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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

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Background: Gene mutations in the RAS family rule out metastatic colorectal carcinomas (mCRCs) from anti-EGFR therapies.

Methods: We report a retrospective analysis by Sequenom Massarray and fast COLD-PCR followed by Sanger sequencing on 240 mCRCs.

Results: By Sequenom, *KRAS* and *NRAS* exons 2-3-4 were mutated in 52.9% (127/240) of tumours, while *BRAF* codon 600 mutations reached 5% (12/240). Fast COLD-PCR found extra mutations at *KRAS* exon 2 in 15/166 (9%) of samples, previously diagnosed by Sequenom as wild-type or mutated at *RAS* (exons 3-4) or *BRAF* genes. After UDG digestion results were reproduced in 2/12 analysable subclonally mutated samples leading to a frequency of true subclonal *KRAS* mutations of 1.2% (2.1% of the previous Sequenom wild-type subgroup). In 10 out of 12 samples, the subclonal *KRAS* mutations disappeared (9 out of 12) or turned to a different sequence variant (1 out of 12).

Conclusions: mCRC can harbour coexisting multiple gene mutations. High sensitivity assays allow the detection of a small subset of patients harbouring true subclonal *KRAS* mutations. However, DNA changes with mutant allele frequencies $<3\%$ detected in formalin-fixed paraffin-embedded samples may be artifactual in a non-negligible fraction of cases. UDG pre-treatment of DNA is mandatory to identify true DNA changes in archival samples and avoid misinterpretation due to artifacts.

The threshold of 'precision medicine' in oncology is continuously rising. In the past few years, many molecular players involved in oncogenesis have been exploited as potential drug targets in the ever-growing approach of patient-tailored care (Jurgensmeier *et al*, 2014; Stevens and Rodriguez, 2015). The results of several clinical trials (Harbeck and Wuerstlein, 2013; Landi and Cappuzzo, 2014; Khattak *et al*, 2015) have shown that often only specific subgroups of patients benefit from molecular-targeted treatments. Thus, the development of highly sensitive and specific companion diagnostic tests is needed to improve the characterisation of the responsive patients and to intercept molecular alterations with negative or positive predictive value (Dienstmann *et al*, 2015;

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Hovelson *et al*, 2015; Zhao *et al*, 2015). Moreover, in the current era of increasing healthcare costs colliding with shrinking budgets, the precise identification of who could benefit the most from a specific treatment is of paramount importance.

In colorectal tumours, the subset of patients amenable to targeted therapies with anti-EGFR drugs underwent few revisions (Van Cutsem *et al*, 2015) as new detrimental somatic point mutations have been found to hamper the response to these treatments, in addition to the more common mutations at codons 12 and 13 of *KRAS*. The additional mutations span codons 61, 117 and 146 of *KRAS* and codons 12, 13, 61, 117 and 146 of *NRAS* (extended *RAS*).

Nevertheless, both the improved understanding of tumour heterogeneity and the high sensitivity of the assays introduced in molecular diagnostics open new questions focusing on which cutoff value makes an alteration a significant marker leading to a different outcome or a different clinical response. Few and contradictory data are available on the predictive role of a low mutational load in tumour samples (defined as a low number of neoplastic cells harbouring a specific mutation) compared to wild-type lesions (Dono *et al*, 2013; Kimura *et al*, 2012; Laurent-Puig *et al*, 2015; Molinari *et al*, 2011; Normanno *et al*, 2015; Tougeron *et al*, 2013; Van Cutsem *et al*, 2015). To date, these questions are being explored especially in the field of lung adenocarcinomas (Wang *et al*, 2014) and more recently facing the issue of quantitatively monitoring the emergence of *KRAS* mutations in liquid biopsy samples of metastatic colorectal carcinoma (mCRC) patients treated with anti-EGFR drugs (Toledo *et al*, 2017).

In the present study, we investigated the real nature and true incidence of subclonal mutations detected in formalin-fixed paraffin-embedded (FFPE) samples by increasing assay sensitivities (0.1–5%).

MATERIALS AND METHODS

Patients. The study included 240 mCRC patients whose tumour was analysed at our institution in 2014 to select possible candidates for anti-EGFR therapy based on their mutational profile. As a local reference laboratory for the diagnostic mutational screening in solid tumours since 2010, we have been successfully participating to several EQA schemes of the European Society of Pathology and of the Italian Association of Medical Oncologists/Italian Society of Pathological Anatomy and Cytology (AIOM-SIAPEC-IAP) for *KRAS* and *BRAF* (data not shown).

The cohort was composed of 108 women and 132 men, and the median age at diagnosis was 62 (range: 34–84) and 67 years (range: 22–92), respectively. Tumour grading and staging data, according to World Health Organization and AJCC Staging criteria (VII Edition, 2010), were available for the 160 patients treated at our Institution (Table 1). The tissues analysed were mainly histological samples from primary CRCs (98%), locally resected at diagnosis (81%) or at relapse (6%). Most cases displayed moderate-to-low differentiation (92.6% G2-G3), locally advanced disease (94.5% pT3-4), lymph nodal involvement (74.4% pN1-2) and perineural and/or vascular invasion (78.4%). The series also included distant metastases (13% of the cases, including seven pulmonary, 17 liver, two bone, one peritoneal, one cerebral and one pelvic metastases).

