Review Article on Improving Outcomes in Lung Cancer Through Early Diagnosis and Smoking Cessation

KRAS mutations testing in non-small cell lung cancer: the role of Liquid biopsy in the basal setting

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Abstract: In advanced stage non-small cell lung cancer (NSCLC) patients, Kirsten Rat Sarcoma Viral Oncogene Homolog (*KRAS*) testing may soon acquire a predictive significance to select patients for AMG510 treatment. Since tissue samples are not always available, liquid biopsy may represent a viable option for *KRAS* testing. Here, we review the last three years clinical practice performed on 194 plasma based liquid biopsies by next generation sequencing (NGS) SiRe® panel. In particular, 36 (18.6%) *KRAS* mutated cases were identified, with an overall median allelic frequency of 5.0% (ranging between 0.2% and 46.8%). No concomitant mutations were observed in the other NSCLC clinical relevant genes included in the SiRe® panel, such as epidermal growth factor receptor (*EGFR*) and v-Raf murine sarcoma viral oncogene homolog B (BRAF). Exon 2 p.G12C was the most common detected mutation (13/36, 36.1%). In conclusion, our data update and confirm that SiRe® NGS panel represents a robust analytical tool to assess *KRAS* mutational status on circulating tumor DNA. Further investigation is required to design more cost-effective diagnostic algorithms to harmonize clinical relevant biomarker testing on tissue and blood in advanced stage NSCLC clinical practice.

Keywords: Kirsten Rat Sarcoma Viral Oncogene Homolog (*KRAS*); next generation sequencing (NGS); lung cancer; AMG510; G12C; basal setting; liquid biopsy

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Introduction

Lung cancer is the most frequent cause of cancer death, worldwide (1). In the last years, several clinical trials have defined the pivotal role of the molecular assessment of different biomarkers, such as epidermal growth factor receptor (*EGFR*), anaplastic lymphoma kinase (*ALK*), ROS proto-oncogene 1 receptor tyrosine kinase (*ROS1*), v-Raf murine sarcoma viral oncogene homolog B (*BRAF*), and programmed death-ligand 1 (PD-L1), in order to administrate either tyrosine kinase inhibitors (TKIs) or immune-checkpoint inhibitors to improve survival and quality of life of advanced stage non-small cell lung cancer (NSCLC) patients (2-14). For this reason the international guideline from the College of American Pathologists (CAP), the International Association for the Study of Lung Cancer (IASLC), and the Association for Molecular Pathology (AMP), the National Comprehensive Cancer Network

(NCCN) and the American Society of Clinical Oncology (ASCO) guidelines established a minimum panel of "must test genes", represented by mutations in *EGFR*, and *BRAF*, and gene fusions affecting *ALK* and *ROS1*, in addition to evaluation of the expression of PD-L1 (15-17).

Beyond these biomarkers, other genes are currently being evaluated as predictive indicators (18-21). In particular, Kirsten Rat Sarcoma Viral Oncogene Homolog (*KRAS*) mutations, occurring in 25–30% of NSCLC patients, have recently acquired a relevant interest, not only as a negative prognostic biomarker, but also as actionable targets (22). As a matter of the fact, a great deal of interest was raised by *KRAS* exon 2 p.G12C (occurring in about 13% of NSCLC patients). In fact, the preliminary results, presented at the 2019 ASCO annual meeting, on the performance of the small molecule AMG510 (Amgen, Thousand Oaks, CA) in a phase 1 clinical trial were promising both in terms of efficacy and safety (23,24). Currently, also other drugs targeting different *KRAS* mutations are under investigation (25).

In order to simultaneously detect all these relevant biomarkers, high throughput devices should be implemented in molecular predictive pathology laboratories. Next generation sequencing (NGS) is a fascinating tool that enables the analysis of different biomarkers for different patients at the same time (26). In our Institution we routinely adopt NGS to assess NSCLC biomarkers; to this end, we designed a customized NGS panel, that makes cost-effective the batching of different tumor samples. In fact, this panel, termed SiRe®, is meant to provide a unique workflow of samples for different specimen types (tissue and liquid biopsies) (27-30). Thus, whenever tissue is not adequate or available for NGS testing, which is a common occurrence for advanced stage NSCLC patients, SiRe® NGS plasma based analysis is a viable option to assess *EGFR* mutational status (31). In a previous study, we showed that SiRe® panel analytical parameters, such as number of total reads, mean read length, number of mapped reads, percentage of reads on target, average reads per amplicon and uniformity of amplicon coverage, are more than satisfactory to reliable assess *EGFR* mutational status (29). Beyond *EGFR* testing, the SiRe® panel also generate data on *KRAS* mutational status. Thus, in this study, we reviewed our NGS records, generated in the last three years from plasma specimens. While for *EGFR* testing a well defined diagnostic algorithm has been established, no specific data are available to evaluate quality benchmark for plasma based *KRAS* testing. This study was undertaken firstly to assess whether, in patients without available or adequate tissue, liquid biopsy is a feasible and reliable tool; secondly, to evaluate the overall *KRAS* mutational rate and the occurrence of single hotspot alterations in exon 2 and 3. The final goal is to build data derived from clinical practice to be useful to designed specific diagnostic algorithm that could harmonize *KRAS* tissue and blood based assays.

