


## MINI REVIEW

# Oncogene addiction and tumor mutational burden in non-small-cell lung cancer: Clinical significance and limitations

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Thoracic Cancer **11** (2020) 205–215**Abstract**

Lung cancer incidence has increased worldwide over the past decades, with non-small cell lung cancer (NSCLC) accounting for the vast majority (85%) of lung cancer specimens. It is estimated that lung cancer causes about 1.7 million global deaths per year worldwide. Multiple trials have been carried out, with the aim of finding new effective treatment options. Lately, special focus has been placed on immune checkpoint (PD1/PD-L1) inhibitors which impact the tumor immune microenvironment. Tumor mutational burden (TMB) has been found to predict response to immune checkpoint inhibitors. Conversely, recent studies have weakened the significance of TMB as a predictor of response to therapy and survival. In this review article, we discuss the significance of TMB, as well as possible limitations. Furthermore, we give a concise overview of mutations frequently found in NSCLC, and discuss the significance of oncogene addiction in lung cancer as an essential driver of tumorigenesis and tumor progression.

**Key points**

1. Tumor mutational burden (TMB) predicts response to immune checkpoint inhibitors
2. Recent studies have shown, however, that TMB has significant limitations
3. Oncogene addicted cancers are driven by one predominant driver mutation
4. Targeted agents are used as first-line treatment in certain types of NSCLC

**Introduction**

Non-small cell lung cancer (NSCLC) constitutes approximately 85% of lung cancer specimens. Increasing incidence rates of lung cancer have been observed in recent years worldwide, rendering this disease the second most common newly diagnosed cancer in men, and the fourth most common in women.<sup>1–4</sup> Throughout the last few decades, lung cancer has been the most fatal of all cancers,<sup>5</sup> accounting for most cancer-related deaths in both males and females, leading to 1.7 million global deaths per year.<sup>2</sup> Worldwide, the age-standardized incidence-to-mortality rates are 1.14/100 000 in men and 1.23/100 000 in women.<sup>6</sup> It is estimated that nowadays 12.9% of all new cancer diagnoses is lung cancer.<sup>6</sup> Mortality ranges between 25%–30%, and late diagnosis due to oligosymptomatic presentation is common.<sup>7,8</sup> Hence, five-years survival rates range from

16%–20% on average, with around 73% in stage IA disease to 13% in stage IV disease.<sup>9</sup> In the last three decades, numerous trials have been conducted across the globe, focusing on the establishment of new treatment options for NSCLC, and several promising results have emerged.<sup>10,11</sup> The field of prognostic biomarkers in lung cancer, especially to determine the effectiveness of various targeted treatment options, is quickly evolving.<sup>10</sup> Tumoral immune microenvironment is a major component of solid cancers. Thus, in NSCLC cytotoxic T CD8+ lymphocyte density in association with the immunologic microenvironment, ie. tertiary lymphoid structures, impacts survival. Research has recently focused on targeting these tumor-surrounding immune cells by PD1/PD-L1 inhibitors. Immune checkpoint inhibitors have proven especially useful in advanced NSCLC so far, showing better effectiveness than second-line chemotherapy.<sup>12</sup> Pembrolizumab, for instance, has

outperformed first-line chemotherapy in NSCLC with strong positivity for PD-L1.<sup>13</sup> In recent years, more and more personalized treatment options for NSCLC have been developed as an add-on to existing conventional chemotherapies, and thereby median overall survival has increased significantly within the past decade,<sup>14–16</sup> ie. from 12.4 months for patients diagnosed in 2004–2009 to 14.8 months for patients diagnosed in 2010–2013 ( $P < 0.001$ ), according to the recent literature.<sup>17</sup>

