Awareness of mutational artefacts in suboptimal DNA samples: possible risk for therapeutic choices

Sara Mariani(1)*, Luca Bertero(1)*, Vittoria Coppola(1), Giorgio Saracco(2), Alberto Arezzo(3), Paola Francia di Celle(4), Jasna Metovic(1), Caterina Marchiò(1) and Paola Cassoni(1).

*SM and LB equally contributed to the study

Affiliation:

(1) Department of Medical Sciences, University of Turin and Pathology Unit, Città della Salute e della Scienza Hospital, Torino, Italy

(2) Department of Medical Sciences, University of Turin and Gastroenterology Unit, Città della Salute e della Scienza Hospital, Torino, Italy

(3) Department of Surgical Sciences, University of Turin and Surgical Unit, Città della Salute e della Scienza Hospital, Torino, Italy

(4) Pathology Unit, Città della Salute e della Scienza Hospital, Torino, Italy.

Running title: Mutational artefacts in suboptimal samples

Corresponding author:

Dr. Luca Bertero

Pathology Unit, Department of Medical Sciences, University of Turin

Via Santena 7, Torino 10126, Italy

E-mail: luca.bertero@unito.it; phone: +39-011-6335438; fax: +39-011-6635267

Abstract

BACKGROUND: Technical biases due to PCR artefacts could represent an insidious obstacle for mutational analysis and precision medicine.

METHODS: We report a retrospective analysis by fast COLD-PCR and sequencing of 31 suboptimal tumor DNA samples obtained from FFPE tissues and liquid biopsies.

RESULTS: In FFPE tumor tissues and plasma liquid biopsies of patients with lung and colorectal adenocarcinoma, we observed a significant rate of artefactual *KRAS* mutations, unveiled by repeated analysis following UDG pretreatment as well as by simple repetition without UDG pretreatment step, thus suggesting a DNA damage different from cytosine deamination. UDG pretreatment was not only unnecessary to contrast artefacts occurrence, but also hampered the efficiency of mutational screening, reducing the analytical sensitivity. Taken individually or considered together, the reduced DNA input per reaction and UDG pretreatment limited the detection of “real” mutated alleles, decreasing PCR sensitivity enough to hamper distinction between artefactual and true subclonal mutations of *KRAS*.

CONCLUSIONS: Careful validation of analytical sensitivities should always be carried out through standard controls, and strategies other than UDG pretreatment need to be identified to avoid both amplification of artefactual mutations and failure to identify real subclonal mutations.

Keywords: UDG, mutational artefacts, PCR, liquid biopsy, sequencing, FFPE

INTRODUCTION

Cancer is a heterogeneous disease, as documented by morphological assessment, biomarker evaluation and changes in tumor molecular profile over time and between different tumor sites (primary *versus* metastatic lesions). Molecular characterization of different cancer types based on next generation sequencing (NGS) analysis recently provided evidence of such heterogeneity, even at base-pair level. In the era of precision medicine, tumor heterogeneity may significantly hamper therapy efficacy and limit response to treatments (McGranahan *et al*, 2017). “Liquid biopsy” is one of the recently introduced approaches aimed at recapitulating intratumoral heterogeneity by analysis of tumor-derived cell-free DNA (cfDNA) circulating in different biological fluids as plasma, urine and cerebrospinal fluid (CSF) (Siravegna *et al*, 2017).

Several studies have demonstrated a high concordance between mutational profiles of candidate genes in matched tumor and plasma DNA samples from patients with breast cancer (Bettegowda *et al*, 2014; Higgins *et al*, 2012; Dawson *et al*, 2013), colorectal cancer (Diehl *et al*, 2008; Misale *et al*, 2012, Thierry *et al*, 2014), and non-small cell lung cancer (NSCLC) (Narayan *et al*, 2012; Khoo *et al*, 2016). Brain metastases of different primary tumor-types are well recapitulated in their DNA alterations by cfDNA circulating in CSF rather than in plasma, supporting the idea that in some circumstances tumour-shed DNA is preferentially confined to specific biological fluids (Brastianos *et al*, 2015; De Mattos-Arruda *et al*, 2015). Therefore, liquid biopsies can be exploited for diagnostic purposes, to identify and track tumour-specific alterations during the course of the disease, and to address therapeutic choices (Siravegna *et al*, 2017). Furthermore, several experimental studies proved the efficacy of liquid biopsy samples in monitoring minimal residual disease after neoadjuvant treatment (Venesio *et al*, 2017) as well as in early tumor detection (Phallen *et al*, 2017).

Besides this wide range of potentialities, liquid biopsies hold some possible drawbacks. A major limiting concern resides in the low amount of cfDNA available: ultra-sensitive assays have to be employed, which are intrinsically more prone to produce mutation artefacts, especially when applied to scarce DNA quantities (Wong *et al,* 2014). In this scenario, it is mandatory to assure the non-artefactual nature of the detected mutations. In most of cases in which DNA is fragmented or degraded, mutation artefacts have been reported to depend on cytosine deamination (Williams *et al*, 1999), and seemed accordingly preventable by uracil DNA glycosylase (UDG) pretreatment of DNA.

In this study, we aimed to assess the extent of artefactual PCR amplification due to cytosine deamination in DNA derived from FFPE tissues and liquid biopsies (plasma or CSF) in samples previously determined as *KRAS* mutated. The hypothetical occurrence of artefactual mutations not due to stable DNA damages was investigated by simple repetitions of PCR amplification and sequencing without UDG, to assess whether and to which extent the latter was effective in preventing artefacts.

MATERIALS AND METHODS

Patients and specimen characteristics

We retrieved 31 DNAs previously extracted from formalin fixed and paraffin embedded (FFPE) tumor tissues (n = 22), plasma (n = 7) or CSF-derived liquid biopsies (n = 2). Selection of DNA specimens has been based on positivity for *KRAS* mutations at exon 2 (one of the most frequent hotspot sequence involved in mutational events in lung and in colorectal adenocarcinomas as well) and on belonging to "borderline" samples in terms of: i) suboptimal neoplastic/normal cells ratio or overall neoplastic cells number (according to the Italian guidelines for enrichment of tumor tissue samples to perform DNA sequencing, https://testbiomolecolari.it/), ii) tumor heterogeneity (very frequent in colorectal cancer) or iii) low tumor DNA enrichment (like in liquid biopsies). These conditions were supposed to favor false positive results due to PCR artefacts.