The study was submitted to and approved by the Ethic Institutional Review Board (IRB) responsible for 'Biobanking and use of human tissues for experimental studies' of the Department of Medical Sciences at the University of Turin. The IRB approved a verbal consent procedure due to the retrospective design of the study, which had no effect on their care.

Material selection. We retrieved the 240 DNA samples that were earlier banked when the diagnostic analysis was performed on one representative FFPE tumour block. For six selected cases additional FFPE samples corresponding to alternative tumour blocks of the primitive tumour and/or regional lymph-node/distant metastases were submitted to DNA extraction.

In all cases a selection of the tumour area was performed as previously described (Mariani *et al*, 2015). Morphological adequacy criteria were derived from the Biogate portal (https://testbiomolecolari.it/) of the AIOM-SIAPEC-IAP. Specimens were

Table 1. Clinicopathological features of tumour specimens in relation to *KRAS*, *NRAS* and *BRAF* gene mutation status

	KRAS mut *n* (%)	*NRAS* mut *n* (%)	*BRAF* mut *n* (%)	*RAS/BRAF* WT *n* (%)	Total *n* (%)
Sex					
F	52 (48.1)	6 (5.6)	10 (9.3)[a]	40 (37.0)	108 (45.0)
M	61 (46.2)	8 (6.1)	2 (1.5)	61 (46.2)	132 (55.0)
Sub total	113 (47.1)	14 (5.8)	12 (5.0)	101 (42.1)	240 (100)
Age (years)					
Median	64	68	65,5	63,5	—
Range	34–84	40–87	51–73	22–92	—
Sub total	101 (47.2)	13 (6.1)	10 (4.7)	90 (42.0)	214 (100)
G					
1–2	57 (48.7)	5 (4.3)	1 (0.9)	54 (27.3)	117 (71.8)
3–4	21 (45.6)	4 (8.7)	5 (10.9)[a]	16 (34.8)	46 (28.2)
Sub total	78 (47.8)	9 (5.5)	6 (3.7)	70 (42.9)	163 (100)
pT					
1–2	5 (55.5)	1 (11.1)	0 (0)	3 (0)	9 (5.5)
3–4	73 (47.4)	8 (5.2)	6 (3.4)	67 (43.5)	154 (94.5)
Sub total	78 (47.8)	9 (5.5)	6 (3.7)	70 (42.9)	163 (100)
pN					
0	19 (46.3)	3 (7.3)	2 (4.9)	17 (41.5)	41 (25.6)
1	36 (56.3)	3 (4.7)	0 (0)	25 (39.1)	64 (40.0)
2	21 (38.2)	2 (3.6)	4 (7.3)	28 (50.9)	55 (34.4)
Sub total	76 (47.5)	8 (5.0)	6 (3.8)	70 (43.7)	160 (100)
Vascular and/or perineural invasion					
Yes	52 (44.8)	6 (5.2)	4 (3.4)	54 (41.7)	116 (78.4)
No	17 (53.1)	2 (6.3)	1 (3.1)	12 (37.5)	32 (21.6)

Abbreviations: mut = mutated; WT = wild-type; F = female; M = male.
[a]*BRAF* mutations are significantly more prevalent in female patients (F) and G3-G4 tumours rather than in males (M) and G1-G2 tumours ($P = 0.013$).

recorded as adequate when tumour enrichment was higher than the 50% and at least 100 neoplastic cell were present.

DNA extraction. DNA extraction from FFPE tissues was performed as previously described (Mariani *et al*, 2015) and concentrations/purity were measured by a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Mass spectrometry technology for DNA sequencing. The DNAs were amplified according to the manufacturer's instructions on a Labcycler (SensoQuest GmbH, Germany), using the CE-IVD Myriapod Colon Status kit (Diatech Pharmacogenetics, Jesi, Italy) based on a multiplex-PCR. The amplified DNAs were submitted to a Shrimp Alkaline Phosphatase digestion (SAP reaction) to remove the excess of free nucleotides. Finally, the purified DNAs underwent a reaction of primer extension (iPLEX) with oligonucleotides flanking each gene position under study and with dideoxynucleotide terminators of known mass to discriminate wild-type from mutated genotypes, by using a matrix-assisted laser desorption ionisation time-of-flight mass spectrometry. Amplified DNAs were dispensed on a supplied solid chip prior to be scanned on a MassARRAY instrument (Sequenom Inc, San Diego, CA, USA).

The Myriapod Colon Status kit identifies 58, 54, 23 and 66 nucleotide substitutions in the *KRAS*, *NRAS*, *BRAF* and *PIK3CA* genes, respectively. Among them, the mutations related to the clinical response to anti-EGFR treatments are those affecting codons 12, 13, 59, 61, 117 and 146 in both *KRAS* and *NRAS*, while the mutations at codon 600 of *BRAF* have a prognostic significance. This assay also explores mutations at multiple codons of the *PIK3CA* gene.

