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ORIGINAL ARTICLE

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

S. Siena[1,2]*, A. Sartore-Bianchi[1,2], R. Garcia-Carbonero[3], M. Karthaus[4], D. Smith[5], J. Tabernero[6], E. Van Cutsem[7,8], X. Guan[9], M. Boedigheimer[9], A. Ang[9], B. Twomey[9], B. A. Bach[9], A. S. Jung[9], & A. Bardelli[10,11]*

[1]Department of Hematology and Oncology, Niguarda Cancer Center, Grande Ospedale Metropolitano Niguarda, Milan, Italy; [2]Department of Oncology and Hemato-Oncology, Università degli Studi di Milano, Milan, Italy; [3]Department of Medical Oncology, Hospital Universitario 12 de Octubre, CNIO, CIBERONC, Universidad Complutense, Madrid, Spain; [4]Department for Hematology and Oncology, Staedt Klinikum Neuperlach and Harlaching, Munich, Germany; [5]Department of Gastroenterology, University Hospital of Bordeaux, Hopital Haut Levaeque, Bordeaux, France; [6]Department of Medical Oncology, Vall d'Hebron University Hospital, Vall d'Hebron Institute of Oncology (VHIO), CIBERONC, Universitat Autònoma de Barcelona, Barcelona, Spain; [7]Department of Gastroenterology and Digestive Oncology, University Hospitals Leuven, Leuven, Belgium; [8]KU Leuven, Leuven, Belgium; [9]Amgen Inc., Thousand Oaks, CA, USA; [10]Candiolo Cancer Institute-FPO, IRCCS, Candiolo, Italy; [11]Department of Oncology, University of Torino, Candiolo, Italy

**Correspondence to*: Prof. Salvatore Siena, Niguarda Cancer Center, Grande Ospedale Metropolitano Niguarda and University of Milano, Piazza Ospedale Maggiore, 3, 20162 Milano, Italy. Tel: +39-02-64442291; Fax: +39-02-64442957; E-mail: salvatore.siena@unimi.it

Prof. Alberto Bardelli, Candiolo Cancer Institute-FPO and University of Torino, SP 142 km 3.95, Candiolo, 10060 Torino, Italy. Tel: +39-011-9933235; Fax: +39-011-9933225; E-mail: alberto.bardelli@unito.it

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.

ClinicalTrials.gov Identifier: NCT00891930

Key words: gastrointestinal cancers, colorectal, phase I–III trials, biomarkers and intervention studies, panitumumab

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Introduction

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/m^2 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],

