

The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers

Luis A. Diaz Jr^{1,2}, Richard T. Williams³, Jian Wu^{1,4}, Isaac Kinde¹, J. Randolph Hecht⁵, Jordan Berlin⁶, Benjamin Allen⁷, Ivana Bozic⁷, Johannes G. Reiter^{7,8}, Martin A. Nowak⁷, Kenneth W. Kinzler¹, Kelly S. Oliner³ & Bert Vogelstein¹

Colorectal tumours that are wild type for *KRAS* are often sensitive to EGFR blockade, but almost always develop resistance within several months of initiating therapy^{1,2}. The mechanisms underlying this acquired resistance to anti-EGFR antibodies are largely unknown. This situation is in marked contrast to that of small-molecule targeted agents, such as inhibitors of ABL, EGFR, BRAF and MEK, in which mutations in the genes encoding the protein targets render the tumours resistant to the effects of the drugs^{3–6}. The simplest hypothesis to account for the development of resistance to EGFR blockade is that rare cells with *KRAS* mutations pre-exist at low levels in tumours with ostensibly wild-type *KRAS* genes. Although this hypothesis would seem readily testable, there is no evidence in pre-clinical models to support it, nor is there data from patients. To test this hypothesis, we determined whether mutant *KRAS* DNA could be detected in the circulation of 28 patients receiving monotherapy with panitumumab, a therapeutic anti-EGFR antibody. We found that 9 out of 24 (38%) patients whose tumours were initially *KRAS* wild type developed detectable mutations in *KRAS* in their sera, three of which developed multiple different *KRAS* mutations. The appearance of these mutations was very consistent, generally occurring between 5 and 6 months following treatment. Mathematical modelling indicated that the mutations were present in expanded subclones before the initiation of panitumumab treatment. These results suggest that the emergence of *KRAS* mutations is a mediator of acquired resistance to EGFR blockade and that these mutations can be detected in a non-invasive manner. They explain why solid tumours develop resistance to targeted therapies in a highly reproducible fashion.

One major barrier to testing any hypothesis about the nature of acquired resistance to anti-EGFR antibodies is limited access to post-treatment tumour tissue. Even when post-treatment tumour tissue is available, sampling bias confounds interpretation because only a small portion of one tumour is usually biopsied, precluding assessment of genetic heterogeneity within or among lesions. To circumvent the tissue access problem, we have examined circulating, cell-free DNA—a form of ‘liquid biopsy’. It has been previously shown that circulating tumour DNA (ctDNA) can be found in the majority of patients with metastatic colorectal cancers^{7–9}. Analysis of ctDNA is informative because not only it can identify a specific mutant genotype, but it can also provide a measurement of the total tumour burden⁷. If tumours became resistant to anti-EGFR antibodies as a result of the emergence of *KRAS* mutations in their tumours, we expected that mutant *KRAS* genes would be released into the circulation in a time frame consistent with the emergence of resistance.

We retrospectively analysed longitudinal serum samples from 28 patients with chemorefractory metastatic colorectal cancer (CRC) receiving single-agent therapy with panitumumab¹⁰. Four patients with *KRAS* mutant tumours, who never achieved disease control, were

selected as controls. As expected, these four patients were found to have progressive disease at the time of first tumour assessment, 7 ± 2 weeks (mean ± 1 standard deviation) after initiating treatment with panitumumab (Supplementary Table 1)¹². The other 24 patients with wild-type *KRAS* tumours achieved a partial response ($n = 8$), had prolonged stable disease ($n = 14$), or had retrospectively-determined progressive disease but remained on study for an extended period ($n = 2$). These 24 patients developed clinically evident progressive disease 25 ± 10 weeks (mean ± 1 standard deviation) following initiation of treatment (Supplementary Table 1) as determined by radiographic imaging.

Serum samples obtained from patients before the initiation of therapy were evaluated for all common mutations at codons 12 and 13 of *KRAS* using a digital ligation assay with a detection limit of one mutant molecule per ml of serum (examples in Supplementary Fig. 1)¹¹. Mutations were independently confirmed in a second aliquot of the same serum and the results quantified via a PCR assay that can digitally enumerate the fraction of rare variants in a complex mixture of DNA template molecules (examples in Supplementary Fig. 1 and Supplementary Table 2)¹².

Of the four cases whose archival tumours harboured *KRAS* mutations, three had detectable levels of mutant *KRAS* in the serum before treatment with panitumumab (Supplementary Table 2). In these three patients, the *KRAS* mutations found in the circulation were identical to those found in the patients’ tumour tissues even though the time of serum assessment was, on average, 88 weeks after the diagnosis of metastatic disease and even longer after the initial tumour excision (Supplementary Tables 1 and 2). No mutations in *KRAS* were detected in the pre-treatment serum DNA from patients whose archival tumour tissue was wild type for *KRAS* (Supplementary Table 2).

Next, we examined 169 serially acquired serum samples from the 28 patients for the presence of mutant *KRAS* fragments (Supplementary Fig. 2). These samples were collected at approximately 4-week intervals until disease progression (Supplementary Table 2). Serum was assessed for mutations at *KRAS* codons 12 and 13 as described above. When sufficient serum was available (23 of 28 patients), it was assessed for *BRAF* mutations at codons 600 and 601 using the identical assay. Of the 24 patients who did not have *KRAS* mutations at baseline, nine (38%) were found to develop *KRAS* mutations during the course of therapy (Supplementary Table 2), whereas none developed *BRAF* mutations. In three cases (patients no. 1, 12 and 22), several *KRAS* mutations appeared in the circulation—two different mutants in one case and four different mutants in the two other cases (examples in Fig. 1). In each of these cases, the time to appearance of all mutations in the circulation was very similar (Fig. 2). Circulating mutant *KRAS* templates were identified before radiographic evidence of disease progression in three of the nine cases (patients no. 1, 10 and 24). The lead time (that is, the interval between detectable ctDNA and radiographic

¹Ludwig Center for Cancer Genetics and Therapeutics and Howard Hughes Medical Institute at Johns Hopkins Kimmel Cancer Center, Baltimore, Maryland 21287, USA. ²Swim Across America Laboratory at Johns Hopkins, Baltimore, Maryland 21287, USA. ³Amgen Inc., Thousand Oaks, California 91320, USA. ⁴State Key Laboratory of Cancer Biology, Cell Engineering Research Center & Department of Cell Biology, The Fourth Military Medical University, Xi'an 710032, China. ⁵Division of Hematology/Oncology, David Geffen School of Medicine, University of California, Los Angeles, California 90095, USA. ⁶Vanderbilt University Medical Center, Nashville, Tennessee 37232, USA. ⁷Program for Evolutionary Dynamics, Harvard University, Cambridge, Massachusetts 02138, USA. ⁸IST Austria (Institute of Science and Technology Austria), Klosterneuburg 3400, Austria.

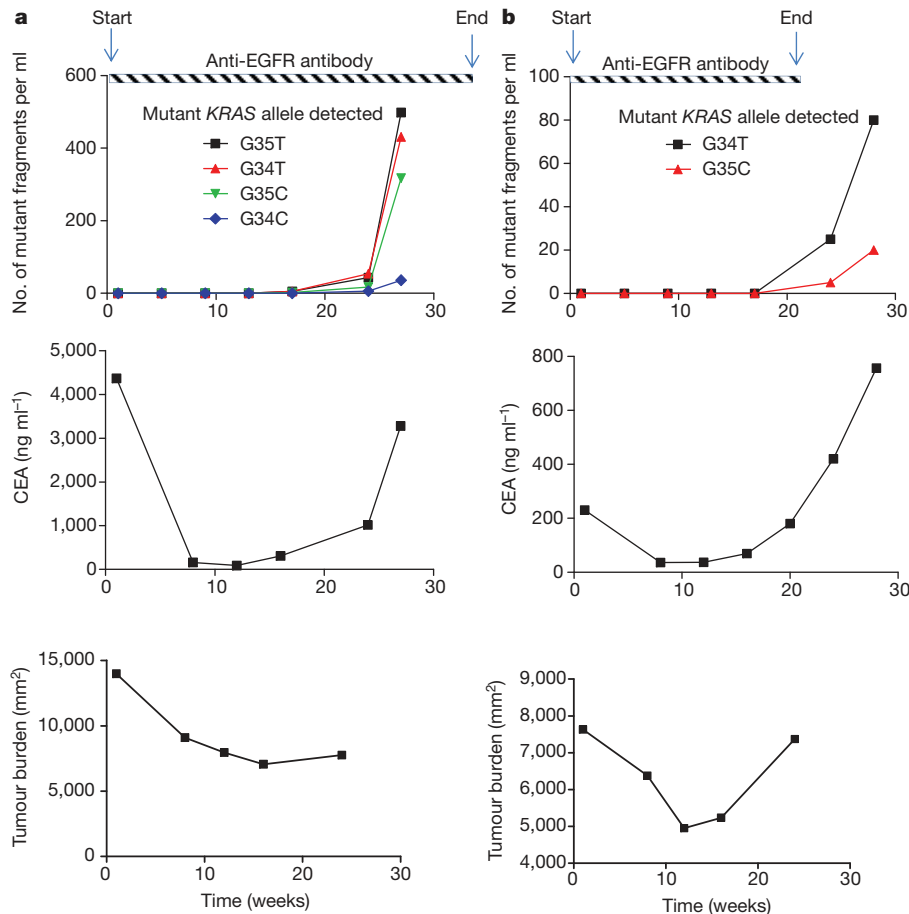


Figure 1 | Emergence of circulating mutant *KRAS*. **a, b,** The time course of circulating mutant *KRAS* alleles, CEA and tumour burden are depicted in two patients where fragments of circulating DNA containing mutant *KRAS* were detected. The emergence of four different mutant *KRAS* alleles in codon 12

(cDNA nucleotides 35T, 34T, 35C and 34C) in the serum of patient 1 is shown in **a**, and the emergence of two different mutant *KRAS* alleles in codon 12 (cDNA nucleotides 34T and 35C) in the serum of patient 12 is shown in **b**. Tumour burden refers to the aggregate cross-sectional diameter of the index lesions.

evidence of disease progression) averaged 21 weeks (Supplementary Table 2). The level of ctDNA generally paralleled that of CEA (carcinoembryonic antigen), the standard biomarker used for following disease progression in metastatic CRC (Supplementary Table 3 and Supplementary Fig. 2).

