Dynamic molecular analysis and clinical correlates of tumor evolution within a phase II trial of panitumumab-based therapy in metastatic colorectal cancer

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Background: Mutations in rat sarcoma (RAS) genes may be a mechanism of secondary resistance in epidermal growth factor receptor inhibitor-treated patients. Tumor-tissue biopsy testing has been the standard for evaluating mutational status; however, plasma testing of cell-free DNA has been shown to be a more sensitive method for detecting clonal evolution.

Materials and methods: Archival pre- and post-treatment tumor biopsy samples from a phase II study of panitumumab in combination with irinotecan in patients with metastatic colorectal cancer (mCRC) that also collected plasma samples before, during, and after treatment were analyzed for emergence of mutations during/post-treatment by next-generation sequencing and BEAMing.

Results: The rate of emergence of tumor tissue RAS mutations was 9.5% by next-generation sequencing (n = 21) and 6.3% by BEAMing (n = 16). Plasma testing of cell-free DNA by BEAMing revealed a mutant RAS emergence rate of 36.7% (n = 39). Exploratory outcomes analysis of plasma samples indicated that patients who had emergent RAS mutations at progression had similar median progression-free survival to those patients who remained wild-type at progression. Serial analysis of plasma samples showed that the first detected emergence of RAS mutations preceded progression by a median of 3.6 months (range, −0.3 to 7.5 months) and that there did not appear to be a mutant RAS allele frequency threshold that could predict near-term outcomes.

Conclusions: This first prospective analysis in mCRC showed that serial plasma biopsies are more inclusive than tissue biopsies for evaluating global tumor heterogeneity; however, the clinical utility of plasma testing in mCRC remains to be further explored.

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Key words: gastrointestinal cancers, colorectal, phase I–III trials, biomarkers and intervention studies, panitumumab
Activating mutations in the rat sarcoma (RAS) gene family (e.g. KRAS and NRAS, exons 2, 3, and 4) are well-established biomarkers for lack of response in patients with metastatic colorectal cancer (mCRC) receiving anti-epidermal growth factor receptor (EGFR) antibody therapy [1]. Although patients who have wild-type RAS tumors before treatment initiation may derive benefit from anti-EGFR therapy, most will ultimately progress [1]. This progression may be attributed to continued clonal evolution and diversification, including the possibility of secondary (acquired) resistance to therapy resulting from selective pressure [2]. Mutations in RAS genes have been implicated as one mechanism of secondary resistance in EGFR inhibitor-treated patients, and mutation emergence has been detected in plasma samples during anti-EGFR therapy [3–5]. Tumor driver alterations in genes other than RAS have also been shown to be prognostically important for tumor growth and may play a role in clonal diversification and acquired resistance during therapy [6].

Tumor-tissue biopsy testing has been the standard for evaluating tumor mutational status. However, biopsies are invasive, contain only a fraction of the total tumor heterogeneity, and may not capture the full spectrum of potential additional genetic changes that occur during treatment [7]. More recently, plasma testing using next-generation sequencing (NGS) techniques or BEAMing (beads, emulsion, amplification, magnetics) of cell-free DNA (cfDNA) have been shown to be sensitive, reproducible methods of detecting clonal evolution of mutations [8]. Sequential plasma sample analysis may allow for the assessment of cfDNA for global mutation status in real time [9] and may reflect a more global sample of total tumor heterogeneity.

Few studies have evaluated paired tumor biopsy and plasma samples for mutation detection and prospective studies in this setting are lacking. We present results from the prospective, phase II, 20070820 study of patients with KRAS exon 2 wild-type mCRC who received treatment with panitumumab plus irinotecan (ClinicalTrials.gov, NCT00891930). Designed in 2007 and completed in July 2013, the study was initially designed to test whether KRAS mutation emergence was associated with the development of secondary resistance to panitumumab therapy. With additional findings reporting the impact of extended RAS mutations [1], the exploratory hypothesis extended to include other RAS family genes.

