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Circulating tumor DNA for the detection and monitoring of human malignancies

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Abstract

The development of non-invasive methods to detect and monitor tumors continues to be a major challenge in oncology. We used digital PCR-based technologies to evaluate the ability of circulating tumor DNA (ctDNA) to detect tumors in 643 patients with various cancer types. We initially identified sequence or structural alterations in tumor tissues of 409 patients using targeted, exomic, or whole genome sequencing. We found that the mutations identified in the tumor were present in the plasma of more than 75% of patients with advanced ovarian, colorectal, bladder, gastroesophageal, pancreas, breast, melanoma, hepatocellular, head and neck cancers but in less than 50% of primary brain, renal, prostate, or thyroid cancers. In patients with localized tumors, ctDNA was present in 73%, 57%, 48% and 50% of patients with colorectal cancer, gastroesophageal cancers, pancreatic, and breast adenocarcinomas, respectively. Circulating tumor DNA was often present in patients without detectable circulating tumor cells, suggesting that these two biomarkers are distinct entities. In a separate panel of 206 patients with metastatic colorectal cancers, we showed that the sensitivity of ctDNA (“liquid biopsy”) for the detection of clinically relevant *KRAS* gene mutations was 87.2% while the specificity was 99.2%. Finally, we assessed whether ctDNA could provide clues into the mechanisms underlying resistance to epidermal growth factor receptor (EGFR) blockade in 28 patients who objectively responded to therapy but subsequently relapsed. Twenty-seven (96%) of these patients developed one or more mutations in genes involved in the mitogen-activated protein kinase (MAPK) pathway. Remarkably, nearly half (42%) of the mutations observed were in codon 61 of either the *KRAS* or *NRAS* gene. Taken together, these data suggest that ctDNA is a broadly applicable, sensitive and specific biomarker that can be used for a variety of clinical and research purposes in patients with several different types of cancer.

Background

Cancer will occur in more than 1.6 million individuals this year in the United States alone, but a clinically proven circulating biomarker that can be used to help guide patient management will be available for only a minority of them, even in the setting of widespread metastasis (1-6). While serum-based protein biomarkers such as carcinoma antigen-125 (CA-125) carcinoembryonic antigen (CEA) and prostate specific antigen (PSA) are commonly used for this purpose, these markers are not truly tumor-specific as they are found in the circulation of individuals without cancer, limiting specificity (2-4). Additionally, these markers are not found in a substantial portion of patients with advanced cancers, limiting their sensitivity (5, 6).

A new generation of biomarkers has become available with the discovery of the genetic alterations that are responsible for the initiation and progression of human cancers (7-11). With the influx of genomic information from recent cancer genome sequencing studies, it is now known that virtually all cancers of every type harbor somatic genetic alterations. These alterations include single base substitutions, insertions, deletions and translocations (the latter including those associated with the creation of gene fusions, gene amplifications or losses of heterozygosity). These somatic mutations occur at negligible frequencies in normal cell populations and therefore provide exquisitely specific biomarkers from a biologic perspective (9).

There are two sources of tumor DNA that can be non-invasively assessed in the circulation: free-floating circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs) (12, 13). Circulating tumor DNA is comprised of small fragments of nucleic acid that are not associated with cells or cell fragments (14). In contrast, circulating tumor cells represent intact, often viable, cells that can be purified from blood by virtue of physicochemical characteristics or cell surface molecules that distinguish them from normal blood cells (15). Many studies have shown that both ctDNA and CTCs are present in advanced neoplasia, though only a few studies have compared the amounts of CTCs and ctDNA templates in the same patients (16-19). The studies comparing the two approaches have reached opposing conclusions, likely due to technical issues that limited interpretation of either the ctDNA or CTC content. Furthermore, the mechanism by which CTCs or ctDNA are released into the circulation are unclear, although one theory is possible that ctDNA actually comes from CTCs. One of the purposes of the current study was to compare the quantities of ctDNA and CTCs using an unbiased approach.

Most studies of ctDNA published to date have each evaluated patients with a single tumor type. This has made it difficult to directly compare the amounts of ctDNA in various tumor types in light of considerable differences in DNA preparation and analytic techniques (16, 20-26). Comparison among studies is also challenging because of differences in the types of data that are reported. For example, it is often impossible to compare real-time PCR results with those reporting the fraction of mutant template molecules assessed, or to compare results based on the analysis of serum with those based on plasma. To directly compare different tumor types and to determine the spectrum of cancers in which ctDNA measurements could prove clinically useful, we evaluated a large number of tumor types in the current study. We purified plasma and tumor DNA using regimented protocols for all samples, and used digital technologies to evaluate ctDNA levels from various tumor types in a comparable fashion, reporting the number of mutant templates per milliliter (ml) of plasma in each case (Fig. 1). This digital paradigm also allowed us to compare directly the two most commonly used types of tumor-specific genetic alterations found in the circulation – single base substitutions and rearrangements.

One of the most immediate applications of ctDNA has been termed the “liquid biopsy” (20). In research studies as well as in clinical practice, it is often difficult to obtain tumor samples for genetic analyses. Some tumors are only accessible through fine needle aspirates (lung cancer, for example) with insufficient material available for genotyping, while in other cases it can be challenging or time-consuming to acquire samples from different medical centers (27). Additionally, once targeted therapy is initiated in patients with multiple metastases and investigators are searching for early evidence of recurrence or mechanisms underlying resistance, liquid biopsies are particularly valuable. For example, they can provide temporal measurements of the total tumor burden as well as identify specific mutations that arise during therapy (16, 20, 21, 23). Though the liquid biopsy approach has been shown to be promising, its sensitivity and specificity with respect to conventional tumor biopsies has not been evaluated in a large, clinically relevant cohort. In the current study, we evaluated the sensitivity and specificity of this approach in patients with colorectal cancers who were candidates for EGFR blockade. We also used liquid biopsies to identify mutations that were responsible for recurrence in patients who initially responded to EGFR blockade. In aggregate, these studies provide a wealth of information on the potential utility, as well as the limitations, of ctDNA measurements for the assessment of patients with various cancers.

Results

Patients with metastatic cancers. We began this study with an evaluation of the tumors of 136 does this include the 7 patient in Table S2 patients with metastatic disease originating from 17 different tissue types and an additional 41 patients with primary brain tumors. The clinical characteristics of these patients are summarized in Table 1. Targeted sequencing, exomic sequencing, or whole genome sequencing was used to identify these mutations, as described in the Materials and Methods. At least one genetic alteration - a point mutation (141 cases) or genetic rearrangement (36 cases) - was found in each of the tumors studied (**table S1**). Except for mutations at the known hotspots of the *KRAS*, *NRAS*, *PIK3CA*, and *BRAF* genes (which are well-known to be somatic), all other genetic alterations were demonstrated to be somatic through evaluation of DNA from non-neoplastic cells of the same patients.

Circulating tumor DNA was found in the majority of the studied patients with solid tumors outside the brain (112 of 136; 82.3%). However, the fraction of patients with detectable ctDNA varied with tumor type. As shown in Fig. 2A, most patients with metastatic cancers of the ovary, pancreas, bladder, colon, stomach, breast, liver, esophagus, head and neck, as well as of patients with neuroblastomas and melanomas, harbored detectable levels of ctDNA. In contrast, less than 50% of patients with medulloblastomas or metastatic cancers of the kidney, prostate, or thyroid, and less than 10% of patients with gliomas, harbored detectable ctDNA.

Though the number of patients with some of the tumor types depicted in Fig. 2A was small, limiting statistical significance of comparisons among tumor types, it was clear that patients with gliomas (low or high grade; table S1) were less likely to harbor ctDNA than patients with metastatic cancers of the pancreas, colon, breast, esophagus/stomach, or ovary.

