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Proteomic biomarkers of non-small cell lung cancer patients

Abstract

Lung cancer is a disease with a very low 5-year survival rate (6–13%) worldwide. The most frequently diagnosed histological type of this cancer is non-small cell lung cancer (NSCLC). Poor prognosis for lung cancer — including NSCLC — is mainly related to the fact that patients are diagnosed in the advanced stages of the disease. The aim of this study is to summarize data that concerns new directions of research regarding diagnostic biomarkers that could be used to support the routine diagnosis of this cancer. In recent years, proteomic analysis has become an important tool for cancer biology research, complementing genetic analysis. Among the numerous methods of proteomic analysis, mass spectrometry techniques enable the extremely accurate qualitative and quantitative identification of hundreds of proteins in small volumes of various biological samples. Such analyses may soon become the basis of improvement in lung cancer diagnostic procedures. This study presents the latest reports in proteomic research concerning the diagnosis of NSCLC. New potential proteomic biomarkers, whose presence indicates the development of a neoplastic process at an early stage, are presented. We describe biomarkers whose altered expression levels correlate with different stages of cancer. We also present protein biomarkers that help differentiate NSCLC subtypes. In the clinical workup of NSCLC patients, it is important not only to make an early diagnosis, but also to monitor the development of the neoplastic disease. Considering this fact, we also present examples of biomarkers whose abnormal expression may indicate a high risk of metastasis to the lymph nodes. This paper also emphasizes the need to conduct further research that would confirm the usefulness of the described biomarkers in clinical practice.

Key words: non-small cell lung cancer, proteomic biomarker, mass spectrometry

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Introduction

Lung cancer is one of the most commonly diagnosed cancers and is also the leading cause of cancer-related mortality. In 2018, 2,093,876 new cases of lung cancer were diagnosed, which accounts for 11.6% of total cases. 1.8 million people died (18.4% of total cancer-related deaths) [1] with a focus on geographic variability across 20 world regions. There will be an estimated 18.1 million new cancer cases (17.0 million excluding nonmelanoma skin cancer. This high mortality is mainly caused by a late diagnosis in patients with advanced-stage cancer. The early stage of the disease is characterized by a poor clinical manifestation or occurrence of unspecific symptoms, which makes the diagnostic procedure difficult. Therefore, there is an urgent need to discover a highly sensitive and specific biomarker in order to diagnose non-small cell lung cancer (NSCLC) patients at an early stage of the disease process.

Proteomic-based lung cancer biomarker search

Proteomic analysis is a powerful tool in the global assessment of protein expression and has been extensively applied to biomarker discovery in clinical diseases [2]. The rapid development of mass spectrometry techniques allows to efficiently identify hundreds of differentially expressed proteins in small quantities of various biological samples [3]. Mass spectrometry identifies unknown biomolecules based on their accurate mass and fragmentation pattern. However, for proteomic studies, this is possible only if a sample is a simple mixture or has been previously divided into simpler parts by high resolution separation methods such as two-dimensional electrophoresis, protein microarrays, and liquid chromatography [4]. Quantitative proteomics provides information about relative and absolute protein expressions within a sample [5].

The most common histological type of lung cancer is NSCLC, which accounts for 85% of all