With this review of the literature we aim to give an overview on oncogene addiction and mutational burden. The association of oncogene addicted NSCLC and a high TMB with survival and response to therapy is discussed in-depth. Nowadays it is assumed that all cancers are “oncogene addicted,” meaning that a single driver mutation is the primary cause of tumor progression and metastasis. However, the primary driver mutation is not always known. According to recent investigations, TMB also influences prognosis in patients whose tumors harbor classical driver mutations, such as *EGFR*-mutations.<sup>18</sup> For instance, it was found that *EGFR*-mutant lung cancers featured lower overall TMB as compared to *EGFR*-wild-type cancers. High TMB in *EGFR*-mutant lung cancers was linked to a significantly shorter overall survival in patients treated with *EGFR*-TKIs.<sup>18</sup> This finding stands in contrast to what is observed for TMB and treatment with immune checkpoint inhibitors. A meta-analysis including the Embase, PubMed and Cochrane library databases, found that high TMB predicts better efficacy of immunotherapy in several cancer specimens.<sup>19</sup> According to this large analysis of 103 078 cancer patients, high TMB correlated with better overall survival and better overall response rates to immunotherapy, independently of cancer type and TMB detection method.<sup>19</sup>

In addition, this article details the most frequent mutations found in NSCLC, and the respective indications for targeted therapeutics. We combine the subjects of TMB, somatic mutations within the tumor tissue, oncogene addiction and therapeutic implications. Although the role of oncogenic driver mutations in carcinogenesis is undoubted, their detection in noncancerous tissue, however, to a much lesser degree as opposed to cancer tissue raises new questions.<sup>20–22</sup>

Table 1 gives an overview of targeted therapeutics currently available for NSCLC treatment, with respect to the mutations found in a given cancer specimen (Table 1).

### **Tumor immune microenvironment as an anchor point for cancer therapy**

Immunological aspects in the process of tumor formation, disease progression and metastasis have become ever more important for the development of new therapeutic options

in recent years. Nonsynonymous somatic mutations in the coding region of a gene have been shown to generate presentable neoantigens, which are consecutively recognized by T-cells with structurally divergent and antigen-specific T-cell receptors. When activated by antigen stimulation, T-cells expand within the tumor tissue and execute their cytosolic immune function, controlling tumor growth. Intratumoral T-cell repertoires are enriched with tumor antigen-specific and clonally expanded T-cells.<sup>23</sup> TMB has recently been identified as a genetic signature associated with a favorable response to immunotherapies, especially immune checkpoint inhibitors.<sup>24</sup> Higher TMB favors neoantigen-specific T-cell infiltration and oligoclonal expansion.<sup>25</sup> Neoantigens were found to be widely heterogeneous between the investigated patients.<sup>26</sup> High-frequency T-cell receptors were also distinct for each individual patient. Summing up this investigation, the link between the immunologic tumor microenvironment and TMB becomes evident.

TMB impacts treatment response to immune checkpoint inhibitors.<sup>24</sup> Although the effectiveness of immune checkpoint inhibitors has clearly been demonstrated in the past, response rates vary greatly among individuals. Hence, TMB is a valid tool to predict whether a good response rate is expected. TMB is defined as the overall number of nonsynonymous mutations per coding areas of a tumor genome. TMB was previously determined by means of whole exome sequencing; however, due to high costs, targeted panel sequencing is currently being explored as a measuring tool for TMB. TMB has been demonstrated to correlate with the response to immunotherapy treatment according to several studies.<sup>27–29</sup> It has been hypothesized that neoplasms with a higher mutational burden also express neoantigens more frequently, and usually induce a more pronounced immune response upon treatment with immune checkpoint inhibitors.<sup>30</sup> The recently published CheckMate 227 trial showed a longer progression-free survival (PFS) of NSCLC patients with tumors featuring a TMB of  $\geq 10$  mutations per megabase, independent of PD-L1 expression.<sup>31</sup> However, new data stemming from the CheckMate 227 trial which was presented at the ESMO 2019 congress, has weakened the significance of TMB as a predictor of treatment response.<sup>32</sup> This data shows that TMB, whilst being a predictor of PFS upon therapy with ipilimumab/nivolumab, does not predict overall survival with the same reliability.<sup>31,32</sup> Similarly, the Keynote 189 study showed that TMB was not significantly associated with the efficacy of pembrolizumab plus chemotherapy, as compared to placebo plus chemotherapy in NSCLC.<sup>33,34</sup> Garassino *et al.* randomized 616 patients to either pembrolizumab plus chemotherapy or pembrolizumab in combination with placebo. TMB, as determined by whole-exome sequencing, was not significantly associated with

