Liquid biopsy: monitoring cancer-genetics in the blood

Emily Crowley, Federica Di Nicolantonio, Fotios Loupakis and Alberto Bardelli

Abstract | Cancer is associated with mutated genes, and analysis of tumour-linked genetic alterations is increasingly used for diagnostic, prognostic and treatment purposes. The genetic profile of solid tumours is currently obtained from surgical or biopsy specimens; however, the latter procedure cannot always be performed routinely owing to its invasive nature. Information acquired from a single biopsy provides a spatially and temporally limited snap-shot of a tumour and might fail to reflect its heterogeneity. Tumour cells release circulating free DNA (cfDNA) into the blood, but the majority of circulating DNA is often not of cancerous origin, and detection of cancer-associated alleles in the blood has long been impossible to achieve. Technological advances have overcome these restrictions, making it possible to identify both genetic and epigenetic aberrations. A liquid biopsy, or blood sample, can provide the genetic landscape of all cancerous lesions (primary and metastases) as well as offering the opportunity to systematically track genomic evolution. This Review will explore how tumour-associated mutations detectable in the blood can be used in the clinic after diagnosis, including the assessment of prognosis, early detection of disease recurrence, and as surrogates for traditional biopsies with the purpose of predicting response to treatments and the development of acquired resistance.

Crowley, E. *et al. Nat. Rev. Clin. Oncol.* advance online publication 9 July 2013; doi:10.1038/nrclinonc.2013.110

Introduction

Biopsies have been used by clinicians to diagnose and manage disease for 1,000 years.[1] In patients with cancer, biopsies allow the histological definition of the disease and, more recently, have revealed details of the genetic profile of the tumour enabling prediction of disease progression and response to therapies. As the techniques that have enabled us to analyse a biopsy become ever more sophisticated, we have realised the limitations of looking at this single snap-shot of the tumour. This single-biopsy bias was highlighted in a study by Gerlinger *et al.*[2] in which it was demonstrated that a portion taken from different parts of a primary tumour and its metastases showed extensive intertumoural and intratumoural evolution. This tumoural heterogeneity highlights the difficulty of dictating a therapeutic course of action based on a single biopsy, as it is likely to underestimate the complexity of the genomic landscape of the tumour.

Having established that there is considerable tumour heterogeneity, taking multiple biopsies from the patients' primary tumour and metastases would seem to be the most obvious next step, so why is it not routinely done? There are many difficulties in obtaining a tissue biopsy—including the discomfort suffered by the patient, inherent clinical risks to the patient, potential surgical complications and economic considerations—meaning that multiple or serial biopsies are often impractical. In addition, some tumours are not accessible for biopsy, the procedure itself might increase the risk of the cancer 'seeding' to other sites,[3] and the procedure might not be recommended for patients receiving antiangiogenic treatment.[4] Even in an ideal situation where several metastatic sites can be biopsied simultaneously, the analysis of the samples can delay the initiation of treatment, and might irremediably jeopardise it. These limitations are particular restraints in the setting of acquired resistance to therapy, as the ability of a clinician to detect therapeutic biomarkers at an early stage would allow a potentially successful change in treatment course.

In light of these limitations on the use of single biopsies, new ways to observe tumour genetics and tumour dynamics have evolved. In 1948, the publication of a manuscript that described circulating free DNA (cfDNA) and RNA in the blood of humans was, without knowing it, the first step towards the 'liquid biopsy'.[5] Furthermore, the levels of cfDNA were higher in diseased than healthy individuals, indicating that it is possible to screen for the presence of disease through a simple blood test.[6] Indeed, the specific detection of tumour-derived cfDNA has been shown to correlate with tumour burden, to a change in response to treatment or surgery, and to indicate that subpopulations of tumour cells that are resistant to treatment can proliferate in response to therapy.[7,8] With the development of sensitive techniques that enable the detection of rare mutations, it is now possible to understand the heterogeneous landscape of the tumour using a blood sample.[9] Nevertheless, key challenges lie ahead and not all results consistently support the application of circulating tumour DNA (ctDNA) in the clinic.

Competing interests
The authors declare no competing interests.

Department of Oncology, University of Turin, Institute for Cancer Research and Treatment, Strada Provinciale 142 Km 3.95, 10060 Candiolo, Turin, Italy (**E. Crowley**, **F. Di Nicolantonio**, **A. Bardelli**). Unit of Medical Oncology 2, Azienda Ospedaliero-Universitaria Pisana, Via Roma 67, 56126 Pisa, Italy (**F. Loupakis**).

Correspondence to:
A. Bardelli
alberto.bardelli@unito.it

© 2013 Macmillan Publishers Limited. All rights reserved

Key points

- Under representation of the heterogeneity of a tumour and poor sample availability means tissue biopsies are of limited value for the assessment of tumour dynamics in the advanced stages of disease
- Extended periods between sampling and clinical application of the results, as well as additional lines of treatment between sampling, might result in an altered genetic composition of the tumour
- Circulating free DNA can be extracted from the blood and tumour-specific aberrations assessed to provide a genetic landscape of the cancerous lesions in a patient
- Tracking tumour-associated genetic aberrations in the blood can be used to assess the presence of residual disease, recurrence, relapse and resistance
- Monitoring the emergence of tumour-associated genetic aberrations in the blood can be used to detect the emergence of resistant cancer cells 5–10 months before conventional methods
- To implement circulating tumour DNA testing in the clinic, standardization of techniques, assessment of reproducibility and cost-effectiveness is required as well as prospective validation in clinical trials

The early diagnosis of cancer is one application for cfDNA assessment and has been covered extensively in previous reviews.[10,11] This Review will explore how tumour-associated genetic alterations detectable in the blood can be used in the clinic, including the assessment of prognosis, early detection of disease recurrence, and as surrogates for traditional biopsies with the purpose of predicting response to treatments or the development of acquired resistance. It will also cover technical and logistical challenges that might represent hurdles for clinical implementation.

Discovery and detection of ctDNA

There are at least two potential, but not mutually exclusive, mechanisms by which DNA can enter into the circulation. These mechanisms can be broadly categorized as passive and active. The passive mechanism suggests that apoptotic and necrotic cells release nuclear and mitochondrial DNA into the circulation in the process of cellular destruction.[12] Under normal physiological conditions, infiltrating phagocytes clear apoptotic and necrotic debris and, therefore, levels of cfDNA in healthy individuals are low.[13] However, certain conditions, such as within a tumoural mass or in the instance of exhaustive exercise or inflammation, clearance does not happen efficiently,[13] leading to an accumulation of cellular debris, including DNA, that is then released into the blood (Figure 1).[14,15] Alternatively, active DNA release has been reported in studies with cultured cell lines of different origins and is believed to involve the spontaneous release of DNA fragments into the circulation.[16–19] Multiple hypotheses have been formulated to explain why living cancer cells would actively release DNA into the circulation, including the possibility that cancer cells release oncogenic DNA to affect the transformation of susceptible cells at distant sites.[20,21] Several reviews are available that detail the biology and mechanisms of DNA release into circulation.[10,12,22]

ctDNA might also be released by circulating tumour cells (CTCs) present in the blood.[23] Definitive evidence for this mechanism has not been reported, and there is a discrepancy between the number of CTCs and the quantity of cfDNA in the blood. A single human cell contains 6 pg of DNA[24] and there is an average of 17 ng of DNA per ml of plasma in advanced-stage cancers;[25] therefore, if CTCs were the primary source of ctDNA it would require over 2,000 cells per ml of plasma. In reality, there are, on average, less than 10 CTCs per 7.5 ml blood.[26]

Presently, the most commonly used protocols to obtain cfDNA require approximately 1 ml of serum or plasma (3 ml of blood) and preparation should not exceed 4–5 h following the blood draw. For plasma preparation, blood must be collected in a tube treated with an anticoagulant, preferably EDTA (ethylenediaminetetraacetic acid). Cells are then removed by centrifugation and the supernatant, or plasma, is removed.[27] Serum is collected after the blood is allowed to clot and following centrifugation the supernatant, or serum, is removed.[28] Circulating DNA is then extracted from the plasma or serum using commercially available kits.

The analysis of ctDNA from the blood offers an exciting clinical application in patients, that is, the detection of tumour-specific genetic aberrations (Figure 1). This approach has greater dynamic range compared to total circulating DNA,[29] is more specific, and has many potential applications in the clinic. However, it is also technically more challenging owing to the presence of high levels of DNA originating from tissue of non-tumour origin. A proportion of cfDNA is derived directly from the tumour (ctDNA) and this fraction can be quantified.[30–35] Furthermore, an *in vivo* study has shown a direct correlation between tumour burden and the quantity of ctDNA released.[33]

High-analytical sensitivity and specialized equipment are required for detection of ctDNA because the quantity and quality of tumour-derived DNA can vary dramatically. Techniques are available that allow reliable monitoring of tumour-associated genetic aberrations, including somatic mutations, loss of heterozygosity and chromosomal aberrations in the blood (Table 1), at frequencies as low as 0.01%.[8,9,36–38] The fraction of circulating DNA that is derived from the tumour can range between 0.01% and 93%.[36,39] Tumour-specific genetic aberrations in cfDNA might offer intriguing insights into tumour dynamics. Nevertheless, there are clinical situations, including early stage disease, where it is unlikely to have broad applications as well as clinical settings where cfDNA levels are below optimal levels for the detection of mutations.[25,40,41] According to a study by Perkins *et al.*,[25] the optimal DNA concentration for the detection of mutations (using a Sequenom® MassARRAY® platform) is approximately 30 ng/ml in the plasma. As certain cancers, including melanoma, have a low average total cfDNA level (7 ng/ml in the plasma) the accuracy of detection could be suboptimal. However, with improving technique sensitivities, the ability to detect genetic aberrations at lower frequencies will undoubtedly improve, as exemplified by the improvement in *PIK3CA* mutation detection in studies by Board *et al.*[40] and Higgins *et al.*,[42] which showed a concordance between primary tumour tissue and plasma DNA of 95% and 100%, respectively.