In the three patients (patients no. 8, 20 and 28) with detectable mutant *KRAS* in their tumours as well as in their circulation, no new mutations in *KRAS* emerged (Supplementary Table 2).

The progression-free survival (hazard ratio 0.9; 95% confidence interval 0.3366 to 2.453; $P = 0.85$; log-rank test) and overall survival (hazard ratio 0.42; 95% confidence interval 0.1599 to 1.144; $P = 0.09$; log-rank test) of patients was not significantly different whether they developed secondary *KRAS* mutations or not (Supplementary Fig. 3).

The availability of serially collected serum samples provided a unique opportunity to model the tumour evolutionary process in those patients that responded to panitumumab. The first question we addressed was whether *KRAS* mutations were likely to be present before the initiation of therapy with panitumumab. To estimate the average tumour growth rate, we used the ctDNA data (Supplementary Table 2) from the nine patients that developed *KRAS* mutations during therapy. The average tumour growth rate was found to be 0.069—that is, the number of tumour cells resistant to panitumumab doubled approximately every ten days (doubling time = $\ln 2 / 0.069$ days; see Supplementary Appendix). The growth rate represents the difference between the cell birth rate b and the cell death rate d , that is, $b - d$. Previous studies¹³ have shown that $b \approx 0.25$ for colorectal cancer cells, corresponding to one cell division every 4 days, yielding a value of 0.18 ($= 0.25 - 0.069$) for d .

Using these data-derived estimates of b and d , a branching process model was used to test the null hypothesis that there were no cells with *KRAS* mutations in the tumours before the initiation of therapy. We calculated the probability that the number of mutant *KRAS* cells could have grown to at least the observed levels at the times they were measured, assuming this null hypothesis (see Supplementary Appendix). Using the known tumour burdens and pre-treatment ctDNA levels measured in patients 8 and 20 who harboured *KRAS* mutations in their tumours before therapy, as well as data obtained on previously studied patients with metastatic disease⁷, we calculated that one mutant *KRAS* template per ml of serum corresponds to a tumour containing 44 million *KRAS*-mutant cells. We performed the statistical valuation separately for each patient. In the three patients that developed more than one circulating *KRAS* mutation, we assumed that each of the detected *KRAS* mutations originated from a different lesion (see below). Thus a total of 16 lesions from nine patients were analysed. For each lesion, the possibility that the observed mutations were absent at the start of treatment could be rejected (thus confirming the presence of pre-existing *KRAS* mutations) with >95% confidence (in most cases, >99.9% confidence; Supplementary Table 4)^{14,15}. Furthermore, we varied the birth rate b around the previously calculated value of 0.25, allowing it to be as small as 0.15 or as large as 0.35, and found that this did not affect our conclusion that the mutations were present before therapy, although for one lesion the confidence fell below 95% as the birth rate was increased (Supplementary Table 4).

Next, we estimated how many mutations gave rise to resistance to panitumumab in patients *in vivo*. The total number of lesions large enough to detect by imaging averaged 7.0 lesions per patient in the 24

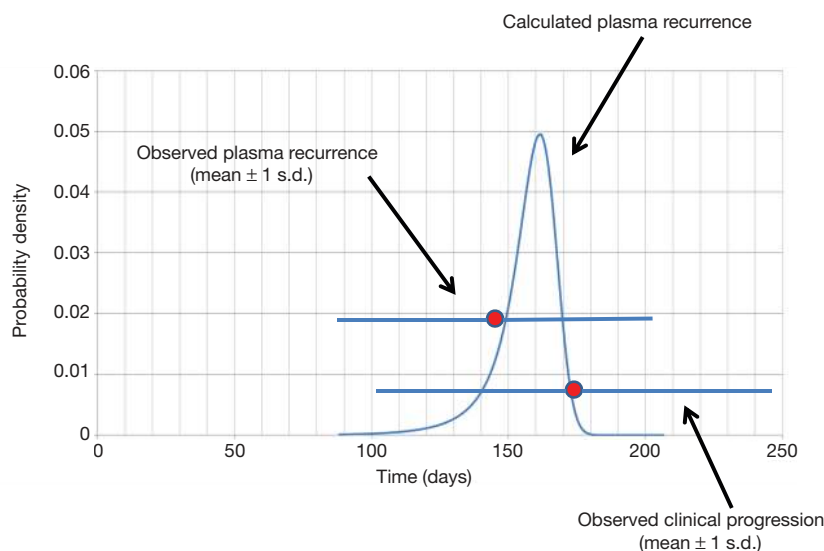


Figure 2 | Predicted probability distribution of times from when treatment starts until resistance mutations become observable in circulating DNA. The observed time to detection of mutant *KRAS* fragments (21 ± 8.5 weeks, mean ± 1 standard deviation) is overlaid with time to clinical progression

(25 ± 10 weeks, mean ± 1 standard deviation) in the patients studied. Predictions were based on the Luria–Delbrück model with death, as introduced in ref. 14, or equivalently, the branching process model from ref. 16.

patients studied with wild-type *KRAS* tumours (Supplementary Table 1). Assuming that each individual *KRAS* mutation detected in the serum emanated from a single metastatic lesion, the 16 lesions responsible for contributing ctDNA (Supplementary Table 2) accounted for 9.6% of the total 167 lesions. The other 90% of lesions presumably had developed genetic alterations that were not assessed in our study. As we assessed four different mutated nucleotides of *KRAS* (at codons 12 and 13, Supplementary Table 5), this analysis suggests that a total of approximately 42 ($= 4 \times 167/16$) nucleotides gave rise to resistance in our 24 patients. These remaining 38 nucleotides (42 minus 4) would represent a maximum of 38 genes (one potential mutant nucleotide per gene), or approximately 10 genes if, like *KRAS*, there was an average of four nucleotides per gene that could give rise to resistance when mutated.

Would at least one of these 42 mutant nucleotides be expected to be present in a metastatic lesion initiated by a single cell that was wild type at all 42 nucleotides? We analysed this question using a Luria–Delbrück model generalized to incorporate cell death^{14–16}. If we conservatively assume that cancer cells have the same mutation rate as normal cells and the same birth rate described above (0.25)^{15,17}, then a tumour detectable by computed tomography scanning (one billion cells) is almost certain (probability $> 1 - 10^{-32}$) to contain at least one cell with a mutation at one of these 42 nucleotides. The expected number of such cells is about 3,200 (Supplementary Appendix)^{16,17}. These 3,200 cells are distributed among various clones that arose during the growth of the metastatic lesion before therapy with panitumumab. The largest clonal subpopulation is in most cases the progeny of the subpopulation containing the first resistance mutation to arise and survive stochastic drift. Using the equations described in the Supplementary Appendix, we calculated the expected size of this clonal subpopulation as 2,200 cells; thus, 69% ($2,200/3,200$) of the resistant cells should be derived from a single clone. As described in the Supplementary Appendix, varying the number of possible mutations conferring resistance from 4 to 100 did not change this expectation: there are hundreds to thousands of cells with resistance mutations in each metastasis before the initiation of panitumumab therapy and more than half of these resistant cells are expected to be derived from a single clone.

Our mathematical model also accounts for the very similar times at which circulating *KRAS* fragments were observed across patients and lesions. We calculated the probability distribution for the times at

which circulating *KRAS* fragments should become detectable (Supplementary Appendix). As shown in Fig. 2, the mean of this distribution was 22 weeks with a 95% confidence interval ranging from 18 to 25 weeks. These predicted times are strikingly similar to those actually observed in the patients analysed in this study (21 ± 8.5 weeks, mean ± 1 standard deviation) and just slightly sooner than when clinical progression was observed (25 ± 10 weeks, mean ± 1 standard deviation). Finally, we performed simulations to further validate these analytical findings. The results were similar: mutant *KRAS* fragments were predicted to become evident at 22 ± 1.5 weeks (mean ± 1 standard deviation) following treatment (Supplementary Appendix).

Our study indicates that the resistance mutations in *KRAS* and other genes were highly likely to be present in a clonal subpopulation within the tumours prior to the initiation of panitumumab therapy. Although we base this conclusion on new data and mathematical analyses, it is consistent with earlier experimental data on other targeted agents^{18–21} as well as theoretical predictions^{22–24}. As noted in several previous studies of targeted therapeutic agents^{18–21}, most lesions recur at approximately the same time following therapy. This was also true in our selected group of patients, in which disease progression occurred at the same time in patients who did or did not develop circulating *KRAS* mutations (Supplementary Fig. 3).

Our study suggests that only a limited number of nucleotide mutations and genes are likely to be able to exert a resistance phenotype. If there were many more genes with this capacity, then it would be unlikely that such a high fraction of patients (38%) and lesions (9.6%) would develop mutations in a single gene (*KRAS*). This finding is consistent with earlier studies in that only a small number of genes has been found to be expressed differentially in resistant tumours and an even smaller number to be mutated—none as frequently as *KRAS*^{18,25,26}. That only a small number of genes can confer resistance is encouraging; if there were a very large number of genes (and nucleotides) that had the capacity to produce resistance to panitumumab, there would be little hope of combining this drug with others to circumvent resistance.

In sum, our results suggest the following scenario for the development of resistance to panitumumab. Each relatively large metastatic lesion is expected to contain a subclone containing hundreds or thousands of cells with one of about 42 mutations conferring resistance to the antibody. Resistance is therefore a fait accompli—the time to recurrence is simply the interval required for the subclone to repopulate the lesion. This generally takes 5 to 6 months (Fig. 2) and is due to

the rapid expansion of the resistant subclone immediately following treatment initiation. To make these remissions last longer than 5 to 6 months, combination therapies targeting at least two different pathways will be required.

METHODS SUMMARY

Patients and specimens. Patients with chemorefractory metastatic colorectal carcinoma were enrolled into one of two panitumumab monotherapy studies (NCT00089635 and NCT00083616)¹⁰. The study protocols were approved by the institutional review boards and all patients signed a written consent form. A subset of patients was selected for this analysis from a total of 388 patients. Patients received panitumumab 6 mg kg⁻¹ every two weeks until disease progression. Tumour scans were read centrally by a panel of at least two blinded independent radiologists using a modification of the WHO criteria. Assessments were performed at 4-week intervals through week 28 and every 3 months thereafter until progression of disease. Responses were confirmed at least 4 weeks after response criteria were first met. *KRAS* mutational status in the tissue was predetermined using the DxS assay (Qiagen).