**Table 1** Targeted treatment options for mutations frequently found in NSCLC

TYPE OF MUTATION	EPIDEMIOLOGY	CLINICAL IMPLICATIONS	TARGETED THERAPEUTICS	REFERENCES
a) <i>EGFR</i>	In NSCLC, <i>EGFR</i> mutations have been observed in 43%–89% of cases, globally. 1/4 of NSCLC harbor mutations in the <i>EGFR</i> tyrosine kinase domain, leading to an increased receptor expression in 75% of cases. >90% of <i>EGFR</i> tyrosine kinase domain mutations are found as short in-frame deletions in exon 19 or as point mutations in exon 21. <i>EGFR</i> mutations are more common in women and people who have not smoked.	<i>EGFR</i> mutations result in constitutive activation of signal transduction pathways, promoting cell proliferation and evasion of apoptosis. Recently, <i>EGFR</i> -targeting agents have become standard first-line treatment options for selected NSCLC patients with <i>EGFR</i> mutations. Common side effects of all <i>EGFR</i> inhibitors include: skin disorders, diarrhea, mouth sores, and loss of appetite	<i>EGFR</i> -targeting TKIs: Gefitinib (Iressa); Erlotinib (Tarceva) – can also be used in advanced-stage patients without <i>EGFR</i> mutations if chemotherapy does not work; Afatinib (Giotrif); Osimertinib (targets T790M mutation as well; Tagrisso); Dacomitinib (Vizimpro) Monoclonal antibodies: Nectinmumab (only used for squamous cell NSCLC; Portazza)	11, 41, 43.
ALK	About 5% of NSCLCs feature a rearrangement of the <i>ALK</i> gene. This change is most often seen in non-smokers (or light smokers), and in the adenocarcinoma subtype.	<i>ALK</i> inhibitors are used after chemotherapy, or instead of chemotherapy in patients with <i>ALK</i> gene rearrangements. Several <i>ALK</i> inhibitors are also useful when <i>ROS-1</i> gene mutations are present. Common side effects of <i>ALK</i> inhibitors are: nausea and vomiting, diarrhea, constipation, fatigue, changes in vision	Crizotinib (Xalkori), the first-class <i>ALK</i> TKI showed superiority to platinum-pemetrexed chemotherapy in <i>ALK</i> -positive NSCLC. Crizotinib has shown >60% overall response rate in <i>ALK</i> -positive NSCLC, improving PFS significantly as compared to second-line chemotherapy. Ceritinib (Zykadia), a next-generation <i>ALK</i> inhibitor, has shown durable response in NSCLC patients pretreated with another <i>ALK</i> TKI, and in <i>ALK</i> TKI naive patients. Alectinib (Alecensa) has shown systemic and central nervous system effectiveness in NSCLC Brigatinib (Alunbrig) Lorlatinib (Lorbrena).	11, 62–65.
b) <i>ROS-1</i>	<i>ROS</i> proto-oncogene 1, receptor tyrosine kinase ( <i>ROS-1</i> ) mutations occur in about 1%–3% of NSCLC specimens.	<i>ROS-1</i> shares certain characteristics with the <i>ALK</i> oncogene. Side-effects of Crizotinib include anemia, leuko-/neutropenia, nausea, vomiting, diarrhea, dizziness and impaired vision. Lorlatinib (Lorviqua) may cause hyperlipidemia, headache, diarrhea, nausea, pneumonitis, joint pain, edema and fatigue.	Crizotinib (Xalkori) has been proven a therapeutic option in <i>ROS-1</i> mutated NSCLC specimens. Crizotinib is FDA-approved for patients with advanced, <i>ROS-1</i> positive NSCLC. Lorlatinib, a novel brain-penetrant <i>ALK</i> and <i>ROS-1</i> TKI, showed systemic and intracranial activity in <i>ALK</i> - and/or <i>ROS-1</i> positive patients. Entrectinib (Ignyta) was FDA-approved only for molecularly defined subsets of NSCLC,	52, 57, 69.

