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ORIGINAL ARTICLE

Comprehensive genomic profiling by liquid biopsy in refractory metastatic colorectal cancer patients who are candidate for anti-EGFR rechallenge therapy: findings from the CAVE-2 GOIM trial

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Background: Biomarker-guided therapies are needed for patients with refractory metastatic colorectal cancer (mCRC). Liquid biopsy (LBx) circulating tumor DNA (ctDNA) comprehensive genomic profiling (CGP) could contribute to the clinical tailoring of molecular targeted therapies for these patients.

Patients and methods: The CAVE-2 GOIM trial is a randomized phase II trial assessing the efficacy of adding avelumab to cetuximab compared with cetuximab monotherapy as anti-epidermal growth factor receptor (EGFR) rechallenge in refractory ctDNA *RAS/BRAF*^{V600E} wild-type mCRC patients. Baseline plasma ctDNA CGP was carried out using the Foundation One Liquid CDx.

Results: LBx-based CGP was available for 324 patients out of 328 screened. A total of 1969 pathogenic variants (PVs) were detected. One hundred and thirty-five cases (41.7%) had single *RAS/BRAF*^{V600} PVs, with 24 tumors (17.6%) having multiple *RAS/BRAF*^{V600} PVs. Of 166 *RAS/BRAF*^{V600} PVs, 95 (57.2%) consisted of subclonal alterations {median variant allele frequency [VAF] of 0.37% [interquartile range (IQR) 0.18%-1.0%] versus median VAF of 4.0% [IQR 0.65%-11.25%] in 71 cases of clonal PV; $P < 0.0001$}. Single *RAS/BRAF*^{V600} alterations had higher clonality compared with co-occurring *RAS/BRAF*^{V600} alterations [12.8% (IQR 1.93%-56.0%) versus 0.67% (IQR 0.37-3.35), $P < 0.0001$]. Among other genes potentially involved in anti-EGFR drug resistance, 174 non-*RAS/BRAF*^{V600} PV were observed with 73 cases having both co-occurring *RAS/BRAF*^{V600} and non-*RAS/BRAF*^{V600} PV, while in 43 cases only non-*RAS/BRAF*^{V600} PV were detected. Tumors harboring co-occurring non-*RAS/BRAF*^{V600} PV had significantly lower clonality as compared with *RAS/BRAF*^{V600} PV [1.52 (0.46-8.88) versus 17.0 (3.75-71.0), $P < 0.0001$]. Finally, a total of 332 genomic alterations with therapeutic relevance according to the European Society for Medical Oncology (ESMO) Scale for Clinical Actionability of Molecular Targets (ESCAT) were detected for 171 patients (52.7%). Of these, 120

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tier I alterations were detected among 111 (34.3%) patients, which included high tumor mutational burden (≥10 mut/Mb, $n = 101$), $BRAF^{V600E}$ ($n = 11$), $KRAS^{G12C}$ ($n = 7$), and RET^{fusion} ($n = 1$).
Conclusions: LBx-based CGP might guide the appropriate use of anti-EGFR drug rechallenge or of other therapeutically actionable gene alterations in refractory mCRC.
Key words: colorectal cancer, liquid biopsy, cetuximab, avelumab, comprehensive genomic profiling

INTRODUCTION

Activation of the epidermal growth factor receptor (EGFR) signaling has a fundamental role in colorectal cancer (CRC) cell proliferation, tumor progression, invasion, and metastasis.[1] Therapeutic targeting of the EGFR pathway at different molecular levels represents a key strategy for the treatment of more than half of patients with metastatic CRC (mCRC), including tumors with *RAS/BRAF* wild-type (WT) status or harboring $BRAF^{V600E}$ mutation and $KRAS^{G12C}$ mutation.[2,3] Emerging evidence supports the concept that a subset of EGFR-dependent tumors that maintain *RAS/BRAF/EGFR*-extracellular domain (ECD) WT status might still benefit from EGFR blockade even upon initial progressive disease (PD) after first-line therapy.[4] It has been hypothesized that after a second-line therapeutic window without the selective pressure of anti-EGFR-targeted therapies, mutated resistant cancer cell clones or subclones might decay over time, thus potentially restoring therapeutic sensitivity to anti-EGFR monoclonal antibodies (mAbs).[5]

Based on this theory, different groups have explored retreatment with anti-EGFR mAbs (cetuximab or panitumumab) in patients with refractory mCRC.[6-13] Of note, these rechallenge strategies have shown promising clinical activity with an overall response rate of up to 30%, median progression-free survival (mPFS) of 4-6 months, and median overall survival (mOS) >1 year in patients whose tumor retained *RAS/BRAF* WT status, as assessed at baseline before treatment by liquid biopsy analysis of plasma circulating tumor DNA (ctDNA).[14]

So far, available data are mainly based on *post hoc* exploratory analysis of single-arm trials including a limited number of patients, by using in most cases PCR-based platforms for the detection of hotspot *RAS* and *BRAF* mutations.[11,15] So far, the CHRONOS trial is the only study that explored prospectively the role of a liquid biopsy-guided use of rechallenge with anti-EGFR mAbs in patients with ctDNA *RAS/BRAF/EGFR* ECD WT tumors.[11]

Nevertheless, it is well known that mCRC is characterized by a significant spatial and temporal tumor heterogeneity that might impair the efficacy of targeted therapies.[16-18] It has been shown that liquid biopsy-based plasma ctDNA analysis of other genes, beside *RAS*/*BRAF* and *EGFR* ECD, that confer resistance to anti-EGFR therapies, which is generally defined as molecular hyperselection, could better identify patients who are more likely to benefit from first-line anti-EGFR drugs.[19-21] However, limited data are available for patients who received anti-EGFR rechallenge therapy.[10] In this respect, an exploratory analysis of the randomized phase II VELO trial has demonstrated that, in molecularly hyperselected patients with baseline liquid biopsy-based ctDNA comprehensive genomic profiling (CGP) by next-generation sequencing (NGS), anti-EGFR drug rechallenge in the third-line treatment of refractory mCRC patients with panitumumab plus trifluridine/tipiracil led to mPFS of >6 months, with a subset of patients experiencing PFS >1 year.[10]

We are currently conducting the CAVE-2 Gruppo Oncologico dell' Italia Meridionale (GOIM) randomized phase II trial.[22] In this multicenter study, refractory mCRC patients who have benefited from first-line chemotherapy plus anti-EGFR drug treatment are treated with anti-EGFR rechallenge therapy in third- or later-lines with cetuximab or with cetuximab plus the anti-programmed death-ligand 1 mAb avelumab. As inclusion criteria for the CAVE-2 GOIM trial, at baseline before study enrollment and randomization, liquid biopsy-based plasma ctDNA analysis, which defines CGP, is carried out with FoundationOne Liquid CDx (324 genes).

In this article, we report the results of molecular screening with the definition of the pathogenic variants (PVs) landscape of 324 refractory mCRC patients who had *RAS/BRAF* WT, had microsatellite stable (MSS) disease, had a major response to first-line treatment with a combination of chemotherapy, and an anti-EGFR mAb, and were treated for at least a subsequent line with an anti-EGFR drug-free therapy, before being candidate to anti-EGFR rechallenge within the CAVE-2 GOIM trial. We conducted an analysis of potential anti-EGFR drug resistance mutations, their co-occurrence in the same patient plasma ctDNA as well as the identification of cancer cell clonality and subclonality for these mutations. Furthermore, we report the analysis of PV with potential therapeutic relevance according to the European Society for Medical Oncology (ESMO) Scale for Clinical Actionability of Molecular Targets (ESCAT).