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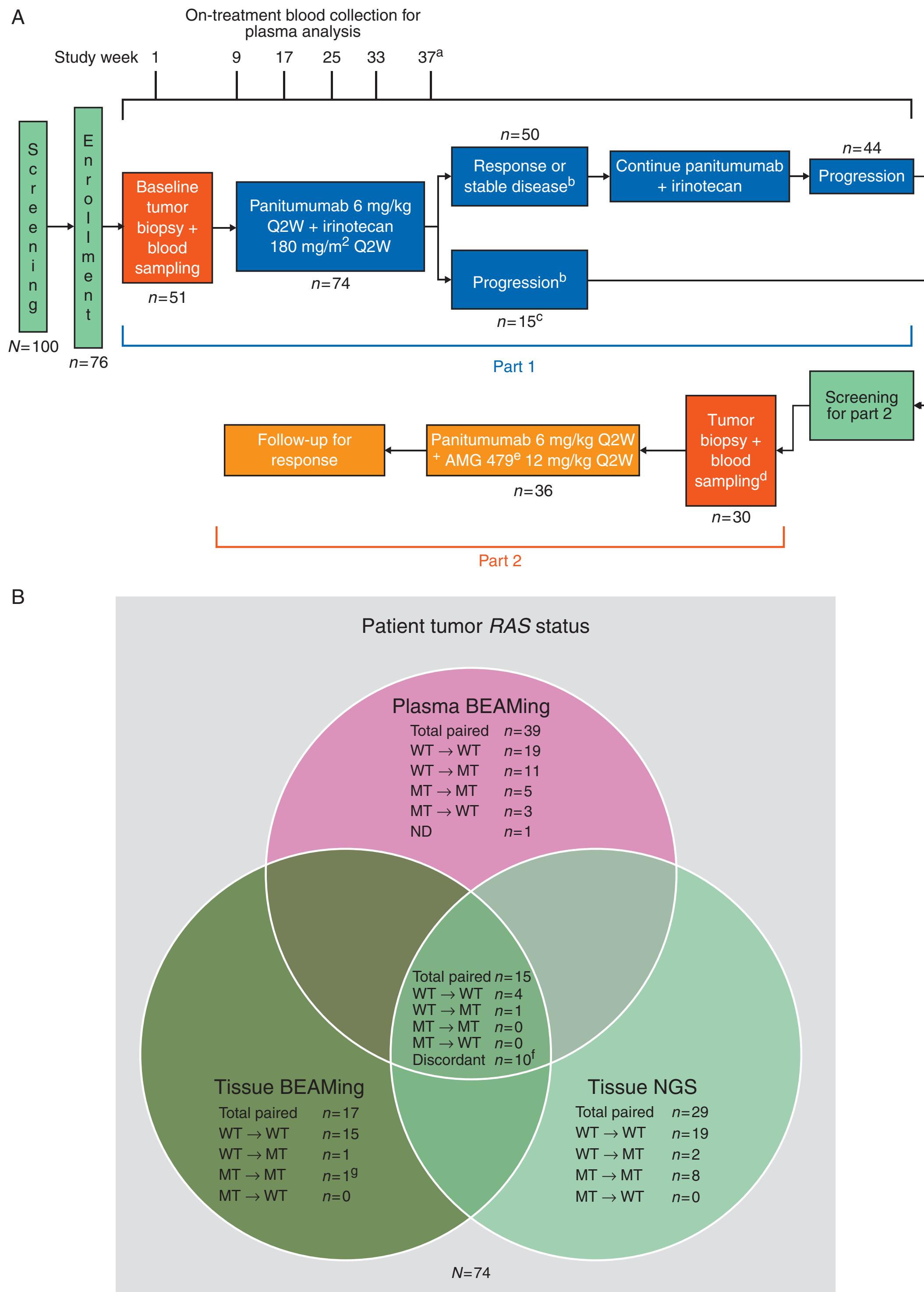

Figure 1. (A) Study schema and (B) patient tumor *RAS* status results determined by tissue and plasma testing biomarker analyses. The *RAS* status of pre-and post-treatment patient tumor samples were analyzed by each testing method. Total patient samples and the number of patients whose *RAS* status 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, magnetics; CT, computed tomography; IGF-1 R, 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. [a]Blood collection for plasma analysis at week 37 and every 4 weeks thereafter until disease progression intolerability. [b]Based on first restaging CT or MRI. [c]Nine patients were either not doing or unevaluable for tumor response assessment. [d]Only patients who had an objective response or stable disease after part 1 underwent a second biopsy. [e]A monoclonal antibody that inhibits the insulin-like growth factor 1 (IGF-1 R) pathway. [f]Patients without both before-treatment and after-treatment samples for both tests were not characterized as discordant. [g]Patient was characterized as *RAS* wild-type at baseline by NGS.

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Table 1. Cancer genes sequenced in tumor samples using the SuraSeq 51-gene panel

Cancer genes sequenced			
ABL1	*FES*	*JAK2*	***PIK3R1***
AKT1	*FGFR1*	***KIT***	***PTCH1***
AKT2	*FGFR3*	***KRAS***	***PTEN***
BRAF	*FLT3*	***MEN1***	*PTPN11*
CDH1	*FOXL2*	*MET*	***RB1***
CDK4	*GATA1*	*MPL*	*RET*
CDKN2A	*GNA11*	***NF2***	***SMAD4***
CEBPA	*GNAQ*	***NOTCH1***	***SMARCB1***
CREBBP	***HIF1A***	*NPM1*	***SMO***
CTNNB1	*HRAS*	*NRAS*[a]	*SRC*
ERBB2	***IDH1***	*PAX5*	***STK11***
EGFR	*IDH2*	*PDGFRA*	***TP53***
IKBKB	*PIK3CA*	***VHL***	

Genes in bold represent whole exon sequencing; non-bolded genes represent hotspot sequencing. Genes marked with a gray box were wild-type across all sample types.
[a]NRAS exon 4 was not included.