DNA purification. DNA was purified from 0.2–1 ml of banked serum using the QIAamp circulating nucleic acid kit (Qiagen, catalogue no. 55114) following the manufacturer's recommendations. Amplifiable DNA was quantified with quantitative PCR, using primers and conditions as previously described²⁷.

Assessments of circulating mutant *KRAS* DNA. The ligation assays were performed as previously described¹¹ using the primers and probes indicated in Supplementary Table 5. In brief, *KRAS* fragments containing codons 12 and 13 were amplified with primers designed to yield a small PCR product (106 base pairs) to accommodate the degraded DNA found in serum²⁸. Note that some of the probes contained locked nucleic acid (LNA) linkages (Exiqon). To confirm and further quantify samples containing *KRAS* mutations, BEAMing assays were used¹² with the primers and probes listed in Supplementary Table 5. The number of mutant fragments per ml of serum was determined from the fraction of alleles containing the mutant allele (determined by BEAMing⁷) and the number of alleles assessed per ml of plasma (determined by qPCR²⁷ and reported in Supplementary Table 6).

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions L.A.D., K.S.O. and B.V. designed experiments, analysed data and wrote the paper. B.V., I.K. and J.W. performed experiments, analysed data and provided input to the manuscript. R.T.W., J.B. and J.R.H. provided critical materials, reagents, analysed data and provided input to the manuscript. B.A., I.B., J.G.R. and M.A.N. analysed data, performed the mathematical modelling and provided input to the manuscript.

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Supplementary Table 1. Clinical characteristics

Clinical and Demographic Info							Prior Therapy					Metastatic lesions prior to Panitumumab therapy	Response to Panitumumab		
Patient #	Age	Gender	ECOG PS ¹	Primary Site	Race/Ethnicity	Weeks since diagnosis of metastatic CRC	Lines of Prior Therapy	Chemotherapeutic agents	Biologic Therapy	Surgery (Intent)	Radiation Therapy	Number of lesions detectable by CT	WHO ² RESPONSE	Progression Free Survival (weeks)	Overall Survival (weeks)
1	64	M	1	Colon	White/Caucasian	93	3	Cape, OXAL, IRT		Resection (Curative)		12	SD	33	33
2	53	M	0	Colon	White/Caucasian	96	3	5FU, LV, Cape, OXAL, IRT		Resection (Staging)		6	PR	22	76
3*	60	M	1	Colon	White/Caucasian	97	2	5FU, LV, IRT		Resection (Curative)		3	PD	7	119
4	67	F	0	Colon	African American	105	3	5FU, LV, OXAL, IRT		Bypass (Palliative)	Pelvis (49 Gy)	2	SD	11	51
5	78	F	0	Colon	White/Caucasian	156	2	5FU, LV, OXAL, IRT	Bevacizumab	Resection (Curative)		4	PR	23	97
6	63	F	1	Colon	White/Caucasian	92	3	5FU, LV, OXAL, IRT	Bevacizumab	Bypass (Palliative)	Pelvis (58 Gy)	4	SD	35	35
7	55	M	1	Rectum	White/Caucasian	194	2	5FU, LV, Cape, OXAL, IRT	Bevacizumab	Resection (Curative)		7	SD	23	77
8*	48	F	1	Colon	White/Caucasian	68	2	5FU, LV, OXAL, IRT	Bevacizumab	Resection (Palliation)		3	PD	11	50
9	50	M	1	Rectum	White/Caucasian	115	2	5FU, LV, Cape, OXAL, IRT		Resection (Palliation)	Pelvis (54 Gy)	9	PD	31	86
10	67	M	1	Colon	White/Caucasian	71	2	5FU, LV, OXAL, IRT		Resection (Palliation)		3	SD	23	113
11	52	M	1	Rectum	African American	48	2	5FU, LV, OXAL, IRT			Rectum (65 Gy)	9	PD	7	58
12	49	M	0	Colon	White/Caucasian	129	3	5FU, LV, Cape, OXAL, IRT	Bevacizumab	Resection (Palliation)		4	SD	23	75
13	59	F	1	Colon	White/Caucasian	61	2	5FU, LV, OXAL, IRT	Bevacizumab	Resection (Palliation)		7	SD	23	57
14	73	M	0	Colon	White/Caucasian	97	3	5FU, LV, OXAL, IRT		Resection (Curative)		4	PR	23	133
15	58	M	0	Rectum	White/Caucasian	151	3	5FU, LV, OXAL, IRT			Pelvis (51 Gy)	14	PR	25	25
16	72	F	1	Colon	White/Caucasian	86	3	5FU, LV, Cape, OXAL, IRT		Resection (Curative)		5	PR	23	45
17	57	F	1	Colon	White/Caucasian	67	2	5FU, LV, OXAL, IRT	Bevacizumab	Resection (Curative)	Pelvis (34Gy)	7	PR	31	83
18	47	F	0	Rectum	White/Caucasian	84	2	5FU, LV, OXAL, IRT	Bevacizumab			6	PR	28	88
19	42	M	0	Rectum	White/Caucasian	99	2	5FU, LV, Cape, OXAL, IRT	Bevacizumab	Resection (Curative)	Pelvis/Rectum (49 Gy)	2	PR	15	120
20*	56	F	1	Colon	African American	62	2	5FU, LV, OXAL, IRT	Bevacizumab	Resection (Curative)		5	PD	7	55
21	57	M	0	Colon	White/Caucasian	106	2	5FU, LV, Cape, OXAL, IRT	Bevacizumab	Resection (Palliation)		10	SD	15	74
22	59	F	1	Colon	White/Caucasian	102	3	5FU, LV, Cape, OXAL, IRT	Bevacizumab	Resection (Palliation)		13	SD	15	25
23	69	M	1	Rectum	White/Caucasian	199	3	5FU, LV, Cape, OXAL, IRT		Resection (Curative)	Pelvis (50Gy)	9	SD	49	49
24	57	M	0	Unknown	African American	52	2	5FU, LV, OXAL, IRT	Bevacizumab	Resection (Curative)	Pelvis (50Gy)	12	SD	52	52
25	47	F	0	Colon	White/Caucasian	96	2	5FU, LV, OXAL, IRT	Bevacizumab	Resection (Palliation)		9	SD	20	67
26	59	F	0	Colon	White/Caucasian	132	3	5FU, LV, OXAL, IRT	Bevacizumab	Resection (Staging)		3	SD	23	130
27	73	M	0	Rectum	White/Caucasian	134	3	5FU, LV, Cape, OXAL, IRT	Bevacizumab	Excision (Curative)	Pelvis (54Gy)	6	SD	23	102
28*	78	F	1	Colon	White/Caucasian	232	3	5FU, LV, OXAL, IRT	Bevacizumab	Resection (Curative)		8	PD	7	36

*Denotes patients (#3, 8, 20 and 28) whose tumor tissue was found to be KRAS mutant

¹ECOG PS - Eastern Cooperative Group Performance Status (0 - Fully active without restriction; 1 - Restricted in physically strenuous activity and able to carry out work of a light or sedentary nature;

2 - Ambulatory and capable of all selfcare but unable to carry out any work activities; 3 - Confined to bed or chair more than 50% of waking hours; 4 - Totally confined to bed or chair; 5 - Dead)

²WHO Tumor Response Criteria (SD - Stable Disease; PD - Progressive Disease; PR - Partial Response; CR - Complete Response)

Supplementary Table 2. *KRAS* assessments

Patient #	Baseline <i>KRAS</i> Status	Circulating Mutant <i>KRAS</i> Status*		Circulating Mutant <i>KRAS</i> (fragments/mL)*							Time to detection of secondary <i>KRAS</i> mutation (weeks)	Time from detection of secondary <i>KRAS</i> mutation to Disease Progression (weeks)
	Tumor Genotype	Mutant <i>KRAS</i> Alleles Detected at Baseline	Secondary Circulating Mutant <i>KRAS</i> Alleles Detected	Week 1	Week 5	Week 9	Week 13	Week 17	Week 25	Follow-up (week 26 to 52)		
1	WT	NMD	G12V	NMD	NMD	NMD	NMD	5	43	498	17	16
			G12C	NMD	NMD	NMD	NMD	5	54	431		
			G12A	NMD	NMD	NMD	NMD	2	17	317		
			G12R	NMD	NMD	NMD	NMD	NMD	6	36		
2	WT	NMD	NMD	NMD	NMD	NMD	NMD	NMD		NMD		
3	G12D	NMD	NMD	NMD	NMD	NMD	NMD	NMD		NMD		
4	WT	NMD	G12R	NMD	NMD	NMD	NMD	NMD	NMD	4	34	Concurrent
5	WT	NMD	G12D	NMD	NMD	NMD	NMD	NMD		13	26	Concurrent
6	WT	NMD	NMD	NMD	NMD	NMD	NMD	NMD		NMD		
7	WT	NMD	NMD	NMD	NMD	NMD	NMD	NMD		NMD		
8	G13D	G13D	NMD	23	NMD	100	119			385		
9	WT	NMD	NMD	NMD	NMD	NMD	NMD	NMD	NMD	NMD		
10	WT	NMD	G12V	NMD	23	46	3	46	12	37	5	19
11	WT	NMD	NMD	NMD	NMD	NMD	NMD	NMD		NMD		
12	WT	NMD	G12C	NMD	NMD	NMD	NMD	NMD	25	80	25	Concurrent
			G12A	NMD	NMD	NMD	NMD	NMD	5	20		
13	WT	NMD	NMD	NMD	NMD	NMD	NMD	NMD	0	NMD		
14	WT	NMD	NMD	NMD	NMD			NMD	0	NMD		
15	WT	NMD	G12V	NMD	NMD	NMD	NMD	NMD		127	22	Concurrent
16	WT	NMD	NMD	NMD	NMD	NMD		NMD	NMD	NMD		
17	WT	NMD	NMD	NMD	NMD		NMD	NMD	NMD	NMD		
18	WT	NMD	NMD	NMD	NMD	NMD	NMD	NMD		NMD		
19	WT	NMD	NMD	NMD	NMD	NMD	NMD	NMD				
20	G13D	G13D	NMD	411	146		1215			2484		
21	WT	NMD	G12V	NMD	NMD	NMD	NMD			16	18	Concurrent
22	WT	NMD	G12S	NMD	NMD	NMD	NMD	24			17	Concurrent
			G12C	NMD	NMD	NMD	NMD	3				
			G12A	NMD	NMD	NMD	NMD	8				
			G12D	NMD	NMD	NMD	NMD	4				
23	WT	NMD	NMD	NMD		NMD	NMD	NMD		NMD		
24	WT	NMD	G12A	NMD	NMD	NMD	NMD	NMD		3	26	29
25	WT	NMD	NMD	NMD	NMD	NMD	NMD	NMD				
26	WT	NMD	NMD	NMD	NMD	NMD	NMD	NMD	NMD	NMD		
27	WT	NMD	NMD	NMD	NMD	NMD	NMD	NMD	NMD	NMD		
28	G12D	G12D	NMD	810	518	806				984		

