A new generation of cancer genome diagnostics for routine clinical use: overcoming the roadblocks to personalized cancer medicine

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Received 15 October 2014; revised 24 February 2015; accepted 7 April 2015

The identification of ‘druggable’ kinase gene alterations has revolutionized cancer treatment in the last decade by providing new and successfully targetable drug targets. Thus, genotyping tumors for matching the right patients with the right drugs have become a clinical routine. Today, advances in sequencing technology and computational genome analyses enable the discovery of a constantly growing number of genome alterations relevant for clinical decision making. As a consequence, several technological approaches have emerged in order to deal with these rapidly increasing demands for clinical cancer genome analyses. Here, we describe challenges on the path to the broad introduction of diagnostic cancer genome analyses and the technologies that can be applied to overcome them. We define three generations of molecular diagnostics that are in clinical use. The latest generation of these approaches involves deep and thus, highly sensitive sequencing of all therapeutically relevant types of genome alterations—mutations, copy number alterations and rearrangements/fusions—in a single assay. Such approaches therefore have substantial advantages (less time and less tissue required) over PCR-based methods that typically have to be combined with fluorescence in situ hybridization for detection of gene amplifications and fusions. Since these new technologies work reliably on routine diagnostic formalin-fixed, paraffin-embedded specimens, they can help expedite the broad introduction of personalized cancer therapy into the clinic by providing comprehensive, sensitive and accurate cancer genome diagnoses in ‘real-time’.

Key words: cancer genome diagnostics, FFPE, cancer, personalized medicine

introduction

The discovery of activating EGFR mutations in lung adenocarcinoma was the starting point for several important discoveries uncovering the vulnerability of selected solid tumors to specific kinase inhibitors [1–3]. Following the clinical success of treating EGFR-mutant tumors with EGFR kinase inhibitors, the concept of ‘oncogene addiction’ (i.e. the dependency of the tumor on a genetically activated oncogene) and molecular testing were soon transferred to ALK rearranged tumors, thus shifting away from treatments based solely on histopathological examination [4, 5]. Today, a broad range of genome alterations in lung cancer is known to induce oncogene addiction, of which several can be treated with targeted therapies [6–10]. Even though newly discovered oncogenes are usually present in only a small fraction of tumors of a given cancer type, some of these (e.g. rearrangements affecting the ROS1 or RET kinase genes) are highly susceptible to already available or newly developed kinase inhibitors [11–14]. These developments are not limited to lung cancer, but have similarly changed routine medical care of patients with other types of tumors. Examples are BRAF-mutant melanomas that are sensitive to BRAF and MEK inhibition [15, 16], gastrointestinal stromal tumors bearing KIT or PDGFRA mutations, which can be treated with inhibitors targeting these kinases [17], or chronic myelogenous leukemia, in which the BCR-ABL kinase fusion can be treated with ABL kinase inhibitors [18]. Considering the ongoing efforts in cancer genome sequencing and drug discovery and development, we expect the number of clinically relevant genome alterations to increase dramatically within the next few years [15, 19–22].

As a consequence of the steadily increasing number of genes that need to be tested across several tumor types, the requirements for comprehensive cancer genome diagnosis to match the right patients with the right drugs are increasing at a dramatic pace. Furthermore, resistance mutations eventually develop in most patients during treatment with targeted therapies. Thus, molecular determination of the mechanism of resistance followed by new therapies that can overcome resistance is essential for long-term tumor control [23–29]. Of note, several of these ‘next-generation’ drugs are based on a precise molecular
understanding of the mechanisms of resistance and show impressive clinical activity [26, 30–33]. Today, EGFR mutations as well as ALK rearrangements are routinely tested in the clinic, thus leading to dramatic improvements in the clinical care of these patients; similar developments are happening in melanoma, colorectal cancer, breast cancer and several other tumor types [15, 21, 34, 35]. Together, these observations (growing number of genome alterations, increasing evidence of unprecedented efficacy of the resulting treatment and the requirements to analyze biopsies obtained at the time of relapse) illustrate the requirement for (i) comprehensive, (ii) scalable and (iii) sensitive, genome diagnostics that can provide a diagnosis within a time frame (iv) that permits first-line treatment of the patient (typically 2 weeks).

challenges

With more and more compounds in clinical development, genomic testing for more and more genes is needed to preselect patients according to their specific pattern of cancer genome alterations. Furthermore, the discovery of more and more oncogenes as well as the inevitable development of resistance mutations after treatment with targeted therapies leads to an expanding panel of genes that need to be tested. The routine clinical testing of such growing number of alterations faces several challenges.