Though ctDNA was found in most patients with metastatic cancers, the concentration of ctDNA varied among patients, even those with the same tumor type (Fig. 2B and table S1). Some of this variability was due to differences in copy number of the genes assayed in different tumors. For example, if the queried gene was amplified 50-fold in the tumor of Patient A, while the queried gene in the tumor of Patient B was present at normal copy number, the amount of ctDNA would be expected to be 50-fold higher in Patient A than in Patient B (see *Comparison of rearrangements with single base substitutions in ctDNA* below). However, great variability was also observed among cancers in which only non-amplified genes (such as *TP53*) were assessed.

Patients with localized disease. We next evaluated ctDNA in patients with localized disease, that is, no clinical or radiographic evidence of distant metastasis at the time of sample collection. Among the 225 patients with localized cancers of all types evaluated, detectable levels of ctDNA were found in 55% (123 of 225 patients; table S1). This fraction was lower than observed in patients with metastatic disease and was true for all tumor types in which a sufficient number of samples were available (breast, colon, pancreas, gastroesophageal; Fig. 3A). Detectable levels of ctDNA were detectable in 48 to 73% of patients with localized tumors and in 84% to 100% of patients with metastatic tumors of these four types (Fig. 3A).

Differences in the fraction of patients with detectable levels of ctDNA also correlated with Stage: 44% of patients with Stage I cancers of any type had detectable ctDNA, while the fraction of patients with detectable ctDNA was 55%, 68%, and 82% for patients with Stage II, III, and IV, respectively (Fig. 3B; table S1; Cochran-Armitage trend test, $p < 0.0001$). The concentration of ctDNA in the plasma similarly increased with Stage (Fig. 3C; table S1).

Comparison of ctDNA with CTCs. For these experiments, DNA was isolated from centrifugal pellets of blood; these pellets contained circulating tumor cells as well as WBCs, platelets, and other cellular fragments. In each case, whole genome sequencing of tumor DNA was used to identify somatic rearrangements. Digital PCR assays were then used to identify these rearrangements in blood pellets (i.e., CTCs) or in the blood supernatants (i.e., plasma) of the same patients. This experiment could be performed with tumor-specific rearrangements, but not with tumor-specific point mutations, for the reasons given in the Discussion. We did not identify any cases in which CTCs were detected but in which ctDNA was absent. However, in the majority of cases in which ctDNA was detected (13 of 16 ; 81%), no CTCs were detectable with the identical assay (**Table 2**). Moreover, in the 3

cases wherein both CTC and ctDNA levels were detectable, the average number of mutant fragments in the plasma was >50-fold higher than analogous levels in CTCs (Table 2).

Comparison of rearrangements with single base substitutions in ctDNA. It was also of interest to compare the quantity of two different types of genetically altered DNA fragments in the circulation of the same patients. Though practical issues precluded us from identifying a rearrangement in all patients in this study (see Discussion), tumor-specific rearrangements as well as tumor-specific point mutations were jointly identified in 20 patients (table S2). The rearrangements were identified by whole genome sequencing of tumor DNA and the point mutations identified by targeted sequencing. In each case, the alteration was shown to be somatic via evaluation of normal DNA from the same patients. In 19 of the 20 patients harboring a circulating point mutation, a circulating rearrangement was also detectable (table S2). The one exception was a patient with a circulating point mutation in *TP53* in which the rearrangement identified in that patient's tumor could not be identified in her plasma (table S2). The absolute number of circulating DNA fragments with point mutations vs. rearrangements was highly correlated (Fig. 4A; correlation coefficient = 0.96). However, in 6 patients, the number of circulating fragments containing rearrangements was > 10-fold than that of the queried point mutation (Fig. 4B). The reason for this was that the rearrangements we chose for analysis often arose as a result of gene amplification in the tumor, whereas the point mutations were generally present only once per tumor genome (see Methods).

The sensitivity and specificity of liquid biopsy. The results described above were obtained by first identifying a mutation in a tumor and then determining whether that same mutation was present in the plasma. For liquid biopsy applications, the mutation in the tumor is not known *a priori* and all mutations of interest are queried at once. To determine the sensitivity of the liquid biopsy approach, we evaluated the plasma and tumors of 206 metastatic colorectal cancer patients in a blinded fashion (table S3). This cohort of patients was completely separate from the 409 patients described above and in tables S1 & S2. For each case, we determined whether mutations at codons 12 or 13 of *KRAS* were present in either the plasma or the primary tumor. The *KRAS* gene was chosen for this study because of its clinical relevance; as mentioned in the Introduction, the absence of a *KRAS* gene mutation in the primary tumor is a prerequisite for treatment of metastatic CRC patients with antibodies that block EGFR. We identified 70 patients (34% of the 206) who harbored circulating mutant *KRAS* in their plasma. Circulating *KRAS* mutations were not detected in 127 of 129 patients with *KRAS* wild-type tumors, yielding an uncorrected specificity of 98.4%. Importantly, the mutation identified in the 70 plasma samples was always identical to that identified in the tumors, further emphasizing the specificity of the liquid biopsy. In addition to these 70 tumors, we identified ten cases (of 206) in which mutations were present in the

primary tumors but not in the plasma, yielding a sensitivity of 87.2%. Percent concordance between KRAS mutation status in the plasma and tumor tissue was 94% and the agreement was highly significant (Kappa statistic 0.87, $p < 0.0001$).

We next evaluated 30 clinical and pathologic characteristics to better understand the observed false positive or false negative results (table S4 and S5). The strongest association for a false negative ctDNA result (i.e. mutant KRAS in the tumor but no mutants detectable in the plasma) was a normal CEA level (≤ 3.0 ng/mL). Normal CEA levels were also negatively correlated with the concentration of mutant KRAS fragments in the plasma (table S6). These observations are consistent with the idea that lower tumor burdens (reflected by normal CEA levels) are associated with lower ctDNA levels (see Discussion). This idea was further supported by the remarkable correlation between the concentration of ctDNA and survival (Fig. 5; Logrank test for trend, $p = 0.0002$). As all the patients in this comparison had extensive Stage IV disease at the time of analysis, the information provided by ctDNA assessment was not captured by staging analysis. Similarly, 33% of the patients with easily detectable levels of circulating KRAS mutations had normal CEA levels of CEA, demonstrating that ctDNA measurements can provide information that is not captured by CEA measurements.

Monitoring patients for resistance-conferring mutations. Liquid biopsies can also be used to monitor patients being treated with targeted agents, providing an early warning of recurrence and information about the genetic basis of resistance. For example, KRAS codon 12 and 13 mutations were shown to develop in 38% of 24 patients who first responded to EGFR blockade, then progressed (20). In each case, the KRAS gene mutation was not present in the primary tumor but had presumably arisen in a small population of cells within a metastatic lesion and expanded under the influence of the EGFR blockade. In the current study, we wished to determine whether other resistance mutations, besides those at KRAS codons 12 and 13, could be identified in liquid biopsies of patients treated with EGFR blockade. We therefore designed a sensitive, multiplex assay that queried known hot-spots of several genes in the EGFR pathway: the regions within and surrounding KRAS codons 12, 13, 59, 60, and 61, NRAS codons 12, 13, 59, 60, and 61; BRAF codons 599 and 600, EGFR codons 712 - 721, 738 - 748, 790 - 800, and 847 - 859 and PIK3CA codons 538 - 549 and 1039 - 1050 (see Methods). The 28 cases assessed included 21 of those previously assessed for KRAS mutations (20) plus seven additional cases of patients who had first responded, then progressed, while being treated with blocking antibodies to EGFR (panitumumab or cetuximab). The primary tumors of 9 of these cases were unavailable, so we used pre-treatment DNA from plasma to assess whether any of the queried mutations were present prior to administration of EGFR antibodies; none of the mutations listed in Fig. 6 were found prior to antibody treatment.

We identified emergent circulating mutations of at least one MAPK pathway gene in 27 of the 28 patients (96%). The number of different mutations identified in the circulation of individual patients averaged 2.8 (range 0 to 12). The development of different mutations in the same patient is not surprising given that each of these patients had multiple lesions; each lesion that responds to EGFR blockade and then progresses is expected to harbor at least one resistance mutation (20, 28).

