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The role of genetic and other biomarkers in NSCLC prognosis

Mini-Review

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Abstract: The development of non-small-cell lung cancer (NSCLC) is a multistep process, which is triggered and maintained by various factors. Many steps of non-small-cell lung carcinogenesis, risk factors and biomarkers have been identified; however no consistent model has been established of personalized medicine for these patients. Distinct various gene expression, products of mutated genes and other markers such as circulating nucleic acids or tumor cells has been proven to be potential biomarkers of non-small cell lung cancer as well as potential targets for new treatment strategies. This article will highlight promising biomarkers in non-small cell lung cancer prognosis.

Keywords: *Non-small cell lung cancer • Biomarkers • Prognosis*

1. Introduction

Primary lung cancer is a frequent and lethal disease worldwide. The high morbidity and mortality are influenced by low sensitivity and specificity of current screening methods such as low-dose computed tomography (CT) scans and the delayed treatment of advanced tumors. Therefore, more often new molecular biomarkers help to describe the non-small cell lung cancer in both predictive and prognostic ways.

Lung cancer development is a multi-stage process, which is influenced by many factors – environmental, molecular and genetic. Various molecular changes occur in primary lung cancer cells during proliferation, differentiation or apoptosis processes. Mutations in the genes of tumor cells affect not only local and distant spread of malignant cells, but also influence resistance to chemotherapy or radiation therapy. To date there are many genes associated with lung tumors, but it is essential to identify their expression rates, determine the frequency of mutations and clinical significance (for example association with smoking, histological type, disease stage, survival rate or response to treatment). It is expected that molecular studies in both healthy and malignant cells will help to identify new biomarkers for lung cancer that would allow clinicians to diagnose the disease quicker, to select more specific treatment and predict the effectiveness of treatment.

In addition to molecular genetic biomarkers, circulating tumor cells and circulating nucleic acids that originate from tumor are being explored. This group of biomarkers has a potential to improve early detection of non-small cell lung cancer as well as monitoring disease recurrence or responses to anticancer therapy. This review presents most promising prognostic biomarkers for non-small cell lung cancer.

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2. Most frequently inactivated tumor suppressor genes

There is a number of tumor suppressor genes (TSGs) which when inactivated become the reason of tumorigenesis or are disabled during cancer progression. Gene *p53* and its encoded protein p53, has been identified as crucial TSG in nearly all cells of the human body. This gene is involved in lung cancer pathogenesis. Studies show that abnormal *p53* status can be associated with poorer survival of NSCLC patients [1]. It must be noted, that EGFR positive NSCLC patients with *p53* mutations treated with *erlotinib* had shorter overall survival compared with patients without *p53* mutations (15 months *vs.* 31 months, p=0.04) [2].

MDM2 (murine double minute 2) binds with the transactivation domain of *p53*, induce its nuclear export and mark it for proteosomal degradation by ubiquintination. This permits the division of unstressed cells. However *MDM2* overexpression reduces the quantity of available p53 protein, enabling damaged cells to escape the cell cycle control [3,4]. *MDM2* upregulation is seen much more frequently in NSCLC. *MDM2* gene promoter polymorphisms have been implicated in reducing or increasing susceptibility to lung cancer. There are controversial reports on *MDM2* T309G polymorphism and its association with lung cancer. Pine *et al.* [5] in their study showed that *MDM2* T309G is not a major factor in lung carcinogenesis, whereas Zhuo *et al.* [6] in their study confirmed that the same polymorphism might be a risk factor for lung cancer among never-smokers. Nonetheless, MDM2 inhibitors seem to have some promising effect in treatment of NSCLC. It has been reported that MDM2 inhibitor-219 (MI-219) selectively inhibits growth of wild-type *p53* containing lung cancer cells [7].

$p16^{INK4A}$ (also known as *p16*) is a cyclin-dependent kinase (CDK) inhibitor, which is found to be aberrant in up to 58% of NSCLC. Inactivation of $p16^{INK4A}$ most frequently occurs through homozygous deletions or aberrant promoter methylation and is an early event in carcinogenesis. It has been suggested that $p16^{INK4A}$ detection assays might be used to ascertain cancer risk in selected patients and serve as biomarker of earliest stages of cancer development [8,9]. In NSCLC patients with stage I and II higher expression of $p16^{INK4A}$ has been associated with unfavorable outcomes [10].