Materials and methods

Experimental design

Patients enrolled in part 1 received panitumumab 6 mg/kg plus irinotecan 180 mg/m² every 2 weeks until progression. After progression, patients were eligible for part 2, which required a second biopsy from the same tumor lesion (e.g. paired biopsy) for those with an objective response or stable disease under treatment with panitumumab in part 1, with the underlying rationale of studying mechanisms of acquired resistance and excluding primary resistant tumors (Figure 1A). The primary end point was objective response rate (supplementary Table S1, available at Annals of Oncology online); secondary end points were progression-free survival (PFS), overall survival (OS), and safety, which have been previously reported [10]. The coprimary objective was to determine whether acquired resistance to panitumumab in patients with wild-type KRAS exon 2 mCRC correlated with the emergence of mutant KRAS tumors.

Biomarker analysis

The objective of the biomarker analyses was to evaluate whether the DNA mutation status of potential tumor-related biomarkers (including RAS) changed in clonal populations of tumor cells during therapy or at progression. The mutation status of a panel of cancer driver gene mutations was evaluated using pretreatment (archival primary or metastatic biopsy tissue) and post-treatment samples from the same lesions where possible. RAS mutation status was determined by examining KRAS exons 2 (codons 12 and 13), 3 (codons 59 and 61), and 4 (codons 117 and 146); NRAS exons 2 (codons 12 and 13) and 3 (codons 59 and 61); and HRAS exons 2 (codons 12 and 13), 3 (codons 59 and 61), and 4 (codon 117 but not codon 146) from tumor tissue. Plasma biopsies collected at screening and weeks 9, 17, 25, 33, and 37 and once every 4 weeks thereafter until progression or study drug intolerability were also analyzed.

Tissue and plasma biomarker testing was carried out according to the analysis plan (Figure 1B); analysts were blinded to clinical data and patient outcomes (see supplementary materials, available at Annals of Oncology online). Because progressive disease as best response suggests primary resistance and the study was aimed at understanding molecular mechanisms of secondary resistance, patients with a best response of progressive disease were not analyzed. For patients with wild-type RAS tumor tissue at baseline with a best response of at least stable disease, plasma samples were analyzed at baseline and time of progression. For patients with wild-type RAS tumor tissue at baseline with mutant RAS tumor results at time of progression, all available plasma sample time points were analyzed to determine the timing and change in allele fraction of mutation emergence.

The study protocol was approved by an independent ethics committee at each study site; all patients provided written informed consent before study entry.

Results

Patients

Overall, 76 patients with wild-type KRAS exon 2 mCRC were enrolled; 74 patients received ≥1 dose of panitumumab and were included in the tumor tissue analysis. Twenty-nine patients had paired evaluable RAS status at baseline and progression and were included in the NGS tissue analysis. Of the 39 patients with evaluable plasma samples at baseline, 30 had wild-type RAS at baseline and paired post-treatment samples and were included in the BEAMing plasma analysis. Overall, 15 patients had both evaluable paired tissue and plasma samples (Figure 1B). Baseline demographics and disease characteristics were generally similar between tumor tissue and plasma testing groups (supplementary Table S2, available at Annals of Oncology online).

Paired tumor biopsies revealed limited emergent mutations

Baseline mutations were determined using DNA extracted from FFPE tumor tissue from 74 patients with wild-type KRAS exon 2 tumors. Mutation rates were determined by NGS of a 51-gene panel (Table 1) with the subset of EGFR pathway-related genes prespecified in the study protocol. At baseline, the most commonly mutated gene was TP53 (61%). Consistent with previous studies [11],
On-treatment blood collection for plasma analysis

**Study week 1**
- Baseline tumor biopsy + blood sampling

**Part 1**
- Panitumumab 6 mg/kg Q2W + irinotecan 180 mg/m² Q2W
- Response or stable disease: Continue panitumumab + irinotecan
- Progression: Progression

**Part 2**
- Plasma BEAMing
- Follow-up for response
- Panitumumab 6 mg/kg Q2W + AMG 479 12 mg/kg Q2W

**Patient tumor RAS status**

- **Plasma BEAMing**
  - Total paired: N=39
    - WT → WT: N=19
    - WT → MT: N=11
    - MT → MT: N=5
    - MT → WT: N=3
    - ND: N=1

- **Tissue BEAMing**
  - Total paired: N=17
    - WT → WT: N=15
    - WT → MT: N=1
    - MT → MT: N=19
    - MT → WT: N=0

- **Tissue NGS**
  - Total paired: N=29
    - WT → WT: N=19
    - WT → MT: N=2
    - MT → MT: N=8
    - MT → WT: N=0

