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From mutation to methylation – molecular markers in lung cancer

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Abstract

Lung cancer is one of the most common types of malignant neoplasms in Poland and in the world, and is associated with the worst prognosis. Within recent years, significant progress has been made when it comes to high-throughput and high-resolution technologies in the field of molecular biology, i.e. next-generation sequencing, microarrays, and mass spectrometry, which contributed to the development of research in the fields of genomics, transcriptomics and epigenetics in lung cancer. Implementing the results achieved by these disciplines quickly allows for the analysis of somatic point mutations or translocations to be used in molecular cancer diagnostics, and, thus, for the personalization of treatment for patients with non-small cell lung cancer (NSCLC). Unfortunately, certain markers and prognostic predictors that could facilitate the detection of lung cancer in its early stages are yet to be found.

Key words: molecular biomarkers, personalized oncology, molecular diagnostics.

Streszczenie

Rak płuca jest najczęstszym oraz najgorzej rokującym nowotworem złośliwym na świecie oraz w Polsce. Na świecie z jego powodu umiera ok. 2 mln osób rocznie, w Polsce odnotowuje się ok. 14,5 tys. zachorowań u mężczyzn, a u kobiet rak płuca zajmuje trzecie miejsce z ponad 6 tys. zachorowań. Przyczyną powstawania raka płuca u 85% mężczyzn i u 47% kobiet jest palenie papierosów. W ciągu ostatnich lat nastąpił ogromny postęp wysokozaawansowanych i wysokorozdzielczych technologii z zakresu biologii molekularnej, takich jak sekwencjonowanie nowej generacji, mikromacierze, spektrometria mas, co przyczyniło się do rozwoju badań z zakresu genomiki, transkryptomiki i epigenetyki w raku płuca. Coraz szybsze wdrażanie wyników tych dyscyplin pozwala na wykorzystanie analiz somatycznych mutacji punktowych czy translokacji do molekularnej diagnostyki onkologicznej, a tym samym na personalizację leczenia pacjentów z niedrobnokomórkowym rakiem płuca. Niestety, nadal brakuje zarówno markerów predykcyjnych, jak i prognostycznych, które pomogłyby w wykrywaniu raka płuca we wczesnym etapie rozwoju choroby.

Słowa kluczowe: markery molekularne, personalizowana onkologia, diagnostyka molekularna.

Introduction

Lung cancer is one of the most common types of malignant neoplasms in Poland and in the world, and is associated with the worst prognosis [1]. In Poland, lung cancer occupies the third place (after breast and colon cancer) with over 6 thousand female patients and the first place with 14.5 thousand male patients. Within recent years, significant progress has been made when it comes to high-throughput and high-resolution technologies in the field of molecular biology, i.e. next-generation sequencing, microarrays, and mass spectrometry, which contributed to the development of research in the fields of genomics, transcriptomics and epigenetics in lung cancer. Implementing the results achieved by these disciplines quickly allows for the analysis of somatic point mutations or translocations

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to be used in molecular cancer diagnostics, and, thus, for the personalization of treatment for patients with NSCLC. Unfortunately, certain markers and prognostic predictors that could facilitate the detection of lung cancer in its early stages are yet to be found.

Genomics

Genomics study the genome and deals with both the physical traits and the properties of the genome. Numerous studies are being carried out to identify the genetic changes related to the process of carcinogenesis. The results of genomic studies on lung cancer appear promising and may be applicable in clinical practice in the future. The identification of specific genetic changes in cancer tissue is not only a part of the diagnostic process, but is also an important factor in prognostication and "to tailor the treatment" [2]. For example Thomas et al. in their work on the molecular profile of lung cancer, underlined thirteen genes with translocations in *ALK*, *ROS1*, *KIF5B-RET* , amplifications in the *MET* and *FGFR1* genes and other mutations in following genes *EGFR*, *HER2*, *PI3K*, *AKT1*, *KRAS*, *BRAF*, *MEK*, *DDR2* [2].

