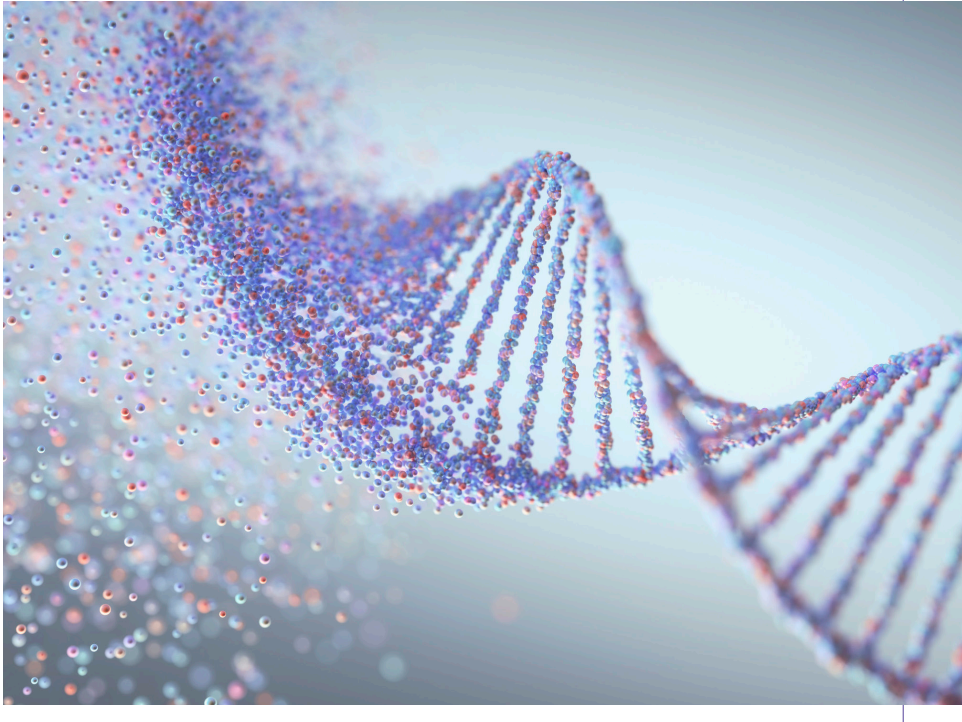
FAST FACTS

Comprehensive Genomic Profiling

Bernardo L Rapoport, Giancarlo Troncone, Fernando Schmitt and Simon J Nayler



Making precision medicine possible

FAST FACTS

Comprehensive Genomic Profiling

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Declaration of Independence
This book is as balanced and as practical as we can make it.
Ideas for improvement are always welcome: fastfacts@karger.com

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Glossary and list of abbreviations

Note that for genes and proteins, the protein name is shown here. In the text, a gene symbol for a gene encoding a protein is usually the same as the abbreviation for that protein, but italicized. For up-to-date gene symbols, please see www.ncbi.nlm.nih.gov/gene

ABL: ABL proto-oncogene 1, non-receptor tyrosine kinase

ACRIN: American College of Radiology Imaging Network

Adapters: sequences of DNA ligated onto DNA/RNA fragments to allow PCR and sequencing

AKT kinases: three closely related serine/threonine-protein kinases (AKT1, AKT2 and AKT3)

ALK: anaplastic lymphoma kinase

AMP: Association for Molecular Pathology

APC: APC regulator of Wnt signaling pathway

ASCO: American Society of Clinical Oncology

Barcode: specific sequences added to DNA/RNA fragments to allow sample identification in multiplexed reactions

BCR: BCR activator of RhoGEF and GTPase

bp: base pair

BRAF: serine/threonine-protein kinase B-raf

BRAF **p.V600E:** *BRAF* gene with missense mutation that causes a glutamic acid, rather than a valine, to occur at amino acid 600 in the polypeptide chain

BRCA1/2: BRCA1 DNA repair associated/BRCA2 DNA repair associated

BREAK-3: Phase II trial of dabrafenib versus acarbazine in patients with BRAF p.V600E-positive mutation metastatic melanoma

CAP: College of American Pathologists

CCDC6: coiled-coil domain containing 6

ccf: circulating cell-free

CDK4/6: cyclin-dependent kinase 4/6

CDKN2A: cyclin-dependent kinase inhibitor 2A

cDNA complementary DNA, synthesized from single-stranded RNA, such as mRNA

CDx: companion diagnostic

CGP: comprehensive genomic profiling; the evaluation of all the genes within a person, a specific cell type or disease

CheckMate: a series of trials of nivolumab, an anti-PD-1 agent, in various malignancies

ChIP-seq: chromatin immunoprecipitation sequencing

CIMP: CpG island methylator

CIN: chromosomal instability

c-KIT: KIT proto-oncogene, receptor tyrosine kinase

CNV: copy-number variant, a variation in the number of copies of a particular gene among people

CRC: colorectal cancer

CTCs: circulating tumor cells

ctDNA: circulating tumor DNA

CTLA-4: cytotoxic T-lymphocyte antigen 4

CTPs: circulating tumor products

dMMR: deficient mismatch repair

DNA: deoxyribonucleic acid

DRUP: Drug Rediscovery Protocol

ECOG: Eastern Cooperative Oncology Group

EGFR: epidermal growth factor receptor

EMA: European Medicines Agency

EMBRACA: Phase 3 trial assessing efficacy and safety of talazoparib versus physician's choice of therapy in patients with advanced breast cancer and a germline BRCA mutation

EML4: EMAP like 4

EORTC: European Organisation for Research and Treatment of Cancer

ER: estrogen receptor

ERBB2: see HER2

ERK: extracellular signal-regulated kinase

ESCAT: ESMO Scale for Clinical Actionability of molecular Targets

ESMO: European Society of Medical Oncology

ESR1: estrogen receptor 1

ETV6: ETS variant transcription factor 6

FDA: US Food and Drug Administration

FFPE: formalin-fixed paraffin-embedded (tissue); a sample that has been fixed in formalin, sequentially processed and then embedded in paraffin. It is the standard tissue block used in histopathology laboratories to generate tissue sections

FGFR: fibroblast growth factor receptor

FNA: fine needle aspiration

Fragmentation: breaking up of DNA/RNA into 200–500-bp fragments

Gene fusion: chimeric genes generated from the fusion of two different genes on the same or different chromosomes

GIST: gastrointestinal stromal tumor

GNAQ: guanine nucleotide-binding protein (G protein), q polypeptide

HER2: human epidermal growth factor receptor 2; the up-to-date gene symbol is *ERBB2*

HERACLES: HER2 Amplification for Colo-rectaL Cancer Enhanced Stratification (trial)

HGVS: Human Genome Variation Society

HR: hormone receptor

HRAS: HRas proto-oncogene, GTPase

HRD: homologous recombination deficiency

IASLC: International Association for the Study of Lung Cancer

IHC: immunohistochemistry

Indel: insertion or deletion mutation; an addition (insertion) or loss (deletion) within a gene. Indels are more difficult to detect using NGS, especially at the edges of sequences

INI1: integrase interactor 1

I-PREDICT: observational study of molecular profile-related evidence to determine individualized therapy for advanced or poor prognosis cancers

ISH: in-situ hybridization

KEYNOTE: a series of trials of pembrolizumab, a PD-1 inhibitor, in various malignancies

***KIT*:** gene encoding c-KIT

KRAS: KRAS proto-oncogene, GTPase (previously known as Kirsten rat sarcoma viral oncogene homolog)

Library: all the DNA fragments from a sample with adapters and barcodes added

lncRNA: long non-coding RNA

LOD: limit of detection; represents the lowest amount of analyte that can be reliably detected

MAPK: mitogen-activated protein kinase

MATCH: Molecular Analysis for Therapy Choice (study)

mBC: metastatic breast cancer

MDM2: MDM2 proto-oncogene

MEK: mitogen-activated protein kinase kinase (also known as MAP2K, MAPKK)

MET: MET proto-oncogene, receptor tyrosine kinase

Metagenomics: the study of genetic material recovered directly from environmental samples

Methylation analysis: the study of chromosomal patterns of DNA or histone modification by methyl groups

Microsatellites: repetitive, highly preserved DNA sequences that occur through the genome

miRNA: microRNA

MLH1: mutL homolog 1

MMR: mismatch repair

mRNA: messenger RNA

MSH2: mutS homolog 2

MSH6: mutS homolog 6

MSI: microsatellite instability

MSI-H: microsatellite instability high

MSK-IMPACT: Memorial Sloan Kettering Cancer Center Integrated Mutation Profiling of Actionable Cancer Targets

MSS: microsatellite stable

MTB: molecular tumor board; a multidisciplinary group characterized by a wide range of medical professional figures (in particular, molecular pathologists, clinicians, surgeons, radiologists, geneticists, bioinformaticians, biologists) involved in the management of cancer patients

mtRNA: mitochondrial RNA

mTOR: mammalian target of rapamycin

Must test gene: gene for which testing is strongly recommended in advanced stage NSCLC

NCCN: National Comprehensive Cancer Network

NCI: US National Cancer Institute

NF1: neurofibromin 1

NGS: next-generation sequencing (after Sanger sequencing); a high-throughput method based on 'sequencing by synthesis' that allows the simultaneous analysis of several gene alterations in different patients (also called massive parallel sequencing)

NRAS: NRAS proto-oncogene, GTPase (previously known as neuroblastoma RAS viral oncogene homolog)

NRG1: neuregulin 1

NSCLC: non-small cell lung cancer

***NTRK*:** a family of three genes, *NTRK1/2/3*, encoding tropomyosin receptor kinases A, B and C

OlympiAD: olaparib versus chemotherapy treatment of physician's choice in patients with a germline *BRCA* mutation and HER2-negative metastatic breast cancer (trial)

ORR: objective response rate

PALETTE: Phase III trial of pazopanib for metastatic soft tissue sarcoma

PARP: poly(ADP-ribose) polymerase

PAX8: paired box 8

PCR: polymerase chain reaction

PD-1: programmed cell death 1

PDGFR: platelet-derived growth factor receptor

PDGFRA/B: platelet-derived growth factor receptor α/β

PD-L1: programmed death-ligand 1

PFS: progression-free survival

PI3K: phosphatidylinositol 3-kinase

PIK3CA: phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α

PMS2: post-meiotic segregation increased 2

PPARG: peroxisome proliferator activated receptor γ

PR: progesterone receptor

PTEN: phosphatase and tensin homolog

RAC1: Rac family small GTPase 1

RAF: RAF kinases, which include BRAF, are serine/threonine kinases; the acronym derives from rapidly accelerated fibrosarcoma

RAS: signal transduction regulators that cycle from 'on' to 'off' during signal transduction; the acronym derives from rat sarcoma

Read length: the length of sequence recorded from each sequencing reaction; it ranges from 25 bp to 500 bp

RET: rearranged during transfection

RNA: ribonucleic acid

rRNA: ribosomal RNA

ROS1: ROS proto-oncogene 1 receptor tyrosine kinase

ROSE: rapid on-site evaluation; represents the immediate assessment of a cytological FNA specimen

RTK: receptor tyrosine kinase

RT-PCR: reverse transcription PCR; RNA-based assay that detects and quantifies, in 'real time', cDNA amplification, obtained by reverse transcription enzyme, of a known target using fluorescent probes

SDH: succinate dehydrogenase

SNP: single nucleotide polymorphism; single base variation occurring at a frequency of more than 1% in the population (a frequency below 1% is regarded as a mutation)

snRNA: small nuclear RNA

SNV: single-nucleotide variant

SOLAR-1: Phase III study of alpelisib plus fulvestrant in men and postmenopausal women with HR+/HER2– advanced breast cancer progressing on or after prior aromatase inhibitor therapy

Splice variant: variation at the splice site between an exon and intron

STARTRK-2: Studies of Tumor Alterations Responsive to Targeting Receptor Kinase 2; Phase II basket trial of entrectinib for the treatment of patients with advanced solid tumors that harbor an *NTRK*, *ROS1* or *ALK* gene fusion

STK11: serine/threonine kinase 11

Structural variant: occurs as a consequence of a change or changes in large DNA fragments due to translocation, inversion, deletion, duplication or amplification

TAPUR: Targeted Agent and Profiling Utilization Registry

TCGA: The Cancer Genome Atlas; a genomics program that has sequenced and characterized at a molecular level a large number of cancers

TKI: tyrosine kinase inhibitor; a small molecule able to block the tyrosine kinase domain

TMB: tumor mutational burden; the number of somatic, non-synonymous, coding base mutations

TNBC: triple-negative breast cancer

TP53: cellular tumor protein p53

Trk: tropomyosin receptor kinase

tRNA: transfer RNA

Universal healthcare: a healthcare system in which all people of a particular country or region are assured access to healthcare

UTR: untranslated region

VEGFR: vascular endothelial growth factor receptor

VUS: variant of unknown significance. Gene variants can be classed as: pathogenic (known, disease causing); likely pathogenic (novel, likely disease causing); VUS (novel, uncertain whether pathogenic or benign); likely benign (novel, unlikely disease causing); and benign (known, not associated with disease)

WES: whole-exome sequencing; sequencing of only the exons of all genes (2% of the genome) within a person, a specific cell type or implicated in a disease

WGS: whole-genome sequencing; sequencing of the whole genome (3×10^9 bases)

Introduction

The ability to decode DNA sequences is providing scientists with powerful insights into cancer biology. From a molecular perspective, cancer is a multifaceted disease involving multiple genetic variations and subsequent changes in gene expression patterns that induce uncontrolled tumor growth. The genomic characterization of cancer is leading to the identification and use of better diagnostic, prognostic and predictive biomarkers and more effective management.

The introduction of rapid DNA-sequencing procedures has significantly accelerated biological and medical research and discoveries. Academic researchers obtained the first DNA sequences in the early 1970s, using arduous procedures based on two-dimensional chromatography. Frederick Sanger was awarded two Nobel prizes, one for the sequencing of proteins and the other for the sequencing of DNA.

The next step from Sanger sequencing, next-generation sequencing (NGS), enables genome sequencing at high speed and low cost. By increasing the affordability, accessibility and reliability of DNA and RNA high-throughput sequencing platforms, NGS has revolutionized the practice of oncology, enabling clinicians to deliver personalized care to their patients. It is important to emphasize that while NGS provides molecular information, the treating clinician should interpret this in light of the clinical picture of the patient. The NGS platform does not recommend how to treat a patient.

Personalized medicine – an innovative concept – tailors therapeutic approaches to patients based on their genomic, epigenomic and proteomic profiles. The assessment of mutations associated with sensitivity or resistance to various forms of treatments provides oncologists with strong evidence to support treatment strategies for specific cancers.

Fast Facts: Comprehensive Genomic Profiling reminds healthcare professionals of basic DNA and RNA biology and provides an accessible overview of NGS, prognostic and predictive biomarkers for different cancer types and molecular-guided treatment options.

1 Genetic mutations and biomarkers

Basic principles in cell biology

The human body is constituted of trillions of cells, the fundamental building blocks of all living organisms. Cells provide structure for the body, take in nutrients from food, convert those nutrients into energy and carry out specific functions. The nucleus functions as the cell's control center, sending instructions to the cell to grow, mature, divide or die (apoptosis).

DNA is the body's hereditary material – it can be duplicated during the process of cell division. Most of the cellular DNA is packaged into thread-like structures called chromosomes, which are found in the nucleus. Humans have 23 pairs of chromosomes, giving a total of 46 chromosomes. Each chromosome is made up of DNA tightly coiled many times around proteins called histones that support its structure (Figure 1.1). At each end of a chromosome there is a telomere, a region of repetitive nucleotide sequences. Telomeres protect the end of the chromosome from deterioration or fusion with neighboring chromosomes.