**Table 1** Continued

TYPE OF MUTATION	EPIDEMIOLOGY	CLINICAL IMPLICATIONS	TARGETED THERAPEUTICS	REFERENCES
<i>BRAF</i>	<i>BRAF</i> mutations are observed in 4%–8% of NSCLC cases, causing downstream activation of the MAPK signaling pathway.	In <i>BRAF</i> -mutant NSCLC specimens, treatment with selective <i>BRAF</i> inhibitors were effective in 33% of patients, improving median PFS with 5.5 months.  Nearly all patients harboring these mutations are active or former tobacco smokers. Even though V600E substitutions are the most common among <i>BRAF</i> mutations, one series also reported a 39% prevalence of G469A substitutions in lung cancer.  Side effects of Dabrafenib and Vemurafenib include decreased appetite, headache, cough, nausea, emesis and/or diarrhea and joint pain. Skin cancer incidence increases upon treatment with <i>BRAF</i> inhibitors.	and may also be effective in <i>ROS-1</i> mutated cancers.  Dabrafenib (a newer-generation, reversible kinase inhibitor of V600E-mutant <i>BRAF</i> with a higher affinity than the wild-type enzyme for mutant <i>BRAF</i> ).  Vemurafenib, Selumetinib, Binimetinib, PLX8394, RXDX-105, LXH254+LTT462, AU922 and Regorafenib are currently investigated in clinical trials in <i>BRAF</i> -mutant NSCLC.	40, 55, 56, 66.
<i>RET</i>	In about 1%–2% of NSCLC, <i>RET</i> -involving gene rearrangements have been reported.	No <i>RET</i> -selective inhibitors have been developed yet. Multitarget agents featuring anti- <i>RET</i> activity as well are available.  <i>RET</i> -rearrangement was associated with a high overall response rate and long PFS in NSCLC patients upon pemetrexed-based chemotherapy.  Possible side effects of <i>RET</i> -targeting multikinase inhibitors are: upper respiratory tract infections, urinary tract infections, decreased appetite, headache, sleep disturbances and impaired vision	Anti- <i>RET</i> multikinase inhibitors currently available are: Vandetanib (targets VEGFR, EGFR and <i>RET</i> ); Cabozantinib (VEGFR2, MET, AXL, c-KIT, FLT3, TIE2, <i>RET</i> ); Lenvatinib (VEGFR1-3, PDGFRB, c-KIT, FLT3, <i>RET</i> ); Sunitinib (VEGFR1-3, PDGFRB, c-KIT, FLT3, <i>RET</i> ); Sorafenib; Dovitinib; AD80 and Sitravatinib  Trials about <i>RET</i> -inhibitors are still ongoing: Alectinib, Pontatinib and Apatinib are investigated specifically in <i>RET</i> -rearranged NSCLC.	53, 54, 58.

overall survival, PFS or objective response rate in this setting<sup>34</sup> as presented at the 2019 IALC World Conference in Barcelona. Also presented at this congress was data from the Keynote 021 study,<sup>35,36</sup> showing that TMB was not associated with objective response rates, PFS or overall survival in patients suffering from NSCLC who received pembrolizumab,

carboplatin and pemetrexed, or carboplatin and pemetrexed alone. In this cohort of patients with metastatic nonsquamous NSCLC, TMB did not predict treatment efficacy, and TMB was also not significantly associated with PD-L1 expression. Notably, in the pembrolizumab plus chemotherapy-group, objective response rate was high in both the TMB-low and

TMB-high group.<sup>36</sup> A recent study, where microsatellite instability status was determined as another predictor for treatment response to immune checkpoint inhibitors, found that TMB does not correlate very well with microsatellite instability, or with PD-L1 status.<sup>37</sup> Moreover, the relationship between TMB, microsatellite instability and PD-L1 seems to vary greatly among cancer subtypes, showing a distinct overlap in colorectal cancer, but rather poor concordance in endometrial, ovarian, neuroendocrine and cervical cancer.<sup>37</sup>