Mutations in the gene coding the epidermal growth factor receptor are the best known mutations in non-small cell lung cancer (NSCLC), widely used in clinical practice. Deletions in exon 19 of the *EGFR* gene constitute one type of such mutations. These deletions most commonly affect 9, 12, 15, 18 or 24 nucleotides [3]. Some of these deletions share a common feature – they cause the removal of four amino acids in codons 747-750: leucine, arginine, glutamic acid, and alanine. The second most common mutation in the *EGFR* gene is substitution in exon 21, mainly L858R [3].

Identifying the above-mentioned somatic mutations provides significant clinical benefits. Patients with activating mutations in the EGFR tyrosine kinase domain respond very well to tyrosine kinase inhibitors, such as erlotinib or gefitinib. The latter ones inhibit EGFR phosphorylation, which prevents the division of neoplastic cells and leads to their death [4].

Taking into consideration the fact that mutations in exons 18, 19, 20, and 21 take place in about 10-13% of Caucasian NSCLC patients [5, 6], their therapeutic usefulness is significant. Further lung cancer research helped identify the *EML4-ALK* fusion gene. This gene appears in NSCLC cells as a result of inversion in the short arm of chromosome 2 [7]. Nine variants of the *EML4-ALK* fusion gene were identified. Each variant contains exons 20-29 of the *ALK* gene as well as fragments of the *EML4* gene. Depending on the variant, these fragments are: exon 13, exon 20, exon 6, exon 6 with the insertion of 11 additional amino acids, exon 2, exon 15, and exon 18. There are also two variants with exon 14 of *EML4*. In one of them, the beginning of the fusion takes place in nucleotide 13 of exon 20 of the *ALK* gene, while in the other, insertion of 11 additional nucleotides occurs in exon 20 of the *ALK* gene [8]. The resulting fusion gene is an oncogene with the ability to promote neoplastic development, which is related to its catalytic activity [7]. This characteristic is utilized in NSCLC therapy. It has been demonstrated that the use of specific inhibitors, blocking tyrosine phosphorylation in the *EML4-ALK* oncogene, inhibits the activity of tyrosine kinase in this fusion gene. This leads to the death of neoplastic cells in which the expression of this gene is present [7]. Last year, it was reported that other fusion genes can also be found in lung cancer: *KLC1-ALK*, *GOPC-ROS1* [9], *TPM3-ROS1*, *SDC4-ROS1*, *SLC34A2-ROS1*, *CD74-ROS1*, *EZR-ROS1*, *LRIG3-ROS1* [10], and *KIF5B-RET* [11]. The fusion between *KIF5B* and the *RET* proto-oncogene seems to be an interesting new molecular target in the personalization of diagnostics and the treatment of patients with NSCLC, and it was found in a non-smoking patient without mutations in the *EGFR*, *KRAS*, or *ALK* genes (triple-negative). Another patient with identified *KIF5B-RET* rearrangement (10p11.22-q11.21) was in the group of double-negative patients (without mutations in the *EGFR* and *ALK* genes). Since high expression of the tyrosine kinase domain of the *RET* gene was observed in only those lung cancer samples which contained the *KIF5B-RET* fusion gene, it seems that this fusion gene may potentially be a good new therapeutic target. However, more research needs to be conducted on the amplification of the *RET* gene and the assessment of the fusion of the *RET* gene with other genes [11].

The application of genomics in lung cancer molecular diagnostics

The above-mentioned mutation analyses constitute a good example of predictive biomarkers which are currently used in oncological molecular diagnostics. However, there are still no biomarkers that would enable quick and early detection of lung cancer.