Fast COLD-PCR followed by Sanger Sequencing for codons 12 and 13 of *KRAS*. The fast COLD-PCR for the exon 2 of *KRAS* followed by Sanger Sequencing was applied to all samples considered wild-type by Sequenom at exon 2 of *KRAS* and to a subset of those *KRAS* (exon 2) mutated. The assay was used: (1) to confirm the Sequenom results; (2) to identify the presence and incidence of minor tumour clones undetectable by the standard methods; (3) to determine the real nature of the subclonal mutations. In brief, 30 ng of DNA/reaction were added to fast COLD-PCR reagents and preliminary submitted to treatment at 37 °C for 30′ in the presence or absence of 0.5 U of Uracil-DNA-Glycosylase (UDG, Thermo Fisher) (Do and Dobrovic, 2012). The protocol of the fast COLD-PCR analysis was derived from Mancini *et al* (Mancini *et al*, 2010) in absence of fluorophores in the reagent mix, under conditions 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"). The denaturation step was set at a critical dissociation temperature, favouring the amplification of the mutated sequences. The amplified products were then submitted to Sanger Sequencing.

PCR followed by Sanger Sequencing for *KRAS*, *NRAS* and *BRAF* mutational analysis. Targeted-PCRs were followed by Sanger Sequencing (Sanger *et al*, 1977) to confirm the somatic gene mutations with clinical significance demonstrated by Sequenom. Sanger Sequencing was also applied to PCR products of the fast COLD-PCR.

Reagent mixes for targeted-PCRs contained 70 ng of genomic DNA, 1 × buffer, 1.5 mM of $MgCl_2$, 200 μM of dNTPs, 1.2 U of AmpliTaq Gold DNA Polymerase (Invitrogen, Carlsbad, CA, USA) and 0.5 μM of each couple of primers, in a final volume of 50 μl. *KRAS* and *NRAS* primers were designed using the software Primer3 (http://frodo.wi.mit.edu/primer3/) on the human genomic sequence (http://www.ncbi.nlm.nih.gov/*gene*) (ID:3845 and ID:7812, respectively) and are available upon request. The *BRAF* primers were derived from Moroni *et al* (Moroni *et al*, 2005), and generated amplicons of 228 bp. PCR conditions consisted of a unique touchdown scheme (an annealing decrease of 3.0 °C each 3 cycles starting from 64.0 °C down to 57.0 °C) that was repeated for 44 cycles. The purified products of both targeted- and fast COLD-PCR (see above) were then submitted to a cycle sequencing reaction, as previously described (Mariani *et al*, 2015). The Chromas software (www.technelysium.com.au) was used for sequence analysis.

Sensitivities of the assays. The declared sensitivity of the Myriapod Colon Status kit varied from 2.5 to 10%, depending on the mutation under study. Homemade reactions were designed to determine the sensitivities of Sanger Sequencing when preceded by a standard targeted-PCR (*KRAS* and *BRAF*) or by a fast COLD-PCR (*KRAS*). Mutated DNA extracted from specific cell lines were progressively diluted in blood-derived DNA of a healthy donor. Briefly, the A549 cell line, homozygous for the *KRAS* p.G12S (p.Gly12Ser) mutation, and the HT29, heterozygous for the *BRAF* p.V600E (p.Val600Glu) mutation, were purchased from the ATCC (LGC Standards S.r.l., Sesto San Giovanni, Milan, Italy) and tested at different dilution in the wild-type sample DNA. The described mutation at *KRAS* was detected by Sanger Sequencing at a sensitivity near to 10% and 0.1% starting from products of standard targeted-PCRs and fast COLD-PCRs, respectively (Supplementary Figure 1A). The sensitivity for the *BRAF* mutation was set at 5% (Supplementary Figure 1B).

Statistics. Pearson's χ^2-test corrected if necessary for the Fisher's exact test was applied to 2 × 2 Contingency Tables to compare the mutation frequencies: (1) among patients with different characteristics; (2) among tumour DNAs analysed with different degree of enrichment, and with alternative protocols of PCR before sequencing (*www.openepi.com*). *P*-values < 0.05 were considered statistically significant.

RESULTS

***KRAS*/*NRAS*/*BRAF*/*PIK3CA* mutation frequencies by Sequenom in mCRCs.** By Sequenom analysis we demonstrated a point mutation (exon 2 of *KRAS*) in 40.0% (96/240) of mCRC patients (Figure 1A), in line with results previously obtained by pyrosequencing (Mariani *et al*, 2015). The extended survey identified 43 additional patients (43/240, 17.9%) with further tumour DNA mutations, mutually exclusive and distributed as follows: 17/240 (7.1%) at exons 3-4 of *KRAS*, 14/240 (5.8%) at exons 2-3-4 of *NRAS* and 12/240 (5.0%) at codon 600 of *BRAF*. Overall, the 'extended *RAS*' mutated samples represented 21.5% (31/144) of those samples defined as wild-type until the ASCO guideline update (Allegra *et al*, 2016). Finally, by Sequenom, for the codons included in the Colon cancer kit the remaining 101/240 (42.1%) patients were considered *RAS*/*BRAF* wild type.

The same protocol allowed the investigation of additional point mutations occurring at the *PIK3CA* gene, albeit considered not significant at present for therapy decision-making. We found 28/240 (11.7%) patients harbouring a *PIK3CA* mutation, alone (9/28, 32.1%) or in conjunction with *KRAS* (15/28, 53.6%), *NRAS* (3/28, 10.7%) or *BRAF* (1/28, 3.6%) mutations.