© 2013 Macmillan Publishers Limited. All rights reserved

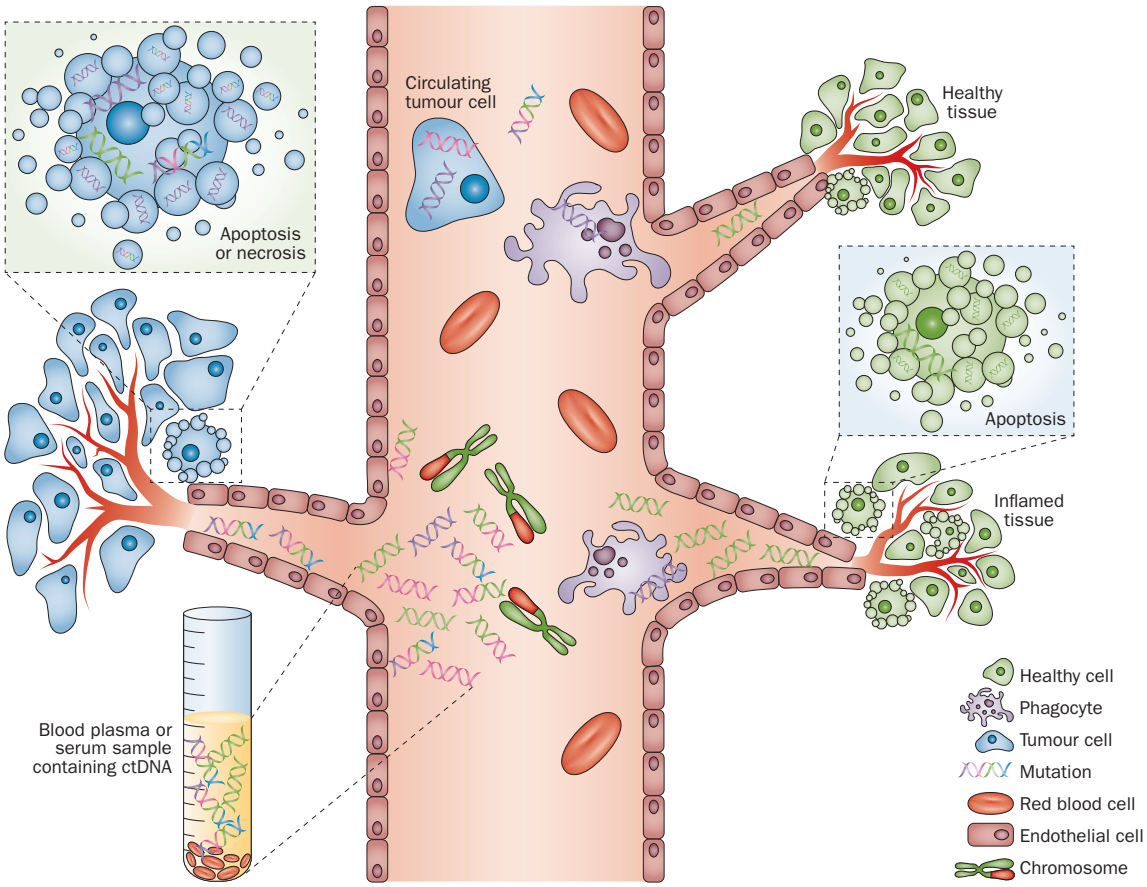


Figure 1 | Release and extraction of cfDNA from the blood. cfDNA is released from healthy, inflamed or diseased (cancerous) tissue from cells undergoing apoptosis or necrosis. cfDNA can be extracted from a blood sample and genetic aberrations in the DNA released from cancerous tissue detected and quantified. Tumour-derived genetic alterations that can be detected in the blood include point mutations (consecutive purple, red, green and blue DNA strands), copy number fluctuations (red portion of chromosomes) and structural rearrangements (green and red DNA strands). Abbreviations: cfDNA, circulating free DNA; ctDNA, circulating tumour DNA.

Liquid biopsies in the clinic

In the past few years there has been considerable focus on the need for 'biomarkers'. These biomarkers should be surrogate indicators for a future event, such as disease recurrence, disease progression, or death, and should indicate if a specific treatment will reduce that risk.[43] Despite extensive investigations, there are no currently approved applications for liquid biopsies in the clinical setting. Nevertheless, recently published data demonstrate that ongoing research holds considerable promise for the future of molecular testing of ctDNA—one of the most promising future tools for clinical application.

Assessment of prognosis

Prognosis

Assessing prognosis for an individual patient involves a combination of clinical observations, staging, and histopathological and biomolecular characterization of different tumour types. This information, derived from imaging and biopsy specimens, allows a clinician to appraise the tumour biology, precisely stage the patient's tumour and differentiate between those patients with more-aggressive or less-aggressive disease. In this context, liquid biopsies are unlikely to supersede current methods, but their use might be important in circumstances where a biopsy is not available or genetic analysis of archived tumour samples is not possible.[44,45]

Assessment of disease stage is one of the most-reliable predictors of prognosis and the relationship between levels of tumour-specific genetic aberrations and stage requires thorough evaluation for all cancer types. Studies have revealed a statistically significant correlation between disease stage and the presence of tumour-associated genetic aberrations (including mutations in *TP53*, *KRAS*, and *APC* and allelic imbalances) in the blood of patients with resectable breast, ovarian, pancreatic and colorectal cancer and oral squamous-cell carcinoma.[27,28,46–53]

Furthermore, following mastectomy as treatment for breast cancer, it was reported that patients with tumours with vascular invasion, more than three lymph-node metastases, and high histological grade at diagnosis had persistent tumour-associated microsatellite DNA alterations as detected by PCR post-surgery in plasma-extracted DNA.[46] Moreover, the presence of tumour-associated genetic aberrations in the blood, including *TP53* mutations[47] and loss of heterozygosity,[48] correlated with overall survival or disease-free survival as assessed

© 2013 Macmillan Publishers Limited. All rights reserved

Table 1 | Tumour-associated genetic aberrations in circulating free DNA

Tumour type	Stage	*n*	Tumour-specific aberration	Tumour burden or stage*	Source	Technique	Reference‡
Colorectal cancer	Early to advanced	33	*APC*	No/Yes	Plasma	BEAMing	Diehl *et al.* (2005)[36]
	Advanced	18	*APC, KRAS, PIK3CA, TP53*	Yes	Plasma	BEAMing	Diehl *et al.* (2008)[27]
	Early to advanced	104	*APC, KRAS, TP53*	NA	Serum	PCR-SSCP	Wang *et al.* (2004)[28]
	Early to advanced	70	*KRAS*	NA	Plasma	ME-PCR	Frattini *et al.* (2008)[34]
Breast cancer	Early to advanced	72	*PIK3CA*	Yes	Plasma and serum	ARMS-Scorpion PCR	Board *et al.* (2010)[40]
	Early to advanced	34 (retrospective) and 51 (prospective)	*PIK3CA*	NA	Plasma	BEAMing	Higgins *et al.* (2012)[42]
	Advanced	30	*PIK3CA, TP53,* structural variation	Yes	Plasma	TAm-Seq and digital PCR	Dawson *et al.* (2013)[29]
Ovarian cancer	Advanced	38	*TP53, PTEN, EGFR, BRAF, KRAS,*	Yes	Plasma	TAm-Seq Digital PCR	Forshew *et al.* (2012)[38]
	Early to advanced	63	*PIK3CA*	Yes	Serum	Fluorescent-PCR	Kuhlmann *et al.* (2012)[52]
Hepatocellular carcinoma	Early	4	SNV	Yes	Plasma	WGS	Chan *et al.* (2013)[9]
Pancreatic cancer	Early to advanced	21	*KRAS*	Yes	Plasma	MASA PCR	Yamada *et al.* (1998)[54]
	Early to advanced	44	*KRAS*	No/Yes	Plasma	RFLP-PCR	Castells *et al.* (1999)[53]
Oral squamous-cell carcinoma	Early to advanced	64	Microsatellite loci	Yes	Serum	PCR	Hamana *et al.* (2005)[50]
	Early to advanced	20	Microsatellite loci	No	Serum	PCR	Kakimoto *et al.* (2008)[157]
Non-small-cell lung cancer	Advanced	246	*KRAS*	Yes	Plasma	ARMS-qPCR	Nygaard *et al.* (2013)[59]
Breast and osteosarcoma	Advanced	3	Genomic alterations	Yes	Plasma and serum	Nested-real time PCR	McBride *et al.* (2010)[154]
Colorectal and breast cancer	Advanced	10	Chromosomal alterations	Yes	Plasma	WGS	Leary *et al.* (2012)[37]

*This column indicates if the study observed a correlation between tumour-associated genetic aberrations and tumour burden or disease stage. ‡The table includes studies in which different tumour-associated genetic aberrations have been detected using a variety of techniques, with different cancer types and at different stages. Abbreviations: ARMS, amplification refractory mutation system; BEAMing, beads, emulsion, amplification, magnetics; MASA, mutant allele specific amplification; ME-PCR, mutant enriched PCR; NA, not applicable; PCR-SSCP, single-strand conformation polymorphism PCR; qPCR, quantitative PCR; RFLP-PCR, restriction fragment length polymorphism PCR; SNV, single nucleotide variants; WGS, whole-genome sequencing.

by multivariate analysis. There are, however, conflicting studies in which a correlation between stage and levels of tumour-associated genetic aberrations (including *KRAS*) are not observed.[34,54] Controversy associated with such studies is often due to limited sample sizes as well as some of the studies not being designed to specifically address this issue,[34] in addition to the obvious technical differences that will be discussed later in this Review.

What are the possible applications of liquid biopsies in the clinic for the assessment of patient prognosis? In the context of resectable tumours, the utility of liquid biopsies is likely to be limited. In this setting, a sample of the tumour itself is usually available and aside from conventional histological, immunohistochemical and molecular analysis, the development of gene-expression classifier assays provide additional prognostic information.[55,56] In the context of breast and colorectal cancer, there are FDA-approved assays that can discriminate, in specific settings, between high-risk and low-risk groups.[55–58] Equivalent panels of genetic aberrations that can be tested using cfDNA samples to accurately stratify patients are not currently available. Therefore, liquid biopsies in the resectable setting are currently unlikely to have significant clinical utility for assessing prognosis.