### Frequent genetic aberrations found in NSCLC: About ALK, EGFR and ROS-1

Molecular testing for epidermal growth factor receptor (*EGFR*) mutations and anaplastic lymphoma kinase (*ALK*) rearrangements have become standard diagnostic procedures in the management of NSCLC.<sup>38,39</sup> DNA mutations in the *EGFR* gene, as detected by polymerase chain reaction (PCR), may occur in regions corresponding to the extracellular or the intracellular portions of the EGFR protein. In NSCLC, mutations affecting the intracellular portions of the protein have been observed in 43%–89% of cases, globally.<sup>40</sup> One quarter of NSCLC harbor mutations in the EGFR tyrosine kinase domain which were associated with an increased receptor expression in 75% of cases.<sup>41,42</sup> Over 90% of EGFR tyrosine kinase domain mutations are found as short in-frame deletions in exon 19 or as point mutations in exon 21; the latter resulting from a replacement of leucine by arginine at codon 858 (L858R).<sup>43</sup> In Asian countries, about 30%–50% of NSCLC harbor activating mutations of the *EGFR* gene according to a recent report, rendering the respective patients suitable candidates for treatment with *EGFR*-targeting tyrosine kinase inhibitors (TKIs).<sup>44,45</sup> All kinds of *EGFR* mutations result in constitutive activation of signal transduction pathways, leading to cell proliferation and evasion of apoptosis. Generally, somatic mutations in NSCLC can lead to oncogene activation by means of various mechanisms, eg. point mutations, insertions, deletions and/or gene rearrangements.<sup>46</sup> In most cases, the gene alterations are mutually exclusive, with one predominant driver mutation in a given cancer.<sup>47,48</sup>

Oncogenic fusion proteins also occur in NSCLC, such as *ALK*, and the ROS1 proto-oncogene receptor tyrosine kinase (ROS-1).<sup>46,49–51</sup> Besides these well-known fusion proteins, other gene rearrangements that involve kinase-encoding genes have also been described in lung cancer, namely the rearranged during transfection gene (*RET*),<sup>52,53</sup> the neurotrophic receptor tyrosine kinase 1 (*NTRK1*), the b-raf proto-oncogene, serine/threonine kinase (*BRAF*),<sup>54,55</sup> and, as mentioned above, *EGFR*.<sup>46</sup> For *ALK* and *ROS-1* there are targeted therapeutics available which have already been well-established in everyday clinical practice. In selected patients, these targeted agents show significant

benefits, leading to tumor reduction and a prolongation in overall survival.<sup>49,50,56</sup>

### Oncogene addiction – a driver of tumorigenesis, but also a predictor of treatment response to ALK-, EGFR- and ROS-targeting agents

The term “oncogene addiction” is a hallmark of cancer, meaning that cancers are primarily driven by divergent signaling pathways of oncogenes. Usually this term refers to a given cancer being driven by one particular mutation, which leads to a cascade of other procarcinogenic events and pathways. In NSCLC particularly, oncogenic gene rearrangement frequently leads to expression of oncogenic fusion proteins by the oncogene formation by a 5' partner creating an in-frame gene fusion with a 3' proto-oncogene.<sup>57–59</sup> Prominent examples are the Echinoderm microtubule-associated protein-like 4 (*EML4*)-*ALK* fusion gene, *ROS-1*- and *RET*-fusions. *ALK*-rearrangements have been discovered recently as an important driver mutation in NSCLC, and especially in advanced-stage NSCLC, *ALK*-mutation status has a major impact on how patients are treated.<sup>60–64</sup> *ALK* rearrangement is found in 3%–7% of patients suffering from stage IIIB or stage IV NSCLC, according to previous data.<sup>65–67</sup> Adenocarcinomas harbor *ALK* mutations more frequently than squamous cell carcinomas. Interestingly, if only non-smoker patients are considered, the prevalence of *ALK* rearrangement is much higher, ranging from 17% to 20%, depending on the case series.<sup>65,67</sup>

Crizotinib is an *ALK*, *ROS-1* and *MET* tyrosine kinase-inhibitor that can be administered orally, and has shown >60% overall response rates in heavily pretreated patients with *ALK*-positive NSCLC in single-arm phase I and phase II trials.<sup>61,68</sup> Crizotinib led to significantly higher response rates and longer PFS as compared to conventional chemotherapy as second-line treatment, and also compared to platinum/pemetrexed chemotherapy in untreated advanced NSCLC featuring *ALK*-rearrangement. Hence, crizotinib has become standard-of-care in these subgroups of patients.<sup>69–71</sup>

