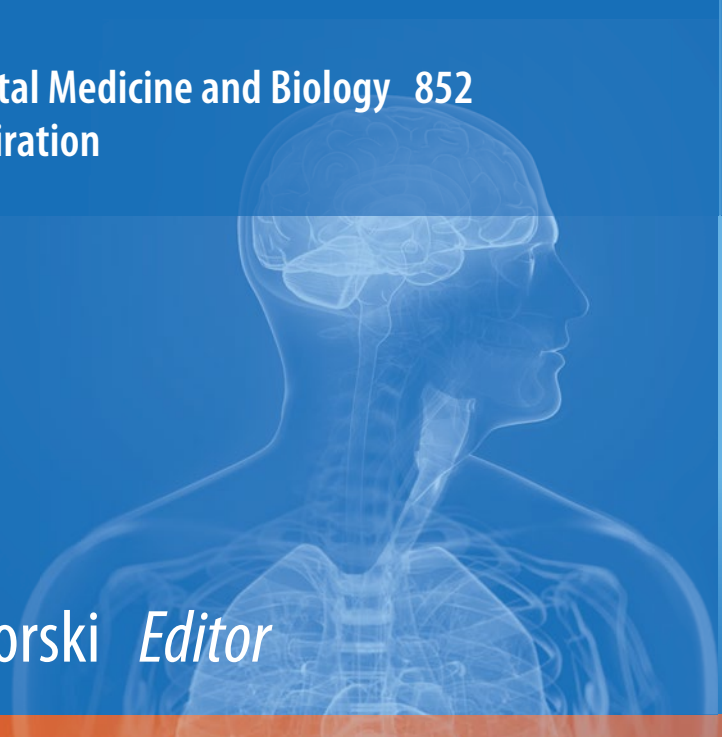
Advances in Experimental Medicine and Biology 852
Neuroscience and Respiration



Mieczyslaw Pokorski *Editor*

Respiratory Carcinogenesis

Springer

Advances in Experimental Medicine and Biology

Neuroscience and Respiration

Volume 852

For further volumes:
http://www.springer.com/series/13457

Mieczyslaw Pokorski
Editor

Respiratory Carcinogenesis

Springer

Editor
Mieczyslaw Pokorski
Institute of Psychology
University of Opole
Poland

ISSN 0065-2598 ISSN 2214-8019 (electronic)
Advances in Experimental Medicine and Biology
ISBN 978-3-319-16921-7 ISBN 978-3-319-16922-4 (eBook)
DOI 10.1007/978-3-319-16922-4

Library of Congress Control Number: 2015938446

Springer Cham Heidelberg New York Dordrecht London

Printed on acid-free paper

Springer International Publishing AG Switzerland is part of Springer Science+Business Media (www.springer.com)

Preface

The book series *Neuroscience and Respiration* presents 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. The authors focus 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. New research is presented 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 also is discussed.

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 therapy of respiratory diseases.

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 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.

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 assume a leading role in the field of respiratory medicine and research and will become a source of reference and inspiration for future research ideas.

I would like to express my deep gratitude to Martijn Roelandse and 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 11: Respiratory Carcinogenesis

The book blends basic and clinical research on respiratory carcinogensis. The contributions tackle a variety of respiratory-related cancers, notably non-small cell lung carcinoma, pleural mesothelioma, mediastinal tumors, or larynx cancer. The focus is on the search for novel molecular markers, derived from easily accessible tissues in clinical settings, such as the serum or bronchoalveolar lavage fluid, which could help diagnose cancer at an early stage and have a prognostic therapeutic value. The transcriptional mechanisms which endow cells with the capacity for unlimited proliferation are considered, with silencing of tumor suppressor genes is the exemplar. Chapters provide insight into a variety of cancer-related disorders of the respiratory tract, novel ways of differential diagnosis and treatment. The aim is to bring the current clinical procedures into alignment with the latest basic research findings. The book is a text for respiratory researchers, clinicians, and pathologists.

Contents

Advs Exp. Medicine, Biology - Neuroscience and Respiration (2015) 11: 1–4
DOI 10.1007/5584_2014_101

Published online: 22 November 2014

The Role of Mediastinoscopy in the Diagnosis of Thoracic Disease: One-Year Single Center Experience

M. Chabowski, A. Szymanska-Chabowska, J. Skotarczak, D. Janczak Jr, L. Pawlowski, and D. Janczak

Abstract

Our experience of using mediastinoscopy for the diagnosis of enlarged mediastinal lymph nodes or mediastinal mass is presented in this study. We reviewed 54 consecutive patients (34 men and 20 women) with mediastinal pathology of varied etiologies who underwent a standard cervical mediastinoscopy from January to December 2012. The histological results were positive in 32 cases (59.2 %), and negative in 22 cases (40.8 %). Transient laryngeal recurrent nerve palsy manifested as prolonged hoarseness of voice was the only minor complication in 3 cases (5.5 %). The sensitivity of the procedure was 72 %, and the specificity was 100 %. We recommend the use of a mediastinoscopy in the staging of lung cancer and the diagnosis of mediastinal mass when other non-invasive procedures are ineffective.

Keywords

Lung cancer • Lymphoma • Mediastinoscopy • Staging • Sarcoidosis

1 Introduction

The standard cervical mediastinoscopy (SCM) was first described by Carlens (1959). This procedure is considered an invasive but safe method for the initial staging of lung cancer, for the diagnosis of mediastinal tumors or granulomatous diseases, i.e. tuberculosis and sarcoidosis (Fibla et al. 2006). Mediastinoscopy is associated with a relatively low postoperative morbidity rate and occasional mortality. The complications include hemorrhage, pneumothorax, recurrent laryngeal nerve injury, esophageal and tracheal perforation, and wound infection (Elsayed 2012; Dunning and Walker 2012). There is little data in the literature reporting the extent to which the surgeon's experience or the implementation of the technique influences the results (Walles et al. 2013).

M. Chabowski (✉), J. Skotarczak, D. Janczak Jr, and L. Pawlowski
Department of Surgery, Fourth Military Teaching Hospital, 5 Weigla St., 50-981 Wroclaw, Poland
e-mail: mariusz.chabowski@gmail.com

A. Szymanska-Chabowska
Department of Internal Medicine and Hypertension, Wroclaw Medical University, Wroclaw, Poland

D. Janczak
Department of Clinical Proceedings, Faculty of Health Science, Wroclaw Medical University, Wroclaw, Poland

2 Methods

The protocol of the study was approved by a local Ethics Committee. We conducted a retrospective single-centre analysis of the records of patients who had undergone mediastinoscopy in our Surgical Department over a 1-year period. The aim of the study was to determine the safety, efficacy, and the current role of mediastinoscopy in the evaluation of mediastinal disease. The diagnostic evaluation included a conventional workup (medical history, physical examination, laboratory tests, and bronchoscopy). All the patients also had preoperative computerized tomography (CT) scans performed. Patients with radiological enlarged mediastinal lymph nodes (diameter >1 cm) or mediastinal masses were subjected to a diagnostic mediastinoscopy. A standard cervical video-assisted mediastinoscopy was performed without an immediate frozen section. General anesthesia and intubation with a single-lumen tube was employed. Patients were in the dorsal decubitus position with a roll under the shoulders. After a transverse cervicotomy, the paratracheal fascia was opened and a blunt finger dissection was done. A spreadable mediastinoscope from Richard Wolf GmbH (Knittlingen, Germany) was inserted. Specimens from lymph node stations 2, 3, 4, and 7 were obtained, the lymph node stations having been classified according to Naruke mediastinal map (Naruke 1993; Naruke et al. 1978). Minor hemorrhaging was successfully controlled by coagulation or gauze packing. The procedures were performed by two consultants in thoracic surgery (MCh and JS). The mortality, morbidity, and sensitivity and specificity of mediastinoscopy for the diagnosis of mediastinal pathology were analyzed. Microsoft Excel XP was used for data collection and basic statistical analysis.

3 Results

Fifty four patients who underwent video-assisted cervical mediastinoscopy within a 1 year period were enrolled in the study. The study population comprised of 34 men (63 %) and 20 women (37 %), with a median age of 63.5 years (range 25–85). On average 2.5 lymph node stations per patient were reached with each mediastinoscopy.

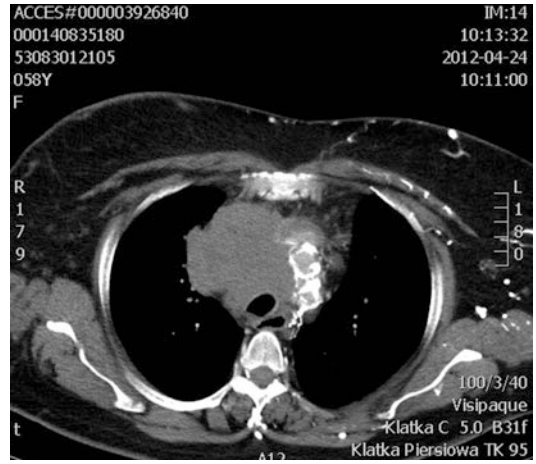


Fig. 1 CT scan showing a large mediastinal mass, which is invading the aortic arch, compressing the right lung parenchyma, and constricting the trachea and the esophagus

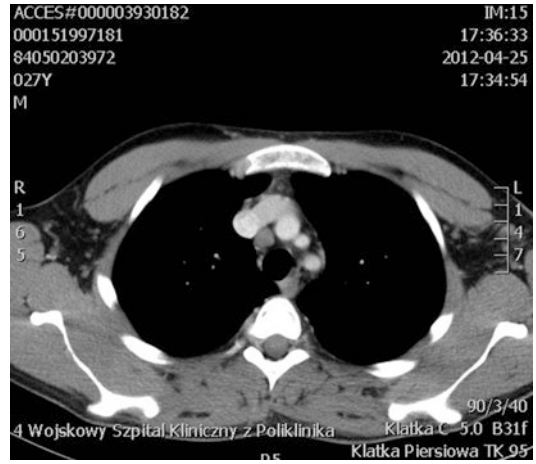


Fig. 2 CT scan showing an enlarged right upper paratracheal lymph node (station 2R)

Mediastinoscopy established a diagnosis in 32 cases (59.2 %): non-small cell lung cancer (NSCLC) (squamous cell carcinoma or adenocarcinoma) in ten patients, small cell lung cancer (SCLC) in 2 (Fig. 1), sarcoidosis in eleven (Fig. 2), B-cell lymphoma in two (Fig. 3), thymic

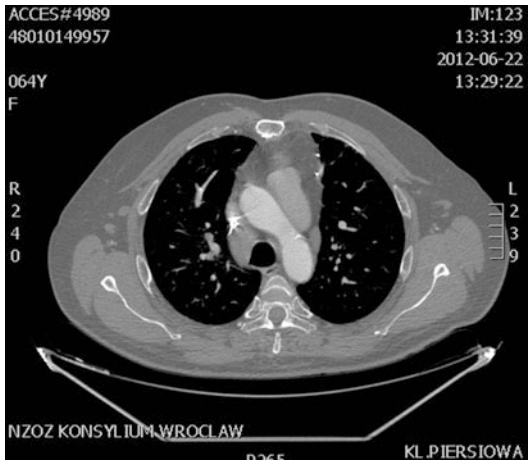


Fig. 3 CT scan showing an enlarged right lower paratracheal lymph node (station 4R)

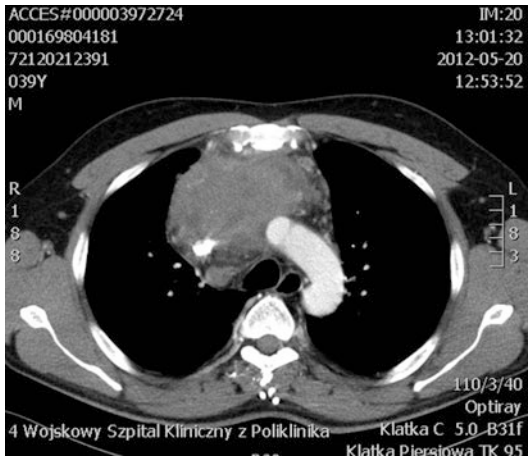


Fig. 4 CT scan showing a large tumor in the anterior mediastinum, which is invading the posterior wall of the sternum and aortic arch, compressing the right and left lung parenchyma, and constricting the trachea

pathology (cancer or thymoma) in four (Fig. 4), and metastases (breast or large bowel cancer) in three.

Negative histological results of mediastinoscopy were obtained in 22 cases (40.8 %). Ten patients did not develop any serious disease, which was confirmed by a positron emission tomography (PET) scans, CT scans, or by further observation (true negative values). Three of these 10 patients underwent a thoracotomy with lymphadenectomy, which proved to be negative. However, 12 patients were discovered to have disseminated cancer, which was confirmed by other diagnostic procedures (false negative values). In our series, the sensitivity of the procedure was 72 %, and the specificity was 100 %.

There were no major complications such as hemorrhaging from major blood vessels. The only minor complication was recurrent laryngeal nerve injury, manifested as prolonged hoarseness of voice, which occurred in three cases (5.5 %). There were no perioperative deaths.

4 Discussion

The role of mediastinoscopy in the evaluation of thoracic disease still remains a debatable subject. Radiological images often do not provide information about the nature of these lesions and consequently fail to enable therapeutic decisions. Surgical exploration of the mediastinum provides a definitive diagnosis as it allows biopsies of lymph nodes or tumors affecting the mediastinum.

The most important question is whether patients with NSCLC will benefit from surgery (Leschber et al. 2008). Mediastinal lymph node involvement (N2 disease), confirming systemic disease, is considered a contraindication for surgery in NSCLC. Mediastinoscopy can obviate an unnecessary thoracotomy. Thoracic CT and PET-CT scans have been widely used for noninvasive tumor staging. Studies have suggested that PET could reduce the need for mediastinoscopy (Hammoud et al. 1999; Vansteenkiste et al. 1998). Invasive mediastinal staging is still necessary as it is based on tissue confirmation. The sensitivity of mediastinoscopy varies between 79 and 93 % (McManus et al. 2008; Fibla et al. 2006). In our series the sensitivity was 72 %. Recently, transbronchial lymph node biopsy by endobronchial ultrasound has been introduced with an approximately 85 % level of sensitivity. The addition of endoscopic ultrasound with fine needle aspiration (EUS-FNA) to mediastinoscopy can increase the sensitivity of detection of mediastinal disease to 93 % (Annema et al. 2010; Annema and Rabe 2006). However, international guidelines regard

the surgical staging as the gold standard. It is suggested that endosonography should be followed by mediastinoscopy if no metastases are found (Annema et al. 2010).

The procedure is safe with minimal morbidity. We noticed only three cases of recurrent laryngeal palsy in our series. However, hemorrhage, vocal cord dysfunction, tracheal injury, pneumothorax, and vascular injury are other major complications that have been described in literature (Chauchan et al. 2012; Minowa et al. 2011; Iskender et al. 2011).

5 Conclusion

Mediastinoscopy, given its safety and efficacy, should be routinely used for the evaluation of mediastinal pathology. The safety of mediastinoscopy safety depends on the experience of the thoracic surgeon. The video-assisted technique allows bi-manual working and improves the efficacy of this procedure.

Conflict of Interests The authors declared no conflicts of interest in relation to this article.

References

Annema JT, Rabe KF (2006) State of the art lecture: EUS and EBUS in pulmonary medicine. Endoscopy 38:118–122

Annema JT, Meerbeeck JP, Rintoul RC, Dooms C, Deschepper E, Dekkers OM, De Leyn P, Braun J, Carroll NR, Praet M, de Ryck F, Vasteenkiste J, Versteegh MI, Veselic M, Nicholson AG, Rabe KF, Tournoy KG (2010) Mediastinoscopy *vs* endosonography for mediastinal nodal staging of lung cancer. JAMA 20:2245–2252

Carlens E (1959) Mediastinoscopy: a method for inspection and tissue biopsy in the superior mediastinum. Dis Chest 36:343–352

Chauchan A, Kapoor S, Ganguly M, Nath P (2012) Cervical mediastinoscopy: re-evaluation of an old technique in era of new imaging technology. Indian J Chest Dis Allied Sci 54:169–173

Dunning J, Walker WS (2012) Troublesome bleeding at mediastinoscopy. Ann Cardiothorac Surg 1:111–112

Elsayed H (2012) Haemothorax after mediastinoscopy: a word of caution. Eur J Cardiothorac Surg 41:138–139

Fibla JJ, Molins L, Simon C, Perez J, Vidal G (2006) The yield of mediastinoscopy with respect to lymph node size, cell type and the location of the primary tumor. J Thorac Oncol 1:430–433

Hammoud ZT, Anderson RC, Meyers BF, Guthrie TJ, Roper CL, Cooper JD, Patterson GA (1999) The current role of mediastinoscopy in the evaluation of thoracic disease. J Thorac Cardiovasc Surg 118:894–899

Iskender I, Tezel C, Kadioglu SZ, Kir A (2011) The role of standard and extended cervical mediastinoscopy in the staging of non-small cell lung cancer patients. Turk J Thorac Cardiovasc Surg 3:405–409

Leschber G, Sperling D, Klemm W, Merk J (2008) Does video-mediastinoscopy improve the results of conventional mediastinoscopy? Eur J Cardiothorac Surg 33:289–293

McManus TE, Haydock DA, Alison PM, Kolbe J (2008) Isolated mediastinal adenopathy: the case for mediastinoscopy. Ulster Med J 77:97–101

Minowa M, Chida M, Eba S, Matsumura Y (2011) Pulmonary artery injury during mediastinoscopy controlled without gauze packing. J Cardiothorac Surg 6:15. doi:10.1186/1749-8090-6-15

Naruke T (1993) Significance of lymph node metastasis in lung cancer. Semin Thorac Cardiovasc Surg 5:210–218

Naruke T, Suemasu K, Ishikawa S (1978) Lymph node mapping and curability of various levels of metastases in resected lung cancer. J Thorac Cardiovasc Surg 76:832–839

Vansteenkiste JF, Stroobants SG, De Leyn PR, Dupont PJ, Bogaert J, Deneffe GJ, Nackaerts KL, Verschakelen JA, Lerut TE, Mortelmans LA, Demedts MG (1998) Lymph node staging in non-small-cell lung cancer with FDG-PET scan: a prospective study on 690 lymph node stations from 68 patients. J Clin Oncol 16:2142–2149

Walles T, Fiedel G, Stegherr T, Steger V (2013) Learning mediastinoscopy: the need for education, experience and modern techniques – interdependency of the applied technique and surgeon's training level. Interact Cardiovasc Thorac Surg 16:450–454

Advs Exp. Medicine, Biology - Neuroscience and Respiration (2015) 11: 5–10
DOI 10.1007/5584_2015_113

Published online: 28 February 2015

Shoulder Ring Complaints as a Rare First Symptom of Malignant Pleural Mesothelioma

J. Lorkowski, O. Grzegorowska, A. Kotela, W. Weryński, and I. Kotela

Abstract

The prevalence of malignant pleural mesothelioma is often encountered in the areas highly exposed to asbestos. The aim of this paper was a retrospective analysis of shoulder pain as a rare, first symptom of pleural mesothelioma, which constitutes an interdisciplinary diagnostic problem concerning both orthopedics and pulmonology. The research was based on a retrospective review of the patients' medical records. The considered period of time included the years 2006–2012. The study group included a total of 49 patients. Seven patients (14.3 %) presented a complain of shoulder pain, as the first symptom of mesothelioma. The remaining 42 mesothelioma patients, without this symptom, constituted a reference group. The intensity of shoulder pain was, on average, 4/10 on an analog scale. A concomitant limitation of mobility was observed in five out of the seven subjects. In one case, limitation of motion and dysfunction of the shoulder joint were at an advanced stage. Neuralgia of upper limbs was found in two cases. We conclude that shoulder pain may be a manifesting symptom of malignant pleural mesothelioma. The neoplasm appears to have a pleiotropic effect on human body, reflected in different ways of its primary manifestation which may also include the motor system.

J. Lorkowski (✉)
Department of Orthopedics and Traumatology, Central Clinical Hospital of Ministry of Interior, 137 Wołoska St., 02-507 Warsaw, Poland

Rehabilitation Center 'Health', Cracow, Poland
e-mail: jacek.lorkowski@gmail.com

O. Grzegorowska
Rehabilitation Center 'Health', Cracow, Poland

A. Kotela
Department of Orthopedics and Traumatology, Central Clinical Hospital of Ministry of Interior, 137 Wołoska St., 02-507 Warsaw, Poland

W. Weryński
Department of Internal Medicine with a Subdivision of Pulmonology, Hospital in Dąbrowa Tarnowska, Dąbrowa Tarnowska, Poland

I. Kotela
Department of Orthopedics and Traumatology, Central Clinical Hospital of Ministry of Interior, 137 Wołoska St., 02-507 Warsaw, Poland

Division of Musculoskeletal Rehabilitation, Physiotherapy Institute, The Jan Kochanowski University in Kielce, Kielce, Poland

Private Practice of Orthopedics and Rehabilitation, Szczucin, Poland

Keywords
Asbestos • Environmental exposure • Pleural malignancies • Pulmonology • Orthopedics

1 Introduction

The incidence of mesothelioma reaches 20/1,000,000 people. However, the estimates are variable as the standardized mortality rate from mesothelioma in the years 1994–2008 was 4.9/1,000,000 people, which makes it even a rarer neoplasm. There also are differences among countries concerning the incidence of mesothelioma (Opitz 2014). It is estimated that about 39,000 cases in the countries of Russia, Kazakhstan, China, India, and Thailand have not been reported, which suggests that the available data may not be up to date (Park et al. 2011). It is known that, in general, the incidence of mesothelioma has an upward trend (Carbone et al. 2012; Robinson 2012) and men are more often affected than women (3.6:1.0) (Delgermaa et al. 2011). Pleural mesothelioma, along with lung cancer and other pleural diseases, is lined to asbestos exposure (Konieczko et al. 2013; Carbone et al. 2012; Skammeritz et al. 2011). There were 4,253 cases of asbestos-linked diseases reported in Poland in the years 1974–2010; pleural mesothelioma was 6.4 % of them (Szeszenia-Dabrowska et al. 2011). Although medicine is still looking for an effective method of treatment, the prognosis in this neoplasm is bad. The survival is about 1 year, despite the application of different methods of treatment - chemotherapy, radiotherapy, aggressive surgical treatment, or phototherapy (Weder and Opitz 2012; Aziz et al. 2002). There are also articles on autoimmune phenomena that arise in response to asbestos exposure (Lee et al. 2014; Pfau et al. 2014) and later accompany pleural mesothelioma if it develops. That is why immune-modulatory drugs have also been tried.

There are a number of non-specific symptoms that accompany mesothelioma. The most frequent are the following: chest pain, dyspnea, cough, weight loss, hyperhidrosis, or fever. Some of them are caused by hydrothorax (NCCN 2013; Mott 2012). Due to the non-specific or asymptomatic course the disease runs, particularly at the beginning stage, it is often detected too late for an effective treatment. It is rather rare when a presaging symptom, like shoulder ring pain, on which this article focuses, can be observed. So far, only two articles have been written, describing single cases of such pain (Verpeut et al. 1999; Mazel and Roolvink 1997). The present study focuses, retrospectively, on describing pleural mesothelioma as one of the shoulder ring pain causes, which should alert diagnostic attention of orthopedicians, patients may sick help from, in this basically pulmonary disease.

2 Methods

The study was accepted by an institutional Review Board for Human Research and was performed in accord with the Declaration of Helsinki for Medical Research Involving Human Subjects. The records of patients with pleural mesothelioma diagnosed over the years 2006–2012 were reviewed. All patients came from the Szczucin municipality in Poland, an area consisting of a population of about 14,000 inhabitants and of known increased risk for environmental asbestos exposure due to the presence of an asbestos-cement establishment. The patients were living in the area for at least

15 years. Seven patients (4 women and 3 men; mean age 51 ± 10, range 41–66 years) who visited orthopedicians because of shoulder ring pain were assessed. A reference group consisted of 42 patients (25 women and 17 men; mean age 64 ± 12, range 38–86 years) with pleural mesothelioma, without any pain in the area of the motor system at the moment of medical consultation. One patient of the seven with the shoulder ring pain and six patients of the reference group were workers at the local asbestos-cement establishment. The shoulder ring pain was diagnosed on the right side in three and on the left side in four patients. Initially, pleura only on one side of the chest was affected. Four of the patients were smoking cigarettes for at least a few years. Two patients presenting with the shoulder ring pain also suffered from asbestosis and Hodgkin's lymphoma.

Chest X-rays and, if necessary, ultrasonography and computerized tomography were used for initial diagnostics. Furthermore, thoracentesis and bronchoscopy were carried out in each case. An average pleural tap amounted to 2,093 ml (range 500–4,600 ml) of fluid in the shoulder ring pain group and 1,250 ml (range 200–3,700 ml) in the reference group. All mesothelioma cases were histopathologically confirmed. Radiograms of two patients presenting with shoulder ring pain are exemplified in Figs. 1 and 2.

After the initial diagnostics at a regional hospital, further treatment took place at the Thoracic Surgery Clinics in the cities of Cracow and Zakopane in Poland. Surgeries of different extend were made, from thoracoscopy with biopsy up to lung resection. Chemotherapy was part of standard treatment. In the shoulder ring pain group videothoracoscopy was carried out in four patients, thoracotomy in two, chemical pleurodesis in three, and pleuropneumonectomy with partial resection of the diaphragm and pericardium in another two patients. In six cases, chemotherapy, involving cisplatin, pemetrexed, or adriablastin, and in three cases radiotherapy were used.

Overall, cancer dissemination was found after a few months in all mesothelioma patients

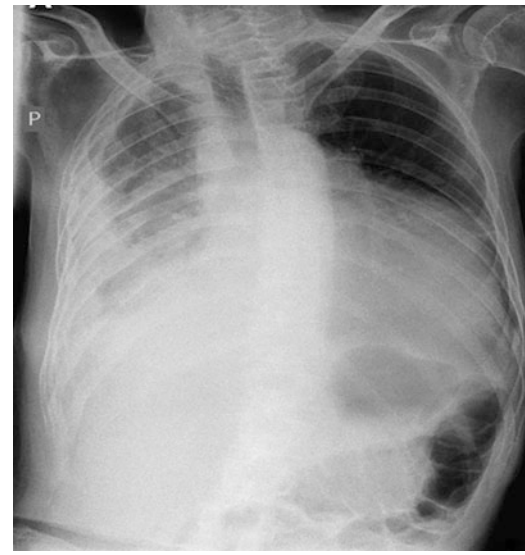


Fig. 1 Chest radiogram of a patient with pleural mesothelioma in the anterior-posterior projection. The examination confirmed hydrothorax on the right side up to the fourth rib, thickened parietal rib pleura and parietal mediastinum pleura. Cardiac silhouette is enlarged with pericardial effusion – neoplastic infiltration of pericardium. Reflexive left bend of the spinal axis due to shoulder ring pain

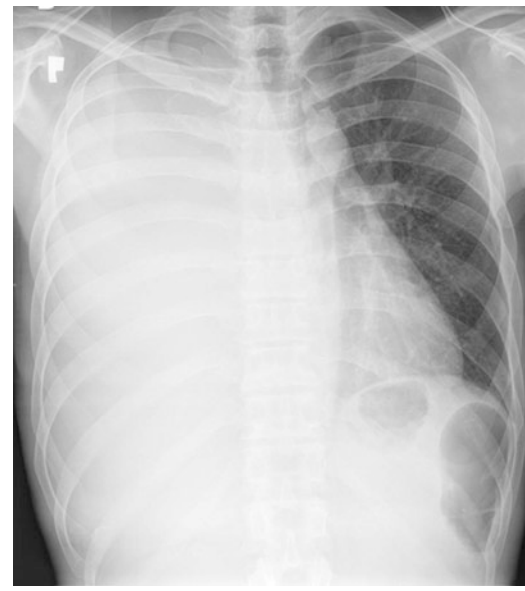


Fig. 2 Chest radiogram of a patient with pleural mesothelioma in the anterior-posterior projection. Right lung completely shadowed by accumulating fluid

presenting with shoulder ring pain. Metastases to the second lung were found in five cases. In two cases, neoplasmatic infiltration of the mediastinum was confirmed, including one patient with an additional infiltration of the esophagus. Metastases to the peritoneal cavity, with accompanying ascites, were present in two patients. One of them also had liver metastases and the other had liver and colon metastases. The adrenal gland was infiltrated in one case. Concerning the motor system, pelvic bone metastases were confirmed in one case, muscles in the area of the pelvic girdle were infiltrated in two patients. Three of the patients had metastases to the abdominal integuments. One patient suffered from the armpit metastases with brachial plexus compression, which resulted in the subluxation of the shoulder joint. The location of metastases is summarized in Table 1. The neoplasm progression and dissemination also were found in the reference group, although it was mostly limited to the lung and pleura on the other side.

Table 1 Metastases in mesothelioma patients with shoulder ring pain

Localization	Number of patients
Lung on the other side	2
Mediastinum	2
Esophagus	1
Peritoneal cavity	2
Liver	2
Colon	1
Adrenal glands	1
Pelvis	1
Pelvis muscles	2
Abdominal integuments	3
Armpit	1

3 Results

The shoulder pain was a presenting complain in all seven patients and made them see an orthopedician. The pain was characterized as diffuse, radiating to the scapula or the neck. Its intensity was rated between 3 and 6 on an analog pain scale, where 0 was no pain and 10 denoted unbearable pain. Three patients rated the pain intensity at 3 points, three other at 4 points, and one at 6 points. Five patients suffered from reduced mobility of the shoulder ring, which was confirmed in physical examination. In one case, the mobility limitation and shoulder joint dysfunction were significant. The ulnar nerve neuralgia of the upper limb was found in two patients; with an additional involvement of the medial and radial nerves in one of them. The following accompanying symptoms were found during the initial work-up: chest pain (5 cases), dyspnea (4 cases), cough (4 cases), weight loss (2 cases), appetite loss (2 cases), and fatigue (3 cases). The exact distribution of symptoms is shown in Table 2. Physical examination showed a pain-related left bend of the spinal axis in one patient, which was confirmed by chest X-ray (Fig. 1). The average survival time, from the time of diagnosis, of the patients with the shoulder ring involvement was 25.4 months (range 12–48 months).