The loss of heterozygosity in the 3p region may potentially represent such a biomarker. This type of deletion occurs in over 70% of NSCLC cases and over 90% of cases of small cell lung cancer. The change takes place very early on, before the structure of the epithelium is significantly affected. Mutations of the *TP53* gene are also present at the early stage of respiratory system changes. It is important to note that the diagnostic value of the molecular analysis is greatly enhanced when a combination of several mutation is evaluated [12]. Thus, the identification of mutations appearing in neoplastic tissue provides significant benefits, both in the diagnostics and the therapy of NSCLC. It is, therefore, important for the methods of assessing mutations in lung cancer to be quick, efficient, and accurate [13]. Depending on the type of mutation (point mutation, translocation), these conditions are met by the dideoxy and next-generation (NGS) sequencing methods, real-time polymerase chain reaction (PCR), fluorescence in situ hybridization (FISH), reverse transcriptase PCR, or genotyping with the use of mass spectrometry [43].

Transcriptomics

Detailed knowledge about the genomescale gene expression profile in thousands of clincal samples were ob-

tained by high throughput microarray technology. It allows precise molecular classification of neoplasms and making more reliable disease development predictions, stratifying patients, as well as developing and implementing new therapies directed at specific targets characteristic for different types of neoplasms.

One of the first evaluation of NSCLC transcripts was performed using a microarray. This study compared the gene expression between normal and malignant lung tissue samples in various stages. This study enabled the identification of 50 genes whose differences in expression allow for the classification of patients with first stage NSCLC into groups with higher and lower chances of survival. That 50 genes signature includes genes related to apoptosis (*CASP4*, *P63*), cell structure and adhesion (*KRT7*, *LAMB1*), cell cycle and growth regulation (*BMP2*, *GAPD*, *CDC6*, *STX1A*), and cell signaling (*ADM*, *AKAP12*, *ARHE*, *GRB7*, *VEGF*), as well as genes coding chaperones (*HSPA8*), receptors (*ERBB2*, *FXYD3*, *SLC20A1*), enzymes (*CSTB*, *CYP24*, *FUT3*, *MLN64*, *SLC1A6*), and proteins related to transcription and translation (*COPEB*, *CRK*, *RELA*) [15].

Another study, also employing microarrays, has enabled the identification of 133 genes, whose expression profile performs the function of a predictive signature, allowing for the prediction of disease recurrence risk in patients at the early stages of NSCLC [16, 17]. The signature of these genes is especially helpful in making decisions concerning the necessity of implementing supplementary chemotherapy in patients with stage 1A tumors after resection. During the first stage of the experiment, a predictive model was created with 93% accuracy, while the accuracy of the predictive model based on clinical data was only 64%. During the second stage, two validation studies were conducted, the predictive accuracy of which was 72% and 79%, respectively [16]. Further research on the signature helped to identify 5 genes whose level of expression was correlated with the overall survival time and recurrence-free survival time of patients with NSCLC: *DUSP6*, *MMD*, *STAT1*, *ERBB3*, and *LCK* [18]. Signatures of high and low risk were identified based on the expression of these five genes. Patients with high risk signature exhibited median overall survival of 20 months, while patients with low risk signatures had median overall survival of 40 months. Moreover, the high risk signature was related to shorter median recurrence-free survival in comparison to the low risk signature (13 months and 29 months, respectively) [18].

The published results of research on gene expression in primary lung tumors samples (adenocarcinoma and squamous cell carcinoma), performed using microarray platforms (Affymetrix), helped identify 91 genes which may serve as survival indicators [19]. Selected genes play a rolein different biological processes, the main ones including: the communication of signals, regulation of transcription, cell cycle, adhesion and proliferation of cells. Some of these genes have previously been identified as predictive markers: *DUSP6* and *ERBB3* [18], *SLC2A1* and *MEF2C*, *AKAP12*, *CYP24A1*, *CSTL*, *SLC2A1*, *GAPD* [15, 19].