What could be the utility of liquid biopsies for advanced-stage, unresectable diseases? In a multivariate analysis, *KRAS* mutations present in the plasma of 246 patients with advanced-stage non-small-cell lung cancer (NSCLC) was shown to predict poor prognosis in patients receiving first-line chemotherapy.[59] *KRAS* mutations in circulation have also been assessed in a cohort of 44 patients with pancreatic cancer to determine patient prognosis and, despite the poor sensitivity of the test (27%), it was shown that patients with *KRAS* mutations had a significantly reduced probability of survival compared to those patients without *KRAS*

© 2013 Macmillan Publishers Limited. All rights reserved

mutations (17% versus 41% at 6 months, and 0% versus 24% at 12 months in *KRAS* mutant and wild type, respectively).[53] However, a parallel study, conducted in 308 patients with advanced-stage NSCLC, showed no correlation between *KRAS* mutations in the plasma and prognosis.[60] *BRAF* mutations as assessed in serum samples have also been shown to effectively stratify 103 patients with melanoma in both early stage and advanced-stage settings.[61] Nevertheless, results remain contradictory in these small patient populations.

Neuroblastoma is a special case where amplification of the gene MYC-related oncogene (*MYCN*) has been identified as a genetic hallmark of aggressive disease, and this abnormality provides important information for patient treatment.[62,63] Patient stratification according to *MYCN* status is important because improved treatment approaches, such as immunotherapy,[64] were shown to significantly increase event-free and overall survival compared to standard treatments and might improve currently unsatisfactory long-term survival rates in patients with high risk *MYCN* amplified neuroblastomas.[65]

It is estimated that for 29% of all patients with neuroblastoma,[65,66] *MYCN* status is unknown despite guidelines recommending genetic testing for patient stratification and treatment tailoring.[67,68] Liquid biopsies are able to accurately detect *MYCN* amplification in the serum of stage III to IV patients with neuroblastoma with a sensitivity and specificity of 75–85% and 100%, respectively.[69–71]

A different approach involves the quantification of the level of multiple tumour-associated genetic mutations present in the blood—this approach might be more sensitive than the assessment of total cfDNA or measuring single tumour-specific aberrations for the stratification of patients with cancer according to their probability of survival.[29] For example, the number of copies of DNA harbouring specific somatic mutations when detected in the plasma were calculated in a cohort of 30 women with metastatic breast cancer, and an inverse relationship (P <0.001) was identified between plasma copy number and survival.[29] Whether this method can be used to accurately stratify patients of all cancer types[25] and at different disease stages[40,72] remains to be validated.

Detection of recurrence

A promising clinical application for liquid biopsies is the early detection of relapse after potentially curative treatments (Figure 2a). After treatment with curative intent, patients are monitored for signs of residual disease and local or distant recurrences using radiological imaging during post-treatment follow-up.[73–75] These methods are costly, often requiring contrast media (exposing patients to doses of radiation), and thus cannot be used for frequent monitoring. Another important consideration is that these imaging techniques have limited sensitivity for the detection of micrometastases.[76,77]

In a landmark paper, Diehl *et al.*[27] showed that by monitoring tumour-specific aberrations (including *APC*, *TP53* and *KRAS*) in the plasma of patients with colorectal cancer post-surgery it was possible to identify disease recurrence with almost 100% sensitivity and specificity. Patients with residual disease were also identified based on the persistence of tumour-associated genetic aberrations in cfDNA immediately after surgery in each case of incomplete resection. Other reports, including studies in breast and lung cancer, and oral squamous-cell carcinoma, demonstrated a consistent relationship between disease recurrence and the reappearance of certain tumour aberrations, including *KRAS*, *APC* and *TP53* mutations as well as allelic imbalances.[34,50,51,78] Monitoring single tumour-associated aberrations in the blood is not only effective for the stratification of patients with minimal residual disease and to identify patients likely to relapse, but also to identify those with dormant disease. Dormancy is a common feature of many cancers, including breast, melanoma, renal cancer and non-Hodgkin lymphoma, and cannot be detected by standard methods.[79] In 50 patients with breast cancer, tracking tumour-specific copy-number aberrations in the plasma pre-operatively and post-operatively demonstrated that tumour-specific copy-number aberrations persisted up to 12 years post diagnosis.[80] As DNA is cleared from circulation within 30 min,[81] the presence of DNA might reflect persistent dormant cells cycling between replication and cell death; however, relapse was not detected in any of the 50 patients. Nevertheless, prospective validation in a larger cohort and in other cancer models is warranted.

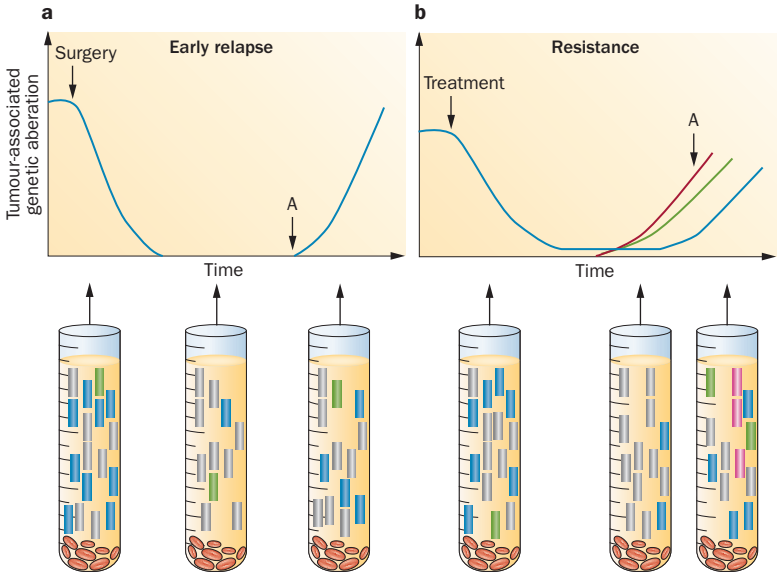


Figure 2 | Monitoring tumour-specific aberrations to detect recurrence and resistance. The figure represents two hypothetical clinical scenarios in which genetic alterations in the blood plasma or serum can be tracked following **a** | surgery, to monitor signs of early relapse and **b** | treatment with a targeted agent (such as anti-EGFR monoclonal antibodies in CRCs) to monitor the emergence of resistant clones. 'A' represents the time of clinically-detectable recurrence or relapse. The blue line represents a 'founder' (early occurring) mutation that is present throughout the bulk of the tumour and reflects overall tumour burden. The red and green lines represent genetic aberrations associated with the growth and expansion of resistant clones.

© 2013 Macmillan Publishers Limited. All rights reserved

The sensitivity of detecting single tumour-specific genetic aberrations in serum or plasma is very variable, particularly in settings of low tumour burden.[30,40] Monitoring tumour-specific genetic aberrations using next-generation sequencing (NGS) technology might offer the opportunity to increase such sensitivity. Genome-wide sequencing of plasma cfDNA has been carried out in patients diagnosed with hepatocellular carcinoma,[9] and by calculating the fractional concentration of tumour-derived DNA (mutations or copy-number aberrations) with respect to total DNA, the researchers were able to demonstrate a significant reduction in tumour-derived DNA following surgery. This finding highlights one of the inherent technical difficulties of using NGS platforms in that the study demonstrated a reduction (threefold to 60-fold), not eradication, of tumour-derived DNA following surgery. As none of the patients relapsed during the course of the study it was assumed that surgery was curative and persistent tumour-associated aberrations were related to sequencing errors. Although residual disease is a possible explanation, tumour-related mutations have also been observed in healthy individuals and smokers suggesting that genetic aberrations might be present at low frequencies even in the absence of cancer.[82–84] This possibility will be clarified with the use of increasingly sensitive techniques.

Difficult-to-diagnose cancers

Liquid biopsies can be used to assist in the clinical management of difficult-to-diagnose patients with advanced-stage cancer, as is the case for bone metastases,[29] some pancreatic cancers and deep pelvic masses.[38] For example, in a study in which targeted deep sequencing of cancer-related genes (including *TP53*, *PIK3CA* and *KRAS*) was carried out on cfDNA in a patient who had previously undergone surgery to resect synchronous cancers of the bowel and ovary, it was shown that on relapse the metastasis was derived from the original ovarian cancer (owing to the presence of a R273H *TP53* mutation and absence of other mutations).[38] A biopsy in this case was not possible and had the information derived from ctDNA been available immediately unnecessary delay or uncertainty over treatments might have been avoided.

Open issues

In theory, the use of liquid biopsies to identify early relapse or progression is very attractive, but there are still some open issues. The most intriguing is the possibility that tumour-associated aberrations can be lost or gained over the monitoring period or in response to drug pressure.[29,42,85] This variation has been ascribed to changes in clonal sub-populations and might occur in response to treatment, adaptation to environmental changes or an increase in metastatic potential.[86] This observation is also interesting in cancers that have long latency periods and liquid biopsies could be used to establish genetic markers of relapse.

Attempts to monitor early relapse or progression require the selection of appropriate 'founder' genetic aberrations (such as *APC* in colorectal cancer or *KRAS* in pancreatic cancer) that are likely to be present from the initiation of tumorigenesis, but provide little to no selective advantage (so-called 'passive' mutations).[87–90] For example, *EGFR* mutation frequency decreases following chemotherapy treatment and would, therefore, not represent a suitable marker of overall tumour burden in a patient receiving chemotherapy.[85] The less responsive the tumour-specific aberration is to therapy the more suitable the marker. The plethora of passive and active mutations that accumulate throughout the lifetime of a tumour means that selecting multiple candidates is not simple.[91] Monitoring relapse or progression using tumour-associated genetic aberrations in the blood requires detailed knowledge of the genes most frequently mutated in each type of cancer. These are now available from large cancer genomics projects such as The Cancer Genome Atlas[92] and the International Cancer Genome Consortium.[93] A rational way to guide the selection of marker lesions might be to choose those that occur early during tumour progression, as they would presumably be present in the whole population as opposed to a clonal sub-population.[2]

Prediction of response to treatment

The presence or absence of a single genetic alteration in tumour DNA is currently employed to guide clinical decision making for a number of targeted agents (for example, *EGFR* mutations for gefitinib in NSCLC, *BRAF* mutations for vemurafenib in melanoma, *KRAS* mutations for cetuximab or panitumumab in colorectal cancer, *ALK* rearrangements for crizotinib in NSCLC).[94–96] Ever-increasing numbers of genomic alterations are being tested as putative predictive biomarkers in clinical trials of novel anticancer therapies.[97]

Targeted agents are often used or tested in patients with advanced-stage disease (often with multiple metastases). However, the associated molecular test is often performed on archived tumour tissues collected years before. This situation is suboptimal as it might not reflect the genomic landscape of current disease. In the context where a new biopsy cannot be obtained, ctDNA might provide superior molecular information compared to archival tissue DNA for determining the current cancer molecular status. Potentially, a 'liquid biopsy' could obviate the need for tumour tissue DNA in metastatic patients. Gevensleben *et al.*[98] have recently described the optimization of a digital PCR method to detect *HER2* amplification in ctDNA of patients with metastatic breast cancer using a development cohort (65 patients) and an independent validation cohort (58 patients). The potential clinical use of such a tool would be in the analysis of *HER2* status in patients who might not have received a biopsy of recurrent breast cancer as part of their routine care, as is often the case. Such patients with metastatic disease that are identified as acquiring HER2-positive status might subsequently benefit from HER2 targeted therapeutics, such as trastuzumab. The researchers also showed that three patients with metastatic breast cancer acquired a HER2-positive status on disease recurrence

© 2013 Macmillan Publishers Limited. All rights reserved

despite being previously classified as HER2 negative by tissue biopsy.[98] The assessment of HER2 status is critical for patient management in breast cancer and current methods for the assessment of the HER2 content of tumours, primarily immunohistochemistry and fluorescence *in situ* hybridization, have limitations.[99] Therefore, a liquid biopsy with an agreed cutoff value could have a considerable impact on the treatment of this patient population.