PATIENTS AND METHODS

Study design and population

The CAVE-2 GOIM study (NCT05291156) is an academic, multicenter, phase II, open-label, randomized trial investigating the combination of avelumab plus cetuximab compared with cetuximab alone as anti-EGFR rechallenge strategy in refractory patients with *RAS*/*BRAF* WT, MSS mCRC.

Eligible patients should have received first-line treatment with anti-EGFR mAbs (cetuximab or panitumumab) in combination with chemotherapy and should have had a complete or partial response, and then should have progressed and, therefore, received at least one anti-EGFR

drug-free subsequent line of therapy. At baseline during the screening phase, a blood sample was obtained using FoundationOne Liquid CDx collection tubes. Plasma ctDNA is measured by the FoundationOne Liquid CDx assay (https://www.foundationmedicine.com/test/foundationone-liquid-cdx) to identify patients without resistance mutations of *RAS/BRAF* to be enrolled in the trial.[17,23,24] The same procedure is carried out at PD. Eligible patients were randomized (2 : 1) to receive cetuximab (400 mg/m^2, as a loading dose, and, subsequently, 250 mg/m^2 weekly), and avelumab (flat dose of 800 mg, every 2 weeks, arm A) or cetuximab (400 mg/m^2, as a loading dose, and subsequently, 250 mg/m^2 weekly, arm B). Tumor assessment by computed tomography scan or magnetic resonance imaging was scheduled every 8 weeks for 40 weeks and every 12 weeks thereafter to determine response to treatment. Response was evaluated using RECIST 1.1. Treatment continued until PD, significant clinical deterioration, or in the presence of any criterion for withdrawal from the trial. The full study protocol with details on the primary (OS) and secondary (PFS, safety) endpoints and on the statistical hypotheses has been reported and is also available in the Supplementary Material, available at https://doi.org/10.1016/j.esmoop.2025.105491.[21]

The study is conducted in accordance with the principles of the Declaration of Helsinki and the International Conference on Harmonisation and Good Clinical Practice Guidelines and has been approved by the Ethics Committee of Università degli Studi della Campania Luigi Vanvitelli/Azienda Ospedaliera Universitaria Luigi Vanvitelli/AORN Ospedale dei Colli and by the ethics committees of the other centers involved in the study. All patients have provided written informed consent to participate.

Statistical analysis

In the current study, only pathogenic or likely pathogenic mutations were considered for analysis. Clonality of PV was calculated by normalizing the variant allele frequency (VAF) with the estimated tumor fraction (TF) as assessed on ctDNA (VAF ÷ ctDNA fraction). Subclonal alterations were defined as mutations showing a clonality <10%. Mutations previously described and belonging to clonal hematopoiesis of indeterminate potential (CHIP) with clonality <25% were excluded from analyses.[25]

Continuous variables were reported as medians and interquartile range (IQR), and categorical variables were summarized as frequency counts and proportions. Categorical variables were compared using Fisher's exact test, while continuous variables between groups were tested using the Wilcoxon−Mann−Whitney test or linear models estimated using the ordinary least squares. Correlation tests were carried out using Kendall's test assuming a non-bivariate normal distribution of ctDNA.

All statistical analyses were carried out using a two-sided significance level of <0.05 using R Software, version 4.3.2.

RESULTS

A total of 328 patients with refractory mCRC were screened for the CAVE-2 GOIM trial. For 324 cases, blood sample was available for baseline liquid biopsy analysis. Of these, 208 (64.2%) patients were considered eligible for randomization and treatment, while 118 patients were excluded for the presence of clonal *RAS/BRAF^{V600}* PVs. Of note, 20 (9.7%) patients were considered eligible despite the presence of *RAS/BRAF^{V600}* PVs at very low VAF (median VAF 0.2%, IQR 0.14%-0.53%) (Supplementary Figure S1, available at https://doi.org/10.1016/j.esmoop.2025.105491). For the eligible patients, baseline clinicopathological characteristics were recorded and are reported in Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2025.105491.

Plasma ctDNA tumor fraction influences the detection of subclonal pathogenic variants

Among 324 cases, a total of 1969 PVs were detected, with *TP53* ($n = 277$, 84.5%), *APC* ($n = 269$, 82.5%), and *KRAS* ($n = 110$, 33.5%) representing the most common mutated genes (Figure 1A). We have previously shown that the sensitivity of liquid biopsy-based CGP is significantly influenced by plasma ctDNA TF.[17] Overall, the median ctDNA TF was 19% (IQR 1.78%-47.0%), with 67 (20.6%) cases showing ctDNA TF ≤1%. We observed a positive correlation between ctDNA TF and the number of detected genomic alterations (Kendall's tau = 0.50, $P < 0.0001$) (Figure 1B) and between tumor mutational burden (TMB) and ctDNA TF (Kendall's tau = 0.42, $P < 0.0001$).

Thus, we hypothesized that ctDNA TF might influence the detection rate of subclonal mutations, considering that these latter PVs originate from a lower proportion of cancer cells. In this regard, a positive correlation between ctDNA TF and the number of detected subclonal mutations was observed (Kendall's tau = 0.42, $P < 0.0001$), which was not shown for clonal PV (Kendall's tau = −0.09, $P = 0.13$) (Figure 1B).

Acquired RAS and BRAF genomic alterations at baseline liquid biopsy analysis of patients who were screened for the CAVE-2 GOIM trial

Among 324 patients who were tested at baseline with liquid biopsy-based CGP, 135 (41.6%) cases had plasma ctDNA with acquired *RAS/BRAF^{V600}* PVs (Figure 2A). Of these 135 patients, 24 (17.6%) patients had tumors with multiple *RAS/BRAF^{V600}* alterations. A total of 166 PVs involving *RAS/BRAF^{V600}* were detected, including *KRAS* ($n = 110$), *NRAS* ($n = 43$), *BRAF^{V600}* ($n = 12$), and *HRAS* ($n = 1$) (Figure 2B; Supplementary Figure S2, available at https://doi.org/10.1016/j.esmoop.2025.105491). Of 166 PVs, 159 (95.7%) consisted of single-nucleotide variants (SNVs), with the remaining 7 PVs consisting of gene amplifications, all involving *KRAS* without a clear association between the