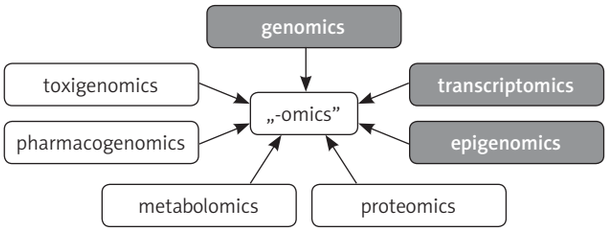

Fig. 1. List of molecular disciplines involving research on lung cancer with the use of high-throughput technologies. The disciplines marked in grey are described in detail in this article

Guo *et al.* proposed a 35-gene signature whose prognostic value enables the stratification of stage 1A NSCLC patients into groups of high and low risk of cancer recurrence [20]. Moreover, this signature was independent of clinical prognostic criteria. No significant relations were found between the level of expression of the 35 studied genes and the age of the patients (over 60 years), tumor differentiation or gender. What is more, it was demonstrated that the 35-gene signature had better prognostic value than other signatures previously used in lung cancer, including the 5-gene signature proposed by Chen *et al.* and the 133-gene signature proposed by Potti *et al.* In this study, the 35-gene signature was validated as an independent prognostic factor for NSCLC [20].

Another example of a molecular signature in NSCLC is the three-gene prognostic classifier. It is based on the expression of mRNA genes: *STX1A*, *HIF1A*, and *CCR7*. Verification of the usefulness of the classifier demonstrated that it enables the stratification of patients into groups of various prognosis, and that it is more effective in establishing prognosis than assessment based on histopathological examination [21]. The expression analysis of five genes – *ERCC1*, *BAG-1*, *BRCA1*, *RRM1*, and *TUBB3* – showed that the ERCC1 and BAG-1 signatures provide both prognostic and predictive information [22]. Patients with reduced ERCC1 expression exhibited longer overall survival by over 21 months and progression-free survival longer by over 27 months. Meanwhile, patients with lowered BAG-1 expression exhibited longer overall survival by over 25 months and progression-free survival longer by over 29 months. Moreover, patients with negative expression of both ERCC1 and BAG-1 responded better to therapy combined with cisplatin or carboplatin [22].

The latest transcriptomic trends utilizes next-generation sequencing technologies (RNA-seq) [23]. Expression profiles of mRNA acquired from bronchoscopy material were compared between healthy smokers and healthy individuals who had never smoked, as well as between healthy smokers and smokers suffering from lung cancer. Smokers exhibited increased expression of the genes related to the xenobiotic metabolic pathway including cytochrome P450, retinol metabolism and oxidoreductase activity. In smokers with lung cancer, the increased expression concerned the genes related to the chemokine signaling pathway and to the interaction of cytokine-cytokine receptors,

as well as the genes coding cell adhesion molecules. Apart from RNA-seq, gene expression was additionally assessed using the microarray and qRT-PCR methods. However, only the RNA-seq technique allowed for the identification of additional transcripts whose changes in expression were correlated with smoking: S100A8 and S100A9 (in the group of healthy non-smokers and healthy smokers), as well as of those correlated with the occurrence of lung cancer: MUC5AC and SCGB3A1 (in the group of smokers with and without lung cancer) [23]. Recent studies put also attention to importance of the tumor microenvironment for cancer development, progression, and ability to metastasize. One of the conducted experiments compared the gene expression in a primary culture of fibroblasts acquired from an NSCLC tumor stroma and in normal fibroblasts collected from the same patient [24]. Thanks to microarray analysis, 46 genes involved in the TNF signaling pathway were identified; the expression of these genes was different in the normal fibroblasts than in the fibroblasts associated with the tumor. Repeated validation of the microarray technology and the correlation of expression results with patient survival helped narrow down the prognostic signature to 11 genes: *ICAM-1*, *THBS2*, *MME*, *OXTR*, *B3GALT2*, *EVI2B*, *MCTP2*, the expression of which increased, and *PDE3B*, *CLU*, *COL14A1*, *GAL*, the expression of which was reduced. In the next step of this experiment, a data-mining comparison of cancer-associated fibroblasts (CAFs) and normal fibroblasts (NFs) as well as NSCLC tumor's stroma stroma and normal lung tissue allowed to create 14-gene classifier. Expression level increased in the *COL11A1*, *MFAP5*, *SULF1*, *ITGA11*, *THBS2*, and *CTHRC1* genes, and decreased in the *GPR126*, *TMOD1*, *PDE3B*, *CCDC102B*, *IGSF10*, *CLU*, *FLRT3*, and *A2M* genes. This study confirms the prognostic function of gene expression changes in fibroblasts from the tumor microenvironment [24].