* NMD = No Mutation Detected in the evaluated sample; blank values represent samples that were not available at the indicated time point

Supplementary Table 3. Tumor Burden and CEA data

CEA Levels:

#	w0	w8	w12	w16	w20	w24	w32	w40	Follow-up
1	4366.0	158.0	87.0	307.0		1014.0			3279.0
2	3526.0	149.0	50.0	42.0		122.0			92.0
3	194.2		268.0	364.7					462.6
4	4.9	0.6		1.8		2.3	4.4		6.4
5	4.8	2.7	2.7	3.5		2.7			3.6
6	154.0	164.0	65.0	61.0		56.0			55.0
7	25.0	2.9	2.1	6.3		29.0			42.5
8	11.0	6.7	10.1						36.8
9	43.6		1.0			1.0	1.9	4.8	10.5
10	40.7	7.2	5.8	8.6		16.6			9.1
11	16578.0	5069.0	2015.0	864.0		1217.0			2252.0
12	231.0	36.3	36.7	69.5	180.0	420.3			756.5
13	51.5	5.1	6.1	6.9		27.6			53.6
14	33.4			1.3		1.1			1.8
15	2454.0	805.0	504.0	569.0	1164.5	1628.0			1584.0
16	389.8		40.6	42.4		137.6			183.0
17	203.0	4.7	2.9	7.3		17.5	34.5		169.1
18	352.2	31.2	23.2	22.9	34.5				114.8
19	1.6	1.2		0.9					
20	190.5		346.5						521.5
21	27.1	4.5	5.4	515.0					9.8
22	73.5	51.6	50.6	35.9					
23	133.6	36.3	19.1	15.5		10.8	22.3		40.4
24	22.6	7.4	6.6	7.1		9.2			23.3
25	5.9	2.5	3.4	3.4					
26	281.1	73.3		48.7		43.5	60.6	116.7	164.9
27	13.9	3.6	3.3	3.0		3.7	4.8		5.8
28	25.8								19.1

Blank cells indicate timepoints for which no data were available

Total cross-sectional area (mm²) of index lesions included in disease response assessment

#	w0	w8	w12	w16	w20	w24	w32	w40	Follow-up
1	13986	9094	7950	7050		7752			
2	24280	9774	9249	8585		10820			9907
3	6225	5904	7968	8316					9212
4	1240	204	342	360		550	594		
5	396	144	144	144		144			
6	13728	15865		9346		11194			
7	3290	1949	2114	2051		4190			
8	1849	1849	2401						
9	5923		3923			3572	3900		
10	1317	864	862	420		635			
11	5649	4411	3853	3506		3360			
12	7631	6375	4954	5236		7367			
13	12351	8274	8210	7612		7822			
14	3310	2039	1331	1265		2610	3386		
15	9657	4487	3865			3600			
16	6619	2964	2124	1911		3722			
17	2102	618	658	792		735	693		
18	12208	7757	7200	5556	4994				5209
19	748	154		121					
20	1714	2300	2959						
21	4484	4082	3847	3578					
22	38006	26596	27359	28512					
23	5580	4282	4444	4095		4025	3987		
24	3706	3594	3480	3285		2887			
25	2196	1749	1508	1687	1909				
26	6450	1864		1129		1730	2244	4620	
27	2799	2181	1892	1701		2088	2620		
28	1312	1698							

Blank cells indicate timepoints for which no data were available

Supplementary Table 4. Probability that the indicated mutation was absent prior to panitumumab therapy

Patient	KRAS mutation	Time (weeks)	<i>p</i> -value (<i>b</i> =0.25)	<i>p</i> -value (<i>b</i> =0.15)	<i>p</i> -value (<i>b</i> =0.35)
1	G12V	25	8E-1199	1E-1997	2E-856
1	G12C	25	2E-1505	2E-2508	2E-1075
1	G12A	25	2E-474	3E-790	5E-339
1	G12R	25	7.E-168	2.E-17	2E-1672
4	G12R	34	4.E-02	5.E-03	1.E-01
5	G12D	26	2.E-223	4E-372	7.E-160
10	G12V	25	4E-335	5E-558	1.E-239
12	G12C	25	3E-697	1E-1161	3E-498
12	G12A	25	5.E-140	6.E-233	3.E-100
15	G12V	22	1E-15203	6E-25399	6E-10860
21	G12V	18	6E-13378	5E-22296	7E-9556
22	G12S	17	6E-32621	9E-54368	7E-23301
22	G12C	17	3E-4078	1E-6796	3E-2913
22	G12A	17	4E-10874	4E-18123	2E-7767
22	G12D	17	2E-5437	7E-9062	4E-3884
24	G12A	26	4.E-52	2.E-86	2.E-37

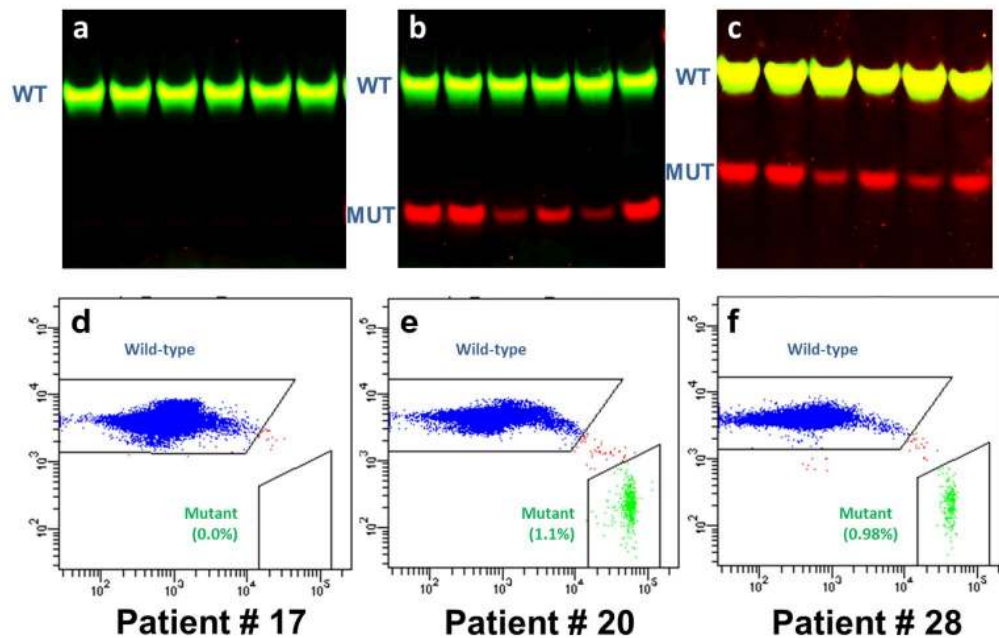
Supplementary Table 5. Oligonucleotides primers and probes