Table 2 Motor system and other symptoms reported to the orthopedician during the initial work-up

	Shoulder ring complaints			Other complaints					
	Pain	Limited mobility	Upper limb neuralgia	Chest pain	Dyspnea	Cough	Weight loss	Appetite loss	Fatigue
Patient 1	Yes	Yes	No	Yes	No	Yes	Yes	Yes	Yes
Patient 2	Yes	No	No	No	Yes	Yes	Yes	Yes	No
Patient 3	Yes	Yes	No	Yes	Yes	Yes	No	No	No
Patient 4	Yes	Yes	No	Yes	No	Yes	No	No	Yes
Patient 5	Yes	No	Yes	No	Yes	No	No	No	No
Patient 6	Yes	Yes	Yes	Yes	No	No	No	No	No
Patient 7	Yes	Yes	No	Yes	Yes	No	No	No	Yes

In all 42 patients of the reference group, no complaints connected with the motor system, and especially with the shoulder ring, were reported. In that group, the first symptoms of pleura mesothelioma were the chest pain on the side of infiltration and dry cough. Thirty one of those patients reported dyspnea and fatigue, and six suffered from significant appetite and weight loss. The average survival time in this group was 15.4 months (range 3–48 months). There were no differences between the patients with occupational or environmental asbestos exposure (those having a direct contact with contaminated agricultural areas). We did not observe a longer survival time than 4 years (the longest were 48 and 36 months). The other patients did not survive longer than 26 months. There were no age or gender-related differences in the survival time.

4 Discussion

There is many a cause of shoulder ring pain. The pain may arise due to congenital and acquired orthopedic disorders, but is also a sequela of neural diseases or damages, vascular diseases, and of other internal causes. When examining a 'painful shoulder' from the orthopedic standpoint, the most frequent causes taken into consideration are the following: bone fractures or luxations, strains or ruptures of tendons or joint capsule structures, degenerative or inflammatory changes, tumors, scalene muscle attachment anomalies, cervical rib pathologies, scoliosis, thorax deformations, brachial plexus anomalies, and brachial artery pathologies. Other pathologies that can cause shoulder joint pain are rarer and thus taken into account much less frequently in orthopedic anamnesis and physical examination. However, the literature shows that other disorders, particularly originating in the thorax, may lead to the shoulder ring pain. These include lung and pleura anomalies, congenital and acquired diaphragm defects, pathologies of the heart and pericardium, and of other mediastinal structures pathologies like esophagus (Ramponi 2011; Adamietz et al. 2008; Caravati et al. 2001). The present review of mesothelioma cases points attention to those relatively infrequent, non-orthopedic reasons of shoulder ring pain, which may be spuriously attributed to an orthopedic pathology. An interdisciplinary approach and unceasingly diligent diagnostic pursuit are thus sometimes required to resolve the diagnostic uncertainties surrounding the underlying cause of the shoulder ring pain. There are additional non-orthopedic symptoms that may increase the awareness toward a malignant underlying reason of the shoulder pain. Some of the most common and non-specific pleural mesothelioma symptoms are dyspnea (90 % of patients), chest pain connected with chest wall or intercostal nerves infiltration, and cough (Neumann et al. 2013). The present study, in general, confirms those findings, although chest pain, in particular, may be misleading as it is encountered in a wide spectrum of cardiac, pneumological, and orthopedic conditions. Concerning the orthopedic conditions, chest pain is a feature of the inflammation of the sternoclavicular joint or rib cartilage, and of cervical discopathies.

In the present study, the primary localization of mesothelioma was the parietal pleura, which lines the inner surface of the chest wall. The metameric innervation comes from the intercostal nerves. There is anastomosing innervation between the parietal pleura and other chest wall layers, which enables the transmission of pain along the dorsal thoracic nerve and the dorsal scapular to the shoulder area. It is therefore reasonable that the first symptom of pleural mesothelioma can be the shoulder pain, radiating to the neck and scapula as we found in the present study. The patients could not precisely localize the focus of pain; it was diffuse, with radiation to the chest. In some instances, pain radiated the other way around, from the chest to the shoulder area; the direction could be interchangable. The pain intensified with inspiration. These complaints were usually vexing and worrisome enough for the patient to seek orthopedician's advice. Mesothelioma remains a relatively uncommon malignancy, particularly taking into account more widely used counter measures against exposure to asbestos. Nonetheless, it remains a difficult to diagnose, deadly

disease, with no effective cure in sight (Mott 2012). Therefore, we believe that the present study's description of the shoulder ring pain as a rear presenting syndrome of mesothelioma is a worthwhile addition to the only two other papers we were able to trace in the literature dealing with the involvement of shoulder pain in the disease recognition (Verpeut et al. 1999; Mazel and Roolvink 1997). This plausibly orthopedic complaint should raise alertness to the possibility of malignancy in the respiratory system, particularly in the areas of known exposure to asbestos.

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

References

Adamietz B, Sauer R, Keilholz L (2008) Radiotherapy for shoulder impingement. Strahlenther Onkol 184(5):245–250

Aziz T, Jilaihawi A, Prakash D (2002) The management of malignant pleural mesothelioma; single centre experience in 10 years. Eur J Cardiothorac Surg 22(2):298–305

Caravatti M, Jan-Wehrle S, Uebelhart D (2001) Conservative treatment of shoulder disorders. Ther Umsch 58(8):475–479

Carbone M, Ly BH, Dodson RF, Pagano I, Morris PT, Dogan UA, Gazdar AF, Pass HI, Yang H (2012) Malignant mesothelioma: facts, myths, and hypotheses. J Cell Physiol 227(1):44–58

Delgermaa V, Takahashi K, Park EK, Le GV, Hara T, Sorahan T (2011) Global mesothelioma deaths reported to the World Health Organization between 1994 and 2008. Bull World Health Organ 89(10):716–724, 724A–724C

Konieczko K, Pałaszewska-Tkacz A, Czerczak S (2013) Chemical carcinogenic and mutagenic agents in the workplace, Poland, 2008–2010. Med Pr 64(2):181–192

Lee S, Matsuzaki H, Kumagai-Takei N, Yoshitome K, Maeda M, Chen Y, Kusaka M, Urakami K, Hayashi H, Fujimoto W, Nishimura Y, Otsuki T (2014) Silica exposure and altered regulation of autoimmunity. Environ Health Prev Med 19(5):322–329

Mazel JA, Roolvink EG (1997) Clinical judgment and decision making in medical practice. A patient with shoulder symptoms. Ned Tijdschr Geneeskd 15:2234–2238

Mott FE (2012) Mesothelioma: a review. Ochsner J 12(1):70–79

NCCN (2013) NCCN clinical practice guidelines in oncology. Malignant pleural mesothelioma. Version I. Available from: http://www.nccn.org/professionals/physician_gls/f_guidelines.asp. Accessed on 31 May 2013

Neumann V, Löseke S, Nowak D (2013) Malignant pleural mesothelioma. Incidence, etiology, diagnosis. Incidence, etiology, diagnosis, treatment and occupational health. Dtsch Ärztebl Int 110(18):319–326

Opitz I (2014) Management of malignant pleural mesothelioma-The European experience. J Thorac Dis 6(suppl 2):38–52

Park EK, Takahashi K, Hoshuyama T, Cheng TJ, Delgermaa V, Le GV, Sorahan T (2011) Global magnitude of reported and unreported mesothelioma. Environ Health Perspect 119(4):514–518

Pfau JC, Serve KM, Noonan CW (2014) Autoimmunity and asbestos exposure. Autoimmun Dis 2014:782045

Ramponi DR (2011) Shoulder pain. Adv Emerg Nurs J 33 (2):114–126

Robinson BM (2012) Malignant pleural mesothelioma: an epidemiological perspective. Ann Cardiothorac Surg 1(4):491–496

Skammeritz E, Omland LH, Johansen JP, Omland O (2011) Asbestos exposure and survival in malignant mesothelioma: a description of 122 consecutive cases at an occupational clinic. Int J Occup Environ Med 2(4):224–236

Szeszenia-Dąbrowska N, Świątkowska B, Szubert Z, Wilczyńska U (2011) Asbestos in Poland: occupational health problems. Intern J Occup Med Environ Health 24(2):142–152

Verpeut A, Vansteenkiste J, Deschepper K, Demedts M (1999) Preoperative work-up of a solitary diaphragmatic mass in a patient with right shoulder pain: a case for diagnosis. Monaldi Arch Chest Dis 54(3):234–236

Weder W, Opitz I (2012) Multimodality therapy for malignant pleural mesothelioma. Ann Cardiothorac Surg 1(4):502–507

Advs Exp. Medicine, Biology - Neuroscience and Respiration (2015) 11: 11–16
DOI 10.1007/5584_2014_104

Published online: 21 January 2015

Primary Prosthetic Voice Rehabilitation in Patients After Laryngectomy: Applications and Pitfalls

V. Calkovsky and A. Hajtman

Abstract

The use of the tracheoesophageal (T-E) silicone rubber voice prosthesis is the most effective and well-established procedure to restore the voice in patients after laryngectomy. The prosthesis is usually well-tolerated with only minor complications. Severe complications are rare. In this article we present our experience with the prosthetic technique at the Clinic of Otorhinolaryngology and Head and Neck Surgery in University Hospital in Martin, Slovakia between the years 2005–2013 and report a case of a 48-year-old man with secondary prosthesis inserted through a T-E shunt 16 months after laryngectomy. On the 6th day after the insertion, the shunt decayed. After prosthesis removal the tissue defect was sutured. Due to repetitive tissue decay, reconstruction of the trachea and esophagus became necessary. On the 10th day, peritracheoesophageal fistula developed and gastrostomy was performed. Because of intense fibrotic and inflammatory changes, further reconstruction was not indicated. After 6 months, esophageal stenosis occurred and endoscopic dilation under local anaesthesia was performed. The T-E voice prosthesis has become one of the choices for voice rehabilitation following total laryngectomy and may improve the patient's long-term quality of life. The overall risk of severe complications seems relatively low. Nonetheless, some complications might be challenging and might require specific management.

Keywords

Alaryngeal speech • Laryngectomy • Prosthesis intolerance • Tracheoesophageal puncture • Voice prosthesis

1 Introduction

Total laryngectomy is the best approach in patients with transglottic tumours. Surgical removal of the larynx affects the major laryngeal functions such as phonation, airway control,

V. Calkovsky (✉) and A. Hajtman
Clinic of Otorhinolaryngology and Head and Neck Surgery, Jessenius Faculty of Medicine, Comenius University and University Hospital Martin, 2 Kollarova St., 036 01 Martin, Slovakia
e-mail: calkovsky1@post.sk

swallowing, and coughing. However, removal of the larynx also results in the loss of laryngeal voice and necessitates a permanent tracheostoma for respiration. The loss of voice is considered a dominant problem after total laryngectomy and an effective voice restoration is critical for prevention of psychosocial consequences (Leonhard and Schneider-Stickler 2015).

Three voice restoration methods after total laryngectomy are currently available: electrolarynx, esophageal voice, and tracheoesophageal puncture. Alaryngeal communication through artificial larynges has a disadvantage of mechanical sound production and has control problems of the laryngeal device with one or both hands. Also, visual distractions to the listener, while the device is being used, may be considered as a disturbance for interactive communication (Wu et al. 2013). Oesophageal speech has an advantage of not necessitating finger closure, but a disadvantage of a long-lasting, complicated learning process. Compared with normal laryngeal speech, esophageal speech has the inherent limitations of slower rate, decreased intensity, lower pitch, stoma blast, and clunking on air intake (Rosso et al. 2012).

Tracheoesophageal puncture (TEP) with voice prosthesis placement is considered a gold standard in alaryngeal patients. Currently, it is the method of choice in Europe and the use of esophageal and electrolarynx voice is in decline (van der Molen et al. 2013). Singer and Blom (1980) have described for the first time one-way silicon voice prosthesis across the tracheoesophageal fistula, thus introducing the most used voice restoration technique. The prosthesis valve protects the airways during deglutition and allows a unidirectional airflow from the trachea across the tracheoesophageal mucosa to produce voice with the vibration of neoglottis. Several voice prostheses have been developed for TEP performed the time of laryngectomy or as a secondary procedure at a later stage (Boscolo-Rizzo et al. 2008; van Weissenbruch and Albers 1993). The prosthesis is usually well-tolerated with only minor complications, e.g. leakage, fungal colonisation, granulation tissue formation, cervical cellulitis, necrosis of the TEP, tracheostomal stenosis, and swallowing impairment. Severe complications are very rare (Laccourreye et al. 1997).

The aim of this article was to present our experience with the prosthetic technique at the Clinic of Otorhinolaryngology and Head and Neck Surgery in University Hospital in Martin, Slovakia between the years 2005–2013 and to report a case of a 48-year-old man with rare complications encountered with secondary prosthesis inserted through T-E shunt 16 months after laryngectomy.

2 Case Report

Between the years 2005–2013, 216 patients with laryngeal cancer were admitted to the Clinic of Otorhinolaryngology and Head and Neck Surgery at the University Hospital in Martin, Slovakia. Of those, 96 underwent total laryngectomy and in 31 patients tracheoesophageal prosthesis was inserted. The study group consists of 28 males and 3 females, with average age of 49 years. The most common complication after puncture was a short-term localized inflammation of varying degrees, requiring treatment with intravenous antibiotics and parenteral nutrition for about 4–5 days ($n = 25$). Among the long-term mild complications, granulation around voice prosthesis due to soft tissue irritation was present in 13 patients.

In the study group, one patient experienced a severe complication. A 48-year-old man with carcinoma on the left hemilarynx, classified as T3 N0 M0 III stage G2 was first admitted to the Clinic in November of 2010. He underwent suprahyoid laryngectomy with revision of the cervical lymphatic system followed by radiation therapy in December of 2010. In February of 2012, 16 months later, the secondary tracheoesophageal prosthesis Provox No. 6 was inserted. Because of prosthesis dislocation on the 1st postoperative day, it was replaced by Provox No. 8. In March of 2012, the prosthesis had to be extracted because of saliva and food leakage through treacheoesophageal shunt (Fig. 1). The shunt was closed to 60 % of its original size. Two weeks later, surgical revision followed, with mobilization of the trachea and suture of the defect at the hypopharyngoesophageal junction (Fig. 2). Subsequently, a new defect of ~10 mm

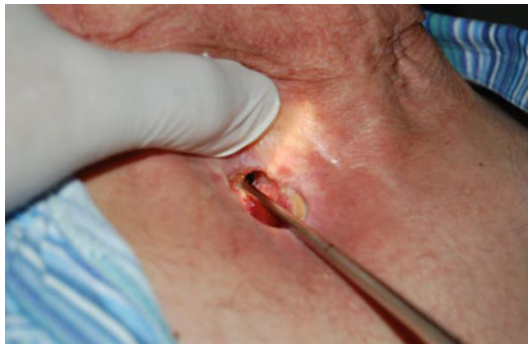


Fig. 1 Tracheoesophageal (T-E) shunt decay at day 6 after secondary TE puncture

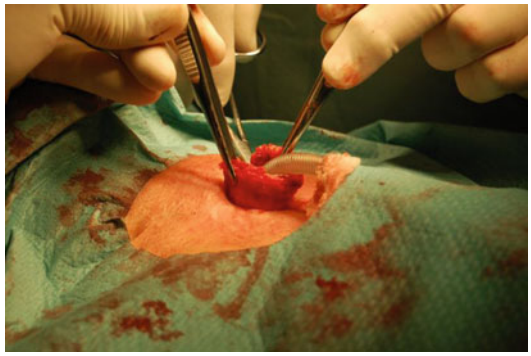


Fig. 2 Mobilization and resection of trachea with reconstruction of esophagus

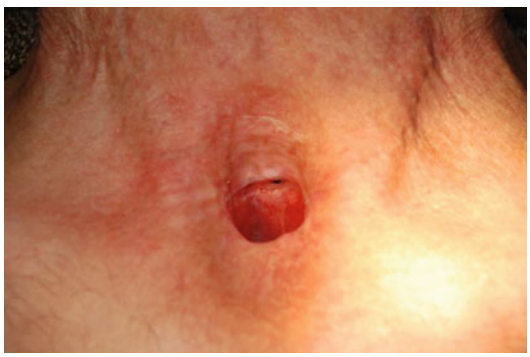


Fig. 3 Persistent treacheoesophageal fistula after reconstruction

in diameter occurred at the upper pole of tracheostomy. Currently, a tracheohypopharyngeal fistula of 2 mm in diameter persists at the upper pole of treacheostomy (Fig. 3).

In April of 2012, the patient underwent the Witzel gastrostomy. The swallowing difficulties developed 6 months later. The esophageal X-ray contrast passage revealed a stenosis at the hypopharyngeal junction. In November of 2012, flexible gastroscopy confirmed the presence of stenosis without mucosal fold in the upper third of the esophagus. Direct repeat esophagoscopy uncovered circular stenosis with ø 2 mm approximately 4 cm above the tracheostomy. The stenosis was gradually dilated up to the probe No. 32. Because of inflammation and edema at the site of stenosis, dilation was discontinued and parenteral anti-inflammatory and anti-edematous treatment was initiated. The patient complained of dyspepsia caused by delay in gastric emptying. He lost 23 kg of body weight between April and November of 2012. The patient's condition improved after introduction of gastric prokinetics. He was finally released in stable condition for outpatient care. Further re-dilation of the stenosis was performed in December of 2012 up to the probe No. 34, after which the swallowing of solid food significantly improved. However, a leak of liquids persisted. A rarely diagnosed material intolerance was suggested as a source of the patient's problems.

3 Discussion

The prosthetic voice rehabilitation developed after a failure of laryngectomized patients to use esophageal voice or electrolarynx (Singer and Blom 1980). The basic condition of successful voice restoration in these patients is an adequate opening pressure in the pharyngoesophageal segment. It seems that anatomical conditions are more important than the patient's mental need to talk (Sebova-Sedenkova 2010).

The insertion of a tracheoesophageal prosthesis may be followed by different adverse effects. A minor consequence is tissue granulation around the puncture site (Imre et al. 2013). Treatment includes antibiotics, antifungals, chemical or electrocautery, and surgical excision of the granulation tissue. Another common complication is biofilms formation. Voice prostheses are

usually made of silicon rubber. A continuous exposure to saliva, food, drinks, and oropharyngeal microflora contributes to the rapid colonization of the prosthesis by biofilms consisting of mixed bacteria and yeast strains leading to failure and frequent replacement (Talpaert et al. 2014). Microbial colonization and biofilm formation can lead to salivary leakage through voice prosthesis and deterioration of the prosthesis due to the blocking of a valve mechanism. Valve failure as well as compromised speech may result in aspiration pneumonia, and repeat valve replacement may lead to the tract stenosis or insufficiency. Prevention and control of biofilm formation will therefore be beneficial not only for the life span of the prosthesis, but also for the general patient's health. A number of different approaches have been suggested to inhibit or minimize biofilm formation (for review see Talpaert et al. 2014).

A circumferential enlargement of the tracheoesophageal puncture is a challenging complication as it results in a leakage around the voice prosthesis into the airway and may eventually lead to aspiration pneumonia and respiratory complications (Mobashir et al. 2014). Several treatment alternatives have been proposed to manage the enlarged tracheoesophageal puncture, with varying success. Surgical options include a submucosal purse-string suture around the enlarged tracheoesophageal puncture and its complete closure. Conservative methods, such as customization of the tracheoesophageal voice prosthesis (Lewin et al. 2012), temporary removal of the voice prosthesis (VP) to facilitate stenosis of the tracheoesophageal tract and tracheoesophageal puncture site injections have often been preferred over surgery (Shuaib et al. 2012).

Imre et al. (2013) have retrospectively analyzed 47 patients with secondary tracheoesophageal prosthesis. Tracheoesophageal puncture and speech valve related complications were observed in 20 patients. Minor complications included granulation tissue formation (2 patients), deglutition of prosthesis (6 patients) and tracheoesophageal puncture enlargement/leakage around the prosthesis (9 patients). Major complications were observed in three patients. Two patients developed life-threatening complications: a mediastinitis and paraesophageal abscess, both in the first month of the postoperative period. The overall complication rate was 42.6 % during a mean follow-up of 15.3 months. Tracheoesophageal fistula enlargement was the most common minor complication and the most common cause of complete closure of tracheoesophageal puncture in that study.

Another retrospective study analyzed 103 patients who underwent total laryngectomy or pharyngolaryngectomy (Bozec et al. 2010). Functional outcomes were recorded 6 months postoperatively. A total of 87 patients (84 %) underwent tracheoesophageal puncture and speech valve placement (79 primary and 8 secondary punctures). A high level of comorbidity was correlated to speech rehabilitation failure. Minor leakage around the valve occurred in 26 % of patients. Late complications occurred in 14 patients, including severe enlargement of the fistula (3 patients), prosthesis displacement (7 patients), and granulation tissue-formation (4 patients).

In the present study, out of the 31 patients with voice restoration by secondary tracheoesophageal puncture, 25 (80.6 %) developed inflammation and 13 (41.9 %) developed granulation, which are considered mild complications. No mediastinitis, bleeding, or prosthesis deglutition were recorded.

Despite short-term complications, the prostheses are considered to be well-tolerated in the long-term view. In a cohort of 100 patients (Lukinović et al. 2012), rehabilitation was successful in 75.8 % of patients. The early complication rate was 4.4 %, and 10.9 % of patients had late complications. Statistical analysis failed to substantiate any differences regarding the complication rate and success rate of rehabilitation between the groups of patients stratified according to age, irradiation status, or timing of prosthesis insertion.

Severe complications, such as bleeding, abscess, or prosthesis aspiration have rarely been reported (Bozzo et al. 2014; Birk et al. 2009;

Denholm and Fielder 1994; Spiro and Spiro 1990; Ruth et al. 1985). In the present study, in a group of 31 patients with tracheoesophageal prosthesis, we observed one severe complication with the secondary prosthesis inserted through a T-E shunt 16 months after laryngectomy. The patient developed shunt decay on the 6th day after the insertion. After prosthesis removal, the tissue defect was sutured and gastrostomy was performed. Due to persisting tissue decay on the 7th postoperative day, reconstruction of the trachea and esophagus was necessary. On the 10th day, peritracheoesophageal fistula developed. Because of intense fibrotic and inflammatory changes, reconstruction was not indicated. After 6 months, esophageal stenosis was revealed and endoscopic dilation in local anesthesia had to be performed. Material intolerance was suggested as a source of the patient's problems, which is a kind of complication not yet reported in the literature on the subject.

4 Conclusions

The tracheoesophageal voice prosthesis has become one of the choices for voice rehabilitation following total laryngectomy and may improve the patient's long-term quality of life. Even the overall risk of severe complications seems relatively low, some of them might be challenging and require specific management.

Acknowledgements The study was supported by VEGA No. 1/0416/12 and Project Virtual and Simulation Tuition as a New Form of Education at JFM CU in Martin (ITMS 26110230071).

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

References

Birk S, Michaeli P, Kapsreiter M, Alexiou C (2009) [Dislocation of voice prostheses. Interdisciplinary management of diagnostics and treatment][Article in German]. HNO 57(11):1126–1130

Boscolo-Rizzo P, Zanetti F, Carpene S, Da Mosto MC (2008) Long-term results with tracheoesophageal voice prosthesis: primary versus secondary TEP. Eur Arch Otorhinolaryngol 265:73–77

Bozec A, Poissonnet G, Chamorey E, Demard F, Santini J, Peyrade F, Ortholan C, Benezery K, Thariat J, Sudaka A, Anselme K, Adrey B, Giacchero P, Dassonville O (2010) Results of vocal rehabilitation using tracheoesophageal voice prosthesis after total laryngectomy and their predictive factors. Eur Arch Otorhinolaryngol 267(5):751–758

Bozzo C, Meloni F, Trignano M, Profili S (2014) Mediastinal abscess and esophageal stricture following voice prosthesis insertion. Auris Nasus Larynx 41(2):229–233

Denholm SW, Fielder CP (1994) Submental abscess: an unusual delayed complication of primary Blom-Singer valve insertion. J Laryngol Otol 108(12):1093–1094

Imre A, Pınar E, Callı C, Sakarya EU, Oztürkcan S, Oncel S, Katılmış H (2013) Complications of tracheoesophageal puncture and speech valves: retrospective analysis of 47 patients. Kulak Burun Bogaz Ihtisas Dergisi 23(1):15–20

Laccourreye O, Mnard M, Crevier-Buchman L, Couloigner V, Brasnu D (1997) In situ lifetime, causes for replacement, and complications of the Provox voice prosthesis. Laryngoscope 107(4):527–530

Leonhard M, Schneider-Stickler B (2015) Voice prostheses, microbial colonization and biofilm formation. Adv Exp Med Biol 830:123–136

Lewin JS, Hutcheson KA, Barringer DA, Croegaert LE, Lisec A, Chambers MS (2012) Customization of the voice prosthesis to prevent leakage from the enlarged tracheoesophageal puncture: results of a prospective trial. Laryngoscope 122(8):1767–1772

Lukinović J, Bilić M, Raguz I, Zivković T, Kovac-Bilić L, Prgomet D (2012) Overview of 100 patients with voice prosthesis after total laryngectomy-experience of single institution. Coll Antropol 36(Suppl 2):99–102

Mobashir MK, Basha WM, Mohamed AE, Anany AM (2014) Management of persistent tracheoesophageal puncture. Eur Arch Otorhinolaryngol 271 (2):379–383

Rosso M, Sirić L, Tićac R, Starcević R, Segec I, Kraljik N (2012) Perceptual evaluation of alaryngeal speech. Coll Antropol 36(Suppl 2):115–118

Ruth H, Davis WE, Renner G (1985) Deep neck abscess after tracheoesophageal puncture and insertion of a voice button prosthesis. Otolaryngol Head Neck Surg 93(6):809–811

Sebova-Sedenkova I (2010) Primary prosthetic voice rehabilitation in patients after laryngectomy – part II. Otorhinolaryngol Head Neck Surg 4(1):15–18

Shuaib SW, Hutcheson KA, Knott JK, Lewin JS, Kupferman ME (2012) Minimally invasive approach for the management of the leaking tracheoesophageal puncture. Laryngoscope 122(3):590–594

Singer MI, Blom ED (1980) An endoscopic technique for restoration of voice after laryngectomy. Ann Otol Rhinol Laryngol 89(6 Pt 1):529–533

Spiro JD, Spiro RH (1990) Retropharyngeal abscess and carotid hemorrhage following tracheoesophageal puncture and voice prosthesis insertion: a case report. Otolaryngol Head Neck Surg 102(6):762–763

Talpaert MJ, Balfour A, Stevens S, Baker M, Muhlschlegel FA, Gourlay CW (2014) Candida biofilm formation on voice prostheses. J Med Microbiol. doi:10.1099/jmm.0.078717-0. [Epub ahead of print]

van der Molen L, Kornman AF, Latenstein MN, van den Brekel MW, Hilgers FJ (2013) Practice of laryngectomy rehabilitation interventions: a perspective from Europe/the Netherlands. Curr Opin Otolaryngol Head Neck Surg 21(3):230–238

van Weissenbruch R, Albers FWJ (1993) Vocal rehabilitation after total laryngectomy using the Provox voice prosthesis. Clin Otolaryngol Allied Sci 18:359–364

Wu L, Wan C, Wang S, Wan M (2013) Improvement of electrolaryngeal speech quality using a supraglottal voice source with compensation of vocal tract characteristics. IEEE Trans Biomed Eng 60(7):1965–1974

Advs Exp. Medicine, Biology - Neuroscience and Respiration (2015) 11: 17–19
DOI 10.1007/5584_2014_96

Published online: 22 November 2014

A Unilateral Hemothorax as a Presenting Manifestation of Mediastinal Spindle Cell Sarcoma

M. Chabowski, A. Szymanska-Chabowska, M. Szolkowska, and D. Janczak

Abstract

The article presents the case of a 73-year-old female injured in a bicycle accident, who was diagnosed with a left hemothorax. Initially, a chest drain was inserted and the pleural hematoma was evacuated. Then a thoracotomy was performed. A hematoma debridement and decortication with a subsequent tissue biopsy was carried out and a final diagnosis of spindle cell sarcoma was made. There is a brief discussion on the differential diagnosis of spontaneous hemothorax and its management.

Keywords

Hemothorax • Mediastinal tumor • Spindle cell lesion • Thoracotomy

1 Introduction

Mediastinal tumors are benign or malignant growths. They mostly consist of reproductive (germ) cells or develop in thymic, neurogenic, lymphatic, or mesenchymal (soft) tissue (Rzyman and Tonnessen 2006). They are usually diagnosed in patients aged 30–50 years, but they can develop at any age. A mediastinal manifestation of a spindle cell sarcoma is a rare occurrence (Varsano et al. 2003).

M. Chabowski (✉)
Department of Surgery, Fourth Military Teaching Hospital, 5 Weigla St., 50-981 Wroclaw, Poland
e-mail: mariusz.chabowski@gmail.com

A. Szymanska-Chabowska
Department of Internal Medicine and Hypertension, Wroclaw Medical University, Wroclaw, Poland

M. Szolkowska
Department of Pathology, National Research Institute of Tuberculosis and Lung Diseases, Warsaw, Poland

D. Janczak
Department of Clinical Proceedings, Faculty of Health Science, Wroclaw Medical University, Wroclaw, Poland

2 Case Report

The patient's informed consent for the publication of material relating to her has been obtained. A 73-year-old female, injured in a bicycle accident, was admitted to the cardiac department of the regional hospital with chest pain symptoms. Her comorbidities were: obesity, arterial hypertension and bilateral carotid artery stenosis. On auscultation the breathing sounds were diminished on the affected side. The chest X-ray revealed a left-sided pleural effusion and an elevated left hemidiaphragm (Fig. 1).

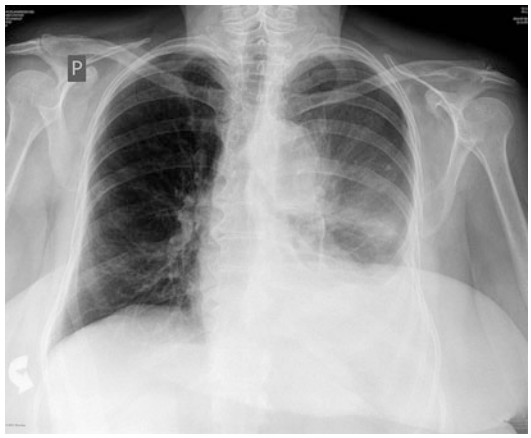


Fig. 1 **Chest X-ray** showing a left-sided pleural effusion and an elevated left hemidiaphragm

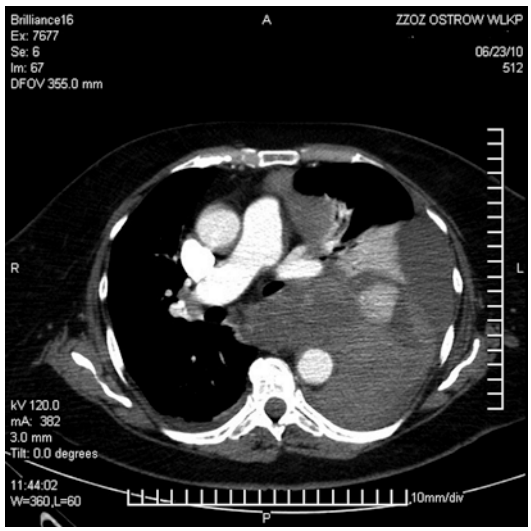


Fig. 2 **Contrast enhanced CT scan** showing fluid accumulation which constricts lung parenchyma and the suspicion of a tumor in the left hilum, measuring 77 × 48 mm in cross section

A thoracic CT scan revealed a large amount of free pleural fluid (50 HU), which constricts lung parenchyma and causes its atelectasis. Moreover, the presence of a tumor in the left hilum, measuring 77 × 48 mm, was suspected (Fig. 2).

