Review Article

Somatic DNA mutation analysis in targeted therapy of solid tumours

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Abstract: Cancer is a disease of the genome with diverse aetiologies including the accumulation of acquired mutations throughout the genome. There has been a flood of knowledge improving our understanding of the biology and molecular genetics of melanoma, lung and colorectal cancer since the genomics era started. Translation of this knowledge into a better understanding of cell proliferation, survival and apoptosis has produced a paradigm shift in medical oncology enabling gene-based cancer treatment (called personalised or precision medicine). Somatic mutation analysis is crucial for a genomics approach since it can identify driver mutations—the "Achilles' heel" of cancer, and support clinical decision-making through targeted therapy. Nevertheless, the applications of somatic DNA testing in cancer face many challenges such as obtaining comprehensive coverage of the cancer genome with limited DNA being available, and delivering an accurate report in a timely fashion without false-negative and false-positive results. Further advances in DNA technologies and bioinformatics will overcome these issues and maximise opportunities for targeted therapy. Somatic mutation analysis will then become an integral part of cancer management for all malignancies.

Keywords: Somatic mutation; DNA testing; targeted therapy; multigene analysis; cancer genome; clinical implication; solid tumours

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Cancer is a global health burden with 13-14 million new cases and 7.6-8.2 million cancer-related deaths per annum (1,2). Great effort and huge resources have been put into "the war on cancer" for the past four decades with the goals to cure cancer or to prolong life and to improve its quality. However, progress has been slow in the overall reduction of cancer mortality. For instance, lung cancer is the leading cause of cancer-related death worldwide (2,3) yet its 5-year survival remains 16.8% in the United States (4). This is essentially unchanged over the past two decades. Similarly, the median survival for patients with advanced melanoma is only 6-9 months (5,6).

The completion of the Human Genome Project in 2003 allowed the era of genomic medicine to start (7). The core goal of the Project was to utilise genomic knowledge for better treatment, prevention and so overall reduction in health costs. Cancer was shown to be a disease of the genome with different hallmarks including genome instability and an accumulation of somatic mutations (8,9). While cancers are characterised by numerous genomic aberrations, some acquired mutation(s) may be sufficient to induce growth and impaired differentiation leading to cancer development. This powerful somatic effect has been commonly described as a *driver* mutation and the overall phenomenon as *oncogene addiction* (9,10).

Oncogene addiction becomes the rationale for targeted therapy of solid tumours enabling a model that delivers treatment with a higher probability of efficacy while at the same time lowers the risk for adverse events (3,10). This biologically consistent approach saves time compared to a more trial-and-error strategy, improves the quality of life for cancer patients, and brings economic benefits by avoiding expensive but ineffective therapies. Targeted therapies for advanced lung cancer, melanoma, colorectal cancer and gastrointestinal stromal tumour (GIST) are examples of success stories that have resulted from the translation of

knowledge gained from the Human Genome Project into informed choices in cancer treatments leading to prolonged survivals (7).

To facilitate the clinical applications of somatic DNA mutation analysis, we review the challenges involving current technologies and discuss the clinical implications of several actionable i.e., relevant to treatment, *driver* mutations in targeted therapy.

Somatic DNA mutation analysis

Clinicians and pathologists should be aware in the modern genomics-based therapeutic era that acquisition of tumour tissue in a biopsy is not just for histological diagnosis and staging, but increasingly for somatic DNA mutation analysis. Molecular characterisation is used to reveal underlying driver mutations and altered pathways which ultimately lead to more personalised or stratified targeted therapies (3).

Tumour DNA source

Somatic DNA mutation analysis requires tumour tissue to source DNA which should be extracted from a relatively pure population of tumour cells and without significant necrosis or inflammation. Surgical resection specimens are generally straightforward since tumour-rich regions are more easily located. However, small biopsies, a common source in practice, are more complex to use. There are different types of biopsies including: core biopsy, fine needle aspiration or cytology samples. The latter may be obtained through bronchoalveolar lavage, bronchial brushing, fine needle biopsy or the use of pleural/peritoneal fluids.

Small biopsies have inherent limitations since they represent a single snapshot in either time or space with the latter leading to selection bias due to heterogeneity. They also usually consist of an admixture of tumour and non-tumour cells, and require macrodissection to enrich for the tumour cell populations. Proper sampling and enrichment is needed to enhance the sensitivity and facilitate detection of low frequency mutations. Multiple sampling at primary and metastatic sites will help to overcome false negative results (11) but this option is not usually available.

Fresh tissue is ideal for somatic DNA testing because it delivers sufficient high quality DNA. While freezing has traditionally been used for storage, fresh tissue can also be kept in a preservative such as RNA*later* (Life Technologies) for a convenient transit at room temperature. In reality, the most common source of diagnostic tumour DNA is from formalin-fixed paraffin embedded (FFPE) tissue which is the traditional method for preparing tissue sections for microscopic examination. It also allows verification of tumour material in the target region and macrodissection, if necessary. FFPE blocks are convenient for transport and long-term storage.