In 34 samples, we were not able to macrodissect tumour areas for their enrichment at levels above 50% before DNA extraction. In this subset of samples, somatic mutations of *KRAS*, *NRAS* and *BRAF* demonstrated by Sequenom occurred in 16/34 (47.1%), 4/34 (11.8%), 1/34 (2.9%) specimens, respectively. The remaining samples (38.2%) were considered wild type. Of note, the distribution of somatic mutations was comparable ($P > 0.05$)

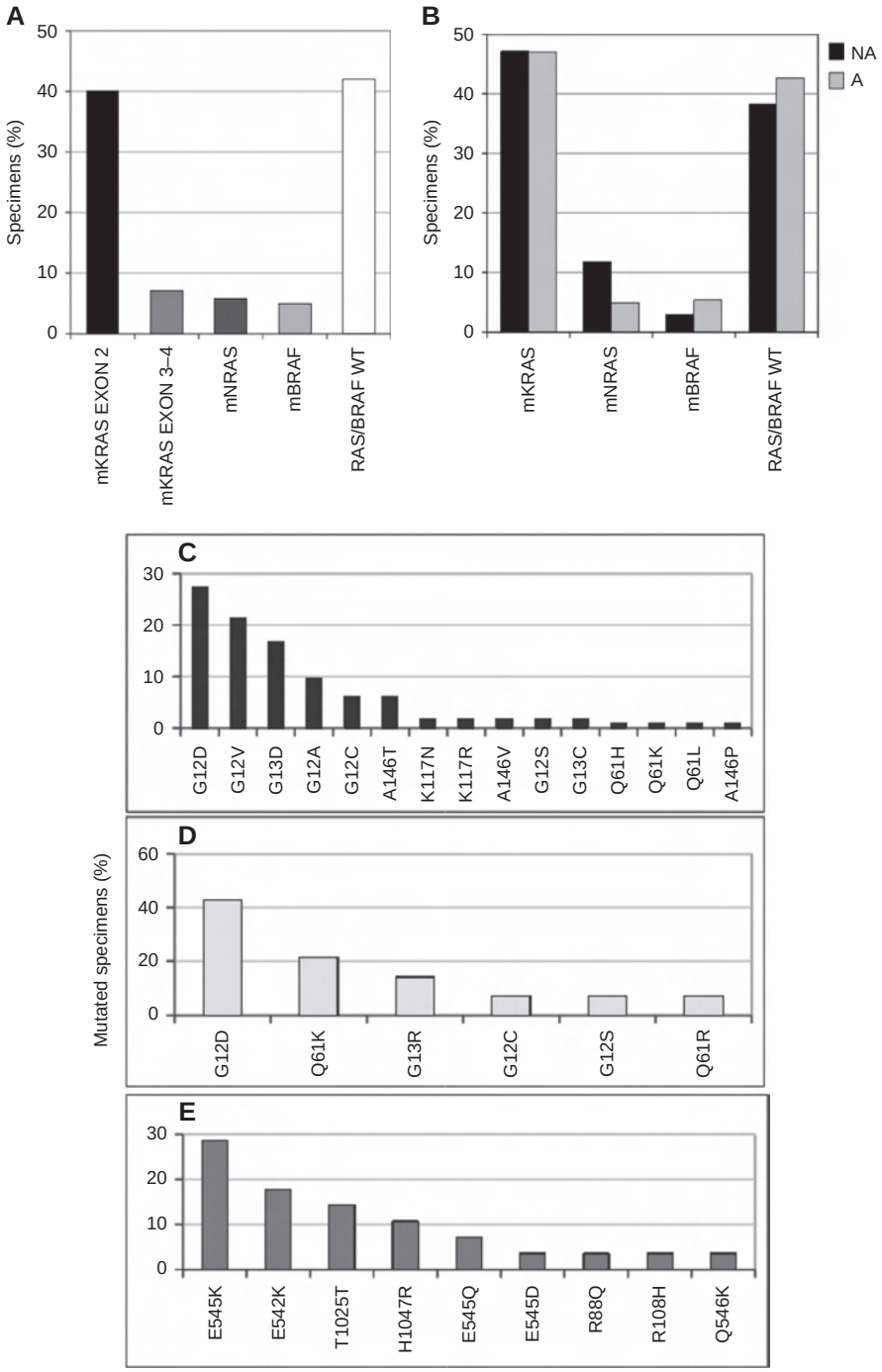

Figure 1. Distribution of gene mutations and allelic variants occurring at *KRAS*, *NRAS*, *BRAF* and *PIK3CA* genes in tumour DNAs of mCRC patients by Sequenom analysis. (**A**) Distribution of mutated (m) and wild-type (WT) specimens at exon 2 of *KRAS*, at exons 3 and 4 of *KRAS*, at exons 2, 3 and 4 of *NRAS* and at codon 600 of *BRAF*. (**B**) Distribution of mCRC patients with tumour DNA sequences either WT or mutated (m) at the *KRAS*, *NRAS* and *BRAF* genes, according to a tumour/normal cell ratio below (NA, not adequate) and above (**A**, adequate) the 50% cutoff. Panels **C**–**E** represent the allelic variant distribution belonging to the mutated *KRAS* (**C**), *NRAS* (**D**) and *PIK3CA* (**E**) genes.

between tumours above and below the 50% enrichment cutoff (Figure 1B) and therefore it did not represent a possible bias.

Frequency distribution and clinicopathological features of *KRAS*, *NRAS*, *BRAF* and *PIK3CA* mutated samples. *KRAS* was confirmed as the most frequently mutated gene in our cohort. In particular, the nucleotide substitutions affected codons 12, 13, 146 and 117 with decreasing frequency (Figure 1C). The most frequent *NRAS* point mutations occurred at codons 12, 61 and 13 (Figure 1D). No exon 4 *KRAS* mutations were detected. *BRAF* was mutated exclusively at codon 600 (p.V600E) (data not shown). Finally, *PIK3CA* mutations mainly mapped to exon 9 (18/240, 7.5%), followed by exon 20 (8/240, 3.3%) (Figure 1E).