Detection of increased gene copy number could also be used to assess amplification of other genes to predict response to treatment, such as the situation of *MET* amplification in lung cancer to predict response to anti-EGFR therapies.[100] Furthermore, as a proof of principle it has been shown that high levels of *KRAS* mutant alleles in the plasma is a clear indicator of response to treatment with third-line cetuximab and irinotecan in the metastatic colorectal cancer setting.[101] In this study, mutant *KRAS* levels were analysed by quantitative PCR in the plasma of 41 patients in whom a *KRAS* mutation was detected in primary or metastatic tumours. It was observed that of those patients with mutated *KRAS* levels in the plasma lower than 75%, 42% had obtained disease control, whereas in patients with a plasma level of >75% no response was observed. Therefore, the level of mutated *KRAS* in the plasma was a strong indicator of poor outcome. This observation suggests that some patients with low plasma levels of mutated *KRAS* might achieve some level of disease stabilization with an anti-EGFR treatment. Similar results were observed in a study that assessed 64 patients with metastatic colorectal cancer who were treated with temsirolimus and irinotecan as a monotherapy or combination therapy.[102] Multivariate analysis demonstrated that patients with high mutant *KRAS* levels in the plasma had a 77% risk of early disease progression when receiving monotherapy compared to 43% risk in patients who were categorized as having low mutant *KRAS* levels in the plasma.[102] In addition, an erlotinib-sensitizing *EGFR* mutation was shown to completely disappear in the blood of a patient with NSCLC who was treated with erlotinib, consistent with therapy benefit.[103] Of course, additional studies are needed to validate ctDNA as a source material to ascertain those biomarkers that have already entered clinical practice, including *EGFR*, *BRAF*, *ALK*, *KIT*, *PDGFR*, *HER2* and *KRAS*.

Acquired resistance

Targeted therapies were heralded as 'magic bullets' that would sound the death knell for cancer.[104] Unfortunately, the development of secondary (acquired) resistance is a common outcome for the majority of patients treated with targeted agents. Conceptually, identification of the mechanisms of secondary resistance can lead to the design of additional lines of therapy. Defining the mechanisms of resistance to targeted agents can often be achieved using preclinical models, but it is generally more difficult to confirm these findings in clinical samples. In principle, every patient who becomes resistant to a particular therapy should undergo a tissue biopsy, and molecular tools (including NGS), should be implemented to compare tissue collected pretherapy and post-therapy. This analysis would typically be carried out in advanced-stage cancers and will at best offer only a snap-shot of the predominant resistant clone of the lesion under examination. Furthermore, archived samples taken months, if not years, before treatment with a targeted therapy might not be representative of the genetic landscape of the disease at the time of treatment owing to clonal divergence.[2]

Research has shown that the detection of the emergence of resistant clones, by the presence of tumour-associated genetic aberrations in the blood, identifies treatment resistance up to 10 months before radiological methods.[7,8] It is also possible to detect mutations in the blood that were not observed in the archived formalin-fixed, paraffin-embedded (FFPE) samples.[38] This rapid detection of resistance (Figure 2b) represents one of the most immediate applications of liquid biopsies in the clinic and would allow clinicians to halt expensive and toxic treatment regimens in patients unlikely to continue responding and to challenge with alternative therapies. As previously mentioned, patients in this setting have advanced-stage cancer with high systemic tumour burden; therefore, a large fraction of the circulating DNA is likely to be derived from the tumour itself.[33,40] In addition, unlike tissue biopsies, liquid biopsies are likely to represent a panorama of tumoural-associated genetic aberrations derived from all cancerous lesions in a patient—although the degree to which this occurs has yet to be fully established experimentally. Furthermore, the serial assessment of cancer genomes in plasma constitutes a novel paradigm for the study of clonal evolution in human cancers under the pressure of targeted therapy.[29,105] Despite such worthy applications there remains the requirement of prior knowledge of the mechanisms of resistance to fully make use of these molecular tools.

The genetic mechanisms of acquired resistance to some targeted agents in solid tumours have been, at least in part, characterized (Table 2). One example is the acquisition of the T790M substitution in the membrane receptor EGFR conferring resistance to gefitinb and erlotinib in lung cancer in approximately 50% of patients.[106–110] The T790M mutation was first observed in relapsed patients and later confirmed through the non-invasive analysis of plasma samples, proving that resistance to targeted therapies can be monitored in the blood.[109,111,112] The T790M substitution increases the affinity of EGFR for ATP and so competes out the binding of the inhibitors.[113] The detection of the T790M allele is of clinical relevance, since third-generation inhibitors have shown activity in the presence of this mutation (such as WZ4002[114]) and novel drug combinations have shown promising preclinical activity.[115–117] With this in mind, and with the development of sensitive techniques that can evaluate the levels of *EGFR* mutations in the plasma, future studies will hopefully show that the cancer can be challenged with novel drugs and drug combinations.[38,111,118,119]

© 2013 Macmillan Publishers Limited. All rights reserved

Table 2 | Mutations responsible for acquired resistance to targeted therapies

Gene	Genetic aberration	Tumour type	Acquired drug resistance	Reference*
EGFR	T790M	Advanced NSCLC	Gefitinib Erlotinib	Yun *et al.* (2008)[113] Murtaza *et al.* (2013)[105]
KRAS	Codon 12, 13 and 61	Colorectal cancer	Cetuximab	Diaz *et al.* (2012)[7] Misale *et al.* (2012)[8]
KIT	T670I	GIST	Imatinib	Tamborini *et al.* (2006)[155]
PIK3CA	NS	NSCLC	Erlotinib Gefitinib	Sequist *et al.* (2011)[134] Murtaza *et al.* (2013)[105]
ALK	C1156Y L1196M	NSCLC	Crizotinib	Choi *et al.* (2010)[156]
MEK1	C121S	Melanoma	Vemurafenib	Wagle *et al.* (2011)[128]
BRAF	Amplification	Melanoma	Vemurafenib	Shi *et al.* (2012)[123] Gevensleben *et al.* (2013)[98]
NRAS	Q61K	Melanoma	Vemurafenib	Nazarian *et al.* (2010)[124]

*References include a selection of studies in which detection of the genetic alteration has been technically achieved in circulating DNA of patients with cancer or proof-of-principle demonstrated.
Abbreviations: GIST, gastrointestinal stromal tumour; NS, not specified; NSCLC, non-small-cell lung cancer.

Analogously, secondary resistance to the anti-EGFR antibodies cetuximab and panitumumab in colorectal cancer is associated with the emergence of *KRAS* mutations.[8] *KRAS* mutation testing on archival FFPE tumoural samples is mandatory in the clinic and anti-EGFR antibodies are prescribed only for the treatment of *KRAS* wild-type patients.[94] The detection of *KRAS* variants in the blood during treatment with cetuximab or panitumumab demonstrated that it is possible to detect the emergence of resistant alleles up to 10 months before conventional methods.[7,8] Diaz *et al.*[7] showed that out of 24 patients with colorectal cancer who were monitored, nine developed detectable *KRAS* mutations using a candidate-gene approach. Remarkably, three patients developed multiple *KRAS* mutations, presumably arising from different sub-clonal populations.[7]

A further example of acquired resistance occurs in patients with *BRAF* V600E mutant melanoma who are treated with vemurafenib. Acquired resistance to vemurafenib is known to occur as a consequence of several mechanisms, including *MAP3K8* overexpression,[120] activation of a MAPK-redundant survival pathway,[121] PDGFRβ upregulation, *BRAF* truncation[122] or amplification[123] and *NRAS* Q61K mutation.[124] Consequently, it is possible to monitor the acquisition of resistance to vemurafenib by the appearance of genetic aberrations (in the same way as *KRAS* mutations are monitored in colorectal cancer[7]) or gene amplifications (in the same way *HER2* amplification is observed in breast cancer).[98,125] Furthermore, promising results for the treatment of acquired and *de novo* resistance to BRAF inhibition in patients with metastatic melanoma have been shown in phase I and II clinical trials in which the BRAF inhibitor dabrafenib and the MEK inhibitor trametinib were used in combination.[126] The median progression-free survival increased significantly (P <0.001) from 5.8 months (in those patients receiving monotherapy) to 9.4 months (in those patients receiving combination therapy). As many of the resistance mechanisms converge on the MAPK pathway, the authors suggested that the combination of both BRAF and MEK inhibitors might be effective for the treatment of *de novo* as well as acquired resistance. In addition, adjusting treatment regimens may also serve to prevent the emergence of drug resistance.[127] Cell lines resistant to vemurafenib also remain sensitive to selumetinib representing a possible alternative therapy albeit not in the presence of all resistance-associated genetic lesions, should the preclinical data be confirmed *in vivo*.[124,128]

Most of the above-mentioned studies are based on a candidate-gene approach requiring prior knowledge of the mechanisms of resistance. Considering the amenability and accessibility of plasma from patients undergoing therapy, future studies will track the emergence of resistant clones in an unbiased fashion. For example, very recently, an unbiased approach was applied to study the acquisition of secondary resistance and to monitor clonal evolution in the blood of patients with cancer. In this comprehensive study, it was shown that genetic markers of resistance can be non-invasively tracked throughout the course of treatment in breast, ovarian and lung cancer.[105] Exome sequencing on six patients' plasma samples obtained pretreatment and post-treatment showed the emergence of resistant alleles. Examples include a T790M *EGFR* mutation[109] in a patient with lung cancer treated with gefitnib and a *MED1* truncating mutation after treatment with tamoxifen and trastuzumab in a patient with breast cancer.[129,130] Other interesting therapeutic resistance-associated genetic aberrations that arose during the course of treatment included *PIK3CA*,[131] *RB1*[132] and *TP53*.[133] The Murtaza *et al.*[105] study demonstrates the applicability of systematic, unbiased sequencing of tumour-associated genetic aberrations in circulating DNA to track the emergence of resistance.