However, formalin fixation causes DNA fragmentation and cross-linkage and may introduce artefacts through stochastic deamination and/or depurination (12,13). In order to reduce these detrimental effects on DNA, tumour tissue should be fixed in 10% neutral buffered formalin under low temperature and without excessive length exposure (14). An alcohol fixative can be excellent for molecular characterisation since cytological smears or Diff Quik (DQ) samples often yield better quality DNA in our and other's experience. Such material is air dried prior to alcohol fixation and does not require the use of formalin (15).

DNA assessment for reliable testing

Limited material remains a common obstacle for somatic DNA mutation analysis. An appropriate and validated DNA extraction method should be used to maximise the chance of obtaining suitable DNA from sparse FFPE tissue. It has been estimated that >1,000 tumour cells could be sufficient for analysis of common mutations in current clinical practice (15). In some cases with very limited material, even what comes from the needle rinse can provide useful material for analysis. Ultimately, the most sophisticated molecular techniques cannot compensate for insufficient and/or poor-quality material. Therefore, both quality and quantity of all target DNA templates should be carefully assessed before analysis.

Spectrophotometric analysis is commonly used for DNA assessment. High ratios of absorbance at 260/280 and 260/230 nm indicate samples are free from significant contaminations from protein, peptide and organic solvents. The quantity of DNA can be estimated based on the absorbance at 260 nm wavelength. However, this method may be inaccurate in FFPE samples since it can overestimate the quantity due to the presence of degraded DNA and RNA. Fluorometric measurement is a better method since it only detects double-stranded DNA. However, even double-stranded DNA may not equate to an amplifiable template due to excessive crosslink and fragmentation during formalin fixation. Various quantitative real time polymerase chain reaction (PCR)

Table 1 Common methods used in somatic DNA mutation analysis

Method	Basic technique	Advantage	Disadvantage
Sanger sequencing	PCR + fluorescent dideoxynucleotides + capillary electrophoresis	"Gold standard"; interrogate base by base	Low sensitivity; difficult for small indels; and sequential analysis (no multiplex)
Pyrosequencing	PCR + incorporated base releases a pyrophosphate that can be detected as visible light via an enzyme system	Sensitive; good for FFPE samples; high resolution without a mononucleotide repeat	Short reads; homopolymer issue and no multiplex capacity
SNaPShot	PCR + fluorescent dideoxynucleotide extension + capillary electrophoresis	Sensitive; good for FFPE samples; modest resolution; limited multiplex	Require careful design & optimisation; pre-defined mutations only
Mass spectrometry MALDI-TOF	PCR + primer extension with nucleotide terminators	Sensitive; good for FFPE samples; high resolution; detects indels; multiplex	Require careful design; pre-defined mutations only
Allele-specific real time PCR	Primers span DNA sites of interest + probes	Very sensitive; no post-PCR process	Require careful design and optimisation; difficult to differentiate nucleotides and no multiplex capacity
High resolution melting	Hetero PCR products melt at different temperatures	Very sensitive, but just for screening; no post-PCR process	No multiplex capacity; difficult to interpret melting curves
Next generation sequencing	Amplification-based library preparation + emulsion PCR + ligation-based sequencing; OR cluster generation + sequencing by synthesis	Very sensitive with deep coverage (more than 1,000×); high multiplex capacity; interrogate base by base	Relatively expensive; slightly long turnaround time; complicated data interpretation

PCR, polymerase chain reaction; MALDI-TOF, matrix-assisted laser desorption/ionization—time of flight; FFPE, formalin-fixed paraffin embedded.

or mass spectrometric methods have been developed to assess the amount and amplification potential of FFPE DNA aiming at efficient use of available but limited DNA. Mass spectrometry analysis using the Exome ID kit (Agena Biosciences) allows different lengths and genomic loci to be assessed in a single assay and consumes only minute amount of DNA (16). Accurate DNA assessment is also essential for the next generation sequencing (NGS) analysis (17).

Challenges in somatic DNA mutation profiling

The ideal method for somatic DNA mutation analysis would: (I) identify a spectrum of genomic aberrations including single nucleotide variants, small indels, copy number aberrations and even structural variants such as re-arrangements or fusion genes; (II) work in a single test and without an excessive amount of DNA; (III) identify somatic DNA mutations in a background of normal with one or more subpopulations of tumour cells i.e., has sufficient sensitivity; (IV) utilise a comprehensive list of targeted genes to maximise the opportunity for targeted therapy; (V) be cost effective and deliver results in a timely fashion to facilitate early initiation of personalised treatment. No one assay currently meets all these criteria, and there remain a number of outstanding technologic challenges (*Table 1*).

The first and foremost challenge is providing reliable and comprehensive results with limited tumour DNA. An international external quality assessment scheme has identified the major analytical methods to be Sanger sequencing and allele-specific real time PCR (*Table 1*) (18). Both amplify one DNA fragment at a time and thus consume significant amounts of DNA. Sequential analysis of various genes and regions with these approaches also limits timely delivery of results. While Sanger sequencing can interrogate every base and identify known and unknown variants, it involves many post-PCR manipulations such as clean-up of PCR and sequencing products. Its most critical disadvantage is

the low sensitivity of this "gold standard", which usually identifies a somatic DNA variant when present at 20% or more. Sanger sequencing can provide a false-negative result or no result in some cases (18-20).