The 31 specimens were derived as follows: 10 FFPE tumor tissues, 7 plasma and 2 CSF samples belonged to 18 patients with lung adenocarcinoma [the latter 2 with central nervous system (CNS) metastases]. The remaining 12 FFPE tumor tissues belonged to patients described in the recent Mariani and coll. paper (Mariani *et al*, 2017) and they consisted of metastatic colorectal cancers (mCRCs) subclonally mutated at *KRAS,* whose mutations resulted artefactual after pretreatment of DNA with UDG.

Clinical characteristics of all patients and specimens, including their *KRAS* sequence at exon 2 hotspots, are reported in Table 1.

Sample preparation

The analyses were repeated on residual DNA originally extracted and tested, thus excluding a possible bias due to different dissection.

In FFPE tissues, selections of tumor areas were originally performed as previously described (Mariani *et al*, 2015) on archival slides used for cytological or histopathological diagnosis. The criteria of morphological adequacy to perform DNA sequencing were derived from the Biogate portal (https://testbiomolecolari.it/) of the Italian Associations of Medical Oncologists and Pathologists (AIOM-SIAPEC-IAP). Specimens were recorded as acceptable when tumor enrichment was above the 50% (neoplastic/normal cells ratio) and at least 100 neoplastic cells were present. The DNA of the FFPE blocks was derived from two 10 μm thick sections that were macrodissected and kept in Eppendorf tubes.

For the analysis of liquid biopsies, 7 ml of blood or 1-2 ml of CSF from lumbar puncture were obtained in the context of routine clinical management of patients. Blood was collected in tubes containing EDTA. Both types of liquid biopsies were centrifuged twice at 2000g at 4°C for 10' to obtain plasma and tumor cells,

respectively. CSF supernatant, usually representing a waste material, was collected in new tubes and stored like plasma at -80°C before extraction of circulating DNA. The study was submitted to and approved by the ethic Institutional Review Board for "Biobanking and use of human tissues for experimental studies" of the Department of Medical Sciences, University of Turin. Due to the retrospective approach of the study and since there was no impact on patients' treatment, a verbal informed consent was requested. All the cases were anonymously recorded. The IRB approved this consent procedure.

DNA extraction from tissue and liquid biopsies

FFPE tumor sections were submitted to DNA extraction (Maxwell16 FFPE Tissue LEV DNA Purification Kit, Promega, Madison, WI, USA) on a Maxwell16 Instrument (Promega, Madison, WI, USA). DNA was eluted in a final volume of 58 µl, and the concentration/purity was measured by a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

cfDNAs circulating in plasma or CSF were extracted from 1 ml of liquid biopsy on a Maxwell 16 instrument (Promega, Madison, WI, USA) using the Circulating Nucleic Acid purification kit (Promega, Madison, WI, USA), according to the manufacturer's instructions and eluted in 50 µl of buffer. Concentration measurement was not required for the following analysis.

***EGFR* and *KRAS* gene mutation analysis**

In suboptimal tumor-enriched tissues and in liquid biopsies of patients with lung adenocarcinoma, *EGFR* gene sequences were evaluated at exons 18-21 with the Easy *EGFR* kit (Diatech Pharmacogenetics, Jesi, Italy), according to the manufacturer's instructions. In colorectal cancers, *KRAS* mutations at exons 2-4 were analyzed by Sequenom mass spectrometry using the Myriapod Colon Status kit, according to the manufacturer's instructions (Diatech Pharmacogenetics, Jesi, Italy). All mutations revealed by mass spectrometry were confirmed by standard PCR and DNA sequencing as reported elsewhere (Mariani *et al*, 2015; Mariani *et al*, 2017). In wild-type samples of both lung and colorectal tumors, *KRAS* mutations at exon 2 were investigated with fast COLD-PCR and DNA sequencing, as described below.

Fast COLD-PCR and DNA sequencing at exon 2 of *KRAS*

Fast COLD-PCR for exon 2 of *KRAS*, followed by DNA sequencing was applied to tumor DNA derived from tissues or liquid biopsies which resulted *EGFR* and *KRAS* wild-type by the previously described, less sensitive methods. The protocol of analysis was derived from Mancini and coll. (Mancini *et al*, 2010). We

amplified 30 ng of DNA or 10 µl of cfDNA, in absence of fluorophores in the reagent mix. Finally, the PCR conditions were modified as follows: 20 cycles of standard PCR (95.0 °C 8'', 60.0 °C 30'', 72.0 °C 30'') followed by 35 cycles of COLD-PCR (82.5 °C 8'', 58.0 °C 30'', 72.0 °C 30''), in which the denaturation step was set at a critical dissociation temperature, favoring the amplification of the mutated sequences. The amplified products were submitted to DNA sequencing as elsewhere reported (Mariani *et al,* 2017). Repetitions of fast COLD-PCR amplifications and sequencing in the presence or absence of UDG pretreatment were applied to DNA samples resulted *KRAS* mutated after a first analysis. UDG pretreatment consisted of an incubation of DNA at 37°C for 30' just before fast COLD-PCR amplification scheme, according to the protocol described by Pierce and coll. (Pierce *et al*, 2004).

Construction of standard DNAs with different mutated allele frequency and concentrations (mimicking DNA input from suboptimal tumor-enriched tissue and liquid biopsy samples)

Positive DNA controls with progressively decreasing mutated allele frequencies (MAFs) were prepared at concentrations of 6 ng/µl mixing a standard FFPE tissue-derived *KRAS* mutated DNA (Horizon, Cambridge, UK) with a control *KRAS* wild-type DNA (Diatech Pharmacogenetics, Jesi, Italy). The expected mutation was at position c.34G>T (p.Gly12Cys) and the six controls generated had the following MAFs: 30%, 10%, 3%, 0.3%, 0.1% and 0%. From each of these six DNA controls, by diluting samples with pure water, we obtained other standards the concentrations of which were 6 pg/µl. The two concentrations obtained (6 ng/µl and 6 pg/µl) were useful to mimic input DNA quantities typical of FFPE- or liquid biopsy-derived samples, respectively. All 12 standard DNAs were prepared for establishing the limit of detection (LOD) of the expected *KRAS* mutation when submitted to fast COLD-PCR and DNA sequencing in conditions of absence or presence of UDG.

