J. Chorostowska-Wynimko

MOLECULAR BIOLOGY METHODS IN THE DIAGNOSTICS OF LUNG CANCER

National Institute of Tuberculosis and Lung Diseases, Warsaw, Poland

Lung cancer is nowadays regarded as one of the key epidemiological issues in the world [1]. The number of newly diagnosed cases in Europe is estimated to be higher than 150 000 per year. In Poland prevalence of lung cancer has risen up to 26 500 newly-diagnosed cases/year representing 27,1 % of all newly-registered neoplasms [2]. Moreover, the standardized prevalence of lung cancer in Polish male population (65,5 per 100 000) is one of the highest in Europe. Meanwhile, many statistics show that only 10 % (5,5-14,3 %) of these patients survive their malignancy [2, 3].

Lung cancer is responsible for more than 33 % of cancer deaths in Poland and more than 1000000 per year in the world. In the USA, the number of deaths caused by lung cancer still exceeds the total number of deaths from breast, colon, prostate and cervical cancer [4]. One of the reasons blamed for such an evocative statistics is the fact that there are no established screening or early detection methods for this type of cancer, especially in the high-risk smoker population. Despite of the progress in the detection techniques for lung cancer, most patients are diagnosed in the late stage, 70 % in stage IIIB or IV, when due to the local tissue involvement or metastatic disease prognosis is poor. It is also acknowledged that survival time of early-diagnosed patients is significantly longer and exceeds 5 years for 80 % of stage I in comparison to 3 % for stage IV group [5]. Therefore, it is obvious that introduction of new screening and early diagnosis methods is nowadays of vital importance, promising to be the most effective way of improving treatment outcomes as well as reducing mortality.

Technical progress substantially increased our knowledge and understanding of the key role that particular genes play in the pathogenesis of lung cancer. Numerous studies confirmed that the modified expression of genes regulating main biological processes like cell cycle, differentiation, maturation, aging and apoptosis is of decisive significance [6]. It is acknowledged that unrestrained growth of tumor tissue results directly from the increased activity of oncogenes as well as down-regulated expression of the *tumor suppressor genes* (TSG) due to the genetic (mutations) or epigenetic (hyperexpression, methylation) modifications [7]. It is possible to effectively detect these alterations in human tissues, therefore some of them might serve as reliable diagnostic markers for cancer screening.

The main mechanisms regulating growth and invasiveness of tumor are reflected in the enhanced or changed expression of particular genes. For example, modified activity of the ERBB gene family encoding EGFR and HER2/Neu is responsible for the non-small-cell-lung-cancer (NSCLC) decreased requirement for the growth factors [6, 7]. Similarly, apoptosis that serves as a physiological mechanism regulating cells liveliness, especially in those with disrupted or abnormal DNA structure, was shown to be inhibited as a result of deregulated expression of p53 and bcl2 genes (respectively in 50 % and 30 % of NSCLC and more than 90 % of SCLC). Other typical alterations of gene expression result in resistance to paracrine growth regulation (loss of heterozygosity (LOH) p53, p16), increased angiogenic activity of cancer tissue (VEGF genes), up-regulated tumor cells replication (telomerase gene) as well as augmented ability to invade neighboring tissue and metastasize (laminin and integrin gene).

The number and type of modifications in gene expression parallels cancer development [8]. What's more, certain molecular markers seem to by characteristic for particular phases of tumor growth and metastasis formation, defining the transition form mild to moderate to severe atypia and subsequently to *carcinoma in situ (CIS)* and microinvasive carcinoma [9].

Early modifications (3p LOH, 9p21 LOH) are present as soon as minor lesions such as hyperplasia or dysplasia occur in the bronchial mucosa [10]. Some of them, mostly promoters metylation, have been observed also in normal mucosa of chronic smokers. More significant changes in biomarker expression are found in preneoplastic lesions, in dysplasia or *carcinoma in situ*. Late modifications, typical for invasive cancer, are more abundant and diverse from loss of genetic material (alleles), spontaneous or induced mutations to epigenetic modifications like genes hyperexpression or methylation. Smoking is particularly effective in inducing multiple genetic modifications in the airways. Active carcinogens present in the cigarette smoke directly interact with the k-ras, p53 and FHIT genes critical for the tumor development and induce the earliest carcinogenic modifications — DNA hipermethylation and deletions in the TSG genes. Chronic exposition to the cigarette smoke is also responsible for the accumulation of these modifications increasing therefore the probability of preneoplastic or neoplastic lesions occurrence in the bronchial mucosa. Thus, smoking cessation is rightly regarded as one of the most important methods of lung cancer prevention.