The use of pyrosequencing significantly improves the sensitivity since detection is based on light emission after nucleotide incorporation (*Table 1*) (21,22). However, this method is limited by a very short sequencing read length (80-140 bp), similar post-PCR manipulations and no capacity for multiplexing.

The allele-specific real time PCR method eliminates post-PCR manipulations and is relatively simple to perform with superior sensitivity (1% detection limit) (20,22,23). However, allele-specific PCR can only identify predefined variants and requires careful design (due to limited primer options) and optimisation because of substantial risk of mispriming. Commercial kits are expensive, particularly if several are required to cover multiple genes. Allele-specific PCR is problematic when it comes to distinguishing different target nucleotides at the same position (20,23).

The accuracy of somatic DNA mutation analysis remains a concern as shown by only 72 of 91 participant laboratories passing an external quality assessment (18). Somatic DNA mutation analysis with multiplex capacity is advantageous since it can profile a multiple genes simultaneously while consuming acceptable amounts of DNA.

Extension-based assays such as SNaPShot® and mass spectrometry are commonly used for somatic DNA testing with acceptable sensitivity (*Table 1*) (24-26). Multiplex amplicons are generally around 80-120 bp, which makes the assay robust for fragmented DNA in FFPE samples. Extension-based assays are able to differentiate various nucleotides at the same position. The mass spectrometric assay has its own unique features including: (I) high resolution without nucleotide labelling or modification; (II) high multiplex capacity (up to 50 targets per reaction); (III) additional specificity related to the extension probe in specific and high multiplex PCRs (24-26). Disadvantages are primer extension assays can only detect pre-defined variants, and undefined ones will be missed. Parallel fluorescence *in situ* hybridisation (FISH) is required for the detection of cancer gene amplifications, rearrangements or gene fusions (27).

A second challenge is distinguishing false-negative results from tumour heterogeneity (11), amplification failure or high background noise. False negative results can be as high as 6.7-7.6% (18) and may deprive patients of the opportunity for targeted therapy. A common cause for this type of result is a low proportion of tumour cells. Therefore, it is important to have the slides reviewed by a tissue pathologist before molecular testing. Working with insufficient DNA templates is not uncommon due to severe fragmentation and cross-linkage even if spectrophotometric or fluorometric values suggest that a defined quantity of DNA is present. Extracted DNA that has contaminants can also lead to false-negative results. For example, melanin is frequently present in melanoma samples and requires additional clean-up to remove this PCR inhibitor.

Many allele-specific PCR assays utilise a template control in addition to the target amplicon to identify when DNA has failed to be added. However, amplification of non-target control does not necessarily mean that target template is available for amplification. Extension-based assays are preferred since they have a built-in template control and any amplicon will be interrogated by a designated extension primer irrespective of the presence or absence of a predefined mutation. Random somatic DNA variants represent another source for amplification failure if they occur at the primer binding sites. Using a set of redundant primers is helpful in all PCR-based somatic DNA tests since they reduce the interference problem due to potential binding site variants.

The third and last challenge is how to avoid false positives found in 0.6-1.4% of molecular assays (18). False-positive findings can be problematic because: (I) they lead to unnecessary treatment with what are likely to be expensive drugs; (II) the patient will be exposed to side effects associated with a drug that is unlikely to work. Treatment with the wrong drug can even accelerate tumour growth as exemplified by giving a patient with melanoma a BRAF inhibitor based on a false-positive *BRAF* p.V600E mutation result when the tumour actually carries a RAS mutation (28-30); (III) patients treated inappropriately could miss out on the optimal time for effective treatment.

As discussed above, formalin fixation can introduce artefactual changes from cytosine to uracil (becoming thymine in PCR product) due to deamination or single base deletion secondary to depurination (12,13). Such artefacts appear to be "convincing" after amplification, particularly when there is insufficient DNA. PCR amplified artefacts will not be prominent if there are sufficient DNA templates since deamination or depurination are stochastic events during formalin fixation. It is also possible to excise the introduced uracil through the uracil-DNA glycosylase digestion (13,31) provided there is sufficient amount of DNA left for somatic mutation analysis.

Role of next generation sequencing (NGS) in somatic DNA mutation analysis

NGS or massively parallel sequencing as it is also called is a promising technology for somatic DNA mutation analysis. FFPE DNA can be amplified directly or after hybrid capture in a highly multiplexed fashion. This amplification strategy overcomes the issue of limited tumour DNA and so to some extent avoids the direct use of poor quality FFPE DNA as a sequencing template. NGS can interrogate every base in particular gene targets at an acceptable coverage (>1,000×) to ensure accuracy and sensitivity (17,22). It automatically controls the target amplification. NGS has significantly high throughput and can cover the long tail pattern for mutation distribution in many solid tumours. It can readily identify single nucleotide variants and small indels, and potentially copy number aberrations and rearrangement or fusions in a single assay. NGS saves precious tumour tissue, avoids time-consuming sequential testing, and so maximises the patient's opportunity for targeted therapy. Further improvement can be achieved if redundant hybridisation probes or amplification primers can be introduced to prevent allele dropout(s) due to potential primer binding site variants.