RESULTS

Fast COLD-PCR generates only few artefactual nucleotide substitutions affecting the *KRAS* gene in FFPE-derived DNA of lung tumors

DNAs extracted from 10 archival FFPE tissue samples of patients with lung adenocarcinoma, suboptimal for standard mutational analysis due to their low percentage (<50%) or number (<100) of neoplastic cells, were selected because they previously resulted *EGFR* wild-type by real-time PCR amplification, but *KRAS* mutated at codon 12 (all but one with MAF > 3%) by fast COLD-PCR followed by DNA sequencing (Table 2a, row A). When fast COLD-PCR and DNA sequencing were repeated in the samples (n = 9, one sample excluded because of failed analysis) in presence (Table 2a, row B) or absence (Table 2a, row C) of UDG

pretreatment, 1/9 (11%; the only sample harboring a MAF <3%) resulted wild-type (data not shown) in both conditions. This finding suggests that the originally diagnosed base-pair substitution (c.35G>A, p.Gly35Asp) was artefactual in nature and that it originated from PCR errors rather than from cytosine deamination, since randomly abolished even in absence of UDG. In the remaining 8/9 (89%) samples the original mutations were confirmed, including one sample showing a G>T substitution at a MAF of 3%. Nevertheless, in confirmed samples the mutated/wild-type allele ratio seemed to be reduced by the UDG pretreatment (Figure 1).

Plasma-circulating cell-free DNA, unlike CSF cell-free DNA, is prone to PCR artefacts

Since characterized by low amount and quality, we reasoned that cfDNA obtained by liquid biopsy was particularly interesting to be analysed for mutational artefacts. Therefore, *KRAS* gene sequence at codon 12 was investigated by fast COLD-PCR in 7 plasma-derived cfDNA samples extracted from blood of patients with lung adenocarcinoma, having suboptimal/unavailable FFPE tissue samples at diagnosis. In 5/7 samples a KRAS mutation was found (Table 2b, row A). In 4 out of these 5 positive samples (80%), PCR and sequencing repetitions in presence or absence of UDG pretreatment did not confirm the presence of a *KRAS* mutation. The previously identified KRAS mutation was therefore revealed as artefactual and independent of cytosine deamination (Table 2b, rows B-C).

Recently, we reported that in two patients with *KRAS* mutated lung tumors metastatic to the CNS the tumor DNA recirculated preferentially in CSF than in plasma (Table 2c, row A) (Marchiò *et al*, 2017). In the present work we performed a re-evaluation by fast COLD-PCR and DNA sequencing in presence or absence of UDG pretreatment of both cfDNAs previously extracted from CSF samples. The aim of the experiment was to evaluate the real or artefactual existence of *KRAS* mutations in this low DNA-yield fluid, since it has never been investigated before. Both CSF liquid biopsies were confirmed to be *KRAS* mutated (Table 2c, rows B-C), excluding in these samples the occurrence of artefacts due to stable DNA damage or to random errors of PCR amplifications.

Independence of mutation artefacts from tissue of origin or UDG prevention

We explored whether the occurrence of these artefactual mutations could be tissue-specific. Since we recently demonstrated the occurrence of UDG-sensitive *KRAS* mutated artefacts in 9/12 (75%) colorectal cancer samples (Mariani *et al*, 2017), a re-evaluation of those DNA sequences at exon 2 of *KRAS* was conceived in absence of UDG pretreatment to better understand the origin of those artefacts. The new experiment confirmed the artefactual nature of the *KRAS* mutations even in absence of UDG pretreatment

(data not shown), suggesting the same randomly PCR-associated origin of mutation artefacts in colorectal cancers as observed in lung cancers, thus excluding cytosine deamination. Taken together, these data suggest that these types of artefacts are likely to be independent from the tumor site and are not prevented by UDG pretreatment.

The limit of detection of *KRAS* mutations by fast COLD-PCR is influenced by UDG pretreatment and by quantity of input DNA per reaction

As reported above, UDG was not useful to identify falsely mutated samples in our cohorts. In addition, since "real" mutated samples presented decreasing mutated/wild-type allele ratio in presence of UDG (Figure 1), we sought to understand whether the UDG pretreatment step not only was unnecessary, but could be instead deleterious. An experiment was designed, applying UDG pretreatment to standard controls of DNA, constructed with decreasing MAFs and diluted at two concentrations mimicking FFPE- and liquid biopsy-derived DNA quantities, respectively. As reported in Figure 2, without UDG (UDG- column) the maximum LOD reached using different DNA input per reaction (Panel A, 30 ng; Panel B, 30 pg) was 0.3% and 10%, respectively. After UDG pretreatment (Figure 2, UDG+ column), the respective LOD at each DNA concentration (Panel A, 30 ng; Panel B, 30 pg) was 3% and more than 10% (likely 20%, indicative value estimated by comparing the mutated/wild-type allele ratios in UDG+ and UDG- columns of Figure 2B), respectively. Taken together, these results demonstrate that the procedure of fast COLD-PCR for *KRAS* followed by DNA sequencing was less effective in detecting the expected and truly present mutation as a consequence of UDG pretreatment and when the quantity of DNA per reaction was decreased from the order of ng (as for FFPE-tissue derived DNA) to that of pg (as for plasma- or CSF-derived cfDNA).

DISCUSSION

Spatial and temporal molecular heterogeneity represents an intrinsic characteristic of solid tumors (Wei *et al*, 2017) hampering the efficacy of personalized medicine and mandating new and diversified schemes of targeted therapies (Senft *et al*, 2017).

Resistance to a targeted treatment may stem from selection of tumor subclones with specific mutations detectable by liquid biopsy: this approach is currently used in lung adenocarcinoma for prompt adjustments of treatment. Of note, the relatively scarce quantities of circulating tumor DNA (ctDNA) in liquid biopsies require highly sensitive analytical methods for mutational analysis, thus increasing the possible emergence of artefacts during PCR (Wong *et al*, 2014; Lamy *et al*, 2011). If on one side the effect of formalin fixation on DNA and the consequent outcome on PCR artefacts is well known (Wong *et al*, 2014; Lamy *et al*, 2011; Do

et al, 2012; Do *et al*, 2013), on the other side the order of magnitude of this phenomenon is yet to be clarified, both in ctDNA and in FFPE tissue-derived DNAs.

Since artefactual DNA calls may affect the selection of patients treatable with specific targeted therapies and confound the evaluation of response to treatment, in the present study we sought to: i) track the existence and nature of false *KRAS* mutations in tissue samples and liquid biopsies of lung tumors; ii) investigate whether artefact occurrence could be tissue-type specific, comparing lung and colorectal cancer DNAs. In particular, we focused on *KRAS* mutations at exon 2 due to their high incidence in both diseases and because a particularly sensitive assay was available to detect even subclonal *KRAS* mutations, which could be suspected to have an artefactual origin.

