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Mieczyslaw Pokorski *Editor*

Lung Cancer and Autoimmune Disorders

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Mieczyslaw Pokorski
Editor

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Editor
Mieczyslaw Pokorski
Institute of Psychology
University of Opole
Poland

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Preface

This is a new book series entitled Neuroscience and Respiration, a subseries of Springer's renowned Advances in Experimental Medicine and Biology. The book volumes present contributions by expert researchers and clinicians in the field of pulmonary disorders. The chapters provide timely overviews of contentious issues or recent advances in the diagnosis, classification, and treatment of the entire range of pulmonary disorders, both acute and chronic. The texts are thought as a merger of basic and clinical research dealing with respiratory medicine, neural and chemical regulation of respiration, and the interactive relationship between respiration and other neurobiological systems such as cardiovascular function or the mind-to-body connection. In detail, topics include lung function, hypoxic lung pathologies, epidemiology of respiratory ailments, sleep-disordered breathing, imaging, and biomarkers. Other needful areas of interest are acute respiratory infections or chronic inflammatory conditions of the respiratory tract, exemplified by asthma and chronic obstructive pulmonary disease (COPD), or those underlain by still unknown factors, such as sarcoidosis, respiratory allergies, lung cancer, and autoimmune disorders involving the respiratory system.

The prominent experts will focus their presentations on the leading-edge therapeutic concepts, methodologies, and innovative treatments. Pharmacotherapy is always in the focus of respiratory research. The action and pharmacology of existing drugs and the development and evaluation of new agents are the heady area of research. Practical, data-driven options to manage patients will be considered. The chapters will present new research regarding older drugs, performed from a modern perspective or from a different pharmacotherapeutic angle. The introduction of new drugs and treatment approaches in both adults and children will be discussed. The problem of drug resistance, its spread, and deleterious consequences will be dealt with as well.

Lung ventilation is ultimately driven by the brain. However, neuropsychological aspects of respiratory disorders are still mostly a matter of conjecture. After decades of misunderstanding and neglect, emotions have been rediscovered as a powerful modifier or even the probable cause of various somatic disorders. Today, the link between stress and respiratory health is undeniable. Scientists accept a powerful psychological connection that can directly affect our quality of life and health span. Psychological approaches,

by decreasing stress, can play a major role in the development and course of respiratory disease, and the mind-body techniques can aid in their treatment.

Neuromolecular aspects relating to gene polymorphism and epigenesis, involving both heritable changes in the nucleotide sequence and functionally relevant changes to the genome that do not involve a change in the nucleotide sequence, leading to respiratory disorders will also be tackled. Clinical advances stemming from basic molecular and biochemical research are but possible if the research findings are "translated" into diagnostic tools, therapeutic procedures, and education, effectively reaching physicians and patients. All that cannot be achieved without a multidisciplinary, collaborative, "bench-to-bedside" approach involving both researchers and clinicians, which is the essence of the book series Neuroscience and Respiration.

The societal and economic burden of respiratory ailments has been on the rise worldwide leading to disabilities and shortening of life span. COPD alone causes more than three million deaths globally each year. Concerted efforts are required to improve this situation, and part of those efforts are gaining insights into the underlying mechanisms of disease and staying abreast with the latest developments in diagnosis and treatment regimens. It is hoped that the books published in this series will fulfill such a role by assuming a leading role in the field of respiratory medicine and research and will become a source of reference and inspiration for future research ideas.

Titles appearing in Neuroscience and Respiration will be assembled in a novel way in that chapters will first be published online to enhance their speedy visibility. Once there are enough chapters to form a book, the chapters will be assembled into complete volumes. At the end, I would like to express my deep gratitude to Mr. Martijn Roelandse and Ms. Tanja Koppejan from Springer's Life Sciences Department for their genuine interest in making this scientific endeavor come through and in the expert management of the production of this novel book series.

Opole, Poland Mieczyslaw Pokorski

Volume 2: Lung Cancer and Autoimmune Disorders

Lung cancer and autoimmune diseases are complex entities in that they involve gene disturbance, gene polymorphism, and impaired gene repair mechanisms. The volume focuses on altered gene expression in tumor processes and in chronic autoimmune disorders. The chapters discuss the biological rationale for novel disease protein markers, present relevant clinical results, and give some diagnostic and therapeutic tips.

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Polymorphisms of DNA Repair Genes and Lung Cancer in Chromium Exposure

M. Sarlinova, L. Majerova, T. Matakova, L. Musak, P. Slovakova, M. Škereňová, E. Kavcová, and E. Halašová

Abstract

Chromium is a well known carcinogen involved in the lung cancer development. Polymorphism of some of the DNA repair genes may be associated with elevated risk of cancerous transformation. In the present study, we investigated the polymorphisms of the following selected members of the base and nucleotide excision repair genes: *XPC* (Lys939Gln), *XPD* (Lys751Gln), *XRCC1*(Arg399Gln), and *hOGG1* (Ser326Ser), and the risk they present toward the development of lung cancer, with emphasis on the effect of chromium exposure. We analyzed 119 individuals; 50 patients exposed to chromium with diagnosed lung cancer and 69 healthy controls. Genotypes were determined by a PCR-RFLP method. We found a significantly increased risk of lung cancer development in *XPD* genotype Lys/Gln (OR = 1.94; 95 % CI = 1.10–3.43; p = 0.015) and in the gene combinations: XPD Lys/Gln+XPC Lys/Gln (OR = 6.5; 95 % CI = 1.53–27.49; p = 0.009) and XPD Lys/Gln+XPC Gln/Gln(OR = 5.2; 95 % CI = 1.07–25.32; p = 0.04). In conclusion, gene polymorphisms in the DNA repair genes may underscore the risk of lung cancer development in the chromium-exposed individuals.

M. Sarlinova and T. Matakova
Department of Medical Biochemistry, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, 4 Malá Hora St., 036 01 Martin, Slovakia

L. Majerova, P. Slovakova, and E. Halašová (✉)
Department of Medical Biology, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Martin, Slovakia
e-mail: halasova@jfmed.uniba.sk

L. Musak
Clinic of Occupational Medicine and Toxicology, Comenius University in Bratislava, Jessenius Faculty of Medicine in Martin and University Hospital in Martin, Martin, Slovakia

M. Škereňová
Department of Clinical Biochemistry, University Hospital in Martin, Martin, Slovakia

E. Kavcová
Clinic of Pneumology and Phthisiology, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Martin, Slovakia

Keywords
Chromium exposure • DNA repair genes • Lung cancer • Single nucleotide polymorphism

1 Introduction

Lung cancer is the most common form of cancer worldwide with 1.61 million new cases in 2008. In the Slovak Republic, the incidence and mortality in men show a downward trend, whereas the mortality rate has increased in women (GLOBCAN 2008). Lung cancer represents the most common cause of cancer-related deaths in particular due to occupational exposure to carcinogens and smoking (Yin et al. 2009, 2011; Bray and Weiderpass 2010; Jemal et al. 2009; Molina et al. 2008; Alberg et al. 2007). The respiratory tract is a major way of entry of occupational chromium, beside oral and dermal exposure. Previous studies have revealed that hexavalent chromium (Cr(VI)) compounds could induce DNA damage (Zhang et al. 2011; Halašová et al. 2001, 2005). Exposure to Cr (VI) significantly increases the risk of respiratory tract cancer. Thus, IARC (1990) has classified Cr (VI) as a group I carcinogen. In cells, chromium induces the formation of reactive intermediates resulting in enhanced oxidative stress (Kubrak et al. 2010). Reduction of Cr(VI) to Cr(V) is required for the induction of DNA damage and mutations (Quievryn et al. 2002). Genotoxic effects of chromium are predominantly represented by the formation of oxidative adducts and apurinic/apyrimidinic lesions, eventually resulting in DNA breaks (Balachandar et al. 2010; Beveridge et al. 2010; Figgitt et al. 2010; Tajima et al. 2010; Tsaousi et al. 2010; Velma and Tchounwou 2010). It is notable that Cr also exhibits epigenetic effects in carcinogenesis, an inhibition of the nucleotide excision repair (NER) (Hodges and Chipman 2002). Humans have developed many protective mechanisms against DNA damage, such as xenobiotics biotransformation and DNA damage repair (Dai et al. 2009; Zhang et al. 2006). Within DNA repair mechanisms, the NER is involved in the removal of a wide variety of DNA lesions. XP proteins play a substantial role in NER (Chen and Suter 2003; Christmann et al. 2003). XPD, XPG, and XPC NER proteins, in particular, participate in preventing DNA mutability and cancer development (Zafereo et al. 2009; Berwick and Vineis 2000). Oxidative stress is represented by the formation of several kinds of oxidative DNA damage, the best characterized of which is 8-oxo-7,8-dihydroguanine (8-oxoG) (Vidal et al. 2001). The biological relevance of 8-oxoG in DNA relies in its displaying, which results in a GC→TA transversion during DNA replication (Hu and Ahrendt 2005; Hashiguchi et al. 2004; Yamane et al. 2004). The major pathway for 8-oxoG removal from DNA is the base excision repair mediated by *hOGG1* protein (Kohno et al. 2006; Mambo et al. 2005). Another protein involved in the base excision repair is *XRCC1* protein which is coded by the *XRCC1* gene and acts in repair of single-strand breaks caused by alkylation agents or radiation. The role of *XRCC1* and its polymorphic forms in lung cancer susceptibility and treatment response to cytostatics is well-documented (Li et al. 2011, 2012; Qian et al. 2011; Yin et al. 2011; Kim et al. 2010). Despite the foregoing, there is still no established diagnostic for screening among the populations at risk, which is additionally complicated by lack of no clear warning clinical signs. However, disease progression can influence appropriate treatment and eventual survival of patients. In this context, a new approach based on molecular genetic analysis, such as gene polymorphisms, could be helpful in the identification of persons at elevated risk of developing lung cancer. Genetic variations in DNA repair genes might be associated with an altered DNA repair capacity, which if reduced due to inherited polymorphisms may increase the susceptibility to cancer (Mandal et al. 2012). The present study investigated the possible effects of polymorphisms in selected repair genes and whether they could form a basis for anticipating the propensity toward developing cancer.

2 Methods

2.1 Study Population

The study was approved by the Ethics Committee of the Jessenius Faculty of Medicine in Martin, Slovakia and all individuals sign informed consent. This study included 50 chromium-exposed lung cancer patient (mean age 65.4 ± 9.5, range 43–88 years; F/M – 6/44) and 69 control subjects (mean age 63.5 ± 10.2, range 50–90 years; F/M – 15/44). The mean exposure time to chromium in the patients was 9.3 ± 1.7 years. Lung cancer cases were collected in a hospital in Dolný Kubín, Slovakia in the period of 2005–2013. Blood samples for control group were collected from volunteers with no previous malignant disease in medical records and family history. The volunteers corresponded with lung cancer patients in age, gender and ethnicity.

2.2 Genotyping Analysis

Single nucleotide polymorphisms in *XPC (rs2228001), XPD (rs13181,) XRCC1 (rs25487),* and *hOGG1 (rs1052123)* genes were determined by PCR-RFLP. PCR reactions were carried out according to the protocol of Musak et al. (2008). The amplified fragments were digested with appropriate restriction endonucleases and analyzed. The digested PCR products were resolved on 3 % (w/v) agarose gels containing ethidium bromide and visualized under UV light. Genotype screening was performed simultaneously for cases and controls.

2.3 Statistical Analysis

The Chi-square (χ^2) and Fischer tests were used to determine the significance of differences from the Hardy-Weinberg equilibrium and the independence of genotype frequency between cases and controls. Odd ratio (OR) and 95 % confidence interval (95 % CI) were calculated to estimate the strength of associations between different genotypes in patients and controls. A value of $p < 0.05$ was considered as statistically significant. All statistical calculations were performed using Microsoft Excel and MedCalc ver. 5 software.

3 Results

Distribution of genes and genotypes of analyzed repair genes *XPC939*, *XPD751*, *hOGG1326*, and *XRCC1399* in lung cancer patients and controls is shown in Tables 1, 2, and 3.

We did not find significant changes in distribution of alleles and genotypes of *XPC, XRCC1*, and *hOGG1* polymorphisms. However, significantly increased risk of lung cancer development was found in the *XPD* gene heterogeneous constitution Lys/Gln (OR = 1.94; 95 %

Table 1 Distribution of alleles of the *XPD, XPC, XRCC1,* and *hOGG1* genes in the chromium-exposed lung cancer patients and controls

Alleles	Exposed n (%)	Controls n (%)	OR (95 %CI)	p	χ^2
XPD					
Lys	63 (63 %)	106 (77 %)	Ref.		
Gln	**37 (37 %)**	**32 (23 %)**	**1.94 (1.10–3.43)**	**0.015**	–
XPC					
Lys	52 (52 %)	85 (62 %)	Ref.		
Gln	48 (48 %)	53 (38 %)	1.48 (0.87–2.49)	0.08	1.81
XRCC1					
Arg	58 (58 %)	87 (63 %)	Ref.		
Gln	42 (42 %)	51 (37 %)	1.24 (0.73–2.09)	0.25	0.42
hOGG1					
Ser	84 (84 %)	108 (78 %)	Ref.		
Cys	16 (16 %)	30 (22 %)	0.68 (0.35–1.34)	0.17	–

Table 2 Distribution of genotypes of the *XPD, XPC, XRCC1*, and *hOGG1* polymorphisms in the chromium-exposed lung cancer patients and controls

Gene and genotypes	Controls n (%)	Exposed n (%)	OR (95%Cl)	p	χ^2
XPD					
Lys/Lys	40 (57 %)	16 (32 %)	Ref.		
Lys/Gln	**26 (38 %)**	**31 (62 %)**	**2.18 (1.37–6.05)**	**0.01**	**1.75**
Gln/Gln	3 (4.4 %)	3 (6 %)	2.50 (0.45–13.72)	0.14	1.17
XPC					
Lys/Lys	29 (42 %)	14 (28 %)	Ref.		
Lys/Gln	27 (39 %)	24 (48 %)	1.84 (0.79–4.25)	0.11	1.48
Gln/Gln	13 (19 %)	12 (24 %)	1.92 (0.70–5.26)	0.15	1.10
XRCC1					
Arg/Arg	23 (33 %)	17 (34 %)	Ref.		
Arg/Gln	41 (59 %)	24 (48 %)	0.79 (0.35–1.77)	0.36	–
Gln/Gln	5 (7 %)	9 (18 %)	2.44 (0.69–8.60)	0.14	1.19
hOGG1					
Ser/Ser	43 (62 %)	36 (72 %)	Ref.		
Ser/Cys	22 (32 %)	12 (24 %)	0.65 (0.28–1.49)	0.21	–
Cys/Cys	4 (6 %)	2 (4 %)	0.60 (0.10–3.45)	0.44	–

CI = 1.10–3.43; p = 0.015) (Table 1). Mutual combinations of genotypes of *XPD, XPC, XRCC1*, and *hOGG1* and their associations with lung cancer are shown in Table 2. In total, significantly increased risk of developing lung cancer was found in the following combinations of genotypes: *XPD* Lys/Gln+XPC Lys/Gln (OR = 6.5; 95 % CI = 1.53–27.49; p = 0.009) and *XPD* Lys/Gln+XPC Gln/Gln (OR = 5.2; 95 % CI = 1.07–25.32; p = 0.04) (Table 3).

4 Discussion

Lung cancer is a multifactorial disease, with many factors contributing to its development. Since the respiratory tract is in direct contact with inhaled, potentially carcinogenic agents, the bronchial epithelial cells are attacked in the first-line. Huang et al. (2013) published a statistically significant dose-response relationship for the incidence of lung squamous cell carcinoma caused by exposure to Cr. These authors also found a dose-response relationship between a soil heavy metal concentration and lung cancer occurrence by specific cell-type; however, the relevant mechanism should be explored further.

An interesting finding was presented by Huvinen and Pukkala (2013). They investigated the cancer incidence among Finnish ferrochromium and stainless steel production workers in the years 1967–2011 and found that the number of lung cancer cases decreased by a fifth, and even more than that among those who had been working in the same department for more than 5 years. However, it seems that increased exposure to mutagenic and carcinogenic agents that cause DNA damage, in combination with some genetic polymorphisms of genes that code the DNA repair genes, may lead to elevated cancer risk. A positive association between the repair genes *XRCC1* Arg399Gln, *XPC* Lys939Gln, *XPD* Lys751Gln, and *hOGG1* Ser326Cys polymorphism and risk of lung cancer has been demonstrated in several studies (Qian et al. 2011; Raaschou-Nielsen et al. 2008; De Ruyck et al. 2007; Halašová et al. 2001, 2005; Vogel et al. 2005). In most multifactorial diseases, single polymorphisms in single genes are unlikely to alter the expression or function of specific proteins to the extent of producing a pathological phenotype. It is most likely that the combined effect of different single nucleotide polymorphisms (SNPs) in a gene produce a change in protein expression or function (Matullo et al. 2006).

The present study is the first dealing with the influence of gene polymorphism on lung cancer risk in chromium-exposed individuals. The most important results of our work are the detection of

Table 3 Combinations of genotypes of the *XPD*, *XPC*, *XRCC1*, and *hOGG1* polymorphisms in the chromium-exposed lung cancer patients and controls

Combinations of genotypes	Controls n (%)	Exposed n (%)	OR (95%Cl)	p
XPD+XPC				
Lys/Lys+Lys/Lys	13 (19 %)	4 (8 %)	Ref.	
Lys/Lys+Lys/Gln	19 (28 %)	8 (16 %)	1.37 (0.43–5.51)	0.46
Lys/Lys+Gln/Gln	8 (12 %)	4 (8 %)	1.62 (0.31–8.39)	0.44
Lys/Gln+Lys/Lys	14 (20 %)	9 (18 %)	2.09 (0.51–8.46)	0.24
Lys/Gln + Lys/Gln	**7 (10 %)**	**14 (28 %)**	**6.50 (1.53–27.49)**	**0.01**
Lys/Gln+Gln/Gln	**5 (7 %)**	**8 (16 %)**	**5.20 (1.07–25.32)**	**0.04**
Gln/Gln+Lys/Lys	2 (3 %)	1 (2 %)	1.62 (0.11–22.99)	0.60
Gln/Gln+Lys/Gln	1 (1 %)	2 (4 %)	6.5 (0.45–91.98)	0.20
Gln/Gln+Gln/Gln	–	–	–	–
XRCC1+hOGG1				
Arg/Arg+Ser/Ser	15 (22 %)	12 (24 %)	Ref.	
Arg/Arg+Ser/Cys	5 (7 %)	4 (8 %)	1.00 (0.21–4.57)	0.65
Arg/Arg+Cys/Cys	3 (4 %)	1 (2 %)	0.41 (0.04–4.54)	0.43
Arg/Gln+Ser/Ser	25 (36 %)	17 (34 %)	0.85 (0.32–2.26)	0.46
Arg/Gln+Ser/Cys	16 (23 %)	6 (12 %)	0.46 (0.14–1.57)	0.17
Arg/Gln+Cys/Cys	–	1 (2 %)	–	–
Gln/Gln+Ser/Ser	3 (4 %)	7 (14 %)	2.92 (0.62–13.76)	0.16
Gln/Gln+Ser/Cys	1 (1 %)	2 (4 %)	2.50 (0.20–31.02)	0.44
Gln/Gln+Cys/Cys	1 (1 %)	–	–	–
hOGG1+XPC				
Ser/Ser+Lys/Lys	19 (28 %)	10 (20 %)	Ref.	
Ser/Ser+Lys/Gln	18 (26 %)	17 (34 %)	1.79 (0.65–4.94)	0.19
Ser/Ser+Gln/Gln	6 (9 %)	9 (18 %)	1.26 (0.35–4.58)	0.48
Ser/Cys+Lys/Lys	7 (10 %)	3 (6 %)	0.81 (0.17–3.85)	0.56
Ser/Cys+Lys/Gln	9 (13 %)	6 (12 %)	1.26 (0.35–4.59)	0.48
Ser/Cys+Gln/Gln	6 (9 %)	3 (6 %)	0.95 (0.20–4.63)	0.64
Cys/Cys+Lys/Lys	3 (4 %)	1 (2 %)	0.63 (0.06–6.91)	0.59
Cys/Cys+Lys/Gln	–	1 (2 %)	–	–
Cys/Cys+Gln/Gln	1 (1 %)	–	–	–
hOGG1+XPD				
Ser/Ser+Lys/Lys	23 (33 %)	12 (24 %)	Ref.	
Ser/Ser+Lys/Gln	17 (25 %)	21 (42 %)	2.37 (0.92–6.10)	0.06
Ser/Ser+Gln/Gln	3 (4 %)	3 (6 %)	1.92 (0.33–10.99)	0.38
Ser/Cys+Lys/Lys	13 (19 %)	4 (8 %)	0.59 (0.16–2.21)	0.44
Ser/Cys+Lys/Gln	9 (13 %)	8 (16 %)	1.70 (0.52–5.55)	0.28
Ser/Cys+Gln/Gln	–	–	–	–
Cys/Cys+Lys/Lys	4 (6 %)	1 (2 %)	0.47 (0.05–4.78)	0.47
Cys/Cys+Lys/Gln	–	1 (2 %)	–	–
Cys/Cys+Gln/Gln	–	–	–	–
XRCC1+XPC				
Arg/Arg+Lys/Lys	9 (14 %)	6 (12 %)	Ref.	
Arg/Arg+Lys/Gln	10 (14 %)	9 (18 %)	1.35 (0.34–5.32)	0.47
Arg/Arg+Gln/Gln	4 (6 %)	2 (4 %)	0.75 (0.10–5.47)	0.59
Arg/Gln+Lys/Lys	18 (26 %)	5 (10 %)	0.42 (0.10–1.74)	0.20
Arg/Gln+Lys/Gln	16 (23 %)	11 (22 %)	1.03 (0.28–3.74)	0.61
Arg/Gln+Gln/Gln	7 (10 %)	8 (16 %)	1.71 (0.40–7.29)	0.36

(continued)

Table 3 (continued)

Combinations of genotypes	Controls n (%)	Exposed n (%)	OR (95%Cl)	p
Gln/Gln+Lys/Lys	2 (3 %)	3 (6 %)	2.25 (0.29–17.77)	0.40
Gln/Gln+Lys/Gln	1 (1 %)	4 (8 %)	6.00 (0.53–67.69)	0.15
Gln/Gln+Gln/Gln	2 (3 %)	2 (4 %)	1.50 (0.16–13.75)	0.57
XRCC1+XPD				
Arg/Arg+Lys/Lys	12 (17 %)	8 (16 %)	Ref.	
Arg/Arg+Lys/Gln	10 (14 %)	9 (18 %)	1.35 (0.38–4.80)	0.44
Arg/Arg+Gln/Gln	1 (1 %)	–	–	–
Arg/Gln+Lys/Lys	25 (36 %)	7 (14 %)	0.42 (0.12–1.43)	0.14
Arg/Gln+Lys/Gln	14 (20 %)	16 (32 %)	1.71 (0.54–5.39)	0.26
Arg/Gln+Gln/Gln	2 (3 %)	1 (2 %)	0.75 (0.06–9.72)	0.66
Gln/Gln+Lys/Lys	2 (3 %)	1 (2 %)	0.75 (0.06–9.72)	0.66
Gln/Gln+Lys/Gln	3 (4 %)	6 (12 %)	3.00 (0.57–15.62)	0.18
Gln/Gln+Gln/Gln	–	2 (4 %)	–	–

associations between certain genotype combinations and lung cancer risk. The presence of *XPD* Lys/Gln+*XPC* Lys/Gln and *XPD* Lys/Gln +*XPC* Gln/Gln seems to be associated with increased cancer risk.

