KRAS mutations in the circulating free DNA (cfDNA) of non-small cell lung cancer (NSCLC) patients

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Abstract: Circulating free DNA (cfDNA) is obtained from serum or plasma by non-invasive methods such as a simple blood draw, a technique known as “liquid biopsy”. Genetic analyses of driver alterations in cfDNA have proved very effective to predict survival and treatment response of cancer patients according to tumoral cfDNA burden in blood. Non-small cell lung cancer (NSCLC) patients with higher concentration of tumoral cfDNA in blood have, on average, shorter progression-free survival (PFS) and overall survival (OS). Regarding specific genetic alterations, KRAS proto-oncogene, GTPase (KRAS) is one of the main genes involved in NSCLC and several studies have been performed to determine its value as a predictive and prognostic biomarker in liquid biopsy. Unfortunately, to date no strong conclusions can be drawn since they have yielded contradictory results. Therefore, further investigations are necessary to establish the value of KRAS testing in liquid biopsy as prognostic or predictive factor in NSCLC. Herein, we review the current knowledge on the importance of KRAS as prognostic and predictive biomarker using non-invasive approaches and the scientific data available regarding its application in clinical practice for treatment of NSCLC.

Keywords: Liquid biopsy; circulating free DNA (cfDNA); KRAS; lung cancer; non-small cell lung cancer (NSCLC)

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KRAS in non-small cell lung cancer (NSCLC)

Lung cancer is the most common cancer and the first cause of cancer deaths worldwide (1). NSCLC is the predominant subtype of lung cancer, being adenocarcinoma the most common histology. Unfortunately, almost half of NSCLC patients are diagnosed at advanced stage and have poor prognosis and limited options for treatment, traditionally restricted to chemotherapy (2). However, in recent years, the identification of prognostic and predictive biomarkers has led to improvements in outcome and has set allowed the application of personalized medicine approaches in NSCLC patients. More than 50% of advanced NSCLC patients harbor a driver genetic alteration that, if targetable, changes the therapeutic panorama (3). For this reason, implementing resources for quick, cost-effective, multiplex detection of alterations has recently gained importance for cancer diagnostics.
Numerous mutations have been identified in NSCLC which vary depending on whether histology is adenocarcinoma or squamous-cell carcinoma (SCC), as well as with smoking history and status. The two most important alterations in the carcinogenesis of the lung are somatic mutations in the epidermal growth factor receptor (EGFR) and KRAS proto-oncogene, GTPase (KRAS) genes (4). These mutations are more frequent in lung adenocarcinoma than in SCC (5) and have implications for treatment selection. Patients with EGFR mutations can be treated with EGFR tyrosine kinase inhibitors (TKIs); however, for no drugs have yet been developed to specifically target KRAS alterations or show an increased efficacy when a KRAS mutation is present.

KRAS mutations were first identified over 30 years ago but it is only in recent years that there have been significant advances in the understanding of the biology of KRAS and its downstream effectors (6). The majority of KRAS-mutant cases in NSCLC present single point mutations at codon 12, while mutations in others positions are relatively rare (in codons 13 and 61) (7). Within codon 12, the most frequent point mutations are G12C (42%), G12V (21%), G12D (17%) and G12A (7%) (8). Current or former smokers have a significantly higher frequency of KRAS mutations than never smokers (9) and it is possible to identify the primary mutagenic signature of DNA damage by tobacco smoke. Smoker patients show substitutions GGT>GGT (G12V) and GGT>TGT (G12C) (purine for a pyrimidine transversions) in comparison with never-smoker patients in whom changes in GGT>GAT (G12D) or GGT>AGT (G12S) (purine for purine transitions) are more common. This suggests that, although some KRAS mutations are associated with history of cigarette smoking, others can also occur in never-smokers.

**KRAS mutations as a prognostic factor**

Prognostic biomarkers can be used as indicators of the natural history of the disease. Traditionally, KRAS mutations detected in biopsies of NSCLC patients have been associated with negative prognosis and poor outcomes (10).