Compelling evidence[8,29,105] shows that clones or mutations that emerge during treatment are already present in a small fraction of the original tumour population. Tracking the dynamics of resistant sub-populations might also allow selection of patients that can be rechallenged with the same, or similar, drugs owing to loss of the resistance-associated allele in the absence of drug pressure. For example, as has been observed in the treatment of NSCLC, the release of drug pressure resulted in the loss of the genetic mechanism of resistance in three patients providing a window of opportunity within which these patients could be re-treated with EGFR inhibitors.[134,135] Although these patients would inevitably relapse, it might provide a period of disease stabilization.

Finally, techniques described in this Review can also be used for monitoring of acquired resistance as a result of increased gene copy number including *BRAF* or *MET* amplification following targeted therapy treatment in melanoma or lung cancer, respectively.[123,125,136]

Understanding and being able to pharmacologically counter the clonal evolution of a tumour under the selective pressure of therapy is a great challenge and a tremendous opportunity. Selection of a specific molecular phenotype through exposure to one anticancer agent

© 2013 Macmillan Publishers Limited. All rights reserved

might lead to the expression of molecular targets for other drugs. Indeed, sequential treatments using drugs that inhibit specific molecular targets and, in parallel, tracking the emergence of resistance-associated genetic aberrations would allow the oncologist to pre-empt the most effective subsequent treatments.[137] In principle, this knowledge could be applied to provide the early initiation of alternate therapies before relapse is detected by clinical or radiological examinations.

Conclusions

Although liquid biopsies have a great potential, many hurdles must be overcome before proceeding to the clinic. One of the major concerns in this field is the lack of standardization of techniques. This lack of consistency extends to each step in the process starting from the material from which DNA is extracted (plasma versus serum) to the techniques used for the quantification of tumour-associated genetic mutations (digital PCR, NGS, BEAMing). Currently, multiple procedures to store and collect samples (blood, plasma, serum or DNA), and to extract DNA are employed (Table 1);[138–140] however, a consensus has yet to be met.[33,141,142] In addition, serum and plasma DNA have been suggested to be related in different ways to diagnosis and prognosis.[143] Nevertheless, studies have shown that plasma provides greater sensitivity for the detection of *KRAS* mutations using highly sensitive quantitative PCR assays.[33,144,145] It has also been shown that the separation of circulating DNA into low-molecular weight or high-molecular weight fractions might enhance the sensitivity of detecting tumour-associated genetic aberrations as mutated alleles preferentially associate with low-molecular-weight fragmented DNA.[48,52,140,146,] This association might be particularly important in situations where levels of circulating DNA are low, such as early stage cancer.[40,72]

Techniques used for the detection of tumour-associated genetic aberrations include BEAMing,[27] NGS (targeted[38] or whole genome[9,37]), digital PCR,[29,119] cold-PCR (co-amplification at lower denaturation temperature-PCR),[147] MAP (MIDI-Activated Pyrophosphorolysis)[148] and mass spectrometry genotyping assay-mutant-enriched PCR.[118] Each technique is able to detect mutant allele frequencies with a sensitivity of at least 2%; however, the cost and practicality of developing each for use in the clinic is a significant consideration and poses diverse technical difficulties. Consider BEAMing technology that is able to detect a single mutant allele in a background of 10,000 wild-type alleles.[36] This technique has a particular technical limitation as probes for the detection of a mutated allele must be personalized for each patient with the prerequisite that a tumour biopsy has been screened in advance to capture the mutational profile.[29] As genetic screening of tumour samples is now routinely carried out in the clinic and an initial tumour sample is almost always available through either surgery or biopsy, this limitation might pose only a minor issue.

The use of NGS for the detection of tumour-associated mutations in the blood opens up exciting possibilities.[9,29,38,105] There are, however, three major limitations to its general application in the clinic including the cost, the requirement for high-quality DNA (not characteristic of cfDNA) and extensive data analysis requiring a dedicated bioinformatician.[9] An intrinsic problem posed by NGS, in terms of an immediate application in the clinic, is the management of the data. This problem is particularly pertinent for NGS as there is fundamental difficulty in distinguishing the intrinsic background noise of deep sequencing from true tumour-associated aberrations. Problems with stochastic noise were highlighted in the study by Chan *et al.*,[9] in which aberrations were detected in 16 healthy individuals and these were suggested as being due to sequencing errors. Both the expense (reagent costs), extensive workflow and data analysis in applying next-generation platforms to cfDNA are ameliorated somewhat by applying targeted sequencing of specific cancer-related genes as outlined by Forshew *et al.*[38] compared to the extensive, yet expensive, genome-wide profiling.[9] Attempts to reduce the costs and speed of whole-genome sequencing for the analysis of circulating tumour-associated genetic aberrations have also been reported.[149] Consequently, improvements in technology and reductions in the cost of sequencing mean the cost is unlikely to remain a limiting problem.

Another important consideration is how tumour markers should be selected, that is, whether multiple mutational panels should be adopted or personalized panels based on the sequence of the cancer of an individual. Of course, this remains very dependent on the technology that is developed for clinical use, for example, BEAMing for known hotspots in selected genes (or single probes for rare variants designed on a 'personalized' basis), whereas NGS can be used to screen large panels of genes.[29,38,150] Furthermore, personalizing the technique on a patient-by-patient basis is very costly. It is also important to consider that although pathologists are familiar with DNA extraction from FFPE tumour specimens for genomic analysis, deep-sequencing of panels of somatic mutations in the clinic is not yet routinely performed.[151] Nevertheless, familiarity with cancer panel kits able to detect hundreds of mutational hotspots from FFPE samples in cancer genomes at less than 5% frequency, are likely to provide the next step towards deep-sequencing in the clinic. Screening panels of somatic mutations that are commonly mutated in cancer will also enable identification of infrequent mutations (such as *KIT* mutations in breast cancer that occur at a frequency of approximately 1%),[152] and for which therapies are available.

Logistical considerations involved in running a facility necessary for the tracking and monitoring of tumour-associated genetic aberrations in the blood include whether samples should be processed in the clinic, agreement over clinical guidelines for processing and storage and whether this should involve local or central facilities. Such a study should be approached in a similar manner to the study by Press and colleagues[153] in which accuracy and reproducibility of *HER2* testing was assessed in external and local laboratories.

© 2013 Macmillan Publishers Limited. All rights reserved

The last big hurdle between theoretical robustness, laboratory data, translational experience and the real possibility of a clinical application involves 'formal validation'. Rigorous concordance studies designed to address some of the issues raised in this Review are now required. Most of the studies conducted so far are so-called 'convenience studies' in which available samples were analysed retrospectively. This methodology produced promising hypothesis-generating data, but with very low levels of evidence. Indeed, studies in which cfDNA testing is the primary end point to assess the applicability of monitoring for tumour-associated genetic aberrations in the cfDNA are now needed. In parallel, the current applications of ctDNA testing have so far been developed for research or investigational purposes only and should proceed to CLIA (Clinical Laboratory Improvement Amendments) certification before implementation in clinical trials.

Review criteria

The PubMed, MEDLINE, Google Scholar and Web of Science databases were searched using the terms: "circulating DNA", "circulating free DNA", "circulating mutated DNA", "circulating plasma DNA", "circulating serum DNA", "extracellular occurring DNA", "cfDNA", "ctDNA", "cpDNA" and "cirDNA". Only articles published in English were considered (excluding the study by Mandel *et al.*[5] that was selected for historical purposes). Although every effort has been made to collect information using various annotations, lack of labelling uniformity may have impaired retrieval of important studies. The following conference abstracts were also assessed 24th EORTC–NCI–AACR Symposium on Molecular Targets and Cancer Therapeutics, 6–9 November 2012, Dublin, Ireland, as well as The Cancer Genome Atlas (http://cancergenome.nih.gov) website, and International Cancer Genome Consortium (http://icgc.org/).