Tissue-derived tumor DNAs already identified as *KRAS*-mutated by highly sensitive fast COLD-PCR amplification and sequencing were retested: a simple PCR replica without UDG pretreatment was sufficient to identify a mutation artefact in one of the analysed samples harbouring a MAF below 3%. Similarly, PCR repetitions without UDG pretreatment unveiled the artefactual nature of *KRAS* mutations detected in colorectal cancers and previously attributed to cytosine deamination (Mariani *et al*, 2017). PCR repetitions without UDG pretreatment also demonstrated 80% of the *KRAS* mutations detected in plasma liquid biopsies to be mere artefacts. Thus, the presence of *KRAS* mutational artefacts encompassed different tumor tissues (lung and colon) and sample-types (FFPE tissues and plasma liquid biopsies).

As revealed even by a simple PCR replica, false G>A transitions, primarily found in plasma DNA samples, and false G>T transversions, which we observed as particularly frequent in FFPE-tissue samples, were not stable damages as one would have expected (Lamy *et al*, 2011). The latter can usually derive either from a guanine oxidation to 8-hydroxyguanine (Lindahl *et al*, 1993) or from randomly generated errors by Taq DNA polymerases during PCR (Stiller *et al*, 2006), through a process called “PCR jumping or template switching” (Pääbo *et al*, 1990). The random nature of the detected artefacts seems to suggest the second hypothesis. It should be noted that different Taq polymerases may have the property of avoiding PCR errors or correcting chemical-induced base substitutions during PCR (Stiller *et al*, 2006) by a 3'-5' exonuclease activity, i.e. a proofreading activity. Our Taq polymerase did not feature this function.

Based on the very low DNA amount and high fragmentation (Wong *et al*, 2014; Lamy *et al*, 2011), plasma ctDNAs produced a higher percentage of artefactual *KRAS* mutations compared to FFPE tissue-derived DNAs. Conversely, liquid biopsies from CFS samples seemed to be devoid of *KRAS* mutation artefacts. Although these data were accrued on a limited number of samples and should be confirmed on a larger scale, we envisage, as already reported (Marchiò et al, 2017), that CSF is representative of a closed anatomical space in which ctDNA, although at low quantity, is presumably homogeneous in sequence, due

to a relative absence of a normal DNA background. This condition apparently favors a good and reliable PCR amplification of ctDNA templates.

In a way akin to colorectal carcinomas (Mariani *et al*, 2017), in lung carcinoma samples a MAF cutoff value of 3% seemed effective in discriminating artefactual from real *KRAS* mutations. Data reported by independent groups (Molinari *et al*, 2011; Kimura *et al*, 2012; Dono *et al*, 2013; Tougeron *et al*, 2013; Laurent-Puig *et al*, 2015; Normanno *et al*, 2015, Van Cutsem *et al*, 2015) suggested that low frequency mutations are not detrimental for clinical response to cetuximab or panitumumab in patients with colorectal cancers, and a MAF of *KRAS* below 3% seems to lack any negative impact on response to treatment and survival, thus most likely representing a grey zone attributable to artefacts. The impact of subclonal *KRAS* mutations to TKI therapy has not been clarified in lung cancers to date (McGranahan *et al*, 2015).

Although in our cohort artefacts were not due to stable damages of DNA, UDG pretreatment remains the only useful method to account for mutations secondary to cytosine deamination. Nevertheless, in our hands UDG pretreatment reduced the efficiency of fast COLD-PCR in identifying real KRAS mutations, completely hampering their detection at allele frequencies below 3% and 20% in tissue- and liquid biopsy-mimicking samples, respectively. Below these frequencies UDG pretreatment turned into wild-type both subclonal PCR artefacts and true mutations. Our results suggest that UDG employment requires a word of caution because of its potentially deleterious effects, conversely to the previous observations concerning its use before different PCR techniques ranging from real-time PCR or high-resolution melting analysis (HRMA) to NGS (Pierce et al, 2004; Lamy et al, 2011; Do et al, 2013; Chen et al, 2014; Pérez-Báez et al, 2017; Kim et al, 2017). None of the previously published studies reported specific experiments aimed at assessing the effect of UDG on PCR efficiency, in particular at low MAFs. In view of the increasing interest in adopting NGS methodologies into the daily routine diagnostic practice, samples with scarce amounts of fragmented DNA amplified by PCR at low coverage (Wong et al, 2014) may be still reliably tested provided that a robust program of “reads” analysis is guaranteed (Kockan et al, 2017).

In conclusion, our results demonstrate that mutational artefacts frequently occur in plasma-derived cfDNAs obtained from patients with lung tumors. This is of particular relevance in these patients because liquid biopsy is currently preferred to tissue biopsy at disease relapse, for the detection of mutations (i.e. *EGFR* p.Tyr790Met) associated with resistance to TKIs of first and second generation, in order to guide the therapeutic choices.

From a technical standpoint, our data suggest that PCR should be repeated in absence and presence of UDG pretreatment to prevent false mutations and to discriminate stable from random artefacts. Stable artefacts, but not real mutations beyond an allele frequency threshold (which we have demonstrated to vary

based on assay type and DNA quantity and quality), would disappear only in presence of UDG. Random artefacts are prevented by simple PCR replica or by comparing results from multiple assays and their occurrence should also be easily avoided by using Taq DNA Polymerases with proofreading activity.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by the Italian Ministry of University (ex-60% 2016-2017 to SM and ex 60% 2016 and 2015HAJH8E to CM) and by Rete Oncologica Piemonte e Valle d'Aosta (to PC).

Author contributions

Conception and design: SM, LB, CM, PC. Analysis and interpretation of the data: SM, LB, VC, GS, AA, PFdC, CM, PC. Drafting of the paper or revising it critically for intellectual content: SM, LB, VC, GS, AA, PFdC, JM, CM, PC. Final approval of the version to be published: SM, LB, VC, GS, AA, PFdC, JM, CM, PC. All authors agree to be accountable for all aspects of the work.

KEY ISSUES

- *KRAS* artefactual mutations seem to occur frequently in suboptimal FFPE tissue samples and plasma liquid biopsies of different tumor types, but not in CSF liquid biopsies.
- These artefactual mutations can be unveiled by simple analysis repetition, even without UDG pretreatment, thus suggesting a DNA damage different from cytosine deamination.
- UDG pretreatment and lower DNA input affected the efficiency of mutational screening by increasing allele limit of detection both in tissue and liquid samples.
- PCR sensitivity can be decreased enough to hamper distinction between artefactual and true subclonal mutations of *KRAS*, with possible consequences on patients' therapeutic management.