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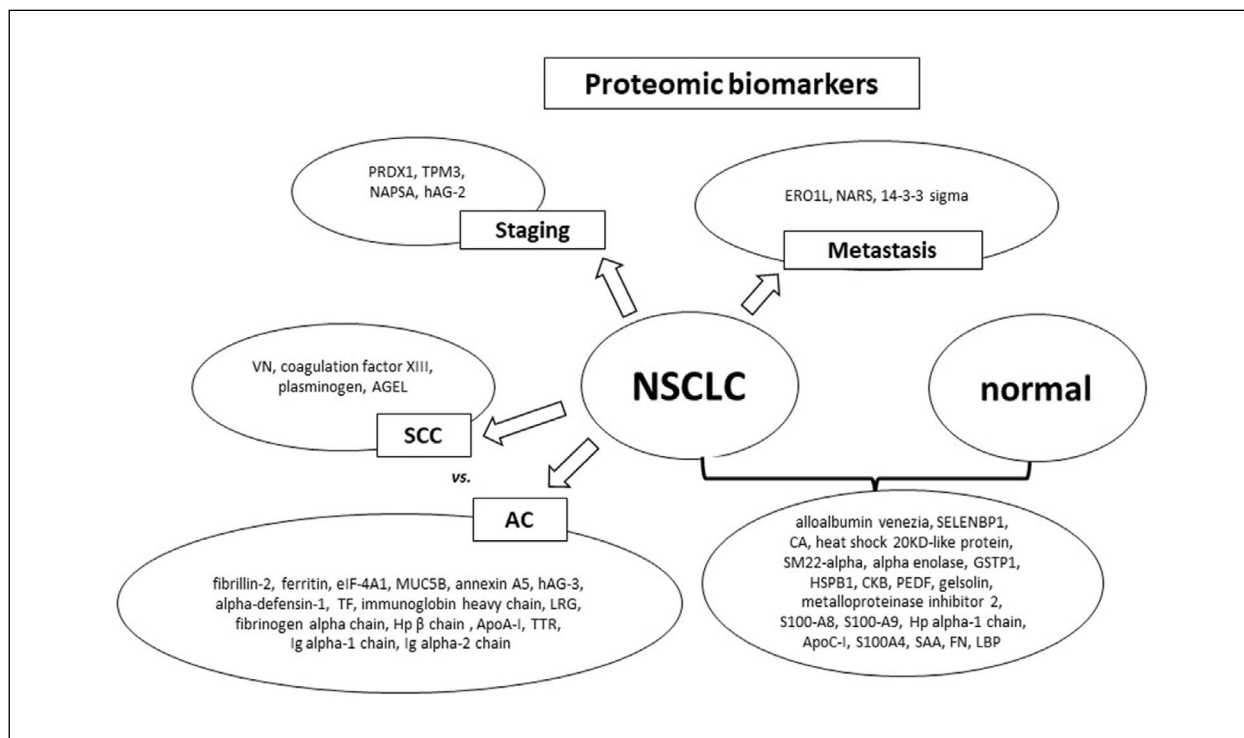


Figure 1. Proteomic biomarkers of NSCLC patients

AC — adenocarcinoma; AGEL — gelsolin; CA — carbonic anhydrase; CKB — creatine kinase brain-type; eIF-4A1 — eukaryotic translation initiation factor 4A1; ERO1L — ERO1-like protein alpha; FN — fibronectin; GSTP1 — glutathione S-transferase P1; hAG-2 — anterior gradient protein 2 homolog; hAG-3 — anterior gradient protein 3; Hp — haptoglobin; HSPB1 — heat shock protein beta-1; LBP — lipopolysaccharide binding protein; LRG — leucine-rich alpha-2-glycoprotein; MUC5B — mucin-5B; NAPSA — napsin-A; NARS — asparagine-tRNA ligase; NSCLC — non-small cell lung cancer; PEDF — pigment epithelium-derived factor; PRDX1 — peroxiredoxin 1; SAA — serum amyloid A; SCC — squamous cell carcinoma; SELENBP1 — selenium-binding protein 1; TF — transferrin; TPM3 — tropomyosin alpha-3 chain; TTR — transthyretin; VN — vitronectin

cases [6]. Histological subtypes of NSCLC include adenocarcinoma (AC) (40%), squamous cell carcinoma (SCC) (25%), and many other subtypes which occur at a very low frequency [7]. The heterogeneity of NSCLC causes difficulty in appropriately diagnosing a patient and subsequently selecting an adequate treatment option, which differs significantly between subtypes [8]. Due to the high prevalence of NSCLC and its diversity, there is a need to identify specific biomarkers that could be used to support routine diagnosis of this cancer. This study presents the latest reports in proteomic research concerning new biomarkers which may be used in the diagnosis of NSCLC (Figure 1).