1. Diamantis, A., Magiorkinis, E. & Koutselini, H. Fine-needle aspiration (FNA) biopsy: historical aspects. *Folia Histochem. Cytobiol.* **47**, 191–197 (2009).
2. Gerlinger, M. *et al.* Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N. Engl. J. Med.* **366**, 883–892 (2012).
3. Robertson, E. G. & Baxter, G. Tumour seeding following percutaneous needle biopsy: the real story! *Clin. Radiol.* **66**, 1007–1014 (2011).
4. Hompes, D. & Ruers, T. Review: incidence and clinical significance of bevacizumab-related non-surgical and surgical serious adverse events in metastatic colorectal cancer. *Eur. J. Surg. Oncol.* **37**, 737–746 (2011).
5. Mandel, P. & Metais, P. Les acides nucleiques du plasma sanguin chez l'homme. *C. R. Seances Soc. Biol. Fil.* **142**, 241–243 (1948).
6. Koffler, D., Agnello, V., Winchester, R. & Kunkel, H. G. The occurrence of single-stranded DNA in the serum of patients with systemic lupus erythematosus and other diseases. *J. Clin. Invest.* **52**, 198–204 (1973).
7. Diaz, L. A. Jr *et al.* The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. *Nature* **486**, 537–540 (2012).
8. Misale, S. *et al.* Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. *Nature* **486**, 532–536 (2012).
9. Chan, K. C. *et al.* Cancer genome scanning in plasma: detection of tumor-associated copy number aberrations, single-nucleotide variants, and tumoral heterogeneity by massively parallel sequencing. *Clin. Chem.* **59**, 211–224 (2013).
10. Jung, K., Fleischhacker, M. & Rabien, A. Cell-free DNA in the blood as a solid tumor biomarker–a critical appraisal of the literature. *Clin. Chim. Acta* **411**, 1611–1624 (2010).
11. Gormally, E., Caboux, E., Vineis, P. & Hainaut, P. Circulating free DNA in plasma or serum as biomarker of carcinogenesis: practical aspects and biological significance. *Mutat. Res.* **635**, 105–117 (2007).
12. Schwarzenbach, H., Hoon, D. S. & Pantel, K. Cell-free nucleic acids as biomarkers in cancer patients. *Nat. Rev. Cancer* **11**, 426–437 (2011).
13. Pisetsky, D. S. & Fairhurst, A. M. The origin of extracellular DNA during the clearance of dead and dying cells. *Autoimmunity* **40**, 281–284 (2007).
14. Mantovani, A., Sozzani, S., Locati, M., Allavena, P. & Sica, A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol.* **23**, 549–555 (2002).
15. Viorritto, I. C., Nikolov, N. P. & Siegel, R. M. Autoimmunity versus tolerance: can dying cells tip the balance? *Clin. Immunol.* **122**, 125–134 (2007).
16. Stroun, M. & Anker, P. Nucleic acids spontaneously released by living frog auricles. *Biochem. J.* **128**, 100P–101P (1972).
17. Anker, P., Stroun, M. & Maurice, P. A. Spontaneous release of DNA by human blood lymphocytes as shown in an *in vitro* system. *Cancer Res.* **35**, 2375–2382 (1975).
18. Stroun, M., Lyautey, J., Lederrey, C., Olson-Sand, A. & Anker, P. About the possible origin and mechanism of circulating DNA apoptosis and active DNA release. *Clin. Chim. Acta* **313**, 139–142 (2001).
19. Rogers, J. C., Boldt, D., Kornfeld, S., Skinner, A. & Valeri, C. R. Excretion of deoxyribonucleic acid by lymphocytes stimulated with phytohemagglutinin or antigen. *Proc. Natl Acad. Sci. USA* **69**, 1685–1689 (1972).
20. Garcia-Olmo, D. C. *et al*. Cell-free nucleic acids circulating in the plasma of colorectal cancer patients induce the oncogenic transformation of susceptible cultured cells. *Cancer Res*. **70**, 560–567 (2010).
21. Trejo-Becerril, C. *et al*. Cancer progression mediated by horizontal gene transfer in an *in vivo* model. *PLoS ONE* **7**, e52754 (2012).
22. Zhou, J., Shi, Y. H. & Fan, J. Circulating cell-free nucleic acids: promising biomarkers of hepatocellular carcinoma. *Semin. Oncol.* **39**, 440–448 (2012).
23. Akca, H. *et al.* Utility of serum DNA and pyrosequencing for the detection of EGFR mutations in non-small cell lung cancer. *Cancer Genet.* **206**, 73–80 (2013).
24. Morton, N. E. Parameters of the human genome. *Proc. Natl Acad. Sci. USA* **88**, 7474–7476 (1991).
25. Perkins, G. *et al.* Multi-purpose utility of circulating plasma DNA testing in patients with advanced cancers. *PLoS ONE* **7**, e47020 (2012).
26. Punnoose, E. A. *et al.* Evaluation of circulating tumor cells and circulating tumor DNA in non-small cell lung cancer: association with clinical endpoints in a phase II clinical trial of pertuzumab and erlotinib. *Clin. Cancer Res.* **18**, 2391–2401 (2012).
27. Diehl, F. *et al.* Circulating mutant DNA to assess tumor dynamics. *Nat. Med.* **14**, 985–990 (2008).
28. Wang, J. Y. *et al.* Molecular detection of APC, K-ras, and p53 mutations in the serum of colorectal cancer patients as circulating biomarkers. *World J. Surg.* **28**, 721–726 (2004).
29. Dawson, S. J. *et al.* Analysis of circulating tumor DNA to monitor metastatic breast cancer. *N. Engl. J. Med.* **368**, 1199–1209 (2013).
30. Sorenson, G. D. Detection of mutated KRAS2 sequences as tumor markers in plasma/serum of patients with gastrointestinal cancer. *Clin. Cancer Res.* **6**, 2129–2137 (2000).
31. Sorenson, G. D. A review of studies on the detection of mutated KRAS2 sequences as tumor markers in plasma/serum of patients with gastrointestinal cancer. *Ann. NY Acad. Sci.* **906**, 13–16 (2000).
32. Mouliere, F. *et al.* High fragmentation characterizes tumour-derived circulating DNA. *PLoS ONE* **6**, e23418 (2011).
33. Thierry, A. R. *et al.* Origin and quantification of circulating DNA in mice with human colorectal cancer xenografts. *Nucleic Acids Res.* **38**, 6159–6175 (2010).
34. Frattini, M. *et al.* Quantitative and qualitative characterization of plasma DNA identifies primary and recurrent colorectal cancer. *Cancer Lett.* **263**, 170–181 (2008).
35. Chen, X. Q. *et al.* Microsatellite alterations in plasma DNA of small cell lung cancer patients. *Nat. Med.* **2**, 1033–1035 (1996).
36. Diehl, F. *et al.* Detection and quantification of mutations in the plasma of patients with colorectal tumors. *Proc. Natl Acad. Sci. USA* **102**, 16368–16373 (2005).
37. Leary, R. J. *et al.* Detection of chromosomal alterations in the circulation of cancer patients with whole-genome sequencing. *Sci. Transl. Med.* **4**, 162ra154 (2012).
38. Forshew, T. *et al.* Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. *Sci. Transl. Med.* **4**, 136ra168 (2012).
39. Jahr, S. *et al.* DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. *Cancer Res.* **61**, 1659–1665 (2001).
40. Board, R. E. *et al.* Detection of PIK3CA mutations in circulating free DNA in patients with breast cancer. *Breast Cancer Res. Treat.* **120**, 461–467 (2010).