Gene	Used for:	5'-Modification	Mutation	Sequence (5'-3')*
PCR Amplification Primers				
KRAS	PCR forward primer	None	KRAS codons 12 & 13	GATCATATTTCGTCCACAAAATGATTTC
KRAS	PCR Reverse Primer	None	KRAS codons 12 & 13	TGACTGAATATAAACTTGTGGTAGTTG
Ligation probes				
KRAS	WT-specific probe	6-FAM	G12S	TCC CGC GAA ATT AAT ACG AG CTA CGC CACC
KRAS	Mutant-specific probe	HEX	G12S	CTC TTG CCT AC GCC AGT
KRAS	Common anchoring probe	Phosphate	G12S	AGC TCC AAC TAC C GG TGT CCA CTA GTC ATG CTT
KRAS	WT-specific probe	6-FAM	G12R	TCC CGC GAA ATT AAT ACG AG CTA CGC CACC
KRAS	Mutant-specific probe	HEX	G12R	CT AC GCC AGG
KRAS	Common anchoring probe	Phosphate	G12R	AGC TCC AAC TAC C GG TGT CCA CTA GTC ATG CTT
KRAS	WT-specific probe	6-FAM	G12C	TCC CGC GAA ATT AAT ACG AG CTA CGC CACC
KRAS	Mutant-specific probe	HEX	G12C	CTA CGC CAC A
KRAS	Common anchoring probe	Phosphate	G12C	AGC TCC AAC TAC C GG TGT CCA CTA GTC ATG CTT
KRAS	WT-specific probe	6-FAM	G12D	ATG GAG AAC TTG ACG TCC T C CTA CGC CAC
KRAS	Mutant-specific probe	HEX	G12D	TGCCT +ACGC+C+AT
KRAS	Common anchoring probe	Phosphate	G12D	CAG CTC CAA CTAC GG TGT CCA CTA GTC ATG CTT
KRAS	WT-specific probe	6-FAM	G12A	ATG GAG AAC TTG ACG TCC T C CTA CGC CAC
KRAS	Mutant-specific probe	HEX	G12A	CCT ACG C C AG
KRAS	Common anchoring probe	Phosphate	G12A	CAG CTC CAA CTAC GG TGT CCA CTA GTC ATG CTT
KRAS	WT-specific probe	6-FAM	G12V	ATG GAG AAC TTG ACG TCC T C CTA CGC CAC
KRAS	Mutant-specific probe	HEX	G12V	CCT ACG CCA A
KRAS	Common anchoring probe	Phosphate	G12V	CAG CTC CAA CTAC GG TGT CCA CTA GTC ATG CTT
KRAS	WT-specific probe	6-FAM	G13D	ATG GAG AAC TTG ACG TCC T C CTT GCCTACGC
KRAS	Mutant-specific probe	HEX	G13D	CTT GCCTACGT
KRAS	Common anchoring probe	Phosphate	G13D	CACGAGCTCCAAC GG TGT CCA CTA GTC ATG CTT
BEAMing probes				
KRAS	Detecting beads containing either WT or mutant sequences	ROX	G12S	TGACGATACAGCTAATTCA
KRAS	WT-specific probe	Cy3	G12S	TGGAGCTGGTGGCGT
KRAS	Mutant-specific probe	Cy5	G12S	TGGAGCTAGTGGCGT
KRAS	Detecting beads containing either WT or mutant sequences	ROX	G12R	TGACGATACAGCTAATTCA
KRAS	WT-specific probe	Cy3	G12R	TGGAGCTGGTGGCGT
KRAS	Mutant-specific probe	Cy5	G12R	TGGAGCTCGTGGCGT
KRAS	Detecting beads containing either WT or mutant sequences	ROX	G12C	TGACGATACAGCTAATTCA
KRAS	WT-specific probe	Cy3	G12C	TGGAGCTGGTGGCGT
KRAS	Mutant-specific probe	Cy5	G12C	TGGAGCTTGTGGCGT
KRAS	Detecting beads containing either WT or mutant sequences	ROX	G12D	TGACGATACAGCTAATTCA
KRAS	WT-specific probe	Cy3	G12D	GGAGCTGGTGGCGTA
KRAS	Mutant-specific probe	Cy5	G12D	GGAGCTGATGGCGTA
KRAS	Detecting beads containing either WT or mutant sequences	ROX	G12A	TGACGATACAGCTAATTCA
KRAS	WT-specific probe	Cy3	G12A	GGAGCTGGTGGCGTA
KRAS	Mutant-specific probe	Cy5	G12A	GGAGCTGCTGGCGTA
KRAS	Detecting beads containing either WT or mutant sequences	ROX	G12V	TGACGATACAGCTAATTCA
KRAS	WT-specific probe	Cy3	G12V	GGAGCTGGTGGCGTA
KRAS	Mutant-specific probe	Cy5	G12V	GGAGCTGTTGGCGTA
KRAS	Detecting beads containing either WT or mutant sequences	ROX	G13D	TGACGATACAGCTAATTCA
KRAS	WT-specific probe	Cy3	G13D	AGCTGGTGGCGTAGGC
KRAS	Mutant-specific probe	Cy5	G13D	AGCTGGTGACGTAGGC

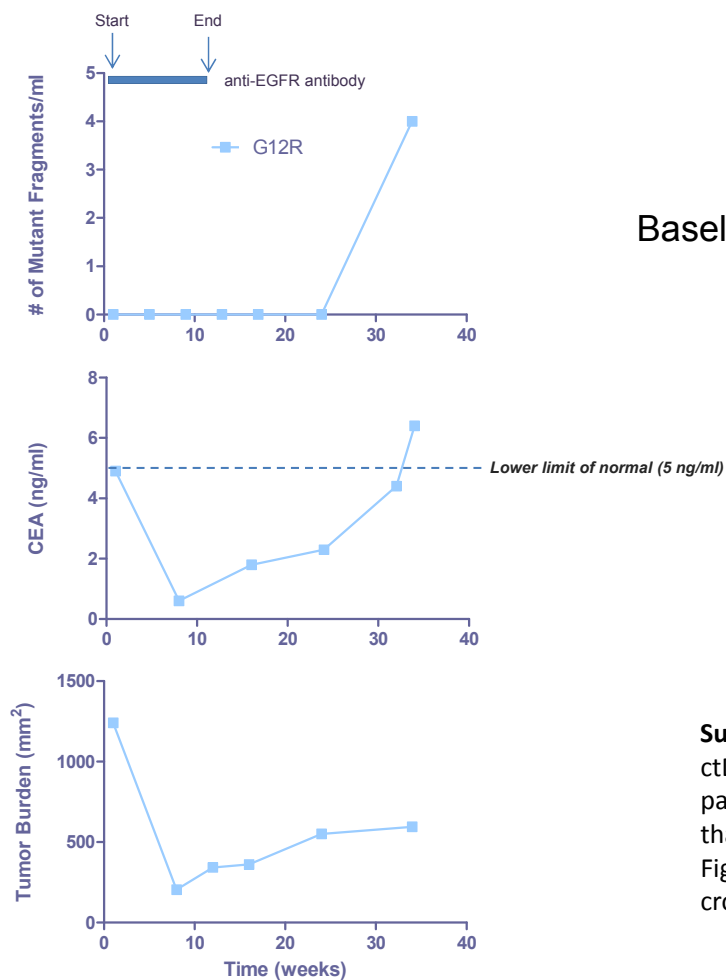
* indicates LNA linkages; red font indicates additional nucleotides appended to the ends of the common anchoring or WT-specific probes. Upon electrophoresis, the extra nt on the 5' end of the WT probes render the WT-specific ligation products larger than the mutant-specific ligation products.

Supplementary Table 6. Total circulating cell-free DNA levels (ng/ul)

Subject	Week 1	Week 5	Week 9	Week 13	Week 17	Week 25	Follow-up (week 26 to 52)
1	1.43	5.23	0.10	0.60	0.27	1.09	5.07
2	2.18	0.70	0.09	0.18	0.56		0.26
3	2.25	0.56	1.03	1.11			3.11
4	1.12	0.34	0.32	0.09	0.08	0.20	0.16
5	0.09	0.05	0.07	0.11	1.02		0.56
6	0.56	0.84	0.18	0.22	0.28		1.94
7	0.10	0.31	2.74	0.24	0.52		0.16
8	0.22	0.32	2.63	1.35			15.97
9	1.17	0.14	0.12	1.12	0.30	1.85	0.30
10	5.51	0.10	0.05	0.06	0.03	0.08	0.11
11	2.05	0.32	0.16	0.07	0.05		0.22
12	0.02	0.13	0.09	0.21	0.47	0.06	1.08
13	0.13	0.08	0.16	0.06	0.14	0.12	0.12
14	0.88	0.91			0.41	0.42	0.19
15	0.95	0.25	0.75	0.74	0.94		2.68
16	0.02	14.39	4.05		0.70	0.36	0.09
17	0.09	0.21		0.15	0.06	0.15	0.19
18	0.20	0.66	0.08	0.74	0.42		0.45
19	0.31	0.14	0.32	0.40	0.42		
20	0.39	0.07		1.04			0.06
21	0.39	0.39	0.07	0.05			0.41
22	0.52	0.38	0.26	0.16	0.14		
23	0.55		0.17	0.08	0.27		0.04
24	0.32	0.79	0.28	0.07	0.04		0.10
25	1.38	0.07	0.22	2.06	0.37		
26	0.16	1.41	0.92	1.53	0.58	0.34	0.39
27	0.52	0.12	0.07	0.07	1.70	0.17	0.62
28	1.11	0.25	1.73				0.64



Supplementary Fig. 1. Ligation and BEAMing assays used to detect circulating *KRAS* mutations. **a, b, c,** Each lane represents the results of ligation of one of six independent *KRAS*-specific PCR products, each containing 100 template molecules from the indicated patients' pre-treatment serum samples. The wild-type ligation products contain 6-carboxyfluorescein-labeled oligonucleotide probes that ligate to an unlabeled oligonucleotide only when wild-type alleles are present. The mutant (MUT) ligation products contain hexachlorofluorescein-labeled oligonucleotide probes that ligate to the same unlabeled oligonucleotide only when mutant alleles are present. The fluorescent images of denaturing acrylamide gels in which the ligation products were size-separated are shown. **d, e, f,** *KRAS*-specific PCR products were used as templates for BEAMing in which each template was converted to a bead containing thousands of identical copies of the template¹². After hybridization to Cy3- or Cy5-labeled oligonucleotide probes specific for wild-type or mutant sequences, respectively, the beads were analyzed by flow cytometry. Beads whose fluorescence spectra lie between the wild-type and mutant-containing beads result from inclusion of both wild-type and mutant templates in the aqueous nanocompartments of the emulsion PCR. See Materials and Methods for additional details. **a, d:** Patient #17; **b, e:** Patient #20; **c, f:** Patient #28. The mutant probes used in **a, b, d,** and **e** were specific for *KRAS* cDNA nt 38A and the mutant probes used in **c** and **f** was specific for *KRAS* cDNA nt 35A. The fraction of beads representing mutant templates are indicated for each patient.