Materials and methods

Patient characteristics

To establish the rate of *KRAS* mutated cases in NSCLC as detected by the SiRe® panel on blood, a computerized search was carried out. Patients who performed SiRe® NGS plasma based analysis, from January 2016 to December 2018, were selected by using the following inclusion criteria: (I) cyto-histological evidence of advanced stage lung adenocarcinoma, (II) unavailability of adequate tissue samples for molecular tests, and (III) naïve to any treatment. This search yielded a total of 194 patients, with a preponderance of male (123/194, 63.4%); the overall *KRAS* mutation rate and the distribution of the single mutation occurring in exon 2 and 3 was assessed in this population.

All information regarding human material was managed using anonymous numerical codes, and all samples were handled in compliance with the Helsinki Declaration (http://www.wma.net/en/30publications/10policies/b3/). According to the aforementioned National Guidelines, the study did not require an Ethical Committee approval since it did not modify the patient clinical management.

NGS analysis

As a general rule, in our clinical practice, liquid biopsy is performed upon oncologist request. Patients are received directly in our Institution where a nurse, who is a permanent member of our molecular laboratory staff, collect in EDTA Vacutainer tubes (BD, Plymount, UK) ten mL of peripheral blood, as previously described (27,29,30). By this in-house approach, samples are immediately processed in order to avoid the risk of circulating tumor DNA (ctDNA) degradation. Two centrifugation steps (2,300 rpm for 10 min) are performed to obtain at least 1.2 mL of plasma for each sample. Subsequently, ctDNA is extracted by adopting the QIAsymphony DSPVirus/Pathogen Midi Kit on the QIAsymphony robot (Qiagen, Venlo Limburg), following the manufacturer instructions. After the extraction, ctDNA

Table 1 Detection of *KRAS* mutational status by next generation sequencing approach on liquid biopsy in basal setting

KRAS mutated cases/total cases (%)	p.G12A (%)	p.G12C (%)	p.G12D (%)	p.G12S (%)	p.G12V (%)	p.G13D (%)	p.G13S (%)	p.A59V (%)	p.Q61H (%)
36/194 (18.6)	1/36 (2.8)	13/36 (36.1)	5/36 (13.9)	3/36 (8.3)	3/36 (8.3)	4/36 (11.1)	4/36 (11.1)	1/36 (2.8)	2/36 (5.6)

KRAS, Kirsten Rat Sarcoma Viral Oncogene Homolog.

Table 2 *KRAS* mutated patients characteristics evaluated in all instances

Characteristics	Value
Patients, n	36 (100.0%)
Male	24 (66.7%)
Female	12 (33.3%)
Age range	36–89
Median age	66.9

KRAS, Kirsten Rat Sarcoma Viral Oncogene Homolog.

is analyzed by NGS. A customized NGS panel, called SiRe®, that covers 568 clinical relevant mutations in six genes [*EGFR*, *KRAS*, Neuroblastoma RAS Viral Oncogene Homolog (*NRAS*), *BRAF*, KIT Proto-Oncogene, Receptor Tyrosine Kinase (*KIT*) and Platelet Derived Growth Factor Receptor Alpha (*PDGFRA*)] is adopted to perform molecular analysis, as previously reported (27-30). In addition to automatic variant calling analysis, carried out by using SiRe® panel specific optimized variant caller plug-in (v.5.0.2.1) parameters, BAM files are visually inspected with the Golden Helix Genome Browser v.2.0.7 (Bozeman, MT, USA).

In this current study, to assess the robustness and the reliability of SiRe® panel on blood, cases showing *KRAS* mutations were evaluated from an analytical point of view, taking into account several run metric parameters; in particular, number of total reads, mean read length, number of mapped reads, percentage of reads on target, average reads per amplicon and uniformity of amplicon coverage were evaluated for each *KRAS* mutated case.