Nevertheless, there are disadvantages with NGS when it is used for clinical diagnosis: (I) the turnaround time will be longer; (II) it is relatively expensive considering the need for specialised equipment, maintenance, sequencing costs and computer infrastructure for analysis and data storage (17); and (III) more effort and bioinformatics skills are required for data analysis. Whole exome NGS lacks the potential to detect rearrangements or fusions, while whole genome NGS is not suitable for high coverage rates with current costs. At present, these problems will make it difficult to use the NGS applications of whole exome or genome sequencing. If NGS is used, it will be targeted to particular gene(s).

Implications of molecular characterisation

In the past decade, extensive research into molecular genetics of lung cancer has identified different *driver* mutations, deciphered the underlying pathways involved in pathogenesis, and from this has emerged the concept of targeted therapy (3,14,32-34). The translation of this knowledge into clinical practice has changed the management of advanced non-small cell lung carcinoma (NSCLC) and altered its nature course. It has led to a paradigm shift in clinical oncology from organ- and morphology-based to gene-based practice. The detection of somatic DNA involving solid tumours is increasingly playing a key role by allowing more biologically relevant diagnoses to be made leading to more effective therapies to be selected (3). The same applies to detection of somatic cell based RNA and chromosomal aberrations although these are not the subject of this review.

Somatic DNA mutations in the tyrosine kinase receptors

EGFR—epidermal growth factor receptor

EGFR encodes a transmembrane receptor tyrosine kinase (*Figure 1*) and binds several ligands including the epidermal growth factor, transforming growth factor-alpha and amphiregulin. In responding to the ligand binding, the receptor forms a homodimer or heterodimer, followed by autophosphorylation in the activation loop of the catalytic tyrosine kinase domain. The active (autophosphorylated) kinase state controls downstream MAPK, PI3K and STATs pathways, regulating cell proliferation and enhancing cell survival (*Figure 1*) (34).

In NSCLC, somatic DNA mutations can occur in or close to the *EGFR* kinase domain (exons 18 to 21) (18) and activate kinase activity by abrogating autoinhibition. The most abundant mutations are small in-frame deletions in exon 19 encompassing the leucine-arginine-glutamate-alanine motive (45-50% of mutations) and the single nucleotide missense variant p.L858R (40-45%) (24,32,33). Exon 19 deletions remove residues from the activation loop and structurally impair the ability of the protein to adopt its inactive position (35-37). The p.L858R variant mutation occurs within the activation loop and leads to a shift in the kinase towards an activated state (36). Both mutation classes result in a decreased affinity for ATP, but enhanced affinity for the tyrosine kinase inhibitors (TKIs, discussed below) compared with the wild-type receptor (35-37). They represent the classical activating mutations and have oncogenic capability for transforming fibroblast and lung epithelial cells (38,39). Interestingly, activating mutations are observed in 10-15% of NSCLC in western Europeans but up to 25-30% in East Asians, particularly female non-smokers.

Currently there are two classes of *EGFR* antagonists: (I) anti-EGFR monoclonal antibodies such as cetuximab and panitumumab (40). They bind specifically to the extracellular domain of the receptor and block ligand binding, thus preventing ligand-induced *EGFR* activation. These antibodies also promote receptor internalisation and