In total, we observed 76 somatic mutations that were not present in the tumor or in the plasma prior to EGFR blockade and only appeared after therapy was initiated (table S6; Fig. 6). Half of the mutations (38 of 76) occurred in *KRAS* codons 12 or 13. These mutations are known to cause resistance to EGFR blockade when present in the primary tumor, and have been observed to arise after EGFR blockade *in vitro* as well as *in vivo* (20, 28). Two mutations in *BRAF* were observed, each in a different patient. Though we surveyed codons 599 to 600 of *BRAF*, only V600E mutations were observed, supporting the specificity of our sequencing approach. Previous studies have shown that *BRAF* V600E mutations, when present in primary tumors, are associated with failure to achieve a response to EGFR blockade (29-31). Two other patients developed mutations in the kinase domain of EGFR (codons 714 and 794; table 6; Fig. 6). Mutations at these residues have been previously observed in primary CRC, albeit infrequently, and resistance to EGFR blockade has been shown to result from genetic alterations in the *EGFR* gene (32, 33). We did not identify treatment-related mutations in the known *PIK3CA* gene hot spots (exon 9 and 20).

The most surprising observation in the EGFR blockade component of our study was the large number of mutations of codon 61 in either the *KRAS* or *NRAS* gene (table S6; Fig. 6). Sixteen of the 28 patients (60%) harbored at least one codon 61 mutation, and the 32 mutations in these 16 patients comprised 42% of the total (76) mutations observed. Fifty percent of the codon 61 mutations were in *NRAS* and the remainder were in *KRAS* (table S6; Fig. 6). Codon 61 mutations of *KRAS* and *NRAS* have previously been observed to occur in primary colorectal cancers, but very infrequently compared to the prevalence at which we found them in patients progressing after EGFR blockade(31). *KRAS* codon 61 mutations have been observed to be associated with primary resistance to EGFR blockade when they occur in primary colorectal cancers (30, 31, 34). There are no prior studies indicating that *NRAS* codon 61 mutations are associated with such resistance, but the results in table S6 leave little doubt as to their role.

Discussion

Through the study of 643 patients, we have learned that mutant DNA fragments are found at relatively high concentrations in most patients with metastatic cancer and at lower but detectable concentrations in a

substantial fraction of patients with localized cancers. These results have several translational implications and suggest important avenues of future research.

Monitoring disease in advanced cancer patients. A genetic alteration could be identified in the circulation of all 643 patients evaluated in this study, making ctDNA a widely applicable biomarker for cancer patients. Moreover, >80% of patients with metastatic disease had detectable levels of ctDNA, higher than that reported for most conventional biomarkers(35). Another advantage of ctDNA is its high specificity compared to protein-based markers. Unlike proteins such as CEA or CA19-9, which are expressed in normal cells as well as in neoplastic cells, genetic alterations of a clonal nature are only found in neoplasms. Measurements of ctDNA can provide therapeutic, predictive and prognostic information, even in patients with metastatic disease. As shown in Fig. 5, metastatic colorectal cancer patients with relatively low levels of ctDNA lived significantly longer than patients with higher levels, and there was a striking correlation between ctDNA levels and survival.

Though these advantages of ctDNA render it promising for monitoring patients, there are potential limitations. The specific mutations are defined by evaluation of the primary tumor, adding both time and expense to patient management. This may be less of an obstacle in the future as more cancer patients have their tumors genetically analyzed to guide therapeutic decisions. The genetic alterations obtained used to guide therapies can be used for ctDNA analysis. A more serious issue relates to the utility of monitoring patients with advanced cancers, with ctDNA or with other biomarkers (36, 37). On one hand, patients and their physicians are anxious to know, as soon as possible, whether disease has progressed. Imaging studies are often non-informative or slow to reflect progression. Repeated imaging also subjects patients to radiation, while monitoring ctDNA is non-invasive. On the other hand, it has not yet been shown that monitoring patients with advanced disease with *any* biomarker provides clinical, rather than psychological, benefits. Knowing that progression (or response) has occurred prior to changes in clinical symptoms may not prolong survival or improve their quality of life.

Methodological comparisons. There are two sources of tumor DNA accessible in the blood (CTCs and ctDNA), and two types of genetic alterations that can be most easily assessed in either source (point mutations and translocations). Previous studies that compared ctDNA with CTCs reached mixed conclusions. One group concluded that ctDNA was present less often than CTCs (17); this group used state-of-the-art methods to detect CTCs, but did not use a highly sensitive method to detect ctDNA. The second group concluded that ctDNA was present more often than CTCs (16); this group used a sensitive method for analyzing ctDNA but used a relatively insensitive method for analyzing CTCs. More recently, much higher levels of ctDNA than CTCs were found in 2 of 3 pediatric patients with pediatric neuroblastomas (19).

To investigate this issue further, we assessed both ctDNA and CTCs in the same blood sample from patients with typical solid tumors. We simply separated WBCs from plasma and determined the fraction of cells or cell equivalents, respectively, in which tumor-specific rearrangements could be identified. Because we did not attempt to purify tumor cells away from normal WBCs, technical issues related to the efficiency of CTC purification were eliminated. The comparison between DNA from CTCs and ctDNA cannot easily be performed with point mutations because the background levels of point mutations is too high; even with the sensitive methods used in our study, this background precludes the detection of point mutations at levels less than 1 in 100,000 cells (38, 39). Because there are several million cells per ml of blood, a technology that is more sensitive is required. The detection of rearrangements is well suited for this task, as it has been shown that one mutation can be reliably detected among millions of wild-type template molecules; PCR errors do not generate rearrangements (40).

Using patient-specific rearrangements as a tool, we were able to show that the level of ctDNA was always higher than the level of CTCs. In 13 of the 16 patients assessed, ctDNA levels were relatively high while no CTCs at all could be detected. This does not mean that ctDNA is preferable to CTCs for the detection or monitoring of cancer. Rather, the optimal technology depends on many other factors, including cost and throughput, for which CTC detection has advantages. But this comparison does indicate that the vast majority of ctDNA is not derived directly from CTCs. As the half-life of ctDNA is short (<1.5 hours) (21), in fact shorter than that of CTCs (41), our work suggests that the mutant molecules in the plasma are generally not derived from the circulating tumor cells.

Another comparison of interest concerns translocations and point mutations. Our results show that the levels of translocations and point mutations were similar in the majority of cases. However, in 1 of 22 cases, a point mutation was detected in a plasma sample in which the studied rearrangement was absent. The likely reason for this was that the point mutation was in a driver gene that occurred relatively early in tumorigenesis while the rearrangement was sub-clonal, perhaps not contributing to the development of the tumor. In 5 other cases, rearrangements were present at ten-fold higher levels than the point mutations (table S2). In these cases, the rearrangements were found to be components of somatically amplified genes.

From a practical perspective, these data suggest the following conclusions: Maximal sensitivity for detecting a genetic alteration can be achieved by using a rearrangement present within an amplicon. Many tumors, particularly advanced ones, contain such amplifications, making them relatively easy to detect with low coverage (10x) genome sequencing. As with the comparison between CTCs and ctDNA, however, this greater sensitivity does not mean that rearrangements are preferred over point mutations for clinical use. The

discovery of a rearrangement in a patient's tumor, and the work and time required to develop and test primer pairs that can efficiently detect the rearrangement(s) in the degraded DNA characteristic of plasma, is considerable. In contrast, a panel of assays detecting the most commonly mutated point mutations is currently simpler and less expensive to implement in the clinical setting.