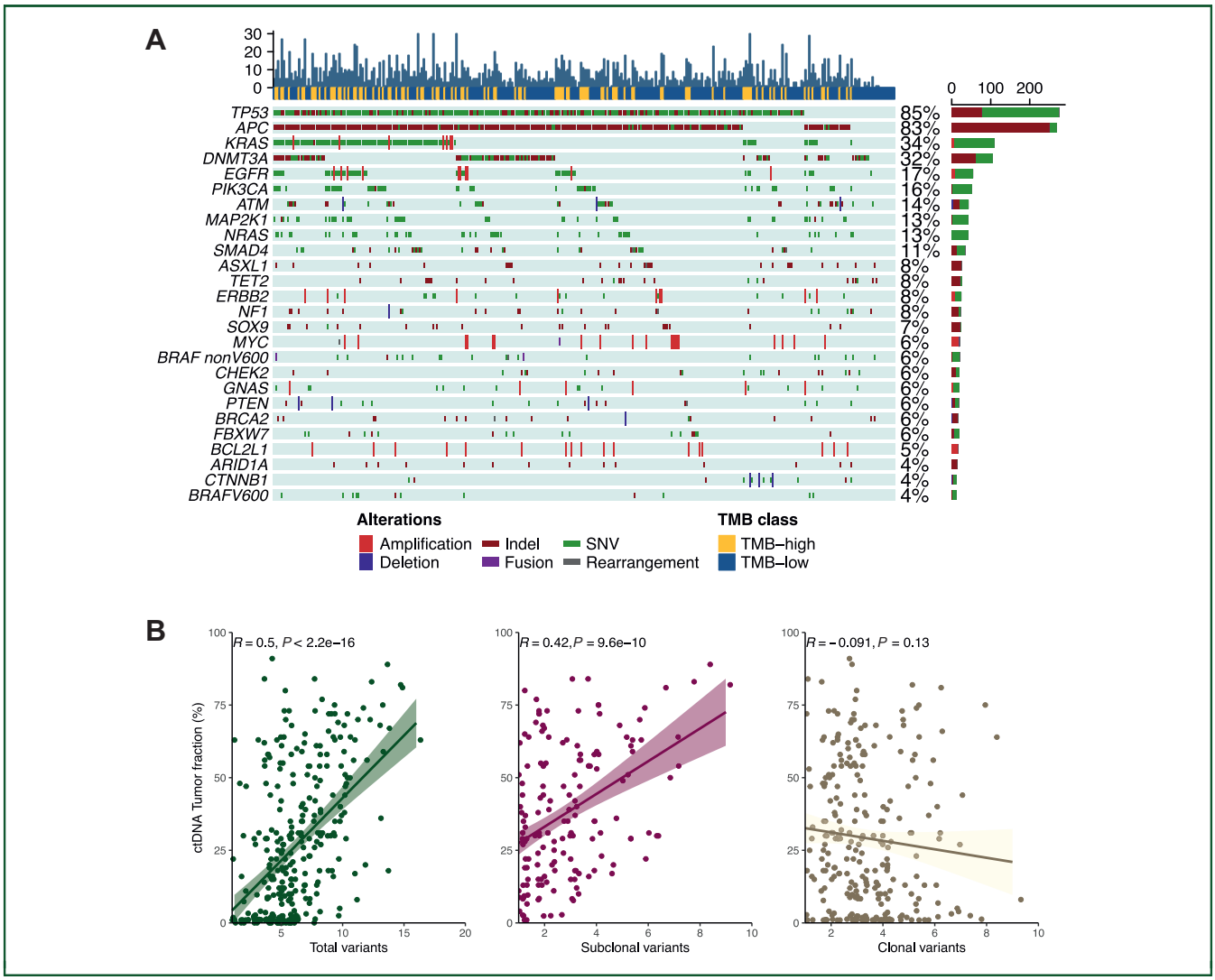

Figure 1. (A) Oncoprint of most common genomic alterations observed in the study cohort, with top annotation showing tumor mutational burden (TMB) and TMB classes. (B) Correlation between TMB and the number of detected pathogenic variants (left), between TMB and subclonal pathogenic variants (middle), and between TMB and clonal pathogenic variants (right). ctDNA TF correlated with the detection of subclonal mutations ($P < 0.0001$) but not with clonal pathogenic variants ($P = 0.13$).

ctDNA, circulating tumor DNA; SNV, single-nucleotide variant; TF, tumor fraction.

clonality of mutations and specific amino acidic residues (Figure 2B; Supplementary Figure S2, available at https://doi.org/10.1016/j.esmoop.2025.105491). Furthermore, 21 *BRAF*^{nonV600} PVs were observed (Supplementary Figure S3, available at https://doi.org/10.1016/j.esmoop.2025.105491).

We next examined the impact of tumor clonality on the occurrence of acquired *RAS/BRAF*^{V600} mutations. Of 166 *RAS/BRAF*^{V600} PVs, 95 (57.2%) were subclonal alterations [median VAF 0.37% (IQR 0.18%-1.0%) versus 4.0% (IQR 0.65%-11.25%), $P < 0.0001$] (Figure 3A), which were detected among 71 (53.3%) patients whose tumors exhibited *RAS/BRAF*^{V600} alterations. Subclonal PVs were detected for *KRAS* ($n = 56$), *NRAS* ($n = 30$), and *BRAF*^{V600} ($n = 9$) (Figure 3B). Overall, acquired *RAS/BRAF*^{V600} PVs had lower clonality compared with background alterations, such as *TP53* or *APC* [median clonality of 3.79% (0.7%-28.7%) versus 47.1% (2.1%-90.7%), respectively, $P < 0.0001$] (Figure 3C).

Subsequently, we investigated whether anti-EGFR resistance by means of acquired *RAS/BRAF*^{V600} PVs could occur either by co-existing subclones or by single *RAS/BRAF*^{V600} alteration. In this respect, single *RAS/BRAF*^{V600} alterations were observed with higher clonality compared with co-occurring *RAS/BRAF*^{V600} alterations [12.8% (IQR 1.93%-56.0%) versus 0.67% (IQR 0.37-3.35), $P < 0.0001$], which was confirmed after correction for ctDNA TF ($P = 0.01$) and when considering dominant *RAS/BRAF*^{V600} PVs alone [12.8% (IQR 1.93%-56.0%) versus 2.81% (IQR 0.71%-13.1%), $P = 0.008$] (Figure 3D). Further, co-occurring *RAS/BRAF*^{V600} PVs did not demonstrate bimodal distribution as it was observed for single *RAS/BRAF*^{V600} PVs (Figure 3E). Collectively, these results support the hypothesis that acquired anti-EGFR drug resistance *RAS/BRAF*^{V600} mutations can derive either by a single resistant cancer cell clone, which represents the majority of cancer cells, or by multiple co-occurring subclones of resistant cancer cells.

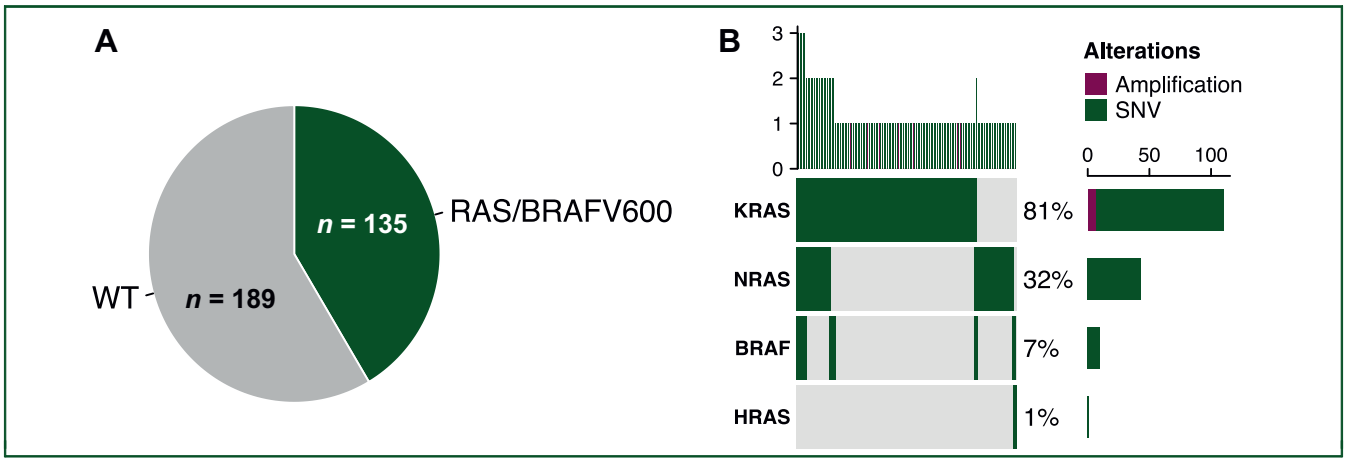

Figure 2. **(A) Pie chart representing the number of cases ($n = 135$, 41.6%) harboring *RAS/BRAF*^{V600} pathogenic variants. (B) Oncoprint of frequency, co-occurrence, and alterations classes of detected *RAS/BRAF*^{V600} mutations. Overall, 166 pathogenic variants involving *RAS/BRAF*^{V600} were detected, involving *KRAS* ($n = 110$), *NRAS* ($n = 43$), *BRAF*^{V600} ($n = 12$), and *HRAS* ($n = 1$).**
SNV, single-nucleotide variant.