Epidemiological data clearly show that in general lung cancer is diagnosed at the late clinical stage when treatment effects are poor [5]. Therefore, it is believed that early diagnosis of lung cancer might be the only way to improve disease outcomes and patients survival. Thus, introduction of new highly efficient, reliable and specific diagnostic methods is of great importance for both patients and clinicians.

It was clearly shown in the 70-ies that screening and diagnostic programs based on the classical chest X-ray and sputum cytological evaluation do not alter lung cancer detectability and mortality rates (11). The poor sensitivity of both methods was considered the main problem responsible for the failure in providing sufficient diagnostic effectiveness. (Brambilla et al. have observed positive sputum cytology only in two per 1500 evaluated subjects. [12])

Therefore, new diagnostic tool low dose computer tomography (CT), characterized by the sensitivity four times higher than classical chest X-ray, promised to open new era in the diagnostics of lung cancer [13]. Preliminary reports of American and European researchers were very optimistic (2,7 % positive tests per 1000; 85 % in stage I) [14, 15]. However, high sensitivity considered the main advantage of low-dose CT was also responsible for considerable number of false positive results (61,5 %) [16, 17]. American Cancer Society guidelines and US Preventive Services Task Force statement emphasized fact that high sensitivity and low specificity of low-dose CT considerable increases the number of invasive diagnostic procedures, that are associated with higher risk of side effects, as well as both time- and funds consuming [18]. The most important are however psychological consequences of false positive diagnosis for patient and his family.

Another diagnostic method that might help to increase the detectability of early lung cancer is fluorescence bronchoscopy. 520 mm light wave rebounds from the pathological bronchial mucosa in slightly different way, so any abnormal lesions of the mucosa: severe dysplasia, *carcinoma in situ* and invasive tumor are clearly visible. Moreover, this method is characterized by considerably higher sensitivity (4,7 higher bronchial dysplasia and 2,3 lung cancer detectability) and average specificity (~55 %), due to the non-specific inflammatory (~55 %) autofluorescent reaction [12, 19]. Additionally, adequate lung tissue sampling is very difficult and its pathological interpretation might be complicated [12].

Fluorescent bronchoscopy is extremely efficient in identifying preneoplastic and carcinogenic lesions in the bronchial wall. However, numerous studies have shown that the most effective approach includes combination of few different diagnostic methods that usually is characterized by the considerable increase in combined tests specificity.

In this context, biomarkers are considered the promising new tool that combined with mentioned above methods might revolutionized the clinical approach to lung cancer diagnostics. Molecular biology techniques effectively estimate expression of particular, appointed genes in tumor cells, but also in other tissues like sputum, bronchoalveolar lavage (BAL) and serum/plasma. Therefore, efficiency of biomarker application in the real-life environment i.e. lung cancer diagnostics depends on the sensitivity and specificity of selected marker but also on the type of biological material used as its source [9, 20].

Sputum examination is a good example of advantages that biomarker examination offers, especially when combined with other diagnostic methods. One of the most promising biomolecular techniques is the nuclear image analysis based on stoichiometric, DNA-specific, nuclear staining with a Feulgen-Thionin reaction which results in a linear relation between the degree of stain and the amount of DNA [21]. The group of Palcic et all. analyzed malignancy-associated changes in non-malignant cells in sputum by means of semi-quantitative nuclear image analysis. Reported sensitivity in stage I lung cancers was 45 % while specificity 90 % in comparison to respectively 14 % and 90 % for standard Saccomano cytology method [22]. Recently, Marek et al. used automated quantitative modification of this method with sensitivity of 75 % and specificity of 98 % [23]. Other molecular techniques with possible potential for routine use for early lung cancer detection are — immunocytochemical staining with monoclonal antibodies against heterogeneous nuclear riboproteins hnRNP A2/B1 and hnRNP B1, DNA-methylation of certain DNA promoters ($p16^{INKA}$, MGMT) and FISH analysis (fluorescence in situ hybridization).