A suction chest drain was inserted because of the hemothorax. Over 1,000 ml of blood was removed. The patient was then transferred to the regional pulmonary center. A bronchoscopy was performed and no pathology in the bronchial tree was discovered. A chest ultrasound revealed a 59 mm thick layer of fluid in the left hemithorax. A thoracotomy was recommended in order to perform a hematoma debridement and a decortication with a subsequent tissue biopsy. Therefore, the patient was admitted to the department of thoracic surgery in July 2010 (No 33270/2010). The patient was hemodynamically stable (BP 110/90 mmHg). Blood tests revealed a hemoglobin level of 8.4 g/dl. The patient received two units of packed red blood cells (pRBC). Informed consent was obtained. The patient was administered a general anesthesia with a double lumen endotracheal intubation. A left anterolateral thoracotomy approach was used. Approximately 2,000 ml of semi-clotted blood was aspirated. As there were dense intrapleural adhesions, a decortication was performed. The mediastinal pleura below the aortic arch was incised and it seemed to the surgeon that there was an organized blood clot which was 6 cm in diameter. When the "clot" was removed from the mediastinum, active arterial bleeding started. The source of the hematoma was in this way identified and ligated. Two thoracostomy tubes were inserted. Routine monitoring of vital signs was performed postoperatively. A second-generation cephalosporin was administered for 24 h. The postoperative course was uncomplicated. The patient was discharged on the 7th postoperative day. Surprisingly, the final pathology examination of the formalin-fixed paraffin-embedded (FFPE) tissue revealed malignant spindle cell neoplasm, probably a sarcoma (Fig. 3).

Immunohistochemically, the tumor cells were vimentin positive and negative for cytokeratin (CK), epithelial membrane antigen (EMA), human melanoma black (HMB45), smooth muscle actin (SMA), and desmin (Fig. 4). The patient underwent palliative radiotherapy to her spine due to bone metastasis. The patient died two months after the operation (in September 2010) due to a rapid progression of the disease.

3 Discussion

The most common cause of a hemothorax is trauma, either blunt or penetrating. An upright chest X-ray and spiral CT are the standard diagnostic studies in the evaluation of hemothoraces

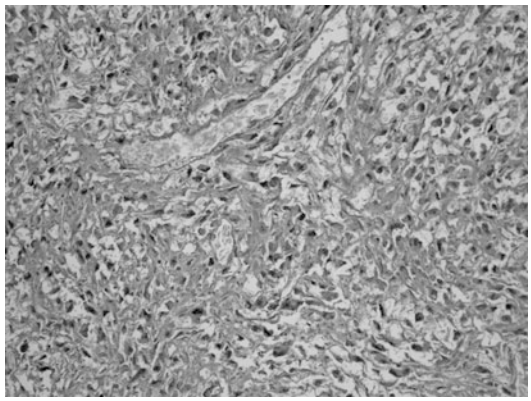


Fig. 3 Malignant spindle cell neoplasm, probably a sarcoma. There are necrotic masses and spindle-shaped cells with nuclear polymorphism, atypia, and nets of reticular fibers (hematoxylin and eosin stain, magnification 200×)

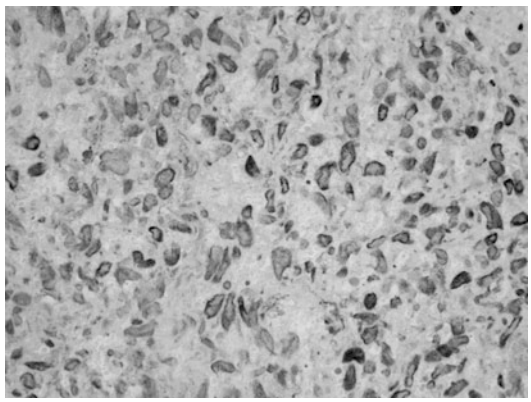


Fig. 4 Positive immunohistochemical staining for vimentin in the tumor cells (magnification 400×)

(Meyer 2007). A measurement of 35–70 HU may point out a hemothorax. The first physician who advocated drainage of hemothoraces by intercostal incision was John Hunter in 1794 (Meyer 2007). The insertion of a 32 F catheter is recommended. In some cases (only 15 %) video-assisted thoracoscopic surgery (VATS) or a thoracotomy are necessary, mainly for the exploration and removal of pleural fluid retained in chest cavity. However, in the majority of cases (85 %) of trauma, only a tube thoracostomy is required. The earlier the detection of a hemothorax and the sooner the pleural drainage is performed, the better the patient's outcome (Misthos et al. 2004).

Less commonly, hemothorax may be caused by thoracic malignancy or may accompany inoperable lung cancer. However, there are the pleural lesions as well. They are composed of spindle fibroblast-like cells, which comprise both neoplastic and reactive disease. The differential diagnosis is not easy, even in a postoperative specimen (Szolkowska et al. 2012). Spindle cell sarcoma belongs to so called connective tissue (soft-tissue) neoplasm (Ali et al. 2008; Galetta et al. 2004).

4 Conclusion

We conclude that the spindle cells neoplasm of the mediastinum is a rare and aggressive tumor with a poor prognosis. The hemothorax may be the only manifestation of this malignant disease.

Conflict of Interests The authors declare no conflicts of interest in relation to this article.

References

Ali HA, Lippmann M, Mundathaje U, Khaleeg G (2008) Spontaneous hemothorax. A comprehensive review. Chest 134:1054–1065

Galetta D, Cesario A, Margaritora S, Granone P (2004) Primary mediastinal hyalinizing spindle cell tumor with giant rosettes. Ann Thorac Surg 77:2206–2209

Meyer DM (2007) Hemothorax related to trauma. Thorac Surg Clin 17:47–55

Misthos P, Kakaris E, Sepsas E, Athanassiadi K, Skottis I (2004) A prospective analysis of occult pneumothorax, delayed pneumothorax and delayed hemothorax after minor blunt thoracic trauma. Eur J Cardiothorac Surg 25:859–864

Rzyman W, Tonnessen T (2006) Fulminant thoracic sarcoma masked by hemothorax. J Thorac Oncol 1:856–856

Szolkowska M, Langfort R, Burakowska B, Szczepulska-Wojcik E, Maksymiuk B, Rudzinski P, Orlowski T (2012) Spindle cell neoplastic and non-neoplastic lesions of the pleura. Pol J Thorac Cardiovasc Surg 9:340–351

Varsano S, Edelstein E, Gendel B, Smorzik J (2003) Bilateral and unilateral spontaneous massive hemothorax as a presenting manifestation of rare tumors. Respiration 70:214–218

Advs Exp. Medicine, Biology - Neuroscience and Respiration (2015) 11: 21–30
DOI 10.1007/5584_2014_105

Published online: 19 December 2014

Diagnostic Performance of Different Pleural Fluid Biomarkers in Tuberculous Pleurisy

J. Klimiuk, R. Krenke, A. Safianowska, P. Korczynski, and R. Chazan

Abstract

Due to the paucibacillary nature of tuberculous pleural effusion the diagnosis of pleural tuberculosis is challenging. This prospective study was undertaken to evaluate the diagnostic performance of ten different pleural fluid biomarkers in the differentiation between tuberculous and non-tuberculous pleural effusions. Two hundred and three patients with pleural effusion (117 men and 86 women, median age 65 years) were enrolled. Routine diagnostic work-up, including thoracentesis and pleural fluid analysis, was performed to determine the cause of pleural effusion. The following biomarkers were measured in pleural fluid: adenosine deaminase (ADA), interferon gamma (IFN-γ), interleukin 2 soluble receptor (IL-2sRα), subunit p40 of interleukin 12b (IL-12p40), interleukin 18 (IL-18), interleukin 23 (IL-23), IFN-γ induced protein 10 kDa (IP-10), Fas-ligand, human macrophage-derived chemokine (MDC) and tumor necrosis factor alfa (TNF-α). There were 44 (21.7 %) patients with tuberculous pleural effusion, 88 (43.3 %) patients with malignant pleural effusion, 35 (17.2 %) with parapneumonic effusion/pleural empyema, 30 (14.8 %) with pleural transudates, and 6 (3 %) with miscellaneous underlying diseases. Pleural fluid IFN-γ was found the most accurate marker differentiating tuberculous from non-tuberculous pleural effusion, with sensitivity, specificity, PPV, NPV, and AUC 97 %, 98 %, 95.5 %, 99.4 %, and 0.99, respectively. Two other biomarkers (IP-10 and Fas ligand) also showed very high diagnostic accuracy with AUC $\geq$ 0.95. AUC for ADA was 0.92. We conclude that IFN-γ, IP-10, and Fas-ligand in pleural fluid are highly accurate biomarkers differentiating tuberculous from non-tuberculous pleural effusion.

Keywords

Biomarker • Pleural effusion • Pleural fluid • Thoracentesis • Tuberculosis

J. Klimiuk, R. Krenke (✉), A. Safianowska, P. Korczynski, and R. Chazan
Department of Internal Medicine, Pneumonology and Alergology, Medical University of Warsaw, Banacha 1A St., 02-097 Warsaw, Poland
e-mail: rafalkrenke@interia.pl

1 Introduction

Tuberculosis (TB) still remains a major worldwide health problem. It primarily affects populations living in low income countries, but due to population aging and increasing immigration a renewed interest in tuberculosis can also be observed in well-developed world regions. Early diagnosis, proper treatment and prevention of disease transmission are the main determinants of effective disease control. Although the predominant form of tuberculosis is pulmonary disease, extrapulmonary tuberculosis may account for 25–27 % of all TB cases (Krenke and Korczynski 2010; Porcel 2009). The pleura is usually reported as the most common extrapulmonary site of the disease. However, the percentage of patients with TB who have pleural involvement, known as tuberculous pleurisy or tuberculous pleural effusion (TPE), varies markedly through different publications. It seems that due to known difficulties in proving the diagnosis of tuberculous pleurisy, the relative incidence of this form of tuberculosis might be significantly underestimated (Light 2010). This refers to data coming from the US and Poland showing the percentage of tuberculous pleurisy of 3.3–4.0 % or even lower (Krenke et al. 2008; Baumann et al. 2007).

Contrary to pulmonary tuberculosis where microbiological examination of appropriate specimens (sputum, bronchial washing, or bronchoalveolar lavage fluid) plays a key role in confirmation of *M. tuberculosis* infection, the sensitivity of microbiological methods detecting mycobacteria in pleural fluid is low. This is due to paucity of *M. tuberculosis* in pleural fluid. Direct microscopic assessment of stained smears gives positive results in only several percent of patients with tuberculous pleurisy (Krenke et al. 2008). Therefore, this method is not recommended in routine TPE diagnostics. Pleural fluid cultures are more sensitive, but they are able to prove *M. tuberculosis* infection in less than 40–50 % of patients with TPE (Light 2010; Porcel 2009; Krenke et al. 2008).

Better results have been found for nucleic acid amplification tests (NAATs), but a disparity in sensitivity of NAATs demonstrated in different studies was very large (15–85 %, mean 62 %) (Krenke et al. 2008; Trajman et al. 2007; Pai et al. 2004). A combined microbiological and histological examination of pleural biopsy samples is a reliable method to confirm the tuberculous etiology of pleural effusion, but biopsy methods are demanding and require considerable skills and experience. Thus, since the diagnostic sensitivity of microbiological methods is unsatisfactory and biopsy methods have limitations, the analysis of tuberculous biomarkers seems to be an attractive diagnostic option. A variety of different pleural fluid biomarkers have been tested with the best and consistent results found for adenosine deaminase (ADA) and interferon gamma (IFN-γ) (Krenke and Korczynski 2010; Liang et al. 2008; Jiang et al. 2007). We have previously shown a very high diagnostic accuracy of pleural fluid ADA and IFN-γ in discrimination between TPE and non-tuberculous pleural effusion (non-TPE) in the Polish population (Krenke et al. 2008). Numerous other biomarkers have been tested as potential tools in diagnosing TPE (Trajman et al. 2008). Unfortunately, most of them presented limited diagnostic performance. However, the assessment of some new markers released during the immune response triggered by the presence of mycobacterial antigens in the pleural space seems promising. These include cytokines which stimulate IFN-γ release (IFN-γ inducing cytokines), for example IL-12, and those whose production and release depend on IFN-γ activity (IFN-γ inducible cytokines), for example interferon-γ-induced protein of 10 kDa (IP-10). Therefore, we undertook the present study to evaluate the usefulness of different cytokines as a biomarkers differentiating between TPE and non-TPE. The diagnostic performance of IFN-γ, soluble interleukin 2 receptor (IL-2sRα), IFN-γ-induced protein 10 kDa (IP-10), soluble Fas-ligand, tumor necrosis factor alfa (TNF-α), sub-unit p40 of interleukin 12b (IL12p40), human macrophage-derived chemokine (MDC), interleukin 23 (IL-23), and interleukin 18 (IL-18) were compared with that of pleural fluid ADA.

2 Methods

The study protocol was approved by the Institutional Review Board of the Medical University of Warsaw.

2.1 Study Design

This prospective study included 242 patients (aged 18–85) with newly diagnosed pleural effusion treated at the Department of Internal Medicine, Pneumonology and Allergology, Medical University of Warsaw, Poland, between the years 2007–2012. An individualized diagnostic work-up was applied to identify the etiology of pleural effusion. All patients underwent clinical examination which included signs and symptoms and medical history, basic blood laboratory tests, ECG, and chest radiograph. In the vast majority of patients diagnostic thoracentesis was performed with subsequent pleural fluid analysis (chemistry, cytology, including total and differential cell count, and microbiology). Effusions were classified as transudates or exudates using Light's criteria. Other diagnostic procedures were ordered when applicable. These included: expanded diagnostic blood tests, echocardiography, thorax and abdominal CT scan, abdominal ultrasound, bronchoscopy, mammography, or breast ultrasound. Specific treatment was applied after the underlying disease related to pleural effusion had been diagnosed.

Pleural fluid samples for subsequent measurement of biomarkers of *M. tuberculosis* infection (mean pleural fluid volume 100 ml) were collected during the first or second diagnostic thoracentesis. The samples were centrifuged at 2,000 revolutions per min for 10 min and the supernatant was frozen at −70 °C for further assays. ADA activity in pleural fluid samples was measured with colorimetric method by Giusti (1974). Pleural fluid cytokine concentration (IFN-γ, IP-10, TNF-α, Fas-ligand, IL-12 p40, MDC, IL-23, IL-2sRα, IL-18) was measured with respective ELISA kits (R&D System, Minneapolis, MN) according to the manufacturer's recommendations.

Of the 242 patients initially enrolled, 8 refused diagnostic thoracentesis. In 31 patients, the etiology of pleural fluid remained unclear either due to withdrawal of patient's consent for further diagnostic tests, contraindications for invasive diagnostic procedures, or equivocal or negative results of diagnostic tests.

A specific cause of pleural effusion (underlying disease) was diagnosed in 203 patients and these patients constituted the proper study group. The patients were further subclassified according to the cause of pleural effusion: tuberculous pleurisy (n = 44), malignant pleural effusion (MPE) (n = 88), parapneumonic effusion (PPE) and empyema (n = 35), pleural transudates (n = 30), and miscellaneous effusions (n = 6). The pleural fluid ADA and the level of cytokines were compared in following groups: TPE *vs.* all other pleural effusions and TPE *vs.* other pleural exudates.

2.2 Definitions

Pleural effusions which met the following criteria were classified as proved TPE: (i) positive culture for *M. tuberculosis* in pleural fluid or pleural biopsy, or (ii) positive smear of pleural fluid and positive result of NAAT for *M. tuberculosis complex*, or (iii) caseating granulomas in pleural biopsy samples or positive microbiological results of respiratory samples (sputum, bronchial washing, and BALF) and exclusion of alternative causes of pleural effusion, and the resolution of effusion after antituberculous therapy.

Malignant pleural effusion was defined as either: (i) positive pleural fluid cytology or positive histology of pleural biopsy or (ii) pleural effusion in patients with known malignant disease, after the exclusion of non-malignant causes of pleural effusion.

Diagnosis or PPE or pleural empyema required: (i) gross appearance of purulent pleural effusion, or (ii) the presence of microorganisms in pleural fluid, or (iii) signs and symptoms of pneumonia accompanied by pleural effusion with characteristic biochemical features which

resolved following antibiotic treatment and/or local pleural drainage.

Diagnosis of transudative pleural effusion was based on Light's criteria. The underlying diseases (congestive heart failure, liver cirrhosis, and nephrotic syndrome) were diagnosed based on the clinical and laboratory data and on the assessment of the effect of specific treatment.

All pleural effusions caused by less common underlying conditions were classified as the group of miscellaneous pleural effusions (e.g., post-by pass surgery syndrome, trapped lung, and pulmonary embolism).

2.3 Statistical Analysis

All continuous variables were presented as median and interquartile range (IQR), while categorical variables as number (%), as appropriate. The Mann-Whitney U test was used to assess the difference between continuous variables in two groups, while Chi-squared test was applied to test the difference between categorical variables. Receiver operating characteristic (ROC) analysis was performed to determine the cut-off threshold and quantify the accuracy of various parameters to discriminate between TPE and non-TPE. The diagnostic yield of the particular biomarkers was assessed by traditional parameters (sensitivity, specificity, positive and negative predicted value, and the area under curve). A p value <0.05 was considered as statistically significant. Statistical analysis was performed with SAS 9.3 statistical software (SAS Institute Inc. Cary, NC).

3 Results

Comparison of clinical and laboratory data of patients with TPE and non-TPE is presented in Table 1. The patients with TPE were significantly younger and had significantly higher body temperature than those with non-TPE. The concentration of the biomarkers evaluated, except IL-23, were significantly higher in TPE as compared with non-TPE (Table 1).

Table 2 shows the diagnostic performance of various pleural fluid biomarkers differentiating between TPE and non-TPE. The highest diagnostic accuracy was found for IFN-γ. Interferon-induced-protein 10 and Fas-ligand showed only a slightly lower accuracy. AUC of four pleural fluid biomarkers (IFN-γ. IP-10, Fas-ligand, and ADA) was higher than 0.9. Very low diagnostic performance was demonstrated for pleural fluid IL-23, with its sensitivity and specificity below 30 and 60 %, respectively.

Significant differences between patients with TPE and patients with non-tuberculous pleural exudates in some clinical and laboratory parameters still persisted after the exclusion of patients with pleural transudates (Table 3). Younger age, higher body temperature, and male predominance characterized patients with TPE as compared with patients with pleural exudates caused by other underlying diseases. No differences in pleural fluid IL-2sRα and IL-23 concentration were noted. However, the activity or concentration of the remaining pleural fluid biomarkers was significantly higher in TPE as compared with all other pleural exudates (Table 3).

Pleural fluid concentration of IFN-γ was the most reliable biomarker differentiating between tuberculous and non-tuberculous pleural exudates (Table 4). Interferon-induced-protein 10 and Fas-ligand showed only slightly lower accuracy. AUC for these three biomarkers was very high and ranged between 0.95 and 0.99 (Table 4). The diagnostic performance of pleural fluid ADA was also high, with sensitivity, specificity, and AUC reaching 88.4 %, 91.1 %, and 0.91, respectively.

4 Discussion

Our study showed that besides two well recognized biomarkers of tuberculous pleurisy – ADA and IFN-γ – other proteins in pleural fluid can reliably differentiate between tuberculous and non-tuberculous pleural effusion. Although significant differences between pleural fluid concentration of several proteins were demonstrated in patients with TPE and non-TPE, only two of

Table 1 Comparison of clinical and laboratory variables in patients with tuberculous pleural effusion (TPE) and non-tuberculous pleural effusion (non-TPE)

Parameter	Tuberculous pleural effusion (TPE) n = 44	Non-tuberculous pleural effusion (non-TPE) n = 159	p
Age (years)	51.5 (35.5–71.5)	68 (58–77)	0.0001
Sex F/M	11/33	75/84	0.01[a]
Site of pleural effusion (left/right/bilateral)	23/21/0	62/94/3	NS[a]
Body temperature (°C)	38.5 (36.6–39.0)	36.6 (36.6–38.0)	<0.0001
Duration of symptoms (weeks)	4 (2–7)	3 (1–6)	NS
TST (mm)	12 (10–15)	0 (0–9)	<0.001
WBC (10^9/L)	6.8 (5.3–8.7)	9.6 (7.0–12.9)	<0.0001
Pleural fluid protein concentration (g/dL)	5.0 (4.6–5.9)	4.1 (3.0–4.6)	<0.0001
Pleural fluid/serum protein ratio	0.7 (0.7–0.8)	0.6 (0.5–0.7)	<0.0001
Pleural fluid LDH activity (IU/L)	1,078 (604–2,070)	778 (298–1,795)	<0.05
Pleural fluid/serum LDH ratio	2.5 (1.5–3.8)	1.3 (0.6–3.2)	<0.05
Pleural fluid total cell count (10^9/L)	1,700 (1,200–3,300)	1,300 (500–3,180)	NS
Pleural fluid lymphocyte percentage	93 (81–96)	63 (36–81)	<0.0001
Pleural fluid ADA (U/L)	58 (44–91)	5 (0–14)	<0.0001
Pleural fluid IFN-γ (pg/mL)	735 (311–1,000)	8 (8–8)	<0.0001
Pleural fluid IL-2sRα (pg/mL)	2,665 (2,065–4,025)	2,116 (1,353–3,514)	<0.05
Pleural fluid IP-10 (pg/mL)	31,660 (21,570–34,399)	1,303 (359–3,400)	<0.001
Pleural fluid IL-12p40 (pg/mL)	621 (392–981)	203 (83–364)	<0.001
Pleural fluid IL-18 (pg/mL)	463 (57–858)	62 (8–185)	<0.001
Pleural fluid TNF-α (pg/mL)	79 (53–194)	13 (5–24)	<0.001
Pleural fluid Fas-ligand (pg/mL)	86 (64–133)	21 (159–32)	<0.001
Pleural fluid human MDC (pg/mL)	753 (523–1,056)	3,631 (281–585)	<0.001
Pleural fluid IL-23 (pg/mL)	1.5 (0.7–3.5)	1.2 (0.1–2.0)	NS

The data were expressed as median with interquartile range; quantitative variables were analyzed with Mann-Whitney U test

n patients number, *NS* non-significant, *TST* tuberculin skin test, *WBC* white blood count, *F* female, *M* male, *LDH* lactate dehydrogenase

[a]Relationship between qualitative variables was tested in system contingency tables (FREQ) using Chi-square or Fisher test

Table 2 Sensitivity, specificity, positive and negative predicted value, AUC, and cut-off values of different pleural fluid biomarkers discriminating between tuberculous pleural effusion (TPE) *vs.* non-tuberculous pleural effusion (non-TPE)

Pleural fluid parameter	Sensitivity	Specificity	PPV	NPV	AUC	Cut-off value
Pleural fluid ADA (U/L)	88.4	**92.8**	**77.6**	**96.6**	0.92	40.0
Pleural fluid IFN-γ (pg/mL)	**97.7**	**98.7**	**95.5**	**99.4**	**0.99**	118.7
Pleural fluid IL-2sRα (pg/mL)	79.1	48.4	29.6	89.4	0.63	2,047.7
Pleural fluid IP-10 (pg/mL)	**90.7**	**91.1**	73.6	**97.3**	**0.96**	11,420.0
Pleural fluid IL-12p40 (pg/mL)	87.2	72.6	45.9	95.5	0.83	296.0
Pleural fluid IL-18 (pg/mL)	61.9	87.6	60.5	88.2	0.77	327.7
Pleural fluid TNF-α (pg/mL)	89.7	79.3	58.3	96.0	0.87	31.6
Pleural fluid Fas-ligand (pg/mL)	**94.6**	89.6	**87.5**	95.6	**0.95**	45.0
Pleural fluid human MDC (pg/mL)	78.4	72.9	69.0	81.4	0.73	520.8
Pleural fluid IL-23 (pg/mL)	29.7	58.3	35.5	51.9	0.39	0.7

The three best results in each column are shown in bold numbers

Table 3 Comparison of clinical and laboratory variables in patients with tuberculous pleural effusion (TPE) *vs.* non-tuberculous pleural exudates.

Parameter	Tuberculous pleural effusion (TPE) n = 44	Non-tuberculous pleural exudates n = 129	P
Age (years)	51.5 (35.5–71.5)	66 (57–74)	0.0008
Sex F/M	11/33	64/65	0.004[a]
Site of pleural fluid (left/right/bilateral)	23/21/0	59/69/1	NS[a]
Body temperature (°C)	38.5 (36.6–39.0)	36.6 (36.6–38.0)	<0.001
Duration of symptoms (weeks)	4 (2–7)	3 (1–6)	NS
TST (mm)	12 (10–15)	0 (0–10)	<0.01
WBC (10^9/L)	6.8 (5.3–8.7)	10.3 (7.8–13.7)	<0.0001
Pleural fluid protein concentration (g/dL)	5.0 (4.6–5.9)	4.3 (3.9–48.0)	<0.0001
Pleural fluid/serum protein ratio	0.7 (0.7–0.8)	0.6 (0.6–0.7)	<0.001
Pleural fluid LDH activity (IU/L)	1,078 (604–2,070)	1,000 (541–2,415)	NS
Pleural fluid/serum LDH ratio	2.5 (1.5–3.8)	1.8 (0.9–4.0)	<0.0001
Pleural fluid total cell count (10^9/L)	1,700 (1,200–3,300)	1,730 (750–4,670)	NS
Pleural fluid lymphocyte percentage	93 (81–96)	62 (27–79)	<0.0001
Pleural fluid ADA (U/L)	58 (44–91)	8 (2–16)	<0.0001
Pleural fluid IFNγ (pg/mL)	735 (311–1,000)	8 (8–8)	<0.0001
Pleural fluid IL-2sRα (pg/mL)	2,665 (2,065–4,025)	2,598 (1,551–3,790)	NS
Pleural fluid IP-10 (pg/mL)	31,660 (21,570–34,399)	1,744 (450–3,647)	<0.0001
Pleural fluid IL-12p40 (pg/mL)	621 (392–981)	234 (105–449)	<0.0001
Pleural fluid IL-18 (pg/mL)	4,638 (57–858)	948 (11–260)	<0.0001
Pleural fluid TNF-α (pg/mL)	79 (53–194)	13 (7–24)	<0.0001
Pleural fluid Fas-ligand (pg/mL)	86 (64–133)	21 (15–32)	<0.0001
Pleural fluid human MDC (pg/mL)	753 (523–1,056)	363 (281–585)	<0.0005
Pleural fluid IL-23 (pg/mL)	1.5 (0.7–3.5)	1.2 (0.1–2.0)	NS

The data were expressed as a median with interquartile range; the analysis for quantitative variables was conducted by Mann-Whitney U test

n patients number, *NS* non-significant, *WBC* white blood count, *F* female, *M* male, *LDH* lactate dehydrogenase

[a]Relationship between qualitative variables was tested in system contingency tables (FREQ) using Chi-square or Fisher test

Table 4 Sensitivity, specificity, positive and negative predicted value, AUC, and cut off values of different pleural fluid biomarkers discriminating between tuberculous pleural effusion (TPE) and non-tuberculous pleural exudates

Pleural fluid parameter	Sensitivity	Specificity	PPV	NPV	AUC	Cut-off value
Pleural fluid ADA (U/L)	88.4	**91.1**	**77.6**	**95.7**	0.91	40
Pleural fluid IFN-γ (pg/mL)	**97.7**	**98.4**	**95.5**	**99.2**	**0.99**	118.7
Pleural fluid IL-2sRα (pg/mL)	79.1	40.6	30.9	85.2	0.57	2,047.7
Pleural fluid IP-10 (pg/mL)	**90.7**	89	73.6	**96.6**	**0.95**	11,420
Pleural fluid IL-12p40 (pg/mL)	87.2	66.4	45.5	93.9	0.8	296
Pleural fluid IL-18 (pg/mL)	61.9	84.1	60.5	84.9	0.73	327.7
Pleural fluid TNF-α (pg/mL)	89.7	78	63.6	94.7	0.86	31.6
Pleural fluid Fas-ligand (pg/mL)	**94.6**	**89.6**	**87.5**	95.6	**0.95**	45
Pleural fluid human MDC (pg/mL)	78.4	72.9	69	81.4	0.73	520.8
Pleural fluid IL-23 (pg/mL)	29.7	58.3	35.5	46.1	0.39	0.7

The three best results in each column are shown in bold numbers

these biomarkers showed a high discriminative value in ROC analysis. That refers to IP-10 and Fas-ligand. We demonstrated that the diagnostic accuracy of these two biomarkers, measured as the area under ROC curve, was only slightly lower than that of IFN-γ and somewhat higher than AUC for ADA. It should be emphasized that virtually the same diagnostic accuracy of IP-10 and Fas-ligand was found regardless of whether the analysis included all patients with pleural effusion or only patients with exudative pleural effusion. We believe that confirmation of the potential diagnostic application of pleural fluid IP-10 and Fas-ligand measurement in these two common clinical scenarios is one of the strengths of our study. Other advantages of the study include: (1) relatively large study group that included patients with different, but well defined, causes of pleural effusion, (2) prospective data collection, (3) application of selection criteria that excluded patients with unproven pleural TB, (4) the use of several different biomarkers in the same study group. The reliability of our study is emphasized by the fact that the present findings are fully consistent with the results of our earlier study that evaluated the role of ADA and IFN-γ in diagnosing tuberculous pleurisy. The optimal cut-off level for ADA calculated by ROC analysis was almost identical in the previous and present study, 40.3 U/L and 40.0 U/L, respectively. The sensitivity of pleural fluid ADA measurement found in our previous study was very high (100 %); it was somewhat lower (88.4 %) in the present study. There was no difference between the specificity of pleural fluid ADA demonstrated in both studies (93.9 and 92.8 %, respectively). Invariably, both studies showed that IFN-γ is a highly sensitive and specific marker of TP. Although the cut-off level of pleural fluid IFN-γ reported in our previous publication was lower than that used in this study, there was no difference in terms of the diagnostic sensitivity and specificity of IFN-γ in patients with TPE. In both studies, the sensitivity and specificity of pleural fluid IFN-γ ranged between 97.7 and 100 %. Thus, according to our experience, pleural fluid IFN-γ is the most sensitive and specific biomarker of TP. This has also been reported by other authors (Dheda et al. 2009a, b). On the other hand, it should be mentioned that the results of three large metaanalyses which assessed the diagnostic value of pleural fluid ADA and the results of two metaanalyses that evaluated the diagnostic value of IFN-γ in patients with TPE indicated a slightly higher sensitivity of ADA than IFN-γ (92.0–92.2 % *vs*. 87.0–89.0 %, respectively) (Liang et al. 2008; Jiang et al. 2007; Goto et al. 2003; Greco et al. 2003). In contrast to the sensitivity, IFN-γ was superior in terms of its diagnostic specificity (97.0 % in both available metaanalyses as compared to 90.0–92.2 % reported in three metaanalyses that evaluated diagnostic performance of ADA) (Liang et al. 2008; Jiang et al. 2007; Goto et al. 2003; Greco et al. 2003).