Acquired multiple genomic alterations in other anti-EGFR drug resistance genes at baseline liquid biopsy analysis of patients who were screened for the CAVE-2 GOIM trial

Several molecular alterations beyond *RAS/BRAF*^{V600} have been described to confer anti-EGFR drug resistance.[19-21,24,26] Accordingly, we sought to define their prevalence at baseline in the CAVE-2 GOIM study cohort. Overall, 340 PVs (174 PVs after excluding *RAS/BRAF*^{V600}) that could confer anti-EGFR drug resistance were detected in 180 (55.5%) patients. After *KRAS* PVs, the most frequent genomic alterations were detected for *EGFR*ECD, *MAP2K1*, and *NRAS* (Figure 4A). The most frequent EGF ECD alterations were *EGFR ECD*^{G645} (n:13), *EGFR ECD*^{V441} (n:12), *EGFR ECD*^{S464} (n:7), and *EGFR ECD*^{S492} (n:5) (Supplementary Figure S4, available at https://doi.org/10.1016/j.esmoop.2025.105491).

Further, co-occurring *RAS/BRAF*^{V600} and non-*RAS/BRAF*^{V600} PVs were found in 73 patients. Only *RAS/BRAF*^{V600} PVs were detected in 64 patients, whereas only non-*RAS/BRAF*^{V600} PVs were observed in the plasma ctDNA of 43 patients. Similar to *RAS/BRAF*^{V600} PVs, non-*RAS/BRAF*^{V600} had a lower clonality compared with background *TP53* or *APC* mutations (Figure 4B).

It could be hypothesized that anti-EGFR drug resistance non-*RAS/BRAF*^{V600} PVs could be primarily acquired in tumors carrying subclonal *RAS/BRAF*^{V600} mutations. Therefore, we evaluated whether tumor low clonality of *RAS/BRAF*^{V600} PVs could account for the emergence of co-occurring non-*RAS/BRAF*^{V600} PVs. Interestingly, cases that exhibited co-occurring anti-EGFR drug resistance non-*RAS/BRAF*^{V600} PVs had significantly lower clonality of *RAS/BRAF*^{V600} mutations [1.52 (0.46-8.88) versus 17.0 (3.75-71.0), $P < 0.0001$], which was consistent also after correcting for ctDNA TF ($P = 0.002$) (Figure 4C).

Potentially actionable genomic alterations at baseline liquid biopsy analysis of patients who were screened for the CAVE-2 GOIM trial

We have previously demonstrated that patients with *RAS/BRAF* WT, MSS mCRC who were candidate for first-line treatment showed a relevant number of genomic alterations according to ESCAT, supporting the role of liquid biopsy-based CGP as a powerful clinical tool to identify additional cancer vulnerabilities that could be treated with appropriate molecular targeted drugs.[17] Overall, 332 actionable genomic alterations with potential clinical relevance were detected among 171 (52.7%) patients who were candidate for the CAVE-2 GOIM trial, according to ESCAT. Tier I alterations ($n = 120$) were detected among 111 (34.3%) patients, which included high TMB ($\geq$10 mut/Mb, $n = 101$), *BRAF*^{V600E} ($n = 11$), *KRAS*^{G12C} ($n = 7$), and *RET*^{fusion} ($n = 1$). ESCAT tier IIIA alterations ($n = 138$, 41.3%) were also found (Figure 5). No association between tumor sidedness and presence of actionable genomic alterations was found ($P = 0.8$).

DISCUSSION

For unselected patients with refractory mCRC, the clinical efficacy of currently available treatment options is limited and median OS ranges from ~7 to 11 months.[27,28] Liquid biopsy-based CGP at the time of PD after the first two lines of therapies could allow to better define the heterogeneous molecular alterations that characterize refractory mCRC in order to propose biomarker-driven more effective cancer treatment options.

In this respect, anti-EGFR rechallenge strategies have been gaining ground as a potential therapeutic option in refractory mCRC patients who have obtained relevant clinical benefit from previous anti-EGFR therapies, while retaining *RAS/BRAF* WT disease (ESMO Clinical practice guidelines for the treatment of mCRC, evidence III C).[14,29,30] Liquid biopsy is the most effective tool that allows, through the analysis of plasma ctDNA, the potential identification of patients who could take advantage from anti-EGFR rechallenge therapies. Patient clinical characteristics or time from the last anti-EGFR drug exposure are not reliable markers for patient selection.[31] We and others have previously demonstrated that the length of anti-EGFR drug-free interval does not predict the absence of anti-EGFR drug

