Review Article

Molecular testing in lung cancer in the era of precision medicine

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Abstract: The clinical expectations how pathologists should submit lung cancer diagnosis have changed dramatically. Until mid 90-ties a clear separation between small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC) was mostly sufficient. With the invention of antiangiogenic treatment a differentiation between squamous and non-squamous NSCLC was requested. When epidermal growth factor receptor (EGFR) mutation was detected in patients with pulmonary adenocarcinomas and subsequent specific treatment with tyrosine kinase inhibitors (TKIs) was invented, sub-classification of NSCLC and molecular analysis of the tumor tissue for mutations was asked for. Pathologists no longer submit just a diagnosis, but instead are involved in a multidisciplinary team for lung cancer patient management. After EGFR several other driver genes such as echinoderm microtubule associated protein like 4-AL-Kinase 1 (EML4-ALK1), c-ros oncogene 1 , receptor tyrosine kinase (ROS1), discoidin domain receptor tyrosine kinase 2 (DDR2), fibroblast growth factor receptor 1 (FGFR1) were discovered, and more to come. Due to new developments in bronchology (EUS, EBUS) the amount of tissue submitted for diagnosis and molecular analysis is decreasing, however, the genes to be analyzed are increasing. Many of these driver gene aberrations are inversions or translocations and thus require FISH analysis. Each of these analyses requires a certain amount of tumor cells or one to two tissue sections from an already limited amount of tissues or cells. In this respect new genetic test systems have been introduced such as next generation sequencing, which enables not only to detect multiple mutations in different genes, but also amplifications and fusion genes. As soon as these methods have been validated for routine molecular analysis this will enable the analysis of multiple genetic changes simultaneously. In this review we will focus on genetic aberrations in NSCLC, resistance to new target therapies, and also to methodological requirements for a meaningful evaluation of lung cancer tissue and cells.

Keywords: Non-small cell lung carcinoma (NSCLC); molecular pathology; target (driver) genes; tissue based assessment

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Introduction

Within the last decade many important discoveries were made in the regulation of growth, differentiation, apoptosis, and metastasis of lung cancers. These findings have dramatically changed the view of the oncology community about the importance of the classification of lung carcinomas. With the findings of different responses for cisplatin treatment in adenocarcinomas versus squamous cell carcinomas (SCCs) this simple clinical lung carcinoma classification schema small cell lung carcinoma (SCLC) versus non-small cell lung carcinoma (NSCLC) was abolished. In addition, results of recent research show even the category of adenocarcinoma is in fact a heterogeneous group of different tumors with a broad spectrum of molecular changes. The chance of targeting at least some of

the mutations by currently available treatment thus requires much more precise classification of lung tumors based not solely on morphology, but including even detection of various molecular predictive markers.

Therapy relevant molecular changes in pulmonary carcinomas

NSCLC and angiogenesis

In the last decade humanized antibodies have been developed to interfere with the neoangiogenesis in primary as well as metastatic carcinomas (1,2). However, anti-angiogenetic drugs can cause severe bleeding, especially when administered in patients with centrally located NSCLC. However, it is still not clear, if the reported bleeding episodes in these patients are due to the squamous histology or more logically to the central located tumors, which are usually supported by arteries and veins arising from large branches. In addition, it was reported that cavitation within the tumor is prone to hemorrhage, again something more common in central tumors located close to large blood vessels (3). The erroneous perception of oncologists about SCCs most probably is due to the fact that SCCs arise predominantly in central bronchi.

Angiogenesis, better neoangiogenesis is a process by which primary tumors get access to nutrients and oxygen and is characterized by the sprouting of endothelial cells from the preexisting vessels (in contrast to vasculogenesis, which is the process of growth of the vessels de novo—e.g., during embryonic development). The process of neoangiogenesis is still not fully understood. Under normal circumstances endothelial cells are virtually quiescent, therefore a crucial requirement for neoangiogenesis is their stimulation to proliferation by angiogenic factors, such as vascular endothelial growth factors (VEGFs). In some cases are these factors produced by the tumor cells themselves, in other cases are these growth factors produced by elements of the immune system, such as macrophages present in the tumor microenvironment (4). However, once new blood vessels (capillaries, small arteries, veins) are formed, this provides advantage for the tumor cells over their normal neighbor cells in getting better oxygen and nutrient supply. Nutrients and oxygen are not the only important factor for rapid growth, also purine and pyrimidine bases are essential for a dividing tumor cell (5,6). Increased angiogenesis itself in invasive adenocarcinomas has a negative impact on survival and progression of disease in these patients (7).