Results

SiRe® NGS analysis

On the overall, 36 (18.6%) out of the 194 liquid biopsies harbored a *KRAS* point mutation either in exon 2 or in exon 3 (*Table 1*). No concomitant mutations were observed in the other NSCLC clinical relevant genes included in the SiRe® panel, such as *EGFR* and *BRAF*.

Clinical data of *KRAS* mutant cases are reported in *Table 2*. Briefly, patients' age widely ranged (36 to 89 years), with a median value of 66.9 years (*Table 2*). Moreover, *KRAS* mutations were more frequent in male than female patients (24 *vs.* 12; 66.7% *vs.* 33.3%). The overall median allelic frequency (AF) was 5.0% (ranging between 0.2% and 46.8%) (*Table 3*).

Most *KRAS* mutations occurred in exon 2 (33/36, 91.7%) whereas exon 3-point mutations were less frequently (3/36, 8.3%) (*Tables 1* and *3*). *KRAS* exon 2 p.G12C was the most common mutation (13/36, 36.1%). The other mutations are reported in *Tables 1* and *3*. *KRAS* exon 2 p.G12C was identified mostly in male patients (8/13, 61.5%), whereas female were a lower number of mutated cases was evaluated (5/13, 38.5%). The median age of patients harboring *KRAS* exon 2 p.G12C was 63.5 (ranging between 48 and 74 years). The median AF of *KRAS* exon 2 p.G12C was 8.0% (ranging between 0.6% and 46.8%) (*Table 3*).

NGS analytical performance

NGS run parameters in *KRAS* mutated cases were evaluated in order to assess the analytical performance of the SiRe® panel. Taking into account all 36 mutant cases, an average of 152371.03 (ranging from 21306.00 to 477906.00) reads per sample was obtained. The median read length was of 133.92 bp (ranging from 95.00 to 209.00). Concerning the number of mapped reads, an average of 151697.61 (ranging from 21126.00 to 476909.00) was obtained, with a 95.64% reads on target (ranging from 86.60% to 99.08%). Considering amplicon parameters, an average reads per amplicon of 3416.26 (ranging from 488.50 to 10048.00) was evaluated with a 98.28% uniformity of amplicon coverage (ranging from 95.24% to 100.00%) (*Table 4*).

Discussion

Our data obtained in real life, generated from a review of routine clinical reports, underlined the technical feasibility of *KRAS* mutation assessment in plasma samples. In fact, in advanced stage NSCLC treatment-naïve patients, the

Table 3 Correlation among patients' age, sex and *KRAS* mutational status

Samples	Age	Sex	*KRAS* mutation (AF)
1	36	F	p.G12D (1.5%)
2	64	M	p.G12C (1.3%)
3	48	M	p.G12C (0.6%)
4	59	F	p.G12D (1.3%)
5	89	M	p.G12D (7.2%)
6	62	M	p.G12C (5.6%)
7	64	M	p.G13S (0.2%)
8	76	F	p.G13D (0.3%)
9	69	M	p.G12S (6.4%)
10	65	M	p.G12C (3.3%)
11	84	M	p.G13S (0.2%)
12	74	F	p.G12C (24.0%)
13	73	F	p.G12C (0.9%)
14	49	M	p.G12S (1.4%)
15	61	F	p.G12C (4.6%)
16	69	M	p.G12C (46.8%)
17	70	M	p.G12S (7.1%)
18	65	F	p.G12V (24.0%)
19	68	M	p.G12D (0.9%)
20	58	M	p.G12C (3.9%)
21	65	F	p.G12C (2.5%)
22	69	M	p.G13D (0.4%)
23	73	M	p.G12C (2.9%)
24	84	M	p.G13S (0.6%)
25	70	M	p.G13S (0.7%)
26	65	M	p.G13D (4.2%)
27	63	M	p.G12C (4.7%)
28	78	M	p.G12D (2.0%)
29	77	M	p.G12A (0.7%)
30	81	F	p.G12V (1.0%)
31	51	F	p.G12C (3.1%)
32	68	M	p.G13D (0.7%)
33	52	F	p.G12V (4.6%)
34	70	M	p.Q61H (0.2%)
35	77	M	p.Q61H (4.7%)
36	64	F	p.A59V (0.2%)

KRAS, Kirsten Rat Sarcoma Viral Oncogene Homolog; AF, allelic frequency; F, female; M, male.