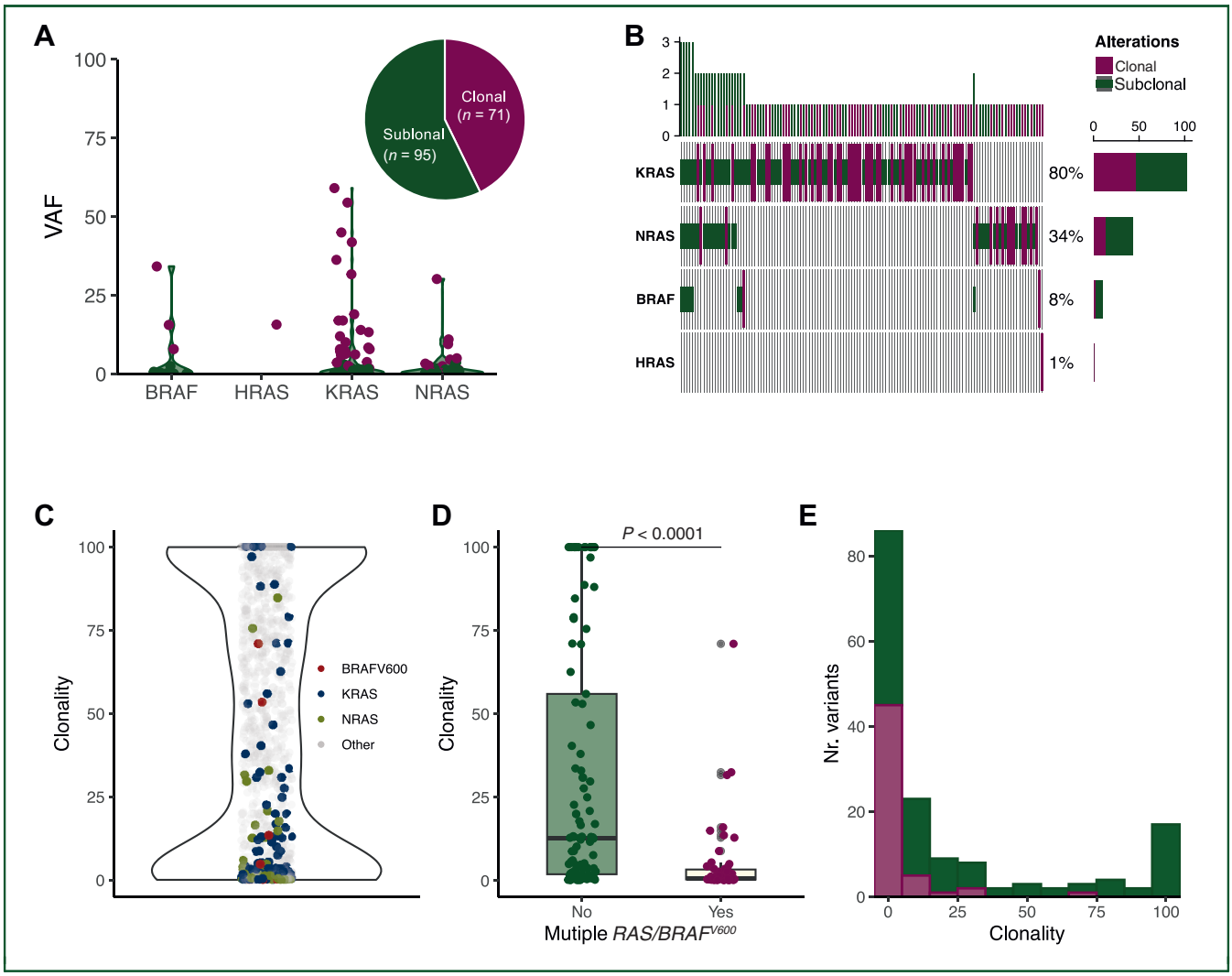

Figure 3. **(A) Frequency of clonal and subclonal *RAS/BRAF*^{V600} mutations. (B) Oncoprint showing frequency and co-occurrence of clonal and subclonal *RAS/BRAF*^{V600} pathogenic variants. Specifically, subclonal mutations involved *KRAS* ($n = 56$), *NRAS* ($n = 30$), and *BRAF* ($n = 9$). (C) Density distribution and comparison of *RAS/BRAF*^{V600} mutation's clonality compared with background (*TP53* or *APC*) alterations. Specifically, *RAS/BRAF*^{V600} mutations had lower clonality [median clonality of 3.79% (0.7%-28.7%) versus 47.1% (2.1%-90.7%), respectively, $P < 0.0001$]. (D) Clonality of co-occurring compared with single *RAS/BRAF*^{V600} pathogenic variants. In detail, single *RAS/BRAF*^{V600} mutations were with higher clonality compared with co-occurring *RAS/BRAF*^{V600} alterations [12.8% (IQR 1.93%-56.0%) versus 0.67% (IQR 0.37-3.35), $P < 0.0001$]. (E) Clonality distribution of single versus co-occurring *RAS/BRAF*^{V600} mutations. Specifically, co-occurring *RAS/BRAF*^{V600} mutations did not demonstrate bimodal clonal distribution as shown by single *RAS/BRAF*^{V600} pathogenic variants.**
IQR, interquartile range; VAF, variant allele frequency.

resistance mutations and, therefore, cannot be predictable of anti-EGFR rechallenge efficacy.[11,15,32] In this scenario, the randomized phase II CAVE-2 GOIM trial is prospectively comparing two anti-EGFR therapies in refractory mCRC patients who are selected by liquid biopsy-based CGP.

In this article, we report the results of the molecular screening of these patients. Overall, 135 (41.7%) patients had a detectable *RAS/BRAF*^{V600} genomic alteration in the plasma ctDNA at baseline and, therefore, were not eligible for the CAVE-2 GOIM trial. Of these, 9.7% of patients had tumors with very low VAF. This proportion is slightly higher than that resulting from a pooled analysis of the CAVE and VELO trials (25%) and from the PARERE trial (34%).[15,32] However, it should be considered that in the CAVE and VELO trials, liquid biopsy-based evaluation of anti-EGFR resistance mutations was carried out with the Idylla™ Biocartis platform, which could detect only specific hotspot genomic alterations with a limit of 1% for *KRAS* exons 2 and 3 or *BRAF*^{V600E}, and of 5% for *KRAS* exon 4 and *NRAS* exons 2, 3, and 4.[15] In the PARERE trial, a small NGS panel was used (Oncomine™ Colon assay), which covers 14 genes in >240 hotspot alterations with a limit of detection of 0.1%.[32] Liquid biopsy-based CGP, as used in the present report with the 324 genes FoundationOne Liquid CDx platform, could have higher sensitivity to identify either uncommon genomic alterations or to detect gene amplifications. In fact, in this article we report that for the *KRAS* gene 7 cases of gene amplification were found out of 110 cases with *KRAS* PVs. We also reported one case with *HRAS* mutation, a very rare finding in mCRC. It is difficult to