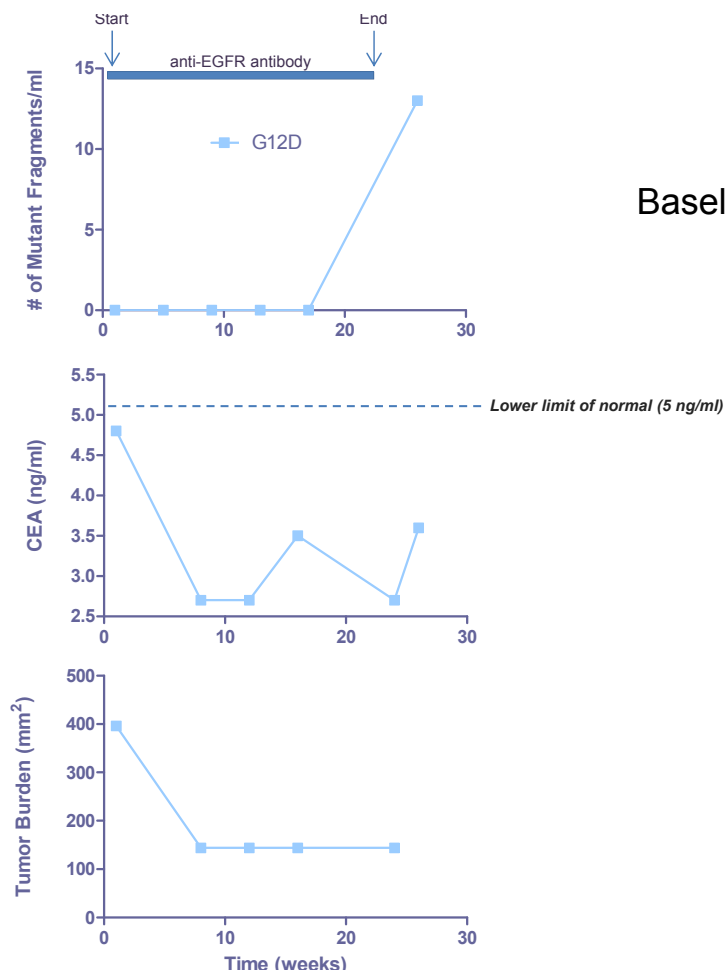
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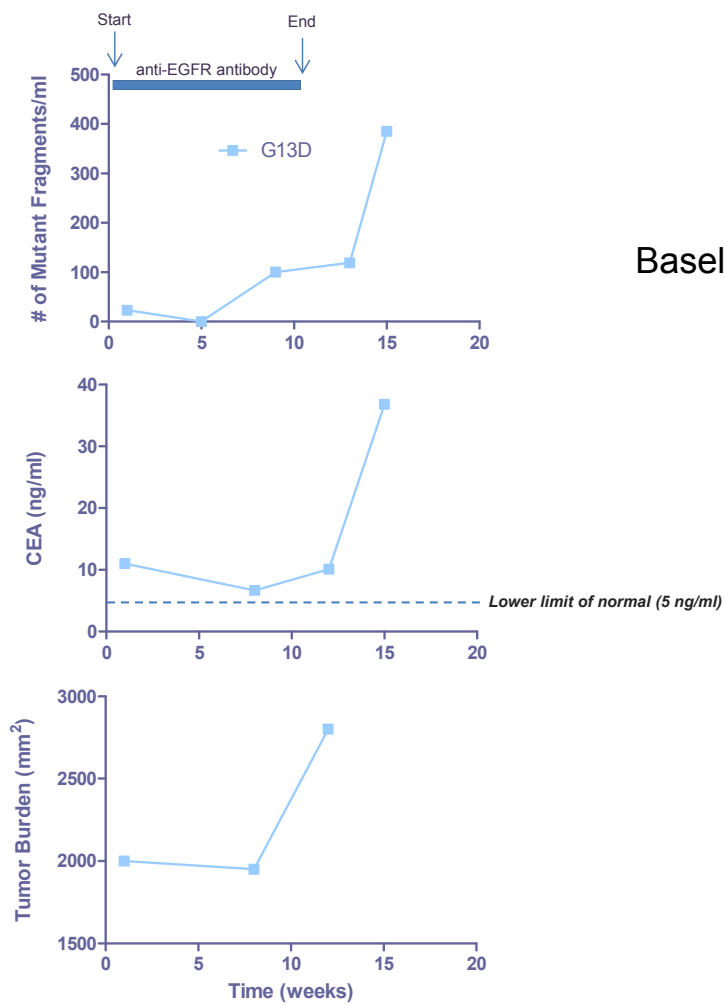
Baseline KRAS Status: WT

Supplementary Fig. 2. Time course of ctDNA, CEA, and tumor burden for all the patients in which ctDNA was detected (other than Patients 1 and 12, which are depicted in Fig. 1). Tumor burden refers to the aggregate cross-sectional diameter of the index lesions.