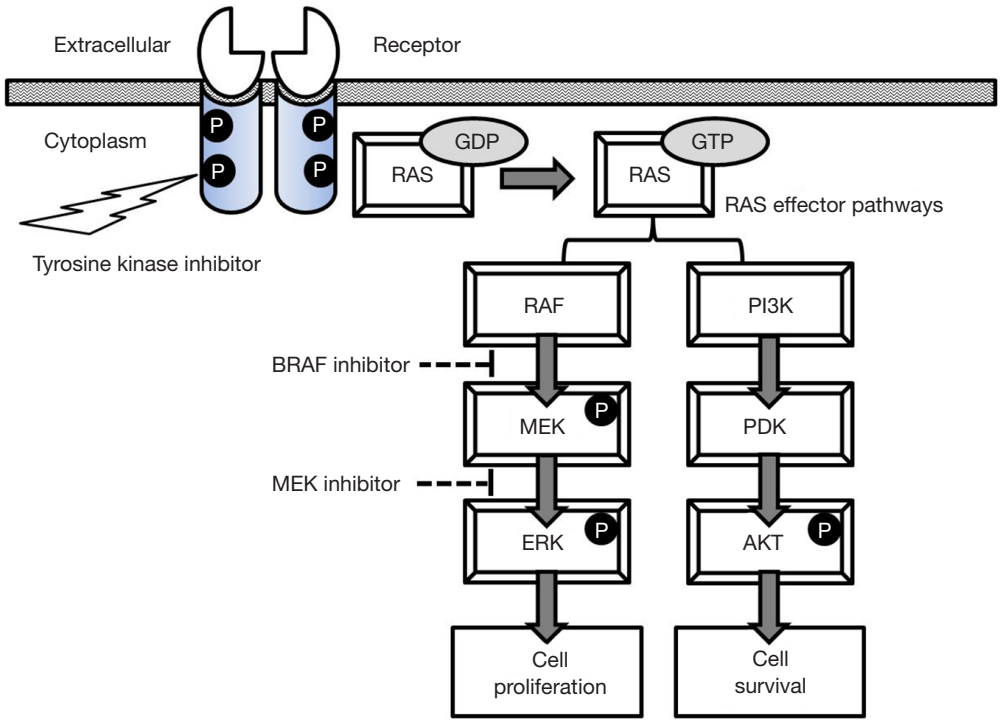

Figure 1 Canonical RAS cascades and point-of action of targeted therapies. Membrane-bound tyrosine kinase receptor: e.g., EGFR (epidermal growth factor receptor) and KIT (tyrosine-protein kinase Kit or CD117). *Mitogen-activated protein kinase (MAPK) pathway*: RAF, rapidly accelerated fibrosarcoma; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinases. *Phophoinositide 3-kinase (PI3K) pathway*: PDK, phophosinositide-dependent kinase; AKT, activate protein kinase B. Tyrosine kinase inhibitor (TKI): EGFR inhibitors include gefitinib and erlotinib as the first-generation (reversible anilinoquinazoine and ATP-mimetic), afatinib as the second-generation and AZD9291 and CO-1686 (covalent pyrimidine inhibitors) as the third-generation. KIT inhibitor is imatinib (2-phenyl-amino-pyrimidine derivative). BRAF inhibitor: sorafenib as a pan-kinase inhibitor, vemurafenib, dabrafenib and LGX818 as the selective inhibitor. MEK inhibitor: MEK162, selumetinib and trametinib as the second- and third-generations.

antibody or complement-mediated cytotoxicity; (II) small-molecule ATP-mimetic TKIs bind to the intracellular catalytic domain of the receptor to inhibit *EGFR* tyrosine phosphorylation and downstream signalling pathways (*Figure 1*). The response rates following treatment with TKIs is >58-70%, with median progression-free survival (PFS) >9 months and overall survival (OS) of 24-30 months in NSCLC patients with activating mutations (41,42). TKIs have become the most robust initial therapy for these patients (43). Other mutations including p.G719A/C/S and p.L861Q (<5%) are also associated with some TKI sensitivity although there are less data about responses in these rare mutations (44).

Several *EGFR* mutations produce resistance to targeted therapy. These include the missense mutation p.T790M, small insertions/duplications of exon 20 and missense mutations at p.S768 and p.V769 (37,39,45). p.T790M at the gatekeeper position of the ATP kinase pocket can mitigate the sensitisation effect of activating mutations (36). The underlying mechanism is due to an increase in the receptor affinity for ATP, and disruption of kinase-drug binding (36,38). Exon 20 indels (5% of mutations) disturb the structural orientation that controls ATP and TKI binding (36), affect the TKI affinity to the receptor and promote the active state of the kinase domain.

The mutation p.T790M is also the major change (50-60%) involved in acquired TKI resistance (37,45,46). TKI Afatinib has some effect on p.T790M-related resistance, but the third-generation TKIs may be even more potent (3,47). Acquired resistance can result from the bypassing of classical signalling pathways (around <15%) involving *MET* (46,48), *ERBB2* (49) and others (47,50) although they are individually uncommon and can be co-identified with p.T790M in same specimens.

KIT—V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog

KIT is a member of the type 3 transmembrane receptor tyrosine kinase family and encodes the human homolog of the proto-oncogene c-kit. Stem cell factor is its ligand. KIT has similar response to ligand binding as EGFR, i.e., receptor dimerization, autophosphorylation, receptor activation (51,52), and it regulates cell proliferation and survival (*Figure 1*).

KIT activating mutations are mostly found in the juxtamembrane exon 11 (65% of mutations) and include in-frame deletions (e.g., around p.W557 and p.K558), missense mutations (e.g., p.L576P) or complex changes. As derived from analog studies (*in silico* analysis of tyrosine receptor analogs allowing inference from known protein functions), the juxtamembrane domain could act as a negative regulator of kinase. These mutations could disrupt KIT conformational integrity and impair its regulation. Other less common clusters are on exon 9 (10%) and exon 13 (2%) (53). *KIT* exon 9 codes for the extracellular domain (the 5th immunoglobulin-like loop) and the mutations in exon 9 can activate the kinase in the absence of ligand via the stabilisation of receptor dimers (54). *KIT* activating mutations are oncogenic with a gain-of-function and have been found in 80-85% of adult GIST as well as chronic myeloid leukaemia (55), seminoma and melanoma (56-58).