A small amount of DNA can be found in the mitochondria – this is referred to as mitochondrial DNA (mtDNA) (see later).

DNA bases. The information in DNA is stored as a code made up of four chemical bases: adenine (A), guanine (G), cytosine (C) and thymine (T). Human DNA comprises about 3 billion bases. It is essential to appreciate that more than 99% of those bases are the same in all people. The sequence of these bases controls the information available for building, developing and preserving the body.

Nucleotides and DNA structure. DNA bases pair up with each other, A with T and C with G, to form a unit called a base pair (bp). Each base is also attached to a sugar molecule and a phosphate molecule. Together, a base, sugar and phosphate are called a nucleotide.

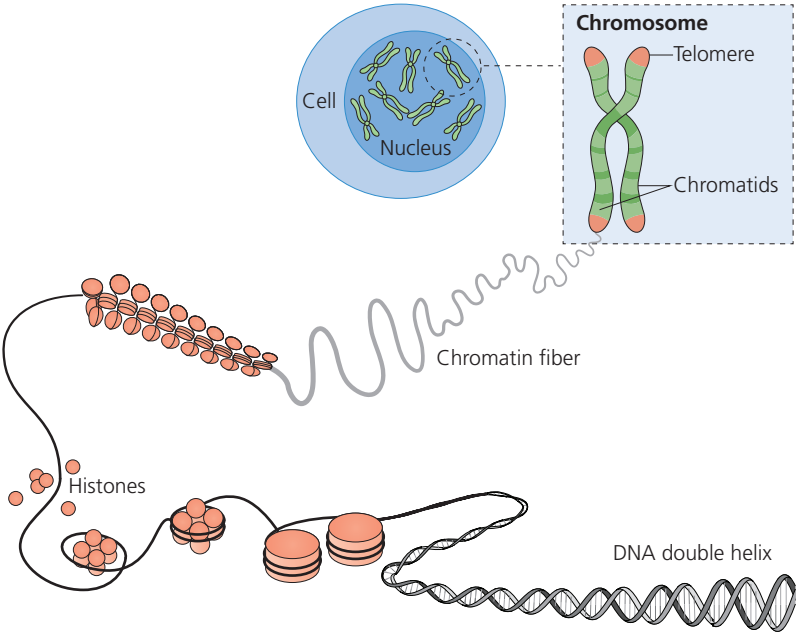

Figure 1.1 The DNA molecules are wrapped around complexes of histone proteins and 'packed' into the chromosomes. Telomeres at the ends of the chromosome comprise long stretches of repeated TTAGGG sequences, which help stabilize the chromosomes.

Nucleotides are arranged in two long strands that form a double helix (Figure 1.2). Although the circumstances of the discovery of DNA structure in the 1950s are fairly well known, they are usefully summarized in a 2019 article on Watson and Crick's original paper in *Nature*.[1]

An essential property of DNA is that it replicates, or makes copies of itself. Each strand of DNA in the double helix can serve as a pattern for duplicating the sequence of bases. This process is vital during cell division because each new cell must have an exact copy of the DNA present in the mother cell.

A codon is a sequence of three DNA or RNA nucleotides that matches a specific amino acid or stop signal during protein synthesis. DNA and RNA molecules are written in a language of four nucleotides; meanwhile,

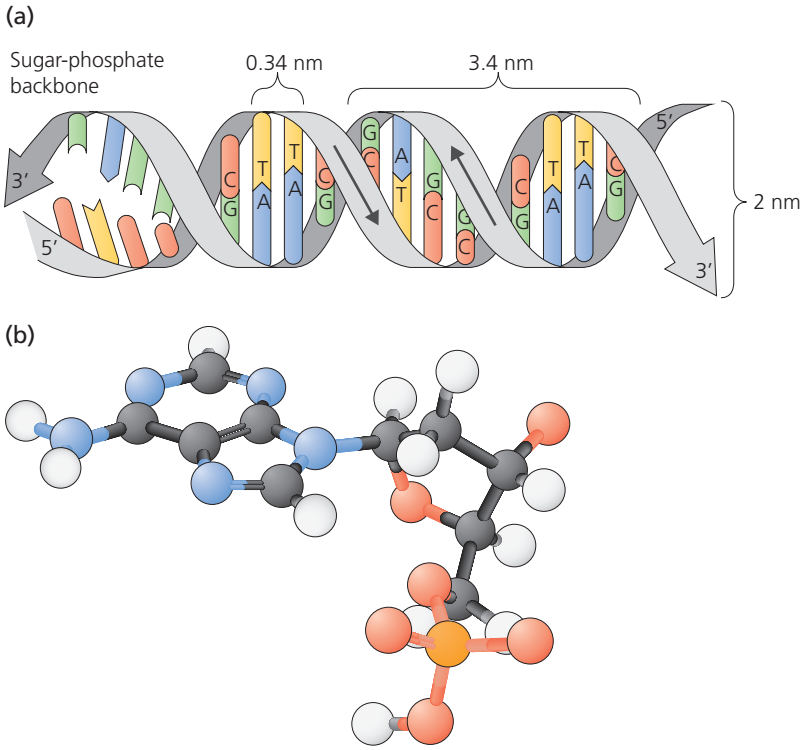

Figure 1.2 (a) Double-stranded DNA forms a double helix. The two strands are joined by hydrogen bonds between the bases. The sugar–phosphate backbones run in opposite directions, so a 3' end on one strand aligns with a 5' end on the other strand. (b) A ball-and-stick model of a single base (adenine in this figure) with part of the helix backbone. Gray, carbon; white, hydrogen; blue, nitrogen; red, oxygen; and orange, phosphate.

the language of proteins includes 20 amino acids. Codons provide the key that permits these two languages to be translated into each other. Each codon corresponds to a single amino acid (or stop signal), and the full set of codons is called the genetic code.

A gene is a sequence of nucleotides in DNA or RNA that encodes the creation of a gene product, either RNA or protein. Genes are built up of DNA. Some genes act as instructions to make proteins. However,

many genes do not code for proteins. In humans, genes can differ in size ranging from a few hundred DNA bases to more than 2 million bases. The Human Genome Project calculated that humans have between 20 000 and 25 000 genes. In most genes, coding regions (exons) are interrupted by non-coding regions (introns) (Figure 1.3).

An intron is any nucleotide sequence within a gene that is removed by RNA splicing during the maturation of the final RNA product. Introns are non-coding regions of an RNA transcript that are eliminated by splicing before translation.

An exon is any part of a gene that will encode a part of the final mature RNA produced by that gene after the introns have been removed by RNA splicing. The term exon refers to both the DNA sequence within a gene and the corresponding sequence in an RNA transcript. In RNA splicing, introns are removed and exons are covalently joined to generate the mature messenger RNA (mRNA).

All exons constitute the exome – the sequences which, when transcribed, remain within the mature RNA after introns are removed by RNA splicing.

Gene expression is the process by which information from a gene is utilized to synthesize a functional gene product. These products are often proteins, but for non-protein-coding genes such as transfer RNA (tRNA) or small nuclear RNA (snRNA) genes, the product is a functional RNA.

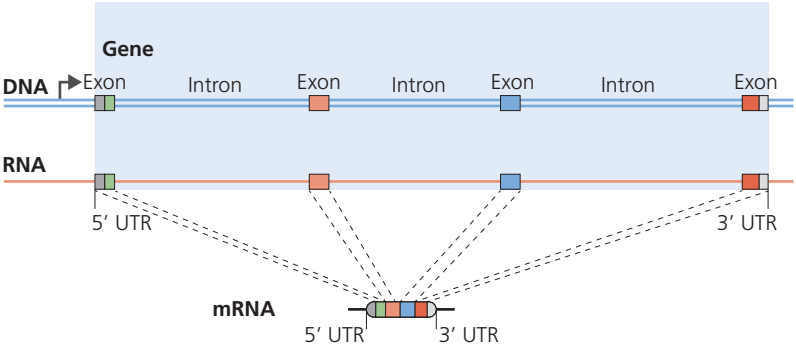

Figure 1.3 A gene comprises exons, which are expressed, and introns, which are spliced from the RNA. UTR, untranslated region.

During transcription, the whole gene is copied into a pre-mRNA, which includes exons and introns. During the process of RNA splicing, introns are removed and exons linked to form a contiguous coding sequence.

Non-coding DNA. Only about 1% of DNA is made up of protein-coding genes; the other 99% is non-coding. Although non-coding DNA does not provide instructions for making proteins, it is essential to the function of cells, particularly the control of gene activity.[2]

Non-coding DNA contains sequences that work as regulatory elements, determining when and where genes are turned on and off. Such elements provide sites for specialized proteins called transcription factors to bind and either activate or repress the process by which the information from genes is turned into proteins during transcription. These processes are very relevant in cancer genetics. Non-coding DNA contains many types of regulatory elements, including promoters, enhancers, silencers and insulators (Figure 1.4).

Promoters provide binding sites for the protein machinery that carries out transcription. Promoters are typically found just ahead of the gene on the DNA strand.

Enhancers provide binding sites for proteins that help activate transcription. Enhancers can be found on the DNA strand before or after the gene they control, or sometimes at a distance from the gene.

Silencers provide binding sites for proteins that repress transcription. Like enhancers, silencers can be found before or after the gene they control and can be some distance away on the DNA strand.

Insulators provide binding sites for proteins that control transcription in several ways. Enhancer-blocker insulators prevent enhancers from aiding in transcription. Barrier insulators prevent structural changes in the DNA that would otherwise repress gene activity. Some insulators can function as both an enhancer blocker and a barrier.

RNA molecules. Other regions of non-coding DNA encode specific types of RNA molecules. Examples include tRNA and ribosomal RNA (rRNA).

- tRNA is an adaptor molecule composed of RNA, typically 76–90 nucleotides in length, which serves as the physical connection between the mRNA and the amino acid sequence of proteins.
- rRNAs help assemble amino acids into a chain that forms a protein.

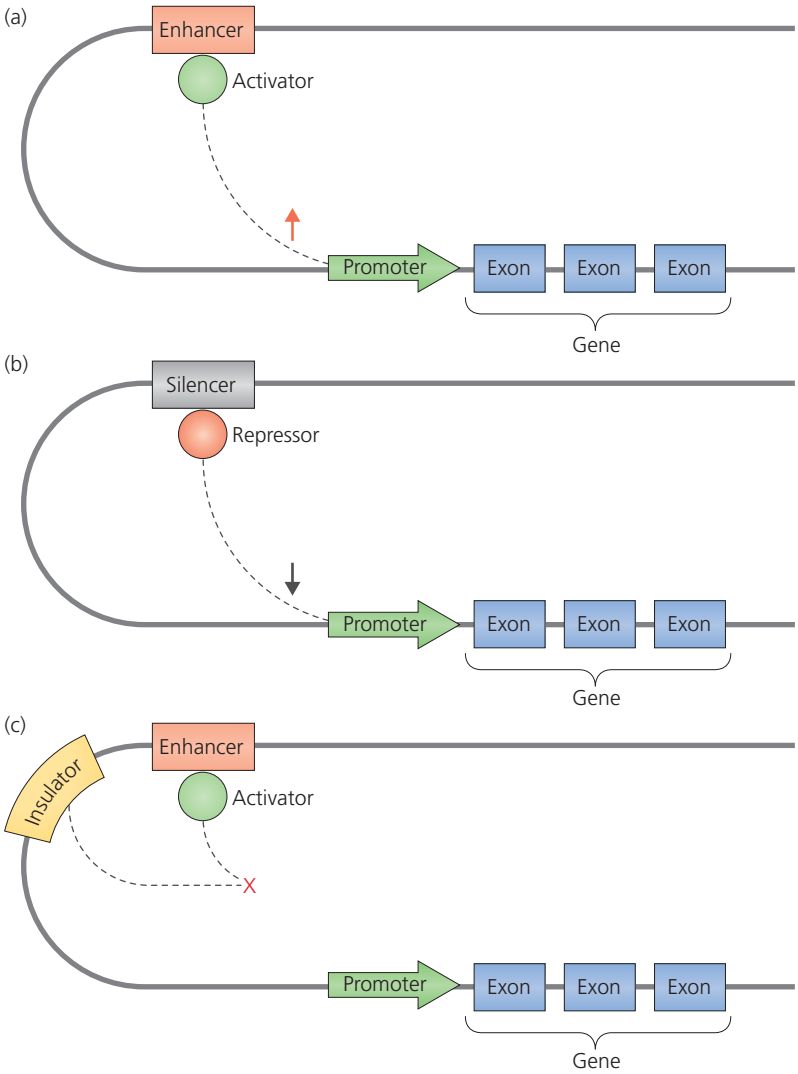

Figure 1.4 Non-coding DNA can control gene activity. (a) Enhancer DNA provides binding sites for activators that increase gene transcription. (b) Silencer DNA binds repressor proteins that reduce transcription. (c) An enhancer-blocker sequence blocks the activity of the activator. The promoter sequence lies in front of the gene.

Other specialized RNA molecules include microRNAs (miRNAs), short lengths of RNA that block the process of protein production, and long non-coding RNAs (lncRNAs), which are more extended lengths of RNA that have various regulatory functions in gene activity.

The cell cycle. When a cell is actively dividing, it goes through a four-stage process called the cell cycle, comprising two G (gap or growth) phases, the S phase and the M phase. Together, the G and S phases are known as the interphase. In the first G phase, G1, the cell grows and proteins and RNA are synthesized. The centromere (see below) and other centrosomal components are made. The chromosomes are duplicated in the S phase, and the cell quality-checks the duplication in the second G phase, G2. The cell then undergoes mitosis – the M phase – with the duplicated genetic material pulled to opposite ends of the cell, which then divides to produce two daughter cells.

The centromere is a unique region of a chromosome, usually near the middle. During mitosis, the centromeres can be observed as a constriction of the chromosome. At the centromeric constriction, the two halves of the chromosome, the sister chromatids, are held together until they are pulled in opposite directions.

When the centromere divides, the chromatids become separate chromosomes. The centromere contains specific types of DNA, which are tandem repetitive sequences (satellite DNA).

Mitochondrial DNA

MtDNA encodes proteins involved in the cell oxidative phosphorylation process, which uses oxygen and simple sugars to create ATP, the primary source of energy. MtDNA contains 37 genes, all of which are essential for normal mitochondrial function. Thirteen of these genes encode enzymes involved in oxidative phosphorylation. The remaining genes provide instructions for the development of tRNA and rRNA, which primarily help assemble amino acids into functioning proteins.

Gene mutations

A gene mutation is a permanent alteration in the DNA sequence that makes up a gene. Mutations can be hereditary or acquired (also

referred to as somatic). These changes can be caused by environmental factors such as solar ultraviolet radiation or can occur if an error is made as DNA copies itself during cell division.

Mutations range in size and can affect a single DNA building block (base pair) to a large segment of a chromosome that comprises multiple genes.

Gene mutations have variable effects on human health, depending on where the mutations occur and whether they modify the primary function of essential proteins. The general types of mutation are described below and/or illustrated in Figure 1.5.

A missense mutation is a change in one DNA base pair that results in the substitution of one amino acid for another in the protein made by the gene.

A nonsense mutation is also a change in one DNA base pair. Instead of substituting one amino acid for another, the altered DNA sequence prematurely signals the cell to stop building a protein – this type of mutation results in a shortened protein that may function wrongly or not at all.

An insertion changes the number of DNA bases in a gene by adding a piece of DNA. As a result, the protein made by the gene may not function properly.