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% CI, 1% 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 ~10% 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% ($n = 1/16$; supplementary Table S4, 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 all had 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 ($n = 14$; supplementary Table S4, 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 ($P = 0.008$, 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 wild-type at progression, 11 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 (HR)=1.08; 95% CI, 0.49−2.38; $P = 0.84$ 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 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).

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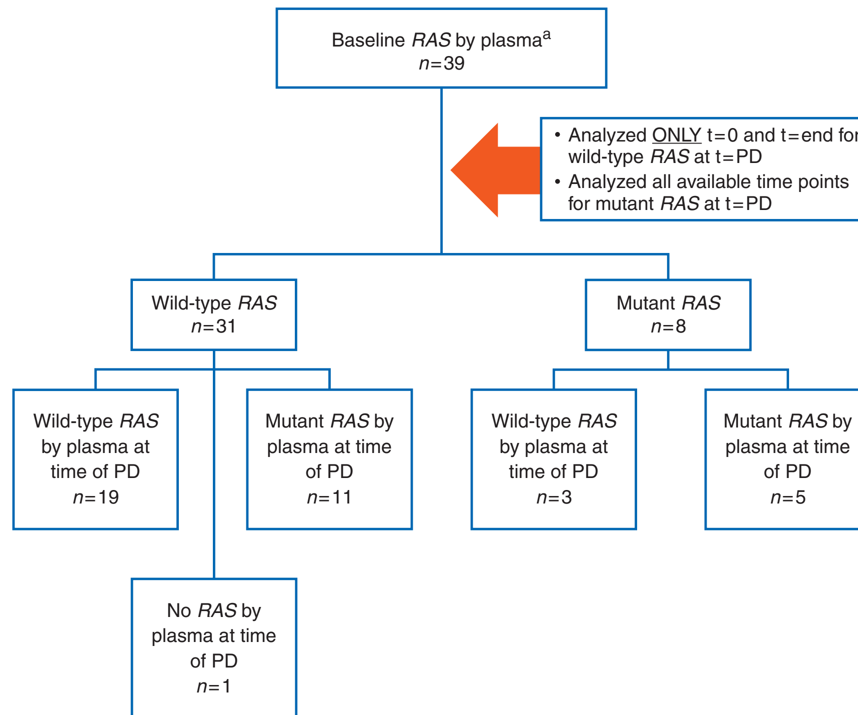

Figure 2. Plasma testing schema and results. PD, progressive disease; *RAS*, rat sarcoma; t, time. [a]All patents included were supposed to have a best response of at least stable disease; however, two patients who were mutant to mutant (progressive disease, $n = 1$; unevaluable, $n = 1$) and one patient who was wild-type to wild-type (progressive disease) were analyzed.

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 ASPECCT, 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.