In conclusion, our study shows susceptibility of some individuals to lung cancer development estimated by the analysis of DNA repair genes polymorphisms. Further research, involving other genes and focusing on gene interactions, is liable to help better identify individuals with increased cancer risk and also may reconcile considerably divergent data on the subject reported in the literature.

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Conflicts of Interest The authors reported no conflicts of interest in relation to this article.

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Circulating Thrombospondin-2 and FGF-2 in Patients with Advanced Non-small Cell Lung Cancer: Correlation with Survival

W. Naumnik, M. Ossolińska, I. Płońska, E. Chyczewska, and J. Nikliński

Abstract

Thrombospondin-2 (TSP-2) is an endogenous negative regulator of vascularization in human cancer. TSP-2 regulates angiogenesis through binding and sequestration of the proangiogenic fibroblast growth factor-2 (FGF-2). However, it is unclear whether TSP-2 and FGF-2 are related to prognosis in non-small cell lung cancer (NSCLC). To study this issue, we measured serum (Elisa) levels of TSP-2 and FGF-2 in 40 NSCLC patients (before chemotherapy) and 22 healthy subjects. Both TSP-2 and FGF-2 concentrations were elevated in the NSCLC group compared with control (TSP-2: 26.72 ± 8.00 *vs*. 18.64 ± 5.50 ng/ml, $p = 0.002$; FGF-2: 11.90 ± 5.80 *vs*. 7.26 ± 3.90 pg/ml, $p = 0.01$). Receiver-operating characteristic (ROC) curves were applied to find the cut-off serum levels of TSP-2 and FGF-2 (NSCLC *vs*. healthy: TSP-2 = 15.09 ng/ml, FGF-2 = 2.23 pg/ml). Patients before treatment with the TSP-2 level <24.15 ng/ml had a median survival of 23.7 months, but those with TSP-2 > 24.15 ng/ml had only 9 months' median survival ($p = 0.007$). Patients with FGF-2 level >11.21 pg/ml had significantly shorter survival than patients with FGF-2 < 11.21 pg/ml (7.5 months *vs*. 16 months, $p = 0.034$). We conclude that NSCLC patients have higher serum concentrations of TSP-2 and FGF-2 than healthy people. High levels of TSP-2 and FGF-2 may predict worse survival.

W. Naumnik (✉)
Department of Lung Diseases, Medical University of Bialystok, 14 Zurawia St., 15-540 Bialystok, Poland

Department of Clinical Molecular Biology, Medical University of Bialystok, 14 Zurawia St., 15-540 Bialystok, Poland
e-mail: wojciechnaumnik@gmail.com

M. Ossolińska and E. Chyczewska
Department of Lung Diseases, Medical University of Bialystok, 14 Zurawia St., 15-540 Bialystok, Poland

I. Płońska
Municipal Hospital in Bialystok, Bialystok, Poland

J. Nikliński
Department of Clinical Molecular Biology, Medical University of Bialystok, 14 Zurawia St., 15-540 Bialystok, Poland

Keywords
Fibroblast growth factor-2 • Lung cancer • Survival • Thrombospondin-2

1 Introduction

Thrombospondins are a group of extracellular glycoproteins that are increasingly being implicated in mechanisms relevant to cancerogenesis (Lawler and Lawler 2012). There are many reports of thrombospondin-1 (TSP-1), an inhibitor of angiogenesis. However, functions and properties of TSP-2 are not well understood. TSP-2 is also known to be an endogenous negative regulator of angiogenesis. The mechanisms by which TSP-2 inhibits angiogenesis can be broadly characterized as direct effects on vascular endothelial cells and indirect effects on the various growth factors, cytokines, and proteases that regulate angiogenesis (Zhang and Lawler 2007). They are also involved in the pathological tissue remodeling that is associated with atherosclerosis (Zhang and Lawler 2007). Recently, Golledge et al. (2013) reported the relation between serum TSP-2 and cardiovascular mortality in older men screened for abdominal aortic aneurysm. Other authors revealed that cancerous, but not stromal, TSP-2 contributes prognosis in pulmonary adenocarcinoma (Chijiwa et al. 2009). However, it is unclear whether serum levels of TSP-2 are related to prognosis in non-small cell lung cancer (NSCLC). Thrombospondins are promising sources of therapeutic agents to treat angiogenesis-driven diseases, including cancer. Colombo et al. (2010) reported that the fibroblast growth factor-2 (FGF-2) binding sequence of thrombospondins might serve as a template for the development of inhibitors of angiogenesis. According to several reports, FGF-2 is an attractive target for new antiangiogenic therapies (Beenken and Mohammadi 2009). As with some other angiogenesis pathways, FGF-2 pathway has been shown to be activated in lung cancer (Behrens et al. 2008). The precise role of this protein in pathogenesis of NSCLC is still unknown. Behrens et al. (2008) reported the overexpression of FGF-2 in NSCLC patients. There is no clear data on the serum concentrations of FGF-2 in NSCLC and their relationship with survival of patients.

2 Methods

The study was performed in conformity with the Declaration of Helsinki for Human Experimentation and the protocol was approved by an institutional ethics board. Written informed consent was obtained from all participants.

Serum samples obtained from 40 patients before treatment of NSCLC were analyzed (squamous cell carcinoma 21, others 19; mean age 63 ± 3 years; F/M-3/37). Serum samples were also obtained from 22 healthy volunteers (mean age 60 ± 4; F/M-2/20). Venous blood samples were drawn into tubes and centrifuged at 3,000× *g* for 10 min. After centrifugation, the serum samples were stored at −80 °C until use. The NSCLC group consisted of 16 patients at clinical Stage IIIB and 24 patients at stage IV of disease. For all patients, the diagnosis of NSCLC was confirmed by the histological examination of biopsy and cytological specimens taken during bronchoscopic examination (Pentax FB 18 V; Pentax Corporation, Tokyo, Japan).

The stage of disease was determined according to the TNM system (Goldstraw et al. 2007). To establish the disease stage, the following investigations were applied in each patient: physical examination, X-ray and CT of the chest, and ultrasonography of the abdomen. All patients received four cycles of chemotherapy (21-day cycle; cisplatin at a dose of 30 mg/m^2 on Days 1, 2, and 3 and gemcitabine at a dose of 1,000 mg/m^2 on Days 1 and 8 of the cycle). The response to therapy was evaluated by diagnostic imaging techniques, including X-ray and CT of the chest, and then the overall response to therapy was analyzed according to the Response Evaluation Criteria in Solid Tumors (RECIST) (Therasse et al. 2000).

Serum samples were assayed for the levels of TSP-2 and FGF-2 by a quantitative sandwich enzyme immunoassay technique (Quantikine HS, R&D System, Minneapolis, MN) according

to the manufacturer's instructions. The minimum detectable levels of TSP-2 and FGF-2 were 0.025 ng/ml and 0.22 pg/ml, respectively. All specimens were assayed in duplicates.

The Shapiro-Wilk test was used for data distribution analysis. All parametrical data was calculated by *t*-test. We used the Mann-Whitney U and Wilcoxon tests for the features inconsistent with the normal data distribution. The Spearman rank test was used to calculate correlations between the parameters. Receiver-operating characteristics (ROC) curves were constructed to find the cut-off levels of TSP-2 and FGF-2. Overall survival was calculated using the Kaplan-Meier Method. The significance of the difference in survival rates was determined by the log-rank test. A value of $p < 0.05$ was considered to indicate statistical significance. Statistica 10.0 software (StatSoft Inc., Tulsa, USA) was used for all analyses.

3 Results

There were no significant differences in age or gender between the patients and healthy subjects. The mean serum TSP-2 and FGF-2 levels were higher in NSCLC group than in healthy people (TSP-2: 26.72 ± 8.00 *vs.* 18.64 ± 5.50 ng/ml, $p = 0.002$; FGF-2: 11.90 ± 5.80 *vs.* 7.26 ± 3.90 pg/ml, $p = 0.01$) (Fig. 1a, b). There were no correlations between the concentrations of TSP-2 and FGF-2.

We constructed ROC curves of serum TSP-2 and FGF-2 to determine the cut-off values (Fig. 2). Specificity and sensitivity of serum TSP-2 in NSCLC patients relative to healthy people were 67 and 96 %, respectively, at a cut-off value of 15.01 ng/ml. Specificity and sensitivity of serum FGF-2 in NSCLC group relative to healthy subjects were 86 and 95 %, respectively, at a cut-off value of 2.24 pg/ml. The area under the curve for serum TSP-2 and FGF-2 were 0.81 and 0.66, respectively.

There were no correlation between the levels of TSP-2 or FGF-2 and the stage of tumor (TSP-2 IIIB *vs.* TSP-2 IV: 25.51 ± 8.10 *vs.* 27.81 ± 8.20 ng/ml, respectively, $p = 0.32$; FGF-2 IIIB *vs.* FGF-2 IV: 9.20 ± 6.00 *vs.* 13.71 ± 9.30 pg/ml, respectively, $p = 0.067$).

We did not find a significant relationship between pretreatment concentrations of TSP-2 or FGF-2, and the effect of chemotherapy. After treatment we found a partial response (PR) in 18 (45 %), stabilisation (SD) in 10 (25 %) and progressive disease (PD) in 12 (30 %) patients (TSP-2: PR *vs.* SD *vs.* PD – 25.10 ± 8.50 *vs.* 25.24 ± 10.40 *vs.* 29.49 ± 6.60 ng/ml, respectively, $p = 0.23$; FGF-2: PR *vs.* SD *vs.* PD – 12.19 ± 11.00 *vs.* 12.76 ± 6.90 *vs.* 10.04 ± 7.20 pg/ml, respectively, $p = 0.13$).

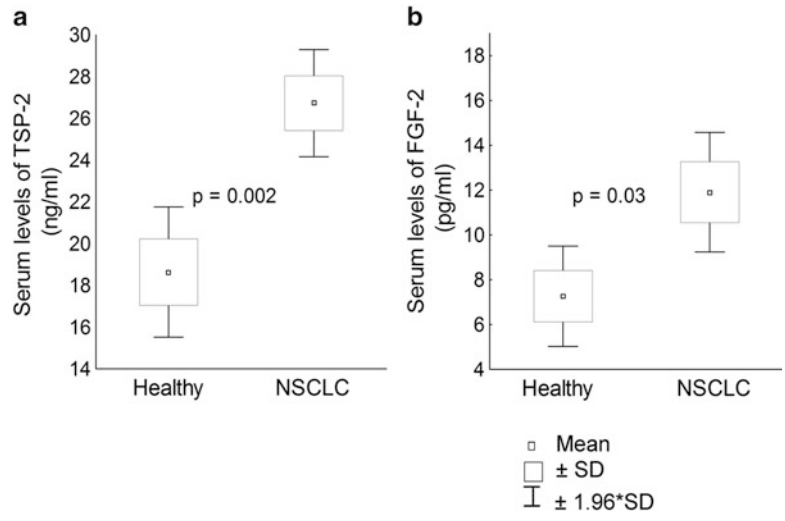

Fig. 1 **Serum concentrations of thrombospondin-2 (TSP-2) (a) and of fibroblast growth factor-2 (FGF-2) (b)** in non-small cell lung cancer (NSCLC) patients and healthy control subjects

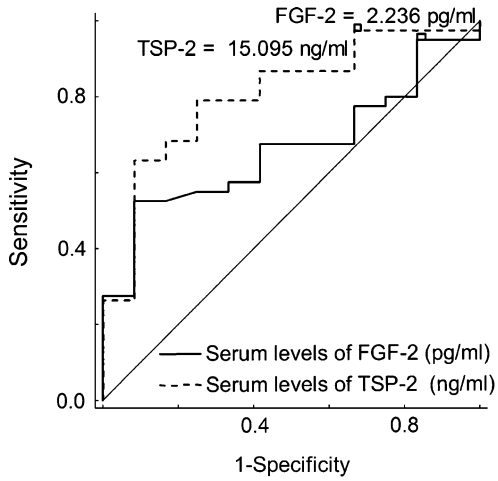

Fig. 2 Receiver operating characteristic (ROC) curves for serum thrombospondin-2 (TSP-2) and fibroblast growth factor-2 (FGF-2) in differentiating non-small cell lung cancer (NSCLC) patients from healthy people

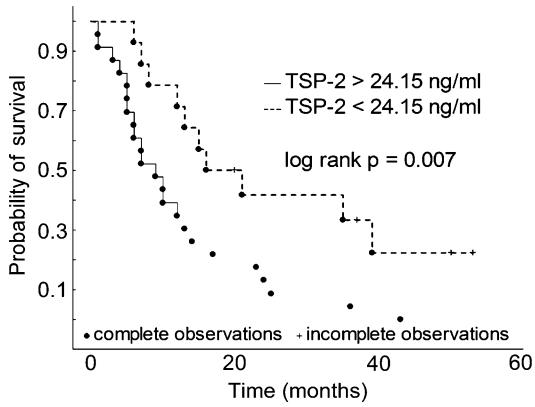

Fig. 3 Kaplan-Meier survival curve. The *solid line* represents survival of patients with thrombospondin-2 (TSP2) >24.15 ng/ml and the *broken line* of those with TSP2 <24.15 ng/ml. Patients with the TSP2 level >24.15 ng/ml had significantly shorter survival

The mean overall survival of all patients was 15.7 ± 13.0 months. The patients with elevated serum TSP-2 levels (TSP2 level >24.15 ng/ml) had a significantly shorter overall survival than those with lower serum TSP-2 levels (TSP2 level <24.15 ng/ml: 9.0 months *vs*. 23.7 months) (Fig. 3). The patients with FGF-2 levels >11.21 pg/ml had a significantly shorter survival than patients with FGF-2 <11.21 pg/ml (7.5 months *vs*. 16.0 months, respectively) (Fig. 4).

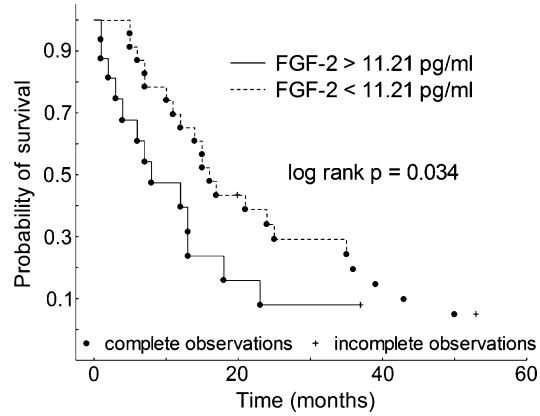

Fig. 4 Kaplan-Meier survival curve. The *solid line* represents survival of patients with fibroblast growth factor-2 (FGF-2) >11.21 pg/ml and the *broken line* of those patients with FGF-2 <11.21 pg/ml. Patients with FGF-2 level >11.21 pg/ml had significantly shorter survival

4 Discussion

TSP-2 has recently attracted attention as an endogenous negative regulator of angiogenesis in tumorigenesis (Lawler 2000). However, functions and properties of TSP-2 are not well understood. Fontanini et al. (1999) reported no statistical differences between TSP-2 mRNA expression and microvessel density in NSCLC by RT-PCR. The opposite results were presented by Tokunaga et al. (1999). They reported that TSP-2 gene expression is correlated with decreased vascularity in NSCLC. Moreover, Chijiwa et al. (2009) reported that patients with pulmonary adenocarcinoma and a pattern of cancerous TSP-2 expression have a good prognosis. On the other hand, pulmonary adenocarcinoma patients with non-cancerous TSP-2 expressions pattern showed poor prognosis (Chijiwa et al. 2009). In a study of Chijiwa et al. (2009), cases with high TSP-2 expression level and good prognosis showed its strong cancerous expression, but not in lymphocytes. On the contrary, cases with high TSP-2 expression, but poor prognosis, showed its low expression in tumor cells, but high levels in lymphocytes (Chijiwa et al. 2009). In the present study, we showed a higher serum level of TSP-2

in NSCLC patients compared with healthy subjects. Moreover, patients with higher levels of TSP-2 had shorter survival. A possible explanation for TSP-2 increased levels in NSCLC group is its releasing from blood cells, endothelial cells, or other cells involved in the pathological tissue remodeling of cancer stroma (Zhang and Lawler 2007). Our results are in accord with those of Chijiwa et al. (2009). They revealed that the stromal TSP-2 expression is not enough to suppress growth of NSCLC, while the cancerous TSP-2 expression directly inhibits growth of the tumor (Chijiwa et al. 2009).

TSP-2 acts at the interface between the cell surface and extracellular matrix to provide contextual cues that regulate matrix structure and cellular phenotype during tumor growth (Lawler and Detmar 2004). One of the main regulators of matrix metalloproteinases production and invasiveness in human cancer cell lines is FGF (Corn et al. 2013).

FGF-2 belongs to a family of ubiquitously expressed ligands that bind to the extracellular domain of FGFRs (FGF Receptors), initiating a signal transduction cascade that promotes cell proliferation, motility, and angiogenesis (Ribatti et al. 2007; Mohammadi et al. 2005). Elevated levels of FGF-2 have been detected in NSCLC cell lines, but the precise role of these molecules in the pathogenesis and progression of this tumor is still unknown (Kuhn et al. 2004).

The first study investigating serum levels of FGF-2 in NSCLC was reported by Brattström et al. (1998). They demonstrated elevated serum of FGF-2 in NSCLC patients (Brattström et al. 1998). We confirmed that findings because NSCLC patients had higher levels of FGF-2 in serum than healthy people. In the present study, patients with higher FGF-2 serum levels had shorter survival than those with lower concentration of FGF-2 (7.5 months *vs*. 16 months). These observations are different from the study of Brattström et al. (1998) who found that elevated FGF-2 at diagnosis was a significant favorable prognostic factor for survival. However, these results disagree with other studies. In a study by Ueno et al. (2001) in 60 NSCLC patients, serum FGF-2 levels did not differ between the clinical stages of NSCLC and showed no correlation with survival. Neither did we find any correlation between the levels of TSP-2, FGF-2 and the stage of tumor. However, our observations on survival are compatible with some other works (Brattström et al. 2002, 2004; Joensuu et al. 2002). In the present study the elevated FGF-2 levels correlated with poor survival in NSCLC.

In summary, data on the prognostic implications of TSP-2 and FGF-2 in serum of NSCLC patients are ambiguous. We believe that the determination of concentrations of TSP-2 and FGF-2 in serum may have a practical significance in predicting survival of patients with lung cancer. Further large scale studies are still needed to define the role of these markers in NSCLC.

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Conflicts of Interest The authors had no conflicts of interest to declare in relation to this article.

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Mismatch Repair Gene Polymorphisms and Association with Lung Cancer Development

P. Slováková, L. Majerová, T. Matáková, M. Škereňová, E. Kavcová, and E. Halašová

Abstract

hMLH1 and *hMSH2* are two of the main members of the mismatch repair (MMR) genes family. Polymorphism of MMR genes is associated with a risk of developing sporadic and hereditary tumors. In the present case-control study, we investigated the promoter polymorphisms of selected mismatch repair genes: *hMLH1* (rs1800734) and *hMSH2* (rs2303425), and the risk they present regarding the development of lung cancer in the Slovak population. The study included 422 lung cancer cases, 511 controls for *hMLH1* gene and 486 controls for *hMSH2* gene. Polymorphism was investigated by a PCR-RFLP method. The risk of cancer development was evaluated in both dominant and recessive genetic models. The evaluation of rs1800734 polymorphism in patients in the dominant model showed a significantly decreased risk of lung cancer in the presence of at least one variant allele A (genotype GA and AA) (OR = 1.40; 95 % CI = 1.08–1.82; p = 0.01). These findings were equally strong expressed in women (OR = 2.00; 95 % CI = 1.23–3.25; p = 0.006). The results for rs2303425 polymorphism revealed an increased risk of lung cancer for variant genotype CC (OR = 2.28; 95 % CI = 1.12–4.63; p = 0.024) in the recessive model. A combination of rs1800734 and rs2303425 polymorphisms was shown to be risky for genotype GGCC; OR = 3.08; 95 % CI = 1.09–8.72; p = 0.03.

P. Slováková (✉)
Department of Medical Biochemistry, Jessenius Faculty of Medicine, Comenius University in Bratislava, Malá Hora 4, 036 01 Martin, Slovak Republic
e-mail: petra.slovakova@gmail.com

L. Majerová, T. Matáková, and E. Halašová
Department of Medical Biology, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Malá Hora 4, 036 01 Martin, Slovak Republic

M. Škereňová
Department of Clinical Biochemistry, University Hospital, Kollárova 2, 036 01 Martin, Slovak Republic

E. Kavcová
Clinic of Pneumology and Phthisiology, University Hospital, Kollárova 2, 036 01 Martin, Slovak Republic

The risk appeared even greater in female gender; (OR = 11.56; 95 % CI = 1.33–100.36, 1.26–94.66; p = 0.005. We conclude that the genotype of mismatch repair genes underscores the risk of lung cancer development in the Slovak population.

Keywords
Lung cancer • Human mutL homolog 1 • Human mutS homolog 2 • Polymorphism • Promoter • Single nucleotide

1 Introduction

Lung carcinoma is a leading cause of cancer mortality worldwide (Ferlay et al. 2013). The biggest unsolved problem is early diagnosis of lung cancer, which would allow to increase the probability of successful treatment from 5 % to more than 70 % (Beržinec 2006). One possibility for early detection of individuals at increased risk for lung cancer development lies in research on genetic polymorphisms. Epidemiological studies often show that subjects with specific gene alterations may be at increased risk of certain forms of cancer. Malignancy involves an interplay of several external and internal factors disrupting the stability of genetic information. One of the internal factors that affects the maintenance of genomic integrity is a mismatch repair (MMR) (Hsieh and Yamane 2008). MMR is one of the five major DNA repair pathways and it is responsible for the elimination of errors that occur during replication or recombination. Genetic inactivation of MMR elevates spontaneous mutability 50–1,000-fold (Ravi et al. 2006). The first clarification of a role of MMR in the pathogenesis of human disease came in 1993 when Fishel et al. (1993) and Leach et al. (1993) demonstrated that mutations in *hMSH2* gene (human MutS homolog 2) predispose individuals to Lynch syndrome. This gene is located on the chromosome 2 and encodes the 934 amino acid long protein MSH2. The protein is part of two heterodimeric protein complexes involved in recognizing of DNA mismatches generated during replication and in initiation of repair (Li 2008). *hMLH1* (human MutL homolog 1) is a next member of MMR gene family, which has been identified on the basis of its locus in frequent mutations observed in the same disease (Papadopoulos et al. 1994). The *hMLH1* gene is located on chromosome 3 and encodes protein MLH1. This protein shows no enzyme activity alone, but it is a part of several dimeric enzymes involved in repair incorrectly paired bases and meiotic crossing over. MLH1 protein heterodimers also perform a role of molecular matchmakers, with endonuclease activity and terminator of mismatch-provoked excision (Li 2008). MMR proteins are multilateral players also involved in DNA signaling damage that ensues in a cell cycle arrest or in apoptosis as a result of a response to certain DNA damaging agents. Therefore, it is possible that mutations and polymorphisms in the sequence of genes for mismatch repair proteins may have an effect on the DNA repair capacity and influence individual susceptibility to the development of lung cancer. To test the existence of such an effect, we performed a case-control study in a Slovak population.

