is further prevailing in men than women, there's scant knowledge regarding potential gender-based variations in the disease's manifestation and its accompanying comorbidities. [23]. Our study showed that most IPF patients were females (63%) in contact to study of Zaman *et al.,* in 2020. In research of Zaman *et al.*, Among the pooled cohort of 1,263 patients with development information, around 71% stayed males. Next altering for age, FVC % forecast, and Dlco % foretold, man gender stayed autonomously linked to a more danger of loss or lung transplantation (danger proportion for males, 1.4; 95% CI, 1.2-1.7; P < 0.001). [24]. Previous research by Han et al. has proposed that women might undergo a gentler illness development, as indicated by the desaturation part on a 6-minute walk examination, in comparison to males. [25]. Additional research has indicated that men diagnosed with IPF face a advanced danger of humanity paralleled to women with the condition. However, the extent of the contrast in survival duration has not been previously documented. [26,27]. The underlying reasons for these outcome variations based on sex—whether they stem from differing risks, disease severity, distinct phenotypes, or inherent biological factors related to sex—remain uncertain. [24].

In study, smoking detected in small numbers of older males and not determine in females and this may be related to earlier respiratory problems of females which prevent cigarette smoking or may inadequate samples that involved in our research. Despite studies indicating a strong correlation between cigarette smoking and the progress of IPF, the apparatus concluded which smoking might contribute to the   IPF pathogenesis remains unknown. [28]. Due to IPF being acknowledged as an age-related ailment, there's speculation that smoking could potentially play a role in the onset of interstitial lung illness in an age-dependent fashion. Additionally, there's a notion that heightened oxidative tension in both existing and previous smokers could accelerate illness advancement. [29].

Immunologically, present study determined high serum concentration of TGF-β in IPF compared to control in our city. Numerous trainings have intensive on investigating the fibrotic process of IPF,

with results representing that TGF-β productions a pivotal part in the disease's progression. [30]. TGF-β1 facilitates the fibrotic progression of IPF concluded multiple pathways of signaling, such as pathways of Smad, MAPK, and ERK. These pathways intersect, presenting novel targets for drug research. Additionally, TGF-β1 influences IPF fibrosis by impacting oxidative stress, epigenetics, and other factors. Ye and Hu's study underscores the critical part of TGF-β1 in idio-pathic pulmonary fibrosis, as it excites fibroblast-to-myofibroblast transformation, epithelial-to-mesenchymal changeover, and enhances collagen, filamentous actin, and α-SMA production. [7]. The fibrogenic function of TGF-β in pulmonary fibrosis stayed validated through the discovery that adenoviral-arbitrated delivery of vigorous TGF-β1 gene induced significant fibrosis in rodent lungs. This was reinforced by findings from a transgenic mouse ideal that overexpresses TGF-β1 by means of a lung-specific organizer. [31]. Furthermore, a absence in Smad3, a crucial intra-cellular signaling molecule specific to TGF-β, mitigates lung fibrosis triggered by bleomycin and by lung-specific overexpression of TGF-β1. [32]. Nevertheless, Deng et al. showed that while Smad3 can be stimulated via TGF-β1 in human lung fibroblasts (HLF), it did not influence the appearance of collagen I or α-SMA. [33]. Exposing fibroblasts to TGF-β1 led to elevated galectin-1 (Gal-1) expression, resulting in the phosphorylation of Smad2 and augmenting Smad2's nuclear retention. This process facilitated myofibroblast differentiation and expedited fibrosis. [34]. Earlier research revealed that TGF-β1/Smad3-prompted NADPH oxidase 4 (NOX4) facilitated the generation of H2O2, a crucial factor for myo-fibroblast distinction in lung mesenchymal cells. This vision offers new perspectives for therapeutic interventions in IPF. [35,36]. Moreover, TGF-β1 was found to expedite lung fibrosis via triggering the generation of reactive oxygen species through NOX-4. These ROS, in turn, facilitated the nuclear transfer of histone deacetylase 4 (HDAC4) and the creation of α-SMA fibers in standard humanoid lung fibroblasts (NHLFs). [37].

Other studied immune marker in our research was OPN that raised in IPF especially smokers. Generally, studies about immunological role of OPN in IPF pathology very limited. Nonetheless, OPN undergoes cleavage and activation by MMP-7, and investigators note that MMP-7 is stimulated by OPN in epithelial cells, implying that this reciprocal reinforcement apparatus likewise applies to OPN and MMP-7. [38This is further reinforced by the co-localization of MMP-7 and OPN in IPF epithelial cells, as well as via the computational correlation between the appearance ranks of OPN. Intriguingly, MMP-7 and OPN are both objective genes of β-catenin. [39]. In earlier work, Chilosi et al. documented significant activation of WNT/β-catenin in IPF lungs and proposed that the interplay between MMP-7 and OPN could show a part in the progressive nature of IPF. [40]. Research conducted through Icer and colleagues demonstrated that the expression of OPN was elevated compared to the control grouping across entirely phases of pulmonary fibrosis in mice. Remarkably, OPN stages in bronchoalveolar lavage fluid (BALF) consistently remained high, while variations in OPN appearance ranks in peripheral blood were significant only during the early stages. [41]. Icer et al. hypothesize that as pulmonary fibrosis progresses, the destruction of lung tissue and failure of alveoli impair the exchange function with blood vessels, causing the accumulation of OPN in the lungs, preventing its entry into the peripheral blood. [41In a recent study, Ji and colleagues revealed that OPN, concealed via macrophages, interacts with alveolar epithelial cells by binding to CD44 receptors on their membrane. This interaction initiates FAK phosphorylation, subsequently leading to AKT phosphorylation and the appearance of EMT-related proteins, ultimately contributing to the onset of pulmonary fibrosis [42] .

Present outcomes found significant increase of OPN in IPF smokers compared with non- smokers. Chipitsyna *et al.*, showed Cigarette smoke has been shown to elevate OPN appearance via the JAK2/STAT3 signaling pathway, facilitating the recruitment of MSC cells and fostering lung cancer metastasis. Likewise, prior research has demonstrated that cigarette smoke boosts OPN appearance in pancreatic ductal adeno-carcinoma cells, consequently advancing cancer progression. [43]. Prior studies have linked cigarette smoking to 17 distinct lung disorders, among them being lung fibrosis.

[44] .On the other hand, we found that TGF-β1 and OPN were affected by the sex and age of patients. We found that these indicators are higher in older patients. The reasons behind this are not clear because studies on the relationship of these immune indicators to gender or age in patients with lung fibrosis are not available. In any case, sex hormones and menopause may production a part in the effectiveness of the immunity system. On the other hand, aging process is accompanied by deterioration of most tissues, including the lungs and the immune system, and thus significant disturbances in the concentration of some mediators as TGF-β1 and OPN are possible [19].

CONCLUSION

The outcomes of our training displayed a upper rate of TGF-β and OPN concentrations in patients (especially the elderly) compared to healthy people, and the concentrations of these indicators varied when distributed according to gender. Our outcomes designate that TGF-β and OPN production a part in mediating lung fibrosis. These results could aid researchers in understanding the recent advancements in IPF pathogenesis associated with TGF-β and OPN, offering new goals and a theoretic foundation for the progress of medical medicines for IPF.

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