As reported in Table 1, the *BRAF* gene was confirmed significantly associated with female gender ($P = 0.013$) and with less differentiated (G3-G4) tumours ($P = 0.013$), as previously reported (Loupakis *et al*, 2016). Node-negative patients resulted more frequently mutated at codon 12 than 13 ($P = 0.04$). The opposite occurred in tumours with pN2 nodal status ($P < 0.05$; Table 2). Among all *RAS* mutations, the p.G12V *KRAS* prevailed in G3-G4 tumours, although this correlation did not reach statistical significance (Table 3).

Table 2. Distribution of *KRAS* mutations according to the involved hotspot gene sequences and to clinicopathological features

	m*KRAS* n (%)	m*KRAS* ex 2 n (%)	m*KRAS* ex 3-4 n (%)	m*KRAS* cod 12 n (%)	m*KRAS* cod 13 n (%)
Sex					
F	52 (46.0)	44 (44.9)	8 (47.0)	35 (46.7)	9 (42.9)
M	61 (54.0)	52 (55.1)	9 (53.0)	40 (52.3)	12 (57.1)
Subtotal	113 (100)	96 (100)	17 (100)	75 (100)	21 (100)
Age (years)					
Median	64	64	65,5	64	63
Range	34–84	34–84	47–76	34–84	49–74
Subtotal	101	85	16	66	19
G					
1–2	57 (73.1)	48 (73.8)	9 (69.2)	36 (72.0)	12 (80.0)
3–4	21 (26.9)	17 (26.2)	4 (30.8)	14 (28.0)	3 (20.0)
Subtotal	78 (100)	65 (100)	13 (100)	50 (100)	15 (100)
pT					
1–2	5 (6.4)	4 (6.1)	1 (7.7)	3 (6.0)	1 (6.7)
3–4	73 (93.6)	61 (93.9)	12 (92.3)	47 (94.0)	14 (93.3)
Subtotal	78 (100)	65 (100)	13 (100)	50 (100)	15 (100)
pN					
0	19 (25.0)	17 (26.6)	2 (16.7)	16 (32.6)[a]	1 (6.6)[a]
1	36 (47.4)	30 (46.8)	6 (50.0)	23 (46.9)	7 (46.7)
2	21 (27.6)	17 (26.6)	4 (33.3)	10 (20.4)[a]	7 (46.7)[a]
Subtotal	76 (100)	64 (100)	12 (100)	49 (100)	15 (100)
Vascular and/or perineural invasion					
Yes	52 (75.4)	44 (75.9)	8 (72.7)	32 (74.4)	12 (80.0)
No	17 (24.6)	14 (24.1)	3 (27.3)	11 (25.6)	3 (20.0)
Subtotal	69 (100)	58 (100)	11 (100)	43 (100)	15 (100)

Abbreviations: m = mutated; ex = exon; cod = codon; F = female; M = male.
[a]The percentage of specimens *KRAS* mutated at the codon 12 is significantly higher in the pN0 than in the pN2 tumours ($P < 0.05$); the opposite occurs in specimens mutated at the codon 13 of the *KRAS* gene.

Table 3. Distribution of the leading *RAS* gene mutations according to clinicopathological features

	KRAS G12A *n* (%)	*KRAS* G12C *n* (%)	*KRAS* G12D *n* (%)	*KRAS* G12V *n* (%)	*KRAS* G13D *n* (%)	*KRAS* A146T *n* (%)	*NRAS* G12D *n* (%)
Sex							
F	7 (63.6)	3 (42.9)	14 (45.2)	11 (45.8)	8 (42.1)	2 (28.6)	4 (80.0)
M	4 (36.4)	4 (57.1)	17 (54.8)	13 (54.2)	11(57.9)	5 (71.4)	1 (20.0)
Subtotal	11 (100)	7 (100)	31 (100)	24 (100)	19 (100)	7 (100)	5 (100)
Age (years)							
Median	60	64	63	64	63	63	63
Range	34–74	34–71	36–84	45–81	49–74	56–73	40–67
Subtotal	11	6	29	18	17	6	4
Tumour							
Primary	7 (63.6)	6 (100)	23 (76.7)	18 (85.7)	17 (89.5)	6 (85.7)	3 (60.0)
Met	4 (36.4)	0 (0)	7 (13.3)	3 (14.3)	2 (11.5)	1 (14.3)	2 (40.0)
Subtotal	11 (100)	6 (100)	30 (100)	21 (100)	19 (100)	7 (100)	5 (100)
G							
1–2	8 (80.0)	2 (66.7)	18 (81.8)	7(53.8)	10 (76.9)	4 (80.0)	1 (50.0)
3–4	2 (20.0)	1 (33.3)	4 (18.2)	6 (46.2)[a]	3 (23.1)	1 (20.0)	1 (50.0)
Subtotal	10 (100)	3 (100)	22 (100)	13 (100)	13 (100)	5 (100)	2 (100)
pN							
0	3 (30.0)	1 (33.3)	8 (38.1)	4 (30.7)	1 (7.7)	1 (25.0)	0 (0)
1	5 (50.0)	2 (66.7)	8 (38.1)	6 (46.2)	7 (53.8)	1 (25.0)	1 (50.0)
2	2 (20.0)	0 (0)	5 (23.8)	3 (23.1)	5 (38.5)	2 (50.0)	1 (50.0)
Subtotal	10 (100)	3 (100)	21 (100)	13 (100)	13 (100)	4 (100)	2 (100)
Vascular and/or perineural invasion							
Yes	8 (88.9)	2 (66.7)	13 (68.4)	7 (70.0)	10 (76.9)	3 (75.0)	1 (100)
No	1 (11.1)	1 (33.3)	6 (31.6)	3 (30.0)	3 (23.1)	1 (25.0)	0 (0)
Subtotal	9 (100)	3 (100)	19 (100)	10 (100)	13 (100)	4 (100)	1 (100)