A deletion changes the number of DNA bases by removing a piece of DNA. Small deletions may remove one or a few base pairs within a gene, while larger deletions can remove an entire gene or several

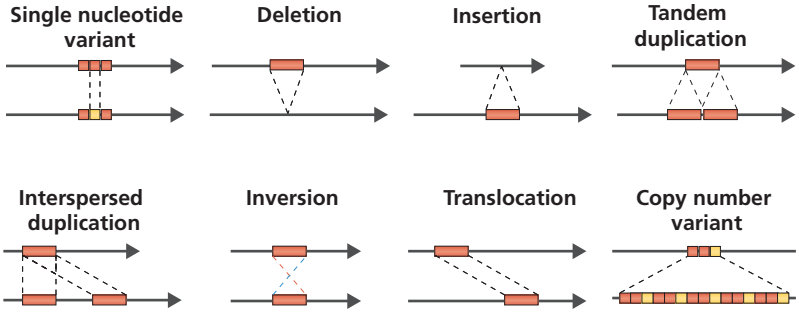

Figure 1.5 Types of genetic mutation.

neighboring genes. The deletion may alter the function of the resulting protein or proteins.

A frameshift mutation occurs when the addition or loss of DNA bases changes a gene's reading frame. A reading frame consists of groups of three bases that each code for one amino acid. A frameshift mutation shifts the grouping of these bases and changes the code for amino acids. The resulting protein is usually non-functional. Insertions, deletions and duplications can all be frameshift mutations.

A duplication consists of a piece of DNA that is abnormally copied one or more times. This type of mutation may alter the function of the resulting protein.

Repeat expansion. Nucleotide repeats are short DNA sequences that are repeated many times in a row. A trinucleotide repeat is composed of three-base-pair sequences, and a tetranucleotide repeat is composed up of four-base-pair sequences. A repeat expansion is a mutation that increases the number of times that the short DNA sequence is repeated. This type of mutation can cause the resulting protein to function inadequately.

A point mutation or substitution is a genetic mutation in which a single nucleotide base is changed, inserted or deleted from a sequence of DNA or RNA. Point mutations have a variety of effects on the downstream protein product. These consequences can range from no effect (for example, synonymous mutations) to effects deleterious to protein production, composition and function. Point mutations in multiple tumor suppressor proteins cause cancer.

A translocation is a type of chromosomal abnormality in which a chromosome breaks and a portion reattaches to a different chromosome. Translocations can be detected with karyotyping of the affected cells.

Genomic alterations in cancer

The comprehensive genomic profiling (CGP) approach distinguishes four classes of genomic alterations: base substitutions, insertions and deletions (indels), copy number alterations, and rearrangements (see Figure 1.5).[3,4]

Base substitutions/single-nucleotide variants are the simplest type of gene-level mutation. As one nucleotide is swapped for another during DNA replication, the overall number of nucleotides in the DNA is unchanged. The *BRAF* p.V600E mutation, which results in glutamic acid (E) being substituted for valine (V) at position 600 of the protein chain, is an example.

Indels are insertions/deletions of nucleotides from a sequence (in contrast to a point mutation, described above). *EGFR* exon 19 deletion is an example (see Chapter 5).

Copy number alterations are somatic changes to the chromosome structure that result in a gain or loss of copies of DNA sections. These are prevalent in many types of cancer. Amplification of *HER2* (also known as *ERBB2*), leading to overexpression of human epidermal growth factor receptor 2, is an example of this type of genomic alteration.

Rearrangements. Many cancers exhibit chromosomal rearrangements. These can be simple, involving a single balanced fusion that preserves the proper complement of genetic information, or complex, with one or more fusions that disrupt this balance. Rearrangements can occur via deletions, duplications, inversions and translocations. An example is an *ALK* gene fusion.

Oncogenic mutations

Tumor suppressor genes. *TP53* is the gene that codes for cellular tumor protein p53. This tumor suppressor protein is a transcription factor that inhibits cell division or survival in response to several stresses. It is a critical failsafe mechanism of cellular anticancer defense.

Tumor protein p53 regulates the expression of genes involved in cell cycle arrest, apoptosis, senescence, DNA repair and metabolism changes. In cancer, its usual roles are not carried out, so cells survive and proliferate and DNA damage accumulates.

TP53 mutations are the most frequent mutations in patients with cancer, occurring in approximately half of all cancers. The mutations are common in ovarian, colon and esophageal cancers, and many other cancer types (as documented in the Catalogue Of Somatic Mutations In Cancer [COSMIC] database – see Useful resources).

Other examples of tumor suppressor genes include the retinoblastoma gene, *RB*, and *PTEN*. RB is a transcriptional regulatory protein encoded by a tumor suppressor gene that was originally recognized by the genetic analysis of retinoblastoma. It is now recognized that RB is also involved in common adult tumors. Mutations of *PTEN*, which encodes phosphatase and tensin homolog (PTEN), are associated with cancer development (see below).

Mitogen-activated protein kinase pathway. The mitogen-activated protein kinase (MAPK) pathway encompasses the signaling molecules RAS, RAF, mitogen-activated protein kinase kinase (MEK) and extracellular signal-regulated kinase (ERK). Activation of the MAPK pathway leads to the transcription of genes that encode proteins involved in regulating essential cellular functions, such as cell growth, proliferation and differentiation.

The MAPK signaling pathway begins with the activation of a RAS family protein by receptor tyrosine kinases (RTKs; Figure 1.6). The activated RAS induces the membrane recruitment and activation of RAF kinases, which phosphorylate MEK, a separate protein kinase in the pathway. MEK phosphorylates ERK, which can, directly and indirectly, activate many transcription factors. The activation of these transcription factors by ERK leads to the expression of genes encoding proteins that regulate cell proliferation and survival.

Dysregulated MAPK signaling occurs in a wide range of cancers. There are multiple mechanisms, including the abnormal expression of pathway receptors with or without genetic mutations, that lead to the activation of receptors and downstream signaling molecules in the absence of appropriate stimuli.

BRAF mutations can drive cancer development. Mutant serine/threonine-protein kinase B-raf (BRAF) can stimulate the MAPK pathway inappropriately, leading to excessive cell proliferation and survival (see Figure 1.6). Tumors with class 1 or 2 BRAF mutations respond to BRAF inhibitor therapy (see Figure 1.6), though the response can be attenuated by intrinsic and adaptive resistance mechanisms (see Chapter 5).

Dysregulated BRAF signaling may play a role in specific malignancies: for example, the *BRAF* p.V600E mutation is present in approximately half of patients with malignant melanoma.

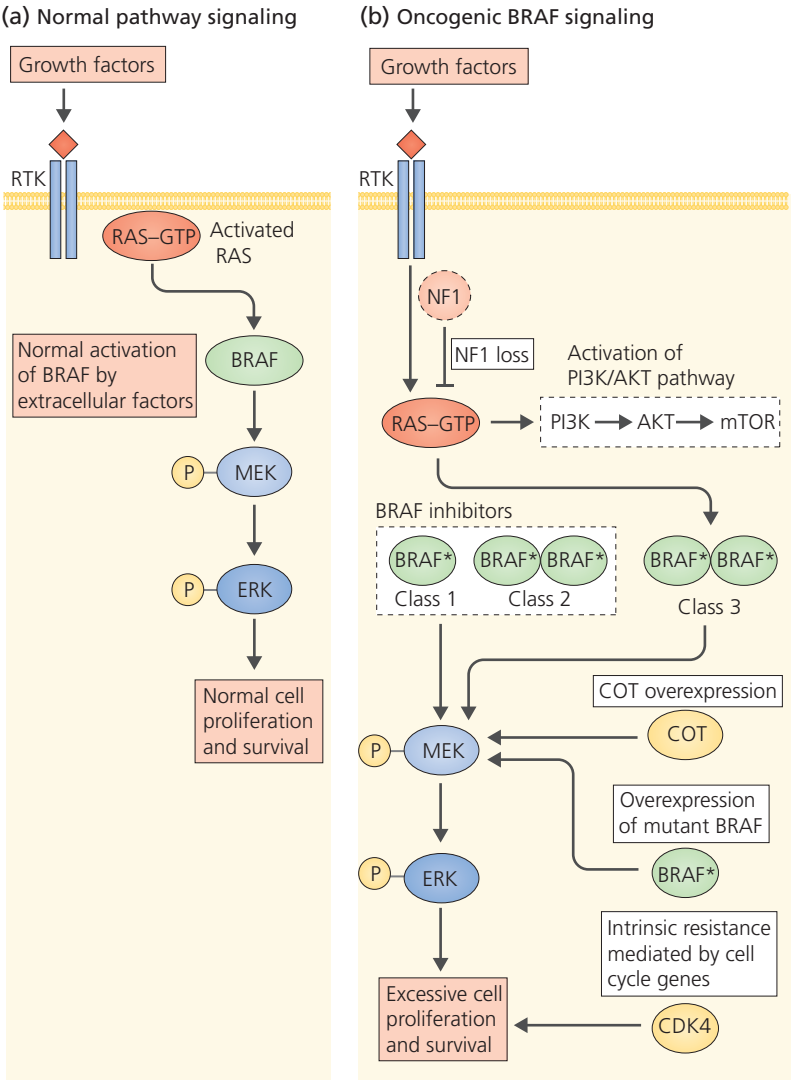

Figure 1.6 In normal cells, external growth stimuli activate RTKs and RAS, which relay growth signals to the MAPK pathway. In BRAF-driven cancers, mutant BRAF (shown here as BRAF*) can stimulate the MAPK pathway either independently of RAS as a monomer (class 1) or a dimer (class 2), or dependently (class 3), leading to excessive cell proliferation and survival. Tumors with class 1 or 2 BRAF mutations respond to BRAF inhibitor therapy. CDK4, cyclin-dependent kinase 4; COT, mitogen-activated protein kinase kinase kinase 8; NF1, neurofibromin 1.

PI3K/AKT/mTOR pathway. The phosphatidylinositol 3-kinase (PI3K)/serine/threonine-protein kinase (AKT)/mammalian target of rapamycin (mTOR) pathway is an intracellular signaling pathway important in regulating the cell cycle. It is directly related to cellular quiescence, proliferation, cancer and longevity. PI3K activation phosphorylates and activates AKT, localizing it in the plasma membrane. AKT can have a number of downstream effects, such as activating mTOR.

This pathway is upregulated in many cancers, reducing apoptosis and allowing proliferation. The pathway is inhibited by various factors, including PTEN and glycogen synthase kinase 3 β (GSK3B).

Biomarkers and prognostic and predictive factors

A biomarker is a biological molecule found in blood, other body fluids or tissues that is a sign of a normal or abnormal process or a condition or disease. A biomarker may be used to see how well the body responds to a treatment for a disease or condition. Biomarkers offer opportunities for improved cancer subtype classification, refined treatment strategies and assessment of response, and recruitment of a more homogeneous population to clinical trials.

A prognostic factor is a measurement associated with clinical outcome in the absence of therapy or with the application of a standard therapy that patients are likely to receive. It can be thought of as a measure of the disease's natural history. A control group from a randomized clinical trial is an ideal setting for evaluating the prognostic significance of a biomarker.

A predictive factor is a measurement associated with response or lack of response to a particular therapy. Response can be defined using any of the clinical endpoints commonly used in clinical trials. A predictive factor implies a differential benefit from the therapy that depends on the status of the predictive biomarker.

An agnostic biomarker is present across many tumor types. Agnostic treatments are based on the cancer's genetic and molecular features, regardless of the cancer type or where the cancer started in the body. Tumor-agnostic therapy uses the same drug to treat all cancer types

that have the genetic mutation or a specific biomarker that is targeted by a particular drug.[5,6]

In May 2017, pembrolizumab became the first tumor-agnostic treatment to be approved by the US Food and Drug Administration (FDA). It was indicated for tumors deficient in mismatch repair or with high microsatellite instability (see Chapter 3).

Circulating tumor products (CTPs) is the collective term for circulating tumor cells (CTCs), circulating tumor DNA (ctDNA) and mRNA. A CTC is a cell that has shed into the vasculature or lymphatic system from a primary tumor and is carried around the body in the blood circulation. ctDNA is tumor-derived fragmented DNA in the bloodstream that is not associated with cells. It is thought to result from apoptosis and necrosis of dying cells or active release from viable tumor cells.

CTPs represent areas of immense interest from scientists' and clinicians' perspectives.[7] CTP analysis may have clinical utility in many areas, including screening patients, diagnosis, clinical decision-making and assessing outcomes at follow-up, and as a source of real-time genetic or molecular characterization. CTP analysis will be helpful in the discovery of new biomarkers, patterns of treatment resistance and mechanisms of metastasis development.

Liquid biopsy. Precision medicine is based on the development of biomarkers.[8,9] Liquid biopsies, which involve analyzing non-solid tissue – usually blood – have been reported to detect biomarkers that carry information about tumor development and progression.[10] Liquid biopsies detect tumor cells or tumor cell products that are released from metastatic sites or the primary tumor. They provide comprehensive and real-time data on tumor cell evolution, therapeutic targets and potential mechanisms of resistance to therapy.

FoundationOne Liquid CDx, approved by the FDA in August 2020, is a companion diagnostic that analyzes guideline-recommended genes from a blood sample. As well as analyzing over 300 genes, this technology provides information on tumor mutational burden (TMB, see page 45), microsatellite instability (MSI, see page 42) and tumor fraction (the fraction of cell-free DNA derived from tumor rather than non-cancerous tissue).

Compared with traditional 'solid biopsy', which cannot always be done to determine tumor dynamics, liquid biopsy has essential advantages, mainly because the procedure is a non-invasive modality that can provide diagnostic and prognostic information before and during treatment and at disease progression.

Key points – genetic mutations and biomarkers

- DNA carries the genetic code in the form of sequences of codons; each codon codes for a specific amino acid or a stop signal.
- Non-coding DNA, which is much more abundant than coding DNA (99% versus 1%), has important functional roles; promoters, silencers, enhancers and insulators are examples of regulatory elements of non-coding DNA.
- Different types of genetic mutation occur. A mutation may result in the production of a protein with altered functional ability, or it may prevent the protein from being produced.
- The most frequent genetic alterations associated with cancer include: base substitutions, indels, copy number alterations, and rearrangements.
- *TP53* mutations are the most frequent mutations in patients with cancer. Mutations affect the ability of cellular tumor protein p53 to regulate cell division and survival.
- Mutations affecting the MAPK signaling pathway are also implicated in cancers. Dysregulation of the pathway results in increased cell division and survival.

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2 Understanding next-generation sequencing

Cancer is a disease driven by genetic mutations. With targeted therapy directed against specific gene mutations, it has become crucial to unravel these genetic abnormalities and thus personalize both the diagnosis and management of cancers. Within the last 15 years, high-throughput gene sequencing has established itself as an indispensable technology for personalizing many cancers and, ultimately, determining the optimal treatment of these tumors. NGS essentially gave birth to precision medicine.

History of gene sequencing

The first major step in sequencing was the introduction, by Maxam and Gilbert, of chemical chain termination in 1977.[1] This was followed closely – in the same year – by Sanger's dideoxy method, also known as the chain-termination method, which generated DNA strands of varying lengths that could be separated by electrophoresis (Figure 2.1).[2] This method, which was the basis of the Human Genome Project, essentially allowed a net signal to be derived from a pool of molecules, to give a collective sequence. Many subsequent improvements allowed for commercialization and widespread use of Sanger sequencing but, as the need for high-throughput technology burgeoned, Sanger sequencing proved to be too labor intensive, time consuming and expensive for routine use.

The drive by the National Human Genome Research Institute to reduce the cost of whole-genome sequencing (WGS) to below $1000 culminated in the 'next-generation' step. Technology was developed that could generate millions of sequencing reactions per run: 'next-generation sequencing'.