Although further validation studies are necessary before these technologies can be recommended for routine use, there are already some clinical data available proving their practical value. McWilliams et al. used automated quantitative sputum nuclear image analysis in association with low-dose CT scanning and fluorescence bronchoscopy to detect lung cancer [24]. They found 14 cancers in 423 subjects (3,3 %) from high risk group. 13 had abnormal sputa, nine of which had positive CT scans and 4 had CIS/microinvasive carcinoma found by fluorescence bronchoscopy. It should be emphasized that four out of 13 patients with lung cancer had negative results of CT scan. It clearly proves that dual screening in the era of high resolution CT provides additional benefits over CT scanning alone and strongly supports the concept that biomarker strategies might improve sensitivity of other methods, are cost-effective and incredibly helpful to patients from psychological point of view (preliminary screening prior to invasive or radiological methods). It is however to be remembered that very common low yield of sputum cytology as well as relatively time-consuming procedure of sputum induction (15—30 minutes) and specific condition for storage and transportation of samples are main technical problems that might seriously affect reproducibility of sputum analysis results and their usefulness for screening and early diagnostic purposes.

Apart from sputum other biological materials have been analyzed as a source of the biomarkers.

Several studies have proven that BAL although of "pulmonary" origin is of limited value for molecular markers analysis. As early as in 1999, Ahrendt et al. demonstrated that frequency of most typical disorders in p53, k-ras genes expression or p16 promoter methylation was significantly lower in BAL material than in NSCLC samples — respectively for all examined markers 53 % vs 100 %, for p53 gene 39 % vs 56 %, for k-ras gene 27 % vs 33 % and for p16 methylation 17 % vs 63 %) [25]. Quite recently, similar conclusions were reported by group that implemented very sensitive, high-tech method of real-time PCR for biomarkers assessment in BAL samples [26]. Modified expression of APC, RASSF1A, MGMT, GSTP1 genes as well as CDH1methylation observed in cancer cells were also seen in ~68 % of BAL material (at least one gene). However, it is not good enough to be considered a valuable diagnostic or screening tool.

While BAL material examination was disappointing, peripheral blood assessment as a reservoir of lung cancer biomarkers was surprisingly efficient [20]. Easily and cheaply accessible, also in the district outpatient clinics outside large hospital centers, with no need for additional personnel training to provide proper sampling, blood seems to be from technical point of view an ideal candidate material for the screening and early diagnostic programs. Therefore, extensive research projects are currently conducted in order to evaluate detailed diagnostic value of multiple biomarkers measured in the peripheral blood. There are nanogram amounts of free serum DNA in healthy subjects, as well as in patients with chronic inflammatory and autoimmune diseases [27]. However, free serum DNA concentration in lung cancer patients is in average four times higher [28]. It was hypothesized that free DNA origins from the tumor tissue undergoing necrosis/apoptosis processes or from circulating cancer cells [20]. Quantitative measurement of free DNA concentration in serum/plasma is considered as a highly promising and very cost-effective biomarker for screening and lung cancer detection. Elevated amounts of free DNA are observed at the early stages of lung tumor development [29]. Recently, Sozzi et al. using sensitive real-time PCR technique demonstrated very satisfactory sensitivity (90 %) and specificity (86 %) with positive predictive value of 90 % and negative predictive value of 90 % (30). DNA level in the plasma of lung cancer patients was 8-times higher than in controls (24,3 vs. 3.1 ng/ml), correspondingly relative risk of lung cancer was 85-times higher in subjects with high DNA concentration. Combination of free DNA assessment with other parameter(s) characterizing gene(s) modification(s) typical for lung cancer might prove to be extremely efficient in its diagnosis, similarly as it was demonstrated for ovarian cancer [31]. It is not yet certain what panel of markers, in what combination will have sufficient sensitivity and specificity to warrant approval for clinical use. The most frequently examined in plasma are early p53 mutations (in plasma of 73 % lung cancer patients), 3pLOH (47,5 %), as well as modified methylation of APC (*adenomatous polyposis coli*) (47 %), $p16^{INK4a}$ (55 %), DAPK (*death-associated protein kinase*) (40 %) and RASSF1A (*Ras association domain family 1a*) (31 %) genes.