The application of transcriptomics in lung cancer molecular diagnostics

High throughput technologies (gene expression microarrays and RNA-seq) open new possibilities in biomarkers discovering. However, the high cost of research and inaccessible equipment in clinical centers [20] severely limit the use of this technology at the moment and prevent its application in routine diagnostic tests [13]. Moreover, at each stage of experiment there are many factors which may influence analysis results and cause them to be difficult to replicate [25]. In this situation, combining the real time-PCR technique with microarray analysis to analyze genome-scale gene expression seems to be an optimal solution [20].

Epigenetics

Although each cell of an organism has the same set of genes, their expression differs in individual types of tissue, which allows the cells to specialize in different specific functions. The modifications the DNA of a cell is subjected to, aimed at changing the way the DNA is decoded, include DNA methylation, covalent posttranslational modification of histones (e.g. acetylation, methylation, phosphorylation, ubiquitination), and changes in miRNA. Abnormalities in the heredity of epigenetic markers may cause abnormal activation or inhibition of various signaling pathways participating in cell growth regulation, cell differentiation, transformation, as well as apoptosis, and may lead to diseases, such as cancer [26, 27].

Cytosine methylation in CG dinucleotides has a significant influence on gene expression. DNA methyltransferases are the enzymes that create and maintain methylation patterns in cells. They transfer a methyl group onto the fifth carbon atom in a pyrimidine ring of cytosine, creating 5-methylcytosine. In the initiation and progression of a neoplasm, DNA methylation plays significant role: DNA methylation in promoter regions or in the first exons of suppressor genes, causes the silencing of their transcription [28], while hypermethylation of intergenic regions and introns of proto-oncogenes affects chromosome instability [29]. What is more, these modifications are often related to changes in DNA methyltransferase (*DNMT*) expression [30].

Recent studies on lung cancer points to the diversification of the occurrence frequency of increased suppressor gene promoter methylation in individual types of neoplasms, which may be applicable for diagnostics purpose. The usefulness of the research increases when specific methylation pattern is evaluated for neoplastic cells and normal lung cells. The comparison of the methylation patterns of *IMP4*, *GATA4*, *SOX18*, and *EGFL7* in the neoplastic tissue of NSCLC patients, the tissue surrounding the tumor, and normal lung tissue showed that the first three genes exhibit excessive methylation in neoplastic cells and in the normal tissue surrounding the tumor. This may point to the early initiation of epigenetic processes, which precede the subsequent genetic and morphological changes leading to the appearance of neoplastically transformed cells [31]. In previous research, a set of genes was defined in which the increase in promoter sequence methylation was characteristic mainly of neoplastic tissue samples. Methylation of the following genes was identified: *RASSF1A* (44% in neoplastic tissue/0% in normal tissue), *p16* (47%/14%), *DLC1* (61%/25%) [32]. Moreover, an increase in *RASSF1A* methylation was observed in the material obtained from patients who were long-term smokers [32]. *RASSF1A* inactivation is associated with progression of the neoplasm and worsening of prognosis. Other suppressor genes characterized by increased methylation of promoter regions in lung cancer are: *FHIT* [28], *CDH13* [33, 34], *KLK10* [33], *EFEMP1* [33], *SFRP1* [33], *RARβ* [33, 34], *APC* [33], and *DAPK* [35]. Silencing these genes causes a decrease in the amount of synthesized protein to a level insufficient for normal cell cycle regulation [36].