**Figure 1.** (A) Study schema and (B) patient tumor RAS status results determined by tissue and plasma testing biomarker analyses. The RAS status of pre- and post-treatment patient tumor samples were analyzed by each testing method. Total patient samples and the number of patients whose RAS status did (e.g. WT→MT, MT→WT) or did not (WT→WT, MT→MT) change pre- to post-treatment are indicated. The numbers in the central intersection of the Venn diagram represent those cases for whom comparisons of analyses by the three indicated methods and source of samples were available. Samples were categorized as discordant if the results varied between testing methods. BEAM, beads, emulsion, amplification, magnetic; CT, computed tomography; IGF-1R, insulin-like growth factor receptor 1; MRI, magnetic resonance imaging; MT, mutant; ND, no data; NGS, next-generation sequencing; Q2W, every 2 weeks; Q4W, every 4 weeks; WT, wild-type.

- aBlood collection for plasma analysis at week 37 and every 4 weeks thereafter until disease progression intolerability.
- bBased on first restaging CT or MRI.
- cNine patients were either not doing or unevaluable for tumor response assessment.
- dOnly patients who had an objective response or stable disease after part 1 underwent a second biopsy.
- eA monoclonal antibody that inhibits the insulin-like growth factor 1 (IGF-1R) pathway.
- fPatients without both before-treatment and after-treatment samples for both tests were not characterized as discordant.
- gPatient was characterized as RAS wild-type at baseline by NGS.
extended RAS analysis revealed that 22% \((n=16/74)\) of the screened wild-type KRAS exon 2 samples had other activating RAS mutations, and 7% had BRAF mutations at baseline (supplementary Table S3, available at Annals of Oncology online).

To study the emergence of drug resistance and tumor evolution with anti-EGFR therapy, we analyzed 29 patients with available tumor tissue samples at baseline and time of progression. Four patients with existing TP53 mutations gained additional coding-region TP53 mutations and six patients had emergent mutations. Of the six with emergent mutations, three gained TP53 mutations (one also had a baseline NRAS [Q61H] mutation that was not detected at progression), one patient gained an SMAD4 mutation, one patient gained both IDH1 and KRAS (G13D) mutations, and one patient gained a KRAS (Q61H) mutation.

Of the 29 paired patient pre- and post-treatment tissue samples, 21 were wild-type RAS at baseline. The mutant RAS emergence rate was 9.5% \((n=2/21); 95\% \text{ CI}, 1\% \text{ to } 30\%\). BEAMing was used to further evaluate the mutant RAS emergence rate as it is reported to have a sensitivity of 0.01% [12] versus \(\sim10\%\) mutant fraction sensitivity of NGS (see supplementary materials, available at Annals of Oncology online). Of the 29 paired patient tissue samples tested using NGS, 17 were evaluated by BEAMing. Because of limited DNA availability and because few emergent mutations were observed using NGS, only RAS status was tested with BEAMing. Sixteen patients were wild-type RAS at baseline and the mutant RAS emergence rate by BEAMing of tissue was 6.3% \((n=1/16); \text{supplementary Table S4}, \text{available at Annals of Oncology online})

Detection for RAS mutations improved with plasma cfDNA profiling

Of the 39 patients with plasma samples at baseline (supplementary Table S4, available at Annals of Oncology online), 31 had wild-type RAS cfDNA and 8 had mutant RAS cfDNA when analyzed by BEAMing (Figure 2). One patient who was evaluated as wild-type RAS at baseline did not have evaluable samples at progression. Of the 30 patients with wild-type RAS cfDNA plasma samples, 19 had wild-type RAS and 11 had mutant RAS at progression. The mutant RAS emergence rate in plasma samples by BEAMing was 36.7% \((n=11/30)\). Of the 8 patients with mutant RAS plasma samples at baseline, 5 retained RAS mutations and 3 had wild-type RAS status at progression. The 3 patients identified as mutant RAS at baseline and wild-type at progression had all low frequencies of a single mutant RAS allele detected at baseline (0.04% KRAS G12A, 0.05% KRAS Q61H, and 0.03% NRAS Q61H, respectively).