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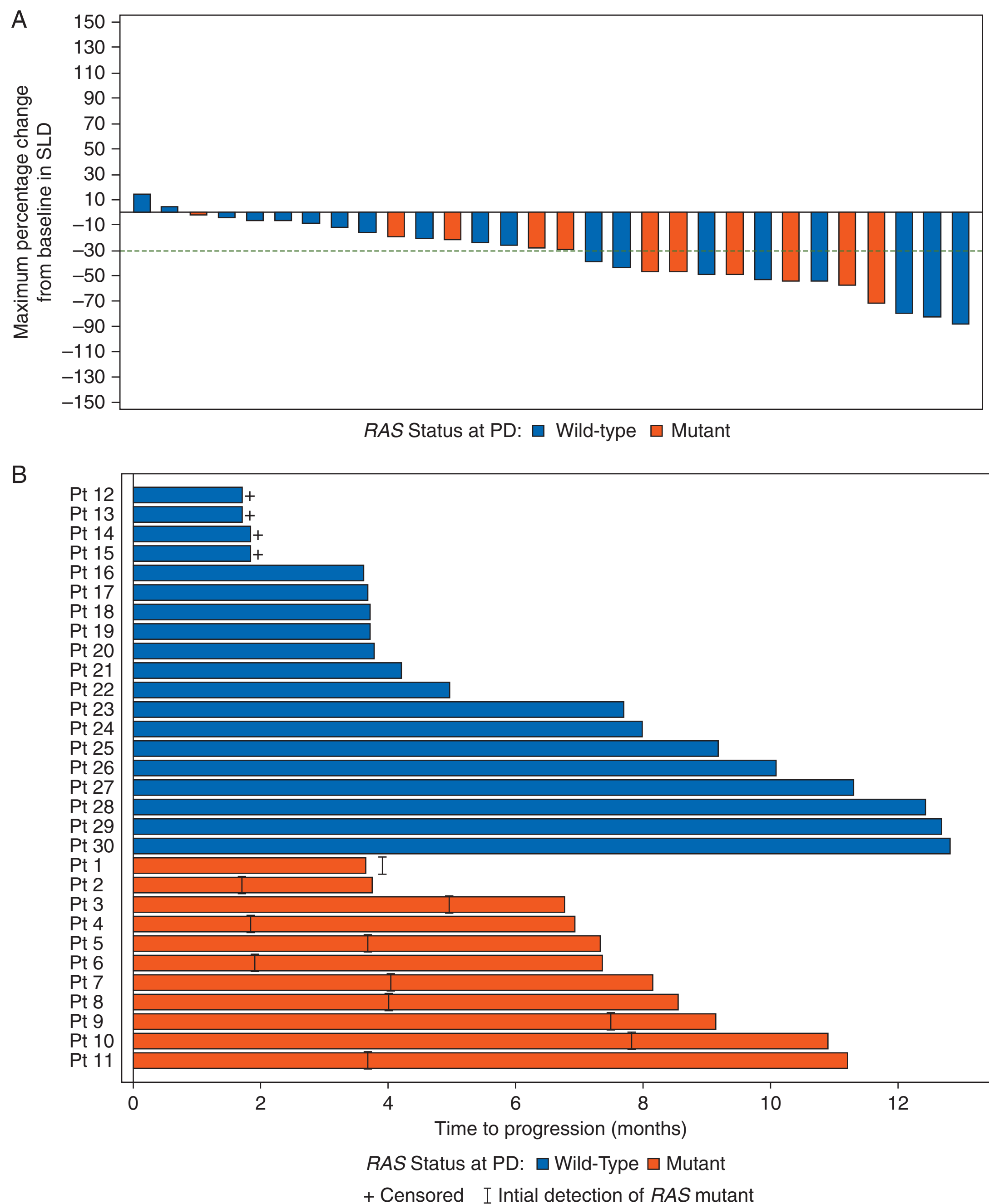

Figure 3. (A) Maximum percentage reduction of the SLD of target lesions in the 30 patients with plasma wild-type *RAS* mCRC at baseline and evaluable *RAS* status post-baseline. (B) Time to progression in the 30 patients with wild-type *RAS* mCRC at baseline and evaluable RAS status post-baseline. (C) 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 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].