2 Methods

2.1 Study Population

The study was approved by the Ethics Committee of the Jessenius Faculty of Medicine in Martin, Slovakia. All subjects enrolled into the study signed written informed consent. The lung cancer patients were recruited from four specialized clinics and hospitals (Martin, Nitra,

Table 1 Characteristics of the study population and type of cancer

	Patients rs1800734	Patients rs2303425	Controls rs1800734	Control rs2303425
Total sample (*n*)	422	422	511	486
Gender				
Male *n* (%)	322 (76)	324 (77)	287 (56)	270 (56)
Female *n* (%)	100 (24)	98 (23)	224 (44)	216 (44)
Age (year; range)				
Total group	64 ± 10 (24–88)		67 ± 7 (22–85)	
Male	64 ± 9 (24–85)		59 ± 8 (22–85)	
Female	63 ± 12 (25–88)		61 ± 10 (23–81)	
Histology *n* (%)	292 (100 %)			
Adenocarcinoma	121 (41 %)			
Small cell carcinoma	50 (17 %)			
Non-small cell carcinoma	22 (8 %)			
Spinocellular carcinoma	65 (22 %)			
Epidermoid carcinoma	34 (12 %)			

Data are means ± SD

Poprad, Vyšné Hágy, and Dolný Kubín in Slovakia) in the period 2005–2013. The control group was made up by healthy individuals, with no previous personal or familial history of malignancy. Controls subjects corresponded with lung cancer patients in age, gender, and ethnicity. Blood samples from both controls and patients were obtained in the same time period. Subjects' characteristics and tumor type are displayed in Table 1.

2.2 Genotyping Analysis

Genomic DNA was isolated using a standard phenol-chloroform extraction. Two SNPs located in the promoter regions of MMR genes were chosen for the study. The SNPs were determined by a PCR-RFLP method. The following primer sequences were used for genotyping of rs1800734 located in *hMLH1* gene: forward – 5′- TGA CTG GCA TTC AAG CTG TC-3′ and reverse – 5′-TTC ACC ACT GTC TCG TCC AG-3′. The PCR reaction was done in a total volume of 25 μl, containing 1 μl of genomic DNA, 1 μl of each primer, 9.5 μl of redistilled water, and 12.5 μl of DreamTaq Green PCR Master Mix (BIOGEN Praha s.r.o., Czech Republic). The PCR cycle conditions consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 40 s each at 94 °C, 40 s at 56 °C, 1 min at 72 °C, and a final extension step at 72 °C for 7 min. The PCR product was digested with an appropriate volume of PvuII restriction endonuclease. Primer sequences used for genotyping rs2303425 located in *hMSH2* gene were: forward- 5′-AGG CAT GCG CAG TAG CTA AA-3′ and reverse-5′-CCC ACA CCC ACT AAG CTG TT-3′. PCR reaction was performed in a total volume of 13 μl, containing 1 μl DNA, and half of the other ingredients of Master Mix used for polymorphism rs1800734. The PCR cycle conditions were the same as those for the polymorphism rs1800734 above outlined. Restriction endonuclease used for PCR product digestion was BseNI. Digested PCR products were analyzed by gel electrophoresis (3 % agarose) and ethidium bromide staining for visualisation under UV light. Following fragment products were seen after visualisation: rs1800734 *hMLH1* gene, wild type genotype WT = 69 bp + 145 bp, heterozygous genotype Het = 69 bp + 145 bp + 214 bp, variant genotype Var = 214 bp, and for rs2303425 *hMSH2* gene WT = 190 bp, Het = 190 bp + 118 bp + 72 bp, Var = 118 bp + 72 bp.

2.3 Statistical Analysis

The Fisher exact test was used to determine the significance of differences from the Hardy-Weinberg equilibrium and the allelic association

between cases and controls. Odds ration (OR) and 95 % confidence intervals (95 % CI) were calculated to estimate the strength of associations between different genotypes in patients and controls. A p-value <0.05 was considered statistically significant. The Fisher exact test was also used to examine the association between genotypes and cancer risk in two genetic models: dominant and recessive. Statistical analysis of the association between a mutual genotype combination of rs1800734 and rs2303425 polymorphisms and risk of lung cancer development was carried out by the Chi square test (χ^2). Statistical calculations were performed using Microsoft Excel and SNP & Variation Suite ver. 7.x6.11 software.

3 Results

Minor allele frequencies (MAF) and their association with lung cancer risk are presented in Table 2. We found only one statistically significant association between minor allele A and cancer risk in the group of women (OR = 0.66; 95 % CI = 0.45–0.99; p = 0.043). The frequency of variant alleles of polymorphisms in the present study were compared with previous published reports. Table 3 describes the main results concerning the associations of genotype rs1800734 and rs2303425 polymorphisms with the risk of lung cancer evaluated in two genetic models: dominant and recessive. The evaluation of cancer risk in relation to genotypes has shown two distinct associations of rs1800734 polymorphism in the dominant genetic model. One was of polymorphism rs18000734 among lung cancer patients, where the presence of at least one variant allele A in genotype (genotype GA and AA) significantly decreased the risk of lung cancer by 1.4 times (OR = 1.40; 95 % CI = 1.08–1.82; p = 0.01). Assessing the risk of lung cancer in regard to gender showed an equally significant result for the rs1800734 polymorphism in women (OR = 2.00; 95 % CI = 1.23–3.25; p = 0.006). No significant association between rs1800734 polymorphism and risk of lung cancer was found in the recessive genetic model.

Concerning the rs2303425 polymorphism, no significant association between genotypes and cancer risk was found in the dominant genetic model. However, there was increased risk of lung cancer for the variant genotype CC (OR = 2.28; 95 % CI = 1.12–4.63; p = 0.024) in the recessive genetic model. Based on the result above outlined, it might be assumed that the presence of both high-risk genotypes GG (rs1800734 polymorphism) and CC (rs2303425 polymorphism) could potentiate the risk of lung cancer development. Indeed, a mutual combination of genotype rs1800734 and rs2303425 polymorphisms showed increased risk of lung cancer (OR = 3.08; 95 % CI = 1.09–8.72; p = 0.03; χ^2 = 4.96). A comparison of gender brought up the fact that the risk of lung cancer was even higher for the mutual combination of genotypes GGCC in women (OR = 11.56; 95 %

Table 2 Minor allele frequencies and their association with lung cancer risk

Group	Gene	Minor allele	MAF in patients	MAF in controls	Fischer's exact *p* allelic associations	Minor allele OR (95 % CI)
	hMLH1	A				
All			0.237	0.268	0.14	0.85 (0.69–1.05)
Male			0.245	0.254	0.74	0.95 (0.74–1.24)
Female			0.210	0.286	0.04*	0.67 (0.45–0.99)
	hMSH2	C				
All			0.192	0.189	0.91	1.02 (0.80–1.29)
Male			0.187	0.198	0.66	0.93 (0.69–1.24)
Female			0.209	0.178	0.38	1.22 (0.80–1.86)

MAF minor allele frequencies, *OR* odds ratio, *CI* confidence interval
*$p < 0.05$

Table 3 Associations between genotypes – rs1800734 and rs2303425 polymorphisms with the risk of lung cancer development in two genetic models

				Dominant model (AA + GA *vs.* GG)		Recessive model (GG + GA *vs.* AA)	
hMLH1 genotypes	Group	Patients *n*	Controls *n*	OR (95 % CI)	*p*	OR (95 % CI)	*p*
	All						
G_G		250	260	1.40 (1.08–1.82)			
G_A		144	228	0.71 (0.55–0.93)	0.01*		
A_A		28	23			1.51 (0.86–2.66)	0.19
	Male						
G_G		185	152	1.20 (0.87–1.65)			
G_A		116	124	0.83 (0.61–1.15)	0.29		
A_A		21	11			1.75 (0.83–3.70)	0.15
	Female						
G_G		65	108	2.00 (1.23–3.25)			
G_A		28	104	0.50 (0.31–0.82)	0.01*		
A_A		7	12			1.33 (0.51–3.49)	0.61
				Dominant model (CC + TC *vs.* TT)		Recessive model (TT + TC *vs.* CC)	
hMSH2 genotypes	Group	Patients *n*	Controls *n*	OR (95 % CI)	*p*	OR (95 % CI)	*p*
	All						
T_T		283	314	1.12 (0.85–1.47)			
T_C		116	160	0.90 (0.68–1.18)	0.44		
C_C		23	12			2.28 (1.12–4.63)	0.02*
	Male						
T_T		220	171	1.23 (0.87–1.72)			
T_C		87	91	0.82 (0.58–1.15)	0.26		
C_C		17	8			1.81 (0.77–4.27)	0.22
	Female						
T_T		63	143	0.92 (0.56–1.52)			
T_C		29	69	1.09 (0.70–1.80)	0.80		
C_C		6	4			3.46 (0.95–12.5)	0.08

OR odds ratio, *CI* confidence interval; *p* for Fischer's exact test
*$p < 0.05$

CI = 1.33–100.36; p = 0.005; $\chi^2 = 7.74$). Comparison of mutual combinations of *hMLH1*and *hMSH2* genotypes in lung cancer patients and controls is shown in Table 4.

4 Discussion

Recently, many a study has been conducted to assess the impact of different genetic variants on health. As a key member of MMR genes, *hMLH1* and *hMSH2*, play a critical role in the process of human cancer formation, including lung cancer. Although initial studies have provided evidence for a role of gene polymorphisms in the etiology of human lung cancer, subsequent studies, carried out in different populations or in a greater number of subjects, disproved the initial evidence. That also concerns the polymorphisms rs1800734 and rs2303425 investigated in the present study. Polymorphism rs1800734 located in the promoter region of *hMLH1* gene has been the subject of several studies, including meta-analyses, which have attained different conclusions. He et al. (2013) studying the relation between rs1800734 polymorphism and the risk of cancer in Asian populations pointed to increased risk of lung cancer formation in the

Table 4 Comparison of mutual combinations of – *hMLH1* and *hMSH2* genotypes in lung cancer patients and controls

Combinations of *hMLH1* and *hMSH2* genotypes	Group	Patients *n*	Controls *n*	OR (95 % CI)	*p*	χ^2
	All					
All other		406	481	Ref.	–	–
GG CC		13	5	3.08 (1.09–8.72)	0.03*	4.96
	Male					
All other		313	266	Ref.	–	–
GG CC		8	4	1.70 (0.51–5.71)	0.39	0.75
	Female					
All other		93	215	Ref.	–	–
GG CC		5	1	11.56 (1.33–100.36)	0.01*	7.74

OR odds ratio, *CI* confidence interval, *p* for Chi^2, *Ref.* reference
*$p < 0.05$

genetic recessive model (OR = 1.69; 95 % CI = 1.30–2.19; p < 0.001), unlike the formation of other types of cancer. In contrast, in the present study we found a reduced risk of lung cancer in a group with at least one variant allele A genotype (genotype GA and AA) (OR = 1.40, 95 % CI = 1.08–1.82; p = 0.01). The risk was even more appreciably reduced in women (OR = 2.00, 95 % CI = 1.23–3.25; p = 0.01). We found a similar risk reduction of lung cancer in women while comparing the frequency of the minor allele (OR = 0.66, 95 % CI = 0.44–0.99, p = 0.04). It is tempting to suggest that the decreased risk in women might indicate a connection between the hormonal stimulation, lung cancer, and *hMLH1* gene. Estrogens have a biological effect on tumors, which are not connected with the reproductive system (Belcher et al. 2009) and the promotor of *hMLH1* gene carries a hemiresponsive estrogen element (Irving et al. 2002). Miyamoto et al. (2006) showed the role of estrogen-mediated transcriptional activation of MMR proteins. The study of Hershberger et al. (2009) confirmed the activation of estrogen response elements by 17beta-estradiol and other estrogen modifiers in lung tumor cells.

The efficiency of mismatch repair processes by rs1800734 polymorphism may be influenced by *hMLH1* gene transcription. Polymorphism of rs1800734 is located in the field of CpG island. It is assumed that the location might affect the binding of methylation machinery and thus weaken the expression of *hMLH1* and *EPM2AIP1* genes. Perera et al. (2011) and Savio et al. (2012) showed that individuals with the wild genotype have appreciably increased methylation of the *hMLH1* promoter gene variant compared with homozygotes and heterozygotes, and that *hMLH1* promoter methylation in peripheral lymphocytes of healthy individuals significantly decreases with aging. Since the level of methylation and estrogen hormonal regulation among women has also been associated with age, it would be useful to carry out a study on a larger group of patients diversified by age to clarify the observed effect on lung cancer development of rs1800734 polymorphism in women.

The promoter polymorphism rs2303425 is localized to the *hMSH2* gene. In the recessive genetic model we found a significant association of the variant CC genotype with increased risk of lung cancer (OR = 2.28, 95 % CI = 1.12–4.63, p = 0.02). In contrast, Jung et al. (2006) did not report any overall risk of lung cancer in the Korean population concerning polymorphism rs2303425. Likewise, Srivastava et al. (2010) did not detect any association of rs2303425 polymorphism with the risk of gallbladder cancer in the Indian population, although the number of tested individuals was small. On the other hand, Mrkonjic et al. (2007) described a strong association between the variant allele C of rs2303425 polymorphism and family history of colorectal cancer in the Caucasian population. Further analysis of this population confirmed the association

of rs2303425 polymorphism with colorectal cancer only in women. That is in contradiction to the present study, as we found an association of the variant genotype CC polymorphism rs2303425 in the whole group of patients, with no inter-gender differences. The discrepancy might have to do with a relatively small number of individuals examined in our study. Functional studies of *hMSH2* gene promoter have shown that rs2303425 polymorphism is located in the binding area of NF-Y transcription factor, which is also known as the estrogen response element (Mrkonjic et al. 2007). As the promoter polymorphisms have a potential to regulate the transcription of genes and thus affect their expression, it is not surprising that a combination of genotypes GGCC of monitored polymorphisms rs1800734 and rs2303425 increased the risk of developing lung cancer as assessed for both the whole group of patients and the group of women alone.

In conclusion, we found out that polymorphism rs1800734 of *hMLH1* gene and polymorphism rs2303425 of *hMSH2* gene were associated with the risk modification of lung cancer in the Slovak population. The effect of polymorphism rs1800734 was clearer expressed in women. Furthermore, other genes included in the mismatch repair should also be investigated, because they might have additional etiological role in the mismatch repair pathway in lung cancer.

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Conflicts of Interest The authors reported no conflicts of interest in relation to this article.

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The Potential of Wharton's Jelly Derived Mesenchymal Stem Cells in Treating Patients with Cystic Fibrosis

D. Boruczkowski, D. Gładysz, U. Demkow, and K. Pawelec

Abstract

Cystic fibrosis (CF) is a life-threatening autosomal recessive multi-organ disorder with the mean incidence of 0.737 per 10,000 people worldwide. Despite many advances in therapy, patients fail to have a satisfactory quality of life. The end-stage lung disease still accounts for significant mortality and puts patients in the need of lung transplantation. Even though the disease is monogenic, the trials of topical gene transfer into airway epithelial cells have so far been disappointing. It is proven that stem cells can be differentiated into type II alveolar epithelial cells. Wharton's jelly-derived mesenchymal stem cells (MSC) from non-CF carrier third-party donors could be an effective alternative to bone marrow or embryonic stem cells. The harvesting process is an easy and ethically uncontroversial procedure. The MSC cell should be applied through repetitive infusions due to rapid lung epithelial cell turnover. However, the low stem cell incorporation remains a problem. Pre-clinical studies imply that even 6–10 % of the wild-type cystic fibrosis transmembrane conductance regulator (CFTR) expression could be enough to restore chloride secretion. The route of administration, the optimal dose, as well as the intervals between infusions have yet to be determined. This review discusses the clinical potential of mesenchymal stem cell in CF patients.

Keywords

Cystic fibrosis • Lung diseases • Stem cell therapy • Umbilical cord

D. Boruczkowski (✉) and D. Gładysz
Polish Stem Cell Bank, 2/41 Grzybowska St., 00-131 Warsaw, Poland
e-mail: dariusz.boruczkowski@pbkm.pl

U. Demkow
Department of Laboratory Diagnostics and Clinical Immunology, Medical University of Warsaw, Warsaw, Poland

K. Pawelec
Polish Stem Cell Bank, 2/41 Grzybowska St., 00-131 Warsaw, Poland

Department of Pediatric Hematology and Oncology, Medical University of Warsaw, Warsaw, Poland

1 Introduction

Cystic fibrosis (CF) is a fatal monogenetic autosomal recessive disease that involves pathology of exocrine gland function and results in multiorgan complications, with pulmonary involvement in most cases and end-stage lung disease as a major cause of death. Due to the advances in patient care, more CF individuals reach adult age; however, they have to cope with long-term consequences including diabetes, bone disease, arthropathy, chronic kidney disease, and depression (Quon and Aitken 2012). Even though survival rates are higher year-by-year and many advances in therapy have been made, lung transplantation often remains the only effective therapeutic approach. Cystic fibrosis is caused by different mutations in cystic fibrosis transmembrane conductance regulator (CFTR) that causes dysfunctional cAMP-mediated chloride transport in exocrine epithelia and deregulated sodium transport. Those pathological changes result in defective mucociliary clearance leading to mucous obstruction, infection, inflammation, and lung scarring (Lubamba et al. 2012). CF, as a monogenic disease, seems to be an ideal candidate for gene therapy. However, intensive pre-clinical experiments have failed to show satisfactory therapeutic outcome due to existing limitations such as the absence of viral vector receptors, the presence of mucus barrier, the rapid cell turnover, the inflammatory response, and the uncertainty of which cell should be chosen as a target. The issue is further confounded by the fact that a single lung stem cell probably does not exist; rather there are several stem cell niches with different cell progenitors depending on the part of the respiratory tract. Moreover, CFTR has been found to be mostly expressed in submucosal glands, not accessible by gene therapy, although likely containing stem cells that could be targeted. Only about 10 % of the stem cell engraftment would be enough to reverse chloride defect; therefore if only chloride transport correction would be enough for symptoms amelioration, a therapeutic effect could be obtained (Spencer and Jaffe 2004).

2 Rationale for Stem Cell Therapy

In recent years, there has been a great interest in stem cell biology and potential therapeutic indications. Several studies have already demonstrated that stem cell therapy could bring benefits in treating patients with pulmonary diseases. An interesting study done by Gilpin et al. (2013) showed increases in the epithelial-like progenitors and differences in plasma cytokines responsible for the cell recruitment in the bone marrow and peripheral blood of CF patients. Those alternations might contribute to the remodeling of the lung architecture seen in cystic fibrosis. The questions concerning whether an increase in the epithelial-like progenitors is a consequence or cause of epithelial hyperproliferation in CF patients and how it might affect stem cell therapy remain to be answered. The stem cells have proved to be capable of differentiating into lung parenchyma and airway epithelial cells both *in vitro* and *in vivo*. They have a potential in treatment of chronic lung diseases with their remarkable characteristics to home into damaged tissue and the ability to contribute to organ regeneration (Loebinger et al. 2008). The idea of using hematopoietic stem cells is supported by Suratt et al. (2003) who revealed the donor-derived cells in the lungs of human female patients after hematopoietic stem cell transplantation from male donors. Until then, that phenomenon had been proven only in the murine model. In the Suratt et al.'s (2003) study, diagnostic evaluation of tissue samples was done retrospectively in patients who underwent lung biopsy or autopsy. Fluorescent *in situ* hybridization showed significant rates of epithelial (8 %) and endothelial (42 %) chimerism. Kleeberger et al. (2003) confirmed these results, detecting chimerism in a short tandem repeat analysis of tissue samples from seven human males, recipients of female lung allograft. The recipient-derived cells were integrated in the bronchial epithelium (5.7–25.5 %), type II pneumocytes (9.1–20.0 %), and seromucosal glands (9.1–24.2 %) with

higher rates in the structures with signs of chronic injury. The exact mechanism of cell incorporation remains unknown and could have to do with cell trans-differentiation, paracrine effects, cell fusion, β-catein signaling pathways, or membrane-derived microvesicles (Wang et al. 2009). A limitation of stem cell therapy in CF may be the abnormalities of immune cells that can be found in these patients. Their neutrophils are unable to cope with bacterial infections, especially with *Pseudomonas aeruginosa*. The patients' inflammatory response is normal, with influx of neutrophils in the airways. However, CF patients secrete less L-selectin and more myeloperoxidase and elastase in comparison with healthy subjects. Conese et al. (2003) suggest that neutrophilic abnormalities might open possibilities of hematopoietic stem cell as a target for therapy. The CFTR is expressed by neutrophilic phagolysosomes; therefore its function could be defective as the loss of chloride secretion impairs the hypochlorous acid production which contributes to bacterial killing (Painter et al. 2006). Some authors claim that genetic factors might cause other neutrophilic dysfunctions as well. Stem cell engraftment into the CF lungs could limit bacterial infection and excessive inflammatory response.

3 Therapeutic Approaches

3.1 Stem Cell Types

The biology of progenitor cells in the lung is complicated and has not yet been fully understood. Science has not determined which type of cells could bring the highest benefit to the patient. A better understanding of the biology of lung injury/repair and the mechanisms involved in lung diseases is needed as well. Studies confirmed that hematopoietic stem cells can convert into the lung cells (Alliota et al. 2005; Surrat et al. 2003); however, they do not seem to carry a regenerative potential. Endothelial stem cells, multipotent adult progenitor cells, induced pluripotent stem cells, mesenchymal stem cells, or circulating fibrocytes are in the area of intensive research. Mesenchymal cells, non-hematopoietic stem cells with the fibroblast-like appearance, with their biological characteristics including the ability to home into inured tissues and proliferation and differentiation potential, seem to be interesting candidates for further studies. Moreover, mesenchymal cells, with a low degree of immunogenicity, are ideal candidates for the allogeneic use (Caimi et al. 2010). Additionally, they possess immunomodulatory and anti-inflammatory properties that could contribute to CF therapy by reducing susceptibility to infections.

3.2 Source Selection

Several therapeutic approaches and different stem cell sources are considered for stem cell therapy. Bone marrow cells are most often used and frequently examined. Loi et al. (2006) hypothesized whether adult bone marrow stem cell with wild-type CFTR could result in functional CFTR expression in a mice model of CF. The engraftment rate was very low at 0.025 %; CFTR expression was detected only in 0.01 % of engrafted cells. The authors also conducted the same experiment with CD3-depleted total marrow cells, but the donor-derived epithelial stem cells were not detected. Bruscia et al. (2006) checked the same hypothesis. CFTR activity was measured in the gastrointestinal and pulmonary tracts by rectal and nasal sample analysis in transplanted mice. CFTR mRNA expression and a modest level of CFTR-dependent chloride secretion could be detected in the examined samples.