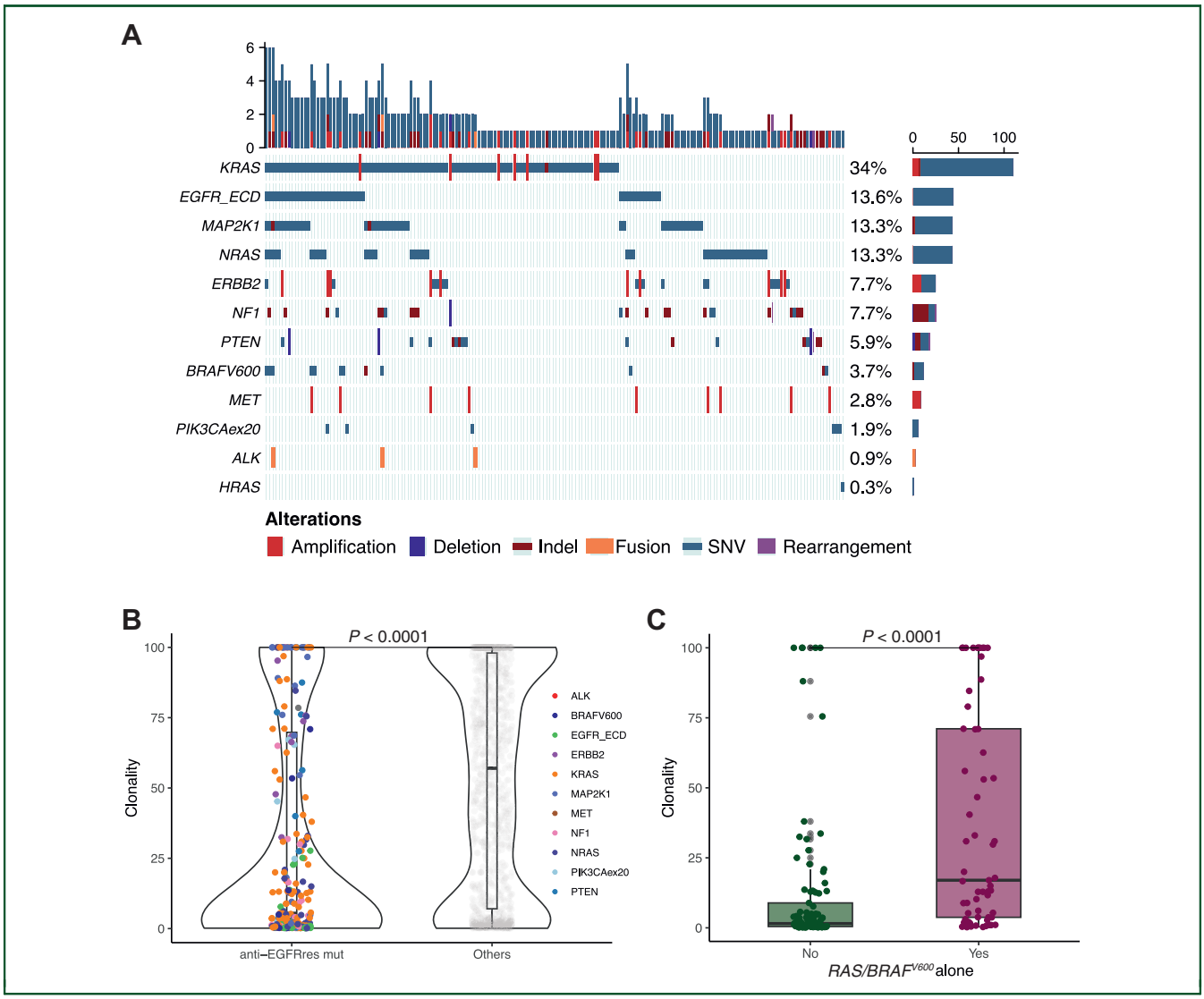

Figure 4. **(A) Oncoprint of anti-EGFR resistance mutations in the study cohort. Specifically, 340 mutations were observed among 180 (55.5%) patients. (B) Clonality of anti-EGFR resistance mutations compared with background (*TP53* and *APC*) mutations, with the former showing a lower overall clonality. (C) Comparison of *RAS/BRAF*^{V600} clonality between cases showing co-occurring non-*RAS/BRAF*^{V600} mutations that cause anti-EGFR drug resistance. Specifically, cases harboring co-occurring non-*RAS/BRAF*^{V600} mutations had significantly lower clonality as compared with *RAS/BRAF*^{V600} mutations [1.52 (0.46-8.88) versus 17.0 (3.75-71.0), *P* < 0.0001].**

EGFR, epidermal growth factor receptor; SNV, single-nucleotide variant.

discern if this PV could be related to clonal hematopoiesis.[25] Moreover, the role of HRAS as a mechanism of resistance to anti-EGFR therapies is not yet established.[33]

It is still debated whether a specific threshold of clonality for a PV, which reflects the percentage of mutated cancer cells, could be used to predict the efficacy of molecular targeted therapies.[34] Further, the simple information regarding the VAF of a PV without the knowledge of the amount of ctDNA in the plasma sample does not permit to evaluate PV clonality. A potential advantage of liquid biopsy-based CGP with a larger number of genes that are evaluated simultaneously by NGS, such as for the 324 genes measured by the assay used in the present report, is the opportunity to estimate plasma ctDNA TF together with PV VAF. These parameters could allow to calculate cancer cell clonality for a given mutation.[20] Interestingly, we observed different patterns of anti-EGFR drug resistance genomic mutations that might represent different trajectories of cancer cell genomic evolution.

A first group of tumors had a single *RAS/BRAF*^{V600} mutation at a clonal level, suggesting that this might represent the main driver of tumor progression. Unfortunately, we do not have the possibility to compare the tumor molecular profiling before the start of first-line anti-EGFR therapies for patients who have been screened for the CAVE-2 GOIM trial when they have progressed from at least two lines of treatment and have refractory mCRC. Therefore, it is difficult to discern if a clonal alteration was pre-existent to anti-EGFR therapy or may represent a true mechanism of acquired resistance. In this regard, we have previously observed in the CAPRI-2 GOIM trial that, in tumors defined as *RAS/BRAF*^{V600} WT and MSS by local laboratory tissue

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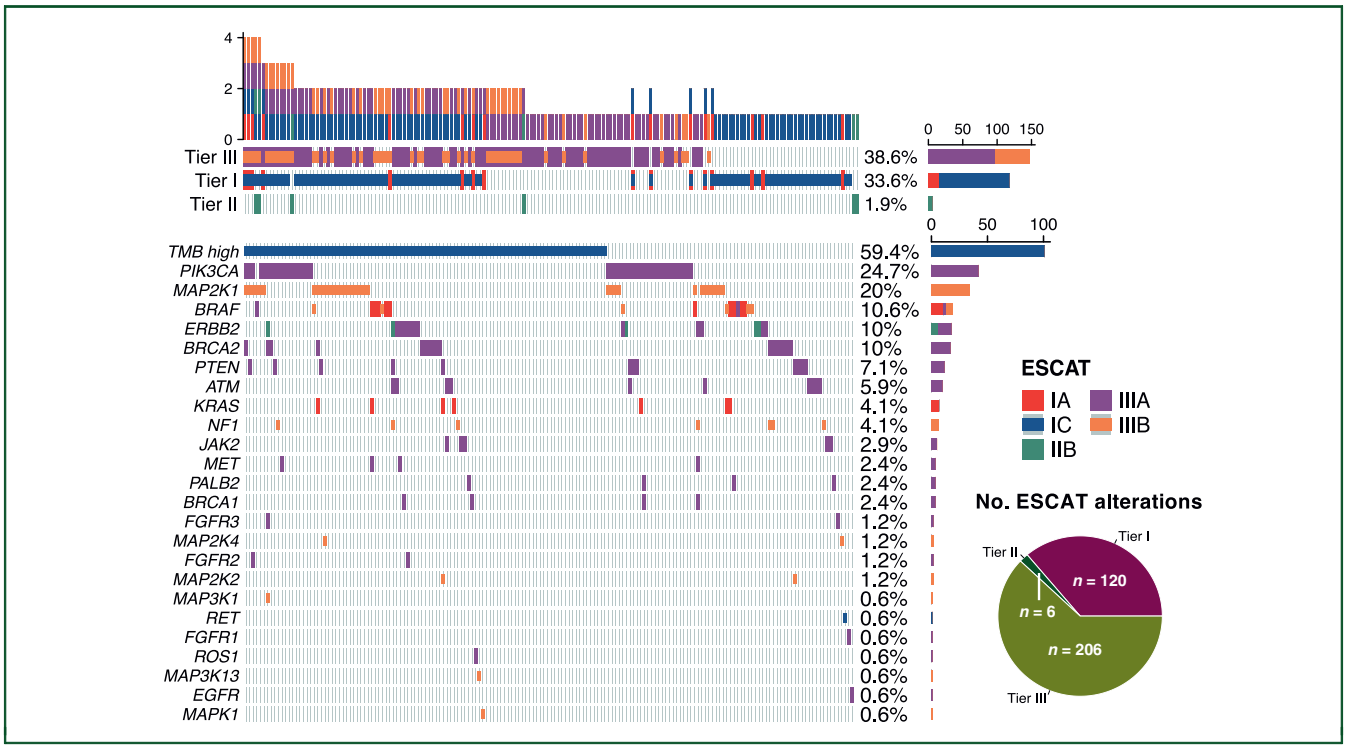

Figure 5. (A) Frequency of ESCAT alterations detected in the study cohort, with the overall ESCAT tier frequency on the top and actionable molecular biomarkers on the bottom. A total of 332 actionable genomic alterations with potential clinical relevance (tier I to III) were detected in 171 cases (52.7%).
ESCAT, European Society for Medical Oncology Scale for Clinical Actionability of Molecular Targets.

analysis before starting first-line treatment, nearly 9% of the cases had *RAS/BRAF*^{V600} PVs.[17] Similar data were reported in an exploratory biomarker evaluation of the PARADIGM trial.[15]