Early detection of localized cancers. Until therapeutic agents with much greater potency and minimal side effects are developed, the current best hope for reducing cancer morbidity and mortality is early detection of neoplastic disease (9). Prior to metastasis, most solid tumors can be cured by extant surgical methods, and even when occult metastasis has occurred, adjuvant therapy or additional surgery can in many cases cure patients. One of the encouraging results of our study is that ctDNA was found in the majority of patients with localized disease, when their chances of a favorable outcome are highest (Fig. 3). Even in patients with Stage I disease, who are nearly always curable by surgery alone, 44% of patients were shown to have detectable levels of ctDNA in their plasma. In Stage III disease, in which many patients with certain forms of cancer are curable, more than two-thirds of patients had detectable ctDNA.

Though early detection strategies based on ctDNA are promising, numerous obstacles must be overcome before they can be applied clinically. The fraction of patients with ctDNA represents the maximum obtainable with the amount of plasma collected in this study. In a screening setting, with the exception of pancreatic ductal adenocarcinomas (where one gene (*KRAS*) is mutated in almost all cases), the mutation of interest would not be known *a priori* and a panel of genes would have to be assessed. Our study of ctDNA in patients resistant to EGFR blockade represents a starting point for such multi-gene panels, demonstrating that mutations present in as few as 1 in 10,000 mutant templates can be detected even when several genes are queried at once in DNA from a single plasma draw.

In addition to these technical challenges, biomedical issues will have to be addressed by any ctDNA-based screening test. False positive findings can be problematic for any screening assay (42). Experience thus far suggests that benign tumors do not generally give rise to ctDNA (43), so the "over-diagnosis" of benign tumors is not likely to pose a major problem. However, other studies suggest that a tumor containing ~50 million malignant (rather than benign) cells releases sufficient DNA for detection in the circulation (20). A cancer of this size is far below that required for definitive imaging at present. How would a patient who had a positive ctDNA test be managed if follow-up imaging tests were negative? A related issue is the fact that the type of mutation does not provide many clues to the tumor type. For example, a patient with a circulating *TP53* mutation, in the absence of other mutations, could have a cancer in any of several organs. Another question concerns the value of detecting early cancers. In pancreatic ductal adenocarcinomas, for example, it might be argued that most

patients with a positive ctDNA test will die from their disease anyway, given the aggressive nature of this form of cancer.

Though these obstacles are formidable, we would argue that the presence of detectable amount of a mutant driver gene is a cause for serious concern given the known causal relationships between such mutations and cancer. Indeed, this point distinguishes mutation-based biomarkers from all other types of biomarkers yet described.

Liquid biopsies. Our studies demonstrate two uses for liquid biopsies. The first - assessing plasma for the presence of specific mutations that can direct patient management - is clinically actionable. We show here that the sensitivity and specificity of the liquid biopsy for testing *KRAS* codon 12 and 13 mutations is 88.2% and 98.5% in patients with mCRC. Though conventional tumor biopsies are preferable, these often cannot be obtained for logistic or medical reasons, as noted in the Introduction. When tumor tissue specimens from metastatic cancer patients are unavailable, liquid biopsies offer an alternative that can be rapidly implemented without the pain, risk, and expense entailed by a biopsy of one of the metastatic lesions. Of note is the fact that ctDNA from neoplasms confined to the CNS (Fig. 2 and table S4) and those with mucinous features (table S4) were not easily detectable. This suggests that physical obstacles such the blood-brain barrier and mucin could prevent ctDNA from entering the circulation.

A second use of liquid biopsies - identifying resistance mutations that occur when patients first respond, then progress, to an agent - is mainly of interest for research purposes at present, though this information could be clinically useful in the future. A good example of this principle is provided by our discovery of remarkably frequent mutations at codon 61 of *NRAS* and of *KRAS*, representing more than 40% of the detected mutations in patients resistant to EGFR blockade. This finding provides unequivocal evidence that these mutations confer resistance to therapy - the probability that recurrent mutations at these positions occurred by chance alone is essentially nil (20). It also strongly supports correlative effects of *KRAS*, *BRAF*, *NRAS*, and *PIK3CA* mutations on the efficacy of EGFR blockade (34). Functional studies suggest that *KRAS* mutations confer primary and secondary resistance to EGFR blockade (29, 31, 44). Our results, the epidemiological correlations of De Roock *et al.* and the mutational and functional studies of Di Nicolantonio *et al.* all suggest that *BRAF* V600E mutations confer resistance (29, 34). Collectively, codon 600 mutations of *BRAF*, codon 61 mutations of *KRAS*, and codons 12 or 61 mutations of *NRAS* occur ~ half as often as mutations in *KRAS* 12 or 13 in primary colorectal cancers (45). These data therefore strongly suggest that patients being considered for treatment with EGFR blocking agents should be tested for these additional mutations. Patients harboring mutations at these positions are unlikely to benefit from these agents and would be better served by other therapeutic approaches.

In summary, we demonstrate that ctDNA can be used as a biomarker for a variety of different solid tumor types and clinical indications. The clinical utility of this biomarker, and the risks and benefits accruing from knowledge of ctDNA levels, can only be addressed through longitudinal studies of ctDNA in appropriate populations of patients. The studies reported here lay the groundwork for such future studies.

Materials and Methods

Samples - All samples were collected after Institutional Review Board (IRB) approval at participating institutions, under full compliance with HIPAA guidelines. Tumors and adjacent normal tissues were either frozen at a minimum of -80 C or formalin-fixed and paraffin- embedded (FFPE) according to standard histopathologic procedures. Tumors were macro-dissected under a dissecting microscope to ensure a neoplastic cellularity of >60%. DNA was purified from the macrodissected frozen tumors using AllPrep (Qiagen, cat #80204) and from macrodissected paraffin-embedded tumors with a Qiagen FFPE Kit (Qiagen cat #56494). Translocations, but not point mutations, were reported previously for three of the CRCs (40, 46). Translocation data, but not all clinical correlates, were previously reported for eight of the nine neuroblastomas recorded in table S1 (47); these cases were included in the current study for comparative purposes only. For white blood cell DNA extraction, cells were pelleted at 1000 g prior to the preparation of plasma. DNA from these cells was purified using AllPrep (Qiagen, cat #80204). Plasma was used for ctDNA measurements in all experiments except in 21 of the 28 cases described in table S6, in which serum was used. DNA from plasma or serum was purified using QIAamp Circulating Nucleic Acid kit (Qiagen cat# 55114). Total plasma DNA levels were measured using quantitative PCR as described in (48).

Tumor Mutational Profiling - A tiered-approach was used to identify somatic mutations in tumors. For pancreatic cancers, genomic regions encompassing *KRAS* codons 12,13, 59, 60 and 61 were amplified and the sequence of the PCR products determined via Digital Ligation (47) or SafeSeqS (38) as it is well known that nearly all pancreatic ductal adenocarcinomas harbor mutations in the *KRAS* gene (49). For colorectal cancers, PCR was used to amplify the *KRAS*, *BRAF*, *TP53*, *SMAD4*, *PIK3CA*, and *APC* genes and the sequence of the PCR products determined, generally using SafeSeqS, as described below. For all other cancers, paired-end libraries were generated and regions encompassing 100 genes commonly mutated in cancers were captured as described previously(47). For tumors that did not contain detectable mutations of these genes, exomic sequencing was performed after capture of the same libraries via SureSelect (Agilent), as previously described (50, 51). In cases in which rearrangements were analyzed, PARE (personalized analysis of rearranged ends), (40, 46), genomic libraries were used directly for whole genome sequencing aiming for a physical coverage of 10x. Whenever

possible, we selected rearrangements within amplified segments of the genome. Such rearrangements would be represented more often in tumor DNA than in DNA from normal cells, theoretically increasing the sensitivity of detection of the altered fragment in plasma. Once putative rearrangements were identified on the basis of sequencing data, PCR primers were designed to amplify PCR products of 100 bp that spanned the rearrangement. The rearrangements were confirmed to be somatic by demonstrating that PCR products were generated from the DNA of the tumor but not from DNA of non-neoplastic cells of the same patient.