First, only limited biopsy tissue might be available to conduct genome analyses. Especially in patients with lung cancer it may be difficult to obtain larger tumor specimens due to the tumor location or the patients’ comorbidity. Thus, fine-needle biopsies are frequently used for primary diagnosis, which are very small and often have low tumor content [36]. Low tumor content represents a major challenge to accurate cancer genome diagnosis because the genes that are mutant in the tumor are not mutated (wild type) in the infiltrating non-tumoral cells (Figure 1). As a consequence, the resulting wild-type signals will inevitably contaminate the DNA of the specimen and thereby mask the mutant signal of the tumor.

Second, routine biopsy tumor specimens are usually formalin-fixed and paraffin-embedded (FFPE), thereby leading to fragmentation of the DNA and to introduction of fixation-related artifacts [37]. These artifacts are in particular challenging when PCR amplification is involved because they can lead to false-positive mutation calls.

Third, the structural differences of the relevant genome alterations that need to be interrogated in a given tumor specimen (i.e. point mutations, small insertions and deletions, somatic copy number alterations (SCNAs) as well as genomic rearrangements/fusions) pose enormous technological challenges to their detection.

Fourth, the broad range of genome alterations needs to be analyzed and interpreted in a way that allows distinguishing the high numbers of functionally irrelevant ‘passenger’ mutations that are found at particularly high numbers in carcinogen-induced tumor types from those ‘driver’ mutations that are oncogenic, clinically relevant and targetable. This challenge is higher in diagnostic sequencing compared with scientific, discovery-oriented, sequencing because a matched normal control DNA sample of the patient is typically not available. Thus, distinguishing rare (mostly irrelevant) germline variants from true somatic mutations is particularly difficult. Broadly, handling and meaningful analysis and interpretation of large genomic datasets clearly represents one of the major bottlenecks to the broad implementation of diagnostic cancer genome analyses. Furthermore, such analytical approaches typically involve massive computational infrastructure to enable the proper storage, processing, analysis and interpretation of the resulting large datasets.

Figure 1. Tumor content and sequencing coverage are critical parameters to reliably detect mutations in clinical specimens. An exemplary schematic of a tumor section containing 30% tumor cells and 70% nontumorous cells is shown. Wild-type alleles are shown as black bars, mutant alleles are shown as red bars (left). Dideoxy-sequencing provides poor mutation signal intensities which are difficult to distinguish from background noise (arrow). The exact proportion of mutant reads in the sample cannot be determined (upper right). Low mean sequencing coverage (as it is often the case in whole-genome sequencing) leads to higher coverage in some but insufficient sequencing depth in every clinically relevant genomic region. The region of interest (indicated by a vertical dotted line) may be covered with only a few or no sequencing reads at all. Thus, a mutation at that position may not be detected reliably (middle). Massively parallel next-generation sequencing affords sequencing coverage of several 100-fold on all clinically relevant regions. Thus, mutation calls can be generated with high confidence and in a statistically meaningful fashion (lower right).
Fifth, to enable a general access of the public to such comprehensive genomic testing, such tests need to be affordable. They should also be scalable to accommodate newly discovered genes and their price should not increase significantly with an increasing number of genes.

Even though the simple process of genotyping tumors for the presence or absence of mutations appears trivial, the complexity of these challenges and their combined impact on the performance on test results pose a substantial roadblock to the broad introduction of personalized cancer therapy into the clinic.

generations of cancer genome analytics

Advances in sequencing technology and computational biology have led to the development of several approaches that are currently used to detect therapeutically relevant genome alterations in cancer specimens in the clinical routine. These may be subdivided into three generations of development.