NGS methods

NGS technologies can be categorized into those that provide long-read sequences of up to 900 kilobase base pairs (kbp) and those that provide short-read sequences of 100–600 bp.

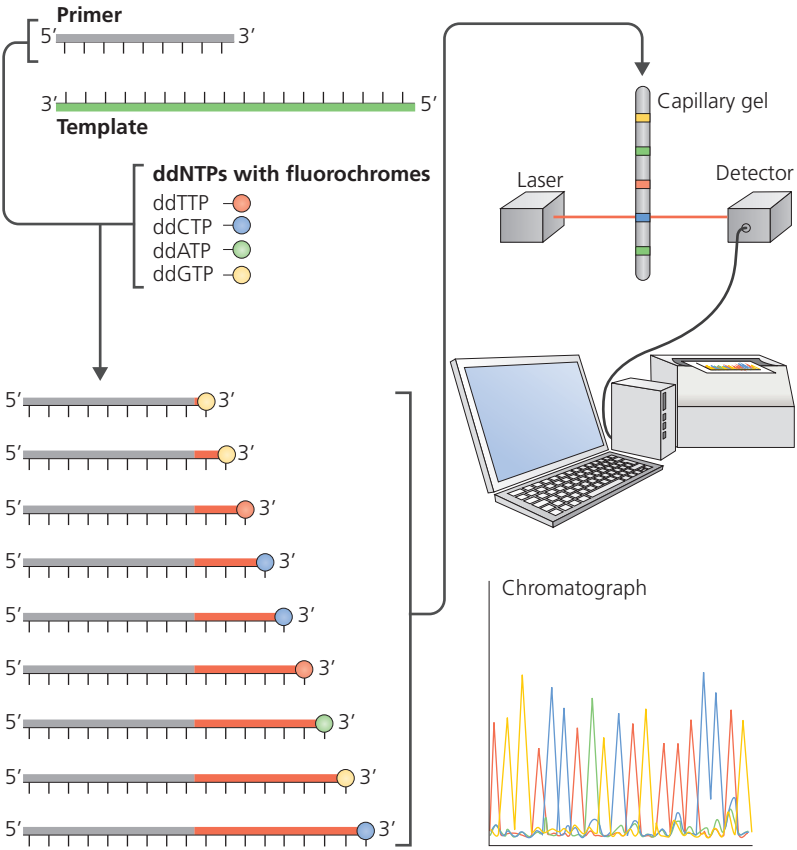

Figure 2.1 The Sanger method of DNA sequencing involves using dideoxynucleotides (ddNTPs), ddATP, ddTTP, ddCTP and ddGTP, each labeled with a different dye color. The DNA sample, a primer, DNA nucleotides, DNA polymerase and a small amount of the ddNTPs are combined. The primer binds to the single-stranded template and DNA polymerase starts to make new DNA, starting from the primer. Nucleotides are added to the chain. Chain elongation stops when a ddNTP is introduced. The process is repeated until it is likely that a ddNTP will have been incorporated at every position in the target sequence. Electrophoresis separates the different DNA fragments. The terminal ddNTP in each chain is detected according to its dye color.

Short-read technologies are more widely utilized as they are more accurate and cheaper than long-read technologies. However, long-read technologies are better for resolving complex regions.

Illumina and Ion Torrent are the major commercially available sequencing platforms utilizing the short-read technology; the other two – 454 Life Sciences and SOLiD (Sequencing by Oligonucleotide Ligation and Detection) – are not widely used nowadays. The MinION system utilizes nanopore technology and is the most widely used long-read platform.

NGS can be utilized to sequence the entire genome (WGS), with all coding (exon) and non-coding (introns, promoters, regulatory and structural elements) regions evaluated. However, this is usually not necessary, and sequencing of the exons of known genes – whole-exome sequencing (WES) – is more appropriate. In some circumstances, information is required only for a limited number of genes or for hotspots in those genes; this is known as targeted sequencing.

NGS can be used for genome-wide analysis of specific modifications, such as DNA methylation (Methyl-seq) or DNA–protein interactions (chromatin immunoprecipitation sequencing [ChIP-seq]). RNA sequencing is also possible – RNA-seq.[3]

At a molecular level, these genetic changes may be copy number variants (CNVs), single-nucleotide variants (SNVs), structural variants, indels (insertion or deletion mutations) and RNA changes (see Chapter 1). Sequencing provides information on cancer biomarkers that may be prognostic, predictive and/or pharmacotherapeutic.

Current methods of clinical NGS. Sequencing involves a common workflow, from DNA extraction, to library preparation, target enrichment, sequencing and bioinformatic analysis. Meaningful information about the relevant genetic changes then leads to clinical decision-making.

DNA extraction from tumor cells can be carried out using virtually any method. Extraction methods for formalin-fixed paraffin-embedded (FFPE) tissue do, however, require special care; macro- or microdissection of viable tumor may be needed to enrich the tumor sample.

Library preparation is the process of preparing DNA for use on a sequencer. All methods ultimately result in DNA fragmentation and the addition of adapters to the fragment ends. Adapters may include molecular barcodes (to allow for pooling of patient samples), a sequence recognized by universal PCR primers, hybridization sequences to bind the DNA fragments to a surface, and recognition sites to initiate sequencing.

The term library refers to these DNA fragments with flanking adapters that are ready for sequencing. The size of DNA fragment between the adapters is the insert size. Insert sizes vary, with shorter and longer inserts having different advantages. It is more likely that both ends of a shorter fragment fall within an exon, which is most often the site of interest (Figure 2.2). Longer fragments are likely to have one end fall in an intron, which will only increase detection of structural rearrangements if exonic regions are selected.

Target enrichment. The resulting library undergoes enrichment for specific regions of interest for whole-exome analysis and/or targeted sequencing of specific genes. Enrichment may occur by hybridization to complementary sequences (sequence capture) or by polymerase chain reaction (PCR). Enrichment by PCR is usually combined with the library preparation step. The choice depends on the clinical use. Sequence capture is preferred for large genomic regions and PCR for smaller regions where greater enrichment is required.[4]

Sequencing is performed using one of the two major platforms, Illumina or Ion Torrent. These are described below and summarized in Figure 2.3; the pros and cons of each platform are summarized in Table 2.1.

The Illumina platform is based on the technique of bridge amplification and uses synthesis with fluorescent detection. DNA molecules of around 500 bp, with appropriate adapters ligated to each end, are utilized as substrates for repeated amplification reactions on a

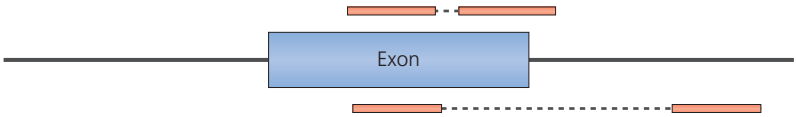

Figure 2.2 Shorter fragments or inserts are more likely to fall entirely within the area of interest than longer fragments.

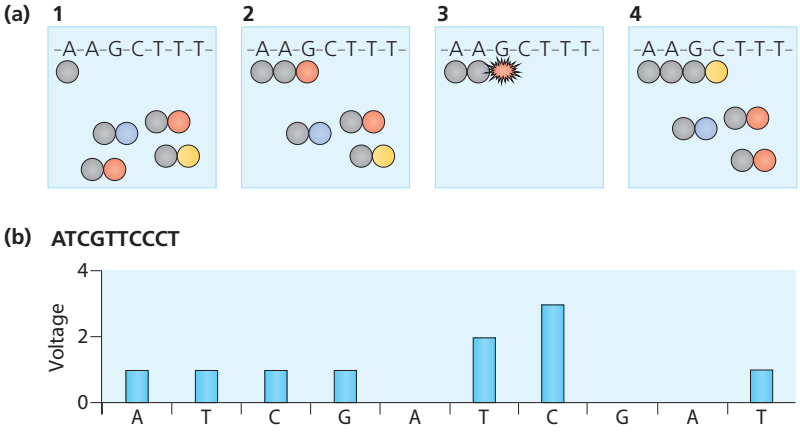

Figure 2.3 (a) Illumina sequencing by synthesis. (1) Nucleotides with fluorescent tags compete for the next space on a DNA strand. (2) A complementary tagged nucleotide is incorporated, blocking further binding. (3) Washing removes the unbound tagged nucleotides, and the signal from a fluorescent emission is captured. (4) The fluorescent tag and blocker are washed away, allowing the process to be repeated in the next cycle. This process happens simultaneously for all DNA strands in a cluster and all clusters on the flow cell. (b) Ion Torrent sequencing. A single base is added in a defined pattern in each cycle. If a base is incorporated, an H^+ ion is released, leading to a pH (voltage) change proportional to the number of bases added in a row. For this example, the order of base additions is T, A, G and C, which then repeats.

solid substrate that contains complementary oligonucleotide sequences. The oligonucleotides are spaced on the solid substrate, usually a glass slide, so that repeated rounds of amplification create clonal clusters of around 1000 copies of the oligonucleotide fragment.

All four of the nucleotides, which are fluorescently tagged, are added and compete for the next space. The complementary tagged nucleotide will bind, but a blocker prevents the binding of more than one nucleotide per round; the remaining non-bound nucleotides are washed away.

Laser excitation leads to a fluorescent emission that is recorded. The fluorescent tag and blocker are cleaved, and then the next round begins. In each round, 1 bp is read from each DNA cluster. This process

TABLE 2.1

A comparison of Illumina and Ion Torrent platforms

Amplification	Detection	Read length (bp)	Pros	Cons
Illumina				
Flow cell	Fluorescent	100–300	Paired end reads	Errors in GC-rich regions
Ion Torrent				
Bead and emulsion	Ion (pH)	100–400	Short run time Paired end reads	Homo-polymer error

Adapted from Yohe and Thyagarajan 2017.[4]

can be repeated on the opposite end of the DNA fragment in a process known as 'paired end reads'. Approximately 300 rounds are repeated.

Illumina-based sequencing is relatively rapid as direct imaging increases the detection speed, in contrast to camera-based imaging.

One downside with Illumina technology is that there can be a lack of synchrony in the synthesis reaction of an individual cluster. This can interfere with the generation of an accurate consensus sequence, thereby reducing the number of cycles that can occur.

Illumina sequencing can be used for genomic sequencing, exomic and targeted sequencing, metagenomics, RNA sequencing, ChIP-seq and methylome methods.[5]

The Ion Torrent platform converts nucleotide sequences directly into digital informatics on a semiconductor chip, as described by Rothberg et al.[6] This is based on the simple principle that an H^+ is generated when the correct nucleotide is incorporated across from its complementary base in a propagating DNA sequence.[5] This changes the pH of the solution, which can be detected as a voltage spike by an ion sensor. If a nucleotide is not incorporated, there is no pH change and no spike in the voltage.

Sequential flooding and washing of a sequencing chamber with reagents containing only one of the four nucleotides at any time results in a voltage change when the specific nucleotide is incorporated. When two adjacent nucleotides incorporate the same nucleotide, two H^+ ions are released, with a doubling of the voltage.

These Ion Torrent reactions occur in millions of wells on a semiconductor chip that converts the chemical reaction information into sequencing information. Initially, the DNA fragments (200–1500 bp), ligated to adapters, are attached to a bead of complementary sequences and then amplified by emulsion PCR (emPCR). This allows millions of beads to have multiple copies of a DNA sequence. These beads are then flowed across the chip, with only one bead entering one well. Thereafter, once the sequencing reagents enter the wells, if the appropriate nucleotide is incorporated, an H^+ is released, the voltage spike occurs and is detected and a signal is recorded.

This process is quick as the nucleotide incorporation is directly recorded, with no requirement for a camera, light source or scanner.

Advantages of NGS over older methods

Sanger sequencing is a very accurate method of sequencing that can analyze relatively large DNA fragments. It is, however, very expensive, with low data output.

All NGS platforms capture the individual sequences of millions of molecules, while standard sequencing methods (Sanger sequencing) deliver a net signal derived from a pool of molecules, a collective sequence. One target is amplified per test (by cloning or PCR) and the net signal from the sequencing of all the amplified molecules is taken for base calling (the process of assigning bases to the peaks on the readout or chromatogram). NGS, in contrast, can sequence multiple targets in one reaction rather than one target/reaction. As NGS sequences multiple fragments, multiple times, it has higher sensitivity and can therefore detect variants that occur at lower frequencies.[1–4,7]

The limit of detection ranges from 5% to 20% for fluorescence-based Sanger sequencing, while the limit of detection of NGS depends on the depth of coverage (how often any particular point in the target region has been sequenced), which can be as low as, or even below, 1%. Having a low limit of detection gives greater flexibility, particularly when the proportion of cancer cells in a tissue sample is low or when there is genetic heterogeneity.[8]

NGS is far more rapid and also cheaper for sequencing multiple targets than standard methods.[9] It permits more comprehensive coverage of genes and increases diagnostic yield. As NGS samples can be multiplexed, NGS has higher throughput.

Limitations

NGS has pre-analytic, analytic and post-analytic limiting factors.[3]

Pre-analytic confounders. Although NGS can be performed on any template, the most common include frozen section materials, FFPE and liquid biopsies (plasma or CTCs).

The content of the tissue is important: the ratio of tumor to non-tumor cells must be above the limits of detection. If RNA sequencing is to be performed, this can be affected by external factors, such as warm ischemia. The best-quality DNA or RNA is achieved with the minimum of cold ischemia. RNA is more severely affected, with even short delays in freezing causing subtle changes in the RNA expression profile. Circulating free DNA is, in general, high quality, though it is often fragmented because of cleavage prior to release from cell nuclei; large fragments (more than 200 bp) are therefore less likely to be successfully sequenced.

The most utilized source of DNA or RNA is FFPE. Formalin fixation causes cross-linking and fragmentation of nucleic acids, resulting in low-quality and low molecular weight DNA. FFPE DNA is more prone to AT/GC drop-out, PCR errors and deamination artifacts. The last of these is caused by deamination of cytosine, resulting in a sequence change to thymidine (C:G to T:A) during PCR. Sequencing platforms analyzing both DNA strands negate such errors.

Analytic confounders. Errors in template concentration (too high or too low) can cause errors to occur in any of the steps of library preparation. Inaccurate dilution of the libraries can lead to errors. Batch variations in reagents may also contribute to errors.

NGS is not optimal for assessing repeat sequences. Those platforms utilized for sequencing very short fragments may miss large deletions (more than 20 bp).

One problem common to all platforms is loss of signal quality due to some of the molecules falling out of phase during the sequencing, with different residues being added. This results in inaccurate linking

of molecules from the same sequence, so that structural variations and splicing changes could be missed.

Post-analytic confounders. Large volumes of data are acquired in NGS, meaning that data need to be optimized and meaningful variations need to be distinguished from non-meaningful (noise) variations.

The data from sequencing give numerous signals, many of which are low quality and need to be filtered out. The effect can be negated by sequencing both strands, with a positive result only being called if both strands show the variant.

Information must be interpreted in the clinical context. A negative result needs to be interpreted in the light of sample adequacy. If there is inadequate tumor, the specimen should be regarded as inadequate rather than negative. Similarly, low or borderline levels need to be evaluated in the same light, depending on the depth of sequencing obtained. Novel abnormalities should be treated with reserve until databases have been populated with sufficient information to determine the pathogenicity of a mutation.

A mutation means a different thing in different tumors: while a mutation may be predictive in some tumors, it may have no clinical implications in others.