3. Epidermal growth factor receptor (*EGFR*)

EGFR is a transmembrane receptor that has tyrosine kinase activity and upon activation takes part in cellular mechanisms responsible for cell proliferation. EGFR is activated by its growth factor ligands, which are found in platelets, macrophages and other cells. In tumor cells EGFR activity is disregulated by oncogenic mechanisms, including *EGFR* gene mutations, increased gene copy number and EGFR protein overexpression [11,12]. *EGFR* is frequently mutated protooncogene. *EGFR* mutations in NSCLC patients are identified in 10-15 % of European population and in 20-50 % of East Asian population [13]. The most often mutations of this gene are in exons 18, 19 and leucin to arginin substitution (L858R) in exon 21 [11,12]. These changes result in a gene product which after activation influences uncontrolled cell proliferation. It must be noted that mutations are more common in women than in men (42% *vs.* 14%), in patients who have never smoked than in patients who have smoked (51% *vs.* 10%), and in patients with NSCLC adenocarcinoma than in those with other histology (40% *vs.* 3%) [12].

Furthermore, *EGFR* is an important biomarker of response to new agents targeting this protein. *Erlotinib* and g*efitinib* are the first generation tyrosine kinase inhibitors (TKIs) that have been proven to be efficient in treatment of NSCLC patients with *EGFR* mutations in tumors. SATURN trial showed a greater benefit from e*rlotinib* therapy for NSCLC patients with *EGFR* mutation-positive tumors [14]. The NEJ 003 trial demonstrated the effectiveness of g*efitinib* as first line treatment of NSCLC patients with *EGFR* mutations in tumors and who are older than 75 years [15]. Similar results have been showed in IPASS trial. NSCLC patients with *EGFR* mutations in tumors who were treated with g*efitinib* had longer progression free survival compared with patients without *EGFR* mutations [16].

Unfortunately development of resistance to TKIs is a common finding in NSCLC patients despite the occurrence of drug-sensitive activating mutations. Most of patients who initially respond to TKIs will develop acquired resistance to it [17]. The secondary mutation T790M in exon 20 of *EGFR* gene accounts for nearly a half of all resistance cases. Second generation TKIs (for example, *BIBW 2992* (Torok), *neratinib* (HKI-272)) bring hope of overcoming this setback, by their ability to irreversibly bind a cysteine residue in position 797 in *EGFR* gene, that enables to inhibit *EGFR* activity even in the presence of T790M mutation [12,17,18].

4. Echinoderm microtubule-associated protein-like 4 with anaplastic lymphoma kinase (*EML4-ALK*)

Reciprocal translocations occasionally result in the creation of fusion proteins that may drive the process of carcinogenesis. An example of such proteins is *EML4-ALK* that occurs most commonly in non-small cell lung cancer, with majority of adenocarcinomas. *EML4-ALK* positive tumors likely represent an independent clinical subgroup of NSCLC patients. The median age of these patients are 54, usually with more advanced NSCLC and with never- or light smoking history [19, 20]. It has been reported that overall survival of these patients is longer compared with patients without *EML4-ALK* expression in tumors (14.7 *vs.* 10.3 months, p=0.009) [21].

The discovery of *EML4-ALK* raised the possibility of more effective treatment of these patients by inhibiting the kinase activity of ALK (anaplastic lymphoma kinase), because patients harboring *EML4-ALK* tend to be resistant to EGFR-TKIs, such as e*rlotinib* or ge*fitinib*, since *EML4-ALK* and *EGFR* mutations are mutually exclusive. Results from the first ALK-targeted phase I clinical trial with c*rizotinib* (orally available, small-molecule inhibitor) has shown a clinical benefit of NSCLC patients with stages III or IV (n=149). 61% of patients had an objective response (complete or partial) and median progression-free survival was almost 10 months (95% CI 7.7-12.8). In this trial 1-year survival rate was 75 %. It must be noted that usually 1-year survival rate for advanced NSCLC is lower than 50% [22]. There are few ongoing trials of c*rizotinib* versus chemotherapy (PROFILE 1007, PROFILE 1014), targeting ALK, and the results of these studies may be promising.

ALK fusion genes represent a new molecular target in the treatment of NSCLC patients, making it the second breakthrough in the treatment of lung cancer patients after the *EGFR* mutations.