Abbreviations: F = female; M = male; Met = metastasis.
[a]In G3-G4 tumours the percentage of the *KRAS*p.G12V (p.Gly12Val) mutated specimens compared to the alternative *KRAS*-mutated specimens is higher although statistically not significant ($P = 0.051$).

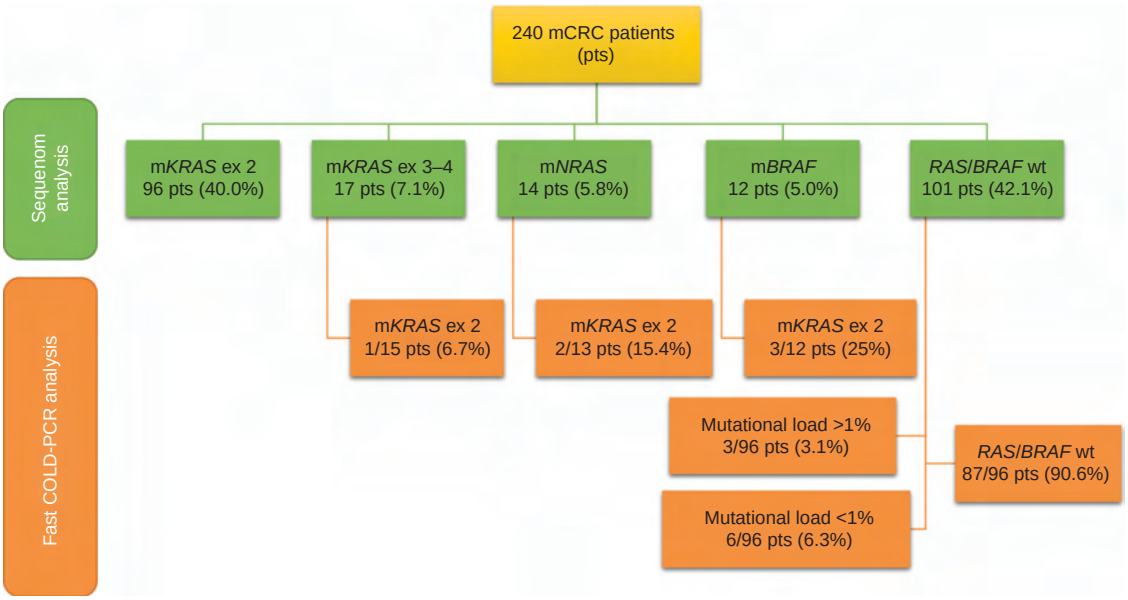

Figure 2. Gene mutations according to the degree of assay sensitivities in 240 mCRC patients. Sequenom found 57.9% of specimens mutated at *RAS/BRAF* (m*RAS/BRAF*), whereas 42.1% were wild-type (WT). A more sensitive fast COLD-PCR method recruited additional mutated sequences in both wild-type samples and in mutated samples. In particular, 90.6% of the samples *RAS/BRAF* WT by Sequenom were confirmed by fast COLD-PCR, whereas the remaining 9.4% of DNAs were mutated at *KRAS* exon 2 (ex 2) with a discrete not negligible (1–5%) or low (<1%) mutational load.

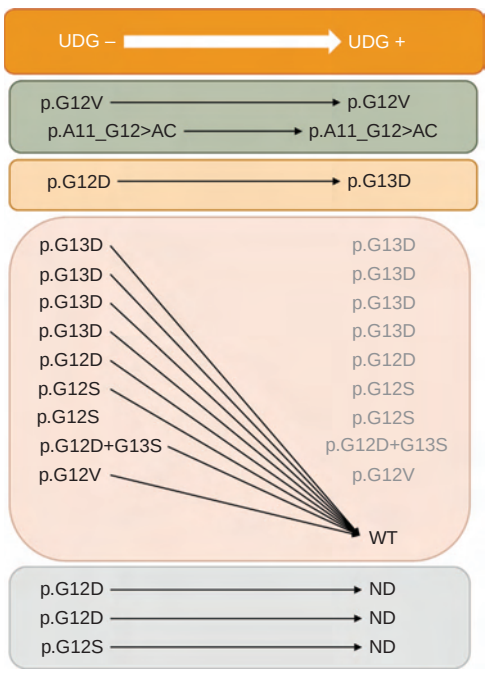

Figure 3. The fate of subclonal *KRAS* mutations after pre-treatment of tumour DNAs with UDG. DNAs belonging to 12/15 mCRC tumours, resulted subclonally mutated at *KRAS* by fast COLD-PCR in absence of UDG pre-treatment (UDG- column), had been re-analysed after UDG digestion (UDG+ column), aimed to avoid PCR artifacts due to formalin fixation injuries on DNA molecules. Eighty-three per cent of the mutated (by fast COLD-PCR, UDG-) cases became WT or changed allelic variant after UDG digestion (UDG+), thus demonstrating their artefactual nature. Only in two tumours (17% of the cases) the mutations were re-confirmed after UDG treatment (UDG+). Both mutations had a mutant allele frequency>3%. ND: not determined cases (due to lack of residual DNA).