The number of studies concerning the adipose tissue-derived stem cells in human lung diseases is limited. The potential of these cells has not been examined in animal models of CF. Suzuki et al. (2008) showed that these cells accelerate neovascularization and epithelialization of regenerated trachea placed in a bioengineered scaffold.

Embryo-derived stem cells originate from a blastocyst embryo stage and can differentiate

spontaneously into tissues of all germ layers. The ability of murine embryonic stem cells to differentiate into type II alveolar epithelial cells, nonciliated secretory epithelial airway cells, and type I collagen has already been demonstrated. Coraux et al. (2005) showed that air-liquid interface-cultured embryo-derived stem cells can form fully differentiated and functional airway epithelium. Leblond et al. (2009) also confirmed the effectiveness of this source; however, still with low engraftment rates. Although the embryo-derived stem cells have high differentiation capabilities, a comprehensive view is rather unpromising. The donation process is ethically controversial and this source of stem cells has the highest tumorigenic potential, so that the perspective of clinical application is low.

Amniotic-derived stem cells can be isolated from the placenta or amniotic fluid, with no harm to the embryo. Moreover, their biology is close to that of the embryonic stem cell. The cells are superior to adult stem cells in the proliferation and differentiation capacity. Paracchini et al. (2012) demonstrated that the human amniotic mesenchymal stem cells express low levels of CFTR mRNA, but after co-culture with CF airway epithelial cells, 33–50 % of them acquired CFTR expression, and thus might contribute to correction of chloride secretion. Moreover, amniotic-derived stem cells were found to differentiate toward the hepatocyte-like cells, which might be of additional benefit considering hepatobiliary complications in CF patients.

Umbilical cord blood can be easily obtained and its harvesting is not ethically controversial; however, it is a poor source of mesenchymal stem cells. Sueblinvong et al. (2008) experimented with mesenchymal stem cells obtained from human cord blood and delivered them intravenously to non-obese diabetic mice with severe combined immunodeficiency evoked by low-dose irradiation, taken as a lung injury model. The cells were stimulated beforehand *in vitro* by culture in airway growth media or with appropriate growth factors to express appropriate markers, and were recombined with the CFTR-expressing lentiviral vector. The MSC were found to express nonciliated secretory epithelial airway cells, CFTR protein, and surfactant protein C. After application, cells of human origin were mainly detected in parenchyma; however, some of them were also found in the airway epithelium 1 day after mesenchymal stem cells injection, with the yield of 3.36 % human β2-microglobulin–positive cells (β2+), the percentage decreased subsequently. Co-staining for cytokeratine with anti-human/mouse pancytokeratin (CK) revealed 4.07 % β2+/−CK + cells in mice lungs, even though mesenchymal stem cells initially did not express cytokeratine. The percentage of these cells rose to 32.89 % 2 weeks after injection, with the most of dual-labeled cells found in the alveolar region, to decrease afterward (Sueblinvong et al. 2008).

Part of the cord tissue, called Wharton's jelly, is rich in mesenchymal stem cells. Wharton's jelly-derived mesenchymal stem cells (WJ-MSC) are more immature than those from bone marrow or adipose tissue, and thus are closer to the potential of the embryonic stem cells. Moreover, the harvesting process is a simple, ethically uncontroversial procedure and due to cells immaturity, the engraftment rates should be higher than that from bone marrow or adipose tissue. As the engraftment is the main limiting issue, the use of WJ-MSC cell could be the gold standard. The cells can be obtained after both natural deliveries and caesarian sections from healthy, non-CF carriers, and unrelated donors. A piece of umbilical cord is collected into a sterile vessel containing physiological saline with an antibiotic and is transported at 18–24 °C. Next, WJ-MSC cells are isolated and cultured in a growth medium with supplements. After the estimation of the cell number and the assessment of their viability, the cells are cryopreserved. Before use, cell counting, viability test, flow cytometric immunophenotyping, and functional *in vitro* differentiation assays should be performed again (Boruczkowski et al. 2012).

3.3 Donor Selection

Stem cells offer a favorable tandem in CF therapy: the ability of homing into injured airways and the possibility of being used as a carrier of a

corrected gene. They can be isolated, expanded, and modified to express wild-type CFTR (Leblond et al. 2009). Wang et al. (2005) hypothesized that mesenchymal stem cells with wild-type CFTR could correct the ion-associated CF defects by contributing to apical chloride secretion. The authors explored the idea of autologous bone-marrow derived mesenchymal stem cells with CFTR gene correction. They demonstrate that the mesenchymal stem cells co-cultured with airway epithelial cells (AEC) assume the epithelia-like shape. Human cytokeratine-18 (CK-18) staining suggested that some of these cells differentiate into AEC, while the mesenchymal stem cells in the control group were CK-18 negative. The authors show, using the CFTR-specific primers, that the co-cultured mesenchymal stem cells expressed CFTR. They also tested the ability of gene-corrected mesenchymal stem cells to contribute to the apical chloride secretion stimulated by cAMP in co-culture with ΔF508 cystic fibrosis AEC in a dose-depended mode. The results show an increase in chloride secretion; being, however, not directly proportional to the mesenchymal stem cell concentration.

The allogenic donor with wild-type CFTR could donate mesenchymal stem cells which are only weakly immunogenic. This may help to avoid genetic manipulation, indispensable for the CFTR gene correction. Stem cells that can be obtained through noninvasive procedures could serve as a source for repetitive infusions to maintain therapeutic effects.

3.4 Delivery Route of Stem Cell

Recent studies suggest that intratracheal application of stem cells might be more efficient since more cells will home into the desired place and a higher percentage might be incorporated. While delivering locally, potential systemic adverse effects can be reduced. Many preclinical studies advocate the intratracheal route, as systemic administration might result in poor homing into the epithelial lining. According to Lebond et al. (2009), the mean engraftment rate after systemic delivery is in a range of 0.01–0.1 %. However, imaging studies of labeled mesenchymal stem cells show that following intravenous delivery most of the cells home into the lung (Love et al. 2007). Yan et al. (2007) conclude that systemic administration of mesenchymal stem cells shortly after lung injury results in adequate engraftment, but their application at a later time might initiate fibrotic changes. Pre-clinical studies comparing the effectiveness of both routes should be carried out.

4 Barriers and Challenges

The most limiting factor for stem cell therapy to move into clinic is a low incorporation rate. Leblond et al. (2009) showed that no mesenchymal stem cells were detected in a healthy airway, but up to 5.5 % survived in damaged tissue, with the engraftment level tapering off as the new epithelium was being formed. Although this characteristic feature of stem cells being able to migrate into damaged tissue is promising, the engraftment rate is too low to restore chloride secretion. Bruscia et al. (2006) showed that bone marrow stem cells from $CFTR^{+}$ mice could engraft as epithelial cells in the lung, liver, and the gastrointestinal tract. Moreover, transplantation to irradiated newborn mice showed an engraftment boost in the respiratory and intestinal tracts in comparison with adult individuals. Both newborn and adult CF mice presented partial restoration of CFTR function. Leblond et al. (2009) also used embryonic and mesenchymal stem cells delivered intratracheally in a murine model of acute airway injury, with 0.4–5.5 % of homing into the airway after 24 h, but the engrafted cells were not detected 7 days later. The immune rejection, as a factor responsible for low homing rate, is not probable as mesenchymal stem cells are minimally immunogenic. Another option is that a newly regenerated airway epithelium might impair stem cell incorporation. The engraftment rate significantly varies in different studies from 0 up to 20 %. However, Loi et al. (2006) suggest that the incorporation rate on the higher side could be

spuriously enhanced due to the overlap with the donor-derived $CD45^+$ leukocytes. Furthermore, methods used for creating models of lung injury vary between studies; which contributes to different engraftment rates. The stem cell incorporation rate, a key parameter for successful therapy, could be improved by determining the best route of application, the optimal dosage, and the stem cell *ex vivo* manipulation and culture with appropriate cytokines or growth factors. Another crucial factor is an adequate method for detection of the donor-derived cells.

Potential adverse effects of stem cell therapy include the possibility of creating fibrotic and malignant lesions. The cells that have been described to contribute to fibrosis are probably hematopoietic stem cells of bone-marrow origin. However, it is hypothesized that systemic infusion of mesenchymal stem cells could contribute to lung fibrosis as well. The malignant changes have been observed in a few studies in animal models, including the development of sarcoma after stem cell application in mice. Additionally, some karyotype changes could occur during a passage of bone marrow stem cells, so that there is a need of monitoring for genetic stability (Loebinger et al. 2008).

5 Conclusions

Stem cell therapy seems potentially beneficial in cystic fibrosis since it is a multi-organ disease with epithelial dysfunction and many pre-clinical studies have already shown that the human mesenchymal stem cells from bone marrow, umbilical cord, and from other sources can differentiate into the airway epithelial cells.

The problem to achieve clinical benefit lies in the low extent of stem cell incorporation. Further studies on specific growth factors and culture conditions that might boost the engraftment rate are being done. Repetitive cell application in a suitable dose could lead to a satisfying engraftment with good clinical results. The determination of a therapeutically effective dose and time interval between consecutive infusions to sustain benefit plays a key role in the present-day stem cell experiments. *Ex vivo* modifications of autologous stem cells to express wild-type CFTR provide satisfactory pre-clinical results; however, the most workable and cost effective procedures are searched for. Third-party donor mesenchymal stem cells can migrate to the airway and engraft in the airway epithelium. Moreover, they are minimally immunogenic. The bone-marrow-derived mesenchymal stem cells have limitations, including a relatively low frequency of these cells, the adult type of cell, and an invasive harvesting procedure. The adipose tissue-derived mesenchymal stem cells share the same drawbacks, except for the collection method, but almost no experimental studies have been conducted using these cells in lung diseases. As the umbilical cord blood is a poor stem cell source, Wharton's jelly-derived mesenchymal stem cells seem to be a natural choice with their favorable properties of a high differentiation capability and an easy, uncontroversial harvesting procedure.

The results of recent studies provide proof of principle for using the mesenchymal stem cells in treating patients with cystic fibrosis. However, further experiments are still needed. Pre-clinical studies, beyond determining the best therapeutic approach, should also focus on the long-term follow-up of stem cell therapy and clinical trials have to establish the safety measures and adequate methods of engraftment monitoring.

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Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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Fatigue in Sarcoidosis and Exercise Tolerance, Dyspnea, and Quality of Life

D. Jastrzębski, D. Ziora, M. Lubecki, K. Zieleźnik, M. Maksymiak, J. Hanzel, A. Początek, A. Kolczyńska, L. Nguyen Thi, A. Żebrowska, and J. Kozielski

Abstract

Fatigue is one of the major symptoms reported by sarcoidosis patients. The relationship between fatigue and clinical course of sarcoidosis remains unclear. The aim of the study was to evaluate the relationship between fatigue and lung function tests, exercise tolerance, dyspnea, and quality of life among sarcoidosis patients. One hundred eleven sarcoidosis patients completed the following questionnaires: Fatigue and Assessment Scale (FAS), Quality of Life Scale (SF-36), and dyspnea scales: Medical Research Council Questionnaire, Baseline Dyspnea Index, and Oxygen Cost Diagram. Clinical parameters (FVC, FEV1, DL_{CO}, VO_2, and VO_2/AT, and work load) were derived from the patients' medical files. The exercise tolerance was the only clinical parameter associated with fatigue (Max. Work Load −0.65, VO_2 −0.42, VO_2/AT −0.51). No correlations were found between FAS and spirometry or diffusing tolerance. Fatigue correlated with all dyspnea domains by means of (r values ranging from 0.47 to 0.77 in multivariate regression analysis) and with quality of life in SF-36 questionnaire (r values ranging from −0.33 to −0.83). We conclude that FAS seems a reliable and valid indicator of dyspnea level, quality of life, and exercise tolerance in sarcoidosis patients.

Keywords

Dyspnea • Exercise ability • Fatigue • Lung function tests • Quality of life • Sarcoidosis

D. Jastrzębski (✉), D. Ziora, M. Lubecki, K. Zieleźnik, M. Maksymiak, J. Hanzel, A. Początek, A. Kolczyńska, L. Nguyen Thi, and J. Kozielski
School of Medicine with the Division of Dentistry, Department of Lung Diseases and Tuberculosis, Medical University of Silesia, 41-803, 1 Koziolka St., Zabrze, Poland
e-mail: darekjdr@poczta.onet.pl

A. Żebrowska
Department of Physiology and Medicine of Sport, Academy of Physical Education, Katowice, Poland

1 Introduction

Sarcoidosis is a disseminated granulomatous disease of unknown aetiology. The clinical course of sarcoidosis is variable, and every organ can be involved. More than 90 % of sarcoidosis patients have changes in the lungs, but many patients also

have extra-pulmonary manifestations, such as ocular, cutaneous, or muscle (Morgenthau and Iannuzzi 2011). Apart from the lung-related symptoms (e.g., coughing, breathlessness, and dyspnea on exertion), patients may suffer from a wide range of non-specific disabling symptoms, such as fatigue, general weakness, fever, anorexia, arthralgia, muscle pain, and muscle weakness. These symptoms do not correspond with the objective physical evidence of disease (Iannuzzi and Fontana 2011). Despite the fact that fatigue is the main problem in sarcoidosis patients (30–90 % reported prevalence) affecting their quality of life, it still remains underestimated and poorly understood (De Vries et al. 2004). The possible factors related to fatigue are general inflammation, sleeping disorders, depression, and small-fibre neuropathy (De Vries and Wirnsberger 2005). Fatigue could be explained by peripheral muscle weakness and exercise intolerance. Although the effects of exercise tolerance on fatigue have not been studied in sarcoidosis yet, reduced tolerance of exercise and general weakness are frequently reported symptoms. The primary aim of our study was to assess the prevalence of fatigue and exercise tolerance in sarcoidosis patients. Additionally, the predictive value of fatigue and the clinical characteristics including lung function tests, exercise tolerance, perceived dyspnea, and general health quality of life were studied.

2 Methods

2.1 Subjects

The study was approved by the Bioethics Committee of the Medical University of Silesia, Poland and written informed consent was obtained from all subjects. One hundred and eleven, non-smoking symptomatic (fatigue, chronic cough) sarcoidosis patients, aged 34–63 (mean 44.8 ± 10.4 years) referred to the Department of Lung Diseases and Tuberculosis of the Medical University of Silesia between January 2011 and December 2012 were included in the study. The patients were diagnosed based on consistent clinical features, bronchoalveolar lavage fluid analysis, and biopsy-proven noncaseating epithelioid cell granulomas, according to the World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) guidelines (Hunninghake et al. 1999). Only patients with Stage II sarcoidosis were included into the study. Forty four percent of the patients received corticosteroids orally in daily doses equivalent to 8–30 mg methylprednisone during the 6–12 months preceding the study. A healthy control group, 52 men and 13 women age-matched, was recruited from hospital employees and the surrounding community.

2.2 Physiological Measurements

An inclusion, forced vital tolerance (FVC) and forced expiratory volume in 1 s (FEV1) were measured with a pneumotachograph (Masterlab; Jaeger, Wurzburg, Germany). The diffusing tolerance of lung for carbon monoxide (DL_{CO}) was measured by a single-breath method. The results were normalized to the reference values proposed by the European Community for Coal and Steel and presented as a percentage of the predicted value (%pred) (Quanjer et al. 1993). Peak exercise tolerance was assessed by a maximal incremental walking-track test. After 2 min of resting period and 3 min of unloaded walking, patients started at 30 W and walked at an incremental workload (+30 Watt/3 min) until symptomatic limitation. At the test end, a Borg score for dyspnea was collected. Peak external work rate and peak oxygen uptake were normalized to height, age, and gender (Wasserman et al. 1999). The results of exercise test were expressed as peak oxygen consumption (VO_2; ml/kg/min), its relation to anaerobic threshold (VO_2/AT; ml/min), and maximal work load (Watt).

2.3 Rating of Fatigue, Dyspnea, and Quality of Life

Fatigue was measured with a 10-item Fatigue Assessment Scale (FAS), which indicates both physical and psychological fatigue. Each item has a five-point rating scale and the FAS score ranges from 10 to 50. A score <22 indicates

non-fatigued patients, 22–34 indicates fatigued patients, and >35 indicates extreme fatigue (Michielsen et al. 2004). Three different methods were used for rating dyspnea: a modification of the Medical Research Council Questionnaire (MRC) (Fletcher 1960), Baseline Dyspnea Index (BDI) (Baddini Martinez et al. 2002), and Oxygen Cost Diagram (OCD) (Baddini Martinez et al. 2002). BDI describes dyspnea in five steps integrated into three categories: functional impairment (FI), and magnitude of task (MT) and effort (ME). Details of methodological aspects of dyspnea rating and quantification using the methods above outlined were described previously (Jastrzębski et al. 2005). The SF-36 questionnaire (Ware et al. 2004) was used to estimate quality of life. The questionnaire consists of 36 questions, which include the following basic domains describing the health status: physical functioning (PF), role physical (RP), bodily pain (BP), general health (GH), vitality (VT), social functioning (SF), role emotional (RE), and mental health (MH). A higher score denotes a higher quality of life. Methodological rules and data analysis of the SF-36 questionnaire have been described previously (Jastrzębski et al. 2005).

Table 1 Characteristics of subjects

	Sarcoidosis ($n = 111$)	Controls ($n = 65$)	p
Age (years)	44.8 ± 10.4	43.3 ± 12.4	
BMI (kg/m^2)	28.2 ± 3.9	26.8 ± 5.6	0.091
FAS	22.9 ± 7.9	17.0 ± 3.3	0.046
MRC	1.3 ± 0.8	1.1 ± 0.3	0.028
OCD	1.6 ± 0.8	1.1 ± 0.3	0.025
Borg's scale	0.7 ± 1.3	0.1 ± 0.1	0.010
FI	1.7 ± 1.1	1.1 ± 0.3	0.007
MT	1.8 ± 0.9	1.2 ± 0.5	0.032
ME	1.9 ± 1.0	1.2 ± 0.5	0.006
FI + MT + ME	5.5 ± 2.9	3.5 ± 1.2	0.004
PF	50.7 ± 7.4	54.8 ± 4.0	0.007
RP	45.2 ± 10.7	53.7 ± 5.4	0.003
BP	46.3 ± 10.0	51.1 ± 10.8	0.106
GH	40.2 ± 11.7	51.0 ± 4.8	0.001
VT	49.9 ± 9.7	54.9 ± 10.7	0.061
SF	47.1 ± 12.1	49.5 ± 9.7	0.160
RE	50.3 ± 12.1	52.0 ± 7.6	0.065
MH	43.7 ± 10.6	47.9 ± 9.0	0.238
PCS	46.4 ± 9.2	54.1 ± 5.1	0.010
MCS	47.3 ± 11.4	49.2 ± 9.9	0.176

Values are means ± SD
BMI Body Mass Index, *FAS* Fatigue Assessment Scale, *MRC* Medical Research Council questionnaire, *OCD* Oxygen Cost Diagram, *FI* Functional Impairment, *MT* Magnitude of Task, *ME* Magnitude of Effort, *PF* Physical functioning, *RP* Role Physical, *BP* Bodily Pain, *GH* General Health, *VT* Vitality, *SF* Social Functioning, *RE* Role Emotional, *MH* Mental Health, *PCS* Physical Cumulative Score, *MCS* Mental Cumulative Score

2.4 Statistical Evaluation

Data are presented as means ± SD. The intergroup correlation coefficients were determined with Pearson's rank order test. Statistical significance was set at $p < 0.05$. Statistical analysis was performed using a commercial Statistica package.

3 Results

Demographic data and questionnaire scores for fatigue, dyspnea, and quality of life for the sarcoidosis and control subjects are presented in Table 1. The mean body mass index (BMI) was in the overweight range for both groups. The mean FAS score was significantly higher in the sarcoidosis group and amounted to >22 points, which indicates fatigue complaints. The complaints were reported in 53 (48 %) patients, and 6 (11 %) of these patients reported extreme fatigue (FAS >35). In contrast, fatigue complaints were observed only in 8 (12 %) control subjects. Woman patients reported more fatigue than men; FAS 25.2 *vs.* 21.1, respectively.

Significant differences between sarcoidosis patients and controls were observed in the perception of dyspnea. These differences were confirmed in all dyspnea questionnaires used (MRC, OCD, Borg's scale, and BDI). The quality of life estimation showed inappreciable differences between sarcoidosis and control subjects in the SF-36 domains that reflect mental aspects: BP, VT, RE, MH and MCS. There were, however, significant differences in the domains that reflect physical aspects: PF (50.7 *vs.* 54.8; $p = 0.007$), RP (45.2 *vs.* 53.7; $p = 0.003$), GH (40.2 *vs.* 51.0; $p = 0.001$), and PCS (46.4 *vs.* 54.1; $p = 0.01$,

respectively) (Table 1). The results of lung function and cardiopulmonary exercise tests were in the patients within normal range.

3.1 Sarcoidosis Patients Stratified by Steroids Consumption

There were no differences in age and BMI between sarcoidosis patients taking and non-taking steroids (Table 2). Nor were there differences in the mean FAS results of sarcoidosis patients regarding steroid consumption. The patients on steroids had a higher dyspnea score than those not taking steroids only in the MRC questionnaire (1.4 *vs.* 1.2, respectively; $p = 0.043$); other dyspnea, quality of life, and lung function results were inappreciably different between these two subgroups of sarcoidosis patients.