5. DNA repair genes. Excision repair cross-complementing 1 (*ERCC1*), ribonucleotide reductase M1 (*RRM1*)

DNA repair genes *ERCC1* and *RRM1* are involved in lung carcinogenesis by influencing DNA repair capacity. *ERCC1* is an enzyme involved in DNA damage recognition and DNA strand incision. *ERCC1* has been investigated both as a prognostic and predictive marker in NSCLC patients [23]. The data concerning the correlation between *ERCC1* expression in tumor and NSCLC patients survival is controversial. Some studies reported that *ERCC1*-positive patients have longer survival than *ERCC1*-negative patients, while other studies showed no significant differences between *ERCC1* expression and survival [24,25].

As a predictive marker in treatment with platinum-based chemotherapy it has been shown that patients with *ERRC1*-negative tumors receiving chemotherapy have longer survival (HR=0.65; 95% CI 0.50-0.86; p=0.002) than patients with *ERRC1*-positive tumors [24].

RRM1 is an enzyme of DNA synthesis, which takes part in conversion of ribonucleoside diphosphates into deoxyribonucleoside diphosphates, the elements of DNA synthesis and repair [23]. Zheng et al. [26] in their study reported that median overall survival is longer for I stage NSCLC patients with high levels of *RRM1* comparing with those patients with low levels of *RRM1* (more than 120 vs. 60.2 months; p=0.02). *RRM1* may contribute to resistance of platinum-based therapy in combination with *gemcitabine*. NSCLC patients with low *RRM1* expression in peripheral blood or tumor showed better response to *gemcitabine* plus *platinum* chemotherapy than patients with high expression (50.0% *vs.* 16.0%; p=0.012), longer median survival (18.5 months *vs.* 13.0 months; p=0.043) and longer progression-free survival (6.0 months *vs.* 4.0 months; p=0.044) [23].

Another study, MADeIT, was conducted in the means of "personalized therapy" based on *ERCC1* and *RRM1* expression. Chemotherapy was assigned for the patients with advanced NSCLC after *ERCC1* and *RRM1* expression measurement in pretreatment biopsy. According to gene expression 4 possible therapy variants were composed: low *RRM1* and low *ERCC1* group received *gemcitabine* and *carboplatin* treatment; low *RRM1* and high *ERCC1* group – *gemcitabine* and *docetaxel*; high *RRM1* and low *ERCC1* group – *docetaxel* and *carboplatin*; high *RRM1* and high *ERCC1* group – *vinorelbine* and *docetaxel*. Although initial data were promising, the final results did not show any advantage in terms of progression free survival or overall survival in tailored chemotherapy group compared to the control group. Despite these results, more studies need to be done to confirm *ERCC1* and *RRM1* clinical significance [27].

6. Kirsten-rous avian sarcoma (*KRAS*)

KRAS oncogene is involved in cellular growth, differentiation and apoptosis. Mutations in *KRAS* gene are found in up to 30% of NSCLCs cases, mostly in

adenocarcinomas and are associated with tobacco smoking history [28]. It has been suggested that *KRAS* mutations could be a negative prognostic factor of overall survival in NSCLC patients, but the data from several studies are conflicting. Guan *et al.* [29] showed shorter overall survival for the NSCLC patients with mutated *KRAS* comparing with patients with wild-type *KRAS* (15.2 months *vs.* 21.3 months, p=0.027). NSCLC patients with early (I-IIIA) and advanced (IIIB-IV) disease stages with mutated *KRAS* had shorter overall survival comparing with wild-type *KRAS* subgroups (28.43 and 7.77 months vs. 75.93 and 12.40 months; early stage p=0.031, advanced stage p=0.039). No association has been demonstrated in other studies between *KRAS* mutation status and survival [30].

It was reported that mutated *KRAS* could serve as predictive marker for NSCLC patients receiving chemotherapy. The phase III TRIBUTE trial reported that patients with mutated *KRAS* tumors and treated with *carboplatin* and *paclitaxel* plus *erlotinib* had shorter time to progression (3.4 months; 95% CI 1.5-6.3) than patients with mutated *KRAS* tumors and treated with *carboplatin* and *paclitaxel* alone (6 months; 95% CI 4.9-7.1) [31]. On the other hand, SATURN trial found no statistically significant differences between *erlotinib* or *cetuximab* therapy in patients with *KRAS* mutations or with wild-type *KRAS* [32].

Overexpression of *KRAS* oncoprotein has been recently observed as a potential target for a new strategy in cancer therapy, namely – vaccination. Vaccination of cancer patients requires a target which would be present exclusively in cancer cells. Carbone *et al.* [33] demonstrated prolonged survival of cancer patients (including lung cancer), which were immunized with cellular vaccine containing mutant p53 and *KRAS*-derived peptides.