Imatinib was initially developed to treat BCR-ABL chronic myeloid leukaemia (59) and has been successfully used in GIST and melanoma patients with *KIT* mutations. Imatinib selectively inhibits tyrosine kinase including ABL, BCR-ABL, KIT and PDGFR (60). It reversely binds to the ATP binding pocket of the KIT or ABL kinase and locks it in a self-inhibited conformation (61). This process inhibits kinase activity, switches off the downstream signalling pathways, leads to growth arrest and eventual apoptosis in tumour cells (62). Imatinib can achieve disease control in 70-85% of patients with KIT mutations with a median PFS of 20-24 months, and an estimated OS >36 months (63,64). TKI therapy has become the standard of care for patients with advanced GIST. Therapy is continued while patients are experiencing clinical benefit. Patients with exon 11 missense changes or insertions have a favourable prognosis, whereas those with *KIT* exon 11 p.W557-K558del and/or exon 9 mutations have a poor prognosis (65).

While TKI therapy induces GIST regression, imatinib rarely achieves complete remissions. Even long-term TKI therapy fails to eradicate tumour cells. Most patients who respond will eventually develop acquired resistance. Antonescu *et al*. reported that the 2-year survival of the imatinib-treated patients with advanced GIST was as high as 72%, but in half the disease progressed within two years (66). Innate resistance occurs in 10-15% of GIST patients. Patients with *KIT* exon 11 or 9 exon mutations or wild-type GIST have a 5%, 16% and 23 % probability of demonstrating innate imatinib resistance (67).

Acquired resistance to imatinib commonly occurs through the emergence of second-site mutations *in cis* with the original *KIT* mutations (9,66,68,69). These mutations are mainly clustered in either the ATP binding pocket (p.V654A and p.670I) or the kinase activation loop (p.C809G, p.D816H, p.D820G/A, p.N822K/Y and p.Y823D) (9,70). The mutations bypass the inhibitory effects of the drug by interference with imatinib binding or direct activation (66). In the minority GIST patients, other mechanisms may be involved such as *KIT* genomic amplification or activation of an alternative tyrosine kinase receptor (71).

Somatic DNA Mutations in RAS proteins

RAS proteins are small GTPases that cycle between an active (GTP) or an inactive GDP bound state (*Figure 1*). There are three human *RAS* genes encoding highly homologous 21 kDa proteins. *KRAS* and *HRAS* were first identified in the Kirsten and Harvey strains of mouse sarcoma virus, whereas *NRAS* represents the neuroblastoma RAS viral oncogene homologue. RAS proteins link the activation of cell surface receptors with a wide variety of cellular processes leading to the control of proliferation, differentiation and apoptosis (*Figure 1*). RAS activation can result from somatic DNA mutations, upstream activation of tyrosine kinase receptors or by loss of function of regulating tumour suppressor genes. Furthermore, oncogenic RAS proteins can interfere with metabolism of tumour cells, microenvironment remodelling, evasion of immune response, and can contribute to the metastatic process. Efforts to target RAS mutants directly have thus far been unsuccessful.

KRAS—Kirsten rat sarcoma viral oncogene homolog

KRAS missense mutations at codon 12 or 13 are most common and account for approximately 85% of somatic DNA mutations in colorectal cancer. Other activating mutations at codons 61, 117 or 146 are found in up to 15% of *KRAS* mutant cases (72-74). The replacement of p.G12 is associated with steric hindrance of GTPase-mediated GTP hydrolysis and thus promotes the formation

of constitutive activation. Glutamine substitution at codon 61 impairs GTPase activity by disrupting a hydrogen bond with GAPp120 (75). *KRAS* p.A146 mutations do not impair KRAS GTPase activity, but confer activity by increasing the rate of guanine nucleotide exchange (76). Their effect may be augmented by frequent conversion to homozygosity and low-level copy number gain of the *KRAS* gene locus (73). Both p.K117 and p.A146 missense mutations are associated with relatively lower levels of GTP-bound RAS compared to the p.G12D mutant and usually predict a more favourable clinical outcome (73,74). *KRAS* somatic DNA mutations have been identified in a variety of human malignancies, most frequently in pancreatic cancer, NSCLC and colorectal cancer.

The efficacy of anti-EGFR monoclonal antibodies is confined to patients with wild-type KRAS in metastatic colorectal patients. Therefore, *KRAS* mutation testing is critical before starting targeted therapy. Patients with *KRAS* mutations, particularly in codon 12 or 13 should not receive this therapy (75). More recently, mutations such as changes at p.K117 and p.A146 in *KRAS* exon 4 have been shown to predict a lack of benefit from anti-EGFR antibody therapy (74). *KRAS* mutations are associated in 35-45% of cases with resistance to anti-EGFR monoclonal antibody.

In NSCLC, substitutions at *KRAS* codons 12 or 13 are more common (>97% of *KRAS* mutations). They are more likely to be found in lung adenocarcinomas from former or current smokers although they are also present in around 15% of never-smokers (77). It has been reported that NSCLC with *KRAS* mutations is more likely to present with locally advanced disease and a poorer survival rate (78). *KRAS*-mutant lung tumours are resistant to EGFR TKIs (79) although *KRAS* testing has not been widely adopted in non-multigene testing in NSCLC.