Protein diagnostic biomarkers detected in NSCLC patients

Proteomic studies allowed to identify a great number of proteins whose expression was diversified between tumor tissue and adjacent macroscopically-unchanged tissue, which constituted the control group in this study. As proteomic biomarkers of NSCLC, Li et al. [9] listed sele-

nium-binding protein 1 (SELENBP1), carbonic anhydrase (CA), heat shock 20KD-like protein, transgelin (SM22-alpha), allobumin venezia (whose expression levels were down-regulated in lung cancer tissue), and alpha enolase (which was overexpressed). SELENBP1, a member of the selenoprotein family, mediates the intracellular transport of selenium [10], whose dietary deficiency is associated with an increased incidence of epithelial cancers, including lung cancer [11]. A progressively decreased expression level of SELENBP1 was observed by Zeng et al. [12] in the human bronchial epithelial carcinogenic process, which indicates its major role in the regulation of cancer development and progression. On the basis of the receiver operating characteristic (ROC) curve analysis, the authors revealed the ability of the SELENBP1 expression level to distinguish the normal bronchial epithelium from preneoplastic lesions with a sensitivity and specificity of 80% and 79%, respectively. Carbonic anhydrases (CAs) are enzymes involved in several fundamental biological processes including respiration, transport of CO₂, pH regulation, and ion transport [13]. It has been shown that CAs are important mediators

of tumor cell pH by modulating the bicarbonate and proton concentrations for cell survival and proliferation. A proteomic study by Nigro et al. [14] concentrated on two CA isoforms, CAI and CAII, revealed a significantly downregulated expression level in the tumor tissue compared to control tissues, which indicates that these proteins could be candidates for use as diagnostic biomarkers in NSCLC patients. SM22-alpha is an actin cross-linking protein that is involved in calcium interactions and regulates contractile properties [15]. It has been found that it may play a role in cell differentiation, cell migration, cell invasion, and matrix remodeling by stabilizing the cytoskeleton through actin binding. However, the data on the abnormal expression level of SM22-alpha in lung cancer is controversial. Contrary to the results of Lie et al. [9], another proteomic study by Rho et al. [16] revealed an upregulated expression level of SM22-alpha in lung AC tissues compared to the control tissue.

Searching for biomarkers to diagnose squamous cell lung carcinoma, Zeng et al. [17] assessed the expression level of proteins in different stages of disease. The combination of three proteins, glutathione S-transferase P1 (GSTP1), heat shock protein beta-1 (HSPB1), and creatine kinase brain-type (CKB) was found to discriminate an invasive stage of cancer from the normal bronchial epithelium with a sensitivity of 92% and a specificity of 91%. Furthermore, they revealed that changes in expression levels of those proteins may be used to diagnose a patient with preneoplastic lesions with a sensitivity of 96% and a specificity of 92%. HSPB1 is a type of small Heat Shock Protein (sHSP) which is produced in cells by stressors such as hypoxia, UV light exposure, and viral agents. There is evidence that HSPB1 plays an essential role in cancer as it protects from programmed cell death (PCD) through interactions with several key regulatory proteins. GSTP1 is an enzyme catalyzing the detoxification of a variety of electrophilic compounds including oxidized lipid, DNA, and catechol products, thereby protecting cells from bioactive xenobiotics and reactive oxidative substances. The down-regulation of GSTP1 enhances the level of harmful substances and the frequency of gene mutations increasing the risk of bronchial epithelial carcinogenesis [17]. The study on human bronchial epithelial line cells revealed that GSTP1 knockdown increased the susceptibility of cell transformation induced by benzo(a)pyrene, the main lung carcinogen within tobacco smoke. CKB is one of two isoenzymes

of creatine kinase which are involved in energy transduction pathways. It is predominantly expressed in the brain as well as in the lung, clearly in airway epithelial cells. The study by Hara et al. [18] demonstrated that CKB expression levels decreased in bronchial epithelial cells in the setting of cigarette smoke exposure, which is the main cause of SCC. According to the results of Zeng et al., upregulation of GSTP1 and CKB expression, and downregulation of HSPB1 expression may indicate the development of squamous cell lung carcinoma.

Pleural effusion, which is produced continuously at the parietal pleural level and reabsorbed through the lymphatic system, is a significant source of NSCLC biomarkers. In a number of disorders, including cancer, it accumulates because the rate of fluid formation exceeds the rate of its removal. It is rich in proteins, either secreted from tumor cells, derived from the circulation, or locally released by inflammation. These can potentially be used as biomarkers.