Although it wasn't proven that *KRAS* is a good target for the therapy, several new agents were introduced to be effective for NSCLC. Molecular chaperone Hsp90 (heat shock protein 90) inhibitor *ganetespib* has been shown to have some efficacy in *KRAS* positive NSCLC cell lines [34]. Another agent – a RAS/RAF pathway inhibitor *sorafenib* – was also proven in a phase II study to be beneficial in terms of progression-free survival and overall survival in NSCLC patients with progressive disease after at least one platinum-based chemotherapy cycle [35].

Also, selective inhibitors of MEK1 and MEK2 have been tested clinically. MEK proteins (also known as MAPK or mitogen-activated protein kinase) participate in the Ras/Raf/MEK/ERK signalling pathway. *Selumetinib* is potent and selective inhibitor of MEK1 and MEK2. Results from randomized, phase II study showed that NSCLC patients with *KRAS* mutated tumors and treated with *selumetinib* had longer progression free survival that patients from *placebo* group (p=0.014). It must be noted that overall survival was also longer in *selumetinib* group, but it wasn't proved to be statistically significant [36].

7. MicroRNAs (miRNAs)

MicroRNAs are small non-coding, single-stranded RNAs composed of 17-22 nucleotides that regulate gene expression and are involved in carcinogenesis. A single miRNA can affect multiple protein coding genes, while a gene can be targeted by more than one miRNA [37]. Cancer-related miRNAs can be both oncogenes and tumor suppressors. For example, there are suggestions that miR-451 acts as tumor suppressor and regulates survival of NSCLC cells through downregulation of ras-related protein 14 (RAB14) [38]; miR-196a may be an oncogene and is associated with proliferation and invasion of NSCLC cells [39].

Current literature data indicate that miRNAs can be useful in overall prognosis of NSCLC patients. Serum miR-125b high expression compared with low expression significantly correlates with poor NSCLC patients survival (18 months *vs.* 26 months, p<0.0001) [40]. Low let-7b and miR-126 expression correlate with shorter progression-free survival of NSCLC patients compared with high expression of mentioned microRNAs (respectively, 84 days *vs.* 243 days, p<0.0001; 66 days *vs.* 243 days, p<0.0001) [41]. Expression levels of miR-16 correlated with disease-free and overall survival. Disease-free survival was shorter for NSCLC patients with high expression compared with patients with low expression (22.4 months *vs.* 55.8 months, p=0.05). Similar results are shown for the overall survival (23.9 months *vs.* 63.5 months, p<0.001) [42].

Yu *el al.* [43] identified five-microRNAs (miR-221, let-7a, miR-137, miR-372, miR-182) which could serve as disease-free and overall survival biomarkers of NSCLC patients. The five-microRNAs risk score was calculated using risk-score formula. NSCLC patients with high-risk five-microRNA set had shorter overall survival and disease-free survival compared with patients with low-risk microRNA set (20 months *vs.* not reached, p<0.001; 10 months *vs.* not reached, p=0.002 respectively).

There is a growing interest in an association between miRNAs and chemo-sensitivity. Cisplatin is commonly used cytotoxic drug in NSCLC treatment and it could cause DNA damage, which is repaired by various DNA repair pathways. Little is known when and how

miRNAs are involved in the regulation of DNA damage repair [44,45]. Sensitivity to cisplatin has been linked to miR-181a expression, while resistance – to miR-630 expression in NSCLC cell line A549 [37,46]. Plasma miR-21 expression may correlate with sensitivity to chemotherapy too. NSCLC patients who underwent 2 to 3 cycles of platinum-based therapy and reached partial response or stable disease effect had higher miR-21 expression compared with patients who had progression of the disease after treatment (p=0.049) [47].

To date it has been demonstrated that miRNAs may be a prognostic NSCLC biomarkers. miRNAs show promising results in association of their expression with prognosis in NSCLC, however the true prognostic value of miRNA is yet to be evaluated.

8. Circulating tumor DNA

An increased circulating DNA concentration in serum or plasma is thought to originate from cancer cells through such processes as apoptosis, necrosis or circulating tumor cells lysis [48,49]. Higher circulating DNA levels are identified in NSCLC patients with disease progression compared with NSCLC patients without disease progression (110.5 ng/ml vs. 82.6 ng/ml; p<0.001) [50].