Thus, the role of IFN-γ and ADA in the diagnosis of TPE is well established. Advances in the understanding of the host immune response to *M. tuberculosis* infection, including cytokine release and cytokine-cell interactions have enabled a targeted search for new biomarkers which could be used in the diagnosis of TPE. The highly elevated level of IFN-γ and its high diagnostic accuracy in patients with TPE raised an interest in other cytokines which are closely related to IFN-γ. These include both IFN-γ inducing cytokines (e.g. IL-12) and IFN-γ inducible cytokines (e.g. IP-10). This was the rationale for the use of these two cytokines in our study. Interferon gamma-induced protein 10 kDa (IP-10) is a pleiotropic molecule capable of exerting potent biological functions: promoting chemotactic activity, inducing apoptosis, regulating cell growth and proliferation as well as angiogenesis in inflammatory diseases. IP-10 plays a role in various infectious diseases, including TB (Guo et al. 2014). It was identified in both resident and infiltrating cells (primarily monocyte/macrophages) present in inflamed tissues. Its expression in stimulated by IFNs released from T-cells and by other proinflammatory cytokines (Guo et al. 2014). We found that IP-10 was a highly sensitive and specific marker of TPE with its diagnostic accuracy, measured as area under ROC curve, only insignificantly

inferior to that of IFN-γ. In fact, pleural fluid level of IP-10 was the second most reliable biomarker of tuberculous pleurisy which showed similar sensitivity and specificity but slightly larger AUC as compared to pleural fluid ADA (0.96 and 0.92, respectively). Other studies comparing the diagnostic performance of IP-10 and IFN-γ in patients with TPE showed similar results or slightly lower sensitivity and specificity of IP-10 (Wang et al. 2012; Supryia et al. 2008). It must be emphasized that the methods of IP-10 measurement in pleural fluid can affect the results. The study by Supryia et al. (2008) showed that ROC analysis based on IP-10 measurement by cytometric bead array calculated sensitivity as 76.3 %, while the ROC analysis that was based on ELISA measurement demonstrated a higher value (89.5 %). In that study, the diagnostic accuracy of IP-10 measured as area under ROC curve was higher than that of IFN-γ (0.920 and 0.905, respectively). Recently, a metaanalysis of studies on the diagnostic yield of IP-10 in patients with TB has been published. Fourteen studies were included, but only five papers reported IP-10 level in patients with TPE. In the remaining studies IP-10 was measured in the whole blood or plasma (Guo et al. 2014). The authors of the metaanalysis calculated pooled diagnostic sensitivity, specificity, positive likelihood ratio, and negative likelihood ratio for pleural fluid IP-10 level in patients with TPE as 0.81 (95 % CI 0.75–0.86), 0.92 (95 % CI 0.84–0.95), 7.01 (95 % CI 4.23–11.61), and 0.22 (95 % CI 0.16–0.30), respectively. Lower values were found for IP-10 level measured in the blood or plasma in the diagnosis of TB.

IL-12 is a cytokine that plays an important role in the immune response to intracellular pathogens such as *M. tuberculosis* (Valdés et al. 2009). IL-12 molecule includes two polypeptide subunits, p40 and p35, linked covalently. The subunit p40 is shared with other member of the IL-12 family-IL-23 that has similar (but also some distinct) biological activities. IL-12 is produced by dendritic cells and other antigen presenting cells. As part of the immune response to *M. tuberculosis* infection, IL-12 activates macrophages by upregulating the production of IFN-γ by activated T cells and natural killer cells. This raised the interest in IL-12 and its subunits as potential markers of TB infection (Valdés et al. 2009). In our present study the diagnostic performance of pleural fluid level of IL-12p40 as a marker of TP was not only inferior to IFN-γ and IP-10 but also to pleural fluid level of ADA. Although the sensitivity of IL-12p40 was relatively high (87.2 %), its specificity for discriminating TPE from all other pleural effusions was significantly lower and was calculated as 72.6 % and 66.4 % when TPE was differentiated from other exudative effusions. The results of other studies on the diagnostic accuracy of IL-12p40 in patients with TPE showed some heterogeneity. Several authors reported a relatively high diagnostic sensitivity of this biomarker (ranging from 85.1 to 97.4 %) but significantly lower specificity (58.3 to 70.2 %) (Valdés et al. 2009; Supriya et al. 2008; Gao and Tian 2005). This is consistent with the results of our study. It might be concluded that, based on the results of the previous and present studies, pleural fluid level of IL-12p40 cannot be regarded as a reliable marker of TPE and should not be used in decision making in patients with pleural effusion. This is particularly important given the fact that significantly more accurate biomarkers are available. It should be underlined that, similar to our findings, several earlier studies which directly compared diagnostic accuracy of IL-12p40 and IFN-γ showed superiority of the latter. Summarizing the discussion on the diagnostic yield of IFN-γ, IL-12, and IP-10 in the diagnosis of TPE, it should be stated that, apart from our study, there are only two other studies which directly compared the diagnostic performance of all these three biomarkers. Based on the results of these studies, IFN-γ and IP-10 should be ranked as the first and the second, respectively, most accurate biomarkers, respectively, while IL-12p40 was found to be less effective.

The present study demonstrates that soluble Fas-ligand was the second-third most valuable marker of tuberculous pleurisy. Fas-ligand is a transmembrane protein expressed by different cells, but it is mainly localized on activated T lymphocytes and natural killer (NK) cells (Caulfield and Lathem 2014). Fas-ligand dependent cell death is involved in a host of inflammatory innate immune responses to many bacterial

pathogens (Caulfield and Lathem 2014). *Mycobacterium tuberculosis* infection may promote apoptosis of macrophages and T lymphocytes through the Fas/FasL pathway (Budak et al. 2008). As matrix metalloproteinases can cleave membrane-bound Fas-ligand, its soluble form might be found in sites of *M. tuberculosis* infection. Our ROC analysis showed that pleural fluid Fas-ligand concentration was a highly accurate marker of TP with an area under ROC curve only slightly lower than that of IFN-γ (0.96 and 0.99, respectively). We are aware of only few earlier publications that reported pleural fluid level of soluble Fas-ligand. The results of these studies are highly discordant. Cui et al. (2010) have demonstrated a significant difference between Fas-ligand concentration in TPE and malignant pleural effusions. However, the absolute difference between Fas-ligand level in these two pleural fluid categories was relatively small. Moreover, the results raise some doubts as the reported pleural fluid Fas-ligand concentration was almost 10^6 fold higher than that found in other studies. In a study by Wang et al. (2002) patients with bacterial empyema had soluble Fas ligand pleural fluid level twice as high as patients with TPE. A Turkish study by Budak et al. (2008) showed a significantly higher pleural fluid level of soluble Fas-ligand in patients with TPE as compared to patients with malignant pleural effusion and other non-tuberculous, non-malignant pleural effusion. However, that study evaluated the relationship between pleural fluid Fas-ligand and other T-helper type 1 cytokines rather than the diagnostic performance of pleural fluid Fas-ligand. The results of our present study are similar to that reported by Wu et al. (2010) who assessed the diagnostic yield of pleural fluid soluble Fas-ligand in 23 patients with TP and 56 patients with non-TPE. They found a very high diagnostic sensitivity and high specificity of this marker (95.7 % and 80.4 %, respectively). In the present study of 44 patients with TP and 159 patients with non-TPE, the sensitivity of pleural fluid Fas-ligand concentration was close to that reported by Wu et al. (2010) (94.6 %), but its specificity was significantly higher and reached 89.6 %. To our knowledge, our study is the first to report such a high diagnostic accuracy of pleural fluid soluble Fas-ligand concentration. This should be validated in further studies.

It might be interesting that albeit Fas-ligand belongs to TNF family, the diagnostic accuracy of TNF in the present study was lower than that of Fas-ligand. The result of other studies were ambiguous. Some papers reported a significant difference in pleural fluid TNF concentration between tuberculous and non-tuberculous (mainly malignant) pleural effusions, some other did not. The effectiveness of pleural fluid TNF-α in differentiation between TPE and non-TPE was moderate, with area under ROC curve 0.89 (0.81–0.97) found in one study and sensitivity, specificity and the diagnostic accuracy 69.2 %, 87.1 %, and 77.1 %, respectively, reported in other study (Daniil et al. 2007; Kim et al. 1997).

We are aware of some potential limitations of the present study. Firstly, the number of patients with different etiologies of pleural effusion was significantly different. This particularly refers to 88 patients with MPE as compared with only 6 patients with miscellaneous causes of pleural effusion. We cannot exclude that these differences could have influenced the cut-off level selection and affect the results of ROC analysis. Secondly, post-test probability of TPE was measured as PPV and NPV only. As both PPV and NPV depend on the prevalence of diseases in the population that were studied, the results cannot be simply extrapolated on the populations with a different prevalence of the conditions which underlie pleural effusion. In this context, the expression of the results as likelihood ratios or relative risk would make our findings more universal.

In conclusion, we demonstrate that pleural fluid IFN-γ is an extremely accurate marker of tuberculous pleurisy. IP-10 and Fas-ligand are two other sensitive and specific biomarkers that can be applied to differentiate between tuberculous and non-tuberculous pleural effusion. In our study the diagnostic accuracy of these three markers exceeded that of ADA. We found that the diagnostic performance of several tested biomarkers was moderate or low, hence there is no justification for the measurement of pleural fluid concentration of these markers in clinical practice.

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

References

Baumann MH, Nolan R, Petrini M, Lee YC, Light RW, Schneider E (2007) Pleural tuberculosis in the United States: incidence and drug resistance. Chest 131 (4):1125–1132

Budak F, Uzaslan EK, Cangur S, Goral G, Oral HB (2008) Increased pleural soluble fas-ligand (sFasL) levels in tuberculosis and its relation with T-helper type 1 cytokines. Lung 186(5):337–343

Caulfield AJ, Lathem WW (2014) Disruption of Fas-Fas ligand signaling, apoptosis, and innate immunity by bacterial pathogens. PLoS Pathog 10 (8):e1004252

Cui HY, Zhang O, Su B, Li W, Tang SJ (2010) Differential levels of cytokines and soluble Fas ligand between tuberculous and malignant effusions. J Int Med Res 38 (6):2063–2069

Daniil ZD, Zintzaras E, Kiropoulos T, Papaioannou AI, Koutsokera A, Kastanis A, Gourgoulianis KI (2007) Discrimination of exudative pleural effusions based on multiple biological parameters. Eur Respir J 30 (5):957–964

Dheda K, van Zyl-Smit RN, Sechi LA, Badri M, Meldau R, Meldau S, Symons G, Semple PL, Maredza A, Dawson R, Wainwright H, Whitelaw A, Vallie Y, Raubenheimer P, Bateman ED, Zumla A (2009a) Utility of quantitative T-cell responses versus unstimulated interferon-{gamma} for the diagnosis of pleural tuberculosis. Eur Respir J 34 (5):1118–1126

Dheda K, van Zyl-Smit RN, Sechi LA, Badri M, Meldau R, Symons G, Khalfey H, Carr I, Maredza A, Dawson R, Wainright H, Whitelaw A, Bateman ED, Zumla A (2009b) Clinical diagnostic utility of IP-10 and LAM antigen levels for the diagnosis of tuberculous pleural effusions in a high burden setting. PLoS ONE 4(3):e4689

Gao ZC, Tian RX (2005) Clinical investigation on diagnostic value of interferon-gamma, interleukin-12 and adenosine deaminase isoenzyme for tuberculous pleurisy. Chin Med J 118(3):234–237

Giusti G (1974) Adenosine deaminase. In: Bergmeyer HU (ed) Methods of enzymatic analysis. Academic, New York, pp 1092–1099

Goto M, Noguchi Y, Koyama H, Hira K, Shimbo T, Fukui T (2003) Diagnostic value of adenosine deaminase in tuberculous pleural effusion: a meta-analysis. Ann Clin Biochem 40:374–381

Greco S, Girardi E, Masciangelo R, Capoccetta GB, Saltini C (2003) Adenosine deaminase and interferon-γ measurements for the diagnosis of tuberculous pleurisy: a meta-analysis. Int J Tuberc Lung Dis 7(8):777–786

Guo SJ, Jia LQ, Hu QJ, Longm HY, Pangm CS, Wen FQ (2014) Diagnostic accuracy of interferon gamma-induced protein 10 for tuberculosis: a meta-analysis. Int J Clin Exp Med 7(1):93–100

Jiang J, Shi HZ, Liang QL, Qin SM, Qin XJ (2007) Diagnostic value of interferon-gamma in tuberculous pleurisy: a metaanalysis. Chest 131(4):1133–1141

Kim YC, Park KO, Bom HS, Lim SC, Park HK, Na HJ, Park JH (1997) Combining ADA, protein and IFN-γ best allows discrimination between tuberculous and malignant pleural effusion. Korean J Intern Med 12 (2):225–231

Krenke R, Korczynski P (2010) Use of pleural fluid levels of adenosine deaminase and interferon gamma in the diagnosis of tuberculous pleuritis. Curr Opin Pulm Med 16(4):367–375

Krenke R, Safianowska A, Paplińska M, Nasiłowski J, Dmowska-Sobstyl B, Bogacka-Zatorska E, Jaworski A, Chazan R (2008) Pleural fluid adenosine deaminase and interferon gamma as diagnostic tools in tuberculous pleurisy. J Physiol Pharmacol 59(Suppl 6):349–360

Liang QL, Shi HZ, Wang K, Qin XJ (2008) Diagnostic accuracy of adenosine deaminase in tuberculous pleurisy: a meta-analysis. Respir Med 102(5):744–754

Light RW (2010) Update on tuberculous pleural effusion. Respirology 15(3):451–458

Pai M, Flores LL, Hubbard A, Riley LW, Colford JM Jr (2004) Nucleic acid amplification tests in the diagnosis of tuberculous pleuritis: a systematic review and meta-analysis. BMC Infect Dis 4:6

Porcel JM (2009) Tuberculous pleural effusion. Lung 187:263–270

Supryia P, Chandrasekaran P, Das SD (2008) Diagnostic utility of interferon-γ-induced protein of 10 kDa (IP-10) in tuberculous pleurisy. Diagn Microbiol Infect Dis 62(2):186–192

Trajman A, Kaisermann C, Luiz RR, Sperhacke RD, Rossetti ML, Féres Saad MH, Sardella IG, Spector N, Kritski AL (2007) Pleural fluid ADA, IgA-ELISA and PCR sensitivities for the diagnosis of pleural tuberculosis. Scand J Clin Lab Invest 67(8):877–884

Trajman A, Pai M, Dheda K, van Zyl SR, Zwerling AA, Joshi R, Kalantri S, Daley P, Menzies D (2008) Novel tests for diagnosing tuberculous pleural effusion: what works and what does not? Eur Respir J 31(5):1098–1106

Valdés L, San José E, Alvarez Dobaño JM, Golpe A, Valle JM, Penela P, Gonzlez Barcala FJ (2009) Diagnostic value of interleukin-12 p40 in tuberculous pleural effusions. Eur Respir J 33(4):816–820

Wang PS, Chen YM, Hsieh YL, Yu CF, Tsai CM, Perng RP (2002) Pleural effusion and serum soluble fas-ligand levels are elevated in different clinical conditions. Lung 180(1):25–32

Wang H, Yue J, Yang J, Gao R, Liu J (2012) Clinical diagnostic utility of adenosine deaminase, interferon-γ, interferon-γ induced protein of 10 kDa and dipeptidyl peptidase 4 levels in tuberculous pleural effusions. Heart Lung 41(1):70–75

Wu SH, Li CT, Lin CH, Chu JJ, Cheng ML, Lin KH (2010) Soluble Fas ligand is another good diagnostic marker for tuberculous pleurisy. Diagn Microbiol Infect Dis 68(4):395–400

Advs Exp. Medicine, Biology - Neuroscience and Respiration (2015) 11: 31–37
DOI 10.1007/5584_2014_107

Published online: 30 January 2015

Low-Dose Computer Tomography as a Screening Tool for Lung Cancer in a High Risk Population

A. Nahorecki, M. Chabowski, T. Kuźniar, B. Kędzierski, P. Jaźwiec, A. Szuba, and D. Janczak

Abstract

The aim of the study was to evaluate the usefulness of low-dose computer tomography as a screening tool for early stage lung cancer. The study was performed in 332 individuals aged 55–70 who were asymptomatic, who had not previously suffered from cancer, and who smoked at least ten packs of cigarettes a year. Baseline and repeated LD-CT scans were performed. Pulmonary nodules were classified according to the size and morphology, and the results were categorized as negative (no nodules observed), semi-positive (nodules of 4 mm or smaller in diameter) and positive (nodules 5 mm or larger). Based on the category of the patient, either a repeat low-dose CT, a bronchoscopy with or without a biopsy, or a PET-CT was performed. The baseline screening showed 59 positive results. Eighteen patients were hospitalized and underwent bronchoscopy and biopsy. One of these patients had Stage I non small cell lung carcinoma (NSCLC) and a lobectomy was performed. Three patients had Stage IV NSCLC and were referred for chemotherapy. We identified 103 semi-positive results. Only 25 of those patients had a repeat scan because of noncompliance. We observed no significant growth of diagnosed nodules in a semi-positive group. Low-dose CT can be used as a screening tool for early stage lung cancer. A high percentage of false-positive results are observed. There are difficulties in diagnosing nodules in patients with post-tuberculosis changes. A high rate of non-compliance was noticed.

A. Nahorecki (✉) and T. Kuźniar
Department of Internal Medicine and Pneumology, Fourth Military Teaching Hospital, 5 Weigla St., 50-981 Wroclaw, Poland
e-mail: a.nahorecki@gmail.com

M. Chabowski and D. Janczak
Department of Thoracic Surgery, Fourth Military Teaching Hospital, 5 Weigla St., 50-981 Wroclaw, Poland

Department of Clinical Proceedings, Faculty of Health Science, Wroclaw Medical University, 5 Bartla St., 51-618 Wroclaw, Poland

B. Kędzierski and P. Jaźwiec
Department of Radiology and Diagnostic Imaging, Fourth Military Teaching Hospital, 5 Weigla St., 50-981 Wroclaw, Poland

A. Szuba
Department of Internal Medicine and Pneumology, Fourth Military Teaching Hospital, 5 Weigla St., 50-981 Wroclaw, Poland

Department of Internal Medicine, Faculty of Health Science, Wroclaw Medical University, 5 Bartla St., 51-618 Wroclaw, Poland

Keywords
Chest • Imaging • Radiology • Tumor

1 Introduction

Lung cancer is the most common cancer in Poland and it is the cancer which causes the highest number of deaths worldwide (Kosacka and Jankowska 2007; Jemal et al. 2004). In 2009, it accounted for 21.4 % of all malignant tumors in Poland among men and 8.5 % among women. It was responsible for 31.2 % of deaths among men and 14.5 % among women according to the National Register of Cancer (2011). In the Lower Silesia region, the morbidity rate totaled 21.2 % for men and 9.8 % for women. Roughly 21,000 people develop lung cancer in Poland each year (morbidity is estimated at 50.0/100,000 for men and 17.2/100,000 for women). Only 13.6 % of new cases undergo surgery in the early stage of the disease, which considerably deviates from the rate present in other well-developed EU countries. Early detection of the disease in its asymptomatic stage increases the recovery rate significantly (the 5-year-long survival rate for Stage I non small cell lung carcinoma (NSCLC) is estimated at 67 %, whereas for Stage IV it is just 3 %) (Grodzki et al. 2009; Chabowski et al. 2008; Henschke et al. 2006). The research carried out in Poland and all over the world has shown that screening tests (tomography) in a population with a higher rate of morbidity shifts patients to lower stages of the disease, which, in turn, enhances the chance of recovery (Henschke et al. 1999; Kubik and Polak 1986).

2 Methods

The study was approved by the Ethics Committee of Lower Silesian Voivodeship in Poland and it was performed at the Department of Internal Medicine and the Institute of Radiology and Imaging Diagnostics of the Fourth Military Teaching Hospital in Wroclaw, Poland.

2.1 Participants

A screening program was created for the population aged 55–70 of the city of Wroclaw who had consistently smoked a minimum of ten packs of cigarettes per year, was asymptomatic, and had not previously been diagnosed with cancer. The individuals volunteered for the study in response to local and regional TV and radio announcements, as well as letters which had been sent to GPs registered in the city. All participants agreed to undergo baseline and subsequent CT examinations. After receiving written information about the study and signing the informed consent, each patient was interviewed according to a structured form. Then, a low-dose CT without contrast was performed. Additionally, each patient filled out a smoking questionnaire (addiction strength, motivation to quit smoking), based on which they were referred to a clinic for nicotine-addicted. Three hundred thirty two participants were enrolled in the trial from December of 2012 till June 2014.

2.2 Imaging

CT scans were performed using Somatom Definition AS (Siemens AG, Erlangen, Germany) with automatic dose adjustment, contrast free, and an average dose of 30 mAs, with a tube voltage of 120 kVp for patients with a body mass index (BMI) >30 kg/m^2 and 100 kVp with a BMI <30 kg/m^2, and 1 mm section width. Scans were performed with spiral data acquisition with the following acquisition parameters: section collimation 64 $\times$ 0.6 mm, pitch 1.2, reconstructions in two ways: 5 mm and 1 mm with a lung and mediastinal window algorithm. All image data were stored in DICOM format. The CT examination performed during one single breath holding included the whole rib cage and the upper part

of the abdomen (reaching the adrenal glands). All scans were reviewed at a computer workstation by radiologists with at least 5 years' experience in chest radiography. Location, size, demarcation, and shape of nodules were registered. We used a modified International Early Lung Cancer Action Program (I-ELCAP 2012) protocol to classify nodules.

The nodules were determined as solid, part-solid, and non-solid; measured and considered to be classified as calcified or non-calcified. The size was defined as the average of length and width. The individuals were classified according to nodule size into three categories: positive, semi-positive, and negative. The result was considered positive if the identified nodule was non-calcified, solid, non-solid or partly solid and 5 mm or larger in diameter. The result was considered semi-positive if the identified nodule was non-calcified, solid, non-solid or partly solid, and smaller than 5 mm in diameter.

2.3 Follow-Up

All scans were evaluated by a pneumologist, who decided on further procedures according to a screening algorithm outlined below. The action taken after a positive result depended on the size of the initially described nodules.

2.4 Baseline Screening

The patients with positive results were divided into three categories.

1. Patients with nodules of 15 mm or larger or with typical signs of malignancy were admitted to the hospital, underwent a bronchoscopy, and those with negative results (no malignant cells detected) were referred to a PET scan.
2. Patients with nodules of 5–15 mm were referred to a repeated low dose CT in 3 months or a PET scan if the nodules were larger than 10 mm but without the presence of typical signs of malignancy.
3. Patients with nodules of 5 mm or larger localized endobronchially were provided with a low dose non-contrast CT scan within 1 month.

The patients with a semi-positive result were referred to CT within 12 months after the initial test. If the result was negative, no further scans were performed.

2.5 Repeat Screening

The stratification of patients with positive results was a follows.

1. Patients with nodules of 5 mm or larger localized endobronchially were provided with a low dose, non-contrast CT scan within 1 month.
2. Patients with nodules of 3–5 mm in diameter were referred for a repeated CT scan in 6 months. If significant growth was observed, the patient underwent a BF with biopsy. The significant growth was determined as the enlargement of the entire nodule, growth of a solid component of a partly solid nodule, or the development of a solid component in a previously non-solid nodule. If no significant growth was observed, no further scans were performed.
3. Patients with nodules of 5 mm or larger in diameter underwent a low dose CT 1 month after the prior one with or without a course of a broad spectrum antibiotic. If the nodule showed significant growth, the patients were admitted to the hospital, underwent a bronchoscopy with biopsy. If the result was negative, a PET scan was provided. If no significant growth was present, the workup stopped.
4. For nodules 10 mm or larger and partly solid nodules whose solid component was 10 mm or larger, an immediate PET scan was provided.

The patients with a semi-positive result (i.e. a new solid or partly solid nodule of less than 3 mm or non-solid nodule of any size) were referred for 12 months after the initial test.

If the result was negative, no further scans were performed.

Of note, incidental findings outside the rib cage were reported and then the patients were referred for further diagnostics. Once there was a suspicion of malignancy, the patient was admitted to the Clinic of Internal Medicine, where they underwent bronchoscopy with transthoracic biopsy when required. When no malignant cells were detected in bronchoalveolar lavage, forceps biopsy, smear brush, or the biopsy specimen, but a nodule was suspected of being malignant, a PET CT scan was performed. Each individual in whom lung cancer was diagnosed was characterized in terms of the stage of the disease, including TNM classification (Mirsadrae et al. 2012), and the cell type.

3 Results

Out of the 332 individuals enrolled it the trial, 165 were men, and 167 were women. Among 332 baseline scans, 59 produced positive results and there were 103 semi-positive results. No pulmonary nodules were observed in 170 CT scans. The classification according to the size of positive findings is included in Table 1.

Table 1 Classification of nodules by size as described in the screening program

Diameter (mm)	1–5	6–9	10–14	15–19	≥20
Number of nodules	103	38	9	6	6
Percentage (%)	63.6	23.5	5.6	3.7	3.7

3.1 Positive Results

Fifty nine individuals were classified as positive (nodule of 5 mm or larger in diameter). Based on the radiologic appearance after baseline screening, ten individuals were admitted to the hospital for diagnostic evaluation. Three of those patients underwent a bronchoscopy with biopsy, as a result of which two cases of NSCLC were diagnosed (Figs. 1 and 2). Nonetheless, no malignant cells were detected in the biopsy specimen of one of the patient, but because of the clinical and radiological changes he was referred to a PET CT in which a malignant tumor with metastases was confirmed. The patient was admitted to the surgical ward, where he underwent mediastinoscopy, with the histopathological result of adenocarcinoma. Another patient underwent lobectomy and Stage I NSCLC was diagnosed (squamous cell carcinoma) (Fig. 3). In another six patients, no sign of malignancy was found. A PET scan was performed in all of them, with negative results. These patients were referred for further evaluation (LD-CT in 6 months). Two of them most likely had post-tuberculotic changes in the lungs (Fig. 4).

Forty nine patients were referred for a repeat scan after 3 months. Thirteen of them were non-compliant and did not turn up for the test. No significant growth was observed in 27 of the remaining 36 cases; a repeat scan after 12 months was preplanned. Six of these patients were referred for a PET scan and all the results were negative. Three patients were submitted to a

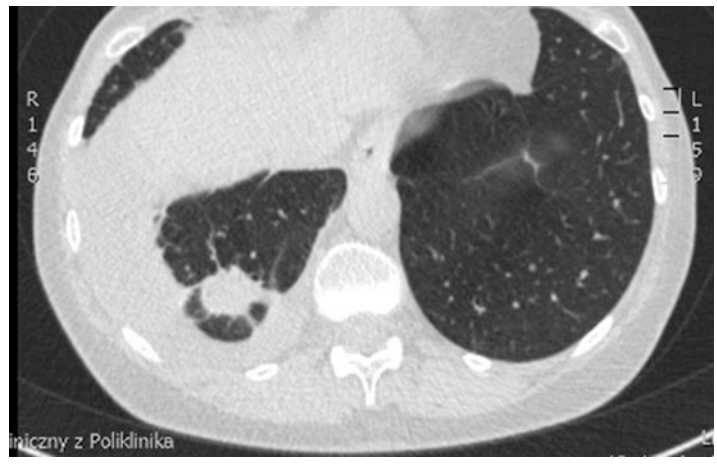


Fig. 1 Non-small cell cancer of right lung

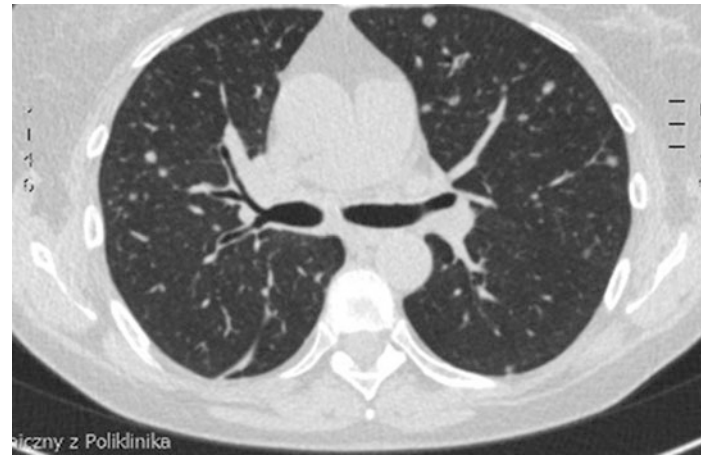


Fig. 2 Pulmonary metastases of non-small cell lung cancer

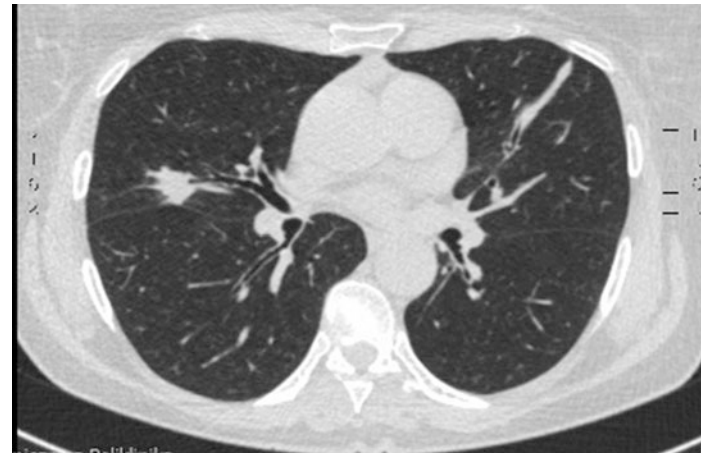


Fig. 3 Squamous cell lung cancer, Stage I, right middle lobe

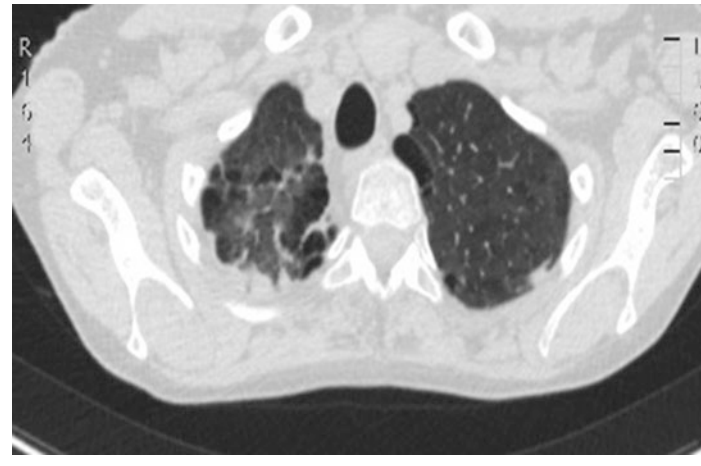


Fig. 4 Post-tuberculotic changes in upper lung lobes

control examination within 3 months after completing a full course of antibiotics. A progression of the disease was noted in one of the patients, after receiving complete antibiotic therapy. This patient and two others with changes in the upper part of the lungs were admitted to the hospital. They underwent bronchoscopy during which the material for a cytologic examination was collected. The examination results were negative and the patients were referred for a PET scan.