Mutation Detection in ctDNA or CTCs - Detection of mutations in plasma was performed in all cases using digital-PCR based technologies. In the early phases of this study, single base substitutions and small insertions or deletions (indels) were assessed either by BEAMing or by Digital Ligation, using methods described previously (21, 47). In the latter stages of this study, mutations were assessed by SafeSeqS, a digital-PCR based approach in which template molecules are individually assessed via massively parallel sequencing (38). In particular, SafeSeqS was used to assess the 206 patients assayed for mutations (Table 2) as well as the 28 patients assayed for resistance mutations following EGFR blockade (Table 4). To control for differences in assay sensitivity, we quantified the level of mutations in 20 plasma samples that had been evaluated by all three digital methods (Digital Ligation(47), BEAMing(52), and Safe-Seq(38) and found that the results differed by less than 2-fold in every case. Additional control experiments were performed to demonstrate that the mutations described in this study were not found at appreciable levels (>0.01% of template molecules) in DNA from non-neoplastic cells. At least one such non-neoplastic DNA control was used for each mutation assessed. For codon 12 or 13 mutations of *KRAS*, DNA from white blood cells (WBCs) or plasma of 50 patients without cancer were employed as non-neoplastic, negative controls.

Rearrangements in ctDNA or CTCs were detected and quantified by digital PCR, as described previously (38, 40) with the following modification. To verify that the PCR fragments of the expected size contained the intended rearrangement, ligation reactions were performed on each PCR fragment. The two oligonucleotides used in the ligation reaction spanned the breakpoint so that ligation only occurred if the PCR products assessed contained the rearrangement (**Fig. S1**). Control experiments with DNA from non-neoplastic cells of the same patients showed that each rearrangement reported in this study was not found in the germ-line of the patient.

Statistical Analyses - Descriptive statistics of clinical characteristics, tissue mutations, and plasma ctDNA values of patients with localized and advanced malignancies were reported. The proportions of patients with detectable ctDNA were compared across stage of disease with the Cochran–Armitage test for trend. For the liquid biopsy cohort of metastatic colorectal cancer patients, the sensitivity and specificity along with 95% confidence intervals, of detecting plasma *KRAS* mutation compared to the detection of a tissue *KRAS* mutation

was reported. We also reported the percent concordance and kappa statistic for the agreement between liquid biopsy and tissue samples. Clinical characteristics were compared between the false positive/negative and true positive/negative, and the false negative and true negative groups with Fisher's exact test for categorical variables and Wilcoxon Mann Whitney tests for continuous variable. In those cases with detectable levels of ctDNA, the association of clinical characteristics with the absolute ctDNA levels was examined using univariate and multivariate linear regression models. Logarithm transformations were made for some continuous predictor variables to correct for skewness. Those variables which were statistically significant in the univariate analysis and which were deemed clinically relevant were added to a multivariate linear model. The final model was chosen using backward selection stepwise regression based on p-values <0.10. Statistical analyses were performed using the R statistical package (version 2.15.1).

Tables

Table 1. Clinical Characteristics of 409 subjects enrolled in this study

Table 2. Comparison of ctDNA with CTCs.

Table S1. Summary of tissue mutations, plasma mutations and clinical information for 409 early and advanced stage subjects

Table S2. Comparison of ctDNA levels in the same specimen assessing point mutation and rearrangements

Table S3. Correlation between plasma and tumor tissue KRAS status in patients with metastatic colorectal cancer (AKA "Liquid Biopsy")

Table S4. Correlation between clinical characteristics and discordant tissue and ctDNA levels in a cohort of metastatic colorectal cancer patients

Table S5. Association between clinical characteristics and absolute levels of ctDNA

Table S6. Patient Characteristics and plasma mutations detected post-EGFR blockade

Fig. Legends

Fig. 1. Depiction of circulating tumor DNA

Fig. 2. Circulating tumor DNA in advanced malignancies: (A) fraction of patients with detectable ctDNA and (B) quantification of mutant fragments. Only tumor types in which we studied more than three cases were included; data on other tumor types is included in table S2. excluded available in supplemental table 1). Stage III ovarian and hepatocellular carcinomas were included because stage IV cases were rare and Stage III disease is more representative of advanced disease in these two tumor types. We also included primary brain tumors in this figure because they rarely metastasize. Error bars represent standard error (SEM) of the mean.

Fig. 3. Circulating tumor DNA in localized malignancies: (A) fraction of patients with detectable ctDNA in localized (stages I-III) and advanced stage(stage IV) colorectal, gastroesophageal, breast and pancreatic cancers and (B) fraction of patients with detectable ctDNA and (C) quantification of mutant fragments in cancer cases categorized by stage. Error bars represent standard error (SEM) of the mean.

Fig. 4. Scatter plot correlating point mutations vs. rearrangements in the same plasma specimens. Additional information is presented in table S2.

Fig. 5. Kaplan-Meier Plot of survival in patients with metastatic colorectal cancers, from date of ctDNA measurement to death. Cohort is segregated into quartiles based on the concentration of mutant fragments in plasma. Shorter survival with increased levels of ctDNA was observed ($p=0.0002$, Logrank test).

Fig. 6. Heat map of acquired resistance mutations to EGFR blockade in ctDNA from patients with metastatic colorectal cancer; additional information available in table S6.

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Applications of Liquid Biopsy