The study by Rodríguez-Piñero et al. [19] compared the proteome of pleural effusion samples as well as serum from NSCLC patients to those from patients with benign lung diseases such as pneumonia or tuberculosis. Their biomarker candidates comprise proteins with an increased expression in malignant pleural effusions such as pigment epithelium-derived factor (PEDF), gelsolin, and metalloproteinase inhibitor 2. Others studied included S100-A8 and S100-A9, although they had a lower expression. PEDF was the only protein found with significantly different levels both in the pleural effusion and the serum from NSCLC patients when compared with benign lung diseases. Recent studies on cell lines showed that PEDF affects migration, invasion, and motility of NSCLC cells by the regulation of thrombospondin 1 expression [20]. Previous investigations revealed that increased expression levels of PEDF were related to a counteracting activity to compensate for increased vascular endothelial growth factor (VEGF) levels [21]. VEGF is a strong angiogenic factor that is overexpressed during tumorigenesis. Therefore, an increase in PEDF would be expected to fight the spread of cancer cells.

Blood proteomic analysis may have a great advantage over the proteomics performed in lung cancer tissue due to the greater availability of blood samples. The proteomic study by Yang et al. [22] identified three serum candidate protein biomarkers for NSCLC: apolipoprotein C-I (ApoC-I), haptoglobin alpha-1 chain, and

S100A4, which can diagnose NSCLC in patients with a sensitivity and specificity of 96.56% and 94.79%, respectively. ApoC-I is a lipid carrier protein and, although previous studies mainly focused on lipoprotein metabolism, it has been reported that the ApoC-I also regulates many cellular functions such as the promotion of growth factor-mediated cell survival and apoptosis [23]. It has been demonstrated that ApoC-I has a certain anticancer effect on tumor cells, as well as the ability to decrease the expression of PCNA, Ki-67 and Bcl-2 proteins, enhance Bax protein expression, and inhibit cell proliferation [24]. S100A4, a member of the S100 family of calcium binding proteins, influences many biological processes including angiogenesis, stimulation of cell motility, upregulation of matrix metalloproteinases (MMPs), and modulation of tumor-related transcription factors [25, 26]. In addition, the tumor suppressor protein p53 has also been identified as a target for the S100A4 protein promoting its degradation and may be central for the stimulation of tumor development [27]. As a serum potential biomarker for NSCLC patients, Sung et al. indicated serum amyloid A (SAA), whose expression level was upregulated when compared to a control group [28]. Considering that SAA is a positive acute phase protein involved in the inflammatory response and that lung cancer is a chronic inflammatory disease, the elevated concentration of this protein is not surprising. However, a further study revealed that serum SAA expression levels in lung AC were significantly higher compared to other diseases of the respiratory system such as idiopathic pulmonary fibrosis and bronchial asthma, as well as in other cancers like stomach and breast cancer. The results of studies conducted by Urieli-Shoval et al. [29] revealed that the production of SAA by the alveolar lining epithelium of the lung occurred without provoking a continuous systemic acute-phase response during carcinogenesis.

In recent years, exosomes have garnered considerable attention from researchers due to their potential utility as circulating biomarkers for cancer. They are small (50–150 nm in diameter), membrane-enclosed particles containing nucleic acid and protein cargo, which have been implicated in a diverse range of physiological functions as well as pathological ones due to their capacity to convey molecules from a donor cell to a recipient cell. Tumor-derived exosomes have been demonstrated to carry the disease-associated molecular cargo and modulate the behavior of recipient cells towards a pro-oncogenic phenotype

[30]. The proteomic studies assessing the profile of proteins in exosomes derived from the serum of NSCLC patients demonstrated a significantly higher expression level of fibronectin [31] and lipopolysaccharide binding protein [32]. It was shown that these proteins may be good biomarkers of NSCLC on the basis of the ROC curve (AUC was 0.833 and 0.713, respectively) and could be applied to diagnose NSCLC patients.

Subtype-specific tumor markers of blood in NSCLC patients

Adenocarcinoma and squamous cell carcinoma are the two most common NSCLC subtypes and have been shown to differ significantly both in terms of their clinical behavior and molecular signatures [33–36].