Angiogenesis is essential for the primary tumor as well as for metastasis. The secretion of VEGFs facilitates most often neoangiogenesis. Tumor blood vessels are immature, with incomplete basement membrane, fragile, and are therefore prone to rupture. Using antibodies against VEGF (bevacizumab) the angiogenesis can be inhibited and regression of the tumor is induced. However, in some cases, mostly in centrally located tumors can this therapy result in severe hemorrhage.

New developments are focusing on the inhibition of the VEGF receptors (VEGFRs) and also on the role of hypoxia inducible factor (HIF) and hypoxia in tumor development and metastasis. In several studies the importance of VEGF and VEGFR axis was stated for vascular invasion and metastasis, mainly involving VEGF-C and VEGFR3 (7-10). Studies aiming to target this axis showed positive results in experimental settings (11-13). Bringing these targeted therapies into clinical trials is still in its infancy (14). A major problem in targeting VEGF-VEGFR is the fact that its regulation is under the major influence of the hypoxia pathway. Hypoxia is an important factor in invasion and angioinvasion, and HIF1-signaling will result in the upregulation of VEGF (15,16). So the hypoxia pathway might constantly overrule a blockade of VEGF-VEGFR unless also HIF1 production is inhibited (17). In addition, several other independent pathways regulate the angiogenesis and thus blocking of just one of them is sooner or later bypassed by another one resulting in resistance and failure of the anti-angiogenic treatment.

NSCLC and cisplatin drugs, the effect of anti-apoptotic signaling

In a large multi-institutional study the effect of cisplatin chemotherapy was investigated. High expression of deoxyribonucleic acid (DNA) repair enzymes, especially excision repair cross complementation group 1 (ERCC1) was found to be responsible for failure of cisplatin chemotherapy and this expression correlated predominantly with squamous cell histology (18). ERCC1 is part of the excision repair machinery involved in the repair of damaged DNA. In NSCLC showing a high expression of this enzyme, the action of cisplatin-based chemotherapeutics is inefficient, most probably because DNA damage induced by the drug is immediately repaired. In a subsequent report the usefulness of ERCC1 immunohistochemistry failed, probably because the antibody clone did not pick up the relevant splice variant of ERCC1. Therefore the authors suggested using messenger ribonucleic acid (mRNA)

quantification instead.

Thymidilate synthase (TS) blocker

Pemetrexed is an inhibitor of TS less for the other enzymes in the thymidine cycle. Thymidine uptake is essential for rapidly dividing carcinoma cells. In tumors with low expression of TS pemetrexed can block the enzyme resulting in growth inhibition. TS expression most often is low in adenocarcinomas, but is highly expressed in many SCCs. Thus pemetrexed is efficient in most adenocarcinomas and not in SCCs (19). However, the action of pemetrexed is still not entirely clear: thymidylate metabolism does not only rely on enzymes of the thymidylate cycle, but also needs active and passive uptake mechanisms; and thymidine uptake might also be influenced by pemetrexed (20).