Patient # 5

Baseline KRAS Status: WT



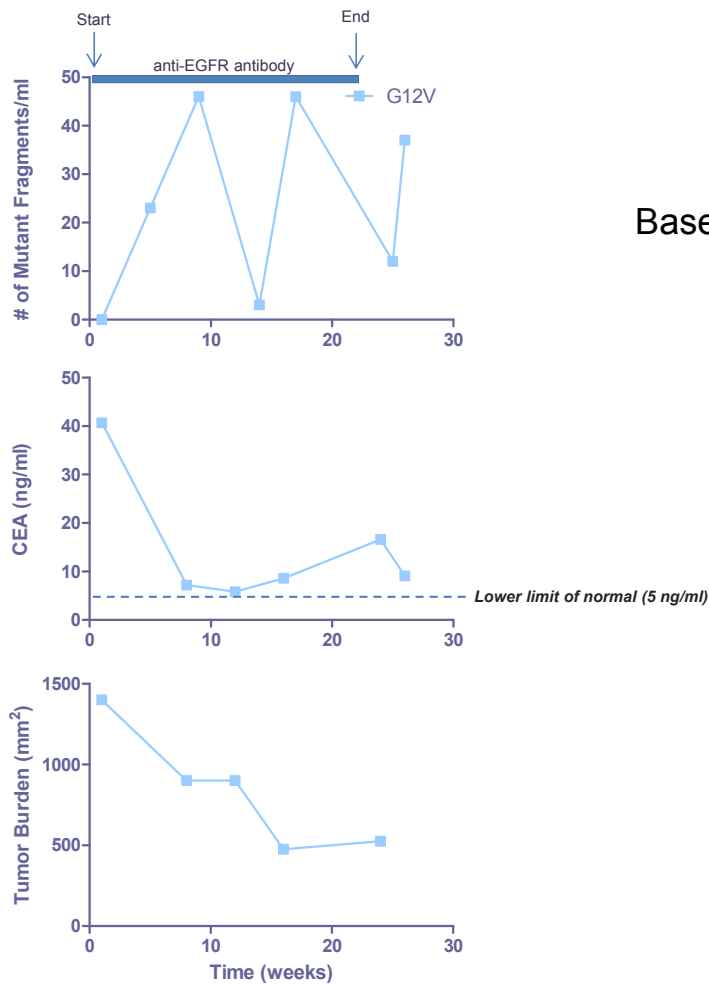


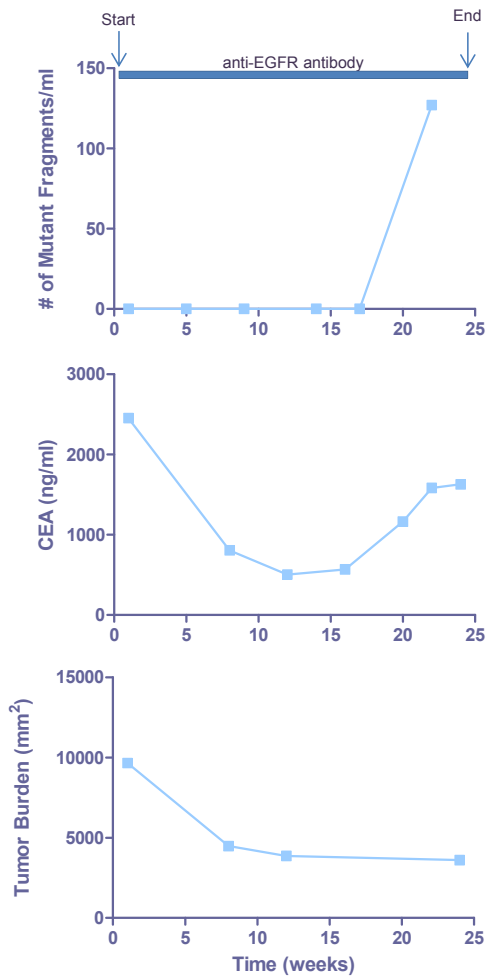
Patient # 8

Baseline KRAS Status: Mutant

Patient # 10

Baseline KRAS Status: WT



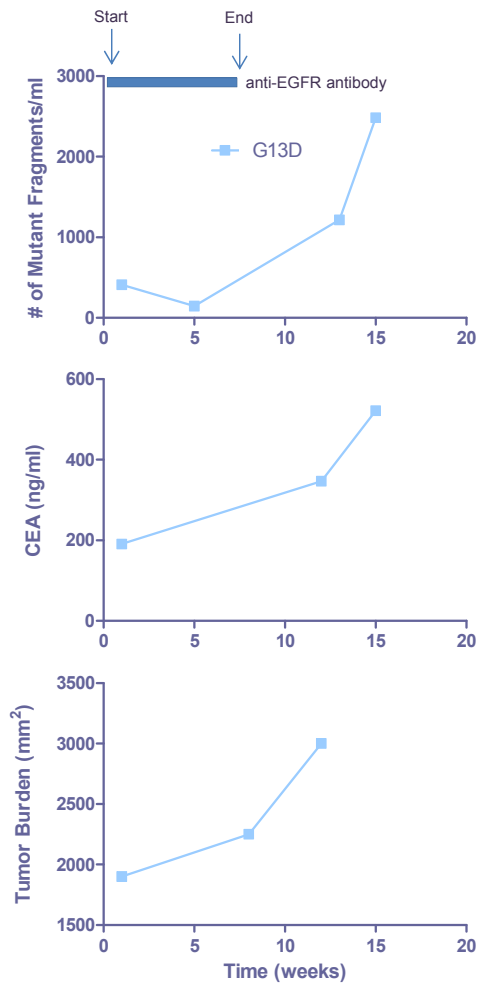


Patient # 15

Baseline KRAS Status: WT

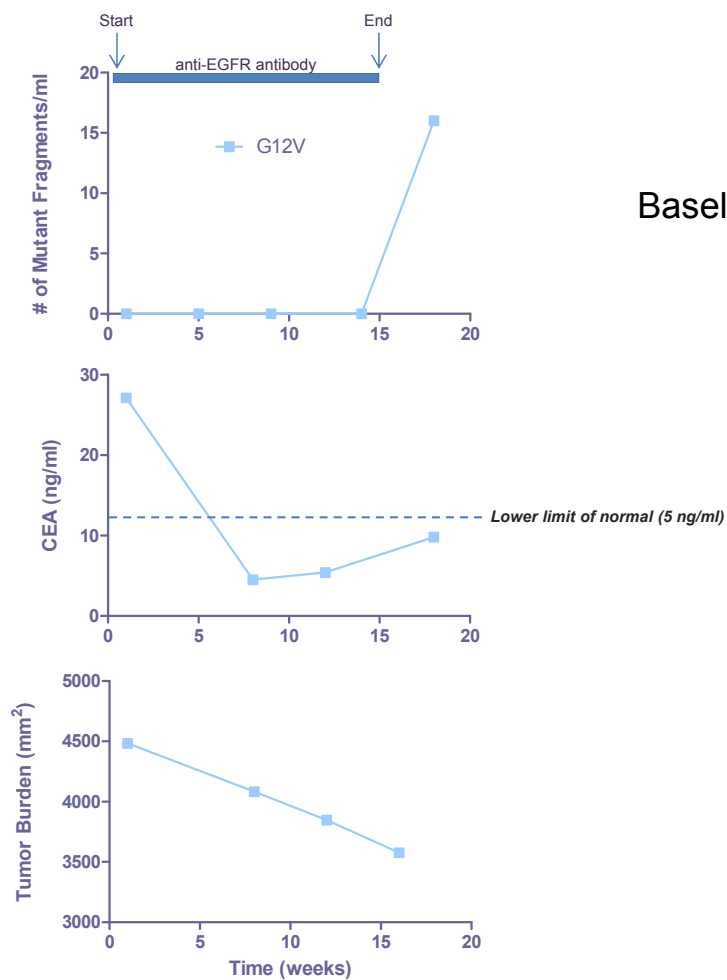
Patient # 20

Baseline KRAS Status: Mutant



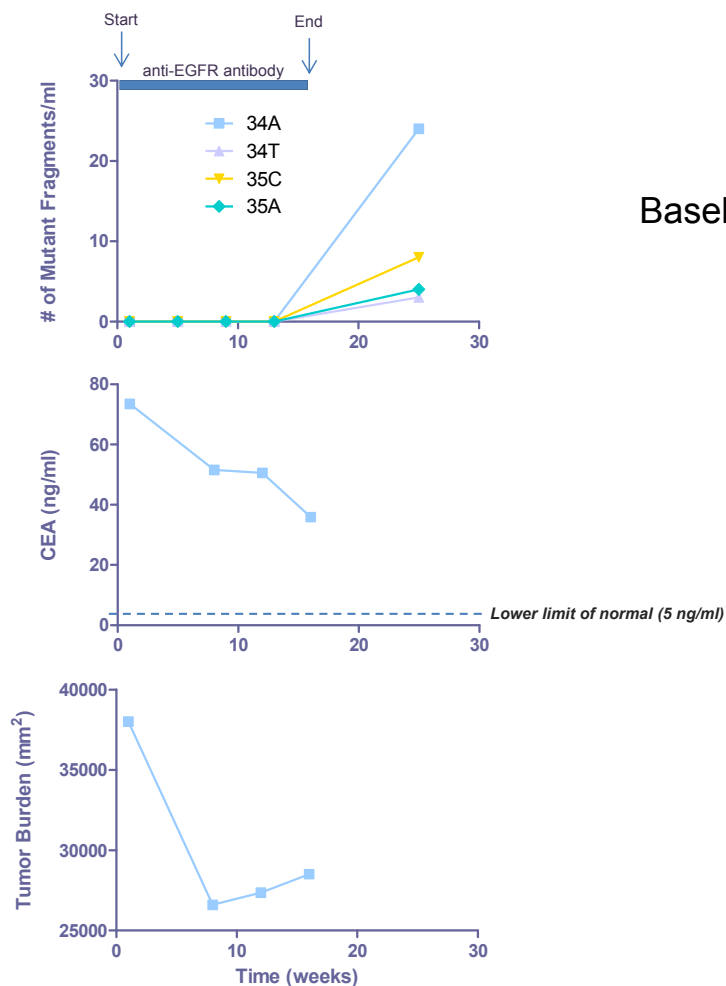
Patient # 21

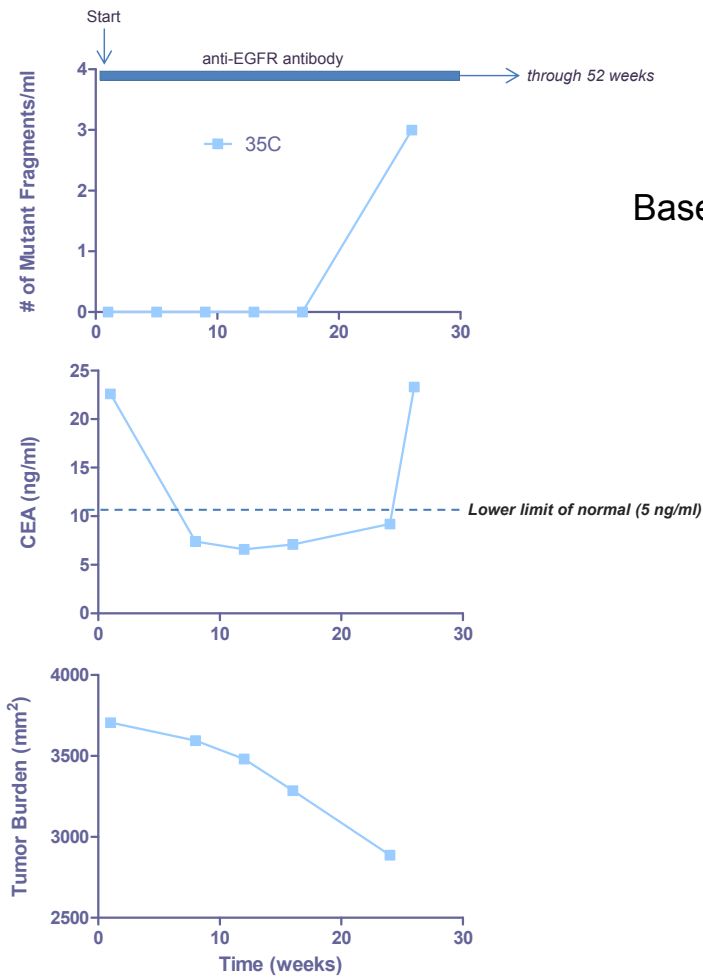
Baseline KRAS Status: WT



Patient # 22

Baseline KRAS Status: WT



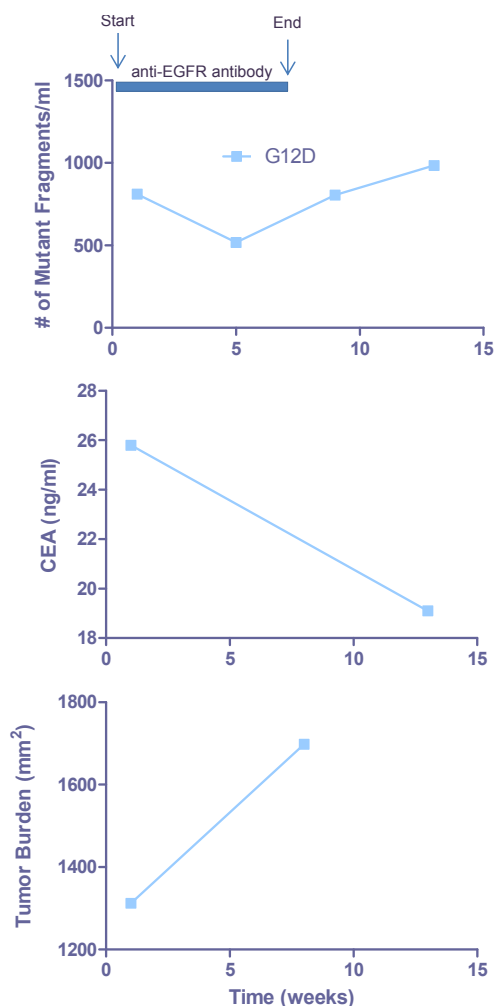


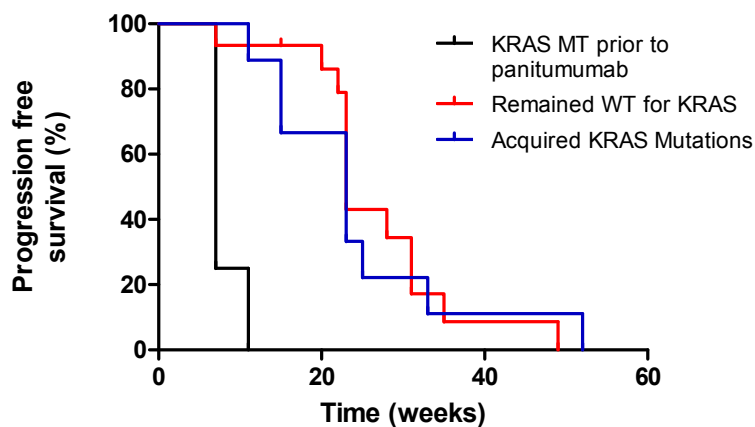
Patient # 24

Baseline KRAS Status: WT

Patient # 28

Baseline KRAS Status: Mutant





Supplementary Fig. 3. Kaplan-Meier Estimates of progression free survival in three groups of patients treated with panitumumab. (Black) patients whose tumors were *KRAS*-mutant prior to initiating therapy, (Red) patients whose tumors were wild-type for *KRAS* prior to starting therapy with panitumumab and *did not* develop detectable secondary mutation in *KRAS*, and (Blue) patients whose tumors were wild-type for *KRAS* prior to starting therapy with panitumumab and *did* develop a detectable mutation in *KRAS*. The progression-free survival was not different among patients who did or did not develop *KRAS* mutations during treatment (Hazard ratio 0.9; 95% CI 0.3366 to 2.453; $p=0.85$; Log-Rank Test).

Supplementary Appendix

1 Statistical test to confirm presence of KRAS-mutated cells at start of treatment

We are interested in the question of whether there were pre-existing KRAS-mutated cells at the time that treatment was started. We take the case that KRAS-mutated cells were absent at the start of treatment as our null hypothesis H_0 ; the alternative hypothesis H_1 is that they were present.

We use a branching process model, described below, to test the null hypothesis. We perform this test separately for each KRAS mutation in each patient. Using our model, we compute an upper bound on the probability $\Pr[\Theta|H_0]$, where Θ is the event that a number of KRAS-mutated cells greater or equal to the observed number are present in the tumor at the time of observation. We use $\Pr[\Theta|H_0]$ as a p -value for this test. We reject H_0 if $\Pr[\Theta|H_0] < 0.05$.