Fast COLD-PCR detection of *RAS* mutations with low mutant allele frequency in candidate patients for anti-*EGFR*-based therapies. We re-analysed *KRAS* by fast COLD-PCR in 30/96 samples with already identified exon 2 mutations, 40/43 samples mutated either at exons 3-4 of *KRAS* or at *NRAS* or *BRAF*, and 96/101 wild-type. All *KRAS* exon 2 mutations by Sequenom were confirmed. A total of 15 additional mutations were detected (15/166, 9%), specifically 9/96 (9.4%), 1/15 (6.7%), 2/13 (15.4%) and 3/12 (25.0%) in mCRC previously categorised by Sequenom as wild-type or already mutated at *KRAS* (exons 3 and 4), *NRAS* or *BRAF* (Figure 2). The range of mutant allele frequency (MAF) of these subclonal mutations was comprised between 0.1% and 5%.

We were able to reanalyse 12/15 subclonally mutated samples after UDG digestion. Results were reproduced in two out of 12 samples, both originally wild type by Sequenom, but harbouring a mutation with a MAF >3% detected by fast-COLD-PCR. One patient had a complex mutation at position c.33_34delTGinsCT (p.A11_G12AC) and the other a mutation c.35G>T (p.G12V, p.Gly12Val), both undetectable with the Sequenom assay. Overall this leads to a frequency of true subclonal *KRAS* mutations of 1.2% (2.1% of the Sequenom wild-type subgroup).

In the remaining 10/12 cases (nine below and one above 3% MAF, respectively) the subclonal *KRAS* mutations either disappeared (9/12, including three cases harbouring a *BRAF* mutation) or turned to a different sequence variant at low MAF (1/12). In the latter case a possible mechanism of DNA damage other than cytosine deamination cannot be excluded (Figure 3).

To deepen our analysis, in six patients originally showing subclonal *KRAS* mutations detected in one representative sample by fast COLD-PCR only, we investigated 6–7 more blocks per patient, deriving from the primary tumour and/or regional lymph-node/distant metastases, for a total of 40 samples. Without UGD pre-treatment, 5/6 cases showed a spectrum of subclonal *KRAS* variants detected in 12/40 of all analysed blocks, of which only one showed the same DNA change originally detected in the representative diagnostic sample. The large majority of PCR amplified DNAs (83.3%) became KRAS wild-type and the remaining (16.7%) showed new mutation sequences, following UDG treatment.

Fast COLD-PCR detects the mutations G > T or G > A of *KRAS* at the codons 12 and 13. In order of frequency, the most represented artifactual mutations were c.35G > A (p.G12D, p.Gly12Asp) and c.38G > A (p.G13D, p.Gly13Asp).

DISCUSSION

The present study (i) confirms the frequency of classical *KRAS* mutations (exon 2) by Sequenom and evaluates the presence of specific additional mutations, as required by the new international guidelines, in a series of 240 mCRCs; (ii) validates fast COLD-PCR method for the analysis of *KRAS* gene exon 2 and determines the frequency of mutations with low MAF, detectable only using highly sensitive assays; and (iii) reveals the likely artifactual nature of *KRAS* mutations present at very low MAF in FFPE samples, especially when below 3%.

The *KRAS* exon 2 mutation rate by Sequenom was 40%, in line with reported data (Rosty *et al*, 2013) and with results previously obtained by our group with assays of similar analytical sensitivities (Mariani *et al*, 2015). Of note, the 'extended *RAS*' test revealed additional mutations in 21.5% of patients who were previously considered eligible to therapies with anti-EGFR molecules. Therefore, these patients would not meet the criteria for eligibility to this targeted therapy according to the new guidelines (Allegra *et al*, 2016). An additional 8.3% of eligible patients were mutated at *BRAF* codon 600, a feature linked with a worse prognosis independently of other clinical parameters (Palomba *et al*, 2016; Richman *et al*, 2009; Souglakos *et al*, 2009; Therkildsen *et al*, 2014; Tol *et al*, 2010).

The Sequenom assay also allowed to explore the prevalence of *PIK3CA* mutations, which were identified in 11.7% of the patients, similarly to what recently reported by Palomba *et al* (2016). In contrast with data reported by Normanno *et al* (2015), we did not observe a significant association between tumours with low *KRAS* mutational load and co-occurrence of mutated *PIK3CA* sequences. This discrepancy may stem from a technical reason, as Normanno and colleagues used a targeted next-generation sequencing approach to assess the *PIK3CA* mutational status. A recent meta-analysis study has pointed out that *PIK3CA* mutations may be detrimental for the response to anti-EGFR molecules, especially those occurring at exon 20 (Huang *et al*, 2014). Nevertheless, this trend needs to be confirmed in clinical trials.