3.2 Correlations Between Fatigue and Clinical Parameters

Correlations between FAS and clinical parameters were summarized in Table 3. To reduce the impact of possible confounding variables, the values of functional tests were normalized for

Table 2 Physical and clinical characteristics of the sarcoidosis patients stratified by steroids consumption

Clinical data	Taking steroids	Non-taking steroids	p
Gender (F/M)	3/45	1/46	0.039
Age (years)	45.1 ± 10.2	43.4 ± 11.3	0.232
BMI (kg/m^2)	27.8 ± 2.9	28.4 ± 4.8	0.414
FAS	21.7 ± 6.9	22.0 ± 7.9	0.819
MRC	1.4 ± 0.6	1.2 ± 0.6	0.043
OCD	1.7 ± 0.9	1.4 ± 0.7	0.357
FI	1.8 ± 1.0	1.5 ± 1.0	0.305
MT	2.0 ± 1.0	1.4 ± 0.8	0.102
ME	2.1 ± 1.0	1.6 ± 0.9	0.125
FI + ME + MT	6.1 ± 2.9	4.6 ± 2.6	0.096
PF	47.5 ± 9.4	53.2 ± 5.0	0.485
RP	38.5 ± 12.0	50.5 ± 6.3	0.505
BP	41.8 ± 8.2	49.9 ± 12.4	0.657
GH	36.7 ± 10.0	43.0 ± 13.3	0.568
VT	48.1 ± 12.3	51.4 ± 8.3	0.800
SF	39.1 ± 14.3	53.5 ± 4.8	0.950
RE	47.1 ± 17.5	52.7 ± 6.9	0.680
MH	43.6 ± 11.1	43.8 ± 11.4	0.704
PCS	41.0 ± 9.6	50.6 ± 7.0	0.590
MCS	45.3 ± 16.0	48.8 ± 7.7	0.547
FVC (%pred)	90.4 ± 16.5	95.2 ± 17.1	0.810
FEV1 (%pred)	90.4 ± 16.5	95.2 ± 17.1	0.850
DLCOSB (%pred)	81.7 ± 29.0	91.0 ± 23.2	0.684
Max. Work Load (Watts)	173.6 ± 46.5	134.3 ± 36.8	0.678
Peak VO_2 (ml/kg/min)	26.3 ± 5.5	28.2 ± 8.7	0.832
VO_2/AT (ml/kg)	53.05 ± 10.2	58.1 ± 16.7	0.765

Values are expressed as means ± SD, except for gender

BMI Body Mass Index, *FAS* Fatigue Assessment Scale, *MRC* Medical Research Council questionnaire, *OCD* Oxygen Cost Diagram, *FI* Functional Impairment, *MT* Magnitude of Task, *ME* Magnitude of Effort, *PF* Physical functioning, *RP* Role Physical, *BP* Bodily Pain, *GH* General Health, *VT* Vitality, *SF* Social Functioning, *RE* Role Emotional, *MH* Mental Health, *PCS* Physical Cumulative Score, *MCS* Mental Cumulative Score, *FEV1* Forced Expiratory Flow in 1 s, *FVC* Forced Vital Tolerance, *DLco* Diffusing Lung Capacity for Carbon Monoxide in single breath technique, *VO_2* Peak Oxygen Uptake, *VO_2/AT* Maximal oxygen uptake in relation to anaerobic threshold

Table 3 Correlation between fatigue (FAS), clinical data, and dyspnea (SF-36) in sarcoidosis patients

Clinical data	FAS	Dyspnea	FAS	SF-36	FAS
DLCO	−0.16	MRC	0.47	PF	−0.76
FEV1	−0.27	OCD	0.65	RP	−0.78
FVC	−0.22	FI	0.72	BP	−0.33
Max. Work Load	−0.65	MT	0.73	GH	−0.64
VO_2	−0.42	ME	0.72	VT	−0.81
VO_2/AT	−0.51	FI + MT + ME	0.77	SF	−0.75
				RE	−0.84
				MH	−0.67

FAS Fatigue Assessment Scale, *DLCO* Diffusing Lung Capacity for Carbon Monoxide in single breath technique, *FEV1* Forced Expiratory Flow in 1 s, *FVC* Forced Vital Tolerance, VO_2 Peak Oxygen Uptake, VO_2/*AT* Maximal oxygen uptake in relation to anaerobic threshold, *MRC* Medical Research Council questionnaire, *OCD* Oxygen Cost Diagram, *FI* Functional Impairment, *MT* Magnitude of Task, *ME* Magnitude of Effort, *PF* Physical functioning, *RP* Role Physical, *BP* Bodily Pain, *GH* General Health, *VT* Vitality, *SF* Social Functioning, *RE* Role Emotional, *MH* Mental Health

age, sex, height, and body weight by expressing them as percent of the predicted values. Neither FVC, nor FEV1 and DLCO were associated with FAS ($r = -0.22$, $r = -0.21$, $r = -0.16$, respectively). On the other hand, the ability to exercise expressed in Watt was significantly associated with FAS ($r = -0.77$), as were VO_2 ($r = -0.42$) and VO_2/AT ($r = -0.51$). Contrary to MRC, perception of dyspnea and quality of life, especially in the domains that indicated the extent to physical health or emotional problems interfering with normal social activities, were strongly associated with FAS. The highest correlation was observed between FAS and the SF-36 domains such as: RP ($r = -0.78$), VT ($r = -0.81$), RE ($r = -0.84$), and MH ($r = -0.67$). No correlation was found between FAS and bodily pain (BP; $r = -0.33$).

4 Discussion

Fatigue is a major symptom in sarcoidosis and it affects different aspects of quality of life. De Vries et al. (2004) reported a wide range of fatigue rates (30–90 %) in sarcoidosis patients. In the present study the prevalence of fatigue was 53 %, out of which 11 % had the FAS score greater than 35 indicating extremely fatigued patients. Marcellis et al. (2011) reported the prevalence of fatigue of 81 %, but patients in that study suffered from severe sarcoidosis. In the present study we included patients with Stage II sarcoidosis without serious complications, which, in all likelihood, was a reason that the observed prevalence of fatigue was less. However, similarly to Marcellis et al. (2011) we found an association between fatigue and exercise tolerance and the lack of correlation between fatigue and lung function results or steroid consumption. These results are in line with other studies (Drent et al. 2012; Michielsen et al. 2006; Spruit et al. 2005; De Vries et al. 2004), where no relations between fatigue and a number of clinical variables, including pulmonary function, metabolic variables, laboratory parameters of inflammation and T-cell activation, and granulomas formations were found.

This study demonstrates that fatigue in sarcoidosis affected the patients' quality of life, which was underscored by the relationships between the FAS score and different types of quality questionnaires. Michielsen et al. (2006) reported a strong correlation between FAS and the quality of life domains: physical and psychological health, as well as the level of independence. A high correlation between fatigue and the level of independence supports the idea that fatigue affects daily life. In addition, we found associations between fatigue and both physical and mental scores of quality of life domains. It is worth noting that mental aspects of life were subject to stronger associations with fatigue than physical ones. Fatigue seems to be a debilitating symptom, affecting one's capability to live a self-governed live. Patients suffering from chronic fatigue syndrome reported a reduced social support network and less participation in social activities.

In conclusion, the present study shows that fatigue is a major clinical problem in sarcoidosis, which affects various domains of quality of life. Moreover, in rapport with other authors (De Vries et al. 2004; Drent et al. 1998; Wirnsberger et al. 1998) the study underlines that regularly performed spirometric measurements or estimation of diffusing tolerance are not helpful in depicting the real impact of the disease on patients' everyday lives. The only true reflection of the level of fatigue in sarcoidosis patients seems the measurement of exercise capacity. Treatment of sarcoidosis should not only concentrate on improving clinical parameters, but it also should take into account the subjective experience of fatigue. In our opinion, fatigue measurement, which is still of interest in follow-up observation, should be introduced into a clinical routine in sarcoidosis patients.

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Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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The rs1800471 Polymorphism of *TGFB1* Gene, Serum TGF-Beta1 Level and Chronic Kidney Disease Progression

K. Kiliś-Pstrusińska, A. Mastalerz-Migas, D. Zwolińska, W. Grzeszczak, K. Zachwieja, J. Zachwieja, K. Madziarska, and L. Hyla Klekot

Abstract

The aim of the study was to investigate whether rs1800471 polymorphism in *TGFB1* gene is associated with the development and progression of non-diabetic chronic kidney disease. Moreover, we examined the serum TGF-beta1 concentration and its association with that polymorphism and progression of the disease. We applied two different methodological approaches. Firstly, a family based study was carried out, comprised of 109 patients with non-diabetic chronic kidney disease and their 218 healthy parents, using the transmission/disequilibrium test. The rs1800471 polymorphism and serum TGF-beta1 level were determined in all subjects. Serum TGF-beta1 concentration was also measured in 40 healthy controls. Secondly, we performed a case-control orientated study to determine whether rs1800471 polymorphism and other factors influence the progression of renal impairment. We found no relationships between rs1800471 polymorphism allele transfer and the incidence or progression of non-diabetic chronic kidney disease. We found, however, that the serum TGF-beta1 was significantly higher in patients than in controls. In conclusion, rs1800471 polymorphism in *TGFB1* gene does not have an impact on the development and progression of non-diabetic

K. Kiliś-Pstrusińska (✉) and D. Zwolińska
Department of Pediatric Nephrology, Wroclaw Medical University, 213 Borowska St., 50-556 Wrocław, Poland
e-mail: katarzyna.kilis-pstrusinska@umed.wroc.pl

A. Mastalerz-Migas
Department of Family Medicine, Wroclaw Medical University, Wrocław, Poland

W. Grzeszczak
Department of Internal Medicine, Diabetology and Nephrology, Silesian Medical University, Zabrze, Poland

K. Zachwieja
Department of Paediatric Nephrology, Dialysis Unit, UJ CM, Kraków, Poland

J. Zachwieja
Department of Pediatric Cardiology and Nephrology, Medical University, Poznań, Poland

K. Madziarska
Department and Clinic of Nephrology and Transplantation Medicine, Wroclaw Medical University, Wrocław, Poland

L. Hyla Klekot
Pediatrics and Oncology Center, Chorzów, Poland

chronic kidney disease caused by primary glomerulopathy and chronic interstitial nephritis. The increased serum TGF-beta1 concentration in such patients suggests its role in the pathomechanism of the disease. Circulating TGF-beta1 level is determined in a multifactorial way, not by rs1800471 polymorphism in *TGFB1* gene.

Keywords
Chronic kidney disease • Family-based study • Modifiable factors • Polymorphism • *TGFB1* gene • Transmission/disequilibrium test

1 Introduction

Chronic kidney disease tends to progress to end-stage renal disease. A wide range of genetic, environmental and modifiable factors such as hypertension and proteinuria may influence the rate of chronic kidney disease progression (El Nahas 2005). Histologically, kidney tissue fibrosis and subsequent sclerosis is a common pathological phenomenon characteristic for end-stage renal disease (Klahr and Morrissey 2003; Border and Noble 1994). Of the cytokines implicated in that process, transforming growth factor beta1 (TGF-beta1) has been singled out as the most fibrogenic (Böttinger 2007; Rao et al. 2007; Babel et al. 2006). TGF-beta1 is a multipotential cytokine that regulates cell growth, its differentiation, processes of apoptosis, extracellular matrix production as well inducing fibrosis in a variety of tissues (Blobe et al. 2000).

The *TGFB1* gene is located at 19q13.1–13.3.9. There have been several polymorphisms in *TGFB1* gene identified to date, among them rs1982073 (+869T/C), rs1800471 (+915G/C) and rs1800469 (−509C/T). It is believed that carriers of G allele of rs1800471 polymorphism and carriers of C allele of rs1982073 polymorphism have a higher amount of plasma TGF-beta1 (Grainger et al. 1999). However, connections have been described between C allele of the rs1982073 polymorphism and lower protein production, and T allele with higher protein production (Mittal and Manchanda 2007).

The role of rs1800469 polymorphism of *TGFB1* gene in the occurrence and progression of renal disease is not clear. In the study performed by Khalil et al. (2005) it was found that among Caucasians with chronic kidney disease of different origin, the TT homozygote carriers of rs1800469 polymorphism and C allele of rs1982073 polymorphism are more likely to develop kidney failure. Moreover, there was an association of rapid progression of chronic kidney disease with GG genotype of rs1800471 polymorphism. Coll et al. (2004), on the contrary, reported no effect of rs1800471 and rs1982073 polymorphisms on the progression of renal disease. Summers et al. (2005), in a study performed among patients with chronic glomerulonephritis, did not report any association between rs1800471 and rs1982073 polymorphisms and the progression of chronic kidney disease either. Patel et al. (2005) found a predisposition to diabetic nephropathy occurrence among Caucasians with type 1 diabetes mellitus carrying TT genotype of rs1982073 polymorphism. Other authors (McKnight et al. 2007; Ng et al. 2003) did not report increased susceptibility to diabetic nephropathy occurrence associated with either rs1982073 or rs1800471 polymorphism of *TGFB1* gene.

Taking into consideration these ambiguous results, our study was aimed at determining whether rs1800471 polymorphism in *TGFB1* gene is associated with the development and progression of non-diabetic chronic kidney disease. Moreover, we analyzed serum TGF-beta1

concentration in patients with chronic kidney disease, its association with the polymorphism outlined above and progression of the disease.

We applied two different approaches in our work. Firstly, a family based study was carried out in families in which children were diagnosed with chronic kidney disease. The family study included the transmission/disequilibrium test (TDT), which evaluates the transmission frequency of specific alleles of a given polymorphism from heterozygous parents to offspring affected with the investigated phenotype (Ewens and Spielman 1995, 2005). Secondly, we performed a case-control orientated study to determine whether rs1800471 polymorphism in *TGFB1* gene and other factors influence the progression of renal impairment.

2 Methods

The parents and children over the age of 16 gave their written informed consent. The study protocol adhered to the Declaration of Helsinki of 1975 as revised in 1997, and was approved by the Ethics Committee of Wroclaw Medical University.

2.1 Subjects

2.1.1 Patients

One hundred nine children and young adults with chronic kidney disease, stages 3–5 (according to K/DOQI guidelines (K/DOQI Clinical Practice Guidelines 2002)) due to primary renal disease and their 218 biological parents with no renal dysfunction (in total, 327 people) were included in the study. The underlying causes of the kidney disease were chronic primary glomerulonephritis (CGN, n = 30) and chronic interstitial nephritis (CIN, n = 79). We excluded family trios where the kidney disease was attributed to diabetic nephropathy, polycystic kidney disease or other heritable conditions (for example, Alport's syndrome), traumatic or drug-induced kidney injury, and unknown etiologies.

Chronic primary glomerulonephritis was diagnosed on the basis of clinical criteria and the renal biopsy in all cases. The histopathological findings in renal biopsy were: minimal change disease in 3 cases, focal and segmental glomerulosclerosis – 9, mesangial proliferative glomerulonephritis – 11, mesangiocapillary glomerulonephritis – 5, and extracapillary glomerulonephitis in 2 cases. Of the 79 patients with chronic interstitial nephritis, 16 had reflux nephropathy, 26 had obstructive nephropathy, 20 had chronic pyelonephritis with other urinary tract defects, and 17 had chronic pyelonephritis without urinary tract anomalies. The patients were treated conservatively (n = 51) or required renal replacement therapy (hemodialysis, n = 17; peritoneal dialysis, n = 33; and transplantation, n = 8). The demographic data of the patients were as follows: 48 females, 61 males, current age 15.4 ± 6.4 (range 0.7–25.0) years, age at the diagnosis of chronic kidney disease (minimum stage 2) 7.8 ± 6.7 (range 0.01–22.0) years, disease duration 8.9 ± 5.6 (range 0.01–21.0) years.

Data concerning the history of kidney disease were collected from all patients. Many features were taken into consideration. To enumerate them: 24-h proteinuria, serum cholesterol levels, and the presence of hypertension at two time points, i.e., at the time of chronic kidney disease diagnosis (minimum stage 2 according to K/DOQI) (K/DOQI Clinical Practice Guidelines for CKD 2002) and at the time of the study. We also analyzed serum creatinine concentrations and their changes during the course of the disease and loss of glomerular filtration rate (GFR) from the initial diagnosis (minimum stage 2) to the time of the study or the development of stage 5 of the disease, requiring dialysis or transplantation. GFR was estimated using the Schwartz formula in children and the Cocroft-Gault formula in young adults. Finally, the patients with chronic kidney disease were divided on the basis of their renal function into two groups: (1) patients with rapid progression of the disease (n = 54), defined as starting renal replacement therapy during the 5-year follow-up period from the diagnosis of chronic kidney disease stage 2 and/or with a

Table 1 Characteristics of patients with chronic kidney disease (CKD)

	Rapid progression ($n = 54$)	Slow progression ($n = 55$)	p
Age at examination (years)	14.6 ± 7.5	16.5 ± 5.1	NS
	(0.7–24.0)	(7.3–25.0)	NS
Age at CKD diagnosis (years)	9.9 ± 6.8	5.6 ± 6.1	**0.001**
	(0.01–22.0)	(0.1–20.0)	
Etiology of CKD:			
Chronic primary glomerulonephritis	24	6	
Chronic interstitial nephritis	30	49	
CKD duration (years)	2.6 ± 1.6	11.3 ± 4.8	**0.0001**
	(0.01–6.0)	(3.0–22.5)	
Proteinuria/CKD (g/24 h)	2.66 ± 4.32	0.59 ± 1.53	**0.00001**
	(0–25)	(0–11)	
Proteinuria/E (g/24 h)	1.23 ± 2.16	0.59 ± 0.99	NS
	(0–10)	(0–6.2)	NS
Serum cholesterol/CKD (mmol/l)	5.55 ± 2.14	4.39 ± 0.95	**0.0015**
	(2.1–12.0)	(3.0–8.5)	
Serum cholesterol/E (mmol/l)	4.90 ± 1.80	4.90 ± 0.90	NS
	(2.9–11.4)	(3.1–6.9)	NS
Presence of hypertension/CKD, n (%)	27 (50)	15 (27)	**0.01**
Presence of hypertension/E, n (%)	27 (50)	15 (27)	**0.01**

Data are means ± SD (range). Parameters were evaluated: at the time of CKD diagnosis, minimum stage 2 by KDOQI (CKD) and at the time of examination (E). Mann-Whitney U test and chi-squared test were used
NS non-significant

doubling creatinine level during the follow-up period, and (2) patients with slow progression of chronic kidney disease (n = 55), i.e., those who had stable renal function. Characteristics of the study groups were shown in Table 1.

2.1.2 Parents of Patients Chronic Kidney Disease

The mean age of the mothers and fathers was 42.3 ± 7.7 and 45.2 ± 7.8 years, respectively. None of the parents presented a documented renal dysfunction, diabetes or impaired fasting glucose, while hypertension (defined as systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg documented in the medical records on at least two separate occasions, or antihypertensive treatment) was present in 3.6 % (n = 4) and 9.8 % (n = 11) of the examined mothers and fathers, respectively.

2.1.3 Control Group

The control group served only for the comparison of the serum TGF-beta1 concentration with that in patients. The group consisted of children and young adults without clinical signs and family history of renal disease (n = 40; mean age 14.6 ± 4.8 years; gender: 50 % male, 50 % female).

2.2 Genotyping

2.2.1 The rs 1800471 Polymorphism

The rs1800471 polymorphism in *TGFB1* gene was genotyped in the family trios: patients affected with chronic kidney disease and their both parents. Genomic DNA was extracted from frozen whole blood samples containing EDTA as an anticoagulant using the MasterPure TM DNA purification kit (Epicentre Technologies; Madison, USA) and resuspended in TE. Genotyping was performed by the method described previously by Wood et al. (2000). Primers (Tip MolBiol; Berlin, Germany) used for amplification were as follows: forward 5′ TTC CCT CGA GGC CCT CCT A 3′; and reverse: 5′ GCC GCA GCT TGG ACA GGA TC 3′. PCR amplification was performer in a

total volume of 25 μl which contained 100 ng of genomic DNA. The reaction mixture contained: 10 mmol/l Tris HCl, pH 8.8, 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 0.1 % Triton X-100, 2.5 mmol/l of each deoxynucleotide triphosphate, 10 pmol of each primer, 1.5 mmol/l magnesium chloride and 0.6 U of thermostable Taq DNA polymerase (DyNAzyme TM II, Finnzymes; Vantaa, Finland). PCR amplification consisted of an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were digested with Bgl I enzyme (Fermentas; Ottawa, Canada) for the detection of polymorphism +915G/C of *TGFB1* gene. All samples were analyzed on 4 % agarose gel electrophoresis and visualized by ethidium bromide staining in UV light (Vilber Loumat transilluminator UV; Marne-la-Vallée, France). Genotyping was performed in a blinded fashion.

2.2.2 Other Data Processing

We tested the transmission of alleles of rs1800471 polymorphism in *TGFB1* gene from heterozygous parents to offspring affected with chronic kidney disease using TDT. We analyzed also whether rs1800471 polymorphism in *TGFB1* gene had any impact on the loss of glomerular filtration rate (rapid *vs*. slow chronic kidney disease progression).

Serum TGF-beta 1 concentration was measured with an immunoenzymatic method using ELISA kits (R&D Systems; Minneapolis, USA). The results were expressed in ng/ml.

2.3 Statistical Analysis

Basic characteristics for qualitative parameters were shown as absolute numbers and percentages. Quantitative data was expressed as means ± SD. The normality of distribution of the continuous variables was checked with the Shapiro-Wilk test. In the statistical analysis, ANOVA Kruskal-Wallis test, Mann Whitney U test, and chi-squared test were used.

The TDT was used in the family-based study. The observed transmission was compared with the transmission expected for no association (i.e., random transmission, 50:50 %), using McNemar's test (Ewens and Spielman 1995, 2005). Alleles transmitted significantly greater than 50 % of the time to an affected offspring provide evidence of disease association.

$P < 0.05$ was considered significant. All calculations were performed using Statistica 7.1.

3 Results

The characteristics of the two study groups according to progression of chronic kidney disease were shown in Table 1. The mean ages, 24-h proteinuria, and serum cholesterol of the patients at the time of the study were similar in both groups. However, patients with fast progression of the disease had higher 24-h proteinuria, serum cholesterol, and blood pressure at the time of diagnosis and higher blood pressure at the time of examination than the patients with slow progression of the kidney disease. Kidney disease caused by chronic glomerulonephritis was more frequently associated with fast progression, compared with that caused by chronic interstitial nephritis.

There were only GG and CG genotypes of rs1800471 polymorphism among patients with chronic kidney disease and their parents. The distributions of genotypes and alleles in the studied groups were shown in Table 2. The differences in genotypes and alleles frequencies between patients from CGN group and CIN group were insignificant (chi-square test; $p = 0.35$). Lack of association between the analyzed polymorphism and loss of glomerular filtration rate (fast *vs*. slow progression of disease) was found in the total group of patients and in the subgroups with different disease etiology (Table 2).

The rs1800471 polymorphism in *TGFB1* gene alleles transmission from heterozygous parents to their affected children was not different from a random proportion expected for no association, in the whole group of subjects and in the

Table 2 Distribution of genotypes and alleles of rs 1800471 (+915G/C) polymorphism in *TGFB1* gene in the groups of patients with chronic kidney disease (CKD) and their parents

	All CKD patients ($n = 109$)		CGN ($n = 30$)		CIN ($n = 79$)	
Genotype, n (%)						
GG	96 (88.1)		25 (83.3)		71 (89.9)	
CG	13 (11.9)		5 (16.7)		8 (10.1)	
Allele, n (%)						
G	205 (94.0)		55 (91.7)		150 (94.9)	
C	13 (6.0)		5 (8.3)		8 (5.1)	
	Rapid progression ($n = 54$)	Slow progression ($n = 55$)	Rapid progression ($n = 24$)	Slow progression ($n = 6$)	Rapid progression ($n = 30$)	Slow progression ($n = 49$)
Genotype, n (%)						
GG	46 (85.2)	50 (90.9)	20 (83.3)	5 (83.3)	26 (86.7)	45 (91.8)
CG	8 (14.8)	5 (9.1)	4 (16.7)	1 (16.7)	4 (13.3)	4 (8.2)
Allele, n (%)						
G	100 (92.6)	105 (95.5)	44 (91.7)	11 (91.7)	56 (93.3)	94 (95.9)
C	8 (7.4)	10 (4.6)	4 (8.3)	1 (8.3)	4 (6.7)	4 (4.1)
	Parents of all children ($n = 218$)		Parents of CGN children ($n = 60$)		Parents of CIN children ($n = 158$)	
Genotype n (%)						
GG	188 (86.2)		50 (83.3)		138 (87.3)	
CG	30 (13.8)		10 (16.7)		20 (12.7)	
Allele n (%)						
G	316 (71.48)		110 (91.7)		296 (93.7)	
C	120 (27.52)		10 (8.3)		20 (6.3)	

CGN chronic primary glomerulonephritis, *CIN* chronic interstitial nephritis

Table 3 Transmission of alleles rs1800471 (+915G/C) polymorphism in *TGFB1* gene from heterozygous parents to offspring with chronic kidney disease (*CKD*) (transmission/disequilibrium test results)

	Allele C transmitted		Allele G transmitted		
	Observed/expected		Observed/expected		p
Groups↓	Yes	No	Yes	No	
All patients	14/16	18/16	18/16	14/16	NS
CGN	5/5	5/5	5/5	5/5	NS
CIN	7/10	13/10	13/10	7/10	NS

CGN chronic primary glomerulonephritis, *CIN* chronic interstitial nephritis, *NS* non-significant

subgroups, depending on the etiology of the kidney disease (chronic primary glomerulonephritis, chronic interstitial nephritis) (Table 3). Based on TDT results, no relationships were found between rs1800471 polymorphism allele transfer and chronic kidney disease incidence.