A number of studies have examined relationship between circulating DNA and its clinical significance for NSCLC patients' prognosis. Results show that a high circulating DNA concentration correlates with poor survival of NSCLC patients compared with low DNA concentration (16.8 months *vs.* 22.4 months; p=0.02) [49]. van der Drift *et al.* [48] in their study demonstrated that overall survival for the NSCLC patients with circulating DNA concentration ≥32 ng/ml was significantly shorter compared to NSCLC patients with lower circulating DNA concentration (11.8 months *vs.* 21.5 months; p=0.03). A DNA cut-off level of >32 ng/ml differentiated with a specificity of 52% and sensitivity of 67%.

Measurement of circulating tumor DNA offers the possibility to follow patients after tumor resection in monitoring disease recurrence. Ludovini *et al.* [51] showed an increase of circulating DNA concentration in NSCLC patients with proven disease relapse compared with disease-free NSCLC patients after 3 months from surgery (32.8 ng/ml vs. 292.7 ng/ml; p=0.0016).

Regarding predictive value of circulating DNA for NSCLC patients' response to treatment is contradictory. Some authors have reported no significant correlations in pre-treatment circulating DNA levels between NSCLC patients responders and non-responders to platinum-based chemotherapy (p=0.09) [50]. Whereas other authors reported significant association between increasing circulating DNA concentrations and tumor progression after chemotherapy with cisplatin or carboplatin and taxanes (p=0.006) [52].

Analysis of circulating DNA in blood with a simple blood test is a promising biomarker. In NSCLC patients elevated circulating DNA concentrations may be prognostic factors, whereas more studies must be done to confirm it as predictive factors.

9. Circulating tumor cells (CTCs)

CTCs disseminate from the primary tumor through the circulatory system and some of them are capable to form distant metastasis. Identification and characterization of CTCs are often named as non-invasive "liquid biopsy", which could represent phenotype and genotype of the primary tumor and/or distant metastases. The CTCs are detectable in blood and they are rare cells even in patients with advanced cancer (~1-100 CTCs per 10^9 blood cells) [53,54].

Several studies have shown that CTCs may be considered as prognostic markers for NSCLC patients. Among NSCLC patients CTCs could be a negative prognostic indicator depending on number of detectable CTCs along with tumor progression. Krebs *et al.* [55] in their study demonstrated that an increasing number of CTCs can be detected in NSCLC patients with more advanced disease (from 0 to 146 CTCs for stage IV patients and from 0 to 3 CTCs for stage IIIB patients). Progression-free survival was 6.8 months for patients with detected less than 5 CTCs in blood compared with 2.4 months for patients with more than 5 CTCs detected in blood (p<0.001). Nieva *et al.* [56] also reported that higher numbers of detected CTCs in blood were associated with an unfavorable prognosis for NSCLC patients with metastatic disease. Time to death was shorter for patients with more than 5 detectable CTCs compared with those patients who had 5 or less CTCs (35±59 days *vs.* 211±207 days, p=0.003).

In consideration of predictive value, CTCs can help in monitoring of disease recurrence and responses to anticancer therapy. Analysis after one cycle of standard cytotoxic therapy showed longer progression-free survival for NSCLC patients with fewer than 5 CTCs compared with patients with more than 5 CTCs (6.9 months *vs.* 2.4 months, p=0.005) [55].

Available technologies for CTCs detection have enabled to analyze various genes expression on individual CTC. It was demonstrated that an increasing *ERCC1* expression in CTCs was associated with significantly shorter progression-free survival compared with decreased *ERCC1* expression in CTCs (266 days *vs.* 172

days, $p < 0.02$) in NSCLC patients with advanced disease stage and receiving platinum-based therapy [57].

Advanced NSCLC lacks reliable validated biomarkers and CTCs may be useful for this purpose as they could indicate metastatic spread or presence of clinically undetectable micro-metastases. Results from several studies demonstrate that CTCs can be a biomarker of disease progression indicating an early metastatic spread as well as guides of treatment decisions. It must be noted that "anti-CTC" therapy may be a future strategy in preventing of metastases formation [53].

10. Conclusions

Individual biomarkers may be applied by various aspects–assessing the risk of cancer, disease diagnosis and prognosis, response to treatment, individual treatment, and so on. Notably, a lot of new biomarkers were clarified in recent decade, but in order to control the disease, it must be a systematic approach to the application of these biomarkers. For example, a biomarker which is suitable to evaluate cancer risk cannot be used for disease prognosis. It is necessary to select the most informative biomarkers for each stage of the disease. Identification and characterization of new biomarkers will lead to new treatment decisions and personalization of NSCLC patients.

Conflict of interest statement

Authors state no conflict of interest.

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