- Careful validation of analytical sensitivities should always be carried out and strategies other than UDG pretreatment need to be identified.

REFERENCES

1) Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, Bartlett BR, Wang H, Luber B, Alani RM, Antonarakis ES, Azad NS, Bardelli A, Brem H, Cameron JL, Lee CC, Fecher LA, Gallia GL, Gibbs P, Le D, Giuntoli RL, Goggins M, Hogarty MD, Holdhoff M, Hong SM, Jiao Y, Juhl HH, Kim JJ, Siravegna G, Laheru DA, Lauricella C, Lim M, Lipson EJ, Marie SK, Netto GJ, Oliner KS, Olivi A, Olsson L, Riggins GJ, Sartore-Bianchi A, Schmidt K, Shih lM, Oba-Shinjo SM, Siena S, Theodorescu D, Tie J, Harkins TT, Veronese S, Wang TL, Weingart JD, Wolfgang CL, Wood LD, Xing D, Hruban RH, Wu J, Allen PJ, Schmidt CM, Choti MA, Velculescu VE, Kinzler KW, Vogelstein B, Papadopoulos N, Diaz LA Jr. (2014) Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med* **6**(224): 224ra24.

2) Brastianos P, Carter S, Santagata S, Cahill DP, Taylor-Weiner A, Jones RT, Van Allen EM, Lawrence MS, Horowitz PM, Cibulskis K, Ligon KL, Tabernero J, Seoane J, Martinez-Saez E, Curry WT, Dunn IF, Paek SH, Park SH, McKenna A, Chevalier A, Rosenberg M, Barker FG, Gill CM, Van Hummelen P, Thorner AR, Johnson BE, Hoang MP, Choueiri TK, Signoretti S, Sougnez C, Rabin MS, Lin NU, Winer EP, Stemmer-Rachamimov A, Meyerson M, Garraway L, Gabriel S, Lander ES, Beroukhim R, Batchelor TT, Baselga J, Louis DN, Getz G, Hahn WC (2015) Genomic characterization of brain metastases reveals branched evolution and potential therapeutic targets. *Cancer Discov* **5**(11): 1164-1177.

3) Chen G, Mosier S, Gocke CD, Lin M, Eshleman JR (2014) Cytosine deamination is a major cause of background noise in next generation sequencing. *Mol Diagn Ther* **18**(5): 587-593.

4) Dawson SJ, Tsui DW, Murtaza M, Biggs H, Rueda OM, Chin SF, Dunning MJ, Gale D, Forshew T, Mahler-Araujo B, Rajan S, Humphray S, Becq J, Halsall D, Wallis M, Bentley D, Caldas C, Rosenfeld N (2013) Analysis of circulating tumor DNA to monitor metastatic breast cancer. *N Eng J Med* **368**(13): 1199-1209.

5) De Mattos-Arruda L, Mayor R, Ng CK, Weigelt B, Martínez-Ricarte F, Torrejon D, Oliveira M, Arias A, Raventos C, Tang J, Guerini-Rocco E, Martínez-Sáez E, Lois S, Marín O, de la Cruz X, Piscuoglio S, Towers R, Vivancos A, Peg V, Ramon y Cajal S, Carles J, Rodon J, González-Cao M, Tabernero J, Felip E, Sahuquillo J, Berger MF, Cortes J, Reis-Filho JS, Seoane J (2015) Cerebrospinal fluid-derived circulating tumour DNA better represents the genomic alterations of brain tumours than plasma. *Nat Commun* **6**: 8839.

6) Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, Thornton K, Agrawal N, Sokoll L, Szabo SA,

Kinzler KW, Vogelstein B, Diaz LA Jr. (2008) Circulating mutant DNA to assess tumor dynamics. *Nat Med* **14**(9): 985-990.

7) Do H, Dobrovic A (2012) Dramatic reduction of sequence artefacts from DNA isolated from formalin-fixed cancer biopsies by treatment with uracil-DNA glycosylase. *Oncotarget* **3**(5): 546-558.

8) Do H, Wong SQ, Li J, Dobrovic A (2013) Reducing sequence artifacts in amplicon-based massively parallel sequencing of formalin-fixed paraffin-embedded DNA by enzymatic depletion of uracil-containing templates. *Clin Chem* **59**(9): 1376-1383.

9) Dono M, Massucco C, Chiara S, Sonaglio C, Mora M, Truini A, Cerruti G, Zoppoli G, Ballestrero A, Truini M, Ferrarini M, Zupo S (2013) Low percentage of *KRAS* mutations revealed by locked nucleic acid polymerase chain reaction: implications for treatment of metastatic colorectal cancer. *Mol Med* **18**: 1519-1526.

10) Higgins MJ, Jelovac D, Barnathan E, Blair B, Slater S, Powers P, Zorzi J, Jeter SC, Oliver GR, Fetting J, Emens L, Riley C, Stearns V, Diehl F, Angenendt P, Huang P, Cope L, Argani P, Murphy KM, Bachman KE, Greshock J, Wolff AC, Park BH (2012) Detection of tumor *PIK3CA* status in metastatic breast cancer using peripheral blood. *Clin Cancer Res* **18**(12): 3462-3469.

11) Khoo BL, Grenci G, Jing T, Kluytenaar J, Kozak KR, Yock TI, Muscato NE, Ugarelli P, Decker RH, Patel AA (2016) Liquid biopsy and therapeutic response: circulating tumor cell cultures for evaluation of anticancer treatment. *Sci Adv* **2**(7): e1600274.

12) Kim S, Park C, Ji Y, Kim DG, Bae H, van Vrancken M, Kim DH, Kim KM (2017) Deamination effects in formalin-fixed, paraffin-embedded tissue samples in the era of precision medicine. *J Mol Diagn* **19**(1): 137-146.

13) Kimura T, Okamoto K, Miyamoto H, Kimura M, Kitamura S, Takenaka H, Muguruma N, Okahisa T, Aoyagi E, Kajimoto M, Tsuji Y, Kogawa T, Tsuji A, Takayama T (2012) Clinical benefit of high-sensitivity *KRAS* mutation testing in metastatic colorectal cancer treated with anti-*EGFR* antibody therapy. *Oncology* **82**(5): 298-304.