customized NGS SiRe® panel offers a valuable option to select, for targeted treatments, patients that despite the morphological evidence of NSCLC do not have tissue availability for molecular diagnosis (*Figure 1*). Diagnostic algorithms are well designed for *EGFR* testing, as liquid biopsy is recommended as the initial procedure whenever tissue is not available (32). Conversely, clinical procedures are less well established to detect *KRAS* exon 2 p.G12C as an actionable biomarker. To date, *KRAS* testing in advanced stage NSCLC patients has been performed either to provide prognostic information (33), or to rule out less common driver alterations, to make lung biomarker testing more effective. More recently, *KRAS* exon 2 mutations also acquired a novel predictive significance. In particular, promising results have been shown by a novel drug (AMG510) administered to advanced stage NSCLC patients harboring a *KRAS* exon 2 p.G12C point mutation (24). Were these very promising data confirmed in a phase III clinical trial, it would be conceivable that *KRAS* mutational testing will soon be mandatory in clinical practice. Currently, in the experience of our laboratory, a referral center for lung cancer biomarker testing, tissue specimens are often (17%) rejected for insufficient cellularity (34). Thus, it is conceivable that *KRAS* testing will be often performed on ctDNA. In this setting, multiplexing is crucial to ensure a complete genomic profile. In addition, when evaluating cases wild type for *KRAS* mutations, the possibility to detect *EGFR* and *BRAF* mutations would be very useful to distinguish between real *KRAS* wild type samples from those tumors that do not shed ctDNA into the bloodstream. In other words, testing plasma for *KRAS* mutations has a double role: first, to select patients for AMG510; second, to make the molecular status assessment of *EGFR* and *BRAF* more cost-effective. In fact, considering our data, a not negligible subset of patients (6.7%; 13/194) of cases showing a *KRAS* exon 2 p.G12C point mutation may undergo a KRAS TKI treatment, while an additional 11.9% (23/194) of cases, showing other common *KRAS* alterations, would not need an *EGFR* and *BRAF* molecular test.

Despite our efforts to provide an accurate and comprehensive evaluation of the role of *KRAS* testing on liquid biopsy, the main limitation of this study, relies on its retrospective design. In addition, it was not possible to correlate the mutational status assessed on plasma with information relative to tissue, since matched cytohistological samples were not available. However, the aim of this study was to provide a snapshot of *KRAS* mutational evaluation on liquid biopsy in advanced stage NSCLC patients in routine

Table 4 NGS parameters

Samples	Reads	Mean read length (bp)	Number of mapped reads	Percent reads on target (%)	Average reads per amplicon	Uniformity of amplicon coverage (%)
1	163,218.00	128.00	162,752.00	98.06	3,800.00	97.62
2	151,445.00	127.00	150,370.00	97.03	3,474.00	97.62
3	103,023.00	128.00	102,573.00	97.79	2,388.00	97.62
4	71,556.00	129.00	71,360.00	97.54	1,657.00	97.62
5	21,306.00	127.00	21,126.00	97.12	488.50	97.62
6	113,202.00	130.00	112,930.00	98.01	2,635.00	97.62
7	348,181.00	135.00	347,148.00	97.91	8,093.00	97.62
8	72,635.00	128.00	72,364.00	97.77	1,685.00	97.62
9	133,275.00	132.00	132,908.00	97.57	3,087.00	97.62
10	92,859.00	126.00	91,962.00	96.90	2,122.00	97.62
11	121,037.00	129.00	120,379.00	97.44	2,793.00	97.62
12	372,007.00	128.00	371,444.00	99.08	8,762.00	100.00
13	261,348.00	128.00	260,930.00	97.99	5,485.00	100.00
14	77,389.00	125.00	77,178.00	98.13	1,735.00	100.00
15	477,906.00	128.00	476,909.00	98.56	10,048.00	100.00
16	246,262.00	146.00	245,509.00	97.77	5,715.00	100.00
17	82,079.00	124.00	81,537.00	94.06	1,826.00	100.00
18	96,798.00	128.00	94,712.00	94.23	2,125.00	100.00
19	77,020.00	123.00	76,284.00	93.81	1,704.00	100.00
20	88,066.00	123.00	87,374.00	95.04	1,977.00	100.00
21	140,493.00	209.00	139,914.00	90.27	3,007.00	95.24
22	167,791.00	204.00	166,706.00	87.79	3,485.00	95.24
23	90,000.00	202.00	89,342.00	86.60	1,842.00	95.24
24	72,172.00	127.00	71,371.00	96.28	1,636.00	100.00
25	37,987.00	95.00	37,234.00	96.33	854.00	100.00
26	123,333.00	129.00	122,705.00	93.95	2,745.00	97.62
27	51,115.00	126.00	50,886.00	95.43	1,156.00	95.24
28	318,977.00	129.00	317,125.00	96.63	7,296.00	95.24
29	175,337.00	128.00	174,508.00	94.37	3,921.00	97.61
30	250,685.00	126.00	249,571.00	92.27	5,483.00	100.00
31	167,650.00	128.00	167,391.00	94.62	3,771.00	100.00
32	97,953.00	126.00	97,331.00	91.84	2,128.00	100.00
33	281,066.00	133.00	280,645.00	92.92	6,209.00	100.00
34	173,021.00	127.00	172,314.00	97.55	4,002.00	97.62
35	47,845.00	130.00	47,653.00	97.32	1,104.00	97.62
36	119,320.00	130.00	118,669.00	97.21	2,747.00	97.62