To investigate the expression of tumor-associated proteins in AC, Li et al. [36] used quantitative proteomic analyses which revealed significant differences in the expression levels of fibrillin-2, ferritin, eukaryotic translation initiation factor 4A1, annexin A5, mucin-5B (MUC5B), alpha-defensin 1, and anterior gradient protein 3, compared to the control lung tissue. Among these proteins, they found that MUC5B may be used as a good candidate biomarker in the detection of AC. MUC5B is a member of a family of high molecular-weight heavily-glycosylated proteins which are involved in the processes of epithelial differentiation, growth regulation, modulation of cell adhesion, cell signaling, and protection of the airway against environmental toxins [37, 38]. However, there is evidence that mucins also play important roles in tumor cell growth, invasion, and metastasis in cancer cells. The study by Nagashio et al. [39] confirmed that the expression level of MUC5B is higher in AC compared to SCC, as well as in patients with an advanced stage of cancer. Moreover, they noticed a correlation between the MUC5B expression level and tumor size, nodal status, and pleural invasion. These results suggest that MUC5B may not only be a useful differential diagnostic marker of AC from other histological types of lung cancer (especially from SCC), but may also serve to be a useful marker for more aggressive AC.

Another proteomic study revealed that the plasma of AC patients was characterized by a higher abundance of transferrin, immunoglobulin heavy chain, and leucine-rich alpha-2-glycoprotein [40]. A recent study by Li et al. [41] reported that the up-regulated leucine-rich alpha-2-glycoprotein expression induced the en-

hancement of cell proliferation, migration, and invasion, and mediated a proangiogenic effect via the activation of the transforming growth factor β pathway. Chang et al. [42] identified eight differentially expressed proteins between lung AC and the control group and these included: fibrinogen beta chain, fibrinogen alpha chain, haptoglobin (Hp), apolipoprotein A-I, transthyretin, serotransferrin, Ig alpha-1 chain, and Ig alpha-2 chain. Of these, haptoglobin had the highest peak ratio. Furthermore, ROC curve analysis revealed that Hp may be used as a good biomarker of AC, especially for males (AUC was 0.929). The main function of Hp is to bind free plasma hemoglobin thus preventing iron loss. However, it was also reported that body iron could accumulate in cancer cells and promote neoplastic cell growth [43]. It has also been shown that Hp has angiogenic [44] and antioxidant properties [45] and plays an important role in cell migration, contributing to cancer progression. Furthermore, Hp acts as a potent immunoreactive modulator protecting tumors against the host's immunity, which may contribute to the immune escape of the tumor [46]. The study by Abdullah et al. [47] revealed extrahepatic expression and synthesis of haptoglobin in lung tumors, especially in ACs, when compared to healthy lung tissues. The proteomic analysis by Kang et al. [49] indicated that a change in the HP molecular structure may be related to tumorigenesis. Hp is a tetrameric protein composed of $\alpha 1$, $\alpha 2$, and β chain polypeptides in differing combinations, which are connected by disulfide bridges. It has been reported that various forms of Hp have different abilities to bind hemoglobin and different properties regarding inflammatory and angiogenic functions [48]. Among the three different chains of Hp, $\alpha 2$ and β chains presented differences in the expression in AC compared to healthy controls. However, only the Hp β chain showed a significant difference between lung AC and other tumors, such as breast cancer and hepatocellular cancers, as well as other respiratory diseases (tuberculosis, idiopathic pulmonary fibrosis, and bronchial asthma) [49]. These studies demonstrated the significant role of Hp in the development and progression of AC and indicated that the Hp β chain could be a potential serum biomarker for AC patients.

The comparative analysis of serum proteome profiles conducted by Ciereszko et al. [39] revealed a higher abundance of vitronectin (VN), coagulation factor XIII, plasminogen, and gelsolin in SCC patients compared to AC patients. Recently, VN has been reported to be a potent migration-en-

hancing factor and plays an important role in the movement of cancer cells to lymphatics and body cavities via the interaction with the uPAR receptor [50]. It may suggest that VN plays a role in spreading cancer cells in SCC patients. Gelsolin acts as a protective protein against apoptosis in NSCLC cells, which is mediated through the inactivation of PI3K/Akt signaling [51], which may contribute to tumor progression.