Tumor heterogeneity remains a major issue, particularly if low-frequency variants are found; these may represent subclones that could confer resistance to certain drugs. The significance of these variants needs to be evaluated in the context of the clinical scenario and the availability of specific therapies. Newer generation drugs targeting these subclones may be indicated. Bioinformatic systems, updated frequently according to international databases and with reference to specific guidelines, can indicate the most appropriate therapies given specific mutations and also which regimen/drugs may be contraindicated.

Developments in sequencing

Technology continues to develop exponentially, and ultimately some of the limitations of current NGS technologies will be overcome. The current platforms rely on clonal PCR to generate a signal. Theoretically, techniques to determine single-molecule sequencing would be preferable.

Third-generation sequencing methods, also termed large-fragment single-molecule sequencing, aim to sequence long DNA and RNA molecules.[7] Pacific Biosystems has developed a method that, although it requires library construction, detects single nucleotides during incorporation into a new strand using immobilized, specifically modified polymerase enzymes.[10]

Nanopore systems for DNA sequencing are being developed as a fourth-generation technology. These include biological membrane systems and solid-state technology and could potentially offer low-cost DNA and RNA sequencing and genotyping.[7]

Oxford Nanopore technology does not require new molecule synthesis. Single-stranded DNA molecules are fed through minute pores in an electrically resistant membrane. Specialized proteins feed single-stranded DNA through the pores, which have current running through them. The molecule disrupts the current and from the pattern of disruption the DNA sequence can be inferred. Analytic devices can be plugged into a USB port and real-time DNA sequences can be read.

While the Pacific Biosystems and Oxford Nanopore technologies are more prone to errors than NGS technologies, they are able to produce much longer reads. This improves the detection of large variants and transcript isoforms generated by alternative splicing, both of which are implicated in carcinogenesis.

Key points – understanding next-generation sequencing

- Sanger sequencing, although groundbreaking, is labor intensive, time consuming and expensive, and hence it has been replaced with NGS.
- The two major methods for NGS use the Illumina and Ion Torrent platforms. Illumina uses fluorescently tagged nucleotides while Ion Torrent detects a pH (voltage) change on nucleotide binding.
- It is vital that the results of NGS are considered in a clinical context. A result from an inadequate sample should not be interpreted as a negative result.
- Methods of single-molecule sequencing, although relatively error prone, have utility in the sequencing of large variants and transcript isoforms.

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3 Elements of comprehensive genomic profiles

Comprehensive genomic profiling is an NGS approach that detects novel and known variants of the four main classes of genomic alterations, and genomic signatures, to provide prognostic, diagnostic and predictive insights that inform research or treatment decisions for individual patients across all cancer types. CGP detects all four main classes of genomic alterations (see Chapter 1) in a comprehensive set of cancer-relevant genes and reports complex biomarkers, such as TMB and MSI. The results of the analyses, including potential treatment options based on the mutations identified, are summarized in a report.

Variants

The recent improvements in terms of available technologies and bioinformatics tools have allowed a better understanding of the pathobiology of different tumor types and the identification of several genomic alterations that may be targetable. CGP offers a complete evaluation of the genomic landscape of each tumor for both prognostic and predictive purposes, helping oncologists make decisions about cancer treatment.[1]

The adoption of broad NGS gene panels (Table 3.1) may support the administration of immunotherapy drugs in patients harboring high TMB (see page 45).[2] Another potentially valuable approach is hybrid capture-based CGP, an ultra-sensitive assay that can be used to detect variants even in specimens with low tumor purity (a low proportion of tumor cells).[3] The implementation of CGP in clinical practice is, however, limited by issues such as costs, turnaround time, sensitivity, specificity and bioinformatics pipelines.[1] Despite increasing knowledge of the cancer molecular landscape, the clinical and pathological significance of several molecular variants, including SNVs, CNVs and indels, remains unknown and unexplored.[4]

As far as variants are concerned and, in particular, when rare or unknown mutations are reported, it is fundamentally important to define the association with the risk of cancer development and

TABLE 3.1

NGS gene panels for CGP

		Type of alteration			
Gene panel	Number of genes	SNVs	CNVs	Indels	Rearrange-ments
FoundationOne CDx	324	✓	✓	✓	✓
MSK-IMPACT	468	✓	✓	✓	✓
Caris Molecular Intelligence	592	✓	✓	✓	
Tempus xT	595	✓	✓	✓	✓
ACE ImmunoID	>20 000	✓	✓	✓	✓
TruSight Tumor 170	170	✓	✓	✓	✓
Oncomine Comprehensive Assay	161	✓	✓	✓	✓
Oncomine Tumor Mutation Load Assay	409	✓	✓	✓	
NeoTYPE Discovery Profile	326	✓		✓	✓
CancerPlex	435	✓	✓	✓	✓

progression. In this setting, it is pivotal to predict the structure and the potential functional impact of the proteins generated to:

- define the frequency of appearance in the healthy population
- identify the inheritance within a family when the same pathological condition is evidenced
- assess the statistical significance of the association with cancer when the same alteration is identified in independent cancer patients.[1]

Single nucleotide variants in coding and intronic regions can significantly alter the functions of cancer-related genes.[4] An interesting field of investigation regarding SNVs involves mutations that affect non-coding regions. Mutations in these sequences in 3'-untranslated regions (3'-UTRs) are frequently implicated in cancer-related oncogenes.[5]

Copy number variants represent an important mechanism of activation and inactivation of oncogenes and tumor suppressor genes, respectively.[4] However, the detection of CNVs is limited by exon-capturing methods that do not cover many recurrent CNV regions and by other computational biases.[4] To overcome these biases, it is possible to define a CNV by the ratio of copies in cancer and normal DNA.[4]

Indels present several issues: in particular, they are not always easy to compare using different variant callers (separating real variants from artifacts), because there are several correct ways of reporting them; this factor may significantly limit reproducibility among laboratories.[6]

Microsatellite instability

Microsatellites are repetitive, highly preserved DNA sequences that occur throughout the genome. Their nature makes them particularly prone to DNA-mismatching errors (MSI) that can occur during DNA replication.[7]

Four genes are involved in the process of mismatch repair (MMR), which is the cell's method of overcoming these errors:[8] *MLH1, MSH2, MSH6* and *PMS2*. These genes encode four proteins, mutL homolog 1 (MLH1), mutS homolog 2 (MSH2), mutS homolog 6 (MSH6) and post-meiotic segregation increased 2 (PMS2), that form heterodimers: MLH1–PMS2 and MSH2–MSH6. These heterodimers work with other proteins to nick the strand around the mutation and repair the sequence. The inactivation of this system leads to a deficient MMR (dMMR) mechanism (Figure 3.1).[7]

Interestingly, only MLH1 and MSH2 are necessary partners in the heterodimers: MSH6 and PMS2 can be replaced by other proteins (such as MSH3, MLH3 and PMS1).[7] To determine the MMR status, both immunohistochemistry (IHC) and PCR-based techniques can be adopted.

Immunohistochemistry uses tagged antibodies to detect specific proteins – MLH1, MSH2, MSH6 and PMS2 in this instance – in tissue slices or samples.[9] The European Society of Medical Oncology (ESMO) recommends IHC as the first-line test to assess MMR status in sporadic cancer belonging to the spectrum of Lynch syndrome (colorectal, endometrial, small intestine, urothelial, central nervous system and sebaceous gland; Table 3.2).[7]

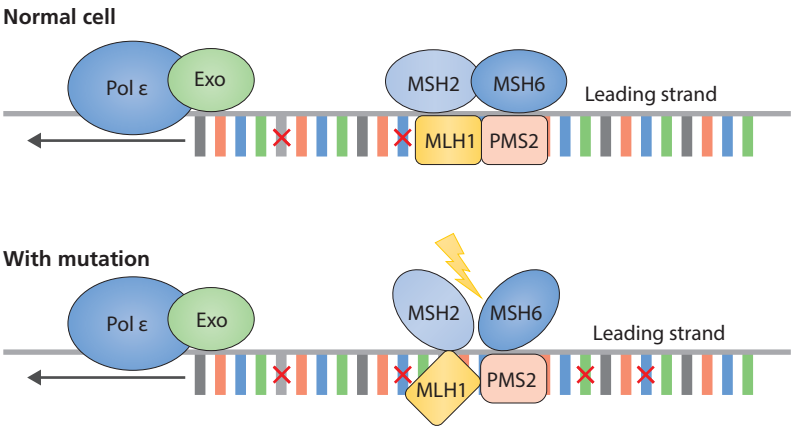

Figure 3.1 In normal cells, MLH1–PMS2 and MSH2–MSH6 support the repair of mutations in the DNA sequence. If this complex does not work because of mutations, the system may break down and mutations accumulate.

TABLE 3.2

ESMO recommendations for MSI testing

Assay	Recommendation
IHC	• First test of choice for analysis of MMR status • Panel to adopt should include evaluation of the expression of MLH1, MSH2, MSH6 and PMS2 proteins
PCR	• PCR confirmatory molecular analysis is mandatory when IHC is doubtful • Two different panels may be adopted – two mononucleotide (BAT-25 and BAT-26) and three dinucleotide (D5S346, D2S123 and D17S250) repeats – five polyA mononucleotide repeats (BAT-25, BAT-26, NR-21, NR-24, NR-27)*
NGS	• May be particularly helpful given its capacity to evaluate both MSI status and TMB

*This panel is preferable as it has higher sensitivity and specificity.

As discussed above, *MLH1* mutations are related to loss on IHC of both MLH1 and PMS2; in the same way, mutations in *MSH2* relate to the loss of both MSH2 and MSH6 on IHC.[7] Overall, it is necessary to adopt all four IHC antibodies to define MMR status.[7] However, IHC can be affected by several issues – for example, false-negative results may relate to problems with tissue fixation.[7] Where there are fixation issues, it is important to focus on positive internal controls (for example, immune cells).[7]

PCR-based techniques involve comparing the profiles of microsatellite loci generated by amplification from neoplastic samples and corresponding normal tissue material obtained from the same patient. If a locus reports a discordance between neoplastic and normal tissue samples, MSI is reported. Two different reference panels are used: one that considers two mononucleotide (BAT-25 and BAT-26) and three dinucleotide (D5S346, D2S123 and D17S250) repeats (an example is shown in Figure 3.2), and the other adopting five polyA mononucleotide repeats (BAT-25, BAT-26, NR-21, NR-24, NR-27).[10,11]

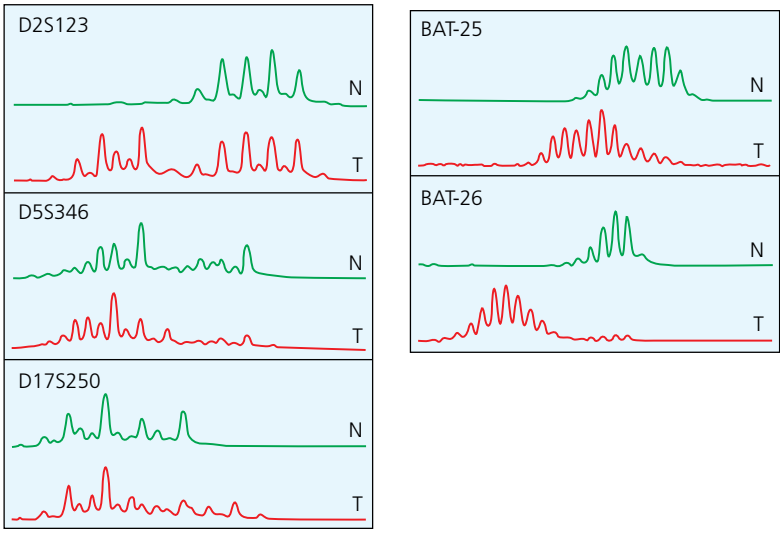

Figure 3.2 Analysis of five microsatellite loci in tumor DNA (T) and paired normal DNA (N). There are differences between the samples for all the loci analyzed. Reproduced, with permission from Oxford University Press, from Mallya et al. 2003.[12]

This latter panel is associated with a higher sensitivity and specificity.[7] MSI status is defined when two or more of five microsatellite loci are unstable.[7]

In indeterminate IHC cases, PCR may be a reliable and highly specific and sensitive tool to define MSI status.[7] ESMO guidance suggests that MSI-low (only one unstable locus) should be considered microsatellite stable (MSS).[7]

NGS may represent a valid alternative for MMR status assessment.[7]

MSI as a predictive biomarker. Because of the development of several mutations, MSI-high (MSI-H) status is a predictive biomarker of response to immunotherapy.[13,14] In May 2017, the FDA approved the use of an immune checkpoint inhibitor (pembrolizumab) in advanced stage solid tumors with MSI-H (evaluated by PCR or NGS) or dMMR (evaluated by IHC) status.[15] Other immune checkpoint inhibitors, such as nivolumab, are now also being used to treat some MSI-H cancers.[14]

Tumor mutational burden

TMB is the number of somatic, non-synonymous, coding base mutations that occur in a defined region of a tumor genome. The accumulation of a high number of mutations is able to generate several neoantigens that can potentially elicit a severe immune response against the tumor.[2] In this setting, CGP may usefully detect advanced stage cancers with high TMB. Rizvi et al. have highlighted the responsiveness of patients with advanced stage non-small cell lung cancer (NSCLC) and high TMB to the anti-programmed cell death 1 (PD-1) antibody pembrolizumab.[2] They emphasized that durable clinical benefit and progression-free survival (PFS) were higher in the cohort of patients with more than 200 mutations compared with patients with low TMB (<200 mutations).

Snyder et al. found similar results using a WES approach in patients with melanoma.[15] The responsiveness and overall survival of patients treated with the anti-cytotoxic T-lymphocyte antigen 4 (CTLA-4) antibody ipilimumab was higher in the high TMB cohort.[15] However, as WES is currently unfeasible in routine clinical practice, with its high costs and long turnaround time, several different approaches are now employed, based on wide NGS panels.

In the CheckMate 227 and 568 clinical trials, TMB was evaluated using the FoundationOne CDx assay to support the administration of an immunotherapy combination (ipilimumab plus nivolumab) in advanced stage NSCLC.[16] Patients were defined as having a high TMB when the tumor DNA harbored at least ten mutations per megabase.[16]

Different gene panels are available to evaluate TMB status (see Table 3.1). For example, Rizvi et al. adopted three different versions of the MSK-IMPACT panel, covering 341, 410 and 468 genes, to assess TMB.[17]

Data interpretation potentially limits the usefulness of TMB analysis, regardless of the approach adopted (WES versus wide gene panels) and gene panel employed. In terms of prediction of suitability for immunotherapeutic regimens, it is important to analyze not only the total number but also the type of the detected mutations.[2] While Rizvi et al. underlined the positive predictive value for immunotherapy when mutations in genes involved in DNA repair and replication are identified, Skoulidis et al. demonstrated that mutations in *STK11* (also known as *LKB1*) were associated with resistance to immunotherapeutic drugs.[18]

RNA

Fusions and splice variants. RNA-based molecular approaches, such as reverse transcription PCR (RT-PCR), NGS and multiplex digital color-coded barcode technology, play a key role in identifying targetable gene fusions (Figure 3.3).[19] These are chimeric genes generated from the fusion of two different genes belonging to the same or different chromosomes.[19]

According to international guidelines, a person with advanced stage NSCLC is potentially eligible for tyrosine kinase inhibitor (TKI) therapy if they have a gene fusion involving *ALK* or *ROS1*.[20] Other gene fusions have also been identified and are now the basis of therapeutic indications and/or are being explored in clinical trials (see Chapter 5).