14) Kockan C, Hach F, Sarrafi I, Bell RH, McConeghy B, Beja K, Haegert A, Wyatt AW, Volik SV, Chi KN, Collins CC, Sahinalp SC (2017) SiNVICT: ultra-sensitive detection of single nucleotide variants and indels in circulating tumour DNA. *Bioinformatics* **33**(1): 26-34.

15) Lamy A, Blanchard F, Le Pessot F, Sesboüé R, Di Fiore F, Bossut J, Fiant E, Frébourg T, Sabourin JC (2011) Metastatic colorectal cancer KRAS genotyping in routine practice: results and pitfalls. *Modern Pathol* **24**(8): 1090-1100.

16) Laurent-Puig P, Pekin D, Normand C, Kotsopoulos SK, Nizard P, Perez-Toralla K, Rowell R, Olson J,

Srinivasan P, Le Corre D, Hor T, El Harrak Z, Li X, Link DR, Bouché O, Emile JF, Landi B, Boige V, Hutchison JB, Taly V (2015) Clinical relevance of *KRAS*-mutated subclones detected with picodroplet digital PCR in advanced colorectal cancer treated with anti-*EGFR* therapy. *Clin Cancer Res* **21**(5): 1087-1097.

17) Lindahl T (1993) Instability and decay of the primary structure of DNA. *Nature* **362**(6422): 709-715.

18) Mancini I, Santucci C, Sestini R, Simi L, Pratesi N, Cianchi F, Valanzano R, Pinzani P, Orlando C (2010) The use of COLD-PCR and high-resolution melting analysis improves the limit of detection of *KRAS* and *BRAF* mutations in colorectal cancer. *J Mol Diagn* **12**(5): 705-711.

19) Marchiò C, Mariani S, Bertero L, Di Bello C, Francia Di Celle P, Papotti M, Rudà R, Soffietti R, Cassoni P (2017) Liquoral liquid biopsy in neoplastic meningitis enables molecular diagnosis and mutation tracking: a proof of concept. *Neuro Oncol* **19**(3): 451-453.

20) Mariani S, Di Bello C, Bonello L, Tondat F, Pacchioni D, Molinaro L, Barreca A, Macrì L, Chiusa L, di Celle PF, Cassoni P, Sapino A (2015) Flexible lab-tailored cut-offs for suitability of formalin-fixed tumor samples for diagnostic mutational analyses. *PloSOne* **10**(4): e0121815.

21) Mariani S, Bertero L, Osella-Abate S, Di Bello C, Francia di Celle P, Coppola V, Sapino A, Cassoni P, Marchiò C (2017) Extreme assay sensitivity in molecular diagnostics further unveils intratumour heterogeneity in metastatic colorectal cancer as well as artifactual low-frequency mutations in the *KRAS* gene. *Br J Cancer* **117**(3): 358-366.

22) McGranahan N, Favero F, de Bruin EC, Birkbak NJ, Szallasi Z, Swanton C (2015) Clonal status of actionable driver events and the timing of mutational processes in cancer evolution. *Sci Transl Med* **7**(283): 283ra54. doi:10.1126/scitranslmed.aaa1408 [published Online First: 15 April 2015].

23) McGranahan N, Swanton C (2017) Clonal heterogeneity and tumor evolution: past, present, and future. *Cell* **168**(4): 613-629.

24) Misale S, Yaeger R, Hobor S, Scala E, Janakiraman M, Liska D, Valtorta E, Schiavo R, Buscarino M, Siravegna G, Bencardino K, Cercek A, Chen CT, Veronese S, Zanon C, Sartore-Bianchi A, Gambacorta M, Gallicchio M, Vakiani E, Boscaro V, Medico E, Weiser M, Siena S, Di Nicolantonio F, Solit D, Bardelli A (2012) Emergence of *KRAS* mutations and acquired resistance to anti-*EGFR* therapy in colorectal cancer. *Nature* **486**(7404): 532-536. doi: 10.1038/nature11156.

25) Molinari F, Felicioni L, Buscarino M, De Dosso S, Buttitta F, Malatesta S, Movilia A, Luoni M, Boldorini R, Alabiso O, Girlando S, Soini B, Spitale A, Di Nicolantonio F, Saletti P, Crippa S, Mazzucchelli L, Marchetti A, Bardelli A, Frattini M (2011) Increased detection sensitivity for *KRAS* mutations enhances the prediction of anti-*EGFR* monoclonal antibody resistance in metastatic colorectal

cancer. *Clin Cancer Res* **17**(14): 4901-4914.

26) Narayan A1, Carriero NJ, Gettinger SN, et al (2012) Ultrasensitive measurement of hotspot mutations in tumor DNA in blood using error-suppressed multiplexed deep sequencing. *Cancer Res* **72**(14): 3492-3498.

27) Normanno N, Rachiglio AM, Lambiase M, Martinelli E, Fenizia F, Esposito C, Roma C, Troiani T, Rizzi D, Tatangelo F, Botti G, Maiello E, Colucci G, Ciardiello F; CAPRI-GOIM investigators (2015) Heterogeneity of *KRAS*, *NRAS*, *BRAF* and *PIK3CA* mutations in metastatic colorectal cancer and potential effects on therapy in the CAPRI GOIM trial. *Ann Oncol* **26**(8): 1710-1714.

28) Pääbo S, Irwin DM, Wilson AC (1990) DNA damage promotes jumping between templates during enzymatic amplification. *J Biol Chem* **265**(8): 4718-4721.

29) Pérez-Báez W, García-Latorre EA, Maldonado-Martínez HA, Coronado-Martínez I, Flores-García L, Taja-Chayeb L (2017) Impact of fixation artifacts and threshold selection on high resolution melting analysis for *KRAS* mutation screening. *Mol Cell Probes* **35**: 34-43.

30) Phallen J, Sausen M, Adleff V, Leal A, Hruban C, White J, Anagnostou V, Fiksel J, Cristiano S, Papp E, Speir S, Reinert T, Orntoft MW, Woodward BD, Murphy D, Parpart-Li S, Riley D, Nesselbush M, Sengamalay N, Georgiadis A, Li QK, Madsen MR, Mortensen FV, Huiskens J, Punt C, van Grieken N, Fijneman R, Meijer G, Husain H, Scharpf RB, Diaz LA Jr, Jones S, Angiuoli S, Ørntoft T, Nielsen HJ, Andersen CL, Velculescu VE (2017) Direct detection of early-stage cancers using circulating tumor DNA. *Sci Transl Med* **9**(403). doi:10.1126/scitranslmed.aan2415

31) Pierce KE, Wangh LJ (2004) Effectiveness and limitations of uracil-DNA glycosylases in sensitive real-time PCR assays. *BioTechniques* **36**(1): 44-46.