3.2 Semi-positive result

A hundred and three patients with a semi-positive result from baseline screening were referred for annual screening. Only did 25 of them turn up for a repeat scan. Among all the annual screenings, no significant growth was reported. No further action was taken.

4 Discussion

Lung cancer is still the leading cause of death due to cancer. The prognosis in lung cancer is very poor with a 5-year survival rate of about 10–15 %. This has remained unchanged over the last two decades. In Poland every year approximately 3,000 out of 20,000 new cases of primary NSCLC are operated on, with a resection rate of 17 % (Chabowski et al. 2008). Trials evaluating chest X-rays and sputum cytology as screening modalities were conducted in the 1970s, but they did not show a reduction in lung cancer mortality (Kubik and Polak 1986). The major breakthrough was the National Lung Screening Trial (NLST) that demonstrated a 20 % reduction in mortality rates in a low-dose CT group compared to the X-ray group (National Lung Screening Trial Research Team 2011). In this trial the number of patients that had to be screened to prevent one death was 320.

A high mortality rate of lung cancer patients is related to delayed diagnosis (Laprus et al. 2011). The majority of lung cancer patients display symptoms in the late advanced stage. The main aim of the screening program was to diagnose lung cancer at an early Stage I or II. The achievement of our program was the detection of one patient at Stage I NSCLC, which constitutes 25 % of lung cancers diagnosed during baseline screening. The lower percentage of Stage I lung cancer patients in comparison with other studies might result from an improper qualification by GPs. Firm conclusions on the benefits of the screening program should not yet be drawn. The disadvantages of our program were the lack of a control group and a limited number of individuals screened. In the trials that have been carried out, a large number of false positive low-dose CT scan results was a major problem. Likewise, the percentage of false positive results was high as it amounted to 47 % in our study. The majority of individuals were not elected for further investigation. However, 19 people underwent some diagnostic tests, e.g. bronchoscopy, transthoracic biopsy, or PET-CT scan, which might have exposed them to the complications connected with these procedures. Fortunately, no patient with a benign tumor underwent surgery. To sum up, low-dose CT showed high sensitivity, but very low specificity in lung cancer detection.

During follow-up visits in the positive group, a significant enlargement of pulmonary nodules was not observed. The nodules qualified for the 2nd and 3rd round CT scan did not prove to be malignant. Therefore, we do not recommend an early CT scan.

Patients with false positive result suffered from the persistent stress due to the necessity of further investigations and the awareness of the presence of a lung lesion suspected of being cancerous. In the following program we are going to introduce a participant discomfort questionnaire. A decrease in false positive results and an increase in sensitivity might be obtained by increasing the size limit of positive and semi-positive lesions and by taking into consideration some morphological features of benign lesions. That could lead to a decrease in the number of CT scan performed and reduction in the total cost of the program. Only did some of our patients (24 %) from the semi-positive group attend the follow-up visits after 12 months. We did not

observe any significant lesion enlargement in any of the 25 patients. However, due to a small study group, we cannot advocate not to perform further CT scans in patients with small, lower than 5 mm lung nodules. Considering a short 12-month observation period, any conclusion on the 5-year survival improvement cannot be drawn either.

Patients' education on the damaging effects of smoking was an additional benefit of this program. During follow-up visits it turned out that six patients from both positive and semi-positive groups stopped smoking. In our opinion that is the best evidence of the strength of nicotine addiction. It should always be remembered that quitting smoking is beneficial to health at any age (Murray and Lopez 1997). At lung cancer screening tests, many other asymptomatic lesions were detected, but the extrapulmonary lesions were not further followed up. However, a large number of patients with coronary artery calcifications should deserve special attention.

In the years 2008–2011, four lung cancer screening programs were conducted in different Polish cities. The enrolled patients were smokers, aged 50–75. In total, 34,810 patients were screened. 324 (0.9 %) lung cancers were detected, including 205 patients (63 %) at Stage I. The overall detection rate was 1 lung cancer per 107 low-dose CT scans (Grodzki et al. 2009), which is grossly similar to the present results of 1.2 % lung cancers being detected, i.e. one lung cancer per 83 low-dose CT scans, and to other trials (Bach et al. 2012).

5 Conclusions

Low-dose CT can be used as a screening tool for early stage lung cancer. A high percentage of false-positive results is observed. There are difficulties in diagnosing nodules in patients with post-tuberculosis changes. A high rate of noncompliance was noticed.

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

References

Bach PB, Mirkin JN, Oliver KT, Azzoli CG, Berry DA, Brawley OW, Byers T, Colditz GA, Gould MK, Jett JR, Sabichi AL, Smith-Bindman R, Wood DW, Qaseem A, Detterbeck FC (2012) Benefits and harms of CT screening for lung cancer: a systematic review. JAMA 307:2418–2429

Chabowski M, Orlowski TM, Rabczenko D (2008) Analysis of prognostic factors and efficacy of surgical treatment for non-small cell lung cancer: department of surgery NTLDRI. Pneumonol Alergol Pol 76:1–10

Grodzki T, Walecka A, Fabian W, Daniel B, Witkiewicz I, Jarmoliński T, Alchimowicz J, Wójcik J (2009) Program of early detection of pulmonary neoplasms by the computed tomography-preliminary Szczecin experience. Pneumonol Alergol Pol 77:521–527

Henschke CI, McCauley DI, Yankelevitz DF, Naidich DP, McGuinness G, Miettinen OS et al (1999) Early lung cancer action project: overall design and findings from baseline screening. Lancet 354:99–105

Henschke CI, Yankelevitz DF, Libby DM, Pasmantier MW, Smith JP, Miettinen OS (2006) Survival of patients with Stage I lung cancer detected on CT screening. N Engl J Med 355:1763–1771

I-ELCAP – International Early Lung Cancer Action Program: Enrollment and Screening Protocol (2012) Available from: http://www.ielcap.org/protocols. Accessed on 2 Nov 2012

Jemal A, Tiwari R, Murray T, Ghafoor A, Samuels A, Ward E, Feuer EJ, Thun MJ (2004) Cancer statistics. CA Cancer J Clin 54:8–29

Kosacka M, Jankowska R (2007) Lung cancer epidemiology. Pneumonol Alergol Pol 75:76–80

Kubik A, Polak J (1986) Lung cancer detection: results of a randomized prospective study in Czechoslovakia. Cancer 57:2427–2437

Laprus I, Adamek M, Kozielski J (2011) The need of lung cancer screening-new evidence, new expectations. Pneumonol Alergol Pol 79:419–427

Mirsadrae S, Oswal D, Alizadeh Y, Caulo A, Van Beek EJR (2012) The 7th lung cancer TNM classification and staging system: review of the changes and implications. World J Radiol 4:128–134

Murray CJL, Lopez AD (1997) Alternative projections of mortality and disability by cause 1990–2020: Global burden of disease. Lancet 349:1498–1504

National Lung Screening Trial Research Team, Aberle DR, Adams AM, Berg CD, Black WC, Clapp JD, Fagerstrom RM, Gareen IF, Gatsonis C, Marcus PM, Sicks JD (2011) Reduced lung-cancer mortality with low-dose computed tomographic screening. N Engl J Med 365:395–409

National Register of Cancer (database online) (2011) Available from: www.pto.med.pl/linki/krajowy_rejestr_nowotworow. Accessed on 11 Nov 2012 (in Polish)

Advs Exp. Medicine, Biology - Neuroscience and Respiration (2015) 11: 39–48
DOI 10.1007/5584_2014_109

Published online: 10 March 2015

Decreased *FAM107A* Expression in Patients with Non-small Cell Lung Cancer

D. Pastuszak-Lewandoska, K.H. Czarnecka, M. Migdalska-Sęk, E. Nawrot, D. Domańska, J. Kiszałkiewicz, J. Kordiak, A. Antczak, P. Górski, and E. Brzeziańska-Lasota

Abstract

Lung cancer is the leading cause of cancer-related death in the world. Early detection, based on molecular markers, could decrease mortality from this disease. Tumor development is often associated with inactivation or loss of tumor suppressor genes (TSGs). The aim of the present study was to analyze the expression level of *FAM107A* gene, a TSG located in 3p21.1, in lung cancer tumors and in tumor adjacent normal lung samples. Promoter methylation status of *FAM107A* was evaluated as the potential mechanism of its epigenetic silencing. The relationship between gene mRNA expression and tumor staging, metastasis status, and non-small cell lung cancer (NSCLC) histopathological subtypes in 60 patients was analyzed. Total RNA was isolated from tissue samples and gene expression was assessed in qPCR assay. Gene promoter methylation status was evaluated in MSP reactions, using bisulfite converted DNA and two pairs of primers: methylated and unmethylated. We found that the expression of the gene was dramatically decreased in all NSCLC samples and was significantly lower than in tumor adjacent normal lung tissue. Promoter methylation of *FAM107A* gene was confirmed only in the minority of NSCLCs. The results highlight the importance of *FAM107A* in lung carcinogenesis, although indicate other than promoter hypermethylation mechanism of the gene decreased expression.

D. Pastuszak-Lewandoska, K.H. Czarnecka, M. Migdalska-Sęk, E. Nawrot, D. Domańska, J. Kiszałkiewicz, and E. Brzeziańska-Lasota (✉)
Department of Molecular Bases of Medicine, Medical University of Lodz, 251 Pomorska St., 92-213 Lodz, Poland
e-mail: ewa.brzezianska@umed.lodz.pl

J. Kordiak
Department of Chest Surgery, General and Oncological Surgery, University Hospital No. 2, Medical University of Lodz, Lodz, Poland

A. Antczak
Department of General and Oncological Pulmonology, Medical University of Lodz, Lodz, Poland

P. Górski
Department of Pneumology and Allergology, Medical University of Lodz, Lodz, Poland

Keywords
Epigenetic mechanisms • Gene expression • Lung cancer • NSCLC • Promoter methylation

1 Introduction

Lung cancer remains the most common cancer in the world, both in term of cases (1.6 million cases, 12.7 % of total) and deaths (1.4 million deaths, 18.2 % of total), being the most common cancer in men worldwide (1.1 million cases, 16.5 % of total), and the fourth most frequent cancer in women (513,000 cases, 8.5 % of all cancers) (Ferlay et al. 2010). According to histopathological verification, lung cancer is classified into two major groups based on its biological phenotype, therapy, and prognosis: non-small cell lung cancer (NSCLC), including squamous cell carcinoma (SSC), adenocarcinoma (AC), and large cell carcinoma (LCC) which account for approximately 80 % of all primary lung cancers, and small cell lung cancer (SCLC) which constitutes about 20 % of malignant cases (Travis et al. 2004). Lung cancer is characterized by high fatality: the ratio of mortality to incidence is 0.86 (Ferlay et al. 2010), mainly because the disease is most often diagnosed at an advanced stage when there are few curative treatment options. The overall 5-year survival for NSCLC does not exceed 15 %, however, in stage I disease it increases up to 83 % (Kathuria et al. 2014). This highlights the importance of detecting lung cancer at an early and potentially treatable stage. It is believed that combining molecular information regarding biomarkers that are highly sensitive and specific with clinical risk features may offer the improved way to identify individuals with the highest risk for lung cancer development or for lung cancer progression.

The *FAM107A* gene, a member of the family with sequence similarity 107 (*FAM107*), also named *DRR1* (downregulated in renal cell carcinoma), and *TU3A* (Tohoku University cDNA clone A on chromosome 3), is localized in chromosomal region 3p21.1, spans ~10 kb of genomic DNA, and the mature RNA (3.5 kb) encodes a 144-amino acid protein (Wang et al. 2000; Yamato et al. 1999). The protein has nuclear localization and contains a coiled-coil domain (Wang et al. 2000). Such structure suggests a role for *FAM107A* in regulating gene transcription. The potential mechanism could be *via* epigenetic regulation, as it interacts with transcriptional adaptor (Tada) 2α, a protein that is part of the histone acetyltransferase (HAT) complex (Nakajima and Koizumi 2014). As found in another study, the gene is involved in cell cycle regulation *via* apoptosis induction (Liu et al. 2009). Although the mechanism is still unknown, it probably acts through indirect mechanism.

FAM107A is considered a tumor suppressor gene (TSG) due to its decreased expression in various types of cancer. The results of studies show no or decreased gene expression in renal cell carcinoma (RCC), ovarian cancer, prostate cancer, as well as in lung cancer cell lines (Liu et al. 2009; Zhao et al. 2007; Kholodnyuk et al. 2006; Wang et al. 2000). Thus, loss of expression of *FAM107A* can play a role in the development of epithelial neoplasms. On the other hand, the highly increased expression of *FAM107A* has been found in the invasive component of glioma and has been associated with tumor invasion (cell migration and expansion) *via* cytoskeleton modulation/rearrangements (Nakajima and Koizumi 2014).

The most frequent mechanisms of TSG inactivation is loss of heterozygosis (LOH) and mutation in the remaining allele or epigenetic silencing, due to promoter methylation. Allelic deletion at human short arm of chromosome 3 – encompassing *FAM107A* locus – occurs frequently in cancers, including lung tumors (Zabarovsky et al. 2002). As no mutations were

identified in *FAM107A*, the hypothesis of gene hypermethylation was tested in several studies. It has been confirmed in several primary cancers and cancer cell lines (Awakura et al. 2008; Vanaja et al. 2006; van den Boom et al. 2006).

Most studies performed to date have been carried out using lung cancer cell lines or only small groups of clinical cases. However, the positive results indicate the role of this gene in lung carcinogenesis. This encouraged us to analyze the expression level and methylation status of *FAM107A* in non-small cell lung cancer patients.

The pre-specified hypothesis tested in the study was that *FAM107A* expression level was decreased in primary non-small cell lung cancer with promoter hypermethylation as the responsible epigenetic mechanism of gene silencing. We tried to elucidate the role of *FAM107A* in early lung carcinogenesis and cancer progression.

2 Methods

The study was approved by the Ethics Committee of the Medical University of Lodz in Poland (permission no. RNN/140/10/KE). Written informed consent was obtained from each patient.

2.1 Characterization of NSCLC Tissue Samples and Patients Clinical Characteristics

Biological material (lung tissue) was obtained from 60 patients admitted to the Department of Thoracic Surgery, General and Oncologic Surgery, Medical University of Lodz in Poland, during July 2010–March 2013. Based on the results of preoperative cytological/histological assessment, the patients were qualified for surgery and were treated by either lobectomy or pneumectomy. Immediately after resection, lung tissue samples (100–150 mg) and the adjacent non-cancerous macroscopically unchanged tissues (100 mg; 10 cm distant from the primary lesion) obtained from the same patients were placed in a stabilization buffer RNAlater® (Qiagen, Hilden, Germany). Each tissue sample was divided into smaller parts (30–50 mg) for individual analysis. All samples were frozen at −80 °C.

The resected tissue specimens were postoperatively histhopathologically evaluated and classified according to the AJCC staging (AJCC 2010) as well as TNM classification (pTNM) (Goldstraw et al. 2007; Mountain 1986). Histopathological assessments of tumor samples were obtained from pathomorphological reports, and were as follows: squamous cell carcinoma (SCC), adenocarcinoma (AC), and large cell carcinoma (LCC).

Histopathological verifications of NSCLC tissues are included in Table 1. The studied group consisted of 24 women and 36 men. All cases were primary tumors without chemo- or radiotherapy treatment. The smoking history was available for all patients and they were divided into groups according to their smoking habits: time of tobacco addiction and amount of cigarettes smoked.

2.2 RNA Extraction, Real-Time PCR (qPCR Method)

Total RNA was extracted from lung samples (cancer tissue obtained from the center of lung lesion and macroscopically unchanged lung tissue obtained from the most distant site from the resected lesion) using Universal RNA Purification Kit (Eurx, Gdansk, Poland) according to the manufacturer's recommendations. The qualitative and quantitative assessments of RNA samples were determined by minielectrophoresis in polyacrylamide gel using RNA 6000 Pico/Nano LabChip kit (Agilent 2100 Bioanalyzer; Agilent Technologies, Santa Clara, CA).

Complementary DNA (cDNA) was transcribed from 100 ng of total RNA, using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) in a total volume of 20 μl per reaction. Reverse transcription (RT) master mix contained: 10× RT buffer, 25× dNTP Mix (100 mM), 10× RT Random Primers, MultiScribe™ Reverse Transcriptase,

Table 1 Clinicopathological features of the studied NSCLC group

Patients' demographics	n = 60	
Women	Mean age: 63.1 ± 7.8 (years)	24 (40 %)
Men	Mean age: 65.8 ± 7.3 (years)	36 (60 %)
Tobacco addiction and consumption	n = 60	
The smoking period	Smokers	55 (92 %)
	< 40 years	26 (47 %)
	≥ 40 years	29 (53 %)
	Non smokers	5 (8 %)
The amount of cigarettes smoked	10–15 cigarettes per day	6 (11 %)
	20 cigarettes per day (1 pack)	39 (71 %)
	30–40 cigarettes per day (1.5–2 packs)	10 (18 %)
Pack Years[a] (PYs)	Up to 40 PYs	26 (47 %)
	≥40 PYs	29 (53 %)
Histopathological verifications of NSCLC samples	n = 60	
Histopathological type of NSCLC	Squamous cell carcinoma (SCC)	34 (57 %)
	Non-squamous cell carcinoma (NSCC)	26 (43 %)
	Adenocarcinoma (AC)	21 (35 %)
	Large cell carcinoma (LCC)	5 (8 %)
AJCC classification[b]	AJCC IA/IB	12 (20 %)
	AJCC IIA/IIB	21 (35 %)
	AJCC IIIA/IIIB	27 (45 %)
pTNM classification[c]	T1	12 (20 %)
	T2	33 (55 %)
	T3/T4	15 (25 %)

[a]PYs were calculated according to the NCI Dictionary of Cancer Terms: 1 Pack Year is equal to 20 cigarettes smoked per day for 1 year (http://www.cancer.gov/dictionary?CdrID=306510)
[b]AJCC (2010)
[c]pTNM – post-operative Tumor Node Metastasis classification (Goldstraw et al. 2007; Mountain 1986)

RNase Inhibitor and nuclease-free water. RT reaction was performed in a Personal Thermocycler (Eppendorf, Hamburg, Germany) in the following conditions: 10 min at 25 °C, followed by 120 min at 37 °C, then the samples were heated to 85 °C for 5 s, and hold at 4 °C.

The relative expression of the *FAM107A* gene was assessed in qPCR reactions using Micro Fluidic Cards with pre-loaded selected assays: Hs00200376_m1 for the *FAM107A* (family with sequence similarity 107A) gene and Hs00382667_m1 for *ESD* (esterase D) as the reference gene. The PCR mixture contained: 50 µl cDNA (50 ng) and 50 µl TaqMan® Universal Master Mix (Applied Biosystems, Carlsbad, CA). TaqMan Array card was centrifuged twice for 1 min at 1,200 rpm to fill the wells with PCR mixture. Then, it was sealed and placed in a 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA). The PCR conditions were as follows: after initial incubation at 50 °C for 2 min and AmpliTaq Gold® DNA polymerase activation at 94.5 °C for 10 min, real-time PCR amplification was processed in 40 cycles of 30 s denaturation at 97 °C, followed by 1 min elongation step at 59.7 °C.

The relative expression of *FAM107A* in the studied samples was assessed using the comparative delta-delta C_T method (TaqMan Relative Quantification Assay software, Applied Biosystems, Carlsbad, CA) and presented as RQ value, adjusted to *ESD* expression level. RNA isolated from normal lung tissue (Human Lung Total RNA, Ambion®, Life Technologies, Carlsbad, CA) served as a calibrator sample. RNAs obtained from macroscopically unchanged lung tissues formed the control group.

2.3 DNA Extraction, Bisulfite Conversion and Methylation-Specific PCRs (MSP Method)

The extraction of genomic DNA from NSCLC specimens was performed using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The quality and quantity of isolated DNA was spectrophotometrically assessed (Eppendorf BioPhotometer™ plus, Eppendorf, Hamburg, Germany). DNA samples with a 260/280 nm ratio in the range 1.8–2.0 were considered as high quality and used in further analysis.

Methylation status of the *FAM107A* gene was assessed by methylation-specific polymerase chain reaction (MSP) using bisulfite converted DNA. Genomic DNA (1 μg) was modified with sodium bisulfite, using the CpGenomeTM Turbo Bisulfide Modification Kit (CHEMICON International, Millipore, Temecula, CA), according to the manufacturer's protocol. Concentration and purity of the modified DNA was spectrophotometrically estimated at 260/280 nm in a biophotometer (Eppendorf BioPhotometer™ plus, Eppendorf, Hamburg, Germany). The conventional MSP method was performed according to Herman et al. (1996), with some modifications. Briefly, MSP was performed for each sodium bisulfite modified DNA sample using AmpliTaq Gold® 360 DNA Polymerase (Applied Biosystems, Carlsbad, CA). Amplifications were conducted in a total volume of 12.5 μl in a Thermocycler SureCycler 8800 (Agilent Technologies, Santa Clara, CA). MSP master mix contained: 1,000 ng DNA, 0.7 μM of each primer (Sigma-Aldrich, Poznan, Poland), 2.5 μM dNTPs mix, 2.5 μM $MgCl_2$, Hot Start AmpliTaq Gold® 360 Polymerase (5 U/μl), 10x Universal PCR buffer and nuclease-free water. PCR conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles involving denaturation at 95 °C for 45 s, annealing temperature – appropriate for a given primer (see Table 2) – for 45 s and elongation at 72 °C for 1 min; the final elongation step was done at 72 °C for 10 min.

The set of primers for the studied gene was flanking the 1 kb 5′ region upstream from the translation start point. Primers for the methylation-specific PCR were designed according to the criteria described by Feltus et al. (2003). Primer sequences for the methylated and unmethylated *FAM107A* promoter regions are given in Table 2.

In each PCR reaction, positive and negative MSP controls were included. CpGenome Universal Methylated DNA (enzymatically methylated human male genomic DNA) served as a positive methylation control and CpGenome Universal Unmethylated DNA (human fetal cell line) was used as a negative control (Chemicon International, Millipore, Temecula, CA). Additionally, blank samples with nuclease-free water were used instead of DNA as a control for PCR contamination.

The MSP products were separated electrophoretically on 2 % agarose gel and their concentration (ng) of MSP products (U and M DNA alleles) was estimated spectrophotometrically, using DNA1000 LabChip Kit on Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Afterwards, the Methylation Index (MI) was assessed for each sample, using the following formula: peak height of methylated products/(peak height of methylated products + peak height of unmethylated product), MI = (M)/(M + U).

Table 2 Characterization of MSP primers used in the study

MSP primers	Forward primer	Reverse primer	Product length (bp)	Annealing temperature (°C)
Methylated (M)	TGTTTTTTTATTTA GGGGTTTTTTAAC	GACTAAACTCGACT ACAACAACGAC	195	67.8
Unmethylated (U)	TTTTTATTTAGGGG TTTTTTAATGT	AACTAAACTCAACT ACAACAACAAC	191	67.3

2.4 Statistical Analysis

The Kruskal-Wallis and Mann-Whitney U tests were used to compare the levels of relative expression (RQ values) between NSCLC subtypes, i.e., SCC, AC, and LCC, shown in box and whisker plots. Spearman's rank correlation coefficient, Mann-Whitney U test, and Kruskal-Wallis test were performed in order to evaluate the relationship between the expression level of the studied gene and examined parameters (patients' characteristics: age, gender, history of smoking and tumor staging according to pTNM and AJCC classifications). The results of relative expression analysis (RQ values) are presented as means ± SE and means ± SD. The accepted level of statistical significance was estimated at $P < 0.05$. Statistica for Windows 10.0 program (StatSoft, Cracow, Poland) was applied for calculations.

3 Results

3.1 Relative Expression of *FAM107A*

The relative expression level of *FAM107A* in the studied tissue samples, determined using delta-delta C_T method, was expressed as RQ values adjusted to the expression of *ESD* (endogenous control) and in relation to the expression level of a calibrator (normal lung tissue), for which RQ = 1. The obtained *FAM107A* RQ values were correlated with histopathological NSCLC subtypes (SCC, AC, and LCC), tumor staging (pTNM and AJCC), patients' age, gender, and smoking history.

FAM107A expression was decreased (RQ value < 1) in all except one (AC) studied NSCLC samples (>98 %), with mean RQ value of 0.14 ± 0.38. The value of RQ < 0.5 is considered significant. In macroscopically unchanged lung tissues (adjacent to lung tumors), the mean *FAM107A* expression was 1.65 ± 1.46. The difference between those two groups was significant (P = 0.0001, Mann-Whitney U test) (Fig. 1).

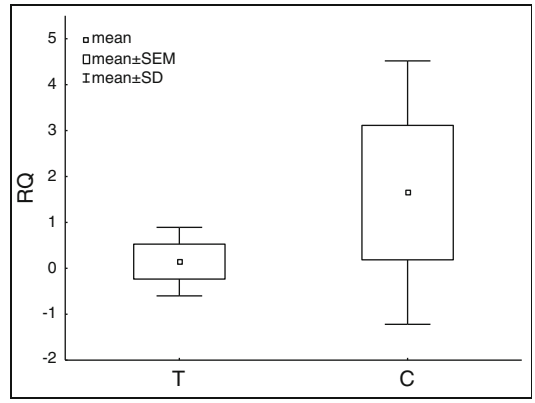


Fig. 1 Expression levels (mean RQ values) of the *FAM107A* gene in NSCLC (T, tumor) and macroscopically unchanged (C, control) tissue groups

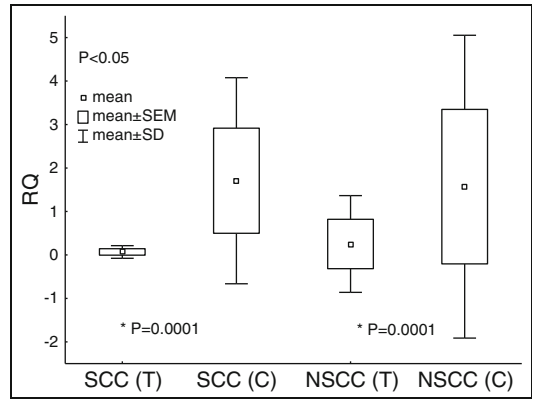


Fig. 2 Expression levels (mean RQ values) of the *FAM107A* gene in NSCLC histotypes (SCC and NSCC) *vs.* matching macroscopically unchanged tissue groups (Mann-Whitney U test)

Regarding the individual three NSCLC histotypes, *FAM107A* expression was not significantly different among them (SCC *vs.* AC *vs.* LCC: 0.07 *vs.* 0.24 *vs.* 0.27; p = 0.36, Kruskal-Wallis test). Due to a small number of cases in the LCC group, statistical analysis was also performed between two groups: SCC and NSCC (non-squamous cell carcinoma involving AC and LCC), but no statistically significant differences were noted (SCC *vs.* NSCC: 0.07 *vs.* 0.24; p = 0.26, Mann-Whitney U test). There were, however, differences in the *FAM107A* expression levels between the individual NSCLC histotypes (SCC or NSCC) and the matching macroscopically unchanged tissue groups as shown in Fig. 2.

There was no significant correlation between RQ values of *FAM107A* and the clinical features of NSCLC patients, i.e., patients' age (three age groups: ≤60 years, 60–70 years, over >70 years: 0.07 *vs.* 0.21 *vs.* 0.08; p = 0.97), gender (men *vs.* women: 0.09 *vs.* 0.22; p = 0.65), and smoking habit (current smokers *vs.* former smokers *vs.* never smokers: 0.10 *vs.* 0.12 *vs.* 0.56; p = 0.88), history of smoking assessed as PY (<25 PYs *vs.* 26–39 PYs *vs.* 40–45 PYs *vs.* ≥45 PYs: 0.23 *vs.* 0.12 *vs.* 0.07 *vs.* 0.14; p = 0.67), as well as histopathological features of tumor, i.e., pTNM classification (T1 *vs.* T2 *vs.* T3/T4: 0.05 *vs.* 0.18 *vs.* 0.14; p = 0.97), AJCC classification (IA/IB *vs* IIA/IIB *vs* IIIA/IIIB: 0.37 *vs* 0.08 *vs* 0.11; p = 0.88) (Kruskal-Wallis test, U Mann-Whitney's test followed by Spearman's rank correlation coefficient).

In the group of smokers (current and former, n = 55), the mean expression level of *FAM107A* was 0.07 in SCC and 0.16 in NSCC; however, no statistically significant difference was reached (p = 0.39, Mann-Whitney U test). Additionally, in the whole group of smokers, analysis was performed to find out if there was a correlation between gene expression and the amount of cigarettes smoked in relation to the length of the smoking (PYs). Spearman's rank correlation revealed rho = −0.17, with no statistical significance (p = 0.21). Likewise, there was no significance in the individual NSCLC subtypes (SCC and NSCC) in smokers; rho values were −0.17 and −0.16, respectively (p > 0.05).

3.2 Methylation Status of *FAM107A*

Based on MSP results, the presence of both methylated alleles of *FAM107A* gene – representing 100 % methylation status (MI = 1) of the studied promoter region – was found only in 7 % of all studied cases, as presented in Table 3. Figure 3 illustrates the examples of totally methylated (MI = 1) and unmethylated (MI = 0) NSCLC samples.