As well as a predictive role, the identification of gene fusions may also have a key diagnostic function for assessing risk of malignancy, such as in thyroid nodules with undetermined cytological diagnosis.[21] In particular, *PAX8*/*PPARG* and *RET*/*CCDC6* gene fusions are more frequently associated with thyroid malignancies.[21]

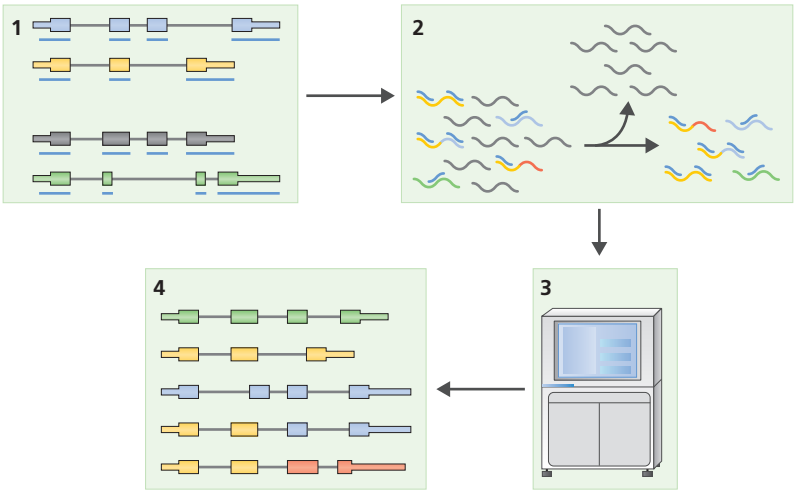

Figure 3.3 Targeted RNA sequencing to detect fusion genes. (1) Probes are designed against target sequences, such as exons, and fusion standards with known diagnostic/prognostic value, and a strand-specific library is prepared. (2) Probes capture (hybridize with) the targeted sequences; the non-targeted transcripts are washed away. (3) The RNA is sequenced. (4) Genes and standards are assembled and novel fusions – those not recognized by the probes – are identified.

Another important field of application for RNA-based molecular approaches is the identification of splice variants. The variation occurs at the splice site between an exon and an intron. It leads to alternative RNA splicing, resulting from the loss of exons or the inclusion of introns, and the generation of a novel protein (Figure 3.4).[22]

As for gene fusions, the presence of a splice variant may have a predictive role for therapy. In advanced stage NSCLC, *MET* exon 14 skipping is associated with responsiveness to crizotinib and cabozantinib.[23]

However, several issues affect RNA analysis. In particular, RNA is less stable than DNA, and for this reason careful attention must be paid to pre-analytic factors that could interfere with RNA stability.[24] In particular, fixation procedures may significantly influence RNA integrity and stability. An alcohol-based fixative gives better results in terms of RNA quality and quantity than a formalin-based fixative.[25]

When RT-PCR is used, careful attention should be paid to the design of primers. In particular, to minimize the risk of false-positive results or overestimation of gene expression, primers should be designed to lie on exon/exon junctions.[26] As far as the complementary DNA (cDNA) input is concerned, the concentration should be between 10 pg and 1 µg.

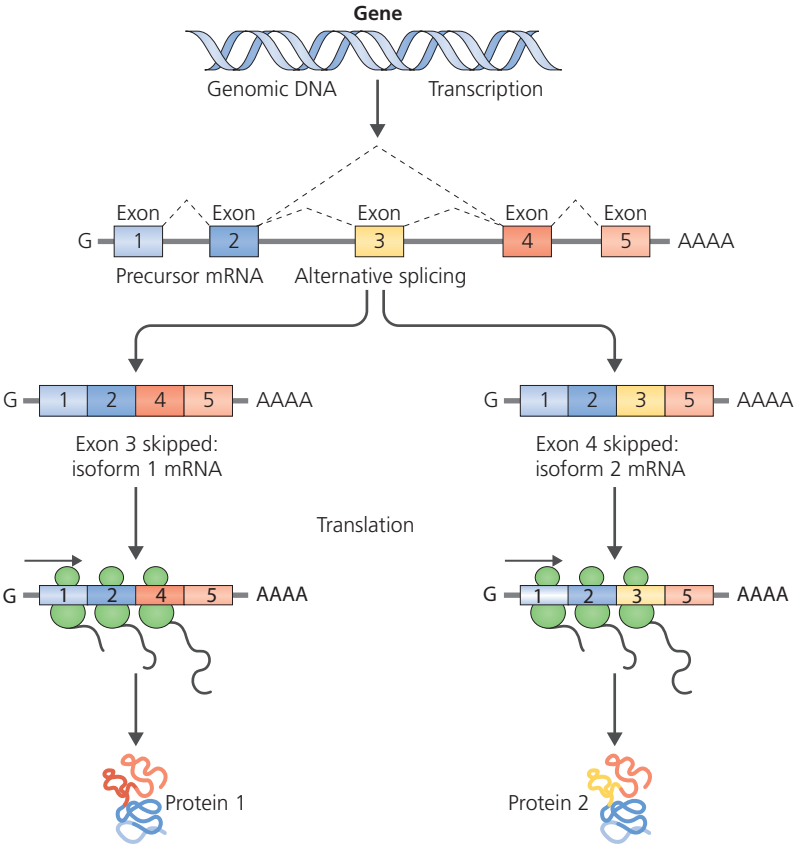

Figure 3.4 Alternative splicing can yield different mRNAs that are translated to protein isoforms that behave differently.

An important limitation is that the RT-PCR method can only identify known alterations. This limitation can be overcome by NGS, which allows the identification of known and unknown gene alterations for different patients simultaneously.[27] However, NGS suffers from several limitations related to high costs and, in particular, data interpretation (see Chapter 6). Strengthening automated data analysis with ad hoc information from the bioinformatics pipeline may reduce this issue.

The nCounter Analysis System is another approach. This is a multiplex digital color-coded barcode technology that can identify gene fusions and splice variants simultaneously.[28] Only a small amount of RNA input is needed (25–250 ng) and the method has a short turnaround time for testing (about 3 working days). In addition, the nCounter technology is able to identify several gene fusions and splice variants, even when RNA is of low quality and target capture amplification fails.[28]

The nCounter workflow is characterized by four main steps:

- pairs of probes (reporter probe and capture probe), designed to be adjacent to one another along the target sequence of a transcript, are hybridized with mRNAs
- excess probes are removed and the hybridized complex is bound to the surface of the sample cartridge
- the probe/target complexes are aligned on the cartridge by an electric current and immobilized for data collection
- the sample cartridge is scanned by a digital analyzer, and each color code is counted and tabulated.

The nCounter system has a number of limitations, however. In particular, results generated by reading the color-coded barcodes are not directly usable for clinical purposes. Thus, additional data processing is required. Furthermore, careful attention needs to be paid to the design of the probe to avoid false-positive results.[29]

Key points – elements of comprehensive genomic profiles

- Comprehensive genomic profiles offer a complete evaluation of the genomic landscape of each tumor. The interpretation of the different variants reported, with attention focused on their clinical significance and usefulness for cancer patients, can support oncologists to make informed cancer treatment decisions.
- The clinical and pathological significance of several molecular variants, including SNVs, CNVs and indels, remains unknown.
- Some therapies are indicated for MSI-H cancer, and others are in clinical trials. Careful attention should be paid to the accuracy of the MMR evaluation (IHC, PCR or NGS).
- WES or wide gene panels may be employed to assess TMB status to guide immunotherapy decisions.
- RNA-based molecular approaches play a pivotal role in the identification of targetable gene fusions and splice variants.

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4 Role in precision oncology

The goal of precision medicine in oncology is to match an individual patient to the correct treatment at the right time, based on the biologic and molecular features of the patient's cancer.

Examples of molecularly targeted agents include imatinib and trastuzumab. Imatinib, a TKI, targets the kinase domain of ABL in the ABL–BCR fusion product that may be identified in, for example, chronic myeloid leukemia. Trastuzumab is a monoclonal antibody that targets the HER2 amplification – for example, in some patients with breast cancer.

The precision medicine approach is now being applied with molecularly targeted treatments and immune-based therapeutics across numerous cancers, including gastrointestinal stromal tumors (GISTs), malignant melanoma and NSCLC.[1–3] In approximately 50% of patients with metastatic NSCLC, the tumor harbors a molecular abnormality that could be treated with targeted therapy (see Chapter 5).

Clinically appropriate biomarkers include genomic modifications in tissue or blood, CTCs and gene expression. Many of these, and the tools that utilize them, are being studied. With the improvement in sequencing technologies, CGP of tissue has developed as the standard of care for sequencing across multiple cancers.

Regulation and approval

The FDA has approved many targeted therapies and immunotherapies with companion diagnostic tests (see next page). NGS is now approved to screen and diagnose many of these targetable abnormalities, and the technologies are steadily being applied to guide therapeutic decision-making.

Given the complexities associated with understanding NGS test results and acting on them, academic and community molecular tumor boards (MTBs) have been developed to implement multidisciplinary expertise (see Chapter 6). NGS test results may

identify FDA-approved therapies, guide clinical trial recommendations or prompt consideration of expanded access to investigational agents or off-label use of therapies approved for other indications. Many clinical trials now include molecular testing to assign treatments to patients based on the molecular profiles of their tumors.

The FDA has publicized mechanisms for the regulatory approval of NGS.[4] These include a pathway for the approval of NGS tests for tumor profiling that involves third-party reviewers of in vitro diagnostics; the New York State Department of Health (NYSDOH) is the first third-party reviewer to work with the FDA in this way.[5]

In the European Union (EU), the European Medicines Agency (EMA) gives a scientific opinion on whether a companion diagnostic for a medicinal product is suitable if the medicinal product has already been approved through its centralized procedure or falls within the scope for approval in this way.

The FDA recognizes three levels of biomarkers and has commented on the analytic and clinical evidence necessary to support NGS tests for these biomarkers (Figure 4.1).[4,6]

Level 1, companion diagnostics. A companion diagnostic is a test that informs the safe and effective use of a corresponding therapy. For approval of the test, there should be evidence of its analytic validity

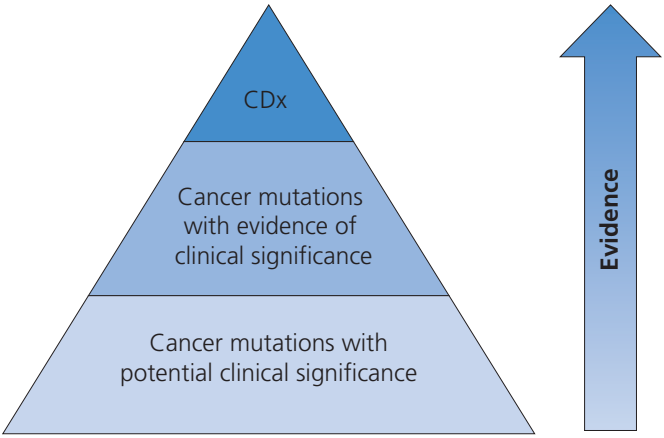

Figure 4.1 FDA pathway for approval of NGS testing platforms. CDx, companion diagnostic.[4]

for each specific biomarker and a clinical study that establishes either the link between the result of that test and patient outcomes or clinical concordance with a previously approved companion diagnostic.

Level 2, cancer mutations with evidence of clinical significance. Tests for cancer mutations allow physicians to make evidence-based treatment decisions. Analytic and clinical validity should be demonstrated, the latter through clinical evidence such as professional guidelines or peer-reviewed publications.

Level 3, cancer mutations with potential clinical significance. These are mutations that do not fall within level 1 or 2. The variants are informational or used to direct patients toward clinical trials. Tests for these biomarkers should be supported by analytic validation and a clinical or mechanistic rationale for their inclusion in the panel.

Molecular tumor boards. Approximately 9% of patients with metastatic cancer harbor targetable genomic alterations for optimal treatment selection with the standard treatments. An additional 27% of patients carry genomic abnormalities with convincing clinical evidence supporting the use of these alterations as predictive biomarkers for response to treatment outside of a therapy's registered indication.[7] Multidisciplinary MTBs, sometimes called multidisciplinary meetings (MDMs) or multidisciplinary teams (MDTs) facilitate the incorporation of molecular diagnostics into the consideration of appropriate therapies for patients with late-stage cancer. See Chapter 6 for further discussion.

Current landscape

FoundationOne CDx (F1CDx) is an NGS-based in vitro diagnostic device for the detection of substitutions, indels and CNVs in 324 genes, selected gene rearrangements and genomic signatures, including MSI and TMB. The test analyzes DNA isolated from FFPE tumor tissue specimens. Programmed death-ligand 1 (PD-L1) IHC can be carried out to provide additional information for immunotherapy selection.

The TMB result, reported as mutations per megabase (mut/Mb), is based on the total number of all synonymous and non-synonymous variants present at 5% allele frequency or higher (after filtering).

FoundationOne Heme is a comprehensive genomic profiling test that combines DNA and RNA sequencing of 406 and 265 genes, respectively. It is used for patients with hematologic malignancies, sarcomas or solid tumors for which RNA sequencing is required. The test allows sensitive detection of translocations and fusions. TMB and MSI are also reported.

TruSight Oncology 500 is a pan-cancer assay that identifies known and emerging biomarkers. The assay uses both DNA and RNA from tumor samples to identify somatic variants, including small variants, gene fusions and splice variants. It can also measure TMB and MSI. The assay covers a large number of genes as well as 1.94 megabases of the genome to measure TMB. It also provides information on MSI.

TruSight Tumor 170 is an NGS-based assay that uses an enrichment-based targeted panel to simultaneously analyze DNA and RNA for alterations in 170 genes associated with common solid tumors. The test assesses fusions, splice variants, indels, SNVs and amplifications with as little as 40 ng of input DNA and RNA, and as low as 5% mutant allele frequency. DNA and RNA are prepared in parallel with an integrated workflow following DNA shearing/cDNA synthesis.

Liquid biopsy is a method of detecting biomarkers in blood for prognostic and predictive purposes. Different analytes, such as CTCs, progenitor and mature endothelial cells, circulating cell-free DNA (ccfDNA), ctDNA and circulating cell-free RNA (ccfRNA), can be identified in blood samples.

ctDNA-based liquid biopsies are currently under investigation.[8,9] Cancer cells release ctDNA by a variety of mechanisms and ctDNA can, therefore, provide information about the genomic nature of the tumor in a specific patient. Using longitudinal ctDNA-based liquid biopsies, tumor mutations may be monitored to predict and assess response to treatment and the presence of minimal residual disease, recurrent or

early metastatic disease and potential resistance or refractory clones. Current challenges include standardization, sensitivity and specificity.

Clinical basket trials and umbrella designs

Traditionally, oncology Phase I clinical trials were conducted with a mixture of solid tumors, and Phase II and III oncology trials were histopathology based. A Phase II oncology study would be designed to answer the question: does a particular treatment at a selected dose in a particular tumor type (from histology) improve clinical outcome (response to treatment, PFS or overall survival)?

Recent developments in molecular profiling of tumors have led to the development of biomarker-driven clinical trials. These can be categorized as basket and umbrella trials, depending on the design (Figure 4.2). A basket trial tests a single treatment and a single biomarker (mutation X in Figure 4.2) in patients with different histologies. In an umbrella trial, participants have a single histology and multiple biomarkers are each matched to different treatments.

Numerous clinical studies are now using novel diagnostics to ascertain the molecular pathways associated with cancer development and progression. Examples are NCI-MATCH, the TAPUR Study, the European Organisation for Research and Treatment of Cancer (EORTC) SPECTA project, DRUP, I-PREDICT and STARTRK-2.

NCI-MATCH is supported by the US National Cancer Institute (NCI) and is coordinated by the Eastern Cooperative Oncology Group (ECOG)–American College of Radiology Imaging Network (ACRIN) Cancer Research Group.[10]

MATCH is a Phase II precision medicine trial that aims to determine whether a drug or drug combination matched to specific genetic aberrations will effectively treat that cancer, regardless of tumor type. The primary endpoint for MATCH is the percentage of patients whose tumors have a complete or partial treatment response.