However, the value of KRAS mutant status as a prognostic marker remains unclear, and seems to depend both on the disease stage at the time of diagnosis and the specific KRAS codon mutation. In terms of staging, the prognostic value of KRAS for resectable disease does not appear to be significant. However, some prospective data have shown that in resected early-stage NSCLC, KRAS mutations were found only in smokers and were associated with worse survival exclusively in stage I disease but not in the whole population (11). By contrast, in stage IV disease, presence of KRAS mutations has been associated with shorter survival (6). In terms of mutation, one study has demonstrated no difference in overall survival (OS) when comparing specific amino acid substitutions on codon 12. An interesting finding was that KRAS codon 13 mutations seemed to be associated with worse survival compared to codon 12 mutations. Unfortunately, these results were not confirmed by independent validation (12). Finally, according to histology, the presence of KRAS mutation in adenocarcinoma subtype appears to be a negative prognostic factor (13).

**KRAS mutations as a predictive factor of resistance**

In tissue, predictive markers can be used as indicators of response or resistance to a specific targeted treatment. Some data show that adjuvant chemotherapy is unlikely to benefit NSCLC patients harboring KRAS mutations. Nevertheless, in a recent study KRAS codon 13 mutations appeared to be deleterious and the patients had significantly worse OS with adjuvant chemotherapy (6,8).

In relation to NSCLC, KRAS mutations were shown to be significantly associated with inferior outcomes to chemotherapy in NSCLC and, consequently, the vast majority of EGFR mutations are present in KRAS wild-type patients (15,16). Therefore, the absence of EGFR alterations, rather than the presence of KRAS mutation, can be a negative predictor of response to EGFR-TKIs (8). At this respect, in advanced NSCLC some studies have also investigated the influence of KRAS mutations on sensitivity to chemotherapy with no significant differences in PFS and OS between KRAS wild-type and KRAS mutated patients (8). By contrast, other reports suggest that in patients treated with first-line platinum-based chemotherapy, KRAS mutations have a negative predictive role. So, all these findings need to be confirmed in a larger population to be of relevance for clinical decision making, highlighting the possibility that
subtype-specific KRAS mutation analysis could identify a subgroup of patients who could benefit more from chemotherapy (10).

Mutations in codon 12 seem to confer different responses depending on treatment. While expression of G12C is associated with reduced response to cisplatin and increased sensitivity to taxol and pemetrexed, G12D is only associated with resistance to taxol treatment and sensitivity to sorafenib. Furthermore, G12V mutants show strong sensitivity to cisplatin when compared with wild-type clones and are slightly more resistant to pemetrexed (10). However, expression of different KRAS mutants did not modify the cellular response to the EGFR inhibitor erlotinib or to gemcitabine (17,18).

Taken together, these findings change the clinical point of view since different KRAS mutations may lead to different signal transduction cascades in NSCLC and to different carcinogenesis and drug sensitivity. Therefore, it is necessary to define the specific KRAS mutation in order to identify those patients with different probabilities of responding to therapy (18). Further research is required to understand KRAS mutations and to develop drugs targeted against them (6). Some recent investigations have generated a renewed interest in the development of direct KRAS inhibitors (19). For instance, Lito and colleagues (20) achieved blockade of nucleotide exchange factors from activating KRAS. They are working with a compound, ARS-853, which is a selective, covalent inhibitor of KRAS\(^{G12C}\) that inhibits mutant KRAS-driven signaling by binding to the GDP-bound oncoprotein and preventing activation. This work could present a significant step toward a direct KRAS inhibitor for the patients with KRAS\(^{G12C}\) mutation, but nevertheless still further optimization is required to generate compound suitable for in vivo studies.

**Circulating free DNA (cfDNA) as prognostic and monitoring technique**

Unfortunately, surgical lung cancer biopsies are ineffective for showing tumor heterogeneity and are not well tolerated by patients, in addition to having certain related risks. Therefore, performing serial tissue biopsies in order to detect and monitor disease progression is extremely challenging. The answer lies in developing more accessible methodologies that facilitate non- or minimally-invasive detection and monitoring of known NSCLC mutations, as well as characterization of metastatic and/or resistant disease mechanisms, when tissue or re-biopsies are unavailable (10).