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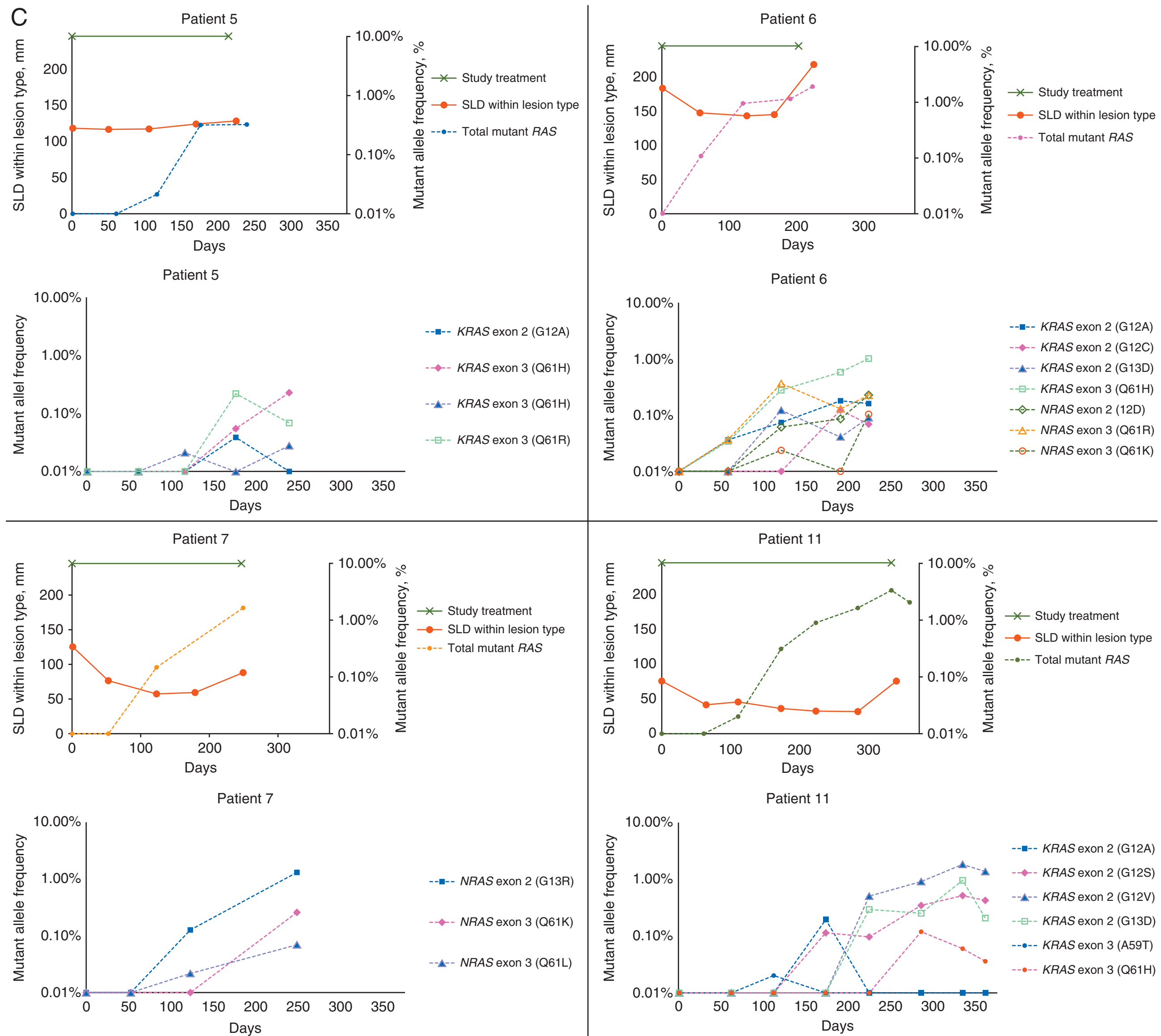

Figure 3. Continued

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 previous work [3–5, 18]. Additionally, the combination with irinotecan 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 panitumumab monotherapy study [13]. The prospectively defined approach 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 analysis 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 resistance to therapy. This study is one of the first prospective analyses 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 mutations (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

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with panitumumab in the third-line setting for mCRC is expected to help determine this threshold.

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Disclosure

SS has participated in advisory boards for Amgen, Roche, Bayer, Merck, Sanofi, Novartis, and Merimack. AS-B has participated on advisory boards for Amgen, Bayer, Lilly, and Sanofi, and has received speaker fees from Amgen, Bayer, and Roche. RG-C's institution has received financial support for the conduct of the clinical trial reported; however, she has no personal conflicts to disclose. MK has participated on advisory boards for Amgen and Roche. DS has participated on advisory boards for Novartis, Servier, Roche, and Ipsen. JT has participated on advisory boards for Amgen, Bayer, Boehringer Ingelheim, Celgene, Chugai, Genentech, Lilly, Merck Sharpe & Dohme, Merck Serono, Novartis, Pfizer, Roche, Sanofi, Symphogen, Taiho, and Takeda. EVC has received research grants from Amgen, Bayer, Boehringer Ingelheim, Celgene, Ipsen, Lilly, Merck, Merck KgA, Novartis, Roche, Sanofi, and Servier. XG is an employee of Amgen. MB is an employee of, owns stock in, and has stock options in Amgen. AA, BT, and ASJ are employees of, own stock in, Amgen. BAB is currently an employee of AbbVie Inc, and was a former employee of, and owns stock in Amgen. AB is a member of the scientific advisory board of Biocartis and Horizon Discovery, has received research funding from Trovagene and Symphogene, and has received paid consultancies from Roche and Novartis.

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