In patients with paired tumor tissue biopsy and paired plasma samples that were wild-type RAS at baseline \((n=14); \text{supplementary Table S4}, \text{available at Annals of Oncology online})\), the mutant RAS emergence rate in tissue samples by BEAMing was 7.1%, \((n=1/14)\), similar to the 9.5% obtained by NGS of tumor tissue. In contrast, the mutant RAS emergence rate by BEAMing in the cfDNA plasma samples was 57.1% \((n=8/14)\). The difference in the emergence rates determined by BEAMing of tissue samples and cfDNA plasma samples was statistically significant \((P=0.008, \text{McNemar’s test})\).

Emergence of detectable RAS mutations in plasma was not associated with immediate resistance to therapy

To further evaluate the potential impact of RAS mutation emergence in plasma, an exploratory analysis of the 30 patients with paired plasma samples who were wild-type RAS at baseline \((19 \text{ wild-type at progression}, 11 \text{ with emergent RAS mutations at progression})\) were evaluated for clinical outcomes. The emergence of RAS mutations at progression was not predictive of the degree of radiologically observed cytoreduction measured as the maximum percentage change of the SLD of target lesions (Figure 3A). In addition, for those who retained wild-type status at progression \((n=19)\), median PFS (95% CI) was 5.0 \((3.7–9.2)\) months versus 7.4 \((6.8–9.1)\) months for those with RAS mutations at progression [hazard ratio \((\text{HR})=1.08; 95\% \text{ CI}, 0.49–2.38; P=0.84 \text{ for mutation effect})].

Given the lack of a correlation between the presence of emergent RAS mutations and both depth of response and the lack of changes in the HR for progression and PFS, analyses to evaluate whether the timing of mutant RAS emergence impacted treatment outcomes were carried out.

Plasma samples collected during treatment were tested by BEAMing in seriatim to conduct a temporal RAS analysis. Time-to-progression analysis revealed that detection of mutant clones during treatment did not correlate with an immediate transition of clinical disease state; however, RAS mutation emergence preceded radiologic relapse for all patients (Figure 3B). Median (range) lead time from the first detection of a RAS mutation to radiologic progression was 3.6 \((-0.3 \text{ to } 7.5)\) months. To better understand the impact of the emergence of RAS mutations on response and relapse to EGFR blockade, we carried out longitudinal assessment of plasma samples in multiple patients (Figure 3C and supplementary Figure S1, available at Annals of Oncology online).
Individual patient analysis illustrated initial detection of RAS mutations was variable, ranging from ~2 to 8 months after therapy initiation (Figure 3B and C; supplementary Figure S1, available at Annals of Oncology online). The time of initial detection of RAS mutations did not necessarily correlate with outcomes (Figure 3B and C; supplementary Figure S1, available at Annals of Oncology online). Once detected, the frequency of individual RAS mutant allele levels fluctuated during treatment but the total RAS mutation levels in a sample increased in most patients. A trend was observed indicating that the highest frequency of total RAS mutations detected were associated with the highest reported SLD of a patient’s tumor (supplementary Figure S1, available at Annals of Oncology online; patients 4 through 11); however, a threshold to predict disease progression could not be established.

Discussion

Results from this study indicate that RAS mutations emerge during panitumumab treatment, that the detected emergence rates are dependent on the DNA source (plasma versus tumor tissue) and analytic technique (NGS versus BEAMing), and that the evaluation of plasma samples can provide a sensitive dynamic picture of global clonal heterogeneity.

Exploratory outcomes analysis of plasma samples from patients with wild-type RAS at baseline revealed that patients with emergent RAS mutations at progression had no significant changes in immediate direction of tumor burden and had similar median PFS to those whose RAS status remained wild-type at progression. Given that all patients eventually relapsed, this indicates that a threshold might be reached for RAS clones to impact clinical outcome and that other potential (non-RAS) mechanisms of resistance may have contributed to progression.

These results analyzing plasma samples at baseline and progression are consistent with the initial analysis of 164 patients from ASPECTCT, a phase III panitumumab monotherapy study, which showed that patients with emergent RAS mutations at progression (detected by plasma NGS; 0.1% detection limit) had similar PFS and OS versus patients whose RAS status remained wild-type at progression [13].

Analysis of serial plasma samples revealed that after initial detection of the mutant RAS allele during treatment, many patients continued to derive clinical benefit from panitumumab plus irinotecan, as determined by radiographic monitoring. The median lead time from initial RAS mutant detection to progression was 3.6 months (range, –0.3 to 7.5 months). Together, this indicates that patient RAS status may be the result of a complex dynamic of heterogeneous clones and that emergent RAS mutations detected in plasma using sensitive assays may not necessarily be associated with worse outcomes in panitumumab-treated patients.