Liquid biopsy is an excellent means of identifying and monitoring alterations using a non-invasive diagnostic method. cfDNA presents the same mutations found in the primitive tumor mass since cellular necrosis and apoptosis cause the release of tumoral DNA into the bloodstream (21). In order to assess cancer disease alterations through the capture and analysis of cfDNA, many highly sensitive and specific techniques have been developed. Among these, our laboratory has extensive experience in detection of melanoma, lung and colon cancer biomarkers in cfDNA by Real-Time PNA PCR, particularly in those advanced NSCLC cases in which tumor tissue cannot be obtained by surgical biopsy. Peptide nucleic acid (PNA) is an artificially synthesized polymer analogue to DNA in which deoxyribose-phosphate backbone is replaced with a peptide of amino-ethyl-glycine unit. It forms highly stable complex with complementary DNA, and we designed to inhibit, in a specific manner, the amplification of the wt allele during the PCR amplification. We currently test serum and plasma from cancer patients for mutations in three genes (EGFR, KRAS and BRAF) (22,23) with 75% sensitivity and 100% specificity. Our experience demonstrates that cfDNA offers an alternative, rapid, minimally-invasive option for accurate mutation testing.

The total amount of cfDNA in the bloodstream has been demonstrated to be an effective biomarker of outcome in NSCLC; patients with higher concentrations of total cfDNA have shorter PFS and OS compared with healthy, high-risk individuals (24). By contrast, tumor regression correlates to decreased ctDNA burden in cfDNA. With regard to specific genetic alterations, one clear example is the clinical utility of the detection of EGFR mutations in the cfDNA of NSCLC patients treated with gefitinib (25) or erlotinib (26). EGFR mutations have also been shown to be of prognostic and predictive value, and patients with an activating mutation in EGFR in cfDNA have been reported to respond significantly better to TKIs (27). KRAS gene alterations detected in cfDNA have also been used as prognostic biomarkers, mainly in colorectal and pancreatic cancer (28,29). However, their predictive and prognostic value in NSCLC remains undefined, and to an extent controversial, due to the relatively few studies performed. Nevertheless, considering that liquid biopsy techniques are still being developed; new data will be generated that, in all likelihood, will clarify the importance of KRAS testing in NSCLC. In addition, all this new information will speed up implementation of potential new treatments. In summary, it can be concluded that the analysis of cfDNA is an essential
KRAS mutations: prognostic and predictive value of cfDNA in NSCLC

As mentioned, KRAS mutations in tissue could be a weak, but valid, predictor of poor prognosis and treatment outcome (14). Therefore, several studies have tried to uncover the same kind of correlation between the presence of KRAS mutations in blood and clinical outcome in order to use KRAS as a biomarker.

We have reviewed the relevant studies related to KRAS mutations in liquid biopsy as predictive or prognostic factors in NSCLC, summarizing all the information in Table 1.

Several studies have evaluated KRAS mutation status in cfDNA and response to chemotherapy. Three were performed using plasma samples and showed worse PFS and OS in KRAS mutated patients (31,33,35) while a study performed in serum did not show any significant differences (30). However, a meta-analysis incorporating data from all the studies concluded that KRAS mutations in cfDNA may not be useful to predict response to chemotherapy (16).

The clinical utility of determining KRAS mutations in liquid biopsy as a marker of sensitivity to EGFR-TKIs in NSCLC has also been studied. So far, two studies, one in plasma and one in serum, failed to show significant differences in terms of OS. However, the plasma study did show that mutant KRAS patients had a worse PFS than wild type subjects (32,34). As mentioned, the discrepancies between these studies might be due to the fact that the vast majority of EGFR mutations occur in KRAS wild-type patients. In consequence, the real value of KRAS as prognostic and predictive biomarker might have been overestimated (34). Another reason could be the small number of studies performed which have assessed the prognostic value of KRAS mutations in NSCLC in cfDNA. In summary, all the evidence suggests that KRAS genotype detected in cfDNA may not be a good prognostic factor of survival in NSCLC patients. However, the predictive or

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Table 1 Survival data according to KRAS status in blood