The concentration of TGF-beta1 in the serum in patients with chronic kidney disease was significantly higher than in the control group and in the group of patients' parents who did not present any features of kidney dysfunction (Table 4). No statistical differences of TGF-beta1 concentration were reported between the groups with the kidney disease, depending on the cause (CGN *vs.* CIN; Table 4) and the disease stage (stage 3: 71.9 ± 2.5 *vs.* stage 4: 71.5 ± 1.7 *vs.* stage 5: 71.5 ± 2.5 ng/ml; $p = 0.91$; ANOVA Kruskal-Wallis test).

No differences of TGF-beta1 concentration in the serum were reported in the groups of patients based on the rate of progression of the kidney disease. Similarly, in the subgroups with chronic kidney disease, depending on etiology, i.e., (CGN *vs.* CIN), no significant differences of TGF-beta1 concentration were reported between children with slow and fast progression of the disease (Table 4).

No statistically significant correlations were reported between the concentration of TGF-beta1 in the serum and concentrations of creatinine ($p = 0.23$) and cholesterol ($p = 0.18$) in the serum, as well as the amount of GFR ($p = 0.58$), daily proteinuria ($p = 0.31$), hypertension ($p = 0.55$) among the group of patients with chronic kidney disease.

Table 4 Serum TGF-beta1 concentration in the tested groups

	TGF-beta1 (ng/ml)
All CKD patients (*n = 109*)	71.6 ± 2.3[a, b]
	(63.8–77.2)
CGN patients (*n = 30*)	71.8 ± 2.3[a, b]
	(67.5–76.4)
CIN patients (*n = 79*)	71.3 ± 2.4[a, b]
	(63.8–77.2)
Control group (*n = 40*)	46.2 ± 5.1
	(38.2–52.4)
Parents of CKD patients (*n = 218*)	38.0 ± 3.4
	(30.9–45.9)
CKD patients with rapid progression of the disease (*n = 54*)	71.8 ± 2.3
	(64.3–77.2)
CGN patients with rapid progression (*n = 24*)	71.7 ± 2.3
	(67.5–76.2)
CIN patients with rapid progression (*n = 30*)	71.7 ± 2.4
	(64.3–77.2)
CKD patients with slow progression of the disease (*n = 55*)	72.5 ± 2.3
	(63.8–76.1)
CGN patients with slow progression (*n = 6*)	72.3 ± 2.4
	(63.8–76.1)
CIN patients with slow progression (*n = 49*)	71.4 ± 2.3
	(63.8–75.9)

Data are means ± SD (range)
CGN chronic primary glomerulonephritis, *CIN* chronic interstitial nephritis
[a] *vs.* control group, $p < 0.01$
[b] *vs.* parents $p < 0.01$ (Mann-Whitney U test)

No association was found between the serum TGF-beta1 concentration and rs1800471 polymorphism of its gene. Serum TGF-beta 1 concentrations were not different in the kidney patients grouped according to the genotype (GG: 71.6 ± 2.2, CG: 71.5 ± 3.2; $p = 0.82$) and alleles of rs1800471 (G: 71.6 ± 2.3, 71.4 ± 3.2; $p = 0.19$).

4 Discussion

In the present study we examined the potential association of rs1800471 polymorphism in *TGFB1* gene with CKD in children and young

population affected with non-diabetic nephropathies. Our results indicate that rs1800471 polymorphism in *TGFB1* gene is not involved in the development and progression of CKD in the examined cohort. These findings are in line with those of Summers et al. (2005) and Coll et al. (2004), but they are in contrast to those reported by Khalil et al. (2005) who showed a connection between rs1800471 polymorphism and the progression of renal disease. The latter authors, however, did not examine other polymorphisms of *TGFB1* gene (Khalil et al. 2005). No studies so far have assessed the transition of genetic material of *TGFB1* in families with a child affected by renal disease. In other research only the test model, which compares unrelated people of a specific phenotype with the control group, was applied. Divergent results indicate a further need for not only cross-sectional population-based studies, but also studies of siblings and families of people affected by renal disease using TDT test and a variety of analysis models.

Contradictory results may arise from the fact that there were some ethnical and demographical differences of the examined populations, there were also groups of different sizes and the criteria by means of which patients were chosen were not uniform. Another important factor may be the kind of renal disease (Locatelli et al. 2000; Hu et al. 2008). The present study was dominated by persons with interstitial nephropathy in the course of congenital malformations of urinary system; a prevailing reason of chronic kidney disease in children.

The present study demonstrates that patients with chronic kidney disease have a higher concentration of TGF-beta1 in the serum than the reference group and the group of parents without signs of kidney dysfunction. This may indicate the importance of the cytokine in the development of the disease. However, serum TGF-beta1 level is not directly related to the progression of chronic kidney disease. These findings are fully consistent with the observations of Khalil et al. (2005). Despite the alleged involvement of TGF-beta1 in conditions associated with fibrosis in different organs, those authors also found no association between plasma levels of TGF-beta1 and morphological changes in kidney biopsies (glomerulosclerosis, interstitial fibrosis) and with immunoreactivity of TGF-beta1. On the other hand, our results confirmed the known observations that in the CKD progression modifiable factors play an important role (Dell'Omo et al. 2009; El Nahas 2005; Klahr and Morrissey 2003). Especially, proteinuria, hypertension, and hypercholesterolemia in the early stages of chronic kidney disease may contribute to the fast progression of the disease.

Scientific literature concerning the relation of TGF-beta1 concentration in the serum with polymorphisms of *TGFB1* gene is ambiguous. The present study demonstrates no correlation between the studied polymorphism in patients with chronic kidney disease and in their healthy parents. Similarly, Gewaltig et al. (2002) did not report the influence of rs1800471, rs1982073, and rs1800469 polymorphisms on the concentration of TGF-beta1 in plasma of healthy people. The authors noted, however, higher serum concentration of this cytokine in patients with liver fibrosis, who are carriers of genotype GG of rs1800471 polymorphism.

According to the literature data, mechanisms (genetic and non-genetic), which determine the concentration of TGF-beta1 in blood are still unclear (Suthanthiran et al. 2009; Grainger et al. 1999). It is also unknown if the circulating TGF-beta1 reflects its concentration in tissues affected with the disease (Khalil et al. 2005). Finally, it is unclear if polymorphism *TGFB1* influences kidney transcription, translation, secretion and/or extra-cellular activation of the coded protein (Gewaltig et al. 2002).

This study has some limitations. The number of patients with chronic kidney disease was relatively small in relation to genetic epidemiological studies. However, it is worth pointing out that we applied a family-based study design, i.e., TDT, to avoid the potential bias of case-control studies. TDT, which evaluates the transmission of associated alleles from heterozygous parents to offsprings affected with the examined phenotype, is more reliable in assessing the genetic predisposition. Familial genetic studies are not numerous in view of the difficulties in gaining

material from both patients and their both parents. In the literature there is no data regarding family studies using TDT to examine the role of *TGFB1* gene polymorphism in the development of chronic kidney disease. The number of examined families was relatively small, which is due to the low prevalence of childhood chronic kidney disease as compared to the adult form. Although in every child the cause of the disease was of non-diabetic origin, the spectrum of diseases was not homogenous, including chronic glomerulonephritis and chronic interstitial nephritis. Finally, there was variation in the size of patient subgroups according to the etiology of the kidney disease, which is connected with the children population. Therefore, the results may not be generalizable for the whole population suffering from chronic kidney disease.

In conclusion, rs1800471 polymorphism in *TGFB1* gene does not have an impact on the development and progression of chronic kidney disease caused by primary glomerulopathy and chronic interstitial nephritis. The increased serum TGF-beta1 concentration in patients with chronic kidney disease may suggest its role in the pathomechanism of the disease. Circulating TGF-beta1 level is determined in a multifactorial way, not by rs1800471 polymorphism in *TGFB1* gene.

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Immunological Characteristics of Children with Hashimoto's Autoimmune Thyroiditis

A.M. Kucharska, E. Gorska, A. Stelmaszczyk-Emmel, U. Demkow, and B. Pyrżak

Abstract

The main cause of autoimmune thyroiditis of Hashimoto's type (HT) is a pathological immune response to thyroid antigens. The aim of the study was to present clinical characteristics and immune profile of children with HT. Ninety five children were examined: 45 with HT (age: 8–18 years) and 50 healthy age-matched controls. The peripheral blood mononuclear cells' (PBMC) phenotype was evaluated using a Beckman Coulter flow cytometer with the following monoclonal antibodies: CD4 – FITC, CD28 – PC5, CD152 – PE and CD8 – FITC, CD28 – PC5, CD152 – PE. The thyroid stimulating hormone, thyroid hormones, and antibodies against thyroid peroxidase (TPO) and thyroglobulin (TG) were evaluated by a microparticle enzyme immunoassay. We found that goiter was present in 53 % of children, the thyroid had an increased density in palpation in 98 %, and hypothyroidism was diagnosed in 11 % of HT patients. The number of CD152+ was lower in HT than in healthy children ($p < 0.05$). CD4+ and CD8+ PBMC subsets did not differ between the groups at baseline. After stimulation with phytohemagglutinine (PHA), CD4+ cells decreased in healthy controls and remained constant in HT children. Anti-TPO and anti-TG antibodies were higher in children with a lower percentage of CD152+. No other markers correlated with the immunological profile of PBMC. The percentages of CD4+ and CD152+ negatively correlated with the anti-TG concentration. We conclude that children with HT have a different PBMC profile than healthy children and show a different pattern of response to stimulation.

Keywords

Autoimmune thyroiditis • Blood mononuclear cells • Clinical course • Children • Goiter • Immune phenotype • Thyroid

A.M. Kucharska (✉) and B. Pyrżak
Department of Pediatrics and Endocrinology, Warsaw Medical University, 24 Marszalkowska St., 00-756 Warsaw, Poland
e-mail: anna.kucharska@litewska.edu.pl

E. Gorska, A. Stelmaszczyk-Emmel, and U. Demkow
Department of Laboratory Diagnostics and Clinical Immunology of the Developmental Age, Medical University of Warsaw, Warsaw, Poland

1 Introduction

Hashimoto's thyroiditis (HT) is a common organ specific autoimmune disease, which is a consequence of the genetic susceptibility and injurious environmental factors (Tomer and Huber 2009). Although the first description of disease was over hundred years ago (Hashimoto 1912), the knowledge on its pathomechanism and on the accompanying dysfunction of the immune system is not yet full. The diagnosis of autoimmune thyroiditis of Hashimoto's type is based on the presence of antithyroid antibodies and hypoechogeneity of thyroid tissue in the ultrasound examination, as the evidence of lymphocytic infiltration of the gland. In the course of disease, the infiltration leads to hypothyroidism as a result of thyroid tissue injury (Pearce 2004; Weetman 2001). The etiology of HT is related to the pathological immune responses to thyroid antigens causing lymphocytic infiltration of the gland and production of organ specific autoantibodies. This process is dependent on multifocal dysfunction of the immune system and insufficient control of self-tolerance. In Hashimoto's thyroiditis, cytotoxic CD8+ T cells play the basic role (Weetman and McGregor 1994). The mechanism for a gene-environment interaction, breaking self-tolerance to thyroid antigens, is largely unknown. The investigation of the genetic background of autoimmune thyroid diseases (AITD) demonstrated that one of the most important factors associated with the disease, except of the *HLA* region, is the *CTLA-4* gene (Tomer and Davies 2003; Ueda et al. 2003; Vaidya et al. 2002). Cytotoxic T lymphocyte antigen-4 (CTLA-4, CD152) is a negative regulator of the immune response (Lenschow et al. 1996). The basic regulatory mechanism related to CTLA-4 is the suppression of T cell activation *via* CD152/B7 signaling and the competition with CD28 antigen (Chambers et al. 1996). Another mechanism is the inhibition of microdomain recruitment in the region of immunological synapse, and destabilization of the contact between T cell receptor and its ligands on antigen presenting cells (Brunner-Weinzierl et al. 2004). Additionally, the differentiation and maturation of natural regulatory T cells (Tregs) (CD4+25+Foxp3 T cells) is dependent on CTLA-4 function (Wing et al. 2008).

The aim of the present study was to describe the clinical characteristics of children with Hashimoto's thyroiditis in relation to the immunophenotype of T lymphocytes from peripheral blood.

2 Methods

The protocol of the study was approved by the Bioethics Committee of the Medical University of Warsaw, Poland. The children's parents signed informed consent concerning the participation in the study. Ninety five children were examined: 45 with chronic Hashimoto's thyroiditis (40 girls and 5 boys of the mean age of 15 ± 2 years; range: 8–10 year) and 50 healthy age- and sex-matched controls. The diagnosis of HT was based on the presence of antithyroid antibodies and a typical ultrasound picture of the thyroid gland.

Physical examination of all patients was performed by the same researcher. Ultrasonography was performed with the use of a Philips iU22 xMATRIX Ultrasound apparatus with a linear sound of 7.5–12.0 MHz (Philips; Bothel, WA). TSH value was evaluated with a Microparticle Enzyme Immunoassay, normal range: 0.49–4.67 mIU/L, free thyroxine, normal range: 0.71–1.85 ng/dl, and antibodies against thyroid peroxidase (anti-TPO), normal value: below 12 IU/ml, and against thyroglobulin (anti-TG), normal value below 34 IU/ml, with MEIA tests (AxSYM kits for all these measurements were purchased from Abbott; Wiesbaden, Germany). The T cell phenotype was evaluated using a flow cytometer (EPICS XL/XL-MCL ver. 2.0, Beckman Coulter Company, Paris, France). The analysis was performed with the use of a

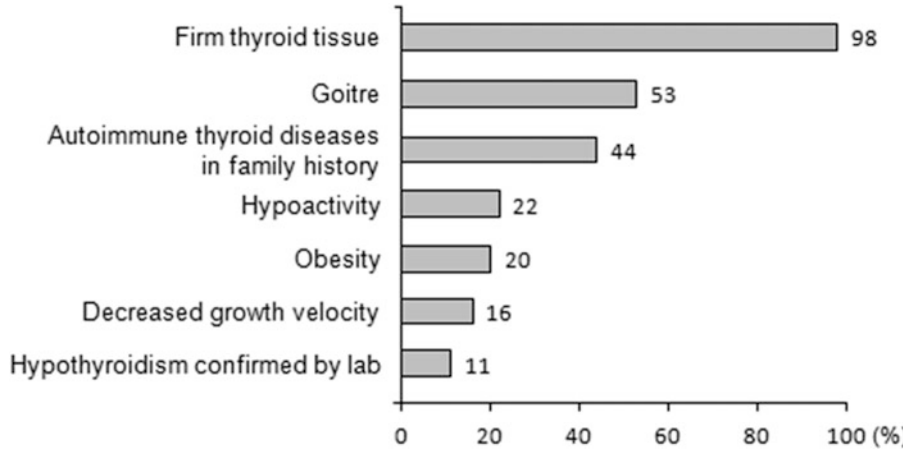

Fig. 1 Clinical characteristics of patients with Hashimoto's thyroiditis

combination of monoclonal antibodies: CD4 – FITC, CD28 – PC5, CD152 – PE and CD8 – FITC, CD28 – PC5, CD152 – PE obtained from Immunotech Beckman Coulter Company (Paris, France). Surface CD antigens were evaluated at baseline and after 48 h of T cell culture with phytohemagglutinine (PHA) as a stimulator of T cell activation. The cell preparation has been described previously (Kucharska et al. 2009).

Data were given as means ± SD. Statistical analyses were performed using the Mann-Whitney U test and Pearson's correlation test.

3 Results

3.1 Patients' History

In the group examined, the ratio of girls to boys was 8:1. This ratio reflects the epidemiology of the disease well. The peak incidence was at the median age of 15.5 years. The most frequent symptom in children diagnosed with thyroiditis, reported by the parents, was hypoactivity – 10 out of the 45 (22 %) children, followed by delayed growth – 7 (16 %) children, and obesity – 9 (20 %) children. In the majority of children, the presence of HT was unraveled accidentally or without any symptoms, but there was a family history for thyroid disorders in 20 out of the 45 (44 %) sick children (Fig. 1).

3.2 Physical Examination and Ultrasound

The most frequent finding in HT children was a firm thyroid gland on palpation: in 44 (98 %) out of 45 patients. The goiter was found only in 24 (53 %) patients. Symptoms of hypothyroidism: bradycardia, dry skin, increased fat tissue, and apathy were present only in 5 children in whom hypothyroidism was confirmed by laboratory tests. In ultrasound examination, thyroid volume exceeded 97th percentile for age and sex in 23 (51 %) out of the 45 patients, but 100 % of children had diffuse hypoechogeneity of the thyroid gland (Fig. 1).

3.3 Laboratory Tests

Primary hypothyroidism in laboratory tests was confirmed in 5 (11 %) out of the 45 patients at the time of diagnosis. The TSH value was between 42.7 and 82.4 mIU/L in five hypothyroid children. All children were positive for anti-TG

antibodies; the mean anti-TG concentration was 366.7 ± 565.3 IU/L, the median was 160.0 IU/L, and the range was 39–2,185 IU/L. The anti-TPO antibodies were found in 41 out of the 45 HT children (91 %); the mean anti-TPO concentration was 626.8 ± 770.0 IU/L, the median was 336.6 IU/L, and the range was 80–2,429 IU/L.

3.4 Cytometric Evaluation of Peripheral Blood Mononuclear Cells (PBMC)

The PBMC subsets CD4+ CD8+ and the co-stimulatory molecules CD152+ and CD28+, which play a key role in T cell activation, were evaluated. Percentages of CD4+ did not differ between the HT and healthy children at baseline before stimulation, amounting to 23.7 ± 10.1 % and 23.9 ± 9.6 %, respectively. Likewise, CD8+ were not different either; 16.6 ± 8.3 % *vs.* 17.0 ± 5.9 %, respectively (Table 1). The percentage of CD8+ PBMC was not influenced by stimulation in either group of children. Stimulation had a differential effect on the CD4+ cells; they remained at a similar level in the HT children, but decreased significantly in the healthy children, from 23.9 ± 9.6 % before to 15.6 ± 5.1 % after stimulation ($p < 0.05$).

Percentages of CD4+ CD28+ cells decreased about uniformly after PHA stimulation in both HT and healthy groups: from 20.6 ± 9.6 % to 8.5 ± 5.1 % and from 21.1 ± 9.5 % to 6.7 ± 2.8 % ($p < 0.05$), respectively, so that there were no differences in the values of CD4+ CD28+ cells between the two groups of children either before or after *in vitro* stimulation. The subset of CD8+ CD28+ cells changed in response to PHA stimulation in like manner (Table 1).

The percentage of PBMC with the surface expression of CD4+ CD152 was lower in the HT children than that in the healthy children; 1.0 ± 0.8 % *vs.* 2.5 ± 1.6 %, respectively ($p < 0.05$). After PHA stimulation, the percentage of CD4+ CD152 cells increased in the HT, from 1.0 ± 0.8 % to 2.9 ± 2.1 %, ($p < 0.001$), as well as in healthy children, from 2.5 ± 1.6 % to 3.4 ± 1.4 % ($p < 0.05$). The increase in CD4+ CD152 cells in response to stimulation was more than twofold greater in the HT children, leveling off the significant differences between the two groups of children present at baseline before stimulation (Table 1).

Finally, the percentage of CD8+ CD152+ also was significantly lower in the HT children compared with the healthy controls at the baseline; 1.2 ± 1.6 % *vs.* 2.5 ± 2.0 %, respectively ($p < 0.05$). Here, PHA stimulation doubled the values of CD8+ CD152+ cells in both groups of children, so that the significant difference between the two groups was sustained; 2.0 ± 1.5 % *vs.* 4.0 ± 1.5 % ($p < 0.05$) in HT and healthy children, respectively (Table 1).

Table 1 Percentages of different subpopulations of peripheral blood mononuclear cells (PBMC) before and after stimulation with phytohemagglutinine (PHA) in Hashimoto's thyroiditis (HT) and healthy children

	PBMC before stimulation		PBMC after stimulation	
PBMC surface phenotype (CD)	HT children	Healthy children	HT children	Healthy children
CD4+	23.7 ± 10.1	23.9 ± 9.6*	21.8 ± 11.4$^{\#}$	15.6 ± 5.1*$^{\#}$
CD8+	16.6 ± 8.3	17.0 ± 5.9	12.2 ± 5.7	14.2 ± 4.4
CD4+CD28+	20.6 ± 9.6*	21.1 ± 9.5*	8.5 ± 5.1*	6.7 ± 2.8*
CD8+CD28+	8.0 ± 5.0*	7.9 ± 3.5*	2.8 ± 2.1*	3.6 ± 0.9*
CD4+CD152+	1.0 ± 0.8*$^{\#}$	2.5 ± 1.6*$^{\#}$	2.9 ± 1.8*	3.4 ± 1.4*
CD8+CD152+	1.2 ± 1.6$^{\#}$	2.5 ± 2.0*$^{\#}$	2.0 ± 1.5$^{\#}$	4.0 ± 1.5*$^{\#}$

Data are mean percentages ± SD

*$p < 0.05$ for before (baseline) *vs.* after stimulation in the same group of patients

$^{\#}p < 0.05$ for HT vs. healthy children in a given condition

3.5 Correlations Between the Level of Autoantibodies and the Lymphocyte Phenotype

The levels of anti-TPO and anti-Tg antibodies were higher in the children with a lower percentage of CD + PBMC cells expressing CD152+ on the surface, but only an association between anti-Tg antibodies and the relative number of CD152 + PBMC reached significance ($r = -0.34$, $p = 0.04$). The hormonal function of the thyroid did not correlate either with T cell phenotypes or anti-thyroid antibody concentrations.