© 2013 Macmillan Publishers Limited. All rights reserved

41. Mead, R., Duku, M., Bhandari, P. & Cree, I. A. Circulating tumour markers can define patients with normal colons, benign polyps, and cancers. *Br. J. Cancer* **105**, 239–245 (2011).
42. Higgins, M. J. *et al.* Detection of tumor PIK3CA status in metastatic breast cancer using peripheral blood. *Clin. Cancer Res.* **18**, 3462–3469 (2012).
43. Brooks, J. D. Translational genomics: the challenge of developing cancer biomarkers. *Genome Res.* **22**, 183–187 (2012).
44. Wang, Y. *et al.* High quality copy number and genotype data from FFPE samples using Molecular Inversion Probe (MIP) microarrays. *BMC Med. Genomics* **2**, 8 (2009).
45. Srinivasan, M., Sedmak, D. & Jewell, S. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. *Am. J. Pathol.* **161**, 1961–1971 (2002).
46. Silva, J. M. *et al.* Persistence of tumor DNA in plasma of breast cancer patients after mastectomy. *Ann. Surg. Oncol.* **9**, 71–76 (2002).
47. Silva, J. M. *et al.* Tumor DNA in plasma at diagnosis of breast cancer patients is a valuable predictor of disease-free survival. *Clin. Cancer Res.* **8**, 3761–3766 (2002).
48. Schwarzenbach, H. *et al.* Loss of heterozygosity at tumor suppressor genes detectable on fractionated circulating cell-free tumor DNA as indicator of breast cancer progression. *Clin. Cancer Res.* **18**, 5719–5730 (2012).
49. Frattini, M. *et al.* Quantitative analysis of plasma DNA in colorectal cancer patients: a novel prognostic tool. *Ann. NY Acad. Sci.* **1075**, 185–190 (2006).
50. Hamana, K. *et al.* Monitoring of circulating tumour-associated DNA as a prognostic tool for oral squamous cell carcinoma. *Br. J. Cancer* **92**, 2181–2184 (2005).
51. Hsieh, J. S. *et al.* APC, K-ras, and p53 gene mutations in colorectal cancer patients: correlation to clinicopathologic features and postoperative surveillance. *Am. Surg.* **71**, 336–343 (2005).
52. Kuhlmann, J. D. *et al.* LOH at 6q and 10q in fractionated circulating DNA of ovarian cancer patients is predictive for tumor cell spread and overall survival. *BMC Cancer* **12**, 325 (2012).
53. Castells, A. *et al.* K-ras mutations in DNA extracted from the plasma of patients with pancreatic carcinoma: diagnostic utility and prognostic significance. *J. Clin. Oncol.* **17**, 578–584 (1999).
54. Yamada, T. *et al.* Detection of K-ras gene mutations in plasma DNA of patients with pancreatic adenocarcinoma: correlation with clinicopathological features. *Clin. Cancer Res.* **4**, 1527–1532 (1998).
55. Salazar, R. *et al.* Gene expression signature to improve prognosis prediction of stage II and III colorectal cancer. *J. Clin. Oncol.* **29**, 17–24 (2011).
56. Paik, S. *et al.* A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. *N. Engl. J. Med.* **351**, 2817–2826 (2004).
57. Maak, M. *et al.* Independent validation of a prognostic genomic signature (ColoPrint) for patients with stage II colon cancer. *Ann. Surg.* **257**, 1053–1058 (2013).
58. van 't Veer, L. J. *et al.* Gene expression profiling predicts clinical outcome of breast cancer. *Nature* **415**, 530–536 (2002).
59. Nygaard, A. D., Garm Spindler, K. L., Pallisgaard, N., Andersen, R. F. & Jakobsen, A. The prognostic value of KRAS mutated plasma DNA in advanced non-small cell lung cancer. *Lung Cancer* **79**, 312–317 (2013).
60. Camps, C. *et al.* The identification of KRAS mutations at codon 12 in plasma DNA is not a prognostic factor in advanced non-small cell lung cancer patients. *Lung Cancer* **72**, 365–369 (2011).
61. Shinozaki, M. *et al.* Utility of circulating B-RAF DNA mutation in serum for monitoring melanoma patients receiving biochemotherapy. *Clin. Cancer Res.* **13**, 2068–2074 (2007).
62. Cohn, S. L. *et al.* The International Neuroblastoma Risk Group (INRG) classification system: an INRG Task Force report. *J. Clin. Oncol.* **27**, 289–297 (2009).
63. Brodeur, G. M. Neuroblastoma: biological insights into a clinical enigma. *Nat. Rev. Cancer* **3**, 203–216 (2003).
64. Yu, A. L. *et al.* Anti-GD2 antibody with GM-CSF, interleukin-2, and isotretinoin for neuroblastoma. *N. Engl. J. Med.* **363**, 1324–1334 (2010).
65. Matthay, K. K. *et al.* Long-term results for children with high-risk neuroblastoma treated on a randomized trial of myeloablative therapy followed by 13-cis-retinoic acid: a children's oncology group study. *J. Clin. Oncol.* **27**, 1007–1013 (2009).
66. Matthay, K. K. *et al.* Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-cis-retinoic acid. Children's Cancer Group. *N. Engl. J. Med.* **341**, 1165–1173 (1999).
67. Ambros, P. F. *et al.* International consensus for neuroblastoma molecular diagnostics: report from the International Neuroblastoma Risk Group (INRG) Biology Committee. *Br. J. Cancer* **100**, 1471–1482 (2009).
68. Schneiderman, J. *et al.* Clinical significance of MYCN amplification and ploidy in favorable-stage neuroblastoma: a report from the Children's Oncology Group. *J. Clin. Oncol.* **26**, 913–918 (2008).
69. Combaret, V. *et al.* Influence of neuroblastoma stage on serum-based detection of MYCN amplification. *Pediatr. Blood Cancer* **53**, 329–331 (2009).
70. Combaret, V. *et al.* Circulating MYCN DNA as a tumor-specific marker in neuroblastoma patients. *Cancer Res.* **62**, 3646–3648 (2002).
71. Combaret, V., Bergeron, C., Noguera, R., Iacono, I. & Puisieux, A. Circulating MYCN DNA predicts MYCN-amplification in neuroblastoma. *J. Clin. Oncol.* **23**, 8919–8920 (2005).
72. Daniotti, M. *et al.* Detection of mutated BRAFV600E variant in circulating DNA of stage III-IV melanoma patients. *Int. J. Cancer* **120**, 2439–2444 (2007).
73. Van Cutsem, E., Oliveira, J. & ESMO Guidelines Working Group. Primary colon cancer: ESMO clinical recommendations for diagnosis, adjuvant treatment and follow-up. *Ann. Oncol.* **20** (Suppl. 4), 49–50 (2009).
74. Kataja, V., Castiglione, M. & ESMO Guidelines Working Group. Primary breast cancer: ESMO clinical recommendations for diagnosis, treatment and follow-up. *Ann. Oncol.* **20** (Suppl. 4), 10–14 (2009).
75. D'Addario, G., Felip, E. & ESMO Guidelines Working Group. Non-small-cell lung cancer: ESMO clinical recommendations for diagnosis, treatment and follow-up. *Ann. Oncol.* **20** (Suppl. 4), 68–70 (2009).
76. Jeffery, M., Hickey, B. E. & Hider, P. N. Follow-up strategies for patients treated for non-metastatic colorectal cancer. *Cochrane Database of Systematic Reviews 2007,* Issue 1. Art. No.: CD002200. http://dx.doi.org/10.1002/14651858.CD002200.pub2.
77. Srikantharajah, D., Ghuman, A., Nagendran, M. & Maruthappu, M. Is computed tomography follow-up of patients after lobectomy for non-small cell lung cancer of benefit in terms of survival? *Interact. Cardiovasc. Thorac. Surg.* **15**, 893–898 (2012).
78. Sozzi, G. *et al.* Analysis of circulating tumor DNA in plasma at diagnosis and during follow-up of lung cancer patients. *Cancer Res.* **61**, 4675–4678 (2001).
79. Uhr, J. W. & Pantel, K. Controversies in clinical cancer dormancy. *Proc. Natl Acad. Sci. USA* **108**, 12396–12400 (2011).
80. Shaw, J. A. *et al.* Genomic analysis of circulating cell-free DNA infers breast cancer dormancy. *Genome Res.* **22**, 220–231 (2012).
81. Tsumita, T. & Iwanaga, M. Fate of injected deoxyribonucleic acid in mice. *Nature* **198**, 1088–1089 (1963).
82. Jiao, L. *et al.* K-ras mutation and p16 and preproenkephalin promoter hypermethylation in plasma DNA of pancreatic cancer patients: in relation to cigarette smoking. *Pancreas* **34**, 55–62 (2007).
83. Magistrelli, P. *et al.* K-ras mutations in circulating DNA from pancreatic and lung cancers: bridging methodology for a common validation of the molecular diagnosis value. *Pancreas* **37**, 101–102 (2008).
84. Huang, X. H. *et al.* Codon 249 mutation in exon 7 of p53 gene in plasma DNA: maybe a new early diagnostic marker of hepatocellular carcinoma in Qidong risk area, China. *World J. Gastroenterol.* **9**, 692–695 (2003).
85. Bai, H. *et al.* Influence of chemotherapy on EGFR mutation status among patients with non-small-cell lung cancer. *J. Clin. Oncol.* **30**, 3077–3083 (2012).
86. Kreso, A. *et al.* Variable clonal repopulation dynamics influence chemotherapy response in colorectal cancer. *Science* **339**, 543–548 (2013).
87. di Magliano, M. P. & Logsdon, C. D. Roles for KRAS in pancreatic tumor development and progression. *Gastroenterology* **144**, 1220–1229 (2013).
88. Powell, S. M. *et al.* APC mutations occur early during colorectal tumorigenesis. *Nature* **359**, 235–237 (1992).
89. Nickerson, M. L. *et al.* Improved identification of von Hippel-Lindau gene alterations in clear cell renal tumors. *Clin. Cancer Res.* **14**, 4726–4734 (2008).
90. Rechsteiner, M. P. *et al.* VHL gene mutations and their effects on hypoxia inducible factor HIFα: identification of potential driver and passenger mutations. *Cancer Res.* **71**, 5500–5511 (2011).
91. Bardelli, A. *et al.* Mutational analysis of the tyrosine kinome in colorectal cancers. *Science* **300**, 949 (2003).
92. National Cancer Institute. *The Cancer Genome Atlas* [online], http://cancergenome.nih.gov (2013).
93. International Cancer Genome Consortium. *ICGC Cancer Genome Projects* [online], http://icgc.org/ (2013).
94. Allegra, C. J. *et al.* American Society of Clinical Oncology provisional clinical opinion: testing for KRAS gene mutations in patients with metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy. *J. Clin. Oncol.* **27**, 2091–2096 (2009).
95. Shaw, A. T. & Engelman, J. A. ALK in lung cancer: past, present, and future. *J. Clin. Oncol.* **31**, 1105–1111 (2013).
96. Gonzalez, D. *et al.* BRAF mutation testing algorithm for vemurafenib treatment in melanoma: recommendations from an expert panel. *Br. J. Dermatol.* **168**, 700–707 (2013).