Table 3 The presence of methylated (M) and unmethylated (U) alleles, expressed as MI, in the studied NSCLC groups

NSCLC subtype	MI = 1 (both M alleles)	MI = 0 (both U alleles)
SCC *(n = 31)*	3 (10 %)	28 (90 %)
AC *(n = 20)*	1 (5 %)	19 (95 %)
LCC *(n = 4)*	0 (0 %)	4 (100 %)
Total *(n = 55)*	4 (7 %)	51 (93 %)

4 Discussion

Lung cancer is the leading cause of death from cancer in the world. The high mortality rate (85 % within 5 years) results, in part, from the lack of effective tools to diagnose the disease at an early stage. In this study we present the expression level of *FAM107A* in lung tissue obtained from patients with diagnosed non-small cell lung cancer. *FAM107A* is a tumor suppressor gene, localized on 3p, with known decreased expression in several cancer cell lines and few primary cancers. However, regarding lung cancer, it was analyzed only in lung cancer cell lines (Liu et al. 2009; Awakura et al. 2008; Wang et al. 2000). In the present study we confirmed the decreased gene expression in primary lung cancer samples. Similar results were obtained by Liu et al. (2009), who, however, reported decreased or undetectable *FAM107A* expression not on mRNA but on the protein level in 15/20 primary lung cancers and a moderate level in 2/2 normal lung tissues. In the present study, the mean *FAM107A* mRNA expression (RQ value) in macroscopically unchanged lung samples oscillated around 1, the value attributed to the normal lung tissue (calibrator) expression level. The significant differences we found between NSCLC samples and tumor-matched macroscopically unchanged lung tissue specimens could suggest an important role of the *FAM107A* gene in lung tumor development. It might be interesting to examine the expression level of this gene in the patient's blood and to find out if it could serve as a biomarker supporting an early diagnosis of NSCLC. Surrogate tissues, such as bronchial

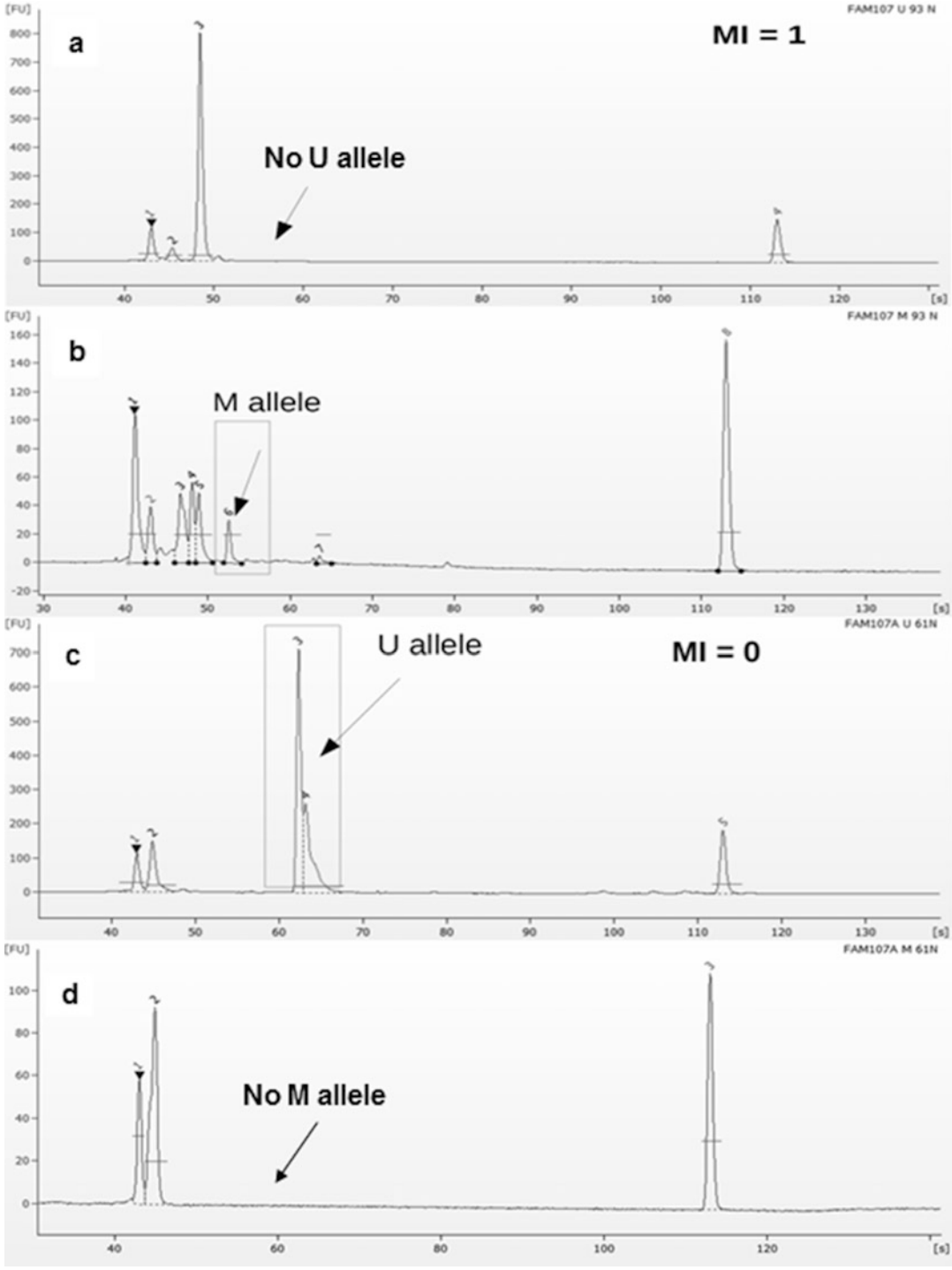


Fig. 3 Examples of methylated (both alleles, MI = 1) and unmethylated (both alleles, MI = 0) NSCLC samples; (**a & b**) –sample with methylated *FAM107A* gene; (**c & d**) –sample with unmethylated *FAM107A* gene

brushings and biopsies, as well as biofluids, such as peripheral blood (all its components: circulating cells, plasma, and serum), exhaled breath condensate (EBC), urine, and sputum offer noninvasive methods to obtain large amount of samples for analysis.

In human lung tumor xenografts, *FAM107A* re-expression has been associated with significantly smaller tumor volumes (Liu et al. 2009). In the present study, gene expression was very low and similar regardless of tumor size. It might additionally support the tumor suppressive role of *FAM107A* in lung carcinogenesis.

Smoking is a proven risk factor in lung carcinogenesis (Proctor 2001). Most patients with lung cancer (80–85 %) have a history of smoking, but only a minority of patients who smoke (10–15 %) will actually develop lung cancer (Kathuria et al. 2014). There is no method to precisely predict which current and former smokers will develop lung cancer. Spira et al. (2004) have shown that smoking contributes to the decreased expression of *FAM107A* in the epithelial cells of the pulmonary airway. In our study group, all NSCLC patients, except five persons, were smokers. It is known that the risk of developing lung cancer increases with accumulated exposure to cigarette smoke, and, on the other hand, individuals remain at high risk decades after they have stopped smoking (Ebbert et al. 2003). In the present study we did not find any significant differences between the smoker groups (in relation to PYs) or the NSCLC subtypes while considering only smokers. However, in all those cases *FAM107A* gene expression was dramatically decreased.

Based on the fact that no genetic alterations were found in the *FAM07A* gene (Wang et al. 2000; Yamato et al. 1999), the possibility of the underlying epigenetic mechanism has been taken into consideration. Indeed, *FAM107A* promoter hypermethylation was analyzed in several studies. It has been confirmed in renal cell cancer, bladder cancer, testis cancer, prostate cancer, and astrocytoma (Lin et al. 2013; Awakura et al. 2008; Vanaja et al. 2006; van den Boom et al. 2006). As far as lung cancer is concerned, Awakura et al. (2008) have shown that methylation of the gene is also present in the lung cancer cell lines. In the present study we did not confirm the pivotal role of promoter methylation of *FAM107A* as the mechanism of gene silencing in our NSCLC patients. However, it should be stressed that epigenetic inactivation of *FAM107A* may occur despite poor methylation. As shown by Zhao et al. (2005), the presence of di-methylation of lysine 9 on histone H3 (H3me2K9) and binding of methyl-CpG binding protein (MeCP2) at the promoter region of TSG are common events leading to gene silencing due to silent heterochromatin state, irrespective of DNA hypermethylation. The heterogeneity of epigenetic regulation should be taken into account. Apart from gene promoter methylation, several other epigenetic mechanisms control gene expression, including post translational modifications to core histones, chromatin remodeling machinery, microRNA (miRNA), and long non-coding RNA (lncRNA) regulation (Gibney and Nolan 2010).

The results obtained in the present study, indicating loss of *FAM107A* expression in NSCLC samples, highlight its importance in lung carcinogenesis. However, it appears that the key *FAM107A* silencing mechanism is not related to the promoter hypermethylation.

Acknowledgements This work was supported by the grant of the National Science Center UMO-2011/01/B/NZ4/04966.

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

References

AJCC – American Joint Committee on Cancer Staging according to the IASCLC Staging Project (2010) Cancer, 7th edn, Springer, New York

Awakura Y, Nakamura E, Ito N, Kamoto T, Ogawa O (2008) Methylation-associated silencing of TU3A in human cancers. Int J Oncol 33:893–899

Ebbert JO, Yang P, Vachon CM, Vierkant RA, Cerhan JR, Folsom AR, Sellers TA (2003) Lung cancer risk reduction after smoking cessation: observations from a prospective cohort of women. J Clin Oncol 21:921–926

Feltus FA, Lee EK, Costello JF, Plass C, Vertino PM (2003) Predicting aberrant CpG island methylation. Proc Natl Acad Sci U S A 100:12253–12258

Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM (2010) Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 27:2893–2917

Gibney ER, Nolan CM (2010) Epigenetics and gene expression. Heredity (Edinb) 105:4–13

Goldstraw P, Crowley J, Chansky K, Giroux DJ, Groome PA, Rami-Porta R, Postmus PE, Rusch V, Sobin L (2007) International Association for the Study of Lung Cancer International Staging Committee, Participating Institutions. The IASLC Lung Cancer Staging Project: proposals for the revision of the TNM stage groupings in the forthcoming (seventh) edition of the TNM Classification of malignant tumours. J Thorac Oncol 2:706–714

Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB (1996) Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U S A 93:9821–9826

Kathuria H, Gesthalter Y, Spira A, Brody JS, Steiling K (2014) Updates and controversies in the rapidly evolving field of lung cancer screening, early detection, and chemoprevention. Cancers (Basel) 6:1157–1179

Kholodnyuk ID, Kozireva S, Kost-Alimova M, Kashuba V, Klein G, Imreh S (2006) Down regulation of 3p genes, LTF, SLC38A3 and DRR1, upon growth of human chromosome 3-mouse fibrosarcoma hybrids in severe combined immunodeficiency mice. Int J Cancer 119:99–107

Lin PC, Giannopoulou EG, Park K, Mosquera JM, Sboner A, Tewari AK, Garraway LA, Beltran H, Rubin MA, Elemento O (2013) Epigenomic alterations in localized and advanced prostate cancer. Neoplasia 15:373–383

Liu Q, Zhao XY, Bai RZ, Liang SF, Nie CL, Yuan Z, Wang CT, Wu Y, Chen LJ, Wei YQ (2009) Induction of tumor inhibition and apoptosis by a candidate tumor suppressor gene DRR1 on 3p21.1. Oncol Rep 22:1069–1075

Mountain CE (1986) A new international staging system for lung cancer. Chest 89(4 Suppl):225S–233S

Nakajima H, Koizumi K (2014) Family with sequence similarity 107: a family of stress responsive small proteins with diverse functions in cancer and the nervous system (Review). Biomed Rep 2:321–325

Proctor RN (2001) Tobacco and the global lung cancer epidemic. Nat Rev Cancer 1:82–86

Spira A, Beane J, Shah V, Liu G, Schembri F, Yang X, Palma J, Brody JS (2004) Effects of cigarette smoke on the human airway epithelial cell transcriptase. Proc Natl Acad Sci U S A 101:10143–10148

Travis WD, Bramble E, Müller-Permalink HK, Harris CC (eds) (2004) Pathology and genetics of tumors of the lung, pleura, thymus and heart. IARC Press, Lyon, pp 9–122, Chapter 1

van den Boom J, Wolter M, Blaschke B, Knobbe CB, Reifenberger G (2006) Identification of novel genes associated with astrocytoma progression using suppression subtractive hybridization and real-time reverse transcription-polymerase chain reaction. Int J Cancer 119:2330–2338

Vanaja DK, Ballman KV, Morlan BW, Cheville JC, Neumann RM, Lieber MM, Tindall DJ, Young CY (2006) PDLIM4 repression by hypermethylation as a potential biomarker for prostate cancer. Clin Cancer Res 12:1128–1136

Wang L, Darling J, Zhang JS, Liu W, Qian J, Bostwick D, Hartmann L, Jenkins R, Bardenhauer W, Schutte J, Opalka B, Smith DI (2000) Loss of expression of the DRR 1 gene at chromosomal segment 3p21.1 in renal cell carcinoma. Genes Chromosomes Cancer 27:1–10

Yamato T, Orikasa K, Fukushige S, Orikasa S, Horii A (1999) Isolation and characterization of the novel gene, TU3A, in a commonly deleted region on 3p14.3p14.2 in renal cell carcinoma. Cytogenet Cell Genet 87:291–295

Zabarovsky ER, Lerman MI, Minna JD (2002) Tumor suppressor genes on chromosome 3p involved in the pathogenesis of lung and other cancers. Oncogene 21:6915–6935

Zhao W, Soejima H, Higashimoto K, Nakagawachi T, Urano T, Kudo S, Matsukura S, Matsuo S, Joh K, Mukai T (2005) The essential role of histone H3 Lys9 di-methylation and MeCP2 binding in MGMT silencing with poor DNA methylation of the promoter CpG island. J Biochem 137:431–440

Zhao XY, Liang SF, Yao SH, Ma FX, Hu ZG, Yan F, Yuan Z, Ruan XZ, Yang HS, Zhou Q, Wei YQ (2007) Identification and preliminary function study of *Xenopus laevis* DRR1 gene. Biochem Biophys Res Commun 361:74–78

Advs Exp. Medicine, Biology - Neuroscience and Respiration (2015) 11: 49–58
DOI 10.1007/5584_2015_115

Published online: 10 March 2015

Clinical Significance of HMGB-1 and TGF-β Level in Serum and BALF of Advanced Non-Small Cell Lung Cancer

K. Jakubowska, W. Naumnik, W. Niklińska, and E. Chyczewska

Abstract

Lung cancer is associated with poor prognosis. The aim of this study was to evaluate the clinical usefulness of HMGB-1 (high-mobility group protein B1) and TGF-β (transforming growth factor beta) in patients with advanced non-small cell lung cancer (NSCLC). We studied 45 patients with NSCLC prior to chemotherapy, 23 patients with Besnier-Boeck-Schaumann (BBS) disease (sarcoidosis), and 15 healthy volunteers. HMGB-1 and TGF-β levels were measured in serum and BALF samples using ELISA method. A higher serum HMGB-1 and TGF-β levels were in NSCLC patients compared with the other groups. TGF-β concentration in BALF was significantly higher in NSCLC than in healthy controls ($p = 0.047$) but lower than in BBS ($p = 0.016$). Serum HMGB-1 in NSCLC correlated with age and gender while its level in BALF was associated with distant metastasis. A higher levels of HMGB-1 in the serum of NSCLC patients with progressive disease was linked with shorter overall survival and disease-free survival. We found a positive correlation between HMGB-1 and TGF-β in BALF of IIIB NSCLC group and overall survival ($p = 0.04$; $p = 0.003$). Our findings confirmed that the measurement of HMGB-1 and TGF-β levels in serum and BALF of patients with NSCLC prior to treatment may have clinical usefulness and predict poor prognosis.

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

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, 13 Waszyngton St., 15-269 Bialystok, Poland
e-mail: wojciechnaumnik@gmail.com

W. Niklińska
Department of Histology and Embryology, Medical University of Bialystok, 13 Waszyngton St., 15-269 Bialystok, Poland

Keywords
Bronchoalveolar lavage fluid • HMGB-1 • Lung cancer • Serum • TGF-β

1 Introduction

Carcinogenesis of lung cancer is a multi-stage, complex process that involves the growth and differentiation of malignant cells, their ability to adhesion, migration, and invasion, as well as the mechanisms involved in the weakening or blocking anti-tumor responses, e.g. apoptosis or immune response (Travis et al. 2004). Significant influence on the fight against this disease plays a capability to develop defense mechanisms against tumor cells. The presence of tumor cells stimulates a variety of factors and inflammatory cells. Tumor cells, equipped with tumor associated antigens (TAA), activate antigen-presenting cells (APCs) such as macrophages, dendritic cells, lymphocytes B and Th cells, epithelial and endothelial cells, and fibroblasts that secrete many cytokines (interferon gamma, tumor necrosis factor, and interleukins 1, 12, 15, 18, and 27), engaging defenses of natural killers (NK) cells, lymphocytes Tc, and macrophages M1 (Liu et al. 2012b). Defensive mechanisms are effective only in the initial stage of cancer development. With the growth of tumor mass, tumor cells acquire various properties allow to lessen the efficiency of defense mechanisms. Because of their heterogeneity tumor cells may activate tumor associated macrophages (TAMs) which release anti-inflammatory cytokines (Liu et al. 2012b). Tumor cells also may lack the ability for apoptosis (Han et al. 2014).

Cytokines act in different directions, depending on the type of secretory cells and the tumor microenvironment. HMGB-1, in chronic hypoxic conditions, released from tumor cells can stimulate neoangiogenesis, cell migration and proliferation, the increase in tumor mass as well as the recruitment of macrophages and endothelial precursor cells type 2 (Smolarczyk et al. 2012). In contrast, HMGB-1 protein, secreted from tumor cells during treatment with weak antigens, can activate anti-tumor response (Smolarczyk et al. 2012). The microenvironment of the tumor cytokines also plays a role in the process of neoplastic development. At early tumor stage, TGF-β stimulates the expression of genes responsible for induction of differentiation, apoptosis, and cell autophagy and for suppression of progression, i.e. angiogenesis, proliferation, and immune response (Wang et al. 2014). These properties are extinguished along with cancer progression. Then, TGF-β determines the migration, invasion of tumor cells, stimulates neovascularization, epithelial-mesenchymal transition and metastatic ability, and also weakens the immune system (Guo et al. 2012). The aim of the present study was to evaluate the clinical usefulness of the measurement of HMGB-1 and TGF-β sera and bronchoalveolar fluid (BALF) of patients with advanced lung cancer.

2 Methods

The study was performed in conformity with the Declaration of Helsinki for Human Experimentation and the protocol was approved by the Bioethics Committee of the Medical University of Bialystok in Poland (permit no. RI-002/151/2013). Written informed consent was obtained from all participants.

2.1 Patients

The study group comprised 45 patients (38 men) with diagnosed non-small cell lung cancer

(NSCLC) and treated in the Department of Lung Diseases, Medical University of Bialystok in Poland in the years 2009–2011. The mean age was 61.7 ± 8.3 years; there were 21 patients under 60 years of age and 24 patients over 60 years of age. Squamous cell carcinoma (SCC) was confirmed in 22 (48.8 %) patients, adenocarcinoma was in 20 (44.4 %) patients, and large cell type only in 3 patients (6.6 %). The study included patients with Stage IIIB (18) and IV (27) not previously treated with any form of immunosuppressive therapy, without signs of infection. In all patients, routine diagnostics were performed such as basic diagnostic laboratory, ECG, spirometry, arterial blood gasometry, X-ray and CT of the chest, bronchofiberoscopy with histopathological tests, transbronchial lung biopsy (TBB), or transbronchial needle aspiration biopsy (TBNA). The clinical staging of NSCLC was evaluated according to TNM classification (IASLC). The response to therapy was estimated after four cycles of chemotherapy according to Response Evaluation Criteria in Solid Tumors (RECIST) (Therasse et al. 2000).

The reference group consisted of 23 patients with respiratory sarcoidosis (Besnier-Boeck-Schaumann disease – BBS), including 20 men and 3 women of the mean age of 58.3 ± 3.0 years. The diagnosis was established on the basis of clinical and histopathological examination of lung slices taken during transbronchial lung biopsy (TBB) according to the consensual (Costabel and Hunninghake 1999). All patients were at stage II of the disease. Basic diagnostic laboratory, ECG, spirometry, plethysmography, DLCO, and gasometry were performed in each case. Another reference group consisted of 15 healthy volunteers (2 women and 13 men; mean age of 60.1 ± 5.0 years) without laboratory and clinical symptoms of infection.

2.2 Collection of Serum and BALF

Six milliliters of venous blood was collected in a closed syringe system with coagulation activator from fasting subjects. After immediate centrifugation at 3,000 rpm, sera were frozen to −80 °C in plastic Eppendorf tubes for further use.

A portion of BALF collected during the lavage procedure was used for phenotypic analysis of the T cell populations: $CD4^+$ and $CD8^+$ by flow cytometry and calculating the CD4/CD8 ratio. Another portion of BALF was filtered through a 2-layer nylon gauze to remove mucus, which particularly occurs in smokers, and was used for the basic cell count performed in a Neubauer chamber, and defined as the number of cells $\times 10^5$/ml of fluid. The remainder of fluid was centrifuged at 800 rpm for 10 min at 4 °C. The cell pellet obtained was washed twice with PBS devoid of Ca^{2+} and Mg^{2+}. Then, preparations were stained with May-Grünwald-Giemsa for cell evaluation. There were at least 400 cells counted and divided into the populations of macrophages, neutrophils, eosinophils, lymphocytes, and epithelial cells under a light microscope (magnification 1 k). Cell evaluation also allowed identifying atypical and cancer cells as well as extracellular components. The supernatant was stored at −20°C for further assays of protein concentration.

2.3 HMGB-1 and TGF-β Analysis

Concentrations of HMGB-1 (ng/ml) and TGF-β in the serum (ng/ml) and in the BALF supernatant (pg/ml) were determined by an enzyme-linked immunosorbent assay (ELISA). All specimens were assayed twice and the average of the two measurements was used for data analysis. The measurement of TGF-β was performed with Quantikine Human TGF-β HS kit (Immunoassay R&D Systems, Minneapolis, MN), with the minimum detectable level of 0.005 ng/ml. HMGB-1 was measured with anti-HMGB-1 immunoassay (IBL International GmbH, Hamburg, Germany), with the minimum detectable level of 0.1 ng/ml.

2.4 Statistical Analysis

The results were presented as the median and range of concentrations, including minimum

and maximum values. Since data were not normally distributed, the Mann-Whitney U test and the Kruskal-Wallis test were used for statistical comparisons. Spearman's coefficient was used to assess the relationship between HMGB-1 and TGF-β content and clinicopathological features. The probability of survival and relapse-free time based on protein concentrations prior to treatment was estimated using the Kaplan-Meier method. Differences were considered statistically significant at $p < 0.05$. Statistical analysis was performed using Statistica ver. 10.0 (StatSoft, Tulsa, OK).

We also assessed the diagnostic usefulness of HMGB-1 and TGF-β measurements in the serum and BALF of NSCLC patients using the receiver operating characteristics (ROC) and evaluating the area under the curve diagram (AUC), which determines the test ability for distinguishing normal from abnormal results.

3 Results

3.1 HMGB-1 and TGF-β Levels in Serum and BALF of Lung Cancer Patients

The highest serum concentrations of HMGB-1 were in NSCLC patients [1.12 (0.21–27.36) ng/ml], lower in healthy subjects [1.17 (0.45–3.58) ng/ml] and in patients with sarcoidosis [0.98 (0.09–4.91) ng/ml]. HMGB-1 concentration in sera of NSCLC patients was significantly higher than that in the control group ($p = 0.04$). Patients with BBS had the highest HMGB-1 concentration in BALF compared with the other groups; healthy control *vs.* NSCLC *vs.* BBS: 3.67 (0.10–44.55) *vs.* 1.58 (0.06–98.86) *vs.* 1.29 (0.10–14.54) ng/ml, but these values were not statistically different. The serum concentration of TGF-β was highest in patients with NSCLC [21.69 (1.14–39.80) ng/ml], lower in healthy subjects [20.82 (11.16–29.70) ng/ml], and the lowest in patients with sarcoidosis [19.13 (1.64–39.47) ng/ml]; but again these values were not statistically different. TGF-β concentration in BALF was highest in patients with sarcoidosis [134.95 (30.77–182.98) pg/ml], lower in patients with NSCLC [91.06 (47.93–209.04) pg/ml], and the lowest in healthy subjects [79.42 (42.61–105.51) pg/ml]. The concentration of TGF-β in BALF in NSCLC patients was significantly higher than that in healthy volunteers, and lower than that in patients with sarcoidosis ($p = 0.047$, $p = 0.016$, respectively). In contrast, TGF-β level in BALF of sarcoidosis patients was significantly higher than that in the control group ($p = 0.003$). Details of the results on protein levels above outlined are shown in Figs. 1 and 2.

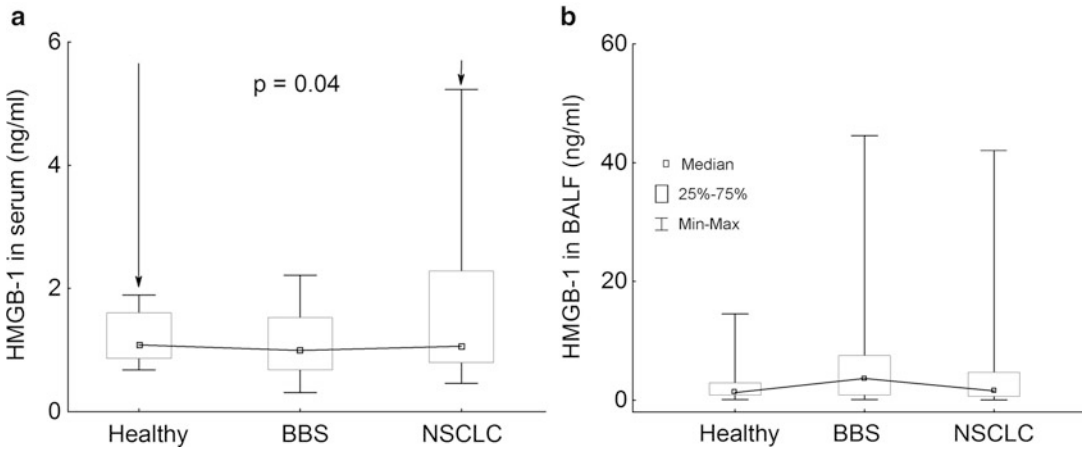


Fig. 1 (**a**) **Serum and** (**b**) **BALF content of HMGB-1 protein** in healthy subjects, and in non-small cell lung cancer (NSCLC) and Besnier-Boeck-Schaumann (BBS) patients

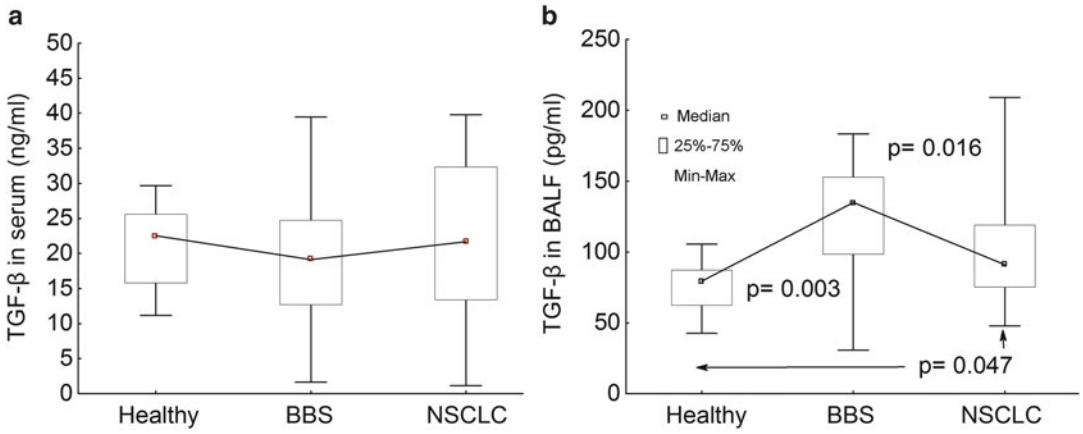


Fig. 2 (**a**) **Serum and** (**b**) **BALF content of TGF-β protein** in healthy subjects, and in non-small cell lung cancer (NSCLC) and Besnier-Boeck-Schaumann (BBS) patients

3.2 ROC Curve Analysis

The lowest values in the AUC were found for HMGB-1 and TGF-β in sera of both NSCLC and BBS patients (NSCLC 0.597/BBS 0.560 and NSCLC 0.667/BBS 0.580, respectively). Power of the diagnostic determination of HMGB-1 and TGF-β in sera of patients with NSCLC and BBS is rather small; on average, AUC = 0.678 and AUC = 0.694 for HMGB-1 in BALF of NSCLC and BBS patients, respectively. With respect to TGF-β in BALF of NSCLC patients, power of the diagnostic determination was significantly higher than that in healthy subjects ($p = 0.012$). In contrast, the AUC of TGF-β in BALF of BBS patients was low (0.502). Differences between the AUC for TGF-β in BALF of patients with NSCLC and BBS were significant ($p = 0.001$) (Fig. 2).

3.3 Correlation of HMGB-1 and TGF-β in Serum and BALF of NSCLC with Clinicopathological Features

In the NSCLC group, a positive correlation was found between the levels of HMGB-1 in BALF and serum ($r = 0.339$, $p = 0.036$). In addition, there were positive correlations between HMGB-1 and TGF-β in sera and in BALF of NSCLC patients; $r = 0.412$, $p = 0.002$ and $r = 0.534$, $p = 0.04$, respectively.

The concentration of HMGB-1 in the NSCLC group was significantly higher in patients younger than 60 years of age ($p = 0.047$) (Table 1). Higher levels of HMGB-1 in sera and TGF-β in BALF were present in men compared with women. HMGB-1 was significantly higher in BALF of NSCLC patients who had distant metastases. HMGB-1 and TGF-β in sera and BALF of NSCLC patients failed to correlate with any other clinicopathological features. However, HMGB-1 in both sera and BALF were higher in clinical stage IIIB than stage IV of NSCLC. Conversely, TGF-β showed a decreasing trend with increasing clinical stage in these patients.

There was a downward trend in HMGB-1 levels with decreasing effectiveness of therapy. HMGB-1 was the highest in BALF of NSCLC patients with partial response to chemotherapy, lower in stable disease, and the lowest in progressive disease, although these differences did not achieve statistical significance. In patients with stable disease, TGF-β level was the lowest in sera and the highest in BALF.