When we put into context our molecular data with respect to tumour features, we observed that node positive (pN2) compared to node-negative tumours were preferentially mutated at codon 13 rather than 12, supporting the hypothesis of a more aggressive behaviour of tumours mutated at codon 13. In line with this assumption, Feng *et al* (2015) demonstrated that *KRAS* mutations at codon 13 and pN2 stage are independent risk factors for distant metachronous metastases.

Interestingly, by deepening our analysis with fast COLD-PCR, we detected up to the 0.1% of mutant/wild-type DNA molecules and demonstrated that ~10% of patients was found to harbour *KRAS* exon 2 mutations with low mutant allele frequency among *BRAF* wild-type individuals and supposed to be responsive to anti-*EGFR* antibodies (*RAS* wild-type by standard assays).

These data are in line with those reported by the CRYSTAL study analysing mCRC patients treated with fluorouracil, leucovorin and irinotecan with or without cetuximab. When tumour DNA sequences of the CRYSTAL series were analysed using a 5% mutant/wild-type cutoff, 14.7% of wild-type *KRAS* exon 2 patients revealed point mutations at the 'extended *RAS*' sequences. The same authors also observed that point mutations other than those occurring at exon 2 of *KRAS* (extended *RAS*) play an adverse role on progression-free and overall survival and objective responses (Van Cutsem *et al*, 2015). The frequency of positive samples increased up to 34.6% using an assay with a 0.1% sensitivity cutoff. Although displaying a detectable level of mutation (0.1–5%), these tumours were not associated with worse clinical responses. Regrettably, the patients harbouring a *KRAS* mutation with low MAF in our study were heterogeneously treated, thus precluding any clinical correlations.

Nevertheless, given that formalin fixation is known to produce several DNA injuries (Do and Dobrovic, 2015; Do *et al*, 2013; Lamy *et al*, 2011), including deamination of the cytosine to uracil which generates artifactual C:G > T:A changes, in our study we also investigated the nature of these subclonal *KRAS* mutations by pre-treating DNAs with UDG. This enzyme removes uracil bases belonging to cytosine deamination and creates disruptions along DNA sequence interfering with the PCR amplification of the artifactual mutated sequences only. Following UDG digestion results were reproduced in two cases only, both originally wild-type by Sequenom and found to harbour mutations with a MAF > 3% by fast-COLD-PCR. This finding leads to a frequency of true subclonal *KRAS* mutations of 2.1% of the Sequenom wild-type tumours.

This evidence on artifactual DNA changes in FFPE samples is of utmost importance, as recently the somatic mutation load has been critically reviewed to determine the association with response or resistance to specific targeted therapies (Dono *et al*, 2013; Molinari *et al*, 2011; Van Cutsem *et al*, 2015). One may wonder which level of mutational load is to be considered clinically predictive of responsiveness/resistance to a specific targeted therapy. Several groups have identified *KRAS* mutations harbouring low MAFs in mCRC patients (Dono *et al*, 2013; Kimura *et al*, 2012; Laurent-Puig *et al*, 2015; Molinari *et al*, 2011; Normanno *et al*, 2015; Tougeron *et al*, 2013; Van Cutsem *et al*, 2015) (Supplementary Table 1); however, distinct cutoffs to define minor subclones have been adopted, heterogeneous cohorts (selected or unselected patients) were analysed and details about the use of UDG pre-treatment were not reported, thus leading to results that are difficult to compare. Nevertheless, based on our data and on results of other studies (Laurent-Puig *et al*, 2015; Normanno *et al*, 2015; Tougeron *et al*, 2013), a MAF range comprised between 1 and 3% seems to emerge as a grey area of mutational loads either with or without clinical significance. Indeed, Tougeron *et al* (2013) were able to show that presence of mutations with a MAF > 2.3% was associated with cetuximab resistance and shorter progression-free survival. Likewise, Normanno *et al* (2015) reported a shorter survival for those patients with a tumour harbouring *KRAS* mutations with a MAF > 3%. Of note, Laurent-Puig *et al* (2015) observed that patients with less than 1% of *KRAS* MAF showed similar progression-free survival and overall survival than those with wild-type *KRAS* tumours, thus suggesting that patients with mCRC with *KRAS*-mutated subclones (at least those with a *KRAS*-mutated subclones fraction lower or equal to 1%) had a benefit from anti-EGFR therapies.

Taken together these data suggest that subclonal mutations with a very low MAF have to be taken with caution and further analysed by including an UDG digestion step in the process to truly identify mutations governing the choice of a therapeutic compound. This is also of importance for an accurate mutational tracking of patients exhibiting emergence of *KRAS*-mutated clones in their plasma during anti-EGFR treatments.

In conclusion, our work further highlights that mCRCs are rather heterogeneous showing coexisting multiple gene mutations, with a small subset of patients (2.1% of the Sequenom wild-type tumours) harbouring *KRAS* mutations missed due to intrinsic limitations of the assay (in terms of sequence coverage and technical sensitivity). In addition, we also unveil the occurrence of artifactual mutations especially when MAFs are below 3%: this evidence strongly supports the importance of UDG pre-treatment

of DNA samples from FFPE to confirm the presence of true DNA changes. Especially in the context of multicenter clinical trials, a proper assessment of pathogenic mutations harboured by tumour subclones (that is, intratumour heterogeneity) is warranted to better understand their biological significance and clinical impact, in terms of response to anti-EGFR molecules and targeted therapies in general.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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