A second pattern of resistance to EGFR mAbs, which was observed in nearly half of the cases, is represented by the presence of subclonal *RAS/BRAF*^{V600} PVs. These alterations were frequently co-occurring with other *RAS/BRAF*^{V600} PVs or with PVs of other genes that are potentially involved in anti-EGFR drug resistance. Interestingly, for tumors harboring a clonal *RAS/BRAF*^{V600} alteration, a lower clonality of non-*RAS/BRAF*^{V600} PVs was observed. These data suggest that in these cases the presence of a clonal *RAS/BRAF*^{V600} PV might constitute the main driver of cancer cell growth. On the contrary, in case of a non-predominant subclonal alteration, multiple PVs coexist and all of them might be necessary for anti-EGFR drug resistance and for tumor progression. However, this hypothesis should be taken with caution and further investigation is required. In this regard, the CAPRI-2 GOIM trial could contribute to shed light on this issue.[34] In fact, the CAPRI-2 GOIM trial is currently evaluating the use of cetuximab-based treatment across three subsequent lines of therapy (first-, second-, and third-line), according to the results of liquid biopsy-based plasma ctDNA CGP using FoundationOne Liquid CDx, which is carried out at baseline and after progression to first- and second-line therapy.[35]

Alongside a biomarker-guided use of patient selection for anti-EGFR rechallenge strategies, liquid biopsy-based CGP could allow the detection of potentially actionable PVs that could represent other therapeutic targets for the refractory mCRC setting. Remarkably, one out of three cases at plasma ctDNA CGP exhibited at least one ESCAT tier I alteration. Of note, high TMB was reported in 31.2% of the cases and, therefore, it might identify a subset of patients with refractory MSS mCRC that could potentially benefit from immune checkpoint inhibitors.[36,37] This percentage is significantly higher than what we have observed in patients with MSS, *RAS/BRAF* WT naive for systemic treatment for metastatic disease in the CAPRI-2 GOIM trial.[17] In this respect, a *post hoc* analysis of the CO.26 trial has reported higher TMB in case of previous exposure to anti-EGFR therapies.[38] Taken together, these data support the rationale of the CAVE-2 GOIM trial in which cetuximab plus avelumab are used in combination.

The current analysis has some limitations. Firstly, clinicopathological information of patients who were not eligible for the study were not recorded. Therefore, we were not able to evaluate whether a correlation between the length of previous anti-EGFR therapy, the type of treatment, and the anti-EGFR drug-free interval exists with the *RAS/BRAF*^{V600} mutation status. Secondly, as discussed earlier, liquid biopsy-based CGP was carried out only at the time of the screening and is then carried out at PD, but no data regarding the molecular profile of chemo-naive patients are available. Thirdly, ~20% of the patients had ctDNA TF <1%. For these cases, the capability of liquid biopsy to detect PVs is suboptimal with the risk of false-negative results.[39] Nevertheless, a liquid biopsy-based CGP approach is the most sensitive technique to identify these cases since PCR

and NGS panels evaluating a limited number of genes could not allow an estimation of ctDNA TF. Further, we report here for the 324 patients who were screened for the CAVE-2 GOIM trial that the median ctDNA TF was 19%. Clinical evidence has been provided suggesting that a value >10% could allow the identification of complex PVs, including gene translocations, copy number variations, and deletions.[17,40]

Conclusion

The results of the molecular screening for the CAVE-2 GOIM trial highlight the complexity and the heterogeneity of refractory mCRC genomic profiles. Liquid biopsy-based CGP could help to identify the molecular heterogeneity. This can be translated in the identification of patients who are suitable for anti-EGFR rechallenge therapies and of patients who could benefit from other actionable genomic alterations. Taken together, these results suggest that plasma ctDNA CGP is a clinically relevant technique for monitoring mCRC molecular evolution through different lines of therapies and for selecting the most appropriate treatment choices in the era of precision medicine.

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DISCLOSURE

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DATA SHARING

Researchers can request access to de-identified individual patient-level data from the corresponding author on a reasonable request.

REFERENCES

1. Ciardiello F, Tortora G. EGFR antagonists in cancer treatment. *N Engl J Med*. 2008;358(11):1160-1174.
2. Napolitano S, Martini G, Ciardiello D, et al. Targeting the EGFR signalling pathway in metastatic colorectal cancer. *Lancet Gastroenterol Hepatol*. 2024;9(7):664-676.
3. Di Nicolantonio F, Vitiello PP, Marsoni S, et al. Precision oncology in metastatic colorectal cancer — from biology to medicine. *Nat Rev Clin Oncol*. 2021;18(8):506-525.
4. Martinelli E, Ciardiello D, Martini G, et al. Implementing anti-epidermal growth factor receptor (EGFR) therapy in metastatic colorectal cancer: challenges and future perspectives. *Ann Oncol*. 2020;31(1):30-40.
5. Parseghian CM, Loree JM, Morris VK, et al. Anti-EGFR-resistant clones decay exponentially after progression: implications for anti-EGFR re-challenge. *Ann Oncol*. 2019;30(2):243-249.
6. Cremolini C, Rossini D, Dell'Aquila E, et al. Rechallenge for patients with RAS and BRAF wild-type metastatic colorectal cancer with acquired resistance to first-line cetuximab and irinotecan: a phase 2 single-arm clinical trial. *JAMA Oncol*. 2019;5(3):343-350.
7. Martinelli E, Martini G, Famiglietti V, et al. Cetuximab rechallenge plus avelumab in pretreated patients with RAS wild-type metastatic colorectal cancer: the phase 2 single-arm clinical CAVE trial. *JAMA Oncol*. 2021;7(10):1529-1535.
8. Ciardiello D, Famiglietti V, Napolitano S, et al. Final results of the CAVE trial in RAS wild type metastatic colorectal cancer patients treated with cetuximab plus avelumab as rechallenge therapy: neutrophil to lymphocyte ratio predicts survival. *Clin Colorectal Cancer*. 2022;21(2):141-148.
9. Napolitano S, De Falco V, Martini G, et al. Panitumumab plus trifluridine-tipiracil as anti-epidermal growth factor receptor rechallenge therapy for refractory RAS wild-type metastatic colorectal cancer: a phase 2 randomized clinical trial. *JAMA Oncol*. 2023;9(7):966-970 [published correction appears in *JAMA Oncol*. 2024;10(4):541].
10. Napolitano S, Ciardiello D, De Falco V, et al. Panitumumab plus trifluridine/tipiracil as anti-EGFR rechallenge therapy in patients with refractory RAS wild-type metastatic colorectal cancer: overall survival and subgroup analysis of the randomized phase II VELO trial. *Int J Cancer*. 2023;153(8):1520-1528.
11. Sartore-Bianchi A, Pietrantonio F, Lonardi S, et al. Circulating tumor DNA to guide rechallenge with panitumumab in metastatic colorectal cancer: the phase 2 CHRONOS trial. *Nat Med*. 2022;28(8):1612-1618.
12. Sunakawa Y, Nakamura M, Ishizaki M, et al. *RAS* mutations in circulating tumor DNA and clinical outcomes of rechallenge treatment with anti-EGFR antibodies in patients with metastatic colorectal cancer. *JCO Precis Oncol*. 2020;4:898-911.
13. Ciardiello D, Martinelli E, Troiani T, et al. Anti-EGFR rechallenge in patients with refractory ctDNA RAS/BRAF wt metastatic colorectal cancer: a nonrandomized controlled trial. *JAMA Netw Open*. 2024;7(4):e245635 [published correction appears in *JAMA Netw Open*. 2024;7(5):e2418437].
14. Ciardiello D, Mauri G, Sartore-Bianchi A, et al. The role of anti-EGFR rechallenge in metastatic colorectal cancer, from available data to future developments: a systematic review. *Cancer Treat Rev*. 2024;124:102683.
15. Ciardiello D, Napolitano S, Famiglietti V, et al. Pretreatment plasma circulating tumor DNA RAS/BRAF mutational status in refractory metastatic colorectal cancer patients who are candidates for anti-EGFR rechallenge therapy: a pooled analysis of the CAVE and VELO clinical trials. *Cancers (Basel)*. 2023;15(7):2117.
16. Normanno N, Rachiglio AM, Lambiase M, et al. Heterogeneity of KRAS, NRAS, BRAF and PIK3CA mutations in metastatic colorectal cancer and potential effects on therapy in the CAPRI GOIM trial. *Ann Oncol*. 2015;26(8):1710-1714.
17. Ciardiello D, Boscolo Bielo L, Napolitano S, et al. Comprehensive genomic profiling by liquid biopsy captures tumor heterogeneity and identifies cancer vulnerabilities in patients with RAS/BRAF^{V600E} wild-type metastatic colorectal cancer in the CAPRI 2-GOIM trial. *Ann Oncol*. 2024;35(12):1105-1115.
18. Zheng Z, Yu T, Zhao X, Gao X, Zhao Y, Liu G. Intratumor heterogeneity: a new perspective on colorectal cancer research. *Cancer Med*. 2020;9(20):7637-7645.
19. Shitara K, Muro K, Watanabe J, et al. Baseline ctDNA gene alterations as a biomarker of survival after panitumumab and chemotherapy in metastatic colorectal cancer. *Nat Med*. 2024;30(3):730-739.
20. Ciardiello D, Boscolo Bielo L, Napolitano S, et al. Comprehensive genomic profiling by liquid biopsy portraits metastatic colorectal cancer mutational landscape to predict antitumor efficacy of FOLFIRI plus cetuximab in the CAPRI-2 GOIM trial. *ESMO Open*. 2025;10(4):104511.
21. Montagut C, Dalmases A, Bellosillo B, et al. Identification of a mutation in the extracellular domain of the epidermal growth factor receptor conferring cetuximab resistance in colorectal cancer. *Nat Med*. 2012;18(2):221-223 [published correction appears in *Nat Med*. 2012;18(9):1445. Somasekar, Seshagiri (corrected to Seshagiri, Somasekar)].
22. Napolitano S, Martini G, Ciardiello D, et al. CAVE-2 (Cetuximab-AVElumab) mCRC: a phase II randomized clinical study of the combination of avelumab plus cetuximab as a rechallenge strategy in pre-treated RAS/BRAF wild-type mCRC patients. *Front Oncol*. 2022;12:940523.
23. Woodhouse R, Li M, Hughes J, et al. Clinical and analytical validation of FoundationOne Liquid CDx, a novel 324-Gene cfDNA-based comprehensive genomic profiling assay for cancers of solid tumor origin. *PLoS One*. 2020;15(9):e0237802.
24. Caputo V, De Falco V, Ventriglia A, et al. Comprehensive genome profiling by next generation sequencing of circulating tumor DNA in solid tumors: a single academic institution experience. *Ther Adv Med Oncol*. 2022;14:17588359221096878.
25. Khoury JD, Solary E, Abla O, et al. The 5th edition of the World Health Organization classification of haematolymphoid tumours: myeloid and histiocytic/dendritic neoplasms. *Leukemia*. 2022;36(7):1703-1719.