3.4 BALF Cells Analysis

There was a positive correlation between HMGB-1 and $CD4^+$ in BALF of BBS patients ($r = 0.766$, $p = 0.027$). Furthermore, in healthy controls, HMGB-1 level was inversely related to the number of lymphocytes ($r = -0.659$,

Table 1 Correlation between HMGB-1 and TGF-β levels in sera and BALF of NSCLC and clinicopathological features

		HMGB-1		TGF-β	
		Serum (ng/ml)	BALF (ng/ml)	Serum (ng/ml)	BALF (pg/ml)
		r	r	r	r
	n	p	p	p	p
Age					
<60 ys	21	−0.303	0.078	−0.031	0.055
>60 ys	24	0.047	0.978	0.714	0.821
Gender					
Male	38	−0.233	−0.273	0.088	0.715
Female	7	0.014	0.391	0.418	0.020
Histological type					
SCC	22	−0.233	0.193	0.064	−0.017
Adenocarcinoma	20	0.660	0.903	0.847	0.430
Large cell carcinoma	3				
Primary tumor site (T)					
1	1				
2	15	−0.165	−0.077	−0.232	−0.253
3	7	0.336	0.203	0.362	0.089
4	22				
Lymph node involvement (N)					
0	2				
1	3	0.302	−0.003	−0.198	−0.010
2	19	0.128	0.291	0.318	0.824
3	21				
Distant metastasis (M)					
0	16	−0.088	0.583	−0.023	−0.043
1a	14	0.135	0.031	0.525	0.618
1b	15				
Distant metastasis					
Opposite lung	14	−0.073	0.017	−0.359	0.425
Liver	3	0.566	0.923	0.037	0.017
Other	12				
Clinical stage					
IIIB	13	0.131	0.035	−0.320	0.039
IV	27	0.153	0.299	0.346	0.678

HMGB-1 high-mobility group protein B1, *TGF-β* transforming growth factor beta, *NSCLC* non-small cell lung cancer, *BALF* bronchoalveolar lavage fluid

$p = 0.05$). In the NSCLC group, there was a positive correlation between the concentration of TGF-β and the number of epithelial cells ($r = 0.347$, $p = 0.030$), neutrophils ($r = 0.530$, $p = 0.001$), and eosinophils ($r = 0.420$ $p = 0.008$), and a negative correlation with the number of macrophages ($r = -0.415$, $p = 0.009$). These results BALF fluid analysis are summarized in Table 2.

3.5 Patients Prognosis

The concentration of HMGB-1 and TGF-β in BALF negatively correlated with survival in stage IIIB of NSCLC ($r = -0.547$, $p = 0.004$; $r = -0.653$, $p = 0.003$, respectively). Likewise, there was a negative correlation between the concentration of HMGB-1 in sera of NSCLC patients who did not respond to chemotherapy

Table 2 Correlation between HMGB-1 and TGF-β in sera and BALF, and cell type in BALF of NSCLC patients

	NSCLC *(n = 45)*		BBS *(n = 23)*		Healthy controls *(n = 15)*	
	HMGB-1 (ng/ml)	TGF-β (pg/ml)	HMGB-1 (ng/ml)	TGF-β (pg/ml)	HMGB-1 (ng/ml)	TGF-β (pg/ml)
	r	r	r	r	r	r
	p	p	p	p	p	p
Cells amount × 10^4/ml	−0.007	−0.116	0.213	0.410	−0.236	0.523
	0.965	0.480	0.396	0.072	0.541	0.098
Macrophages × 10^4/ml	0.027	−0.415	−0.340	−0.440	0.476	0.268
	0.868	0.009	0.167	0.080	0.194	0.425
Lymphocytes × 10^4/ml	0.102	−0.129	0.345	0.420	−0.659	−0.240
	0.529	0.434	0.160	0.065	0.050	0.476
Epithelial cells × 10^4/ml	−0.059	0.347	–	–	–	–
	0.717	0.030				
Neutrophils × 10^4/ml	−0.102	0.530	−0.289	0.059	−0.143	−0.392
	0.531	0.001	0.245	0.805	0.712	0.233
Eosinophils × 10^4/ml	0.006	0.420	−0.011	0.348	0.354	−0.161
	0.969	0.008	0.965	0.132	0.350	0.635
Lymphocytes $CD4^+$ (%)	–	–	0.766	0.486	–	–
			0.027	0.154		
Lymphocytes $CD8^+$ (%)	–	–	−0.186	−0.416	–	–
			0.658	0.232		
$CD4^+/CD8^+$ ratio (%)	–	–	0.473	0.717	–	–
			0.658	0.070		

HMGB-1 high-mobility group protein B1, *TGF-β* transforming growth factor beta, *NSCLC* non-small cell lung cancer, *BBS* Besnier-Boeck-Schaumann disease, *BALF* bronchoalveolar lavage fluid

and survival ($r = -0.734$, $p = 0.006$). However, there was no differences between the probability of survival, as based on low and high protein levels, using the Kaplan-Meier method. The cut-off values, defined as the median level of protein, amounted to 1.129 ng/ml and 1.586 ng/ml for HMGB-1 and 21.690 ng/ml and 91.060 pg/ml for TGF-β in the serum and BALF, respectively. The median survival in all patient groups was 8.4 months (Fig. 3).

4 Discussion

The findings of the present study demonstrate the highest serum levels of HMGB-1 in patients with NSCLC, lower in healthy subjects, and the lowest in patients with BBS. Differences between the NSCLC and healthy groups were

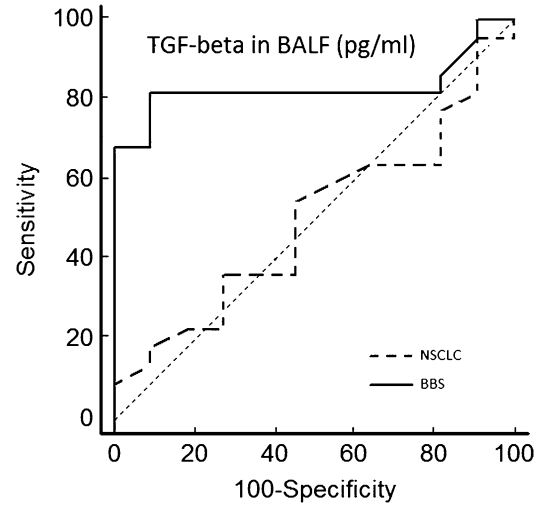


Fig. 3 ROC curve discriminating the diagnostic power of TGF-β protein in BALF in NSCLC and BBS patients

significant. These results are consistent with those of Guo et al. (2012) who found higher levels of HMGB-1 in sera of patients with squamous cell carcinoma of the larynx compared with healthy controls. Somewhat different results were reported by Shang et al. (2009) who found the highest serum concentration of HMGB-1 in NSCLC patients, lower in COPD, and the lowest in healthy subjects. Other studies point to increased serum HMGB-1 concentration at the preoperative stage in patients scheduled for thoracic surgery due to cancer of esophagus (Suda et al. 2006). Discrepancies also exist concerning the level of HMGB-1 in healthy subjects *vs*. patients suffering from other pulmonary diseases. In the present study we found that HMGB-1 was higher in healthy subjects compared with sarcoidosis patients. Hamada et al. (2008), on the other side, reported the following order of decreasing HMGB-1 content: idiopathic pulmonary fibrosis, nonspecific pneumonia, and healthy people. Ebina et al. (2001) investigated the level of HMGB-1 in BALF and its expression in tissue specimens from patients with idiopathic pulmonary fibrosis. The authors noted that the level of HMGB-1 was low at the initial active stage of disease, but it was gradually increasing with disease stabilization. According to these observations, the level of HMGB-1 in BALF of patients with interstitial lung disease may be an index of local, chronic inflammation. In the present study, a positive correlation was found between HMGB-1 and the number of $CD4^+$ T cells in BALF of BBS patients, which underscore the role of $CD4^+$ cells in sarcoid granuloma formation. HMGB-1 protein can be isolated from a variety of inflammatory cells such as neutrophils, lymphocytes, macrophages, or epithelial cells. High concentrations of HMGB-1 in sera of NSCLC patients compared with sarcoidosis patients found in the present study suggest that cancer cells may also be the primary source of this protein.

Likewise, concerning serum TGF-β, its highest concentration was found in NSCLC patients, lower in healthy subjects, and the lowest in BBS. These results are consistent with the observations of other authors (van de Wiele et al. 2008; Hou et al. 2013) who found a higher serum level of the protein in lung cancer patients compared with healthy controls. Hirakata and Kitamura (1996), in turn, reported that serum TGF-β was specifically higher in lung cancer patients with, rather than without, signs of finger clubbing. Concerning TGF-β in BALF, we found that it was significantly higher in BBS than in other cases; the finding in line with that of Kowalska et al. (2010). We also found that TGF-β in BALF was higher in NSCLC patients than in healthy controls. That is also in line with other studies in which the highest level of the protein has been reported in lung cancer of advanced stage (Domagała-Kulawik et al. 2006). Distinct differences in BALF content of TGF-β protein between NSCLC and BBS patients, make this protein potentially useful in initial differential diagnosis.

Pittet et al. (2013) showed that HMGB-1 and TGF-β share a common signaling pathway in alveolar epithelial cells type II. The authors observed that the HMGB-1 protein released from damaged alveolar type II cells causes an increase in the level of IL-1β. They also demonstrated that IL-1β activates the TGF-β signaling pathway through integrin νβ6 and p38 mitogen-activated protein kinase (MAPK), a member of mitogen-activated protein kinases, which results in repair of damaged epithelium. The findings of our present study indicate that the above mentioned mechanism of cellular signaling could also be at play in NSCLC patients. We show that the level of HMGB-1 correlated positively with that of TGF-β in both serum and BALF of NSCLC patients. Thus, assumedly, increase in HMGB-1 is conditioned by secretion of TGF-β in these patients, which, in turn, leads to suppression of the immune system, activation of TGF-β procancer properties, and progression of disease.

In the present study, the serum level of HMGB-1 in NSCLC patients was significantly higher in patients under 60 years of age. Semrau et al. (2014) showed that age actually does not affect the lifespan of cancer patients. However, it should be pointed out that biological activity of

the immune system is higher in younger people, which may slow down or limit cellular responses, reduce reactivity with respect to antigens of inflammatory cells, and suppress the formation of proinflammatory cytokine (Dunn et al. 2004).

Cytokines influence the ability of cancer cells to adhere to the extracellular matrix, cell degradation and remodeling, and migration of cells to blood and lymph vessels, and to body cavities, which all has to do with tumor invasiveness. That seems consistent with the present findings showing that HMGB-1 concentration in BALF of NSCLC patients was associated with the development of metastases. HMGB-1 may promote the spread of NSCLC cells to distant organs. A study of Sun et al. (2013) on HMGB-1 expression in the cell culture of A549 lung cancer demonstrated the association between the overexpression of the HMGB-1 protein and the cell's ability to proliferate and metastasize, probably by extracellular-signal-regulated kinase (ERK1/2) and p83 phosphorylation. Likewise, Wang et al. (2012) found that HMGB-1 along with the CpG (regions of DNA where cytosine nucleotide occurs next to guanine nucleotide) stimulate growth and invasiveness, including the ability to metastasize, in the 95D cell line of human lung carcinoma.

Although both HMGB-1 and TGF-β are involved with cancer progression, they act in different ways (Stenmark et al. 2013; Liu et al. 2012a). HMGB-1 activates neoangiogenesis, migration, and proliferation of cancer cells, and enhances their ability to escape immune surveillance (Smolarczyk et al. 2012). TGF-β, on the other hand, has a role in malignant changes in the epithelial-mesenchymal transition (EMT), inhibition of apoptosis, and suppression of immune response (Guo et al. 2012). In the present study, HMGB-1 and TGF-β in BALF was negatively associated with survival in stage IIIB of cancer. That finding, along with the relationship between the concentration of HMGB-1 in BALF and the appearance of metastases in NSCLC patients, suggests that a high level of HMGB-1 in stage IIIB NSCLC may possibly provide a rapid progression of disease and a reduced survival time. High levels of HMGB-1 in sera of NSCLC patients, who did not respond to treatment also negatively correlated with survival time.

The present study confirms that the level of a protein may determine the response to treatment and also has a predictor value in cancer patients. The analysis of NSCLC survival shows that higher levels of HMGB-1 and TGF-β in sera and BALF in patients before chemotherapy may indicate a shorter survival time. Although this tendency did not reach statistical significance in our study, it is in line with the results of Vazquez et al. (2013) who confirmed the high level of TGF-β in lung cancer patients augurs worse. While the evidence of a prognostic value of HMGB-1 specifically in NSCLC patients has not yet been reported, there are reports that its increase is associated with a decreased survival time of patients with bladder, stomach, nasopharynx, and liver cancers (Stenmark et al. 2013; Liu et al. 2012a).

In conclusion, we believe we have shown in this study clinical usefulness of HMGB-1 and TGF-β measurements in sera and BALF of patients with advanced inoperable non-small cell lung cancer. The observed interdependence of both proteins concentrations in NSCLC patients suggests their acting through a common signaling pathway. These proteins may have an impact on tumor invasiveness, suppression of immune system, and resistance to treatment, which all worsens the prognosis of NSCLC patients.

Acknowledgments We are indebted to Dr. Maria Ossolińska for the help with the ELISA assessment. KJ participated in this study within the doctoral support program co-funded by the European Union Social Funds for the academic year 2013–2014.

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

References

Costabel U, Hunninghake GW (1999) ATS/ERS/WASOG statement on sarcoidosis. Sarcoidosis Statement Committee. American Thoracic Society. European Respiratory Society. World Association for Sarcoidosis and Other Granulomatous Disorders. Eur Respir J 14:735–737

Domagała-Kulawik J, Hoser G, Safianowska A, Grubek-Jaworska H, Chazan R (2006) Elevated TGF-beta1 concentration in bronchoalveolar lavage fluid from patients with primary lung cancer. Arch Immunol Ther Exp 54:143–147

Dunn GP, Old LJ, Schreiber RD (2004) The immunobiology of cancer immunosurveillance and immunoediting. Immunity 21:137–148

Ebina M, Taniguchi H, Miyasho T, Yamada S, Shibata N, Ohta H, Hisata S, Ohkouchi S, Tamada T, Nishimura H, Ishizaka A, Maruyama I, Okada Y, Takashi K, Nukiwa T (2001) Gradual increase of high mobility group protein b1 in the lungs after the onset of acute exacerbation of idiopathic pulmonary fibrosis. Pulm Med 2011:916486

Guo Y, Liu Y, Tan PQ, Li G, Su ZW, Tian YQ, Qiu YZ (2012) Expressions and clinical significance of high mobility group box-1 mRNA and protein in laryngeal squamous cell carcinoma tissues and serum. Zhonghua Er Bi Yan Hou Tou Jing Wai Ke Za Zhi 47:487–490

Hamada N, Maeyama T, Kawaguchi T, Yoshimi M, Fukumoto J, Yamada M, Yamada S, Kuwano K, Nakanishi Y (2008) The role of high mobility group box1 in pulmonary fibrosis. Am J Respir Cell Mol Biol 39:440–447

Han J, Tang FM, Pu D, Xu D, Wang T, Li W (2014) Mechanisms underlying regulation of cell cycle and apoptosis by hnRNP B1 in human lung adenocarcinoma A549 cells. Tumori 100:102–111

Hirakata Y, Kitamura S (1996) Elevated serum transforming growth factor beta 1 level in primary lung cancer patients with finger clubbing. Eur J Clin Invest 26:820–823

Hou YL, Chen H, Dong ZH, Xue CJ, Wu YF, Luo HX, Wu YC, Li CH (2013) Clinical significance of serum transforming growth factor-β1 in lung cancer. Cancer Epidemiol 37:750–753

Kowalska A, Puścińska E, Czerniawska J, Goljan-Geremek A, Czystowska M, Rozy A, Chorostowska-Wynimko J, Grecka D (2010) Markers of fibrosis and inflammation in exhaled breath condensate (EBC) and bronchoalveolar lavage fluid (BALF) of patients with pulmonary sarcoidosis – a pilot study. Pneumonol Alergol Pol 78:356–362

Liu F, Zhang Y, Peng Z, Gao H, Xu L, Chen M (2012a) High expression of high mobility group box 1 (hmgb1) predicts poor prognosis for hepatocellular carcinoma after curative hepatectomy. J Transl Med 10:135

Liu L, Ge D, Ma L, Mei J, Liu S, Zhang Q, Ren F, Liao H, Pu Q, Wang T, You Z (2012b) Interleukin-17 and prostaglandin E2 are involved in formation of an M2 macrophage-dominant microenvironment in lung cancer. J Thorac Oncol 7:1091–1100

Pittet JF, Koh H, Fang X, Iles K, Christiaans S, Anjun N, Wagener BM, Park DW, Zmijewski JW, Matthay MA, Roux J (2013) HMGB1 accelerates alveolar epithelial repair via an IL-1β- and vβ6 integrin-dependent activation of TGF-β1. PLoS One 16:e63907

Semrau S, Zettl H, Hildebrandt G, Klautke G, Fietkau R (2014) Older patients with inoperable non-small cell lung cancer: long-term survival after concurrent chemoradiotherapy. Strahlenther Onkol 12:1125–1132

Shang GH, Jia CQ, Tian H, Xiao W, Li Y, Wang AH, Dong L, Lin DJ (2009) Serum high mobility group box protein 1 as a clinical marker for non-small cell lung cancer. Respir Med 103:1949–1953

Smolarczyk R, Cichoń T, Jarosz M, Szala S (2012) HMGB1-its role in tumor progression and anticancer therapy. Postepy Hig Med Dosw 66:913–920

Stenmark MH, Cai XW, Shedden K, Hayman JA, Yuan S, Ritter T, Ten Haken RK, Lawrence TS, Kong FM (2013) Combining physical and biologic parameters to predict radiation-induced lung toxicity in patients with non-small-cell lung cancer treated with definitive radiation therapy. Int J Radiat Oncol Biol Phys 84:e217–e222

Suda K, Kitagawa Y, Ozawa S, Saikawa Y, Ueda M, Abraham E, Kitajima M, Ishizaka A (2006) Serum concentrations of high-mobility group box chromosomal protein 1 before and after exposure to the surgical stress of thoracic esophagectomy: a predictor of clinical course after surgery? Dis Esophagus 19:5–9

Sun KK, Ji C, Li X, Zhang L, Deng J, Zhong N, Wu XY (2013) Overexpression of high mobility group protein B1 correlates with the proliferation and metastasis of lung adenocarcinoma cells. Mol Med Rep 7:1678–1682

Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom AT, Christian MC, Gwyther SG (2000) New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 92:205–216

Travis WD, Brambilla E, Müller-Hermelink HK, Harris CC (2004) Pathology and genetics of tumours of the lung, pleura, thymus and heart, World Health Organization classification of tumours. ARC Press, Lyon, pp 10–33

Van de Wiele C, Vande Vyver F, Debruyne C, Philipp J, van Meerbeeck JP (2008) FDG uptake by the bone marrow in NSCLC patients is related to TGF-beta but not to VEGF or G-CSF serum levels. Eur J Nucl Med Mol Imaging 3:519–522

Vazquez PF, Carlini MJ, Daroqui MC, Colombo L, Dalurzo ML, Smith DE, Grasselli J, Pallotta MG, Ehrlich M, Bal de Kier Joff ED, Puricelli L (2013) TGF-beta specifically enhances the metastatic attributes of murine lung adenocarcinoma: implications for human non-small cell lung cancer. Clin Exp Metastasis 30:993–1007

Wang C, Fei G, Liu Z, Li Q, Xu Z, Ren T (2012) HMGB1 was a pivotal synergistic effector for CpG oligonucleotide to enhance the progression of human lung cancer cells. Cancer Biol Ther 13:727–736

Wang JL, Wu DW, Cheng ZZ, Han WZ, Xu SW, Sun NN (2014) Expression of high mobility group box – B1 (HMGB-1) and matrix metalloproteinase-9 (MMP-9) in non-small cell lung cancer (NSCLC). Asian Pac J Cancer Prev 15:4865–4869

Advs Exp. Medicine, Biology - Neuroscience and Respiration (2015) 11: 59–69
DOI 10.1007/5584_2014_106

Published online: 10 March 2015

TGF-β and SMADs mRNA Expression in Pulmonary Sarcoidosis

W.J. Piotrowski, J. Kiszałkiewicz, D. Pastuszak-Lewandoska, A. Antczak, P. Górski, M. Migdalska-Sęk, W. Górski, K. Czarnecka, E. Nawrot, D. Domańska, and E. Brzeziańska-Lasota

Abstract

Lung fibrosis is a complication of sarcoidosis, in which TGF-β/Smad pathway may play an important role. We evaluated gene expression of TGF-β_1, SMAD2, 3 and 7 in bronchoalveolar lavage (BAL) cells and peripheral blood (PB) lymphocytes of sarcoidosis patients ($n = 94$) to better understand the mechanisms of sarcoid inflammation. The relative gene expression was analyzed by qPCR method. Selected clinical/radiological features and biochemical markers were taken into account in the analysis. We found that TGF-β_1 and SMAD3 expressions in PB lymphocytes were significantly higher in sarcoidosis patients. Up-regulation of SMAD7 (inhibitory Smad) and down-regulation of SMAD3 in BAL cells in all subgroups were found. The expression of TGF-β_1 in PB lymphocytes was the highest in patients with lung parenchymal involvement and in the insidious onset phenotype. The expression of TGF-β_1 in BAL cells was higher in patients with abnormal spirometry ($p = 0.012$), and TGF-β_1 and SMAD3 in patients with restrictive pattern ($p = 0.034$ and 0.031, respectively). Several statistically significant negative correlations were found between the expression levels of SMAD2 and 3 in BAL cells and various LFT parameters. We conclude that TGF-β/Smad pathway is involved in the pathogenesis of pulmonary sarcoidosis. These biomarkers (especially TGF-β_1, SMAD2 and 3) are of a negative prognostic value.

W.J. Piotrowski, P. Górski, and W. Górski
Department of Pneumology and Allergy, First Chair of Internal Diseases, Medical University of Lodz, 251 Pomorska St., 92-213 Lodz, Poland

J. Kiszałkiewicz, D. Pastuszak-Lewandoska, M. Migdalska-Sęk, K. Czarnecka, E. Nawrot, D. Domańska, and E. Brzeziańska-Lasota (✉)
Department of Molecular Bases of Medicine, First Chair of Internal Diseases, Medical University of Lodz, 251 Pomorska St., 92-213 Lodz, Poland
e-mail: ewa.brzezianska@umed.lodz.pl

A. Antczak
Department of General and Oncological Pulmonology, First Chair of Internal Diseases, Medical University of Lodz, 251 Pomorska St., 92-213 Lodz, Poland

Keywords
Biomarkers • Disease mechanisms • Lung fibrosis • Prognosis • Radiological classification

1 Introduction

Sarcoidosis is a chronic inflammatory disease, presenting – in the majority of patients – as intrathoracic lymph nodes enlargement and parenchymal lung disease, with the possible involvement of many extrapulmonary organs. The formation of non-caseating granulomas and lymphocytic inflammation with the predominance of $CD4^+$ Th1 lymphocytes are the most characteristic histologic features. The overall prognosis is good, as about 60 % of patients presenting various phenotypes may recover without treatment. Lung fibrosis is the most unfavorable outcome, and may occur in 5–25 % of patients. It is difficult to anticipate at the beginning which patients will develop this severe complication (Ianuzzi et al. 2007; ATS&ERS&WASOG 1999).

Transforming growth factor beta (TGF-β) is a key cytokine responsible for tissue regeneration and scarring. Its role in the pathogenesis of experimental lung fibrosis (Shi et al. 2014) and idiopathic pulmonary fibrosis (IPF) (Khalil et al. 1996) is well documented. Biological effects of TGF-β include fibroblast recruitment, activation, proliferation and transformation to myofibroblast phenotype, epithelial-mesenchymal transition (EMT), and epithelial cells apoptosis (Patterson et al. 2012). It is believed to play a role in the late phase of sarcoid inflammation, being responsible either for tissue repair or for pathologic fibrosis. Moreover, it may play an important immunoregulatory role in early stages of sarcoid inflammation. An ambiguous role of TGF-β makes its use as a disease marker difficult.

Activation of TGF-β receptors (TβRI and TβRII) by TGF-β and phosphorylation of the receptor complex activates the Smad intracellular signaling proteins through the Smads (Smad2 and 3) and co-Smad 4 receptors. Activation of Smads 2–4 is inhibited by Smad7 (inhibitory Smad) (Flanders et al. 2002). Therefore, the activity of TGF-β should always be considered in relation to the whole TGF-β/Smad signaling pathway. To our best knowledge, the entire pathway has not been studied in the context of sarcoidosis so far.

Taking into account the information above outlined, the aim of this study was to verify the hypothesis, that TGF-β/Smad intracellular pathway signaling elements (TGF-β_1, Smad2, 3 and 7) are important in the pathogenesis of sarcoidosis. In order to assess their significance as negative prognostic markers we evaluated the expression of TGF-β_1, SMAD2, 3, 7 genes in bronchoalveolar lavage fluid (BALF) cells and peripheral blood (PB) lymphocytes in sarcoidosis patients in relation to signs of lung parenchymal involvement, lung function results, clinical phenotypes, and several biochemical markers.

2 Methods

The study was approved by the Ethics Committee of the Medical University of Lodz (RNN/141/10/KE). Written informed consent was obtained from each patient.

2.1 Study Group

A total of 94 patients with pulmonary sarcoidosis were recruited for the study. Patients were admitted to the Department of Pneumology and Allergy of the Norbert Barlicki memorial University Hospital No. 1 in Lodz (Poland) during the years 2010–2014. The diagnosis was based on the current standards (Ianuzzi et al. 2007; ATS&ERS&WASOG 1999). Consistent clinical and radiological picture of sarcoidosis, with the presence of non-caseating granuloma in tissue biopsy, was confirmed for each patient.

The diagnosis was documented by EBUS-TBNA, bronchial mucosal biopsy, transbronchial peripheral lung biopsy, mediastinoscopy, or extrathoracic biopsy (skin, peripheral lymph nodes). Only in patients with typical clinico-radiological picture (bilateral hilar lymph nodes enlargement) and typical BAL results (increased percentage of lymphocytes with CD4/CD8 > 3.5) the biopsy was not obligatory. The patients were divided based on chest X-ray results into the following radiological subgroups: stage I (hilar lymph nodes enlargement without signs of parenchymal involvement), stage II (signs of parenchymal involvement in addition to hilar lymph nodes enlargement), stage III (parenchymal involvement without visible hilar lymph nodes enlargement), and IV (signs of irreversible extensive lung fibrosis). An independent comparison between the patients with acute onset (Löfgren syndrome with arthritis, erythema nodosum, elevated body temperature – with at least two symptoms present) and patients with insidious onset was done. Clinical and biological characteristics of the study group is presented Table 1.

Control group consisted of 50 non-smokers referred for bronchoscopy due to chronic cough or undefined changes on chest X-ray. These patients after thorough examination were finally diagnosed either with idiopathic cough, or as healthy – when radiological signs were defined as clinically insignificant changes or artifacts.

2.2 Bronchoscopy and Bronchoalveolar Lavage Fluid (BALF) Collection

Bronchoscopy was performed with a flexible bronchoscope (Pentax, Tokyo, Japan) according to the Polish Respiratory Society Guidelines (Chciałowski et al. 2011). Patients optionally received midanium and atropine before the examination, 2 % lidocaine was used as a topical anaesthetic. BAL fluid (BALF) was collected from medial lobe, by instillation and subsequent withdrawal of 4×50 mL of 0.9 % NaCl. The fluid recovery was 52.1 ± 1.2 %. The crude BALF was filtered through a gauze, to clear the thick mucus and other contaminants, next centrifuged, and the pellet was suspended in a phosphate buffer. The total number of non-epithelial cells (total cell count – TCC) was presented as $n \times 10^6$. Cytospin slides were prepared and stained by May-Grünwald-Giemsa stain. The number of macrophages, lymphocytes, neutrophils, and eosinophils was calculated under a light microscope and presented as % of TCC. After the calculations, all fluid was centrifuged (10 min 1,200 rpm), supernatant of BALF was suspended in RNAlater RNA Stabilization Reagent (Qiagen, Hilden, Germany) in a volume of about 350 μl of solution in Eppendorf tubes, marked with an identification number, and was frozen (−80 °C) until further RNA isolation procedures.

Table 1 Clinical and biological characteristics of sarcoidosis patients

	Stage I	Stages II–IV	Acute onset	Insidious onset
	n = 46	n = 48	n = 42	n = 51
Gender (F/M)	25/21	22/26	22/20	24/27
Age (year)	40.06 ± 10.97	42.79 ± 11.64	37.29 ± 9.21	44.65 ± 11.99
FEV_1 (% pred.)	94.30 ± 14.62	87.67 ± 17.31	98.18 ± 13.59	85.10 ± 16.10
FVC (% pred.)	103.54 ± 13.46	96.55 ± 117.16	107.52 ± 11.56	93.93 ± 16.21
FEV_1/FVC	0.76 ± 0.07	0.75 ± 0.07	0.76 ± 0.07	0.74 ± 0.06
DLCOc (% pred.)	–	90.73 ± 16.16	–	91.63 ± 16.69
BAL-L%	30.58 ± 18.00	29.45 ± 17.08	35.12 ± 16.62	25.79 ± 17.32
BALF $CD4^+/CD8^+$	6.84 ± 4.37	4.29 ± 3.66	7.98 ± 4.31	3.89 ± 3.18
$Ca^{2+}S$ (mmol/l)	2.44 ± 0.09	2.42 ± 0.18	2.41 ± 0.16	2.45 ± 0.13
$Ca^{2+}U$ (mmol/24 h)	4.83 ± 2.31	4.44 ± 2.66	4.24 ± 2.54	4.91 ± 2.46

Sarcoidosis patients were grouped according to the absence/presence of lung parenchymal changes on chest X-ray (stage I *vs.* stage II–IV) and clinical phenotype (acute *vs.* insidious onset). F – females, M – males, FEV_1 – forced expiratory volume in 1 s, FVC – forced vital capacity, DLCOc – lung diffusion for carbon monoxide corrected for hemoglobin, BAL-L% – bronchoalveolar lavage – % of lymphocytes

2.3 Lung Function

Spirometry was performed according to the Polish Respiratory Society Guidelines (2006) with a computer-based spirometer (Jaeger, Dortmund, Germany). Forced vital capacity (FVC) and forced expiratory volume in 1 s (FEV_1) were measured, and the Tiffenau index (FEV_1/FVC) was calculated. Lung diffusion capacity for carbon monoxide was measured in patients with lung parenchymal disease only (stage II–IV) with a single breath method, with a Lungtest 1000 SB (MES, Cracow, Poland) according to ATS/ERS standards (European Respiratory Society 1993). The values were corrected for the hemoglobin concentration (DLCOc). All data (except the Tiffeneau index) were presented as % of predicted value.