Early Detection and Monitoring

Brain tumor DNA blocked by blood-brain barrier

Breast cancer

Pancreatic cancer

Colon cancer

Many tumors release DNA fragments that circulate in the bloodstream

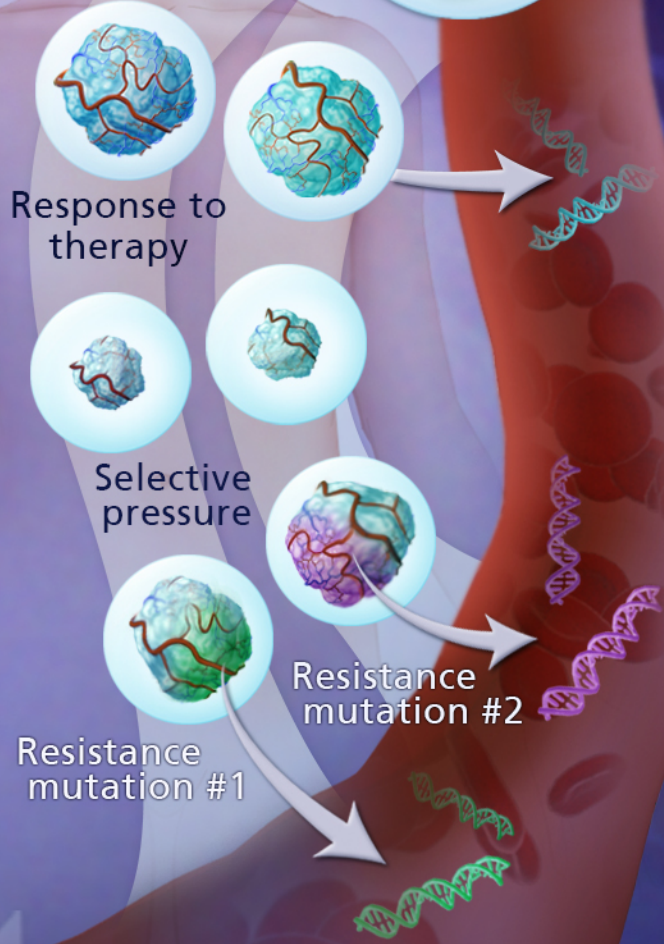
Analysis of ctDNA



Detection of Resistance Mutations



Targeted therapy



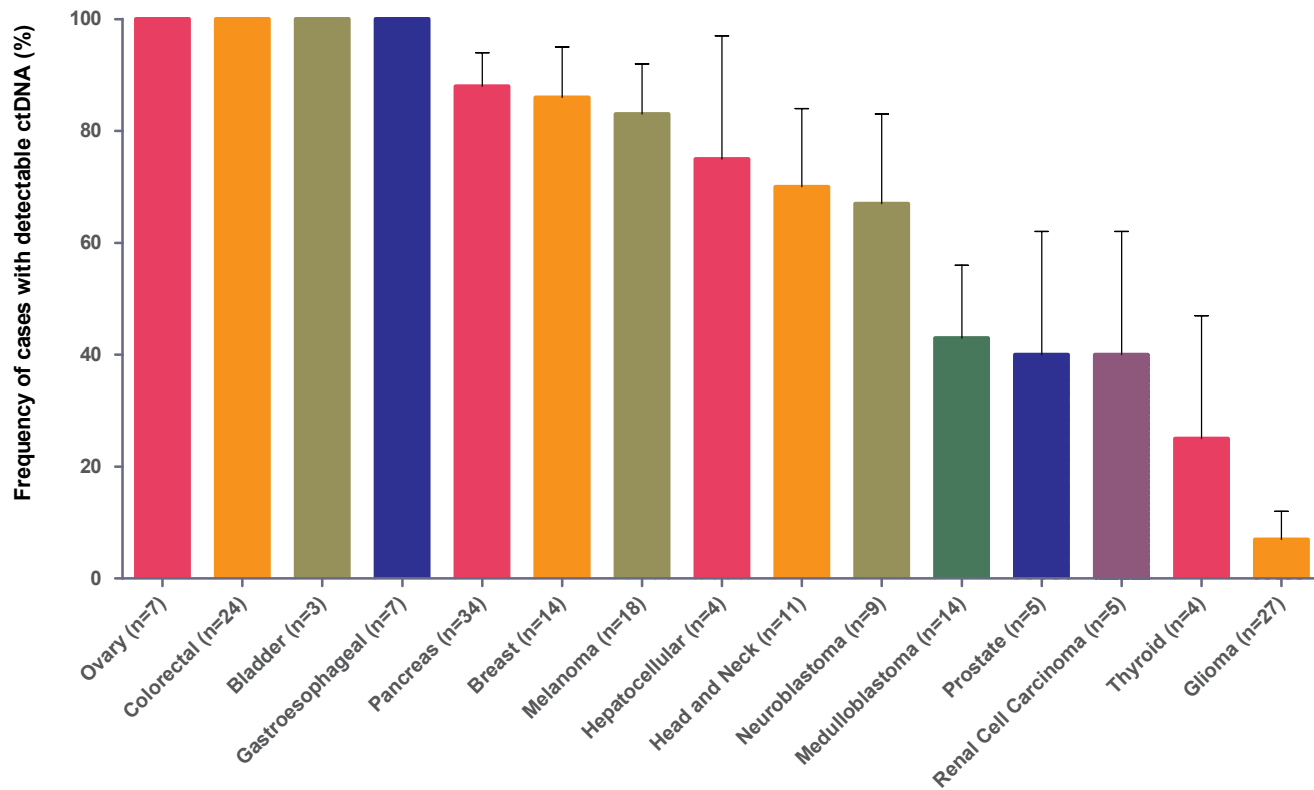
Resistance mutation #1

Resistance mutation #2

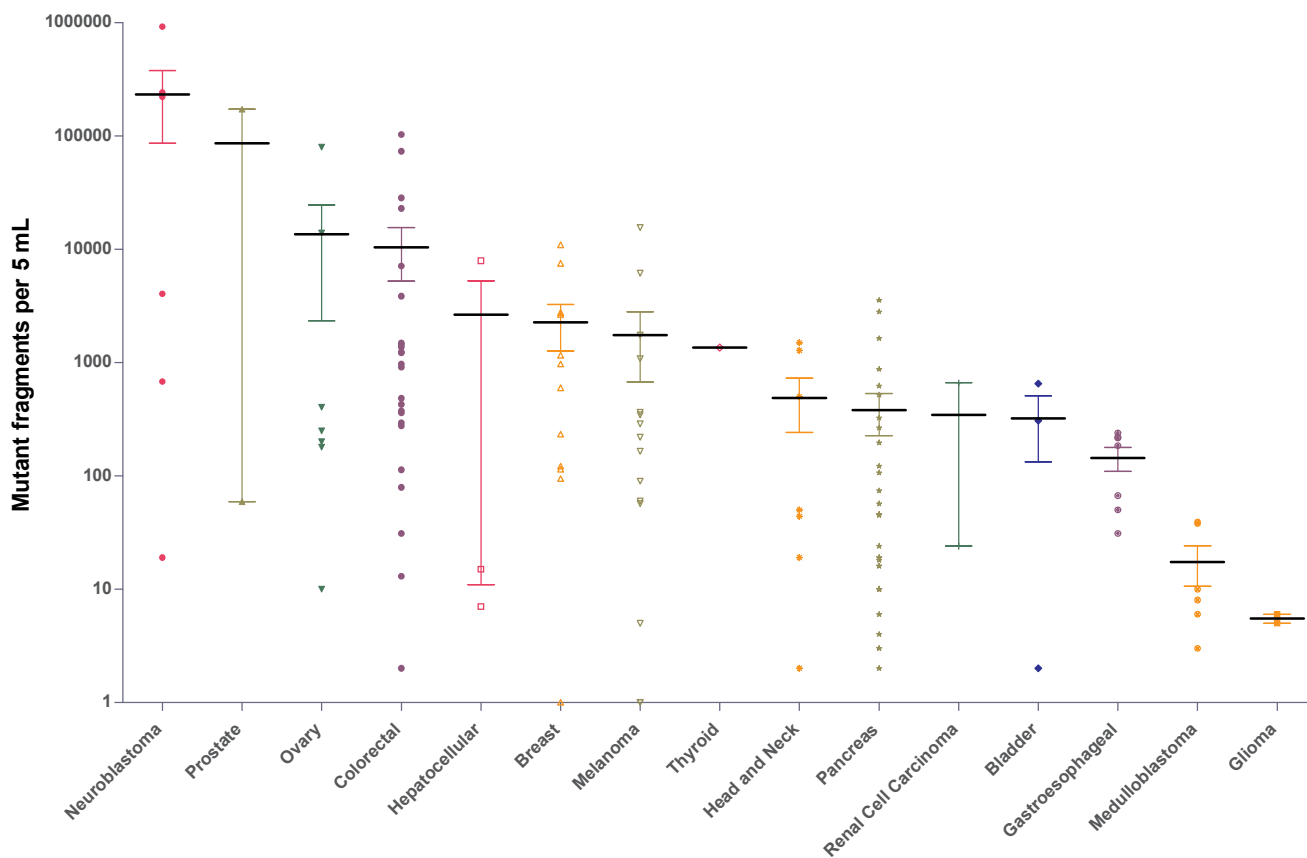
ctDNA of resistance mutations collected in blood sample