Stage-specific tumor biomarkers

Based on the Tumor-Node-Metastasis (TNM) system, NSCLC patients are classified into different stages of disease (stages IA1, IA2, IA3, IB, IIA, IIB, IIIA, IIIB, IIIC, IVA, and IVB) through the assessment of primary tumors (T descriptor), regional lymph node (LN) involvement (N descriptor), and occurrence of distant metastasis (M descriptor) [52]. Depending on the stage of NSCLC disease, the 60-month overall survival rate significantly decreases from 92–68% in patients with stage I disease, to less than 10% in patients with stage IV disease [53]. Therefore, it is extremely important to discover biomarkers in the early-stage of NSCLC development.

In an attempt to find new stage-specific tumor markers, Deng et al. [54] assessed differential protein expression patterns of lung squamous carcinoma tissue collected from patients at different pathological stages. Among all identified proteins, tropomyosin alpha-3 chain (TPM3) demonstrated a decrease in the expression level with malignant progression from stage I to stage IV, while the expression level of peroxiredoxin 1 (PRDX1) was significantly increased from stage I to III and had a slight decrease at stage IV. To the best of our knowledge, this is the only study demonstrating the role of TPM3 in NSCLC progression and requires further investigation. PRDX1 is a member of the redox-regulating protein family of peroxiredoxins and has antioxidant activity protecting against Reactive Oxygen Species damage, which is attributed to its cell survival enhancing function. The study by Chen et al. [55] on cell lines confirmed that PRDX1 I influences cell proliferation by regulating the cell cycle and enhances their metastatic properties by increasing the expression of bcl-2 and VEGF proteins, contributing to tumor progression.

In order to characterize protein expression reflecting clinical stages of individual patients with AC, Kawamura et al. [56] performed proteomic analysis and identified 81 proteins with significantly different expression levels in patients

with stage IA of disease compared to patients with stage IIIA of disease. Further, proteomic analyses by Nishimura et al. [57] demonstrated that napsin-A (NAPSA) expression was significantly reduced in patients with an advanced stage of disease. Furthermore, they found a negative correlation between the expression level of NAPSA and survival time after surgery. The study by Nishimura et al. [57] also revealed that the anterior gradient protein 2 homolog (hAG-2) was highly expressed in patients with stage IIIA in comparison to those with stage IA. The higher expression level of hAG-2 was also related to the development of regional lymph node metastasis. This data suggested that the assessment of the expression level of these proteins can be used to distinguish between early and advanced stages of AC.

Metastasis-specific tumor markers

The presence of metastasis in patients with NSCLC is the major factor which influences lower survival rates. Unfortunately, it is estimated that 30–50% of NSCLC patients present with metastatic disease at the time of diagnosis [58, 59] as part of the Monitoring of Cancer Incidence in Japan (MCIJ). A better understanding of the molecular mechanism that regulates the development of metastasis is needed; such research will also reveal biomarkers predicting the progress of NSCLC.

In terms of determining metastasis-specific tumor markers, Hsu et al. [60] performed a study in lung tissue from AC patients with different extents of lymph node involvement. Their study indicated that the ERO1-like protein alpha (ERO1L) and asparagine-tRNA ligase may have a significant impact on the development of metastasis in this type of cancer [60]. They also suggested that ERO1L overexpression in primary sites of early-stage tumor tissue indicated a high risk for cancer micrometastasis. Previous studies reported that the induction of ERO1L was the key adaptive response in the HIF-1-mediated pathway under hypoxia that operates to improve VEGF secretion, facilitating local tumor progression and the formation of distant metastases [61].

The proteomic study by Li et al. performed on lung squamous carcinoma tissue indicated that 14-3-3 sigma may be a potential lymph node metastasis-related biomarker in SSC patients. The expression of this protein was significantly down-regulated in lymph node metastatic tumors compared to primary SSC. This protein is involved in the negative regulation of cell cy-

cle progression and modulation of cell growth, differentiation, and apoptosis [63]. It has been shown that reducing the 14-3-3 sigma expression by siRNA silencing increased the in-vitro invasive ability of HTB-182 and A549 cells, while the enforced expression of ectopic 14-3-3 sigma decreased these abilities [62]. This data suggests that the downregulation of 14-3-3 sigma expression in tumor tissues may indicate an increased risk of developing lymph node metastases in SCC patients.

The proteomic approach has allowed for large-scale studies of protein expression in different tissues and body fluids which have been applied to discovering cancer biomarkers. This report reviews the major proteomic biomarkers which may be used to diagnose the development and progression of NSCLC.

Conflict of interest

None declared.

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