Mutations of the *EGFR* gene constitute about 43%–89% of NSCLC cases, depending on the case series, rendering the affected patients candidates for targeted treatment with *EGFR*-TKIs.<sup>72–78</sup> Although screening for *EGFR* mutation-status is performed routinely, little is known about possible coexisting carcinogenic mechanisms in the population of patients featuring *EGFR* mutation. Therapeutic benefits by the supplemental use of other biologicals to further improve survival and quality of life have become evident according to clinical trials, and some targeted agents are already used as first-line treatment for selected patients with *EGFR* mutations.<sup>72,79</sup> Gefitinib, erlotinib and afatinib

today constitute the standard of care drugs incorporated in routine clinical management of *EGFR*-mutated lung cancers. Experimental studies have been carried out by several research groups where molecular subtyping using the latest DNA sequencing techniques was performed in NSCLC patients. As a result of these studies, it became clear that there is a high degree of molecular heterogeneity in *EGFR* nonmutated NSCLC.<sup>80</sup> Beyond NSCLC harboring *EGFR*- or other common mutations which can be targeted by biologicals, there is a certain gray zone of tumors as well (about 40% of NSCLC) which are negative for all molecular alterations that can be targeted by clinically approved drugs. Hence, conventional chemotherapy is the only option in these cases.<sup>72,81–83</sup>

Facchinetti *et al.* have reviewed in-depth *ROS-1* inhibition in the context of oncogene addiction in NSCLC.<sup>84</sup> *ROS-1* has been shown to share certain characteristics with the *ALK* oncogene; however, there are also distinct disparities between the two fusion genes.<sup>84</sup> When more common genetic aberrations, such as *EGFR*-, *KRAS*-, and *ALK*-mutations, are absent it is mandatory to search for *ROS-1* rearrangement. According to the literature, *ROS-1* can be found in 1%–2% of lung adenocarcinomas. Crizotinib can have a major positive impact on *ROS1*-mutated tumors, rendering this treatment option the major reason why *ROS-1*-rearrangements must not be overlooked.<sup>84</sup> The authors of the above-mentioned review article suggest careful standardization of molecular diagnostics, relying upon practical and efficient algorithms, and comprising immunohistochemistry as well as fluorescence-in-situ hybridization (FISH). By carefully outlining every *ROS-1*-mutated tumor, larger amounts of data will be gathered to gain even more insight into the tumor biology and molecular behavior in this specimen of oncogene-addicted NSCLC.

The *ALK-EML4* translocation represents another mutation that may be found in NSCLC, influencing treatment and prognosis. Noteworthy, it has been proposed that also the impact of radiotherapy is partly dependent on mutation status.<sup>85</sup> Radiotherapy poses an important therapeutic option in locally advanced lung cancer. Hence, Dai and colleagues sought to investigate whether there are synergistic effects when combining radiotherapy and *ALK*-inhibition via TAE684 in *ALK*-positive versus wild-type lung cancer cells. In this cell culture experiment, three different cell lines were investigated: human NSCLC cell lines harboring wild-type *ALK* (A549), human NSCLC cell lines featuring *ALK-EML4* translocation (H3122), as well as murine Lewis Lung Cancer (LLC) cells. The three cell lines were irradiated with 1–4 Gy X-Rays (320 keV) and carbon ions. Consecutively, TAE684, a potent and selective small-molecule *ALK* inhibitor, was administered at the dose range of 0–100 nM. The authors assessed cell survival, proliferation rates, and apoptosis via caspase 3/7 expression

levels. It was found that TAE684 inhibited the proliferation of H3122 cells in a dose-dependent manner, as opposed to A549 and LLC cells that were more resistant to TAE684 treatment, and the half maximal inhibitory concentration was not reached at all tested concentrations (up to 100 nM); whereas the half maximal inhibitory concentration was reached at about 8.2 nM for the H3122 cells.<sup>85</sup> Those anti-proliferative effects of TAE684 were augmented by radiotherapy in H3122 cells, rendering these cells particularly more vulnerable to particle therapy with carbon ions (sensitizer enhancement ratio of about 1.61;  $P < 0.05$ ). Moreover, activity of caspase 3/7 was evidently enhanced after combining both therapy options in H3122 cells. Summing up this interesting data, synergistic effects of combined TAE684 and radiotherapy in *EML4-ALK* positive lung cancer cells are demonstrated: Not only does *ALK*-inhibition enhance the effect of canonical photon radiotherapy, but also of particle irradiation using carbon ions.<sup>85</sup>