***NRAS*—neuroblastoma viral (V-Ras) oncogene homolog**

Somatic NDA mutations of *NRAS* are commonly encountered in codons 12, 13 and 61. These mutations lock NRAS into a constitutively activated state by eliciting downstream effectors. Activating *NRAS* mutations are reported in 15-20% of advanced melanoma in Caucasian patients (80) and our unpublished data suggest that the prevalence of *NRAS* mutations can be up to 30% in the same ethnic group. In African and Asian populations, there is a lower frequency (12% and 7.2%, respectively) (81,82). Many studies have suggested that *NRAS* mutations are significantly more common in melanomas arising in chronic sun-damaged skin (83,84).

Newly emerged *NRAS* mutations that arise during treatment, such as p.Q61K, represent one of the resistance mechanisms for BRAF inhibitor therapy (85). *MEK* is downstream of *BRAF* in the MAPK pathway (*Figure 1*) and its inhibitor can mediate blockade of *NRAS* mutant signalling. MEK162 is an oral MEK inhibitor, which was tested in patients with advanced melanoma harbouring *NRAS* mutations. The results were encouraging, but the response rate was relatively low (<20%) (86). Most of the patients rapidly develop resistance to the MEK inhibitor. More trials are on the way to test the efficacy of new MEK inhibitors. The results so far suggest that single-agent strategies may prove insufficient in *NRAS* mutant tumours. Instead, combination strategies using a BRAF inhibitor with a MEK or AKT inhibitor may work synergistically to inhibit proliferation of tumour and resistant cells to overcome resistance. Interestingly, *NRAS* mutations in advanced melanoma can be a biomarker for response to immunotherapy since more clinical benefit was observed in those patients with NRAS mutants compared to those with RAF/NRAS wild types (87,88). Lung cancers harbouring *NRAS* mutations are a distinct subset with potential sensitivity to MEK inhibitors (89).

Somatic DNA Mutations in Serine/threonine Kinase

***BRAF*—v-raf murine sarcoma viral oncogene homolog B1**

BRAF encodes a serine/threonine kinase with the monomer representing the "off" state. BRAF can be phosphorylated at p.T599 and/or p.S602 by RAS, followed by dimerization with itself or ARAF and CRAF. RAF dimer is the "on" state and can transmit proliferative and survival signals downstream of the RAS proteins (*Figure 1*).

BRAF p.V600E represents a hot-spot for mutations accounting for at least 75% of driver mutations in this gene, followed by p.V600K (20%) and others. Position 600 and its vicinity are part of the activation loop of the kinase. The replacement of a negatively charged glutamic acid to valine can disrupt the domain conformation and mimic the conformation of the phosphorylated wild-type protein, which is necessary for kinase activation (90). It can dramatically increase BRAF activity and lead to constitutive ERK activation (*Figure 1*) (91). Recently, a crystal structure study has revealed that dimerization is a key step for RAF activation. The p.V600E missense substitution significantly contributes to the destabilisation of the "off" conformation and the stabilisation of the "on" conformation through salt-bridge interactions (92). *BRAF* somatic DNA mutations

have been found in 50% patients with advanced melanoma as well as 10% of colorectal cancer, 40% papillary thyroid cancer and others (93-95).

The outlook for advanced melanoma has been transformed with BRAF inhibitors including vemurafenib, dabrafenib and LGX818 in the past a few years (88). These inhibitors are selective ATP-competitors and can stabilise BRAF mutants in the ATP pocket (96). Vemurafenib and dabrafenib have a response rate of 48% compared to 5% with standard chemotherapy (6,85,97). The median OS increased to 13-16 months, but PFS was only 5-7 months. The limited data have suggested that low-activity *BRAF* mutations such as p.L597S can be suppressed by MEK inhibitor both *in vitro* and in melanoma patient (98).

Early development of resistance is the major drawback of BRAF inhibition therapy. Resistance can be attributed to several factors including induction of alternative splice variants of *BRAF* or *de novo* mutations in *NRAS* or *MEK*. BRAF splice variants lack the RAS-binding domain, but retain RAF kinase activity in the presence of vemurafenib secondary to their enhanced homodimerisaton (99,100). Upregulation of signalling through receptor tyrosine kinase in alternative proliferative pathways is also associated with both innate and acquired resistance (101). Inhibited BRAF can still activate the pathway through dimerization with CRAF (29,102).

A complete inhibition of the MAPK pathway can be achieved by the combination of BRAF and MEK inhibitors (*Figure 1*) (87). Combination treatment can delay or prevent MAPK-dependent resistance and reduce BRAF inhibitor related toxicities as a result of paradoxical activation of the MAPK pathway in non-melanoma *BRAF* wild-type cells (28,87). This strategy has proved effective and reduces the incidence of secondary malignancies arising from off target promotion of RAS mutant cancers such as squamous cell carcinoma (28). The combination of dabrafenib with trametinib in BRAF "addicted" cancers showed a significant higher response rate (76% *vs.* 54%) and significantly longer PFS (9.4 months versus 5.8 months) than monotherapy with dabrafenib (103). It has been proposed that this type of combination therapy along with immunotherapies is likely to replace BRAF inhibitor monotherapy as the preferred first-line MAPK inhibitor treatment for BRAF-mutant metastatic melanoma in the near future (3,88).