The TAPUR Study is a non-randomized clinical trial that aims to identify the performance (both safety and efficacy) of FDA-approved targeted anticancer drugs used for the treatment of advanced disease that potentially has actionable genomic alterations.

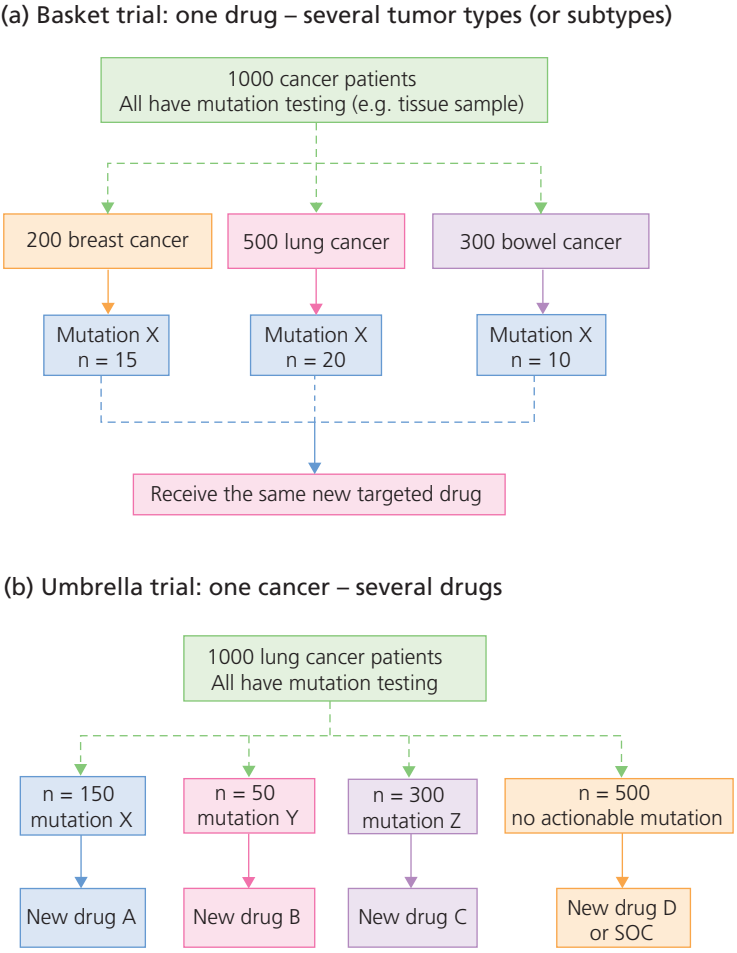

Figure 4.2 A basket trial (a) tests a single drug against a single mutation in cancers at different sites, while an umbrella trial (b) tests a number of drugs against a cancer with a single histology but with different mutations identified. Reproduced from *Fast Facts: Clinical Trials in Oncology*. SOC, standard of care.

The study provides FDA-approved targeted therapies contributed by collaborating pharmaceutical companies, catalogs the choice of genomic profiling tests by clinical oncologists and aims to identify the potential signals of drug activity. A total of 117 clinical sites in the USA are involved in the TAPUR Study, which is the first clinical trial conducted by the American Society of Clinical Oncology (ASCO).

Early data show positive results in three cohorts of patients, described below.[11–13]

Colorectal cancer patients with BRAF p.V600E mutation. The combination of the MEK inhibitor cobimetinib and the BRAF inhibitor vemurafenib showed antitumor activity in a cohort of heavily pretreated patients.

Colorectal cancer patients with an HER2 (ERBB2) amplification. Responses were documented in a cohort of heavily pretreated patients who received the anti-HER2 monoclonal antibodies pertuzumab and trastuzumab.

Colorectal cancer patients with high TMB. Pembrolizumab showed efficacy in a cohort of previously treated patients with colorectal cancer (CRC) and a high TMB (defined as nine or more mutations per megabase by either a FoundationOne test or another test agreed by the MTB).

SPECTA is a collaborative European platform that supports the delivery of high-quality molecular and pathological screening across multiple cancer types to aid patient selection for clinical trials.[14] It provides integrated clinical research support in an international setting involving top-level university hospitals from the EORTC network. SPECTA provides a single entry point to multiple studies, prompt access to patient data and biological specimens for research purposes and longitudinal follow-up of patients to help understand patterns of progression.

SPECTA has a flexible infrastructure comprising a biobank, clinical data collection and genomic analysis that allows researchers to reach outside of clinical trials to access pathologically annotated biological material from cancer patients. The goal of the platform is to support biospecimen-based translational research and biomarker discovery and, ultimately, propose new treatment options for cancer patients.

DRUP, the Drug Rediscovery Protocol launched in the Netherlands in 2016, is trialing FDA- and/or EMA-approved targeted therapies in patients with potentially actionable variants in a tumor type (advanced or metastatic solid tumor, multiple myeloma or B-cell non-Hodgkin lymphoma) outside of the therapy's approved indication.[15] The aim is to increase patient access to potentially effective therapies while building a knowledge base for outcomes in off-label use. This is particularly important for patients with rare cancers, for which Phase II and III trials are unlikely to be conducted.

I-PREDICT utilized tumor DNA sequencing and timely recommendations for individualized treatment with combination therapies.[16] The investigators found that it was viable to administer customized multidrug regimens, with 49% of consented patients receiving personalized treatment. Targeting of a larger fraction of identified molecular alterations, yielding a higher 'matching score', was correlated with significantly improved disease control rates, as well as longer progression-free and overall survival rates, compared with targeting of fewer somatic alterations. The findings indicate that treating molecularly complex and heterogeneous malignancies with combinations of customized agents could offer a means of improving outcomes over those achieved with the current approach, which pairs one driver mutation with one drug. However, additional studies are needed to confirm the findings.

STARTRK-2 is an open-label, multicenter, global Phase II basket trial of entrectinib for the treatment of patients with locally advanced or metastatic solid tumors that harbor *NTRK1/2/3*, *ROS1* or *ALK* gene rearrangements.[17] Patients are allocated to separate baskets according to their tumor type and gene fusion.

Potential cost benefit and reimbursement

The use of personalized medicine is potentially a cost-effective choice in cancer patients if a molecular selection can identify which patients will achieve partial, complete or durable responses to a particular treatment. Models of cost-effectiveness need to be developed so that they can be usefully applied to emerging targeted treatments and

immunotherapy agents. These models should be the basis for obtaining reimbursement approval for the newly developed NGS platforms.

Key points – role in precision oncology

- Precision medicine in oncology matches an individual patient to the correct treatment, based on the biological and molecular features of the patient's cancer.
- The FDA recognizes a pathway of three levels of biomarkers, based on the clinical evidence necessary to support NGS tests.
- Multidisciplinary MTBs, comprising various medical disciplines, facilitate the incorporation of molecular diagnostics and interpretation of results into therapeutic decision-making for a patient with cancer.
- A basket trial is a biomarker-driven study in which a single treatment and single biomarker are tested in patients with different histologies.
- An umbrella trial is a biomarker-driven study in which participants with a single histology and multiple biomarkers are each matched to a particular treatment.

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5 Predictive and prognostic biomarkers

Non-small cell lung cancer

Lung cancer is the principal cause of death from cancer worldwide.[1] The identification of biomarkers that can inform treatment selection is therefore of vital importance.

'Must test' genes. For advanced stage NSCLC, international guidelines by the College of American Pathologists (CAP), the International Association for the Study of Lung Cancer (IASLC) and the Association for Molecular Pathology (AMP), the National Comprehensive Cancer Network (NCCN) and ASCO define a minimum panel of genes – the 'must test' genes – that should be tested to inform treatment decisions.[2–4]

The use of TKIs is guided by the presence of:

- alterations in *EGFR*
- gene fusions involving *ALK* and *ROS1*
- alterations in *BRAF*.

EGFR. Mok et al. demonstrated, for the first time, that gefitinib, a first-generation epidermal growth factor receptor (EGFR) TKI, was more effective than carboplatin–paclitaxel doublet chemotherapy in patients with *EGFR* mutations (12-month PFS 24.9% versus 6.7%, respectively).[5] A similar result was obtained by Rosell et al. for another EGFR first-generation TKI, erlotinib.[6] In this clinical trial, the 1-year PFS with the TKI was also higher than that with chemotherapy (40% versus 10%, respectively).[6] The study authors focused attention on the better response of patients harboring *EGFR* exon 19 deletions rather than point mutations in *EGFR* exon 21.[6]

The second-generation EGFR TKI afatinib has also been shown to be more effective than chemotherapy in patients with *EGFR* mutations.[7,8] More recently, patients receiving the third-generation EGFR TKI osimertinib have been shown to have a higher PFS (18.9 months versus 10.2 months) and a lower number of high-grade adverse events compared with those taking first-generation TKIs.[9]

ALK rearrangements occur in a limited number of patients with advanced stage NSCLC (3–5%). Despite the low number, patients with an *ALK* fusion have been shown to respond well to the first-generation anaplastic lymphoma kinase (ALK)- fusion TKI crizotinib when compared with chemotherapy (PFS 10.9 months versus 7.0 months and objective response rate [ORR] 74% versus 45%), with a significant improvement in quality of life.[10]

The second-generation ALK TKI alectinib was investigated to overcome the limitations of crizotinib (in particular, the inability to act on metastasis in the central nervous system); it has been shown to be more effective than crizotinib in *ALK*-rearranged patients.[11,12]

ROS1 rearrangements. Patients with *ROS1*-positive rearrangements showed responsiveness when treated with crizotinib (median PFS 19.2 months and 3 of 50 participants having a complete response).[13]

More recently, in the STARTRK-1 Phase I clinical trial, the tyrosine kinase multi-inhibitor entrectinib has shown promising results in patients with *ROS1* rearrangements.[14]

BRAF p.V600E. Identification of the *BRAF* p.V600E mutation is increasingly important as it supports treatment with dabrafenib plus trametinib in advanced stage NSCLC.[15,16]

Programmed death-ligand 1. In addition to the must test genes, the guidelines (see above) strongly recommend evaluating PD-L1 expression to inform immunotherapy decisions.

Two clinical trials (KEYNOTE-024 and KEYNOTE-042) have shown pembrolizumab to be an effective first-line treatment when at least 50% of cancer cells express PD-L1, or a second-line therapeutic choice when at least 1% of cancer cells express PD-L1, respectively (Table 5.1).[17,18]

'Should test' genes are other clinically relevant genes that are being investigated in this therapeutic area. In addition to a negative prognostic role, acquired *KRAS* mutations have a predictive value in advanced stage NSCLC. A Phase I study showed promising results in terms of efficacy and safety for a novel small molecule, AMG510, that is able to target, irreversibly, the *KRAS* p.G12C point mutation.[19]

TABLE 5.1

Clinical trials supporting the use of biomarkers in advanced stage NSCLC

Clinical trial	Biomarker	Investigated drug	Standard of care
IPASS[5]	*EGFR*	Gefitinib	Carboplatin–paclitaxel
EURTAC[6]	*EGFR*	Erlotinib	Cisplatin/carboplatin–docetaxel/gemcitabine
LUX-LUNG 3[7]	*EGFR*	Afatinib	Cisplatin–pemetrexed
LUX-LUNG 6[8]	*EGFR*	Afatinib	Cisplatin–gemcitabine
FLAURA[9]	*EGFR*	Osimertinib	Gefitinib/erlotinib
ARCHER 1050[25]	*EGFR*	Dacomitinib	Gefitinib
PROFILE 1014[10]	*ALK*	Crizotinib	Cisplatin/carboplatin–pemetrexed
ALEX[11]	*ALK*	Alectinib	Crizotinib
J-ALEX[12]	*ALK*	Alectinib	Crizotinib
ASCEND-4[26]	*ALK*	Ceritinib	Cisplatin/carboplatin–pemetrexed
ALTA 1L[27]	*ALK*	Brigatinib	Crizotinib
NCT01970865[28]	*ALK, ROS1*	Lorlatinib	–
PROFILE 1001[13]	*ROS1*	Crizotinib	–
NCT01964157[29]	*ROS1*	Ceritinib	–
ALKA-372-001, STARTRK-1 and STARTRK-2[30]	*ROS1*	Entrectinib	–
KEYNOTE-024[17]	*PD-L1*	Pembrolizumab	Carboplatin/cisplatin–pemetrexed/gemcitabine or carboplatin–paclitaxel

(CONTINUED)

TABLE 5.1 (CONTINUED)

Clinical trials supporting the use of biomarkers in advanced stage NSCLC

Clinical trial	Biomarker	Investigated drug	Standard of care
KEYNOTE-042[18]	*PD-L1*	Pembrolizumab	Carboplatin–paclitaxel/ pemetrexed
NCT01336634[15]	*BRAF* p.V600E	Dabrafenib (BRAF inhibitor) + trametinib (MEK inhibitor)	–
LIBRETTO-001[31]	*RET*	Selpercatinib	–
GEOMETRY[32]	*MET*ex14	Capmatinib	–
NAVIGATE[21] SCOUT[33]	*NTRK* rearrangement	Larotrectinib	–
ALKA-372-001, STARTRK-1 STARTRK-2[34]	*NTRK* rearrangement	Entrectinib	–

*MET*ex14, *MET* exon 14 deletion.

Although the mutations are rare, cancers with *RET* and *NTRK* rearrangements show high sensitivity to cabozantinib and larotrectinib, respectively.[20,21] Entrectinib is another drug that targets the products of NTRK rearrangements.

Patients harboring *NRG1* gene fusions respond to afatinib treatment.[22]

A high response rate was found when crizotinib and cabozantinib were administered to patients with a *MET* mutation causing exon 14 skipping.[23] Capmatinib is another MET inhibitor being evaluated as a treatment for NSCLC with *MET* exon 14 skipping.

Tumor mutational burden. As far as immunotherapy decisions are concerned, TMB is another predictive biomarker. A high number of non-synonymous mutations (at least ten) has been shown to be predictive of response to nivolumab plus ipilimumab, regardless of PD-L1 expression.[24]

Breast cancer

Breast cancer remains the most common cancer type among women in the USA and Europe.[1] Despite advances in detection and treatment, breast cancer remains the second leading cause of death for women in the western world, with most deaths attributed to metastatic disease.[35]

With increasing treatment options for metastatic breast cancer (mBC), it is of fundamental importance to know the genomic landscape of the disease and how to incorporate tumor genomic findings into clinical practice.

Primary breast cancer. Besides the classic biomarkers used by pathologists in every case of primary breast cancer, such as estrogen receptor (ER), progesterone receptor (PR) and HER2, which guide prognostication and treatment selection, gene expression profile assays have recently been incorporated into the biomarker assessment of early breast cancer.[36] Commercially available genomic assays that provide these profiles include Oncotype DX, MammaPrint, Predictor Analysis of Microarrays 50 (PAM50), EndoPredict and Breast Cancer Index.[36]

Data from two large randomized clinical trials examining Oncotype DX and MammaPrint have yielded important evidence for use in discussions about potential benefit from chemotherapy in specific patient populations. When using the Oncotype DX assay, chemotherapy is not recommended for patients older than 50 years whose tumors have a recurrence score of less than 26. For those patients younger than 50 years whose tumors have a recurrence score of less than 16, there is little to no benefit from chemotherapy; however, clinicians may offer chemoendocrine therapy to those with a recurrence score in the range 16–25. In addition, oncologists may offer chemoendocrine therapy to any patient with recurrence score of 26–30.[37] There are many guidelines published concerning the use of these assays.[36–38]

Metastatic breast cancer. In breast cancer, CGP is more applicable in the metastatic setting. The recent successes with a PI3K inhibitor for the treatment of *PIK3CA*-mutated hormone receptor (HR)-positive mBC and of poly(ADP-ribose) polymerase (PARP) inhibitors in

deleterious germline *BRCA1/2*-mutated mBC have solidified the role of genomic testing to guide therapy for patients with mBC.[35]

The routine implementation of NGS in many laboratories has allowed the readout of large amounts of DNA, making it possible to detect multiple genetic alterations at the same time, using the same assay, leading to the concept of 'multigene sequencing'. This can be applied to tumor tissue, CTCs, ctDNA and normal tissue (with germline DNA).