### RET-and c-MET proto-oncogenes: Less frequent examples of oncogene addicted NSCLC

The *RET* proto-oncogene encodes a certain receptor tyrosine kinase which is found in tissues that originate from the neural crest. *RET* also plays a role in the development of the kidneys and the enteric nervous system.<sup>86,87</sup> When gene rearrangements involve *RET*, cells acquire oncogenic properties, as has been proven in thyroid papillary carcinoma.<sup>88</sup> In about 1%–2% of NSCLC, *RET*-involving gene rearrangements have been reported, according to the recent literature. Interestingly, NSCLC gene rearrangements involving *RET* are mutually exclusive with other procarcinogenic driver mutations, eg. *ALK* or *ROS1* rearrangements.<sup>57,89,90</sup> Multiple fusion partners with *RET* have been described: According to a global registry where patients with *RET*-rearranged NSCLC were involved, it was reported that among 81 cases with identified *RET*-fusion partners, the kinesin family 5B gene (*KIF5B*) was involved in 72%. This finding supported previous observations where *KIF5B* was also described as the most prevalent fusion partner in NSCLC.<sup>57,89,91</sup> The second most common fusion partner in *RET*-rearranged NSCLC is *CCDC6* (23%). Less frequently, fusion with *NCOA4* (2%), *EPHA5* (1%) and *PICALM* (1%) has been reported.<sup>91,92</sup> Notably, *RET*-rearrangements are found in equal proportion in male and in female NSCLC patients. Among patients in the above-mentioned global registry, a relatively large proportion, namely 63%, were never-smokers, whilst 24% were former smokers and 10% reported to currently smoke. Nearly all the reported cases (98%) were adenocarcinomas. A total of 72% of these *RET*-rearranged tumors were already at stage IV at the time of diagnosis. On the one

hand, this suggests a relatively high metastatic potential, possibly caused by the *RET*-fusion oncogene. On the other hand it could also mean that *RET*-fusion occurs in later stages of carcinogenesis, or that the *RET* fusion-oncogene is more readily found in patients with metastatic disease, since molecular testing is done in nonmetastatic cases less frequently.<sup>46,91</sup> Another example of oncogene addiction is NSCLC featuring aberrant expression of the proto-oncogene *c-MET* in addition to *EGFR* mutation.<sup>93</sup> A study from 2018 revealed that cigarette smoking further augments oncogene addiction to *c-MET* in NSCLC cells, suggesting that *MET*-inhibition is a therapy option especially for lung cancer patients with a smoking history, or who are currently smoking. It is already known that cigarette smoking is associated with the insensitivity of NSCLC to treatment with *EGFR*-TKIs. However, it is yet undetermined by which exact molecular mechanisms tobacco smoke affects *EGFR*-TKI treatment. In this study about *c-MET* in the context of NSCLC and tobacco smoke, it was shown that chronic exposure to cigarette smoke extract or tobacco smoke-derived carcinogen benzo[ $\alpha$ ]pyrene, B[ $\alpha$ ]P, but not nicotine-derived nitrosamine ketone (NNK), diminished treatment response to *EGFR*-TKIs in wild-type *EGFR*-expressing NSCLC cells. Interestingly, TKI-treatment did inhibit *EGFR* tyrosine kinase activity almost completely; however, no inhibitory effect on downstream Akt and ERK pathways was observed in B[ $\alpha$ ]P-treated NSCLC cells.<sup>93</sup> Cigarette smoke extract and B[ $\alpha$ ]P both transcriptionally upregulate the activity of the proto-oncogene *c-MET*, also activating its downstream Akt pathway, which could not be targeted by *EGFR*-TKIs. Consecutively, the authors of this study silenced *c-MET* which effectively reduced B[ $\alpha$ ]P-induced activation of Akt. The cigarette smoke extract-treated NSCLC cells were sensitive to treatment with the *c-MET* inhibitor crizotinib. Thus, cigarette smoke evidently increases NSCLC-oncogene addiction to *c-MET*, but treatment with the *c-MET* inhibitor crizotinib seems to be effective despite ongoing cigarette smoke exposure.<sup>93</sup>