26. Randon G, Maddalena G, Germani MM, et al. Negative ultraselection of patients with *RAS/BRAF* wild-type, microsatellite-stable metastatic colorectal cancer receiving anti-EGFR-based therapy. *JCO Precis Oncol*. 2022;6:e2200037 [published correction appears in *JCO Precis Oncol*. 2022;6:e2200356].
27. Grothey A, Van Cutsem E, Sobrero A, et al. Regorafenib monotherapy for previously treated metastatic colorectal cancer (CORRECT): an international, multicentre, randomised, placebo-controlled, phase 3 trial. *Lancet*. 2013;381(9863):303-312.
28. Prager GW, Taieb J, Fakih M, et al. Trifluridine-tipiracil and bevacizumab in refractory metastatic colorectal cancer. *N Engl J Med*. 2023;388(18):1657-1667.
29. Cervantes A, Adam R, Roselló S, et al. Metastatic colorectal cancer: ESMO Clinical Practice Guideline for diagnosis, treatment and follow-up. *Ann Oncol*. 2023;34(1):10-32.
30. Ciardiello F, Ciardiello D, Martini G, Napolitano S, Tabernero J, Cervantes A. Clinical management of metastatic colorectal cancer in the era of precision medicine. *CA Cancer J Clin*. 2022;72(4):372-401.
31. Rossini D, Germani MM, Pagani F, et al. Retreatment with anti-EGFR antibodies in metastatic colorectal cancer patients: a multi-institutional analysis. *Clin Colorectal Cancer*. 2020;19(3):191-199.e6.
32. Germani MM, Vetere G, Giordano M, et al. Molecular screening with liquid biopsy for anti-EGFR retreatment in metastatic colorectal cancer: preliminary data from the randomized phase 2 PARERE trial. *Front Oncol*. 2024;13:1307545.
33. Boidot R, Chevrier S, Julie V, Ladoire S, Ghiringhelli F. HRAS G13D, a new mutation implicated in the resistance to anti-EGFR therapies in colorectal cancer, a case report. *Int J Colorectal Dis*. 2016;31(6):1245-1246.
34. Nakamura Y, Ozaki H, Ueno M, et al. Targeted therapy guided by circulating tumor DNA analysis in advanced gastrointestinal tumors. *Nat Med*. 2025;31(1):165-175.
35. Martini G, Ciardiello D, Napolitano S, et al. Efficacy and safety of a biomarker-driven cetuximab-based treatment regimen over 3 treatment lines in mCRC patients with *RAS/BRAF* wild type tumors at start of first line: the CAPRI 2 GOIM trial. *Front Oncol*. 2023;13:1069370.
36. Marabelle A, Fakih M, Lopez J, et al. Association of tumour mutational burden with outcomes in patients with advanced solid tumours treated with pembrolizumab: prospective biomarker analysis of the multicohort, open-label, phase 2 KEYNOTE-158 study. *Lancet Oncol*. 2020;21(10):1353-1365.
37. Duvivier HL, Rothe M, Mangat PK, et al. Pembrolizumab in patients with tumors with high tumor mutational burden: results from the targeted agent and profiling utilization registry study. *J Clin Oncol*. 2023;41(33):5140-5150.
38. Loree JM, Titmuss E, Topham JT, et al. Plasma versus tissue tumor mutational burden as biomarkers of durvalumab plus tremelimumab response in patients with metastatic colorectal cancer in the CO.26 trial. *Clin Cancer Res*. 2024;30(15):3189-3199.
39. Rolfo CD, Madison RW, Pasquina LW, et al. Measurement of ctDNA tumor fraction identifies informative negative liquid biopsy results and informs value of tissue confirmation. *Clin Cancer Res*. 2024;30(11):2452-2460.
40. Husain H, Pavlick DC, Fendler BJ, et al. Tumor fraction correlates with detection of actionable variants across $>$ 23,000 circulating tumor DNA samples. *JCO Precis Oncol*. 2022;6:e2200261 [published correction appears in *JCO Precis Oncol*. 2022;6:e2200622].