Somatic DNA mutations identified through multigene analysis

Multigene profiling can provide useful and, at times, unexpected information for clinical decision making. Somatic DNA mutations in *EGFR*, *KRAS* or *NRAS* and *BRAF* are generally mutually exclusive. Identification of one gene mutation provides confidence in a negative result for mutations in other genes. Patients with melanoma or GIST can benefit from simultaneous profiling of *BRAF* and *KIT*. Although rare, *KIT* mutations are found in melanomas from acral melanoma, mucosal melanoma and melanoma located in sun-damaged skin (57,104). It has also been reported that 15% of anal melanomas harboured a *KIT* mutation (104).

KIT TKIs (imatinib and sunitinib) are of interest in terms of melanoma treatment. Sunitinib has shown a clinical response in three of four *KIT*-mutated melanomas, but only in one of six melanomas with KIT amplification only (105). Melanoma patients with *KIT* mutation had a better outcome after imatinib treatment compared with those having *BRAF* inhibitor treatment. On the other hand, a primary *BRAF* mutation can be found in 7-13% of adult GIST patients who lack KIT/PDGFRA mutations (106). *BRAF* mutations can also be associated with acquired resistance when KIT-dependant GIST is treated with imatinib (107).

As discussed in the section on RAS proteins, the presence of wild-type KRAS is required but not sufficient to confer sensitivity to anti-EGFR monoclonal antibodies. The expression levels of EGFR ligands, increased *EGFR* copy number, *NRAS* or *BRAF* mutations and *PTEN* loss may contribute to non-responsiveness with therapy. Wild type status in the above genes is associated with an improved objective response, longer median PFS and OS. The finding of *BRAF* mutations in colon cancer can have different implications, for example, there may be no role for anti-EGFR treatment because of a rapid feedback activation mechanism (108). The inhibitory effect of the driver mutation p.V600E can evoke a rapid feedback activation of *EGFR* and support continued proliferation (108,109).

The activation of multiple pathways may be complementary and interchangeable across different cancers. *BRAF* mutation can trigger resistance to TKIs in *EGFR*-mutant lung cancer (110), while *EGFR* mutation can mediate resistance to vemurafenib in colon cancers with the *BRAF* p.V600E mutation (111). Newly emerged oncogenic RAS mutation has been shown to have high risk for the development of secondary malignancies in patients with selective BRAF inhibition therapy (28,112). Therefore, it is important to use a multigene analysis approach to monitor the underlying molecular changes at different stages during cancer treatment.

Conclusions and future prospects

Current insight into the cancer genome, particularly the identification of driver mutations, has invigorated the campaign against cancer. The stunning initial success in personalised targeted therapy has boosted optimism that cancer can be cured, and success in genomic medicine will continue to gain momentum. Genomic application will extend to paediatric malignancies, particularly to sarcomas (113). NGS screening of enriched targeted transcripts can be developed for more effective and accurate detection of a variety of driver fusion genes (114). Fusion gene identification will help the diagnosis and provide the prognostic information. Ultimately, it may lead to effective targeted therapies such as Crizotinib or Ceritinib for ALK-EML4 fusion gene in NSCLC (115). Cancer somatic DNA mutation analysis is crucial for precision oncology and tomorrow's cancer treatment will be more dependent on mutations in a tumour than on the organ in which the cancer arises. Further research involving single tumour cell analysis might provide insights into inter- and intra-patient tumour heterogeneity, and tumour genomic, genetic and epigenetic evolution (3), which will help to overcome resistance to current targeted therapies.

Multiple sampling of blood from a cancer patient is described as a "liquid biopsy". It allows circulating cell-free DNA derived from tumour to be monitored and analysed before, during and after the treatment (3,22). Tumour burden, residual disease, resistance and early relapse can be objectively detected during treatment. This level of molecular information will assist oncologists to be proactive rather than reactive once resistance or relapse is first detected.

Today, the bottleneck in cancer DNA mutation profiling is less related to data generation but revolves around data analysis, display and integration. More innovative approaches are essential, for example identifying and excluding formalin induced artefacts, detecting low frequency mutations and differentiation of driver mutations from a sea of *passenger* variants. We need the capability to visualise the vast data sets, identify hidden patterns and potential "Achilles' heel" for particular cancers (10) as well as retrieve the relevant data for pharmacokinetic and pharmacodynamic consideration.

Data integration is essential in a multidimensional way to allow a "Google map" view for ease of use and interpretation. From such a map one can find not only the location of a target, but also its function and matched intervention(s). Contemporary knowledge from a variety of databases needs to be integrated in ways that allow better data interpretation, and to assist in decision making for individual cancer patients (3). As the numbers of tumour genome profiles grow, it is inevitable that new targets for treatment will be detected. Ultimately, improvements in patient outcomes will follow.

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Footnote

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