Apart from lung cancer screening and diagnostics, molecular markers transpire as a new hope for improved disease prognosis in patients beginning or currently undergoing chemotherapy [32]. Similarly, it is believed that biomarkers might prove very helpful in early metastasis or disease recurrence detection [33]. The presence of structural mutations of p53 in the tumor cells and lower than usual expression of HIN-1 gene strongly correlates with poor survival of lung cancer patient. Similar, indicative effect has promoter methylation of certain genes, like for example APC. It should be mentioned however, that the best predictive value has been associated with markers evaluated directly in the cancer cells. However, free plasma DNA has been also shown to provide quite valuable information concerning possible disease recurrence or effectiveness of NSCLC surgical treatment. Successful radical tumor resection has for example resulted in significantly (3 times) lower concentration of plasma DNA than in non-surgically treated (7,1 vs 24,7 ng/ml) [29]. Other markers, like EGFR, Her2/Neu have also been very extensively researched.

In addition, molecular diagnostic methods might be very efficiently used for assessment of lung tumor susceptibility to chemotherapy. Presence of cysplatine adducts in the normal cells cytoplasm, as well as the decreased expression of ERCC1 or Ape1 genes seem to be reliable and relatively easy to estimate markers of cancer cells resistance to cytostatic drugs. Rosell et al. have proven that high expression of the RRM1 (ribonucleotide reductase responsible for the DNA synthesis and repair) closely corresponded with better outcome of surgical treatment, lower rate of subsequent tumor relapse and much prolonged patients survival time [34].

It should be also emphasized that research on the molecular biomarkers is closely related to the investigation of the new treatment modalities for lung cancer [35]. Many known biomarkers represent key mechanisms required for consecutive stages of tumor development, such as modified requirement for the growth factors or resistance to cell growth and apoptosis regulation. Better un-

derstanding of these mechanisms due to the intensive search for the reliable early stage biomarkers might significantly help in elaborating of new treatment concepts.

Another, new therapeutic option that might be successfully implemented in future is lung cancer chemoprevention [36]. Although at present neither of experimentally evaluated options (β-carotene, α-tocopherol, retinyl palmitate) proven effective, it should be expected that dynamic progress in molecular biology and cancer research will provide us with new more efficient compounds.

In summary, in spite of all efforts in the conventional diagnostics and therapy in the last decades, lung cancer survival has experienced only minor improvements. Recent developments in research on the molecular biology of cancer give hope that the major goal in the lung cancer treatment — the improvement of long-term survival might be truly achieved by means of new more sensitive molecular methods of screening and early tumor diagnosis, therapy effects and relapse occurrence assessment as well as of introducing new treatment and chemoprevention modalities.

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Summary

Lung cancer is the leading cause of cancer-related death throughout the world. The best prognosis can be expected by diagnosis at an early stage of this disease. Similarly, long-term survival may be improved by increasing the number of early-stage diagnoses. Over last decade, significant advances have been achieved in cancer molecular biology, including identification of genes critical for its growth and metastasizing, which formed basis for new screening and early diagnosis approaches. Number of studies produced intriguing results regarding the detection of biomarkers in tumor samples but also in easily accessible specimens such as sputa and plasma. Recent advances in these aspects of biomarker identification as well as their utility for predicting disease outcome including survival and response to chemotherapy are reviewed.

МОЛЕКУЛЯРНО-БИОЛОГИЧЕСКИЕ МЕТОДЫ ПРИ ДИАГНОСТИКЕ РАКА

Д. Хоростовска-Вынимко

Резюме

Во всем мире рак легких является ведущей причиной смерти от рака. Предполагается, что наилучшие прогностические результаты могут иметь место благодаря установлению диагноза на ранней стадии заболевания. Аналогично, уровни долговременного выживания также могут быть улучшены путем увеличения диагнозцирования на ранней стадии. За последнее десятилетие значительные успехи были достигнуты в области молекулярной биологии рака и, особенно в идентификации генов, играющих критическую роль в его росте и метастазировании. Все это создало базу для применения новых подходов к обследованию больных и ранней диагностике. В ряде работ были получены интересные результаты относительно обнаружения биомаркеров в раковых образцах, а также в легко доступных пробах, таких как мокроты и плазма. В настоящей работе проанализированы последние достижения в вопросах идентификации биомаркеров, а также их использования для предсказания исхода болезни, включая выживания и реакции на химиотерапию.