first generation of molecular diagnostics, dideoxy-sequencing and FISH

Conventional dideoxy- (or ‘Sanger’-) sequencing has been used for many years for the detection of point mutations as well as small insertions and deletions (Figure 2). Dideoxy-sequencing has low associated costs when sequencing only individual gene regions (e.g. frequently mutated exons). However, the heterozygous nature of most mutations, as well as contamination with non-cancerous cells (e.g. fibroblasts and lymphocytes) dilute the mutation signal to a degree that makes reliable detection of mutations across a broad range of clinical specimens impossible for dideoxy-sequencing (Figure 1) [38–40]. Thus, this technique typically lacks the sensitivity required for accurate cancer genome diagnosis. In addition to the relatively low sensitivity of dideoxy-sequencing and the inability to scale the procedure to larger number of genes, the detection of SCNAs and genomic rearrangements is not possible. For this purpose, fluorescence in situ hybridization (FISH)-based microscopy has to be carried out in parallel in most cases. Even though well established and powerful, this technology has to be applied separately for each genomic alteration to be tested and is very laborious and difficult to standardize due to visual analysis and interpretation (Figure 2).

second generation of molecular diagnostics, PCR amplicon multiplex gene panel testing for mutation detection and FISH

To overcome some of the limitations of dideoxy-sequencing, several approaches have been developed over the past several years aimed at analyzing the mutational signature of each tumor sample across several genes (in ‘multiplex’) at high sensitivity. Today, a broad range of different techniques have been established, including mass-spectrometric genotyping, real-time PCR and multiplexed PCR-based massively parallel ‘next-generation sequencing’ [34, 41, 42]. All of these approaches show enhanced analytical sensitivity to enable the detection of somatic mutations at low allele frequency as it often occurs in primary tumor specimens (Figure 1). A broad range of therapeutically relevant mutations can be detected in parallel, thereby providing results faster, with lower amounts of input material across a large number of genes. However, methods based on genotyping (i.e. mass-spectrometric genotyping and real-time PCR approaches) have the inherent disadvantage that each individual mutation has to be prespecified to be detected. Thus, mutations that differ on a nucleotide level but show the same amino acid change as well as rare mutations at other positions will not be detected if they are not part of the prespecified panel. As a result, genotyping approaches are often limited to the most frequent genome alterations and cannot be used to discover novel and rare mutations.

Approaches based on PCR amplification of candidate regions (e.g. frequently mutated exons of candidate genes) followed by massively parallel next-generation sequencing avoid this problem by direct amplicon sequencing (Figure 2). Next-generation sequencing affords high sensitivity for detection of mutation because every base of interest can be independently queried and analyzed multiple times. This ‘massively parallel’ interrogation of candidate bases not only compensates for limited accuracy of an individual sequencing read. It also overcomes problems associated with tumor impurity by providing statistically meaningful representations of all mutant and wild-type alleles in a given sample (Figure 1). The number of sequencing reads covering a given base is the ‘coverage’ and can be seen as a measure of sequencing depth and thus, sensitivity. In diagnostic sequencing, the coverage is typically several 100-fold, whereas in research projects aimed at discovering new mutations, it frequently is as low as only 30-fold (Figure 1).

As a major caveat, PCR-based multiplex sequencing approaches require initial amplification of the region of interest. As with all technologies that rely on high-level PCR amplification, false-positive results due to nucleotide changes introduced by the polymerase may occur. Especially in samples with very low amounts of input DNA, the low library complexity can (i) lead to false-positive results by overrepresenting FFPE and polymerase artifacts and (ii), more importantly, may lead to false-negative results in samples with low proportion of mutant reads in the sample even though a successful DNA amplification might suggest successful performance of the assay [43]. Due to the nucleotide composition of most intronic regions harboring genomic rearrangements as well as the heterogeneity of genomic breakpoints eventually leading to the same gene fusion, amplification by PCR of these regions is extremely challenging to impossible. Thus, in order to enable the detection of genomic rearrangements, FISH analysis has to be carried out in parallel. In light of the often small amounts of tissue, the requirement of storing additional sections for subsequent FISH analyses may thus reduce the total amount of tumor DNA available for PCR amplification further, with the consequences described above (Figure 2).

third generation of molecular diagnostics, hybrid capture-based massively parallel next-generation sequencing