<table>
<thead>
<tr>
<th>Author, year</th>
<th>Study population (n)</th>
<th>NSCLC Stage</th>
<th>Therapeutic regimen</th>
<th>Specimen type</th>
<th>PFS (months)</th>
<th>P value (PFS)</th>
<th>OS (months)</th>
<th>P value (OS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Camps C. et al., (30) 2005</td>
<td>67</td>
<td>IIIB or IV</td>
<td>Chemotherapy</td>
<td>Serum</td>
<td>KRAS +: 7.3</td>
<td>0.2300</td>
<td>KRAS +: 11.4</td>
<td>0.2800</td>
</tr>
<tr>
<td>Gautschi O. et al., (31) 2007</td>
<td>175</td>
<td>I, II, III (A/B) or IV</td>
<td>Surgery + chemotherapy</td>
<td>Plasma</td>
<td>–</td>
<td>–</td>
<td>Worse OS of patients with mutant plasma KRAS</td>
<td>0.0370</td>
</tr>
<tr>
<td>Wang S. et al., (32) 2010</td>
<td>120</td>
<td>IIIB or IV</td>
<td>EGFR-TKI</td>
<td>Plasma</td>
<td>KRAS+: 2.5</td>
<td>&lt;0.0010</td>
<td>KRAS +: 16.9</td>
<td>0.8270</td>
</tr>
<tr>
<td>Nygaard AD. et al., (33) 2013</td>
<td>246</td>
<td>III or IV</td>
<td>Chemotherapy</td>
<td>Plasma</td>
<td>KRAS +: 3.0</td>
<td>0.0043</td>
<td>KRAS +: 4.8</td>
<td>0.0002</td>
</tr>
<tr>
<td>Kim ST. et al., (34) 2013</td>
<td>57</td>
<td>IIIB and IV</td>
<td>EGFR-TKI</td>
<td>Serum</td>
<td>–</td>
<td>–</td>
<td>KRAS +: 3.9</td>
<td>0.4520</td>
</tr>
<tr>
<td>Nygaard AD. et al., (35) 2014</td>
<td>69</td>
<td>III or IV</td>
<td>Chemotherapy</td>
<td>Plasma</td>
<td>KRAS +: 2.1</td>
<td>0.0100</td>
<td>KRAS +: 3.6</td>
<td>0.0300</td>
</tr>
<tr>
<td>Ai B. et al., (16) 2016 meta-analysis (30,31,33,35)</td>
<td>57</td>
<td>III or IV</td>
<td>Chemotherapy</td>
<td>cfDNA</td>
<td>No significant differences</td>
<td>0.4500</td>
<td>No significant differences</td>
<td>0.8900</td>
</tr>
</tbody>
</table>

NSCLC, non-small cell lung cancer; PFS, progression-free survival; OS, overall survival; EGFR, epidermal growth factor receptor; cfDNA, circulating free DNA.
prognostic role of detection of KRAS mutations in cfDNA remains to be confirmed and warrants further investigation (4). Also, serial testing of KRAS mutations in the blood of KRAS positive patients can be useful to monitor the course of the disease, as it has already been demonstrated for EGFR or BRAF mutations. Our laboratory is actively working in this direction, and preliminary results are encouraging (36).

Conclusions

Tissue biopsy is still the gold standard for diagnosis. However, new technologies are improving the isolation and identification of lung cancer-related mutations in blood and therefore leading to new therapeutic options for the management of cancer patients. Currently, the ability to analyze tumoral cfDNA is one the most important breakthroughs in thoracic oncology. Furthermore, liquid biopsy has the important advantage of being a noninvasive procedure, meaning it can be reproduced, facilitating repeated evaluations of tumor genetic alterations and monitoring of their status throughout the course of the disease. Liquid biopsy has also been shown to be a huge boon to oncologists in terms of early identification of the molecular mechanisms responsible for development of acquired resistance to targeted therapies.

Regarding the detection of EGFR or KRAS mutations in cfDNA as predictive and prognostic biomarkers, EGFR T790M mutations are clearly related to acquire resistance to EGFR-TKIs. However, the prognostic and predictive value of KRAS mutations in cfDNA as a biomarker is still a matter of debate. Therefore, prospective studies with larger patient research cohorts are still required to draw definitive conclusions.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

References

Garzón et al. **KRAS mutations in cfDNA of NSCLC patients**