Receptor tyrosine kinases (RTKs) in lung carcinomas

RTKs are membrane-bound protein receptor composed of an extracellular receptor domain, a transmembrane spanning portion, and an internal (intracellular) domain, which at its C-terminal end contains the kinase domain. The external receptor domain has a specific configuration for the binding of growth factors. Such stimulation results in dimerization of the receptor, where two molecules form either homo- or heterodimer. This specific binding changes the configuration of the whole receptor and leads to the phosphorylation and activation of the kinase domain. There are two ways of activation of RTKs in lung cancer: overproduction of ligands either by the tumor cell or by cells within the microenvironment, such as macrophages; or activation by a mutation of the receptor gene, most often within the kinase domain. The receptor kinase itself can act also in two different ways: one is transfer of phosphorylation to transfer molecules (21,22), like GAB1 or Grb2; or the kinase splits into fragments, where one activated protein fragment translocates into the nucleus and binds to specific DNA elements and induces transcription of downstream proteins (23). In lung cancer RTKs can be constantly activated by different mechanisms: amplification of the RTK gene, mutations of the RTK gene, gene rearrangements (translocation/inversion) with constant activation or inactivation of regulatory proteins. Another mechanism is downregulation of regulatory proteins by microRNAs (miRNAs), so a tumor suppressor or a negative feedback protein is not synthesized because of mRNA inactivation by miRNA (24-29).

Adenocarcinomas

Adenocarcinomas in highly industrialized countries are the most common lung carcinoma, representing up to 40% of all lung carcinomas. In addition what was previously regarded as a single entity has become a huge diversity of carcinomas. Adenocarcinomas in never-smokers most probably represent a separate entity with different etiology, pathogenesis, and gene signatures and a slower progression rate compared to adenocarcinomas in smokers. Also recent studies of gene signatures have contributed to a more heterogeneous picture of these neoplasms. Morphologically adenocarcinomas can show a variety of patterns, which in part correlate with gene signatures, although our knowledge in this respect is still in its infancy.

Adenocarcinoma is defined by the formation of papillary, micropapillary, cribriform, acinar, and solid structures, the latter with mucin synthesis-mucin-containing vacuoles in at least 10% of the tumor cells. Adenocarcinomas can be either mucinous or non-mucinous. Both will show the above-mentioned patterns. Some rare variants are fetal, colloid, and enteric adenocarcinomas. Most often a mixed pattern is seen with a predominance of at least one component.

Tumor cells in adenocarcinomas can show differentiations along well-known cell types as Clara cells, pneumocytes type II, columnar cells, and goblet cells. Due to the importance of targeted therapy the exact classification of adenocarcinomas and their differentiation from other NSCLC has become a major task in pulmonary pathology. Differentiation factors are used to prove the nature of the carcinoma especially in poorly differentiated tumors. A variety of useful markers have been tested, the most important ones are thyroid transcription factor-1 (TTF1), cytokeratin 7 and Napsin A.

Epidermal growth factor receptor (EGFR)

In 2004, an EGFR mutation was detected in a patient with lung adenocarcinoma and responded to tyrosine kinase inhibitor (TKI) treatment—a new era of targeted therapy in NSCLC has started (30,31).

Mutation of EGFR has been detected in a small percentage of lung cancer patients in the Caucasian population. These are activating mutations found in exons 18, 19, 20, and 21 of the EGFR gene (kinase domain) (32). Mutations are most often found in never smokers, females, and in patients with adenocarcinoma histology. Mutations change the configuration of the kinase, which does not need anymore the ligand-based activation from the receptor domain. The receptor stays in an activated stage and constantly signals

downstream. Proliferation of neoplastic cells in carcinomas with this activating mutation can be inhibited by small receptor TKIs such as gefitinib, erlotinib, and afatinib. These TKIs bind either reversibly or irreversibly into the adenosine triphosphate (ATP) pocket of the mutated EGFR kinase domain and thus inhibit phosphor-transfer to downstream molecules, thus blocking the signaling cascade (33). The most common mutations are deletions within exon 19 with a variation of 9-18 nucleotides, and a point mutation at exon 21 (L858R). Other less common mutations are point mutations in exon 18, and insertions in exon 20.

However, mainly within exon 20 there are also resistance mutations, the best known is T790M. This type of mutation inhibits or reverses the binding of the TKIs gefitinib and erlotinib and prevents the receptor blockade. The occurrence of T790M is most frequently associated with previous TKI treatment. This mutation can be present in the tumor cells already before the treatment initiation and becomes detectable as a result of clonal selection (overgrowth of resistant cell population) or it originates de novo. The irreversible TKI afatinib might overrule some of these resistance mutations, but more data are needed to prove this (34).