The key advantage of third-generation molecular diagnostics is the ability to detect the full spectrum of clinically relevant genomic alterations (point mutations, small insertions and deletions, copy number alterations as well as genomic rearrangements/fusions) in a single assay (Figure 2). In contrast to
Figure 2. Workflows are shown of first- (top), second- (middle) and third-generation (bottom) molecular diagnostics. Top: Dideoxy-sequencing of each exonic region of interest is carried out separately to detect point mutations and small insertions and deletions with a lack of sensitivity necessary to detect mutations at low allele frequency. Somatic copy number alterations as well as gene fusions cannot be detected using this technique. Thus, fluorescence in situ hybridizations for each genomic alteration of interest have to be carried out in parallel (colored dots in the schematic nuclei on the right). Taken together, both approaches exhaust high amounts of tumor tissue and are time consuming. Middle: PCR amplicon gene panel testing is used to detect mutations in limited amounts of tumor tissue in a multiplexed fashion. Somatic copy number alterations as well as gene fusions need to be analyzed by fluorescence in situ hybridizations, thus exhausting additional tumor tissue and requiring manual inspection and analysis. Bottom: Hybrid capture-based massively parallel next-generation sequencing uses limited tumor material to enrich the genomic regions of interest followed by massively parallel next-generation sequencing and subsequent computational analysis. By enriching the genomic regions of interest without PCR amplification, the full spectrum of clinically relevant genomic alterations (point mutations, small insertions and deletions, copy number alterations as well as genomic rearrangements/fusions) can be detected in a single assay using only limited tumor material in real time. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Genetics [38].
methods that rely on PCR amplification, ‘third-generation’ cancer genome analysis methods involve hybrid capture-based enrichment of the genomic region of interest, followed by massively parallel next-generation sequencing and subsequent computational analysis [44–49]. Sequencing at very high depth (typically, several 100-fold coverage) ensures the necessary sensitivity (Figure 1). At the same time, the use of randomly fragmented (shotgun) libraries followed by enrichment of candidate regions by hybridization to specific oligonucleotides allows (i) capturing of the large intronic regions where rearrangements/fusions occur, (ii) statistically meaningful representation of gene amplifications and deletions. By using long oligonucleotides for hybridization, changes in sequence composition do not prevent efficient enrichment of candidate regions. Thus, all types of cancer genome alterations can be enriched from a single analyte (DNA) (Figure 2).

Unlike technologies that rely on PCR amplification, this approach enables successful enrichment and detection of gene fusions in the same assay. Another major advantage of capture-based massively parallel sequencing is the possibility to detect previously unknown oncogenic gene fusions. By using sequence-specific hybridization to enrich the nonkinase partners of recurrent kinase fusions (e.g. CD74 as the nonkinase partner of CD74-ROS1 fusions), previously unknown fusion partners are also enriched and identified by direct sequencing (e.g. CD74-NRG1, [46, 50]). Copy number alterations can be detected based on the varying abundance of region-specific DNA fragments because when sequencing shotgun libraries—but only then—the number of sequence reads is directly proportional to the copy number at that locus. As a consequence, neither FISH nor other techniques are required to detect copy number alterations and rearrangements/fusions, thereby saving precious tumor tissue and time [51].

With decreasing sequencing costs, the addition of newly discovered oncogenes to a current diagnostic gene panel affords stable assay costs. Furthermore, the amount of input material needed (currently <100 ng of DNA) is independent of the number of genes to be analyzed.

Following sequencing, complex computational algorithms are used to process the large amounts of data and to detect point mutations, small insertions and deletions, copy number alterations as well as genomic rearrangements in a reliable fashion. Compared with the technologies described above, a more substantial IT infrastructure is required to handle the amount of data generated. First, big datasets need to be run through several consecutive processes requiring fast data handling, high-performance computation as well as solid version control and sample tracking to enable smooth high-throughput data analysis. Second, algorithms that call all types of genome alterations need to be tested, improved and validated to generate robust data at clinical confidence levels. Third, due to the considerable size of sequenced regions (several 100 kilobases up to megabases), a large number of candidate variants needs to be analyzed for clinical significance and biological relevance. Data output needs to be implemented in constantly updated databases to allow consistent data interpretation and fast access to available annotations to distinguish polymorphisms and germline mutations from likely somatic events. This aspect is of particular importance in the context of diagnostic sequencing because of the absence of a matched normal control DNA and impacts PCR-based as well as capture-based approaches. Thus, rare germline variants may be suggestive of a mutation, thus causing a misleading diagnosis and possibly, choice of treatment. While such rare germline variants are almost impossible to formally exclude from any analysis, sophisticated computational algorithms are capable of recognizing true mutations by a combination of different features, thereby dramatically limiting the impact of this problem.