4 Discussion

Hashimoto's thyroiditis is a multifactorial and heterogeneous disease. Genetic and environmental factors are known to interplay in the onset and progression of the disease. In the examined group, a significant female predominance in the development of the disease was found, which is consistent with other authors' opinion, irrespective of the ethnic origin of patients. However, the reason for this female predominance is incompletely understood. Hormonal status, genetic and epigenetic differences, and lifestyle have been considered to explain the female predominance in HT. Sex hormones also play a determinant role in the HT pathogenesis. Sex-related differentiation of the immune system is not only dependent on steroid influence (Pennel et al. 2012), but also on the genes located on the X chromosome and its skewed inactivation (Quintero et al. 2012). Recently, Y chromosome's protective function against autoimmune diseases also is being considered (Persani et al. 2012).

A goiter is not commonly seen in HT as confirmed in the present study. Almost half of the patients had the thyroid gland of normal size, whereas almost all (98 %) of them had a firm thyroid gland on palpation. However, the evaluation of this symptom is very subjective and difficult to compare. It is warranted to use a new imaging technique, like elastography, to objectively evaluate thyroid tissue density (Moon et al. 2012).

In most cases, autoimmune thyroiditis of the Hashimoto type is diagnosed accidentally, and symptoms reported by children or parents are not related to thyroid dysfunction. According to other authors, the majority of patients positive for anti-Tg antibodies have normal thyroid function, progression to overt hypothyroidism stands at the low 2–4 % each year (Vanderpump et al. 1995), and it is preceded by subclinical hypothyroidism in many patients (Huber et al. 2002). Hypothyroidism at the time of diagnosis is present in just a handful of patients as was also observed in the present study. Our results confirmed that HT in children is diagnosed in early phase, when the destruction of thyroid tissue is mild or weak. A high family incidence of thyroid disorders in our patients points to the importance of a genetic background. The basic mechanism of thyroid autoimmunity is the loss of tolerance for thyroid specific antigens. The *HLA-DR* and *CTLA-4* genes are crucially involved in the autoimmune thyroid disorders as they are strongly involved in the control of immune responses (Tomer and Davies 2003). The present study shows that children with HT had a different immunological T cell profile than healthy children do, especially concerning CD152+ T cell subsets. The response to stimulation revealed differences concerning CD4+ and CD8+ T cells as well. We also found a relationship between the level of anti-Tg antibodies and the number of CD4+ expressing CD152+ on the cell surface. This observation is consistent with the regulatory function of CD152+. The surface molecule CD152 plays an inhibitory role for T cell activation (Carreno et al. 2000). Therefore, a decrease in CD152+ T cells can be responsible for the autoimmune disorders resulting from a poor control of the effector cell proliferation, generation of activated effector T and B cells, and generation of autoantibodies (Davies 2013). As opposed of what we found in HT children, we noted a decrease in CD28+ and CD4+ cells after stimulation and an increase in CD152+ cells in healthy children. This finding reflects a deficient CTLA-4 (CD152) function in HT patients.

Among the CD4+ cells, there are natural regulatory T cells (Tregs) and other effector cells, like proinflammatory Th17 cells, generated in a diverse cytokine millieu (Jager and Kuchroo 2010). Different properties of these subsets are related to the profiles of cytokines they release. Recently, Th17 cells are considered the basic players in autoimmunity and the link between them and Th1 cells, as well as the balance of Th17 and Tregs are involved with the pathogenesis of an autoimmune process. Natural Tregs and Th17 are recruited from the CD4+ cells. The crucial role of CTLA-4 (CD152) in differentiation and maturation of Tregs is well documented (Wing et al. 2008). Therefore, a decrease in CD152+ T cells we noted in HT in the present study may promote disbalance of crucial molecules controlling autoimmunity leading to the development of an autoimmune disorder. This issue ought to be addressed by the assessment of CD152 expression on the CD4+CD25+Foxp3 and CD8+CD122+ Tregs, which requires an alternative study design.

In conclusion, in the present Hashimoto's thyroiditis was diagnosed predominantly in female children. In the majority of children the disease was diagnosed accidentally. The incidence of hypothyroidism was low and the most characteristic feature was a firm thyroid gland on palpation. Children with Hashimoto's thyroiditis had a different T cell profile and a different response of lymphocytes to stimulation from those in healthy children.

Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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Endostatin and Cathepsin-V in Bronchoalveolar Lavage Fluid of Patients with Pulmonary Sarcoidosis

W. Naumnik, M. Ossolińska, I. Płońska, E. Chyczewska, and J. Nikliński

Abstract

Recently, it has been reported that lack of cathepsins prevent the development of lung granulomas in a mouse model of Besnier-Boeck-Schaumann (BBS) disease, sarcoidosis. There is no data about cathepsin V (Cath V) in bronchoalveolar lavage fluid (BALF) in humans. Endostatin is a novel inhibitor of lung epithelial cells. The role of this protein in BBS is not determined. The aim of this study was to evaluate the concentration of endostatin, Cath V, and IL-18 in BALF of BBS patients. We studied 22 BBS patients (Stage 2). The control group consisted of 20 healthy subjects. Cath V concentration was lower in BBS than in healthy group (16.03 ± 8.60 *vs.* 32.25 ± 21.90 pg/ml, $p = 0.004$). Both endostatin and IL-18 levels were higher in BBS than in the control group (0.88 ± 0.30 *vs.* 0.29 ± 0.04 ng/ml, $p = 0.028$; 40.37 ± 31.60 *vs.* 14.61 ± 1.30 pg/ml, $p = 0.007$, respectively). In BBS there were correlations between the levels of endostatin and IL-18 ($r = 0.74$, $p = 0.001$) as well as endostatin and DL_{CO} (diffusing capacity for carbon monoxide) ($r = -0.6$, $p = 0.013$). Receiver-operating characteristic (ROC) curves were applied to find the cut-off for the BALF levels of Cath V, endostatin, and IL-18. We conclude that Cath V and endostatin may represent an index of pulmonary sarcoidosis activity.

Keywords

Bronchoalveolar lavage fluid • Cathepsin V • Endostatin • Interelukin-18 • Sarcoidosis

W. Naumnik (✉)
Department of Lung Diseases, Medical University of Bialystok, 14 Zurawia St., 15-540 Bialystok, Poland

Department of Clinical Molecular Biology, Medical University of Bialystok, Bialystok, Poland
e-mail: wojciechnaumnik@gmail.com

M. Ossolińska and E. Chyczewska
Department of Lung Diseases, Medical University of Bialystok, 14 Zurawia St., 15-540 Bialystok, Poland

I. Płońska
Municipal Hospital in Bialystok, Bialystok, Poland

J. Nikliński
Department of Clinical Molecular Biology, Medical University of Bialystok, Bialystok, Poland

1 Introduction

Sarcoidosis is a disease of an unknown etiology. It is characterized by a chronic inflammation with creation of granulomas consisted of lymphocytes, macrophages, and epithelial cells. These cells release several chemokines and cytokines that lead to cellular proliferation. The cytokine profile of active BBS is characterized by T helper 1 (Th1) prevalence (Kieszko et al. 2007). The main cytokine involved in the polarization of T-cell response is IL-18, an interferon gamma inducing factor (Kieszko et al. 2007). Remodeling of lung tissues during the process of granuloma formation requires restructuring of the extracellular matrix and cathepsins K, L, and S are among the strongest extracellular matrix degrading enzymes (Samokhin et al. 2011). According to Samokhin et al. (2011), lack of cathepsin activities alters or prevents the development of lung granulomas in a mouse model of Besnier-Boeck-Schaumann (BBS) disease, sarcoidosis. One of the newly discovered proteolytic enzymes is cathepsin V (Cath V). There have so far been no data about concentrations of Cath V in BALF in humans.

There are many reports about increased angiogenesis in patients with pulmonary BBS. Increased angiogenesis-inducing ability of activated alveolar macrophages has been found in bronchoalveolar specimens from patients with pulmonary sarcoidosis (Fireman et al. 2009). Endostatin, an anti-angiogenic peptide, is a novel inhibitor of distal lung epithelial cells and primary type II cells (Richter et al. 2009). This protein has not yet been investigated in BBS patients.

The clinical course of the BBS may be different; from spontaneous remission to severe and chronic form. New markers of sarcoidosis are highly desirable because they can improve the diagnosis and treatment. In our study we examined Cath V and endostatin in BALF of BBS patients. We correlated these proteins with IL-18, increased in bronchoalveolar lavage fluid (BALF) in active sarcoidosis (Kieszko et al. 2007).

2 Methods

The study was performed in conformity with the Declaration of Helsinki for Human Experimentation of the World Medical Association and the protocol was approved by a local Ethics Committee. Written informed consent was obtained from all participants.

2.1 Subjects

The study group consisted of 22 BBS patients (Stage 2, with bilateral hilar lymphadenopathy and pulmonary infiltrations; F/M – 4/18, mean age 47 ± 9 years) consecutively recruited at the Department of Lung Diseases, the Medical University of Bialystok, Poland between 2007 and 2011. The diagnosis of BBS was based on clinical and pathological criteria (Statement on sarcoidosis 1999). The control group consisted of 20 healthy volunteers (F/M – 3/17 men; mean age 49 ± 7) without any acute or chronic inflammatory conditions. Both patients and healthy subjects underwent BALF and lung function tests including spirometry and diffusing capacity for carbon monoxide (DL_{CO}) (Standardization of Spirometry, 1994 Update; American Thoracic Society (ATS) 1995). Bronchoscopy and BALF in all patients was performed as part of a routine clinical workup. Bronchoalveolar lavage was made using fiberoptic bronchoscopy (Pentax FB 18 V; Pentax Corporation, Tokyo, Japan) under local anesthesia with lidocaine, following premedication with intramuscular atropine and hydroxyzine as a sedative. The bronchoscope was wedged in the right middle lobar bronchus and three 50 ml aliquots of sterile 0.9 % saline were gently instilled and recovered by suction. The recovered fluid was collected and stored on ice and processed within 1 h. Recovered fluid was sieved through a layer of sterile gauze and centrifuged at 800 rpm for 10 min at 4 °C. Supernatant was stored at −70 °C until use. BALF samples were analyzed for total and differential cell counts, Cath V, endostatin, and IL-18 levels detected by Elisa. These results were expressed

as cells $\times 10^5$/ml. The differential cell profile was made by counting at least 400 cells under a light microscope (magnification $\times$ 1,000). Another part of the cell suspension was incubated with phycoerythrin-labeled anti-CD4 antibody (Becton Dickinson, Mountain View, CA), and fluorescein isothiocyanate-labeled anti-CD8 antibody (Becton Dickinson, Mountain View, CA) for 20 min, washed twice, and resuspended for flow cytometry. The stained cells were analyzed on a flow cytometer (Becton Dickinson, Mountain View, CA). Lymphocytes were gated on forward and side scatter, and the percentages of positively stained cells were scored to determine the number of CD4 and CD8 cells.

2.2 Concentrations of Cathepsin V, Endostatin and IL-18 in BALF

Cathepsin V, endostatin, and IL-18 were analyzed in BALF with quantitative test kits and concentrations were determined by means of enzyme-linked immunosorbent assay (ELISA) method (R&D System, Minneapolis, MN). All specimens were assayed in duplicates. The minimum detectable levels of Cath V, endostatin, and IL-18 were 0.95 pg/ml, 0.001 ng/ml, and 10.5 pg/ml, respectively.

2.3 Statistical Analysis

Data distribution was checked with the Shapiro Wilk test. A *t*-test for independent or dependent data was used to compare respective groups and pairs. The Wilcoxon and Mann-Whitney U tests were used for the features inconsistent with the normal data distribution. Correlations were calculated by the Spearman rank test. Receiver-operating characteristics (ROC) curves were constructed to find the cut-off levels of Cath V, endostatin, and IL-18. $p < 0.05$ was considered statistically significant. Statistical analysis was performed using Statistica 10.0 software (Stat Soft Inc., Tulsa, OK).

3 Results

There were no significant differences in age or gender between patients and healthy subjects. Pulmonary function analysis revealed that %VC and $\%DL_{CO}$ were significantly reduced in patients with BBS in comparison with the control group (%VC: 87.4 ± 17.1 *vs.* 98.1 ± 4.0, $p = 0.03$; $\%DL_{CO}$: 83.3 ± 30 *vs.* 92.5 ± 12.7, $p = 0.02$).

There was no difference in the fluid recovery rate of bronchoalveolar lavage (BAL) between the two investigated groups. Patients with BBS had a higher percentage of lymphocytes and a lower percentage of macrophages (%lymphocytes: 42.4 ± 21.0 *vs.* 18.0 ± 8.0, $p = 0.0002$; %macrophages: 55.5 ± 21.0 *vs.* 80.1 ± 17.0, $p = 0.004$). The percentage of CD4+ was higher in BALF of BBS patients than in healthy subjects (%CD4+: 46.6 ± 16.0 *vs.* 9.3 ± 0.4, $p = 0.009$). There was a similar percentage of CD8+ in BBS and healthy subjects (%CD8+: 17.8 ± 4.8 *vs.* 19.1 ± 5.0, $p = 0.231$).

The BALF levels of Cath V, endostatin, and IL-18 are shown in Fig. 1a–c. Cath V concentration was lower in BBS than in healthy subjects (16.03 ± 8.60 *vs.* 32.25 ± 21.90 pg/ml, $p = 0.004$). Both endostatin and IL-18 levels were higher in BBS than in control group (0.88 ± 0.30 *vs.* 0.29 ± 0.04 ng/ml, $p = 0.028$; 40.37 ± 31.60 *vs.* 14.61 ± 1.30 pg/ml, $p = 0.007$).

ROC curves for Cath V, endostatin, and IL-18 in BALF were applied to determine the cut-off values. Sensitivity and specificity of Cath V levels in the BBS patients relative to the healthy group were 90 % and 50 %, respectively, at a cut-off value of 28.08 pg/ml. Sensitivity and specificity of endostatin levels in the BBS patients relative to the healthy subjects were 71 % and 8 %, respectively, at a cut-off value of 0.39 ng/ml. Sensitivity and specificity of IL-18 levels in the BBS patients relative to the healthy subjects were 81 % and 28 %, respectively, at a cut-off value of 14.21 pg/ml. The areas under the curve for Cath V, endostatin, and IL-18 in BALF were 0.74, 0.84 and 0.79, respectively (Fig. 2).

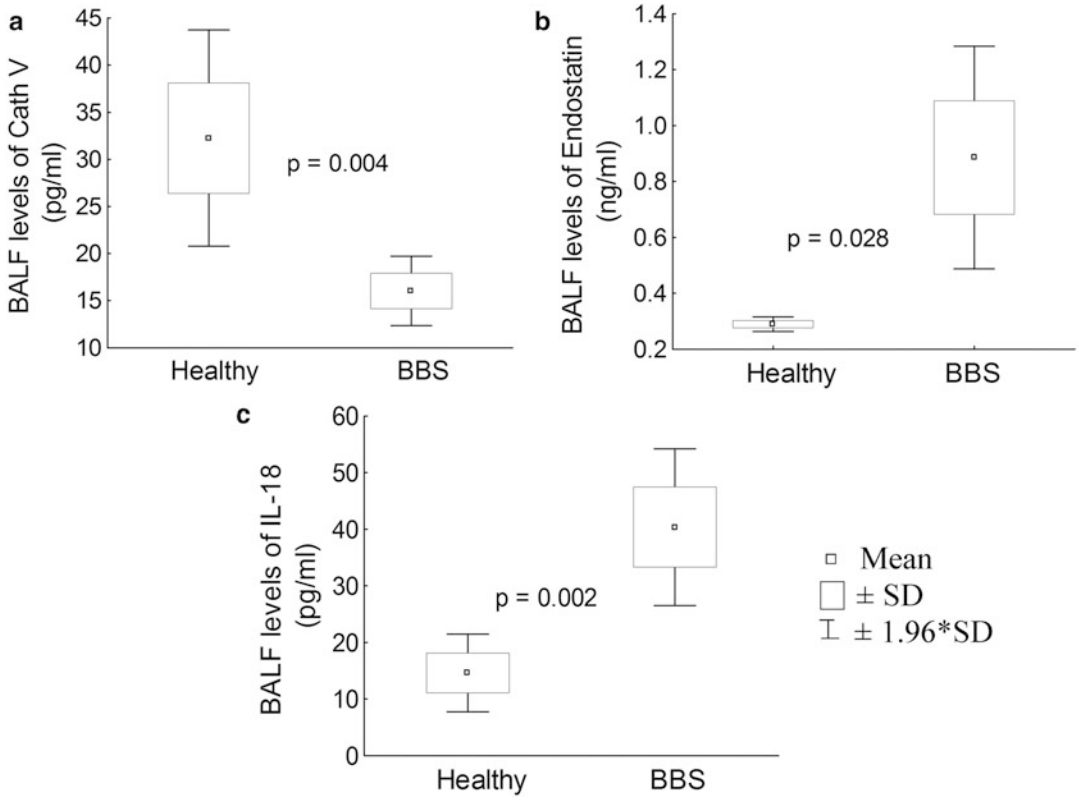

Fig. 1 **Decreased Cath V** (**a**), **increased endostatin** (**b**) **and IL-18** (**c**) in BALF of Besnier-Boeck-Schaumann disease (BBS) patients as compared with healthy volunteers

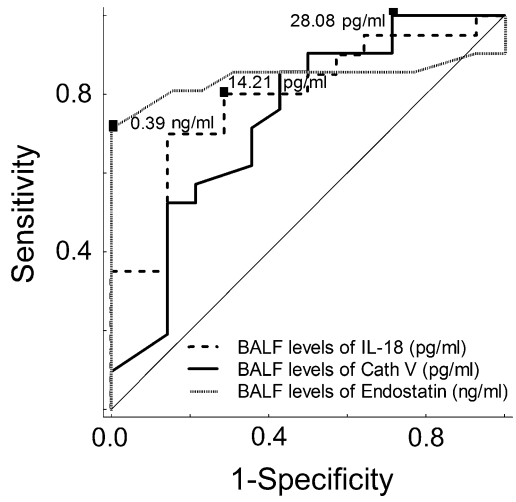

Fig. 2 **Receiver operating characteristic (ROC) curve** for Cath V, endostatin, and IL-18 in BALF differentiating BBS and healthy subjects (AUC 0.74, 0.84, and 0.79, respectively)

In the BBS group, a positive correlation was found between the BALF levels of endostatin and IL-18 ($r = 0.74$, $p < 0.001$) (Fig. 3). We observed a negative correlation between BALF levels of endostatin and $\%DL_{CO}$ in the BBS group (Fig. 4). Moreover, in the BBS group, BALF concentrations of endostatin correlated with following parameters: %lymphocytes ($r = 0.52$, $p = 0.019$), %macrophages ($r = 0.52$, $p = 0.018$) and CD4+/CD8+ ($r = 0.86$, $p = 0.013$). Cath V concentration negatively correlated with CD4+/CD8+ in BALF of BBS patients ($r = -0.83$, $p = 0.003$).

4 Discussion

In the present study we revealed that patients with BBS had a lower level of Cath V in BALF than healthy people. To our knowledge, this

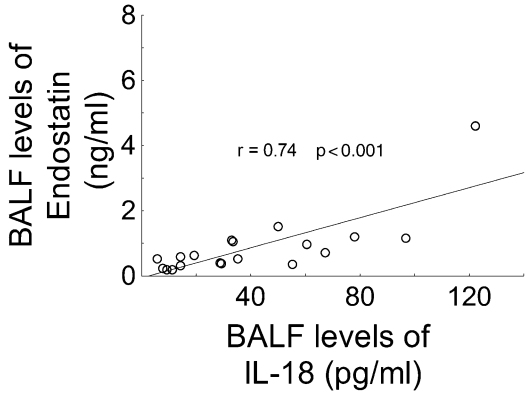

Fig. 3 Correlation between concentrations of endostatin, and IL-18 in BALF of Besnier-Boeck-Schaumann disease (BBS) patients

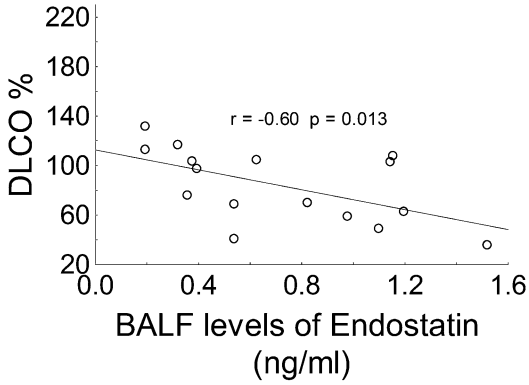

Fig. 4 Correlation between endostatin and DL_{CO} (diffusing capacity for carbon monoxide) in BALF of Besnier-Boeck-Schaumann disease (BBS) patients

study is the first concerning the cathepsin concentration in BALF of humans. Our study is consistent with the findings of Samokhin et al. (2011) in the mouse lungs. That study, which focused on the cathepsin K, L, and S, showed that lack of cathepsin activity prevents the development of lung granulomas. These cysteine proteases degrade major extracellular proteins (Shi et al. 1992) and are involved in immune responses (Hsing and Rudensky 2005; Honey and Rudensky 2003). Cathepsins L and S play a significant role in the antigen presentation and T cell selection (Honey and Rudensky 2003; Nakagawa et al. 1998), and the formation of granulomas has been linked to T cell activation (Gerke and Hunninghake 2008; Grunewald and Eklund 2007; Noor and Knox 2007). The role of Cath V in humans has not yet been clarified, but *in vitro* experiments have demonstrated that it has similar physiological properties to cathepsin S (Berdowska 2004). Possible explanation for the decreased Cath V level, in the present study, is proteolytic degradation. Pulmonary sarcoidosis is associated with chronic lung inflammation; thus, it is possible that pro-inflammatory protease may degrade Cath V and lead to its reduced level in BALF. We presume that a low level of Cath V in BBS patients is due to its being used up during the destruction of the extracellular matrix. This process is essential to the lung granuloma formation (Gerke and Hunninghake 2008; Grunewald and Eklund 2007; Noor and Knox 2007). A negative correlation between the BALF level of Cath V and CD4+/CD8+ is consistent with the hypothesis above outlined. Lymphocytic alveolitis with predominance of CD4+Th cells and macrophages is typical in sarcoidosis (Grunewald and Eklund 2007).

Several studies on cathepsins have demonstrated that these enzymes participate in signaling pathways leading to apoptosis (Turk et al. 2002). Apoptosis plays an important role in the resolution of granulomas (van Maarsseveen et al. 2009). Recently, it has been demonstrated that BBS lymphocytes CD4+ present resistance to apoptosis (Dubaniewicz et al. 2006). Petzmann et al. (2006) described decreased apoptosis of antigen-primed T cells in BALF of BBS patients. These findings are in accord with our study because BBS patients had lower levels of Cath V than healthy subjects.

Shi et al. (2003) described that cathepsins are involved in the controlled extracellular matrix degradation, enabling endothelial cells to penetrate the vascular basement membranes to form new vessels. Several investigators confirmed that in pulmonary BBS the angiogenic/angiostatic balance is also disturbed. Fireman et al. (2009) reported that VEGF in the alveolar space is lower in BBS than in healthy subjects. The authors also revealed that the level of VEGF in patients at Stage 3–4 of disease is significantly lower than that at Stage 1–2, indicating a less fibrotic parenchymal disorder in the latter.