1.1 Branching process model

We model the dynamics of KRAS-mutated cells by a branching process (Coldman and Goldie, 1983, 1986; Iwasa et al., 2006) with birth rate b and death rate d . We let \tilde{X}_t denote the number of KRAS-mutated cells conditioned on nonextinction of this population. A result of Durrett and Moseley (2010) implies that, as $t \rightarrow \infty$, the quantity $e^{-(b-d)t} \tilde{X}_t$ converges to a random variable V , where V is exponentially distributed with mean $b/(b-d)$.

Using this result, we can write

$$\Pr[\tilde{X}_t \geq N] \approx \Pr[V \geq e^{-(b-d)t} N] = \exp\left(-\frac{b-d}{b} e^{-(b-d)t} N\right),$$

where the approximation is accurate for large t . We conclude that

$$\Pr[\Theta|H_0] \leq \exp\left(-\frac{b-d}{b}e^{-(b-d)T}N\right),$$

where T is the time at which the number of KRAS-mutated cells, N , is measured.

1.2 Estimation of parameter values

We use the observed data to estimate the growth rate, $b - d$, of KRAS-mutated cells. This growth rate is equal to the change in the log number of cells per unit time. We therefore estimate this growth rate as

$$b - d \approx \frac{\text{total change in log number of cells}}{\text{total change in time}}.$$

To compute this average, we used the data from Patients 1 and 12, for which there were multiple KRAS mutations that were observed to grow over the course of two or more measurements. This yielded an estimate of $b - d \approx 0.069$ as average growth rate of KRAS-mutated cells per day. We also computed the growth rates separately for each lesion in Patients 1 and 12.

We initially assume $b = 0.25$, which corresponds to one cell division every four days, and obtain d from the estimated value of $b - d$. We also repeat our analysis for $b = 0.15$ and $b = 0.35$ to test the sensitivity of our results to this parameter.

As described in the main text, we estimate that each KRAS-mutated fragment detected per milliliter of blood corresponds to 4.4×10^7 KRAS-mutated cells.

2 Probability that resistant cells exist at start of treatment under generalized Luria-Delbrück distribution

To complement the above hypothesis tests, we also calculate the probability that resistant cells exist at the start of treatment, under a Luria-Delbrück distribution generalized to incorporate cell death (Dewanji et al., 2005). This distribution assumes a particular model of tumor development, in which the

population of sensitive cells grows exponentially, and resistance mutations arise stochastically. A slower pattern of growth (but still leading to the same tumor size at the start of treatment) would be expected to yield more resistance mutations (Luebeck and Moolgavkar, 1991). In contrast, the hypothesis tests described above make no assumptions on the dynamics of the sensitive cell population.

From this point forward we use the following notation and parameter values (unless otherwise specified):

- $M = 10^9$ is number of tumor cells at the start of treatment,
- $b = 0.25$ is the division rate of both sensitive and resistant cells,
- $d = 0.181$ is the death rate, so that the growth rate is $b - d = 0.069$ (as inferred above from the observed dynamics of circulating KRAS-mutated fragments),
- $u = 42 \times 10^{-9}$ is the total rate at which resistance mutations are generated (assuming 42 possible mutations conferring resistance, as estimated in the main text, and a mutation rate of 10^{-9} per cell division).

Using the generalized Luria-Delbrück distribution proposed by Dewanji et al. (2005; or equivalently, the formulas of Iwasa et al., 2006), we calculate the likelihood that no resistant cells exist prior to treatment to be 4×10^{-33} . Thus, under this model, resistance is almost certainly present at the start of treatment.

3 Distribution of waiting times until resistance can be detected

This generalized Luria-Delbrück distribution can also be used to obtain the distribution of waiting times until resistance mutations become detectable in circulating DNA. To obtain this distribution, we first used the methods of Dewanji et al. (2005) to numerically calculate the size distribution of the resistant cell population at the start of treatment. We then assume that the resistant cell population grows exponentially (deterministically) once treatment starts. This deterministic assumption is justified since, for the above parameter values, the number of resistant cells at the start of treatment is

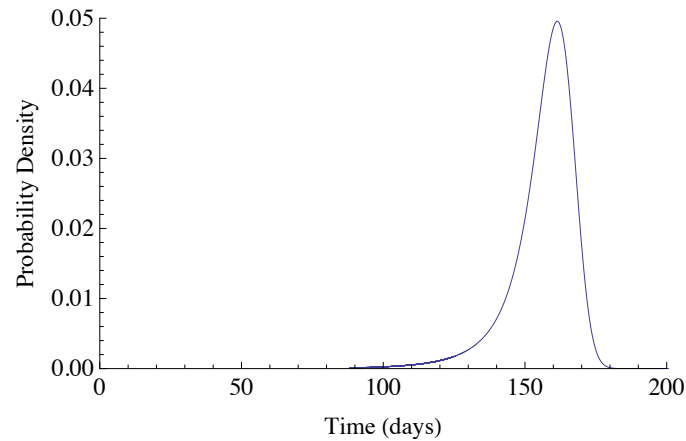


Figure 1: Predicted probability distribution of times from when treatment starts until resistance mutations become observable in circulating DNA. Predictions are based on the Lea-Coulson model with death introduced by Dewanji et al. (2005), or equivalently, the branching process model of Iwasa et al. (2006). Tumor growth rates were inferred from the growth in observed KRAS fragments over time in different lesions; otherwise, parameters were not fit to the data.

likely to be large. (For instance, there is a 99% chance that there are at least 276 resistant cells when treatment starts.) We used 4.4×10^7 for the number of resistant cells for which resistance mutations become observable in circulating DNA.

The resulting distribution has a mean of 156 days (22 weeks), with a standard deviation of 11 days. The 95% confidence interval spans from 126 days (18 weeks) to 172 days (25 weeks). The probability density function for this distribution is plotted in Figure 1.

Our analytic results were confirmed through simulation of the birth-death branching process with mutation (Coldman and Goldie, 1986; Iwasa et al., 2006), using the above parameter values. In these simulations, the exact branching process is followed for clonal populations smaller than 10^4 , and deterministic exponential growth approximations are used for larger populations. These simulations yielded a mean of 157 days and a standard deviation of 12 days for the waiting time until resistance become detectable.

These results are a close match to the observed data, in which resistance mutations were first observed at Week 17 in some cases and Week 25 in others. In particular, the sharpness of this distribution helps explain the striking similarity, across patients and lesions, in the times at which resistance mutations become detectable.

To incorporate the possibility that not all cells in a lesion are actively dividing, we repeated this analysis using $M = 10^8$ as the number of actively dividing tumor cells at the start of treatment (one-tenth of the total number of cells in a detectable lesion). This yielded an expected time of 200 days (29 weeks), with a standard deviation of 17 days. The assumption that all (or almost all) tumor cells are actively dividing—that is, $M = 10^9$ as above—better fits the observed data.

4 Size of the largest clonal subpopulation of resistant mutants

Next we obtain an analytical estimate for the size of the largest clonal subpopulation of resistant cells. We assume that the largest clonal subpopulation is the progeny of the first resistance mutation to arise and survive stochastic drift. This is reasonable, since resistance mutations beyond the first are likely to arise significantly later, after this first subpopulation has grown

substantially.

We use the result of Iwasa et al. (2006) that the collection of tumor sizes at which resistance mutations are produced can be viewed as a homogenous Poisson process on $[1, M]$ with intensity $u/(1 - d/b)$. Each such mutation survives stochastic drift with probability $1 - d/b$, so the tumor sizes at which mutations that survive stochastic drift arise are produced can be viewed as a Poisson process on $[1, M]$ with intensity u . Since M is large and u is small, we can replace the interval $[1, M]$ by $[0, M]$, without losing much accuracy.

Let M_1 denote the size of the tumor when the first mutation that survives stochastic drift is produced. Then M_1 is exponentially distributed with mean u^{-1} (since M_1 corresponds to the first event in a Poisson process with intensity u). By the time that the total tumor cell population reaches size M , the size of the clonal subpopulation initiated by this mutation can be approximated by MV/M_1 , where V is an exponentially distributed random with mean $b/(b - d)$. (This follows from the results of Durrett and Moseley, 2010.)

Let Y_1 denote the size of the clonal subpopulation initiated by the first mutation that survives stochastic drift, conditioned on $M_1 \leq M$ (i.e., this mutation arises before the tumor reaches size M). Using the above results, we obtain the cumulative distribution function $F(y)$ of Y_1 by

$$\begin{aligned}
 1 - F(y) &= \Pr[Y_1 \geq y] \\
 &\approx \Pr\left[\frac{MV}{M_1} \geq y \mid M_1 \leq M\right] \\
 &= \int_{z=0}^M \text{Prob. Density}[M_1 = z \mid M_1 \leq M] \times \Pr\left[V \geq \frac{yz}{M}\right] dz \\
 &= \int_{z=0}^M \frac{ue^{-zu}}{1 - e^{-Mu}} \exp\left(-\frac{yz}{M} \frac{b-d}{b}\right) dz \\
 &= \frac{u}{1 - e^{-Mu}} \int_{z=0}^M \exp\left(-z\left(u + \frac{y}{M} \frac{b-d}{b}\right)\right) dz \\
 &= \frac{Mu}{1 - e^{-Mu}} \left(Mu + y \frac{b-d}{b}\right)^{-1} \left(1 - \exp\left(-Mu - y \frac{b-d}{b}\right)\right).
 \end{aligned}$$

The expected size of this clonal subpopulation can be calculated as

$$E[Y_1] \approx \int_0^M y F'(y) dy \approx 2237,$$

for the given parameter set. Simulations of the birth-death branching process (as described in Section 3 above) confirmed the size of the largest clone to be ~ 2300 .

We can compare this to the total expected number of resistant mutants, $E[Y]$, which can be calculated using Eqn. (13) of Dewanji et al. (2005) or Eqn. (10) of Iwasa et al. (2006):

$$E[Y] \approx 3241.$$

Thus most (69%) of the resistant population is comprised of a single clonal type.

We can also vary the number of possible mutations conferring resistance by varying u ; for example, in the case of four mutations conferring resistance we would use $u = 4 \times 10^{-9}$. The results are shown in the following table:

Number of mutations conferring resistance	$E[Y_1]$	$E[Y]$	$E[Y_1]/E[Y]$
4	252	309	81%
10	584	772	76%
42	2237	3241	69%
100	4575	7717	65%

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