32) Senft D, Leiserson MDM, Ruppin E, Ronai ZA (2017) Precision Oncology: The Road Ahead. *Trends Mol Med* **23**(10): 874-898.

33) Siravegna G, Marsoni S, Siena S, Bardelli A (2017) Integrating liquid biopsies into the management of cancer. *Nat Rev Clin Oncol* **14**(9): 531-548.

34) Stiller M, Green RE, Ronan M, Simons JF, Du L, He W, Egholm M, Rothberg JM, Keates SG, Ovodov ND, Antipina EE, Baryshnikov GF, Kuzmin YV, Vasilevski AA, Wuenschell GE, Termini J, Hofreiter M, Jaenicke-Després V, Pääbo S (2006) Patterns of nucleotide misincorporations during enzymatic amplification and direct large-scale sequencing of ancient DNA. *Proc Natl Acad Sci USA* **103**(37): 13578-13584.

35) Thierry AR, Mouliere F, El Messaoudi S, Mollevi C, Lopez-Crapez E, Rolet F, Gillet B, Gongora C, Dechelotte P, Robert B, Del Rio M, Lamy PJ, Bibeau F, Nouaille M, Loriot V, Jarrousse AS, Molina

F, Mathonnet M, Pezet D, Ychou M (2014) Clinical validation of the detection of *KRAS* and *BRAF* mutations from circulating tumor DNA. *Nat Med* **20**(4): 430-435.

36) Tougeron D, Lecomte T, Pagès JC, Villalva C, Collin C, Ferru A, Tourani JM, Silvain C, Levillain P, Karayan-Tapon L (2013) Effect of low-frequency *KRAS* mutations on the response to anti-*EGFR* therapy in metastatic colorectal cancer. *Ann Oncol* **24**(5): 1267-1273.

37) Van Cutsem E, Lenz HJ, Köhne CH, Heinemann V, Tejpar S, Melezínek I, Beier F, Stroh C, Rougier P, van Krieken JH, Ciardiello F (2015) Fluorouracil, leucovorin, and irinotecan plus cetuximab treatment and *RAS* mutations in colorectal cancer. *J Clin Oncol* **33**(7): 692-700.

38) Venesio T, Siravegna G, Bardelli A, Sapino A (2017) Liquid biopsies for monitoring temporal genomic heterogeneity in breast and colon cancers. *Pathobiology* Published Online First: 15 June 2017. doi: 10.1159/000473882.

39) Wei Q, Ye Z, Zhong X, Li L, Wang C, Myers RE, Palazzo JP, Fortuna D, Yan A, Waldman SA, Chen X, Posey JA, Basu-Mallick A, Jiang BH, Hou L, Shu J, Sun Y, Xing J, Li B, Yang H (2017) Multiregion whole-exome sequencing of matched primary and metastatic tumors revealed genomic heterogeneity and suggested polyclonal seeding in colorectal cancer metastasis. *Annal Oncol* **28**(9): 2135-2141.

40) Williams C, Pontén F, Moberg C, Söderkvist P, Uhlén M, Pontén J, Sitbon G, Lundeberg J (1999) A high frequency of sequence alterations is due to formalin fixation of archival specimens. *Am J Pathol* **155**: 1467-1471.

41) Wong SQ, Li J, Tan AY, Vedururu R, Pang JM, Do H, Ellul J, Doig K, Bell A, MacArthur GA, Fox SB, Thomas DM, Fellowes A, Parisot JP, Dobrovic A (2014) Sequence artefacts in a prospective series of formalin-fixed tumours tested for mutations in hotspot regions by massively parallel sequencing. *BMC Medical Genomics* **7**: 23. doi: 10.1186/1755-8794-7-23.

FIGURE LEGENDS

Figure 1. Fast COLD-PCR efficiency is negatively influenced by UDG pretreatment of *KRAS* mutated samples. A representative *KRAS* mutated tumor DNA sequence, obtained from a colorectal FFPE tissue specimen of the case series. The sample DNA was PCR amplified in absence (Panel A) or presence (Panel B) of UDG pretreatment. Black lines identify nucleotide sequences at codon 12. The mutated (c.34_35GG>TT, p. Gly12Phe)/wild-type allele ratio is significantly decreased by DNA pretreatment with UDG (Panel B), compared to a standard reaction (Panel A).

Figure 2. The efficiency of fast COLD-PCR amplification for *KRAS* gene sequencing is negatively affected by both UDG pretreatment and quantity of DNA input. Panels A and B represent the sequence of *KRAS* arising from fast COLD-PCR amplifications of a standard mutated control (c.34G>T, p.Gly12Cys) diluted in a wild type DNA control at different MAF (10%, 3%, 0.3%) and used in the range of nanograms and picograms, respectively. The standard DNA controls were amplified in absence (UDG-) or presence (UDG+) of UDG pretreatment. The LOD of *KRAS* mutations obtainable by fast COLD-PCR was negatively affected (higher percent values) both by presence of UDG pretreatment (LOD for DNA input of ng: 0.3% *vs* 3%; LOD for DNA input of pg: 10% vs 20%) and decreasing DNA input (LOD in absence of UDG: 0.3% *vs* 10%; LOD in presence of UDG: 3% vs 20%).