Changes in the expression of DNA methyltransferases mainly consist in their increased transcription, which leads to the heightening of these enzymes' activity in neoplastic cells and may be related to the hastening of lung cancer

development and the worsening of prognosis [30]. A correlation was found between *DNMT1* and *DNMT3b* overexpression and the increased methylation of suppressor gene promoters, especially in smokers. This may result from methyltransferases combining with protein 2, which binds methylated CpG (MeCP2). This protein prevents transcription factors from binding to a promoter [37].

Modifications of histones are also conducive to the faster development of the disease and lower probability of survival. Di-methylation of 4 lysine and acetylation of 18 lysine in histone H3 may serve as examples. An increase in the overall histone modification level points to higher aggressiveness of the neoplasm; significant similarities of these relations were found in adenocarcinomas acquired from various types of epithelial tissue, including prostate, lung, and renal cancer [38].

The application of epigenetics in lung cancer molecular diagnostics

Epigenetic changes taking place in neoplastic cells can be detected at the early stage of the disease; therefore, they may be used for screening and diagnostic examinations in the future. Moreover, these changes are not permanently inscribed in the genome and it is possible to reverse or modify them. The currently used biomarker is the assessment of MGMT gene methylation in gliomas; unfortunately, the use of epigenetics in lung cancer is still in the research phase and is not yet employed in routine molecular diagnostics. For example, the research concerning miR-29s, the expression of which is inversely correlated with *DNMT3A* and *-3B* in lung cancer, indicated that restoring the high expression of miR-29s normalizes methylation pattern deviations and inhibits *in vitro* and *in vivo* development of neoplasms [39]. This confirmed the role of miR-29s in the epigenetic normalization of NSCLC and in the development of a new potential strategy for lung cancer treatment [39].

The examination of easily obtainable samples is of great importance in cancer diagnostics. Many scientific studies use fresh neoplastic tissue, usually acquired during surgery or biopsy. The use of blood, saliva, or sputum is less invasive and more beneficial for screening purposes; therefore, many studies follow this direction in the search for biological markers in body fluids. A decrease in the expression of the *p16/INK4* gene, related to the increased methylation of its promoter, can be observed in non-small cell lung cancer [40]. In the aforementioned study, the hypermethylation levels of *p16/INK4a* were compared in the DNA isolated from sputum and bronchial lavage fluid acquired from long-term smokers and lung cancer patients. The cancer patients exhibited an increase in methylation (51%), but this tendency was also found in smokers (28%). The examination of the methylation levels of promoter genes *CDKN2A*, *CDH1*, and *MGMT* in patients with chronic obstructive lung disease and patients with lung cancer, where the used material was sputum, showed an increase in the methylation of CDKN2A and *MGMT* in both patient groups. *CDH1* methylation increased in patients with lung cancer only [41].

Future directions for research and molecular diagnostics

One of the latest trends in genomics, transcriptomics, and epigenetics is the analysis of patient molecular profiles using data mining and correlating these results with the patients' response to particular types of treatment. This research contributed to the development of targeted therapies and personalized medicine [42]. The next step was the use of high-throughput technologies in molecular cancer diagnostics, which allows for the analysis of larger numbers of genes at lower examination costs [13]. For example, the LungCarta™ Panel (Sequenom, Inc.) enables the analysis of 214 somatic mutations in 26 genes in DNA isolated from lung cancer tissue, while OncoDEEP (ONCODNA SA) uses next-generation sequencing, providing an initial analysis of 40 genes or a more detailed analysis of up to 406 genes. The above-mentioned analyses not only further the understanding of the molecular changes taking place in cancer tissue, but also provide key molecular information when selecting a personalized, "made-to-measure" therapy for a patient.

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