By generating high sequencing coverage, all types of genome alterations can be detected even at very low allele frequency in a digital manner and therefore ranked based on the proportion of mutant reads in the sample and clinical relevance [52]. The Next Generation Sequencing: Standardization of Clinical Testing (Nex-StoCT) workgroup, convened by the US Centers for Disease Control and Prevention (CDC), published a summary describing some of these suggested procedures including validation and quality control [53].

After filtering for clinically relevant genomic alterations, physicians can select the right treatment option for each patient individually by considering the complete spectrum of relevant alterations as well as the overall condition of the patient and the line of treatment. This may include approved drugs for the respective tumor type, but also, in some cases, in which no approved drug or suitable clinical trials are available, drugs approved for other tumor types that harbor the same or similar mutations as well as experimental drugs or drug combinations, which should—as a general rule—be tested within controlled clinical trials. Delays in generating the therapeutically relevant diagnosis—an inherent problem when testing genes sequentially (if negative for the first mutation tested)—are thus avoided (Figure 2). Patients with uncommon or rare genome alterations also benefit from such routine comprehensive diagnostic test that can help expanding the choice of available treatment options.

Recent technological improvements suggest that these third-generation cancer genome analyticals can similarly be applied to blood samples taken from cancer patients [54]. This development seems particularly exciting not only because it would help reducing the number of painful biopsies, but also because it may be used to monitor the emergence of acquired resistance and to detect tumors early—at a stage where they may still be curable by surgery.

whole-genome sequencing for routine clinical genotyping?

Whole-genome sequencing, which, in contrast to exome-sequencing, can also reliably detect gene fusions, has been proposed frequently as the future of cancer genome diagnostics. However, a number of challenges associated with this approach preclude its application in the clinical routine in the near future. First, in the upcoming years, such approaches will still be prohibitively expensive. Costs do not only arise from the enormous sequencing costs associated with sequencing an entire genome at diagnostic sensitivity (several 100-fold sequencing coverage, Figure 1), but also from costs associated with the substantial computational infrastructure required for such analyses and data storage. In addition, the vast majority
of mutations detected by whole-genome sequencing occur in noncoding regions of the genome and are therefore most likely passenger mutations or are not informative for clinical practice [47, 55–57]. Furthermore, the sheer number of bases that are sequenced lead to a number of mutations falsely considered as potentially relevant with possibly dramatic impact on diagnostic decision making. Thus, only due to the huge number of sequenced bases, the chances for detecting fixation artifacts, sequencing errors, alignment errors etc. increase the number of mutations which cannot be excluded by rigorous filtering dramatically (see below). Finally, whole-genome sequencing does not permit control over the coverage of the regions that have to be sequenced and thus, clinically relevant regions might be covered by only few sequencing reads, which are insufficient to reliably detect mutations (Figure 1). As an example, while it may not be a problem covering exon 21 of EGFR (containing the frequent L858R mutation) with only one sequencing read in a whole-genome research sequencing study aimed at making new discoveries in a large lung cancer cohort, high and accurate sequencing coverage of this locus is absolutely critical for each patient waiting for a diagnosis.

### clinical variant annotation and prioritization

Common to all sequencing-based diagnostics, the correct notation of each variant, which can reach thousands of variants per sample in the case of whole-genome sequencing, is challenging. First, the differentiation of polymorphisms and germline variants from somatic events is difficult if no matching nontumorous tissue is sequenced. Here, databases containing sequencing data of tumor normal pairs or annotated single nucleotide polymorphisms help to exclude polymorphisms from further analysis. As many polymorphisms are not annotated as such yet, further filtering is needed. Therefore, several analytical steps are required to identify the ‘fingerprints’ of true mutations and distinguish them from rare germline variants. Second, driver and passenger aberrations need to be distinguished to develop a rationale for targeted treatment. All available preclinical and clinical data have to be taken into consideration to define driver mutations of a given tumor. This can be particularly challenging in tumors with high mutation burden and only few known driver mutations (e.g. small-cell lung cancer). Mutations can