Table 1. Clinical and specimen characteristics of the patients

PATIENTS	GENDER	AGE AT DIAGNOSIS	HISTOLOGICAL TYPE	SYNCHRONOUS METASTASTATIC SITES	SAMPLE ANALYZED	SPECIMEN ANALYZED	FIRST KRAS MUTATIONAL ANALYSIS*	REPLICA OF KRAS MUTATIONAL ANALYSIS (WITH OR WITHOUT UDG)
Case 1	M	73	COLORECTAL ADENOCARCINOMA	LOCOREGIONAL LYMPH-NODES	PRIMITIVE TUMOR	HISTOLOGICAL FFPE TISSUE	p.G13D (<3%)	WT
Case 2	F	59	COLORECTAL ADENOCARCINOMA	LOCOREGIONAL LYMPH-NODES	PRIMITIVE TUMOR	HISTOLOGICAL FFPE TISSUE	p.G12S (<3%)	WT
Case 3	F	71	COLORECTAL ADENOCARCINOMA	LOCOREGIONAL LYMPH-NODES	PRIMITIVE TUMOR	HISTOLOGICAL FFPE TISSUE	p.G12D (<3%)	WT
Case 4	F	67	COLORECTAL ADENOCARCINOMA	NA	PRIMITIVE TUMOR	HISTOLOGICAL FFPE TISSUE	p.G13D	WT
Case 5	F	63	COLORECTAL ADENOCARCINOMA	LIVER	LOCAL RELAPSE	HISTOLOGICAL FFPE TISSUE	p.G12S (<3%)	WT
Case 6	M	60	COLORECTAL ADENOCARCINOMA	LOCOREGIONAL LYMPH-NODES AND LIVER	PRIMITIVE TUMOR	HISTOLOGICAL FFPE TISSUE	p.G12D (<3%) + p.G13S (<3%)	WT
Case 7	F	52	COLORECTAL ADENOCARCINOMA	PERITONEUM	PERITONEAL METASTASIS	HISTOLOGICAL FFPE TISSUE	p.G12D (<3%)	p.G13D (<3%)
Case 8	M	59	COLORECTAL ADENOCARCINOMA	LOCOREGIONAL LYMPH-NODES	PRIMITIVE TUMOR	HISTOLOGICAL FFPE TISSUE	p.G12V (3%)	p.G12V
Case 9	F	50	COLORECTAL ADENOCARCINOMA	LOCOREGIONAL LYMPH-NODES	PRIMITIVE TUMOR	HISTOLOGICAL FFPE TISSUE	p.G12V (<3%)	WT
Case 10	M	70	COLORECTAL ADENOCARCINOMA	LOCOREGIONAL LYMPH-NODES	PRIMITIVE TUMOR	HISTOLOGICAL FFPE TISSUE	p.G13D (<3%)	WT

Case 11	M	67	COLORECTAL ADENOCARCINOMA	LOCOREGIONAL LYMPH-NODES, LIVER, LUNG	PRIMITIVE TUMOR	HISTOLOGICAL FFPE TISSUE	p.G13D (<3%)	WT
Case 12	M	71	COLORECTAL ADENOCARCINOMA	NA	PRIMITIVE TUMOR	HISTOLOGICAL FFPE TISSUE	p.A11_G12>AC	p.A11_G12>AC
Case 13	F	73	LUNG ADENOCARCINOMA	BONE	PLASMA cfDNA	PLASMA	p.G12D	WT
Case 14	F	79	LUNG ADENOCARCINOMA	LOCOREGIONAL LYMPH-NODES & SKIN (METACHRONOUS)	PLASMA cfDNA	PLASMA	p.G12T (<3%)	WT
Case 15	M	84	LUNG ADENOCARCINOMA	BONE	PLASMA cfDNA	PLASMA	p.G12C	p.G12C
Case 16	M	68	LUNG ADENOCARCINOMA	BONE	PLASMA cfDNA	PLASMA	p.G12D (<3%)	WT
Case 17	M	53	LUNG ADENOCARCINOMA	BONE	PLASMA cfDNA	PLASMA	p.G12S (<3%)	WT
Case 18	M	67	LUNG ADENOCARCINOMA	LOCOREGIONAL LYMPH-NODES, ADRENAL GLAND, BONE	PLASMA cfDNA	PLASMA	WT	WT
Case 19	M	78	LUNG ADENOCARCINOMA	PLEURA (METACHRONOUS)	PLASMA cfDNA	PLASMA	WT	WT
Case 20	M	58	LUNG ADENOCARCINOMA	CNS	CSF cfDNA	CSF	p.G13C	p.G13C
Case 21	F	64	LUNG ADENOCARCINOMA	CNS	CSF cfDNA	CSF	p.G12F	p.G12F
Case 22	M	78	LUNG ADENOCARCINOMA	PLEURA (METACHRONOUS)	PRIMITIVE TUMOR	HISTOLOGICAL FFPE TISSUE	p.G12D (<3%)	WT

Case 23	M	75	LUNG ADENOCARCINOMA	ADRENAL GLAND	PRIMITIVE TUMOR	CYTOLOGICAL FFPE SAMPLE	p.G12C	p.G12C
Case 24	M	80	LUNG ADENOCARCINOMA	PERITONEUM	PRIMITIVE TUMOR	CYTOLOGICAL FFPE SAMPLE	p.G12V	p.G12V
Case 25	F	65	LUNG ADENOCARCINOMA	ADRENAL GLAND	PRIMITIVE TUMOR	CYTOLOGICAL FFPE SAMPLE	p.G12C	p.G12C
Case 26	F	71	LUNG ADENOCARCINOMA	MEDIASTINUM	PRIMITIVE TUMOR	HISTOLOGICAL FFPE TISSUE	p.G12C	p.G12C
Case 27	M	77	LUNG ADENOCARCINOMA	NA	PRIMITIVE TUMOR	HISTOLOGICAL FFPE TISSUE	p.G12D	p.G12D
Case 28	F	60	LUNG ADENOCARCINOMA	UTERUS	PRIMITIVE TUMOR	CYTOLOGICAL FFPE SAMPLE	p.G12C	p.G12C
Case 29	M	59	LUNG ADENOCARCINOMA	LUNG	PRIMITIVE TUMOR	HISTOLOGICAL FFPE TISSUE	p.G12V	p.G12V
Case 30	F	57	LUNG ADENOCARCINOMA	CNS	PRIMITIVE TUMOR	CYTOLOGICAL FFPE SAMPLE	p.G12V	p.G12V
Case 31	M	70	LUNG ADENOCARCINOMA	LOCOREGIONAL LYMPH-NODES	PRIMITIVE TUMOR	HISTOLOGICAL FFPE TISSUE	p.G12V	p.G12V

F: female; M: male; WT: wild-type; NA: not available; cfDNA: cell-free DNA, CSF: cerebrospinal fluid; FFPE: formalin-fixed and paraffin-embedded.

*Subclonal *KRAS* mutations with mutated allele ratio $\leq$3% had been reported in parenthesis.

Table 2. Mutational results after primary and repeated analysis

	FFPE tissue-derived DNAs (a)										Plasma-derived ctDNAs (b)							CSF-derived ctDNAs (c)	
A																			
B																			
C																			

A: *KRAS* mutational results by fast COLD-PCR; B: Replica of fast COLD-PCR for *KRAS* in presence of UDG pretreatment; C: Replica of fast COLD-PCR for *KRAS* in absence of UDG pretreatment (White boxes: samples wild-type at *KRAS*; Grey boxes: samples mutated at *KRAS*; Black box: failed analysis).