However, when clonal dynamics of individual patients were analyzed, the highest levels of total RAS mutations appeared to be associated with the highest SLD of a patient’s tumor.

In a previous study of mCRC treated with anti-EGFR inhibitors in the first-line setting, the emergence of mutant alleles also preceded progression (limit of detection, at least 15% across methods) [5]. Another study also found that emergence of RAS or EGFR extracellular domain mutations correlated with clinical response after treatment with panitumumab or cetuximab in the first-, second-, or third-line settings.
This study also provides prospective data on the emergence rates of mutations by NGS with a subset of 51 genes during anti-EGFR therapy. When tissue samples were evaluated, remarkably limited changes were found. No new BRAF mutations were found, in contrast with a previous study [14]. Previous studies also reported emergence of MEK as well as amplification of MET and HER2 in patients treated with anti-EGFR antibodies [5, 15]; however, these genes were not evaluated here. The present study reported emergence of an IDH1 mutation in a post-treatment biopsy, suggesting a possible association between its emergence and development of secondary resistance to anti-EGFR therapy [16]. However, it is also possible that the detection of this mutation was caused by clonal hematopoiesis or contaminating white blood cells, as has been previously reported for an IDH2 mutation [17].

Figure 3. (A) Maximum percentage reduction of the SLD of target lesions in the 30 patients with plasma wild-type RAS mCRC at baseline and evaluable RAS status post-baseline. (B) Time to progression in the 30 patients with wild-type RAS mCRC at baseline and evaluable RAS status post-baseline. (C) Select patient temporal analyses of reductions in SLD during the emergence of RAS mutations as evaluated by plasma BEAMing during treatment with panitumumab + irinotecan. Data from all patients with RAS mutations are shown in supplementary Figure S1, available at Annals of Oncology online. mCRC, metastatic colorectal cancer; PD, progressive disease; RAS, rat sarcoma; SLD, sum of longest diameters.
This study had several limitations. First, it was not intended to
determine a RAS mutation threshold that was predictive of a
change in clinical course. Furthermore, although this is one of the
largest datasets of plasma testing of mutant RAS emergence in
mCRC, the study included small patient numbers. Nonetheless,
results from this study track consistently with results from previ-
ous work [3–5, 18]. Additionally, the combination with irinote-
can may have impacted patient sensitivity and/or selection
pressure of emerging clones to panitumumab. However, to our
knowledge, emergent RAS mutations have not been described in
response to irinotecan, and clinical correlation results from our
paired analysis of plasma samples were consistent with a panitu-
mumab monotherapy study [13]. The prospectively defined ap-
proach to testing RAS status at progression may also have limited
analyses; only baseline and progression samples were analyzed for
patients identified as having no emergent RAS mutations,
whereas all samples from all available time points were analyzed
for patients identified as having mutant RAS at progression. Of
note, there were tumors classified as mutant RAS at baseline and
wild-type RAS at progression. It is unknown whether this was
due to reversion to wild-type, the sensitivity threshold of the ana-
lysis method, or the occurrence of false positives.

In summary, RAS mutations emerged in 36.7% of patients who
developed acquired resistance to panitumumab plus irinotecan,
consistent with previous studies [3, 4, 13, 18]. The first detected
emergence of RAS mutations in plasma preceded progression by
a median of 3.6 months (range, 0.3 to 7.5 months) and did not
correlate with outcomes nor was it associated with immediate re-
sistance to therapy. This study is one of the first prospective ana-
lyses to show that liquid biopsies are more comprehensive than
tissue-based studies for understanding the potential mechanisms
of resistance to targeted agents. Additional prospective studies
with other technologies, including the analysis of truncal muta-
tions (e.g. APC variants), are needed to establish threshold values
of mutant allele frequency from plasma to accurately characterize
clinical progression. The planned CHRONOS trial of rechallenge

![Graphs showing mutant allele frequency over time for different patients.](https://academic.oup.com/annonc/article-abstract/29/1/119/4103097)
with panitumumab in the third-line setting for mCRC is expected to help determine this threshold.

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**Disclosure**

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