© 2013 Macmillan Publishers Limited. All rights reserved

97. Simon, R. & Roychowdhury, S. Implementing personalized cancer genomics in clinical trials. *Nat. Rev. Drug Discov.* **12**, 358–369 (2013).
98. Gevensleben, H. *et al.* Non-invasive detection of HER2 amplification with plasma DNA digital PCR. *Clin. Cancer Res.* **19**, 3276–3284 (2013).
99. Shah, S. S. *et al.* Impact of American Society of Clinical Oncology/College of American Pathologists guideline recommendations on HER2 interpretation in breast cancer. *Hum. Pathol.* **41**, 103–106 (2010).
100. Bean, J. *et al.* MET amplification occurs with or without T790M mutations in EGFR mutant lung tumors with acquired resistance to gefitinib or erlotinib. *Proc. Natl Acad. Sci. USA* **104**, 20932–20937 (2007).
101. Spindler, K. L., Pallisgaard, N., Vogelius, I. & Jakobsen, A. Quantitative cell-free DNA, KRAS, and BRAF mutations in plasma from patients with metastatic colorectal cancer during treatment with cetuximab and irinotecan. *Clin. Cancer Res.* **18**, 1177–1185 (2012).
102. Spindler, K. L. *et al.* Phase II trial of temsirolimus alone and in combination with irinotecan for KRAS mutant metastatic colorectal cancer: outcome and results of KRAS mutational analysis in plasma. *Acta Oncol.* **52**, 963–970 (2013).
103. Weber, B. *et al.* Complete pathologic response in lung tumors in two patients with metastatic non-small cell lung cancer treated with erlotinib. *J. Thorac. Oncol.* **6**, 1946–1949 (2011).
104. Martini, M., Vecchione, L., Siena, S., Tejpar, S. & Bardelli, A. Targeted therapies: how personal should we go? *Nat. Rev. Clin. Oncol.* **9**, 87–97 (2011).
105. Murtaza, M. *et al.* Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. *Nature* **497**, 108–112 (2013).
106. Kosaka, T. *et al.* Analysis of epidermal growth factor receptor gene mutation in patients with non-small cell lung cancer and acquired resistance to gefitinib. *Clin. Cancer Res.* **12**, 5764–5769 (2006).
107. Balak, M. N. *et al.* Novel D761Y and common secondary T790M mutations in epidermal growth factor receptor-mutant lung adenocarcinomas with acquired resistance to kinase inhibitors. *Clin. Cancer Res.* **12**, 6494–6501 (2006).
108. Zhang, Z. *et al.* Activation of the AXL kinase causes resistance to EGFR-targeted therapy in lung cancer. *Nat. Genet.* **44**, 852–860 (2012).
109. Pao, W. *et al.* Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Med.* **2**, e73 (2005).
110. Kobayashi, S. *et al.* EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N. Engl. J. Med.* **352**, 786–792 (2005).
111. Nakamura, T. *et al.* Application of a highly sensitive detection system for epidermal growth factor receptor mutations in plasma DNA. *J. Thorac. Oncol.* **7**, 1369–1381 (2012).
112. Taniguchi, K. *et al*. Quantitative detection of EGFR mutations in circulating tumor DNA derived from lung adenocarcinomas. *Clin. Cancer Res*. **17**, 7808–7815 (2011).
113. Yun, C. H. *et al.* The T790M mutation in EGFR kinase causes drug resistance by increasing the affinity for ATP. *Proc. Natl Acad. Sci. USA* **105**, 2070–2075 (2008).
114. Zhou, W. *et al.* Novel mutant-selective EGFR kinase inhibitors against EGFR T790M. *Nature* **462**, 1070–1074 (2009).
115. Li, D. *et al.* BIBW2992, an irreversible EGFR/HER2 inhibitor highly effective in preclinical lung cancer models. *Oncogene* **27**, 4702–4711 (2008).
116. Engelman, J. A. *et al.* PF00299804, an irreversible pan-ERBB inhibitor, is effective in lung cancer models with EGFR and ERBB2 mutations that are resistant to gefitinib. *Cancer Res.* **67**, 11924–11932 (2007).
117. Cortot, A. B. *et al.* Resistance to irreversible EGF receptor tyrosine kinase inhibitors through a multistep mechanism involving the IGF1R pathway. *Cancer Res.* **73**, 834–843 (2013).
118. Brevet, M., Johnson, M. L., Azzoli, C. G. & Ladanyi, M. Detection of EGFR mutations in plasma DNA from lung cancer patients by mass spectrometry genotyping is predictive of tumor EGFR status and response to EGFR inhibitors. *Lung Cancer* **73**, 96–102 (2011).
119. Yung, T. K. *et al.* Single-molecule detection of epidermal growth factor receptor mutations in plasma by microfluidics digital PCR in non-small cell lung cancer patients. *Clin. Cancer Res.* **15**, 2076–2084 (2009).
120. Johannessen, C. M. *et al.* COT drives resistance to RAF inhibition through MAP kinase pathway reactivation. *Nature* **468**, 968–972 (2010).
121. Villanueva, J. *et al.* Acquired resistance to BRAF inhibitors mediated by a RAF kinase switch in melanoma can be overcome by cotargeting MEK and IGF-1R/PI3K. *Cancer Cell* **18**, 683–695 (2010).
122. Poulikakos, P. I. *et al.* RAF inhibitor resistance is mediated by dimerization of aberrantly spliced $BRAF^{V600E}$. *Nature* **480**, 387–390 (2011).
123. Shi, H. *et al.* Melanoma whole-exome sequencing identifies (V600E)B-RAF amplification-mediated acquired B-RAF inhibitor resistance. *Nat. Commun.* **3**, 724 (2012).
124. Nazarian, R. *et al.* Melanomas acquire resistance to B-RAF(V600E) inhibition by RTK or N-RAS upregulation. *Nature* **468**, 973–977 (2010).
125. Page, K. *et al.* Detection of HER2 amplification in circulating free DNA in patients with breast cancer. *Br. J. Cancer* **104**, 1342–1348 (2011).
126. Flaherty, K. T. *et al.* Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. *N. Engl. J. Med.* **367**, 1694–1703 (2012).
127. Das Thakur, M. *et al.* Modelling vemurafenib resistance in melanoma reveals a strategy to forestall drug resistance. *Nature* **494**, 251–255 (2013).
128. Wagle, N. *et al.* Dissecting therapeutic resistance to RAF inhibition in melanoma by tumor genomic profiling. *J. Clin. Oncol.* **29**, 3085–3096 (2011).
129. Nagalingam, A. *et al.* Med1 plays a critical role in the development of tamoxifen resistance. *Carcinogenesis* **33**, 918–930 (2012).
130. Cui, J. *et al.* Cross-talk between HER2 and MED1 regulates tamoxifen resistance of human breast cancer cells. *Cancer Res.* **72**, 5625–5634 (2012).
131. Isakoff, S. J. *et al.* Breast cancer-associated PIK3CA mutations are oncogenic in mammary epithelial cells. *Cancer Res.* **65**, 10992–11000 (2005).
132. Knudsen, E. S. & Knudsen, K. E. Tailoring to RB: tumour suppressor status and therapeutic response. *Nat. Rev. Cancer* **8**, 714–724 (2008).
133. Wu, C. C., Yu, C. T., Chang, G. C., Lai, J. M. & Hsu, S. L. Aurora-A promotes gefitinib resistance via a NF-κB signaling pathway in p53 knockdown lung cancer cells. *Biochem. Biophys. Res. Commun.* **405**, 168–172 (2011).
134. Sequist, L. V. *et al.* Genotypic and histological evolution of lung cancers acquiring resistance to EGFR inhibitors. *Sci. Transl. Med.* **3**, 75ra26 (2011).
135. Watanabe, S. *et al.* Clinical responses to EGFR-tyrosine kinase inhibitor retreatment in non-small cell lung cancer patients who benefited from prior effective gefitinib therapy: a retrospective analysis. *BMC Cancer* **11**, 1 (2011).
136. Turke, A. B. *et al.* Preexistence and clonal selection of MET amplification in EGFR mutant NSCLC. *Cancer Cell* **17**, 77–88 (2010).
137. Di Nicolantonio, F. *et al.* Cancer cell adaptation to chemotherapy. *BMC Cancer* **5**, 78 (2005).
138. Board, R. E. *et al.* Isolation and extraction of circulating tumor DNA from patients with small cell lung cancer. *Ann. NY Acad. Sci.* **1137**, 98–107 (2008).
139. Chen, X. *et al.* Detecting tumor-related alterations in plasma or serum DNA of patients diagnosed with breast cancer. *Clin. Cancer Res.* **5**, 2297–2303 (1999).
140. Wang, M., Block, T. M., Steel, L., Brenner, D. E. & Su, Y. H. Preferential isolation of fragmented DNA enhances the detection of circulating mutated k-ras DNA. *Clin. Chem.* **50**, 211–213 (2004).
141. Page, K. *et al.* The importance of careful blood processing in isolation of cell-free DNA. *Ann. NY Acad. Sci.* **1075**, 313–317 (2006).
142. Guadalajara, H. *et al.* The concentration of deoxyribonucleic acid in plasma from 73 patients with colorectal cancer and apparent clinical correlations. *Cancer Detect. Prev.* **32**, 39–44 (2008).
143. Thijssen, M. A., Swinkels, D. W., Ruers, T. J. & de Kok, J. B. Difference between free circulating plasma and serum DNA in patients with colorectal liver metastases. *Anticancer Res.* **22**, 421–425 (2002).
144. Andersen, R. F., Spindler, K. G., Jakobsen, A., Pallisgaard, N. Plasma is superior to serum for cfDNA mutation detection and monitoring [abstract 479]. *Eur. J. Cancer* **48** (Suppl. 6), 148–149 (2012).
145. Morgan, S. R. *et al.* Comparison of KRAS mutation assessment in tumor DNA and circulating free DNA in plasma and serum samples. *Clin. Med. Insights Pathol.* **5**, 15–22 (2012).
146. Müller, I. *et al.* Identification of loss of heterozygosity on circulating free DNA in peripheral blood of prostate cancer patients: potential and technical improvements. *Clin. Chem.* **54**, 688–696 (2008).
147. Liu, P. *et al.* Potential clinical significance of plasma-based KRAS mutation analysis using the COLD-PCR/TaqMan®–MGB probe genotyping method. *Exp. Ther. Med.* **4**, 109–112 (2012).
148. Chen, Z. *et al.* Analysis of cancer mutation signatures in blood by a novel ultra-sensitive assay: monitoring of therapy or recurrence in non-metastatic breast cancer. *PLoS ONE* **4**, e7220 (2009).
149. Heitzer, E. *et al.* Tumor associated copy number changes in the circulation of patients with prostate cancer identified through whole-genome sequencing. *Genome Med.* **5**, 30 (2013).
150. Li, C. N. *et al.* Cell-free DNA is released from tumor cells upon cell death: a study of tissue cultures of tumor cell lines. *J. Clin. Lab. Anal.* **17**, 103–107 (2003).
151. Haas, J., Katus, H. A. & Meder, B. Next-generation sequencing entering the clinical arena. *Mol. Cell. Probes* **25**, 206–211 (2011).

© 2013 Macmillan Publishers Limited. All rights reserved

152. Santarpia, L. *et al.* Mutation profiling identifies numerous rare drug targets and distinct mutation patterns in different clinical subtypes of breast cancers. *Breast Cancer Res. Treat.* **134**, 333–343 (2012).
153. Press, M. F. *et al.* Diagnostic evaluation of HER-2 as a molecular target: an assessment of accuracy and reproducibility of laboratory testing in large, prospective, randomized clinical trials. *Clin. Cancer Res.* **11**, 6598–6607 (2005).
154. McBride, D. J. *et al.* Use of cancer-specific genomic rearrangements to quantify disease burden in plasma from patients with solid tumors. *Genes Chromosomes Cancer* **49**, 1062–1069 (2010).
155. Tamborini, E. *et al.* Functional analyses and molecular modeling of two c-Kit mutations responsible for imatinib secondary resistance in GIST patients. *Oncogene* **25**, 6140–6146 (2006).
156. Choi, Y. L. *et al.* EML4-ALK mutations in lung cancer that confer resistance to ALK inhibitors. *N. Engl. J. Med.* **363**, 1734–1739 (2010).
157. Kakimoto, Y., Yamamoto, N. & Shibahara, T. Microsatellite analysis of serum DNA in patients with oral squamous cell carcinoma. *Oncol. Rep.* **20**, 1195–1200 (2008).

Acknowledgements
We would like to thank Giulia Siravegna and Elisa Scala (IRCC, Institute for Cancer Research at Candiolo, Italy) for their assistance with technical aspects concerning methods of DNA extraction from blood. Work in the authors' laboratories is supported by 'Fondazione Piemontese per la Ricerca sul Cancro—ONLUS grant 'Liquid Biopsy—5 per mille 2010 Sanità'; AIRC 2010 Special Program Molecular Clinical Oncology 5xMille, Project n. 9970 (A. Bardelli); AIRC, grants MFAG 11349 (F. Di Nicolantonio) and IG 12812 (A. Bardelli); 'Fondazione Piemontese per la Ricerca sul Cancro—ONLUS grant 'Farmacogenomica—5 per mille 2009 MIUR'—(F. Di Nicolantonio); the European Community's Seventh Framework Programme under grant agreement n. 259015 COLTHERES; Intramural Grants Fondazione Piemontese per la Ricerca sul Cancro ONLUS (5 per mille 2008 Ministero dell'Istruzione, dell'Università e della Ricerca) to A. Bardelli and F. Di Nicolantonio. E. Crowley is supported by the Marie-Curie Incoming Fellowship.

Author contributions
E. Crowley researched the data for the article, and all authors made a substantial contribution to discussion of the content, to writing the article and to reviewing or editing the manuscript before submission.

© 2013 Macmillan Publishers Limited. All rights reserved