### Circulating tumor DNA in association with mutational load

Circulating cell-free tumor DNA fragments have been found in the bloodstream of patients suffering from malignant diseases since 2001.<sup>94</sup> As cell-free DNA also occurs in the bloodstream of healthy individuals, however to a much smaller proportion as compared to cancer patients, it is believed that cell-free DNA derives from both tumor cells and nonmalignant cells.<sup>95,96</sup> It has also been reported that higher levels of circulating DNA are found when cells undergo apoptosis or necrosis.<sup>94</sup> Recently, researchers have focused on cancer-specific mutations found in the cell-free

DNA, which are also present in the primary tumor.<sup>97</sup> Since the proportion of necrosis within rapidly proliferating and growing tumors increases, more cell free DNA is released. Generally, the more aggressive a tumor is growing, the more mutations are found, and therefore it is likely that mutated cell-free tumor DNA increases with mutational load.<sup>98</sup> In a study by Winther *et al.* levels of plasma mutant cell-free DNA and metabolic tumor burden, measured by 18F-fluoro-d-glucose positron emission tomography/computer tomography (PET/CT), were correlated. Additionally, the patients' survival times were correlated with the amount of mutated cell-free DNA. Overall, 46 patients suffering from advanced NSCLC were included in the analysis. At the time of inclusion, blood was taken and PET/CT scans were performed. Cell-free DNA was isolated and next-generation sequencing was carried out by means of the AmpliSeq Colon and Lung Cancer Gene Panel V2. Metabolic tumor burden was determined volumetrically via the PET/CT. The authors of this interesting study report a significant correlation between the allele frequency of the most frequent mutations and metabolic tumor burden ( $P < 0.001$ ).<sup>98</sup> When mutated cell free DNA was detected, median overall survival was significantly shorter as compared to patients where no mutated cell-free DNA was found (3.7 vs. 10.6 months,  $P < 0.019$ ). Interestingly, this impact on survival was independent of the metabolic tumor burden, indicating that the mutational load is even more important for prediction of a patient's prognosis. Mutational load detected in circulating cell-free DNA could become an important prognostic parameter in future everyday clinical practice.

A different study, conducted by Reynolds and colleagues, aimed at validating next generation sequencing technology for detecting gene mutations with residual cell pellets by means of liquid-based cytology.<sup>99</sup> *EGFR*-mutation status in NSCLC samples is routinely outlined with allele-specific polymerase chain reaction in liquid-based cytology samples, for instance from endoscopic ultrasound-guided fine needle aspiration. In this study, however, DNA extracted from liquid-based cytology samples was tested with a multiplex-amplified and enriched Ion AmpliSeq Cancer Hotspot sequencing panel, as well as the OneTouch 2 instrument. Consecutively, six NSCLC-related genes covered by this sequencing panel (*BRAF*, *EGFR*, *ERBB2*, *KRAS*, *MET*, and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit a [*PIK3CA*]) were analyzed.<sup>99</sup> Not surprisingly, the common *EGFR* sequence changes, including four *L858R* mutations, three exon 19 deletions and one exon 20 insertion, were found to correspond 100% between the assay platforms. Variants of the mutation hotspots *ERBB2*, *KRAS*, *MET*, and *PIK3CA* genes, commonly found in NSCLC, were also observed. Thus, next generation sequencing can obviously

also be performed successfully in liquid-based cytology samples of NSCLC.<sup>99</sup>

## Conclusion

Mutations frequently observed in NSCLC are of increasing relevance for choosing the most beneficial therapy regimen in a patient. Within the last few years, several new targeted therapies, specifically anchoring at NSCLC hotspot mutations, or at tumor-specific fusion proteins, have been developed. The overall mutational load in a given tumor mass seems to have prognostic relevance, although data is inconclusive in case of oncogene-addicted tumors that are primarily driven by a single mutation. Here, TMB might be associated with a better overall survival and PFS when immune therapy is administered. However, novel data from the CheckMate 227 and the Keynote 021 trial show differential results, suggesting that TMB might not be a reliable predictor of PFS, overall survival and response rate in NSCLC upon immunotherapy. A higher mutational burden is usually associated with an adverse outcome, since high TMB generally occurs in rapidly growing and advanced-stage tumors. It has recently been demonstrated that the tumor microenvironment changes in accordance with overall mutational load, highlighting the connection between mutational status, total mutational load, aspects of the immunological microenvironment and, of course, treatment response and outcome.

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