In the present study, the level of endostatin was higher in the BBS than in control subjects. This may indicate the inhibition of angiogenesis in patients with sarcoidosis. Our results are similar to those reported by Richter et al. (2009). They confirmed that endostatin reduces migration and spreading of endothelial cells, and induces epithelial apoptosis. Moreover, macrophages are also known to secrete anti-angiogenic factors, such as endostatin/collagen XVIII (Kamboucher et al. 2011). We confirmed that report as endostatin correlated with the percentage of macrophages in BALF. Kamboucher et al. (2011) found a correlation between endostatin and lung function impairment. We also observed a negative correlation between the level of endostatin and DL_{CO}. The presence of endostatin within the lung in BBS may result in an alveolar epithelial repair. Another possible explanation for increased endostatin level is proteolytic degradation of proangiogenic stimulators. The relationship between endostatin and IL-18 in BALF indicates a potentially important clinical role of our observations.

Recent studies have reported that IL-18 is closely related to the pathogenesis of pulmonary sarcoidosis (Shigehara et al. 2001). IL-18 plays an important role in the induction of the Th1 response and it may be responsible for BBS progression and granuloma formation. Moreover, it plays a pivotal role in linking inflammatory immune responses and angiogenesis in pulmonary BBS (Amin et al. 2010).

In summary, our findings, although obtained in a small number of patients, show a significant relationship between the level of endostatin and DL_{CO} in BALF of BBS patients. Patients with the more severe impairment of DL_{CO} had a higher concentration of endostatin – an inhibitor of angiogenesis. Plausibly, an endostatin inhibitor may become a therapeutic option in BBS in the future.

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Conflicts of Interest The authors had no conflicts of interest to declare in relation to this article.

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Posturography in Patients with Rheumatoid Arthritis and Osteoarthritis

B. Sokołowska, L. Czerwosz, M. Hallay-Suszek,
T. Sadura-Sieklucka, and K. Księżopolska-Orłowska

Abstract

The purpose of the paper was to investigate the usefulness of posturographic analysis in patients with rheumatoid arthritis (RA) and osteoarthritis (OA). RA is a chronic inflammatory disorder responsible for destruction of active and passive components of joints. It is the most common autoimmune disease, and the second most common form of arthritis after OA. OA is a chronic disorder characterized by irreversible changes in the joint structure developing with advancing age. Both diseases lead to the destruction of many parts of the motor system, cause pain, weakness, and damage of ligaments, muscles, bones, and articular cartilage. The etiology of the diseases remains unknown. In the present study, evaluation of body balance in the standing position was performed by means of Pro-Med force plate system. Three posturographic tests were applied: with eyes open, closed, and with the biofeedback – under conscious visual control of body movements. The following posturographic parameters were measured: the radius of sways, the developed area, and the total length of posturograms, and also two directional components of sways: the length of left-right (in frontal plane) and forward-backward (in sagittal plane) motions. The results demonstrate that the biofeedback test is most useful in the evaluation of instability in rheumatic patients; it is more powerful than the other posturographic tests evaluated.

B. Sokołowska (✉)
Bioinformatics Laboratory, Medical Research Center Polish Academy of Sciences, 5 Pawińskiego St., 02-106 Warsaw, Poland
e-mail: beta.sokolowska@imdik.pan.pl

L. Czerwosz
Bioinformatics Laboratory, Medical Research Center Polish Academy of Sciences, 5 Pawińskiego St., 02-106 Warsaw, Poland

Department of Respiratory Research, Medical Research Center Polish Academy of Sciences, Warsaw, Poland

M. Hallay-Suszek
Interdisciplinary Center for Mathematical and Computational Modeling, Warsaw University, Warsaw, Poland

T. Sadura-Sieklucka and K. Księżopolska-Orłowska
Department of Rheumatologic Rehabilitation, Institute of Rheumatology, Warsaw, Poland

Keywords
Aging • Arthritis • Biofeedback test • Body balance • Joints • Posturogram • Postural stability

1 Introduction

Postural stability is defined as the ability to maintain the balance of the body. Since the human body is not statically stable, an active control system is required for stabilization. This ability is an important indicator of physical functions and is also strongly related to the risk of falls. Aging and many pathologies often reduce postural stability. Posturography is an objective and quantitative method of the assessment of balance and postural stability (Visser et al. 2008). It evaluates the current postural state by measuring postural sways (Murnaghan et al. 2011; Baratto et al. 2002). Posturographic investigations with eyes open (EO) and closed (EC) or biofeedback control (BF) provide information on the balance stability in health and disease. Various computerized posturography systems are widely used in adults (Du Pasquier et al. 2003), in neurological patients (Rocchi et al. 2006), in patients with stroke, head injury, or cerebral palsy (Lehmann et al. 1990), in patients with hydrocephalus, brain atrophy, or enlargement of brain ventricles (Czerwosz et al. 2009, 2013; Szczepek et al. 2012), as well as in rheumatic patients (Park et al. 2013; Kim et al. 2011; Hen et al. 2000).

The present study was devoted to the investigation of patients with rheumatoid arthritis and osteoarthritis. Rheumatoid arthritis (RA) is a specific autoimmune disorder that affects about 1 % of the population worldwide (Scott et al. 2010; Gabriel 2001). RA is approximately 2–3 times more prevalent among women than men, and about 80 % of the total number of cases occurs in the age range of 35–50 years. RA usually begins with synovial inflammation of peripheral joints, distributed relatively symmetrically on both sides of the body, causing their progressive destruction. Osteoarthritis (OA), on the other hand, is a chronic disease characterized by irreversible damage to the joint structure, which develops with advancing old age. The damage starts from the articular cartilage leading to its loss. Rheumatic diseases lead to the destruction of many parts of motor system, cause pain, weakness, and damage of the ligaments, muscles, and bones. The etiology is still not full well known (Westwood et al. 2006; Guidelines 2002).

The main goal of the present study was the evaluation of the balance in rheumatic patients by means of a computerized force plate system, performed in a variety of conditions.

2 Methods

2.1 Subjects

The study was approved by the Ethics Committee of the Institute of Rheumatology in Warsaw, Poland, and the patients gave written informed consent to the procedures. A total of 186 women were enrolled into the study. The participants were divided into two groups: 108 patients with severe multijoint rheumatic symptoms and 78 control subjects. The control group consisted of healthy young women aged 20–25 (median 21 years) showing no signs of rheumatic or arthritic symptoms. The patient group was stratified into two subgroups, according to the American College of Rheumatology diagnostic criteria (Aletaha et al. 2010; Arnett et al. 1988; Altman et al. 1986):

– 50 women with RA, disease duration – from 2 to 40 years (median 10), aged 35–66 (median 57 years);
– 58 women with OA, disease duration – from 1 to 30 years (median 10), aged 45–67 (median 56 years).

2.2 Posturographic Measurements

The measurements of postural sways in the standing position were performed by means of the Pro-Med Posturographic System (Czerwosz et al. 2013). Three measurement conditions (tests) were applied for each patient: eyes open (EO), eyes closed (EC) – both in the unrestrained standing position, and biofeedback (BF) coordination in the standing position under the visual-motor feedback control. The last condition included voluntary movements of the body apart from spontaneous sways.

For each posturographic trajectory the following parameters were calculated:

- average sway radius (R);
- developed are of posturogram (A);
- total length of posturogram (TL);
- directional components of sways: left-right length (LRL) of frontal plane movements and forward-backward length (FBL) in sagittal plane movements;
- biofeedback (BF) coordination, i.e., a percentage of time when the patient's center of pressure (COP) was located within the 10×10 mm^2, in relation to the duration of measurement.

The data analysis consisted of the two steps: estimation of posturographic parameters and statistical analysis.

2.3 Statistical Analysis

Posturographic data were presented as medians and interquartile ranges. Kruskal-Wallis analysis of variance and the Friedman test were used, followed by the Mann-Whitney U or Wilcoxon test for a two-group comparison of unpaired and paired data, respectively. Relationships among parameters were analyzed by means of Spearman's rank correlation coefficients (ρ). Analysis of the receiver operating characteristic (ROC) curves was also performed (Lasko et al. 2005; Zweig and Campbell 1993). The probability value of $p < 0.05$ was considered to be statistically significant. Calculations were performed using a commercial statistical package of Statistica ver. 9.0.

3 Results

The results of the six posturographic parameters (R, A, LRL, FBL, TL, and BF) in the three posturographic tests (EO, EC, and BF) in the three groups studied (controls, OA patients, and RA patients) are summarized in Table 1. The R and A parameters of posturograms for all tests in all patients were significantly larger than those in controls; however, the A was a more sensitive parameter in the patients with RA. The LRL was significantly greater in the RA and OA groups compared with the control one, but only in the BF test. On the other side, significant differences in the FBL were noted in the RA patients in all tests, whereas in the OA patients such differences were present only in the EC condition. The TL parameter was significantly greater in the RA group in all tests compared with controls, and slightly greater in the OA patients for the BF test. The BF coordination was evidently smaller in both patient groups compared with controls. Summing up, the RA patients were characterized by significantly greater differences of sways, compared with the control subjects, than OA patients, but the explicit differences between the OA and RA groups did not reach the demanded statistical significance.

Figure 1 presents the raw recordings of posturogram trajectories in an RA patient in the EO, EC, and BF conditions. This patient exhibited larger sways in the EC condition compared with the EO and BF conditions.

Table 2 and Fig. 2 demonstrate the results of receiver operating characteristic (ROC) curves in rheumatic patients. LRL motions in the EO, EC, and BF tests resulted in larger area under the curve (AUC) values than other motions did, but the maximum of AUC (>0.75) has been obtained for the BFC parameter. The sensitivity and specificity above 0.75 was achieved when the cut-off point of BFC equaled 92 %.

Figure 3 presents the results of correlations among posturographic parameters. In general, the strongest correlations were observed in the healthy control subjects. Comparison of various conditions (columns) in Fig. 3 shows that the strongest correlation was present in the BFC

Table 1 Medians and interquartile ranges of posturographic parameters in the three groups investigated: controls, patients with osteoarthritis (OA) and with rheumatoid arthritis (RA) during posturographic tests: eyes open (EO), eyes closed (EC), and biofeedback (BF) coordination

Parameter	Group	EO	EC	BF
R (mm)	Controls	2.0	1.9	$2.3^{\#,\$}$
		(1.7–2.6)	(1.6–2.6)	(1.8–2.8)
	OA	2.7^{**}	2.8^{***}	$3.2^{***,\#\#}$
		(2.4–3.4)	(2.2–3.8)	(2.5–4.1)
	RA	2.6^{**}	2.8^{**}	$3.3^{***,\#\#\#,\$\$}$
		(2.1–3.4)	(2.1–3.4)	(2.7–4.1)
A (cm^2)	Controls	139	130	$165^{\#\#\#,\$}$
		(97–185)	(99–212)	(107–231)
	OA	178^{*}	$222^{**,+}$	$283^{***,\#\#\#}$
		(138–250)	(141–367)	(192–406)
	RA	186^{**}	$236^{***,++}$	$296^{***,\#\#\#,\$\$\$}$
		(144–240)	(160–302)	(182–508)
LRL (mm)	Controls	111	115^{++}	$117^{\#\#}$
		(96–123)	(103–127)	(104–131)
	OA	110	114^{+++}	$149^{*,\#\#\#,\$\$}$
		(95–123)	(101–133)	(110–194)
	RA	114	122^{++}	$149^{***,\#\#\#,\$\$\$}$
		(99–129)	(107–149)	(116–213)
FBL (mm)	Controls	147	176^{+++}	$173^{\#\#\#}$
		(129–170)	(150–210)	(144–216)
	OA	166	$205^{*,+++}$	$212^{\#\#\#,\$}$
		(138–209)	(162–297)	(162–247)
	RA	176^{***}	$230^{***,+++}$	$225^{**,\#\#\#}$
		(157–212)	(186–284)	(180–265)
TL (mm)	Controls	210	238^{+++}	$234^{\#\#\#}$
		(189–238)	(202–272)	(208–281)
	OA	221	279^{+++}	$285^{*,\#\#\#}$
		(192–280)	(215–349)	(246–368)
	RA	245^{**}	$286^{**,+++}$	$297^{***,\#\#\#,\$}$
		(206–284)	(248–346)	(248–418)
BF (%)	Controls	–	–	97
				(93–99)
	OA	–	–	88^{***}
				(77–94)
	RA	–	–	87^{***}
				(77–92)

R sway radius, *A* developed area, *LRL* left-right length, *FBL* forward-backward length, *TL* total length, *BF* biofeedback coordination

$^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$ for patients *vs*. controls (Mann-Whitney U test)

$^{+}p < 0.05$, $^{++}p < 0.005$, $^{+++}p < 0.0005$ for eyes open *vs*. eyes closed condition (Wilcoxon test)

$^{\#}p < 0.05$, $^{\#\#}p < 0.005$, $^{\#\#\#}p < 0.0005$ for eyes open *vs*. biofeedback condition (Wilcoxon test)

$^{\$}p < 0.05$, $^{\$\$}p < 0.005$, $^{\$\$\$}p < 0.0005$ for eyes open *vs*. biofeedback condition (Wilcoxon test)

test. Spearman's correlation coefficients (ρ) related to BFC with other parameters are presented in Table 3. The smallest correlations of BFC were with FBL and LRL for patients with OA ($\rho = -0.52$ and $\rho = -0.55$, respectively). Correlations of BFC with R were strong in both osteoarthritic and rheumatic patients ($\rho = -0.96$ in OA and $\rho = -0.95$ in RA).

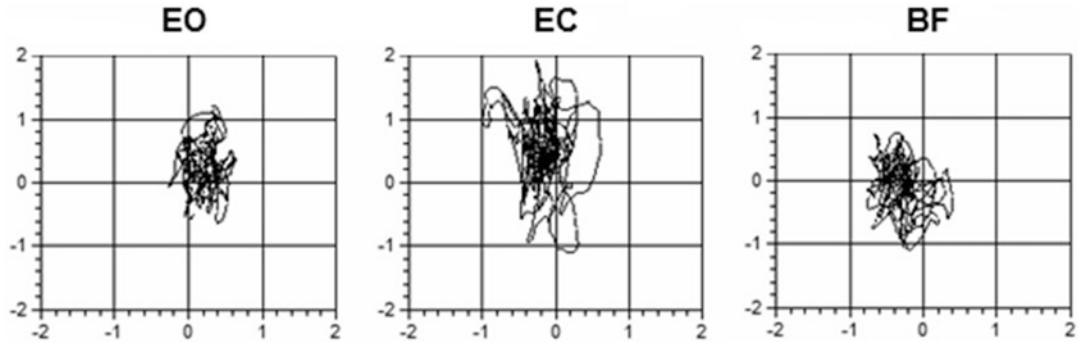

Fig. 1 Representative trajectories of a rheumatoid arthritis patient in eyes open (*EO*), eyes closed (*EC*), and biofeedback coordination (*BFC*) tests

Table 2 Area under curve (AUC) values of ROC analysis

Parameter	Patients	EO test	EC test	BF test
R	OA	0.28	0.26	0.24
	RA	0.30	0.27	0.23
A	OA	0.33	0.29	0.28
	RA	0.29	0.26	0.24
LRL	OA	0.54	0.49	0.33
	RA	0.45	0.41	0.28
FBL	OA	0.36	0.34	0.36
	RA	0.25	0.25	0.28
TL	OA	0.41	0.37	0.32
	RA	0.29	0.26	0.26
BFC	OA	–	–	0.77*
	RA			0.78*

R sway radius, *A* developed area, *LRL* left-right length, *FBL* forward-backward length, *TL* total length, *BF* biofeedback coordination, *EO* eyes open, *EC* eyes closed, *BFC* biofeedback coordination, *OA* osteoarthritis, *RA* rheumatoid arthritis

*$p < 0.005$

4 Discussion

In this study we attempted to choose the best posturographic tests for the evaluation of patients with rheumatoid arthritis and osteoarthritis. Pain, functional disability, and impairment of quality of life are major complaints in such patients. We found that patients exhibited significantly larger postural sways in all tests, compared with healthy subjects, which makes posturography an effectively method to detect disorders of the motor system. Moreover, postural sways were greater in patients with rheumatoid arthritis than in those with osteoarthritis, although the difference did not achieve statistical significance. Rheumatoid arthritis is a systemic disease of connective tissue which contributes to multiorgan complications. Thus, a wider spectrum of rheumatoid symptoms can correspond to larger sways. Park et al. (2013) have studied standing balance, with eyes open and closed, in 37 female patients (mean age of 56 years) with knee osteoarthritis and found no correlation between pain intensity and balance. The authors suggested that worse balance is associated with advanced age rather than disease. However, other studies reveal that worse balance is in fact related to knee pain (Hinman et al. 2002). Our present results are in line with the latter studies, which underscores that, apart from age, the severity and duration of disease can influence the state and activity of rheumatic patients. Kim et al. (2011) studied 80 patients (aged 50 and over) with knee osteoarthritis subdivided into mild and moderate-to-severe disease. The authors showed that patients with moderate-to-severe osteoarthritis had more deficits in the balance control than those with a milder form of disease in various modes of eyes open/closed posturographic tests and concluded that decreases in muscle strength, proprioception, and increased pain contribute to postural instability. Hen et al. (2000) have investigated the influence of double tasks for standing balance in 18 patients with severe rheumatoid arthritis. They estimated postural sways during quiet standing with eyes open/closed and while

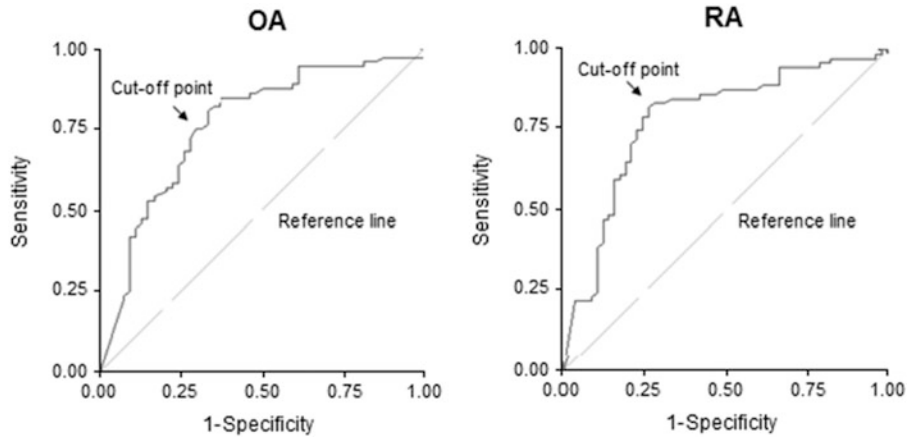

Fig. 2 ROC curves of biofeedback coordination (BFC) parameter for patients with osteoarthritis (*OA*) and rheumatoid arthritis (*RA*)

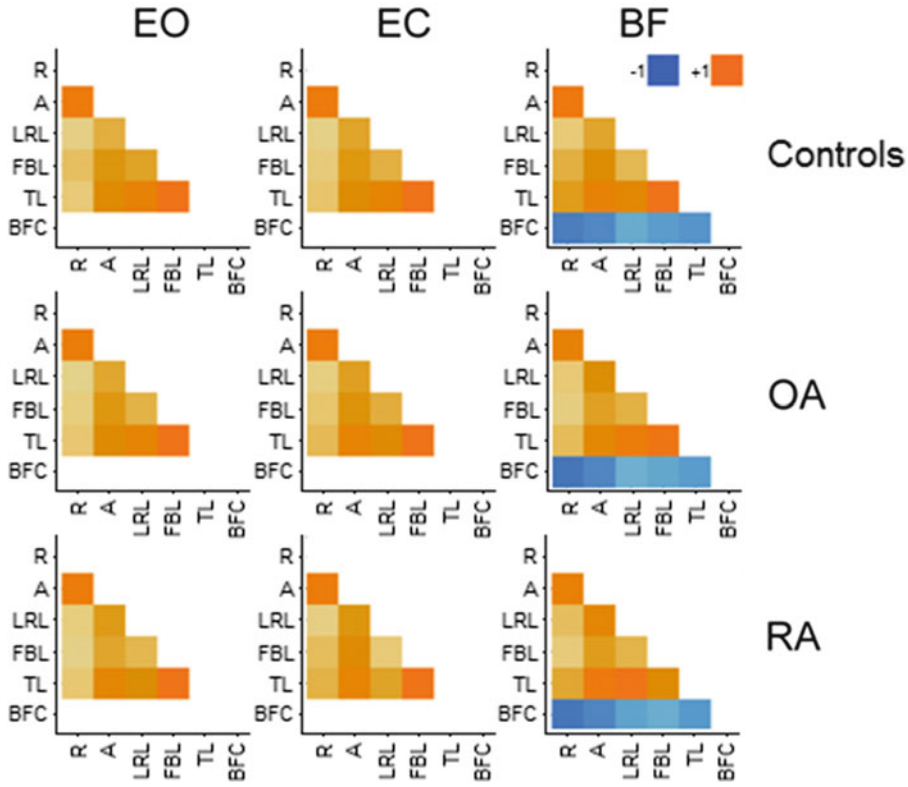

Fig. 3 Correlation matrixes of posturographic parameters for eye open (*EO*), eye closed (*EC*), and biofeedback (*BF*) conditions in the studied groups: healthy controls, osteoarthritis patients (*OA*), and rheumatoid arthritis (*RA*). *R* sway radius, *A* developed area, *LRL* left-right length, *FBL* forward-backward length, *TL* total length, *BF* biofeedback coordination, *EO* eyes open, *EC* eyes closed, *BFC* biofeedback coordination, *OA* osteoarthritis, *RA* rheumatoid arthritis

Table 3 Correlation coefficients of biofeedback coordination (BFC) with other parameters of the biofeedback test

Correlated parameter	Controls	OA patients	RA patients
BFC & R	-0.85^{*}	-0.96^{*}	-0.95^{*}
BFC & A	-0.81^{*}	-0.82^{*}	-0.83^{*}
BFC & TL	-0.74^{*}	-0.63^{*}	-0.68^{*}
BFC & FBL	-0.73^{*}	-0.55^{*}	-0.56^{*}
BFC & LRL	-0.60^{*}	-0.52^{*}	-0.62^{*}

R sway radius, *A* developed area, *TL* total length, *FBL* forward-backward length, *LRL* left-right length
$^{*}p < 0.001$

performing a secondary attention-demanding arithmetic task. Patients had greater postural sways than control subjects. The superimposed effect of arithmetic tasks was negligible and similar in both patients and controls. The authors concluded that rheumatoid arthritis accompanied by severe knee joint impairment causes a substantial basic postural instability. The results of the present study make us share this notion. In addition, our ROC analysis showed that the biofeedback coordination parameter was the best assessment of instability in rheumatic pathology (with sensitivity and specificity above 0.75). The efficient performance of a biofeedback measurement relies on voluntary movements that have to be precise and adequate in power and speed. The rheumatic symptoms of pain and movement limitations can significantly disturb performance during a biofeedback test. On the basis of the present results we submit that simple physical activity under visual-motor control on the force plate can be more sensitive in the assessment of rheumatic pathologies.

In conclusion, computer-driven posturography appears a useful, non-invasive evaluation of the balance and postural stability in patients with rheumatic diseases.

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Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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