Treatment response with TKIs is best in exon19 deletions, followed by exon21 point mutation. Mutations within exon 18 and 20 are less responsive (35).

For targeted therapy with TKIs tissue samples of NSCLC have to be analyzed for these mutations. Within the different subtypes of adenocarcinomas some will show a higher percentage of EGFR mutations, whereas others not. In Caucasian population adenocarcinomas with acinar or papillary pattern are mutated in up to 27%, whereas mucinous adenocarcinomas are constantly negative for EGFR mutations (and show KRAS mutation instead). Carcinomas with biphasic morphology such as adenosquamous carcinomas and mixed small cell and adenocarcinomas can show mutations but usually in a very small percentage of cases.

Another therapy approach was tested with humanized monoclonal antibodies for EGF. By competitive binding to the receptor, this antibody replaces EGF and thus inhibits transactivation of the kinase. This type of therapy seems to be especially promising in EGFR-naïve (wild-type) adenocarcinomas and in addition also in SCCs (36,37).

Echinoderm microtubule associated protein like 4-AL-Kinase 1 (EML4-ALK1) and additional fusion partners

Inversion of the ALK1 kinase gene and fusion with the EML4 gene has been recently shown in patients with NSCLC, especially in solid adenocarcinomas with focal differentiation into signet ring cells. Subsequently other patterns have been associated with this type of gene rearrangement, such as micropapillary. Both genes are on chromosome 2; the chromosomal break is inversely rearranged whereby the kinase domain of ALK and EML4 are fused together. The ALK kinase thus is under the control of EML4, which results in a constant activation of the kinase. ALK similarly to EGFR stimulates proliferation and inhibits apoptosis. Patients with this inversion respond excellently to crizotinib treatment, which is now the second example of targeted therapy in NSCLC (38). Proof of EML4ALK1 inversion can be done with different methods: the most common is FISH where two probes (3' and 5') detecting the ALK gene on both sides of the breakpoint are used. In the normal situation these probes will detect the two portions close together or overlapping within the tumor nucleus (resulting in fused FISH signal). In cases of rearrangement, the probes will highlight each of the splitted portions of the ALK1 gene, so instead of two overlapping signals the signals split apart. In the Caucasian population EML4ALK1 rearrangement is usually found in 4-6% of NSCLC; in adenocarcinomas this might be increased to 8%.

Other genes joining the ALK1 gene in the same way can replace the EML4 gene. If kinesin family member 5B (KIF5B) joins to ALK1, the overexpression of KIF5B-ALK (27) in mammalian cells led to the activation of signal transducer and activator of transcription 3 (STAT3) and protein kinase B and enhanced cell proliferation, migration, and invasion (27). Another fusion partner recently described is ALK-KLC1 (39). These other ALK1 fusions are rare; the incidence is about 1%.

C-ros oncogene 1, receptor tyrosine kinase (ROS1)

ROS1 is another kinase involved as a driver gene in adenocarcinomas of the lung (40). Usually the rearrangement of ROS1 is evaluated by two FISH probes for the 3'- and the 5'- ends. Only few fusion partners have been identified so far, CD74, SLC34A2, EZR, and GOPC/FIG (41,42). This gene rearrangement has no influence on outcome, but similar to ALK1 this is usually a younger population of cancer patients (43). The incidence of ROS1 rearrangement is in the range of 1%. The function of one of the fusion genes EZR-ROS was studied in a mouse model and showed that in this experimental setting the fusion gene acted as an oncogene inducing multiple tumor nodules in mice (44). Most important patients with this type of gene aberrations

responded well to the ALK1 inhibitor crizotinib (45-47).