2.4 Peripheral Blood Samples Collection

Blood was collected into 2 mL EDTA containing tubes (labeled with the identification number). For lymphocyte separation, a density gradient cell separation medium Histopaque-1077 (Sigma-Aldrich, Poznan, Poland) was used. Blood (3 mL) was carefully layered onto 3 mL of Histopaque-1077 in a 15-mL conical centrifuge tube. Next, samples were centrifuged at 400 × *g* for 30 min at room temperature. After centrifugation, mononuclear cells were transferred into a clean conical centrifuge tube. Cells were washed by adding 10 mL of isotonic phosphate buffer and mixed gently, then centrifuged at 250 × *g* for 10 min. The supernatant was discarded. Cells were resuspended in 5 mL of isotonic phosphate buffered saline solution and centrifuged at 250 × *g* for 10 min. The supernatant was discarded and cells were resuspended in 350 µl RNAlater RNA Stabilization Reagent and frozen (−80 °C).

2.5 Gene Expression Analysis

RNA isolation was performed using mirVana™ miRNA Isolation Kit (Life Technologies, Carlsbad, CA), according to the manufacturer's protocol. The quality and quantity of isolated RNA was spectrophotometrically assessed (Eppendorf BioPhotometrTM Plus, Eppendorf, Hamburg, Germany). The purity of total RNA (ratio of 16S to 18S fraction) was determined in the automated electrophoresis using RNA 6000 Pico LabChipplates on Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

cDNA was transcribed from 100 ng of total RNA, using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) in a total volume of 20 µl. Reverse transcription (RT) master mix contained the following: 10 x RT buffer, 259 dNTP Mix (100 mM), 10 x RT Random Primers, MultiScribeTM Reverse Transcriptase, RNase Inhibitor, and nuclease-free water. RT reaction was performed in a personal thermocycler (Eppendorf, Hamburg, Germany) in the following conditions: 10 min at 25 °C, followed by 120 min at 37 °C; then the samples were heated to 85 °C for 5 s, and held at 4 °C. The relative expression analysis was performed in 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA) using TaqMan probes for the studied genes: TGF-β_1 (Hs00998133_m1), SMAD2 (Hs00183425_m1), SMAD3 (Hs00969210_m1), SMAD7 (Hs00998193_m1). The PCR mixture contained: cDNA (1–100 ng), 20 × TaqManR Gene Expression Assay, 2 × KAPA PROBE Master Mix (2x) ABI Prism Kit (Kapa Biosystems, Wilmington, MA) and RNase-free water in a total volume of 20 µl. The expression levels (RQ values) of the studied genes were calculated using the delta delta CT method, with the adjustment to the β-actin expression level and in relation to the expression level of calibrator (Human Lung Total RNA Ambion®), for which RQ value was equal to 1.

2.6 Statistical Analysis

The Kruskal–Wallis test, the Mann–Whitney U test, the Neuman–Keuls' multiple comparison test, and the Spearman rank correlation were used to assess the correlation between the relative gene expression levels and sarcoidosis groups classified on the basis of chest X-ray results (stage I *vs.* II–IV) and disease phenotype

(acute *vs*. insidious onset), spirometric parameters, DLCOc, serum Ca^{2+} concentration, Ca^{2+} in 24 h urine collection, the percentage of lymphocytes in BAL, the phenotype of immune cells ($CD4^+/CD8^+$), age, and sex of patients. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Relative Expression of Genes in BALF Cells

There were significant differences between the expression level of TGF-β_1 and SMAD3, TGF-β_1 and SMAD7, and between all SMAD genes: SMAD2, 3 and 7 in BALF cells ($P < 0.05$, Neuman-Keuls multiple comparison test) (data not shown).

In the patients with radiological stage I, the highest expression level of SMAD7 (mean RQ = 3.41) and the lowest of SMAD3 (mean RQ = 0.26) was found in BALF cells. Likewise, the patients with radiological stages II–IV revealed the highest mean expression level of SMAD7 (mean RQ = 3.54) and the lowest of SMAD3 (mean RQ = 0.33). In both acute and insidious disease onset groups, the highest mean expression level was observed for SMAD7 (mean RQ: 3.49 and 3.40, respectively), and the lowest for SMAD3 (0.36 and 0.23, respectively).

Summarizing, the expression of SMAD3 gene was decreased in the majority of samples (90–94 %), and the highest expression level was observed for SMAD7 gene (82–85 %), regardless of the disease classification (Table 2).

3.2 Relative Expression of Genes in Peripheral Blood Lymphocytes of Patients with Sarcoidosis

There were significant differences between the expression level of TGF-β_1 and SMAD2 in PB cells ($P < 0.05$, Neuman-Keuls multiple comparison test) (data not shown). In the patients with radiological stage I, the highest expression of SMAD3 (mean RQ = 1.060) and the lowest of SMAD2 (mean RQ = 0.494) was found. In the patients with radiological stages II–IV, the highest mean expression of TGF-β_1 (0.92) and the lowest of SMAD2 (0.53) was observed.

In the acute onset group, the highest mean expression of SMAD3 (1.11) and the lowest of SMAD2 (0.47) was revealed. In the insidious onset group, the highest mean expression level

Table 2 Mean RQ values (range) of all studied genes (*TGF-β_1, SMAD2,3,7*) in BALF cells in patients with sarcoidosis

			Number (%) of samples with	
		Mean RQ value (range)	RQ value >1	RQ value <1
TGF-β_1	Radiological stages I	0.854 (0.071–4.569)	11 (24 %)	35 (76 %)
	Radiological stages II–IV	0.953 (0.022–3.281)	15 (31 %)	33 (69 %)
	Acute onset	0.773 (0.155–3.181)	9 (22 %)	33 (78 %)
	Insidious onset	1.004 (0.022–4.569)	16 (31 %)	35 (69 %)
SMAD2	Radiological stage I	0.899 (0.016–4.355)	11 (24 %)	35 (76 %)
	Radiological stages II–IV	0.703 (0.009–6.840)	8 (17 %)	40 (83 %)
	Acute onset	0.716 (0.307–4.312)	6 (15 %)	36 (85 %)
	Insidious onset	0.872 (0.009–6.480)	13 (25 %)	38 (75 %)
SMAD3	Radiological stage I	0.264 (0.003–2.487)	3 (7 %)	43 (93 %)
	Radiological stages II–IV	0.334 (0.002–5.227)	5 (10 %)	43 (90 %)
	Acute onset	0.361 (0.086–5.227)	4 (10 %)	38 (90 %)
	Insidious onset	0.230 (0.002–1.999)	3 (6 %)	48 (94 %)
SMAD7	Radiological stage I	3.414 (0.197–12.208)	38 (82 %)	8 (18 %)
	Radiological stages II–IV	3.535 (0.572–11.416)	41 (85 %)	7 (15 %)
	Acute onset	3.490 (0.197–12.208)	35 (83 %)	7 (17 %)
	Insidious onset	3.400 (0.421–11.416)	43 (84 %)	8 (16 %)

Table 3 Mean RQ values (range) of all studied genes (*TGF-β_1, SMAD2,3,7*) in lymphocytes in patients with sarcoidosis

			Number (%) of samples with	
		Mean RQ value (range)	RQ value >1	RQ value <1
TGF-β_1	Radiological stage I	1.045 (0.142–2.790)	8 (38 %)	13 (62 %)
	Radiological stages II–III	0.917 (0.380–3,108)	4 (23 %)	13 (77 %)
	Acute onset	0.910 (0.142–2.672)	6 (30 %)	14 (70 %)
	Insidious onset	1.075 (0.380–3.108)	6 (33 %)	12 (67 %)
SMAD2	Radiological stages I	0.494 (0.018–1.645)	3 (14 %)	18 (86 %)
	Radiological stages II–III	0.527 (0.049–2.356)	1 (6 %)	16 (94 %)
	Acute onset	0.472 (0.018–1.645)	2 (10 %)	18 (90 %)
	Insidious onset	0.550 (0.049–2.356)	2 (11 %)	16 (89 %)
SMAD3	Radiological stage I	1.060 (0.257–4,489)	7 (33 %)	14 (67 %)
	Radiological stages II–III	0.721 (0.088–1.606)	3 (17 %)	14 (83 %)
	Acute onset	1.107 (0.088–4.489)	7 (30 %)	13 (70 %)
	Insidious onset	0.688 (0.089–1.606)	3 (16 %)	15 (84 %)
SMAD7	Radiological stage I	0.807 (0.157–2.666)	5 (23 %)	16 (77 %)
	Radiological stages II–III	0.854 (0.021–4.883)	3 (17 %)	14 (83 %)
	Acute onset	0.740 (0.021–2.666)	4 (20 %)	16 (80 %)
	Insidious onset	0.943 (0.225–4.883)	4 (22 %)	14 (78 %)

for TGF-β_1 (1.08) and the lowest for SMAD2 (0.55) was found.

The expression of the TGF-β_1 gene increased in 23–38 % of the samples, regardless of the clinical disease classification groups. Decreased expression levels were observed for SMAD2 gene in the majority of patients (86–94 %) (Table 3).

3.3 Expression Levels of Genes (TGF-β_1, SMAD 2,3,7) in Sarcoidosis Patients *vs.* Controls

In BAL cells of the control group, the mean RQ values of the genes were as follows: 0.470 for TGF-β_1, 0.468 for SMAD2, 0.161 for SMAD3, and 2.670 for SMAD7. The mean RQ values of these genes for patients with sarcoidosis are shown in Table 2. No statistically significant differences were found for any of the studied genes between sarcoidosis and control subjects ($P > 0.05$; Mann–Whitney U test).

In PB lymphocytes of the control group, the mean RQ values were as follows: 0.51 for TGF-β_1, 0.49 for SMAD2, 0.41 or SMAD3 and 0.81 for SMAD7. The mean RQ values of these genes for patients with sarcoidosis are shown in Table 3. Significant differences between the patients and controls were observed for the TGF-β_1 gene ($P = 0.004$) and for the SMAD3 gene ($P = 0.002$, Mann–Whitney U test), with a higher gene expression in sarcoidosis patients (Figs. 1 and 2).

3.4 BAL Cells *vs.* Lymphocytes

Significant differences between the BAL cells and lymphocytes for the TGF-β_1 gene and for the SMAD3 gene ($P = 0.001$, Mann–Whitney U test) were observed, with a higher expression level of those genes in lymphocytes. In contrast, The SMAD7 gene was significantly higher ($P = 0.001$) in the BAL cells.

3.5 Expression Levels of Genes in Different Sarcoidosis Subgroups

There were no significant differences regarding the expression of the genes between the patients without and those with parenchymal involvement (stages I *vs.* II–IV) or between the clinical phenotypes (acute *vs.* insidious onset), either in BAL cells or in PB lymphocytes.

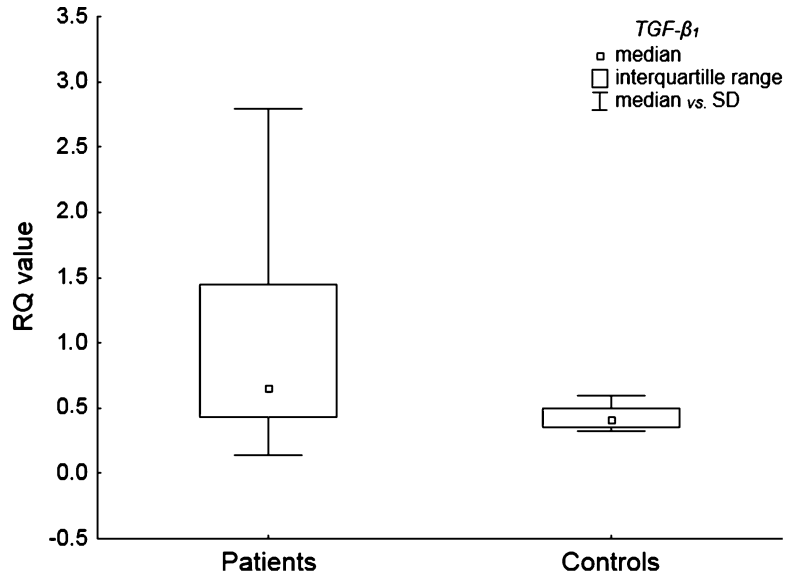


Fig. 1 **TGF-β_1 RQ values in peripheral blood lymphocytes** of patients and controls (P = 0.004)

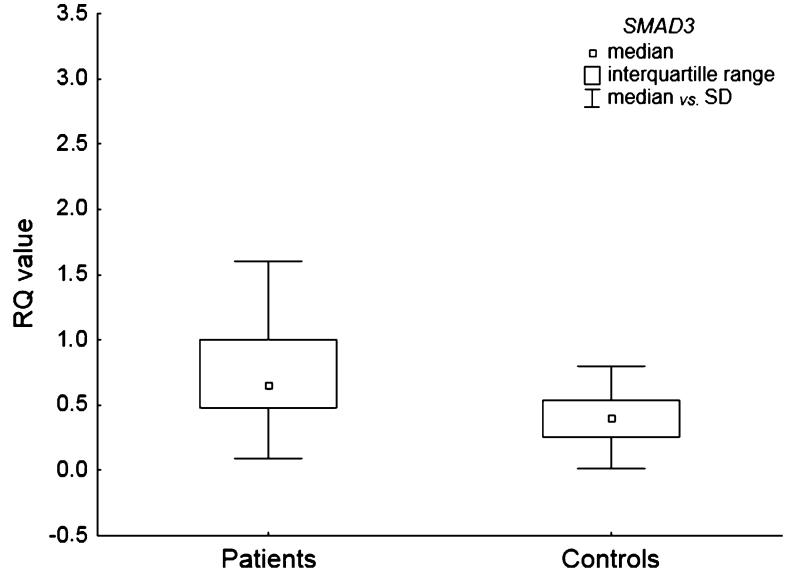


Fig. 2 **Smad3 RQ values in peripheral blood lymphocytes** of patients and controls (P = 0.001)

Significantly increased expression of TGF-β_1 was observed in BAL cells in the patients with abnormal compared with those having normal spirometry (P = 0.012). Likewise, expression of both TGF-β_1 and SMAD3 in BAL cells was higher in a subgroup of patients with restrictive spirometric pattern (P = 0.003 both, Mann–Whitney U test) (Figs. 3 and 4).

3.6 Gene Expression Levels in BAL Cells in Relation to Patients' Gender, and Biochemical and Immunological Markers

There were significant inter-gender differences for the TGF-β_1 gene in a total patient group (P = 0.032), with a higher gene expression level in men. Likewise, in

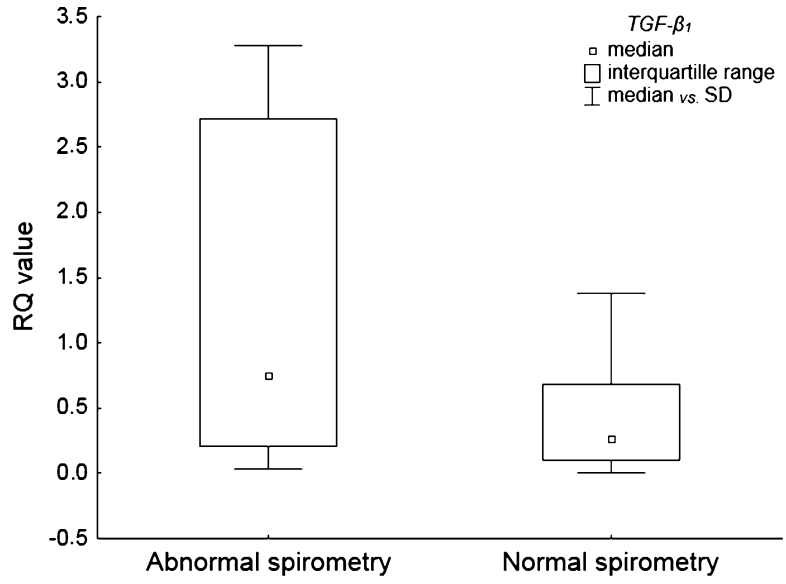


Fig. 3 TGF-β_1 gene RQ values in BAL cells in patients with normal and abnormal spirometry (P = 0.012)

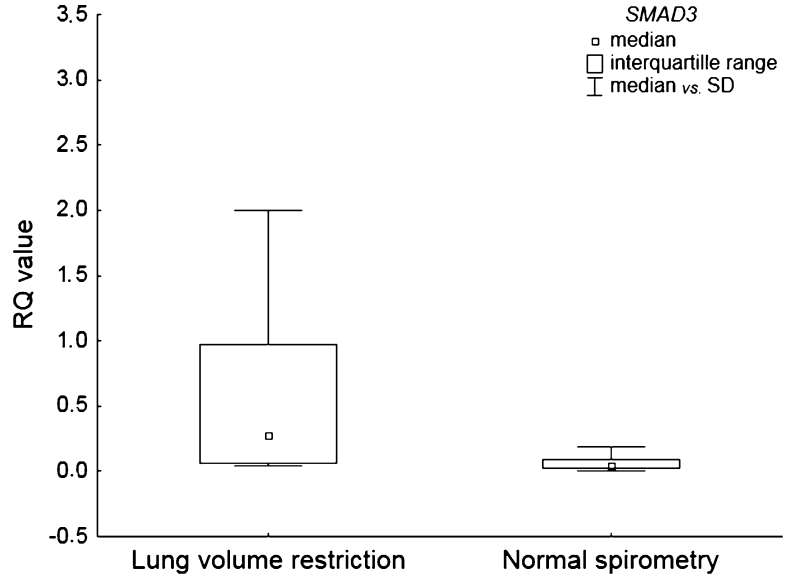


Fig. 4 SMAD3 gene RQ values in BAL cells in patients with normal spirometry vs patients with restrictive spirometric pattern (P = 0.003)

men with radiological stage I, a significantly higher TGF-β_1 gene expression level was observed (P = 0.029). In females, a significantly higher SMAD2 gene expression level was found in the insidious onset phenotype group (P = 0.042, Mann–Whitney U test). We found several negative correlations between the lung function parameters and RQ values of the genes studied. Those and other correlations are presented in Table 4.

3.7 Gene Expression in Peripheral Blood Lymphocytes in Relation to Patients' Gender, and Biochemical and Immunological Markers

Significant inter-gender differences were observed for the SMAD7 gene in a total patient group (P = 0.021; U Mann–Whitney U test), with a higher gene expression level in females.

Table 4 Correlations between the expression of the genes studied (RQ values) and lung function parameters, and selected laboratory markers in sarcoidosis patients

Biological material	Parameter	Gene product	Group/subgroup	Rho value	P value
BAL cells	FVC	SMAD3	Entire	−0.284	0.009
	FVC	SMAD3	Insidious onset	−0.368	0.011
	FVC	SMAD3	Acute onset	−0.327	0.045
	FVC	SMAD3	Abnormal spirometry	−0.510	0.009
	FVC	SMAD3	Restriction	−0.761	0.017
	FVC	SMAD3	Parenchymal involvement	−0.370	0.016
	FVC	SMAD2	Restriction	−0.677	0.045
	FEV_1	SMAD3	Entire	−0.289	0.008
	FEV_1	SMAD3	Insidious onset	−0.433	0.002
	FEV_1	SMAD3	Abnormal spirometry	−0.437	0.029
	FEV_1	SMAD3	Parenchymal involvement	−0.423	0.005
	DLCOc	SMAD2	Entire	−0.268	0.038
PB lymphocytes	FVC	SMAD2	Acute onset	−0.518	0.019
	FEV_1	SMAD7	No parenchymal involvement	−0.478	0.044
	DLCOc	SMAD7	No parenchymal involvement	−0.885	0.019
	$Ca^{2+}U$	TGF-β_1	Acute onset	+0.683	0.042
	$Ca^{2+}S$	SMAD7	No parenchymal involvement	−0.608	0.027
	$Ca^{2+}S$	SMAD7	Acute onset	−0.623	0.030

BAL – bronchoalveolar lavage, $Ca^{2+}S$ – serum calcium concentration, $Ca^{2+}U$ – calcium in 24 h urine collection, DLCOc – lung diffusion capacity for carbon monoxide corrected for hemoglobin, FEV_1 – forced expiratory volume in 1 s of expiration, FVC – forced vital capacity, PB – peripheral blood

Correlations between RQ values of the genes studied in peripheral blood lymphocytes and lung function and laboratory results are presented in Table 4.

4 Discussion

Although we found inappreciable differences between the sarcoidosis and control groups regarding the expression of genes in BAL cells, we did find upregulated TGF-β_1 and SMAD3 genes in peripheral blood lymphocytes in sarcoidosis patients. The TGF-β_1 gene was overexpressed exclusively in peripheral blood lymphocytes in the patients with lung parenchymal involvement and insidious disease onset (both known to be related to increased risk of chronic and progressive disease). Interestingly, BAL fluid cells obtained from the sarcoidosis patients showed signs of overexpression of inhibitory SMAD7, with presumably a subsequent downregulation of SMAD3. The most informative parameters of bronchial and parenchymal distortion and damage – lung function results and diffusion capacity – correlated negatively with SMAD3, SMAD2, and TGF-β_1. These findings were confirmed in the whole sarcoidosis group and in the subgroups of patients with radiological signs of lung parenchymal involvement and insidious disease onset, both features related to chronicity and progression. In addition, increased expression of SMAD3 and TGF-β_1 genes were also confirmed in patients with abnormal spirometry, especially those with restrictive pattern.

Our results are in concordance with other authors (Salez et al. 1998; Ishioka et al. 1996) who reported the lack of significant differences in TGF-β_1 protein/mRNA expression in BALF of sarcoidosis patients in comparison with healthy controls. The possible explanation of this phenomenon is a complicated system of adjustment of the TGF- β_1 activity in lung cells by a set of receptors which reveal serine-threonine kinase activity and collaborate with different cytokines via TGF/Smad signaling and other downstream pathways (e.g., Ras/MEK/ERK signaling)

(Massague 1998). Moreover, according to the earlier published data, a class of intracellular signaling proteins such as Smads has been confirmed as the pivotal mediators of TGF-β_1 activity (Derynck et al. 1998).

These signaling proteins may strongly modulate the TGF-β_1 expression. Especially, Smad3, which interacts with many transcription factors, oncogenes, and with glucocorticoid receptors, may influence the TGF-β_1 synthesis in the inflammatory process. In the present study we confirmed a reduced expression of TGF-β_1 followed by a reduced SMAD3 gene expression in BAL cells of sarcoidosis patients. Consequently, we did not observe any difference in TGF-β_1 expression between the patient and control groups. Additionally, we observed an overexpression of inhibitory SMAD7 in the patients' BAL cells, which possibly is also responsible for the downregulation of TGF-β_1 gene.

It seems interesting that, according to other published results, TGF-β_1 immunoexpression in BAL cells is increased in sarcoidosis patients with altered lung function (Salez et al. 1998). Likewise, in the present study in BAL cells of patients with lung function impairment, the increased expression of TGF-β_1 on the transcriptional level and a negative correlation between TGF-β_1 mRNA level and lung function results were found. The overwhelming source of information on the role of Smads in TGF-β-mediated responses comes from the studies on wound healing. We can learn from these studies that SMAD3 gene and its protein Smad3 may play a key role in the regulation of this process (Ashcroft and Roberts 2000). For instance, the rate of wound healing in $Smad3^{ex8/ex8}$ (null) mice is substantially accelerated when compared to wild-type animals (Ashcroft et al. 1999). Also, in a skin radiation fibrosis model, Smad3 seems to play a modulatory role, as $Smad3^{ex8/ex8}$ mice are protected against cutaneous injury induced by ionizing radiation, and show reduced epidermal acanthosis, reduced influx of neutrophils and mast cells, and, most importantly, reduced accumulation of myofibroblasts and extracellular matrix components (Flanders et al. 2002). Recent studies also point out a critical role of TGFβ/Smad3 signaling in bleomycin-induced lung fibrosis in mice. Tang et al. (2014) have reported that TGF-$\beta_1$wt transgenic mice, showing increased levels of latent TGF-β_1 in plasma and lung tissue, are protected from bleomycin-induced lung inflammation and collagen matrix accumulation. This effect was accompanied by inactivation of TGFβ/Smad3 pathway and increased levels of an inhibitory Smad7. Such studies on the role of the TGFβ/Smad signaling pathway in sarcoidosis have not yet been published. Therefore, we presume that the present data showing the role of SMAD genes in sarcoidosis are the first ones published.

Many studies dedicated to the significance of TGF-β in interstitial lung diseases provide evidence for a negative prognostic value of this cytokine. For instance, the correlation between the concentration of TGF-β_1 in BAL fluid and the high resolution computed tomography (HRCT) score of the lavaged segment has been found (Szlubowski et al. 2010). The authors conclude that this cytokine may be a good, but not specific, marker of fibrosis in the plethora of interstitial lung diseases.

Interestingly, glucocorticosteroids, which are the mainstay of treatment in severe sarcoidosis and other interstitial lung diseases potentially leading to fibrosis, downregulate the TGF-β receptor 1 (tgfbr1)/Smad2/3 pathway in lung fibroblasts *in vitro* (Schwartze et al. 2014). Also, inhibition of extracellular signal-regulated kinase (ERK-5), a MAP-kinase known to play a role in an experimental model of bleomycin-induced lung fibrosis, ameliorates fibrosis *via* inhibition of Smad3 acetylation (Kim et al. 2013). Therefore, Smad3 and SMAD3 gene may be a target for pharmacological modulation. It may be presumed that this pathway will be considered in the development of new antifibrotic drugs.

In conclusion, we provide data showing upregulation of TGF-β_1 and SMAD3 in peripheral blood lymphocytes in sarcoidosis patients, while in BAL cells these genes may be downregulated by inhibitory SMAD7. The increased activity of these genes in patients presenting signs known to be related to worse prognosis (insidious disease onset, parenchymal involvement, lung function

impairment) as well as several negative correlations between lung function results and mRNA levels of these molecules, point to the $TGF\beta_1$/Smad pathway as being critical for the development of such unfavorable outcome in sarcoidosis as lung fibrosis.

Acknowledgements The study was funded by grant 2011/01/B/NZ5/04239 from the National Science Center.

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

References

American Thoracic Society: European Respiratory Society: World Association of Sarcoidosis and Other Granulomatous Disorders (1999) Statement on sarcoidosis. Am J Respir Crit Care Med 160:736–755

Ashcroft GS, Roberts AB (2000) Loss of Smad3 modulates wound healing. Cytokine Growth Factor Rev 11:125–131

Ashcroft GS, Yang X, Glick A, Weinstein M, Letterio JJ, Mizel DE (1999) Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response. Nat Cell Biol 1:260–266

Chciałowski A, Chorostowska-Wynimko J, Fal A, Pawłowicz R, Domagał-Kulawik J (2011) Recommendation of the Polish Respiratory Society for bronchoalveolar lavage (BAL) sampling processing and analysis methods. Pneumonol Alergol Pol 79:75–89

Derynck R, Zhang Y, Feng XH (1998) Smads: transcriptional activators of TGF-beta response. Cell 95:737–740

European Respiratory Society (1993) Standardized lung function testing. Official statement of the European Respiratory Society. Eur Respir J 16(Suppl):1–100

Flanders KC, Sullivan CD, Fuji M, Sowers A, Anzano MA, Arabshahi A, Major C, Deng C, Russo A, Mitchell JB, Roberts AB (2002) Mice lacking Smad3 are protected against cutaneous injury induced by ionizing radiation. Am J Pathol 160:1057–1068

Ianuzzi MC, Rybicki BA, Teirstein AS (2007) Medical progress. Sarcoidosis. N Engl J Med 357:2153–2165

Ishioka S, Saito T, Hiyama K, Haruta Y, Maeda A, Hozawa S, Inamizu T, Yamakido M (1996) Increased expression of tumor necrosis factor-alpha, interleukin-6, platelet-derived growth factor-B and granulocyte-macrophage colony-stimulating factor mRNA in cells of bronchoalveolar lavage fluids from patients with sarcoidosis. Sarcoidosis Vasc Diffuse Lung Dis 13:139–145

Khalil N, O'Connor RN, Flanders KC, Unruh H (1996) TGF-beta 1, but not TGF-beta 2 or TGF-beta 3, is differentially present in epithelial cells of advanced pulmonary fibrosis: an immunohistochemical study. Am J Respir Cell Mol Biol 14:131–138

Kim S, Lim JH, Woo CH (2013) ERK5 inhibition ameliorates pulmonary fibrosis via regulating Smad3 acetylation. Am J Pathol 183:1758–1768

Massague J (1998) TGF-beta signaling transduction. Annu Rev Biochem 67:753–791

Patterson KC, Hogarth K, Husain AN, Sperling AI, Niewold TB (2012) The clinical and immunologic features of pulmonary fibrosis in sarcoidosis. Transl Res 160:321–331

Polish Respiratory Society Guidelines for Spirometry (2006) Zalecenia Polskiego Towarzystwa Chorób Płuc dotyczące wykonywania badań spirometrycznych. Pneumonologia i Alergologia Polska 74(Suppl 1) (in Polish)

Salez F, Gosset P, Copin MC, Lamblin Degros C, Tonnel AB, Wallaert B (1998) Transforming growth factor-β_1 in sarcoidosis. Eur Respir J 12:913–919

Schwartze JT, Becker S, Sakkas E, Wujak ŁA, Niess G, Usemann J, Reichenberger F, Herold S, Vadsz I, Mayer K, Seeger W, Morty RE (2014) Glucocorticoids recruit Tgfbr3 and Smad1 to shift transforming growth factor-β signaling from the Tgfbr1/Smad2/3 axis to the Acvrl1/Smad1 axis in lung fibroblasts. J Biol Chem 289:3262–3275

Shi K, Jiang J, Ma T, Xie J, Duan L, Chen R, Song P, Yu Z, Liu C, Zhu Q, Zheng J (2014) Pathogenesis pathways of idiopathic pulmonary fibrosis in bleomycin-induced lung injury model in mice. Respir Physiol Neurobiol 190:113–117

Szlubowski A, Soja J, Grzanka P, Tomaszewska R, Papla B, Kużdżał J, Ćmiel A, Sładek K (2010) TGF-b1 in bronchoalveolar lavage fluid in diffuse parenchymal lung diseases and high-resolution computed tomography score. Pol Arch Med Wewn 120:270–275

Tang YJ, Xiao J, Huang XR, Zhang Y, Yang C, Meng XM, Feng YL, Wang XJ, Hui DS, Yu CM, Lan HY (2014) Latent TGF-β1 protects against bleomycin-induced lung injury in mice. Am J Respir Cell Mol Biol (Epub ahead of print), 51(6):761–771

Advs Exp. Medicine, Biology - Neuroscience and Respiration (2015) 11: 71–73
DOI 10.1007/5584_2014

Index