NGS, next generation sequencing; bp, base pair.

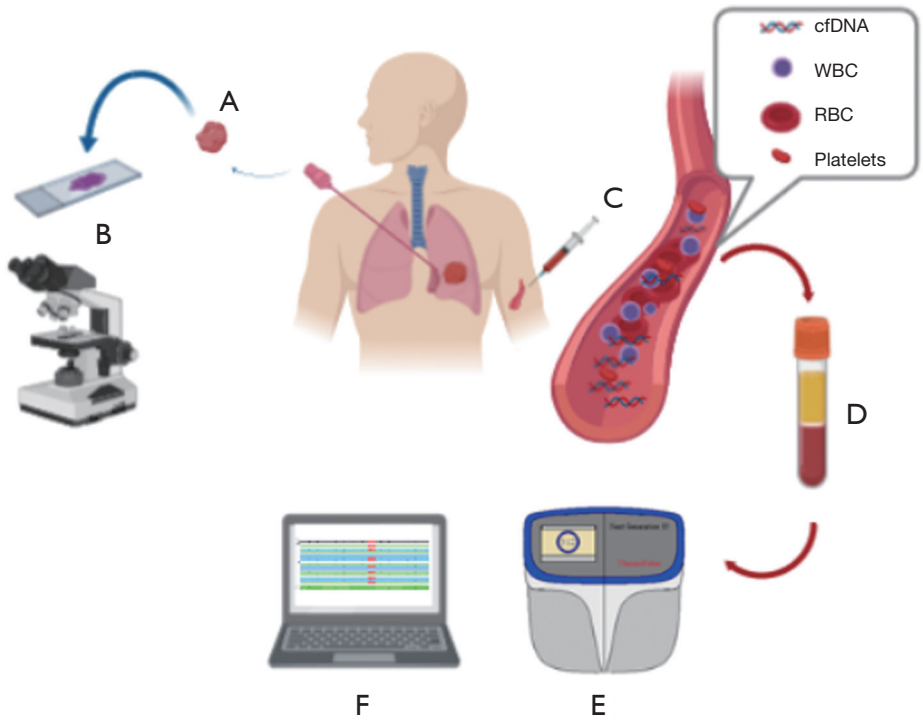

Figure 1 If tissue samples (histological or cytological) were inadequate or insufficient at morphological evaluation for molecular analysis (A, B), liquid biopsy may represent a useful tool (C). In our study, after blood withdrawn (C), plasma was obtained after centrifugation (D) and extracted ctDNA was further analyzed by using our next generation sequencing panel (SiRe®) on S5 platform (Thermo Fisher Scientific, Waltham, MA) (E). Visual inspection of the sequencing reads was performed by using the Golden Helix Genome Browser v.2.0.7 (Bozeman, MT, USA) (F). (Credit: Created with Biorender).

setting. In fact, data relative to the previous validation study performed on matched tissue and blood samples strongly suggested that SiRe® NGS panel can be a reliable tool in clinical practice even when tissue samples are not available.

In conclusion, our data confirm that SiRe® NGS panel represents a robust analytical tool to assess *KRAS* mutational status on ctDNA. Further investigation is required to design more cost-effective diagnostic algorithms to harmonize clinical relevant biomarker testing on tissue and blood in advanced stage NSCLC clinical practice.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All information regarding human material was managed using anonymous numerical codes, and all

samples were handled in compliance with the Declaration of Helsinki (as revised in 2013). According to the aforementioned National Guidelines, the study did not require an Ethical Committee approval since it did not modify the patient clinical management.

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