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**Table 1.**

<table>
<thead>
<tr>
<th>Glossary</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Oncogene addiction</td>
<td>Oncogene addiction describes the phenomenon of cancer cells being dependent on genetically activated oncogenes. A constant oncogene activation is essential for tumor maintenance as well as cell survival.</td>
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<tr>
<td>Copy number alteration</td>
<td>A genomic alteration leading to an increased number (amplification) or loss (deletion) of copies of a small segment of the genome (less than a few megabases).</td>
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<tr>
<td>Massively parallel sequencing</td>
<td>A high-throughput approach to sequencing DNA (or RNA), which is also known as next-generation or second-generation sequencing. Here, millions of sequencing reads are generated and aligned to a reference genome to analyze genomic variants in a statistically meaningful fashion.</td>
</tr>
<tr>
<td>Resistance mutation</td>
<td>A genomic event rendering the cancer cell resistant to treatment. Resistance mutations can occur in cis (e.g. as point mutations within the targeted protein that prevent compound binding) and in trans (e.g. activating another oncogenic signaling pathway rendering the cancer cell independent of the protein targeted by the respective drug).</td>
</tr>
<tr>
<td>Rearrangement</td>
<td>A genomic event juxtaposing genomic regions that are normally separated. Rearrangements are a result of DNA double-strand breaks and can occur in or between chromosomes.</td>
</tr>
<tr>
<td>Sequencing coverage</td>
<td>The number of sequencing reads at a particular position in the genome (e.g. base, exon or region). High sequencing coverage reduces noise and enables high specificity and sensitivity.</td>
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<tr>
<td>Tumor content</td>
<td>The fraction of tumorous cells that are present in a tumor specimen used for molecular diagnostics. Low tumor content leads to a dilution of tumor-specific mutation signals by nontumorous DNA.</td>
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<tr>
<td>Passenger mutation</td>
<td>A mutation, usually induced by external influences like carcinogens that does not influence the growth advantage of the cell in which it occurred. Thus, passenger mutations occur at a relatively low frequency due to the lack of selection pressure.</td>
</tr>
<tr>
<td>Somatic alterations</td>
<td>Genomic alterations that occur after conception; thus are only present in a subfraction of cells within the whole organism. Somatic alterations in tumor biology describe alterations that are tumor specific and are not found in nontumorous tissue.</td>
</tr>
<tr>
<td>Driver mutation</td>
<td>Driver mutations ‘drive’ tumor maintenance and development and are therefore essential for cancer cell survival (see oncogene addiction). These mutations are statistically enriched across many tumor types since they directly or indirectly confer a selective growth advantage.</td>
</tr>
<tr>
<td>Exome</td>
<td>The collection of all exons in the human genome. Exome sequencing refers to an enrichment of all protein coding exons followed by next-generation sequencing.</td>
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</table>
further be ranked based on the knowledge of clinical relevance, thus, by the oncogenic potential of each mutation as well as the therapeutic implications of a given genomic alteration. Third, the defined driver mutation needs to be matched with a certain therapy. Thus, clinical and, if not available, validated preclinical data might be used to predict tumor sensitivity to certain drugs. Certainly, the different levels of confidence associated with such annotation (e.g. data from a prospective clinical trial versus results from a study in cell lines) have to be carefully taken into account. Finally, the clinical impact of treatment selection based on comprehensive molecular diagnostics should ideally be captured in databases in order to monitor the impact on personalized cancer treatment. However, the growing number of targeted drugs as well as the different types of targetable genome alterations within and across different tumor types challenge clinical diagnostics to match drugs to patients. Hybrid capture-based massively parallel next-generation sequencing technologies afford comprehensive, sensitive, fast and cost-effective detection of all different types of genome alterations on routine tumor specimens today. These advances may therefore help to provide broad access to personalized cancer medicine to all patients (Table 1).

disclosure

JMH is a full-time employee of New Oncology/Blackfield AG and is a co-founder and shareholder of the same. RKT is a co-founder and shareholder of New Oncology/Blackfield AG. RKT received consulting and honoraria (New Oncology/Blackfield AG, Sanofi-Aventis, Merck, Roche, Lilly, Boehringer Ingelheim, Astra-Zeneca, Daiichi-Sankyo, Johnson & Johnson, Puma and Clovis) as well as research support (Merck, EOS and Astra-Zeneca).

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