KIF5B and ret proto-oncogene , receptor tyrosine kinase (RET)

KIF5B is one of the fusion partners for either ALK1 or RET. The KIF5B-RET fusion gene is caused by a pericentric inversion of 10p11.22-q11.21. This fusion gene overexpresses chimeric RET RTK, which can spontaneously induce cellular transformation (48). Besides KIF5B, CCDC6, and NCOA4 can form fusion genes with RET. Patients with lung adenocarcinomas with RET fusion gene have more poorly differentiated tumors, are younger, and more often never-smokers. Solid adenocarcinomas predominate, tumors are smaller but lymph node involvement is higher. The incidence of RET fusion is about in 1% of NSCLCs and almost 2% of adenocarcinomas (48-50).

Met proto-oncogene, receptor tyrosine kinase (MET)

MET is another RTK bound to cell membranes in NSCLC. The ligand for MET is hepatocyte growth factor (HGF), originally found in hepatic carcinomas. This receptor came into consideration in NSCLC because amplification of MET or alternatively upregulation of HGF was identified as a mechanism of the resistance in EGFR mutated adenocarcinomas treated by TKI (25,51). A search for the role of MET in other NSCLC excluding EGFR mutated adenocarcinomas showed, that MET amplification was a rare event, but upregulation of MET is relatively common: approximately 20% of NSCLC including adenocarcinomas and SCCs showed high protein expression, but only 2% MET amplification (Popper *et al*. in preparation). Clinical studies are in progress to evaluate the possibility to interfere with MET signaling using monoclonal antibodies. Other studies use small molecule inhibitors for MET. Since MET expression is common in EGFR mutated adenocarcinomas some studies aim to inhibit both EGFR and MET signaling pathways (52). In a phase III trial the combination of EGFR TKI and MET inhibition failed, most probably because the cut-off levels were not properly set (personal experience and Popper *et al*. in preparation).

Squamous cell carcinomas (SCCs)

SCC is defined by a plate-like layering of cells, keratinization of at least single cells, intercellular gaps and bridges (represented by desmosomes and hemidesmosomes), and expression of high molecular weight cytokeratins (CK 3/5, 13/14). There are some morphologic variants as small cell and baseloid SCC, but these have not been associated with specific gene signatures and therefore are only important in diagnostics.

The incidence of SCC has dropped in the last three decades from a major entity representing 35% of lung carcinomas to around 17%. One of the major reasons is the shift from filter-less to filter cigarettes. This has resulted in the reduction of particle-bound carcinogens and increase of vaporized carcinogens, which more easily reach the bronchioloalveolar terminal unit, inducing mainly adenocarcinomas.

In the past, SCC was mainly a diagnosis required to exclude several therapeutic options in the clinic: no pemetrexed therapy, no antiangiogenic drugs, less responsiveness to cisplatin treatment. However, this has changed within the last 3 years, as there are several emerging new targets for treatment of SCC.

Fibroblast growth factor receptor 1 (FGFR1)

FGFR1 was identified being amplified in about 20% of SCCs (53) [M. Sharp *et al*., Poster presentation, American Association for Cancer Research (AACR) meeting 2011]. In experimental studies as well as in ongoing clinical trials it was found that only amplification, proven by *in-situ* hybridization methods identified patients, who respond to small molecule inhibitor treatment (54). In subsequent trials the FGFR1-TKI therapy failed despite amplification: it became clear recently that there are additional genetic changes in some of these patients, specifically CA-PI3K mutations or amplifications. So in future the tumor in these patients will require analysis for several genes.

Discoidin domain receptor tyrosine kinase 2 (DDR2) and FGFR2

DDR2 and FGFR2 mutations are found exclusively in SCCs, however, only in a small percentage, 4% and 2%, respectively (55). In DDR2 mutated SCC patients some TKIs were successfully applied (56,57). For FGFR2 multikinase inhibitors might be an option for specific treatment (58,59).

Large cell carcinoma (LCC)

LCC is defined by large cells (nuclei >25 μm) devoid of any cytoplasmic differentiation, and large vesicular nuclei. They have a well-ordered solid structure. By electron microscopy differentiation structures can be seen such

as hemidesmosomes, tight junctions, intracytoplasmic vacuoles with microvilli, and ill-formed cilia. This fits clearly into the concept of a carcinoma, at the doorstep of adenocarcinoma and SCC differentiation. LCC numbers have dramatically decreased due to the routine use of immunohistochemistry for more precise sub-classification of NSCLC. Using TTF1, low-molecular cytokeratins, as well as p63 and cytokeratin 5/6 most cases of LCC were either reclassified into adenocarcinoma or SCC, respectively (60). These recent changes make an evaluation of genetic aberrations in LCC quite difficult, since genetic studies were based on previous classifications.