Figure 2

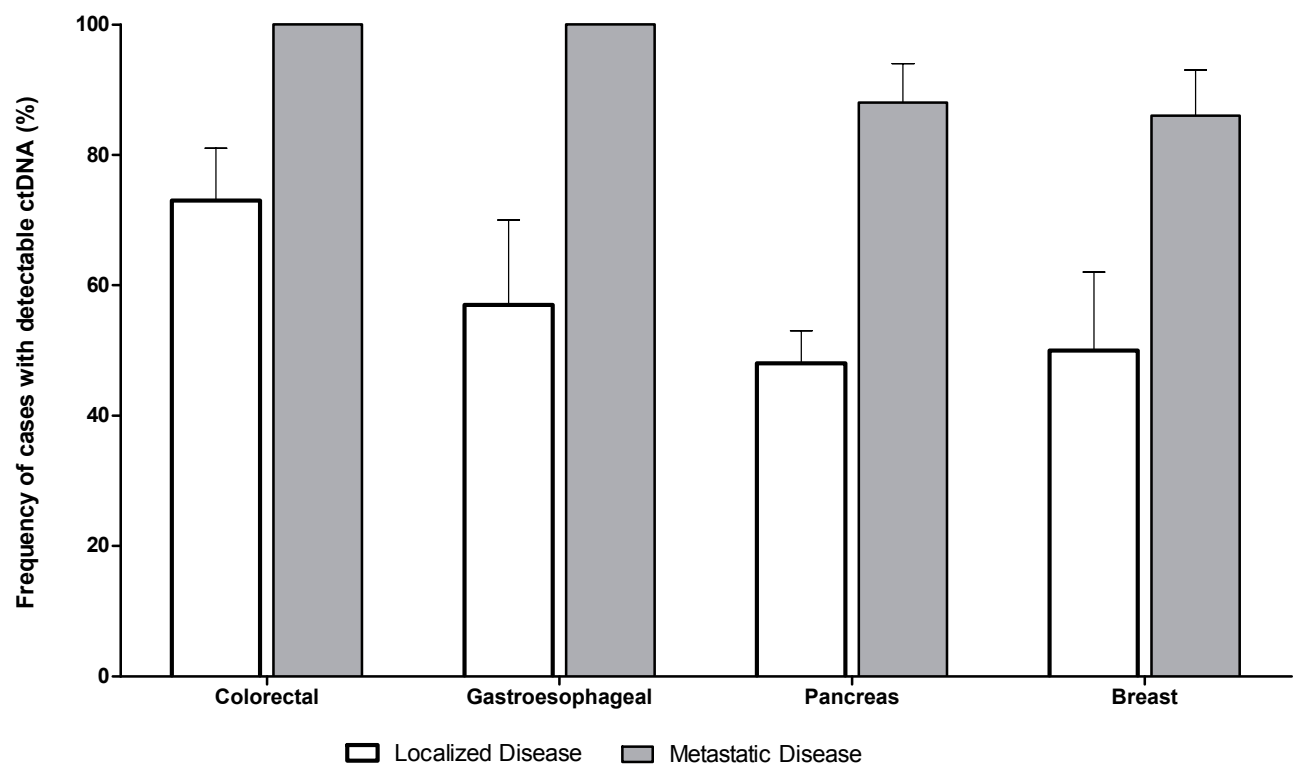
A



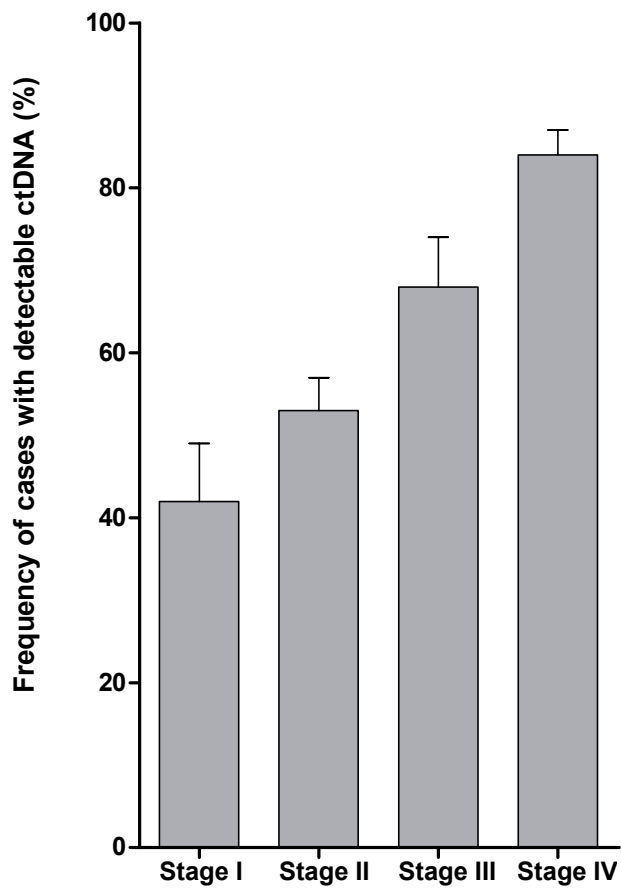
B



A



B



C

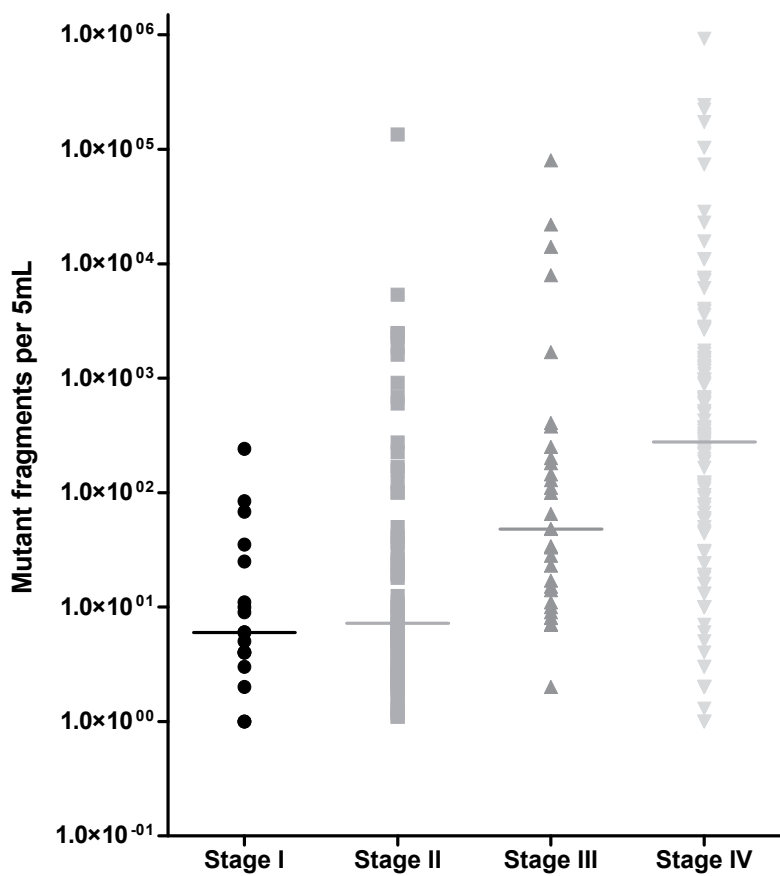


Figure 4

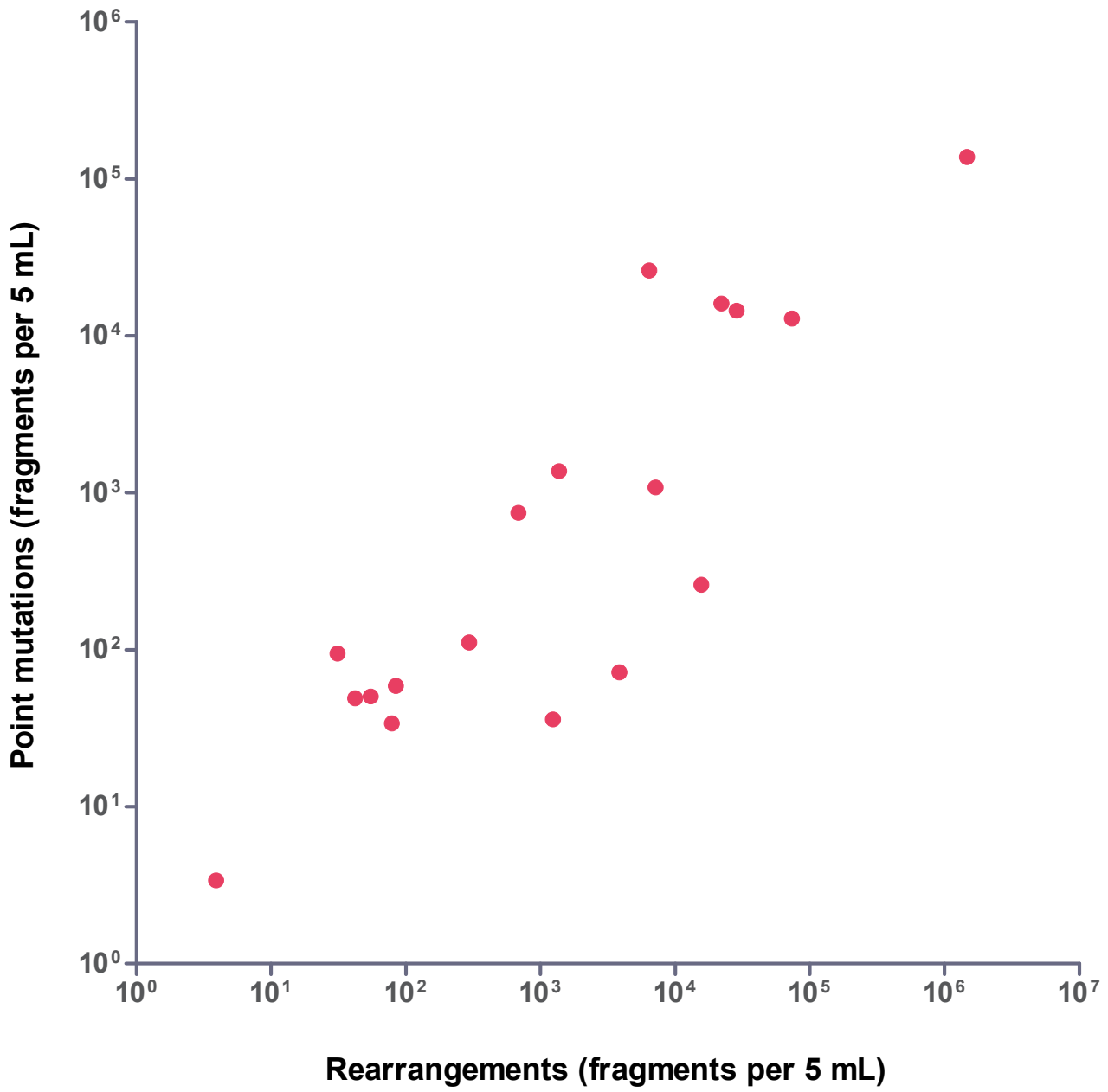


Figure 3

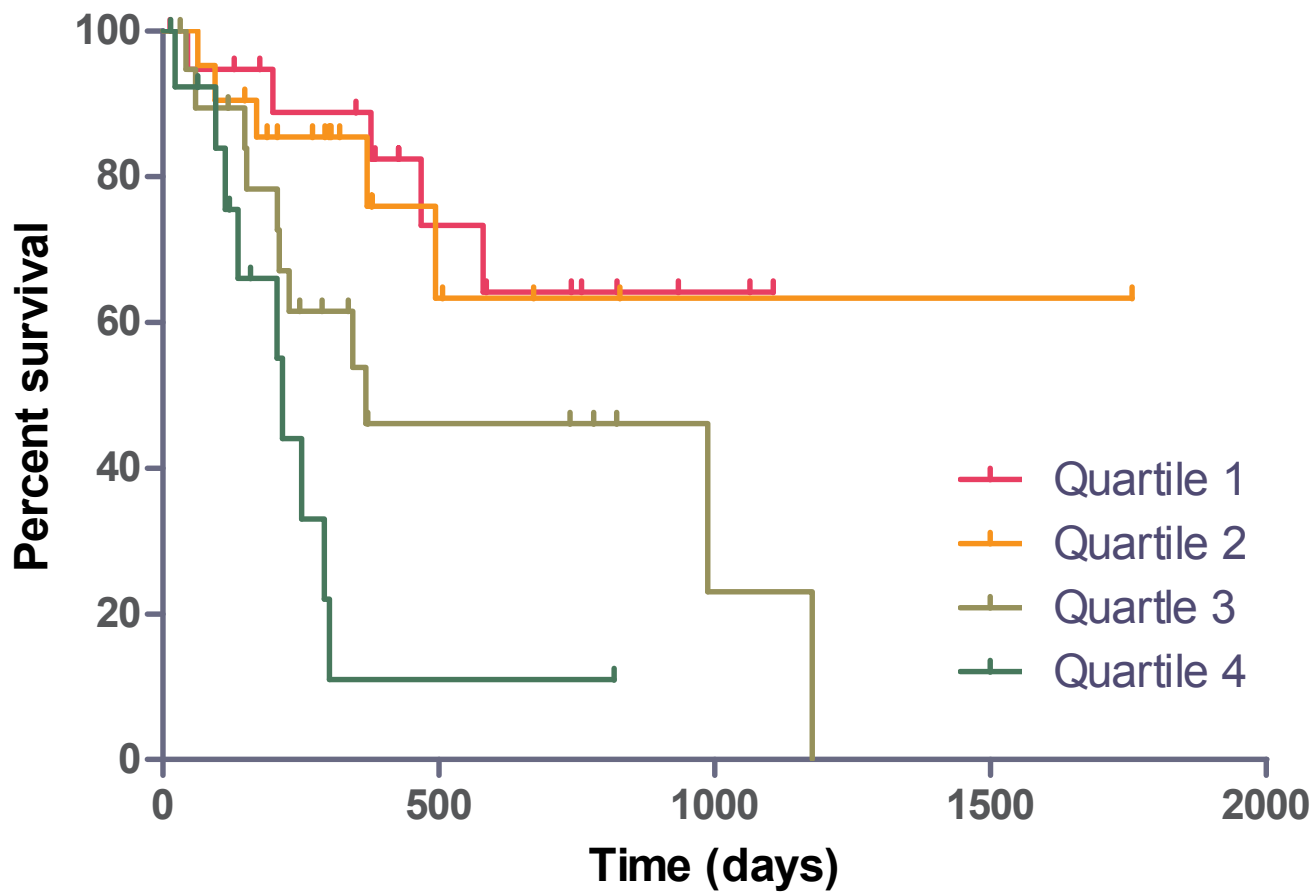




Figure 6

Sample ID	Pre-Treatment									Post-Treatment								
	KRAS 12	KRAS 13	KRAS 61	NRAS 12	NRAS 61	BRAF 600	EGFR 794	EGFR 714	KRAS 12	KRAS 13	KRAS 61	NRAS 12	NRAS 61	BRAF 600	EGFR 714	EGFR 794		
AMG 011									Single mutation									
AMG 022									Single mutation									
AMG 028								Multiple mutations										
AMG 034										Single mutation						Single mutation		
AMG 040																		
AMG 046									Single mutation			Single mutation						
AMG 105													Single mutation					
AMG 109									Single mutation			Multiple mutations						
AMG 114								Multiple mutations							Single mutation			
AMG 121												Single mutation	Single mutation					
AMG 126									Single mutation			Single mutation						
AMG 132									Single mutation									
AMG 140									Single mutation									
AMG 148										Single mutation								
AMG 155								Multiple mutations			Multiple mutations		Multiple mutations					
AMG 161									Single mutation									
AMG 167									Single mutation		Single mutation							
AMG 180									Multiple mutations				Multiple mutations					
AMG 188									Single mutation				Single mutation					
AMG 195									Single mutation		Multiple mutations							
AMG 208									Multiple mutations				Single mutation					
BARD 101 PLS									Single mutation		Single mutation		Single mutation					
BARD 102 PLS									Multiple mutations		Single mutation							
BARD 103 PLS									Multiple mutations									
CRC 188 PLS									Single mutation									
CRC 189 PLS											Multiple mutations							
CRC 190 PLS									Single mutation		Multiple mutations		Multiple mutations	Single mutation				
CRC 191 PLS									Multiple mutations		Single mutation							
Total Number of Cases	0	0	0	0	0	0	0	0	35	3	16	2	16	2	1	1		

 Single mutation
 Multiple mutations