Not surprisingly EGFR mutations, MET amplifications, and EML4ALK1 fusions have been reported in LCC (61). LKB1, a gene mutated in a small percentage of adenocarcinomas was also shown in squamous and large cell carcinomas (62). LKB1, also known as serine/threonine kinase 11 (STK11), is involved in the negative regulation of mechanistic target of rapamycin (mTOR) and closely cooperates with tuberous sclerosis gene (TSC) 1 and 2 genes (63).

Resistance mechanisms

There are general classes of resistance mechanisms to TKI therapy. The target can be altered by a secondary inhibitory mutation or by amplification. The second class is a bypass track, by which the blocked TK is circumvented. Finally the tumor may undergo phenotypic and genotypic changes, which makes TKI-therapy inefficient.

The most frequent resistance mechanisms for EGFR are inhibitory mutations on exons 20 and 19. The most common ones on exon 20 are D770_N771 insertions (up to 3%) and the mutations T790M, V769L, N771T, and the D761Y mutation on exon 19 (64-66). Several of these mutations might be targeted by second and third generation TKIs (67). A common bypass track in EGFR mutated adenocarcinomas is amplification of the MET receptor (64,68,69). A third mechanism is a phenotypic change of the tumor. A transition from adenocarcinoma to small cell carcinoma has been reported. Also re-biopsies have shown a transition from a well-differentiated adenocarcinoma to an undifferentiated carcinoma (57,70-72). Concomitant to this phenotypic change also genotypic changes are seen: a SCLC no longer presents with EGFR mutation but will respond to classical chemotherapy. In transgenic mice an upregulation of pS6 might explain some of these phenomena. Two new resistance mechanisms have been reported on a recent poster session: methylation of PTEN promoter region caused a deactivation of PTEN (similar to PTEN loss) and subsequent upregulation of PI3K-AKT pathway. The second resistance mechanism was an aberrant signaling of EGFR into SRC kinases, thus circumventing the effect of EGFR blockade by TKI (Izumi *et al.*, ERS Congress Munich, Sep. 6th, 2014).

Resistance mechanisms in EML4ALK rearranged lung adenocarcinomas do exist, however, the exact mechanisms are still under investigation (73,74). Most common are secondary mutations in the ALK domain. Most common are L1196M and G1269A, less common are 1151Tins, L1152R, C1156Y, F1174L, G1202R, and S1206Y (75-77). Again bypass mechanisms do occur such as MET activation, but also ALK amplification. Interestingly second and third generation ALK inhibitors can target most of the secondary mutations. However, also these new generation ALK inhibitors will induce secondary resistance mutations, for which new drugs have to be designed (78,79).

Similar to EGFR and EML4ALK also for ROS1, KIF5B, and RET secondary mutations have been reported (80,81). For MET this can be expected, but so far treatment has just started with MET inhibitors.

Resistance mechanisms for FGFR1 inhibition are still not exactly known. The major problem in this setting of SCCs is complicated, because response to treatment might be dictated by the mode of FGFR1 modification in the carcinoma: mutation, amplification, deletion, and/or multiple alterations. In lung SCCs the prevalent alterations are amplification and mutation (53,82). This has largely been ignored, therefore the outcome and response has to be reevaluated. Using TKIs for FGFR1 some carcinomas responded quite well, whereas others not. Another problem in FGFR1 amplified pulmonary SCCs is the coincidence of FGFR1 amplification with PI3K mutations and amplifications (82). These new findings have to taken into account, before resistance mechanisms can be further explored.

Treatment for DDR2 and FGFR2 mutations has been applied in few patients. A resistance mutation has already been shown in cell culture studies using cell lines with DDR2 mutation (83). So far this has not been seen in patients.

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