The genomic landscape of mBC is broad; alterations in multiple genes have been found, many with potentially actionable changes. Here, we will discuss only those classified as tier I-A (prospective randomized clinical trials show the alteration–drug match in a specific tumor type results in a clinically meaningful improvement in a survival endpoint) according to the ESMO Scale for Clinical Actionability of molecular Targets (ESCAT) or level 1 (FDA-recognized biomarker predictive of response to an FDA-approved drug in this indication – see Chapter 4) by the precision oncology knowledge base OncoKB.[39,40] These are *PIK3CA* mutations, germline *BRCA1/2* mutations, *HER2* amplification, MSI and *NTRK* translocations.[35,39,40]

PIK3CA codes for the catalytic subunit of PI3K. A gain-of-function mutation can cause the activation of multiple downstream signaling cascades, including the PI3K/AKT/mTOR pathway that promotes cell survival and proliferation (see Figure 1.6). The Phase III randomized SOLAR-1 trial compared the combination of the PI3K inhibitor alpelisib and fulvestrant with fulvestrant alone in patients with HR-positive *HER2*-negative mBC who had progressed on prior endocrine therapy.[41] Participants receiving alpelisib–fulvestrant had superior PFS compared with those receiving fulvestrant alone, leading the FDA to approve alpelisib for HR-positive mBC.[42] These findings stress the importance of clinical testing for the *PIK3CA* mutation in patients with HR-positive mBC who experience progression on first-line endocrine therapy. Alpelisib is now also authorized for use in the EU.

BRCA1/2 germline mutations result in homologous recombination deficiency (HRD). PARP enzymes are essential for DNA single-strand break repair. Tumors with germline HRD rely more heavily on PARP enzymes for DNA repair;[43] therefore, inhibition of PARP enzymes leads to persistence of DNA single-strand breaks and eventual cell death through synthetic lethality.

The randomized OlympiAD trial compared olaparib, a PARP inhibitor, with single-agent chemotherapy in patients with *HER2*-negative mBC who harbored a germline *BRCA1/2* mutation. The results showed significant improvement in PFS in the olaparib group compared with the chemotherapy group (7.0 months versus 4.2 months). The ORR in the olaparib group was 59.9% compared with 28.8% in the chemotherapy group.[44] On the basis of this study, olaparib received FDA approval for the treatment of *HER2*-negative mBC with germline *BRCA1/2* mutations in patients previously treated with chemotherapy. Olaparib is also approved for use in the EU.

Similarly, the Phase III EMBRACA trial compared the PARP inhibitor talazoparib with single-agent chemotherapy in patients with mBC harboring germline *BRCA1/2* mutations. The median PFS was significantly longer in the talazoparib arm than in the chemotherapy arm (8.6 months versus 5.6 months).[45] On the basis of this study, talazoparib received FDA approval for the treatment of *HER2*-negative mBC with germline *BRCA1/2* mutations, regardless of prior chemotherapy use.[46] Talazoparib is also approved for use in the EU.

The role of PARP inhibitors for the treatment of mBC with somatic *BRCA1/2* mutations is under investigation.[37] Although NGS is able to detect *HER* amplification, it is more commonly determined in the clinical setting using IHC or in-situ hybridization (ISH). Multiple clinical trials have confirmed the role of different HER2-directed therapies in the treatment of *HER2*-amplified breast cancer across multiple settings.[37]

MSI. Evaluation of MSI that leads to defects in DNA mismatch repair has become a standard of care in metastatic solid tumors. Patients with tumors that harbor MSI are candidates for treatment with the immune checkpoint inhibitor pembrolizumab.[47] MSI is rare in breast cancer, with rates between 0.9% and 1.5%, though the frequency is higher in triple-negative breast cancer (TNBC).[35,48]

NTRK1, ***NTRK2*** and ***NTRK3*** genes encode the three transmembrane tropomyosin receptor kinase (Trk) proteins, TrkA, TrkB and TrkC. Fusion of an *NTRK* gene induces constitutively active protein function, resulting in an oncogenic driver.[49] Two Trk inhibitors, larotrectinib and entrectinib, have gained FDA approval for the treatment of solid tumors that harbor an *NTRK* gene fusion. Larotrectinib has also been approved by the EMA.

In breast cancer, *NTRK* fusions are found most commonly in secretory breast carcinomas and mammary analog secretory carcinomas.[50] The frequency of *NTRK* fusions in mBC is low; one study that examined 12 214 consecutive patients with mBC found that 0.13% of tumors harbored *NTRK* gene fusions.[51]

Other mutations. Several other alterations described in multigene sequencing, such as mutations in *ESR1* (related to increased resistance to endocrine therapy), *HER2*, *PTEN* and *AKT1*, are being investigated in clinical trials.[35]

The value of assessing TMB in mBC also remains investigational.

Colorectal cancer

Globally, CRC is the third most commonly diagnosed cancer and the second most common cause of cancer-related deaths in men and women.[1] More than 90% of cases are sporadic, the other 10% resulting from hereditary cancer syndromes.

Prognostic and predictive biomarkers have been well established in CRC, and NGS is adding to this well of information. Three major pathways of carcinogenesis have been elucidated:[52]

- chromosomal instability (CIN), which accounts for ~85% of all CRCs
- MSI, which accounts for ~15% of CRCs
- CpG island methylator (CIMP), which is found in 17% of CRCs and shows overlap with the MSI pathway.

Chromosomal instability is characterized by alterations in chromosomes 17p and 18q. In addition, the tumors acquire mutations in oncogenes and tumor suppressors including *APC*, *TP53*, *KRAS* and *BRAF*. According to the Vogelstein model,[53] there is initial inactivation of *APC*, followed by mutations of *RAS* with inactivation of the *TP53* suppressor gene.

The most clinically relevant pathways affected are the Wnt and MAPK pathways. Alterations in the Wnt signaling pathway, which occur in 93% of all CRC tumors, lead to cell proliferation. The MAPK pathway is activated by RTKs, such as EGFR, though it can be activated by other downstream signaling molecules, such as KRAS proto-oncogene, GTPase (KRAS), NRAS proto-oncogene, GTPase (NRAS) and BRAF, as well as ERK.[54]

Microsatellite instability is due to generalized instability of short tandem repeats of DNA sequences known as microsatellites, resulting from mutations of the MMR genes *MLH1*, *MSH2*, *MSH6*, *PMS2,* or silencing of *MLH1* by hypermethylation of the CpG-rich promoter sequence (see Chapter 3). Mutations resulting in the inability to repair replication errors result in Lynch syndrome. Hypermethylation of the *MLH1* promoter can cause sporadic cancer.

MSI can be determined by IHC: loss of staining of one or more of the MMR proteins indicates MMR deficiency (that is, MSI). PCR can also be used, with commercial kits available to test for the five microsatellite loci, referred to as the Bethesda panel, BAT-25, BAT-26, D2S123, D5S346 and D17S250, as proposed by the NCI. NGS can also be utilized to determine MSI status and allows for analysis of over 100 loci. NGS has 98% sensitivity and 100% specificity as compared with PCR.

MSI-H tumors have been shown to have a better prognosis. Importantly, fluorouracil-based chemotherapy has no benefit in MSI-H CRC.[55]

CpG island methylator phenotype. The CIMP pathway is characterized by hypermethylation of CpG island loci and inactivation of suppressor genes. Sporadic MSI CRCs are associated with CIMP-associated methylation of the *MLH1* promoter which, in turn, is associated with the presence of *BRAF* mutation.

NGS testing for predictive biomarkers. EGFR activation of the RAS/RAF/MEK/ERK pathway (see Chapter 1) plays an important role in oncogenesis in CRC. Up to 50% of CRCs show activating mutations of *KRAS*.

Anti-EGFR antibodies, such as cetuximab and panitumumab, can be beneficial in patients with metastatic CRC provided that there is no downstream mutation activating the RAS/RAF/MEK/ERK pathway.[54] Initially, patients with codon 12/13 *KRAS* mutations did not benefit in clinical trials,[56] and subsequently mutations in other codons of *KRAS* and in *NRAS* were also shown to confer resistance to antibody-based therapies.[57,58] Thus, guidelines require an 'extended' RAS analysis be performed. At present, the minimum testing required to determine

whether anti-EGFR therapy may be of benefit is this extended RAS testing of *KRAS* and *NRAS*:[58]

- codons 12 and 13 of exon 2
- codons 59 and 61 of exon 3
- codons 117 and 146 of exon 4.

Wild-type phenotype for *KRAS* and *NRAS* is thus an indication for anti-EGFR therapy.[59]

Theoretically, activating mutations in the genes for any of the downstream molecules in the RAS/RAF/MEK/ERK pathway should result in a pathological scenario similar to that with RAS mutation.[60] At present, though, there are insufficient data to support differentiating the treatment of wild-type RAS and *BRAF*-mutated cancers and nor is there evidence to support the use of anti-BRAF agents (as there is in melanoma). Nonetheless, a *BRAF* mutation is a negative prognostic indicator.[61]

US and European guidelines mandate extended RAS and *BRAF* testing and MSI analysis using the most appropriate methods (Figure 5.1).[58,62] NGS provides the widest genetic coverage and most cost-effective solution. Where gene chips for multiple genes are utilized, extra information may be gained. In particular, mutations in other genes (*PIK3CA*, *PTEN*) and overexpression of *HER2* (*ERBB2*) may be detected, as may other novel biomarkers.[59,63]

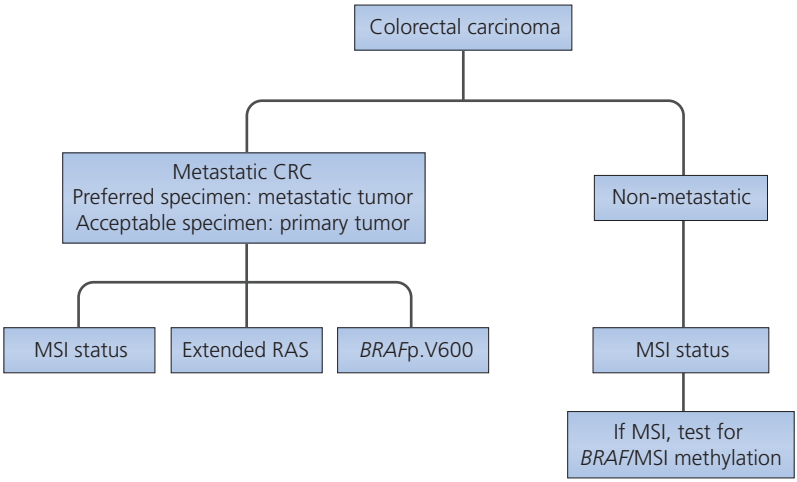

Figure 5.1 Molecular testing algorithm for CRC. Adapted from Harada and Morlote 2020.[52]

HER2 overexpression has been detected in around 3.5% of CRCs.[64] Promising responses to anti-HER2 therapies have been reported in clinical trials (HERACLES-A, HERACLES-B and HERACLES-RESCUE).[65–67]

Patients with MSI-H CRC tumors respond to therapy with pembrolizumab.[68] The group of potentially benefiting patients has now been expanded to include patients with high TMB, which is emerging as an important predictive biomarker for response to immune checkpoint inhibitor drugs in the subset of MMR-deficient (MSI-H) CRC.[69] TMB can be detected by NGS technology (see page 45).

The use of validated biomarkers and those currently being investigated is summarized in Table 5.2.

TABLE 5.2

Predictive and prognostic markers in CRC

Validated	
Predictive	• Extended RAS (*NRAS* and *KRAS*): if wild type, cetuximab or panitumumab is potential therapy • MSI: 5-fluorouracil-based agents contraindicated if MSI present
Prognostic	• *BRAF* p.V600E: poorer prognosis if mutated • MSI: better prognosis if MSI present
Research stage	
Predictive	• *PTEN*: cetuximab and panitumumab contraindicated if mutated • *PIK3CA*: cetuximab and panitumumab contraindicated if mutated • *HER2* (*ERBB2*): anti-HER2 therapies • TMB: immunotherapy

Liquid biopsy. Undoubtedly, liquid biopsies – being minimally invasive – will become more commonly used to determine biomarkers. Already, the presence of ctDNA is a predictor of relapse. Although ctDNA may be utilized predictively in the future to guide patient management, at present it has relatively high false-positive and -negative rates. Current ESMO and ASCO guidelines do not recommend its use for the initial diagnosis of, or as a predictive biomarker for, CRC.[58,62] As NGS and newer technologies overcome the technical shortcomings, it is highly likely that liquid biopsy will find a more significant role in diagnostics, which will necessitate new guidelines.

Melanoma

Melanoma is a highly mutated malignancy, with mutations documented in all subtypes (Table 5.3).[70,71] The *KIT* mutation is associated with chronic sun damage.

The *BRAF* mutation is present in 11% of patients with mucosal melanoma, *NRAS* mutation in 5% and *KIT* mutation in 15–20%.

From a molecular point of view, uveal melanoma is a distinct condition characterized, in 50% of patients, by the presence of a *GNAQ* mutation.[72] The presence of *BRAF* and *NRAS* mutations has not been described in uveal melanoma.[73]

The serine/threonine kinase BRAF is involved in the downstream signaling of the RTK and RAS proteins. Approximately half of melanomas show *BRAF* point mutations (Figure 5.2). In the majority of cases, a valine at position 600 is mutated to glutamic acid or lysine (*BRAF* p.V600E and *BRAF* p.V600K); both mutations are associated with kinase activation that results from relieving an intramolecular autoinhibitory interaction between the activation segment and P-loop of the protein.[74,75]

The events result in abnormal activation of the MAPK pathway, including the serine/threonine kinases MEK1/2, ERK1/2, and many downstream targets, resulting in many hallmarks of cancer, such as proliferation, migration, defense from apoptosis, and cellular metabolism.[76]

BRAF mutations are most common in the nodular and superficial spreading melanoma types, and they are rare in acral lentiginous (5–10% of cases) and non-cutaneous melanomas.[77] *BRAF* mutation

TABLE 5.3

Common genomic alterations in melanoma and prognostic and predictive clinical implications

Mutation	Relative frequency (%)	Clinical features
BRAF p.V600	40–50	• Sensitivity to BRAF/MEK inhibitors
NRAS	15–20	• Poor prognosis • Immunotherapy is a better treatment option
NF1	10–15	• More common on sun-exposed skin • Immunotherapy is a better treatment option
KIT	1–2 More common in mucosal (15–20%) and acral melanomas (15–20%)	• Sensitivity to c-KIT inhibitors
Atypical *BRAF* (non-V600)	4–5	• Sensitivity to MEK or RAF inhibitors
GNAQ/GNA11	80–90 (uveal melanoma)	
TERT promoter	40–50	• Poor prognosis • UV-mediated mutation
CDKN2A	25–35	• Deep deletions more common than mutations
PTEN	4–8	• May correlate with immune resistance • Deep deletions more common than mutations

Adapted from Davis et al. 2018.[71]

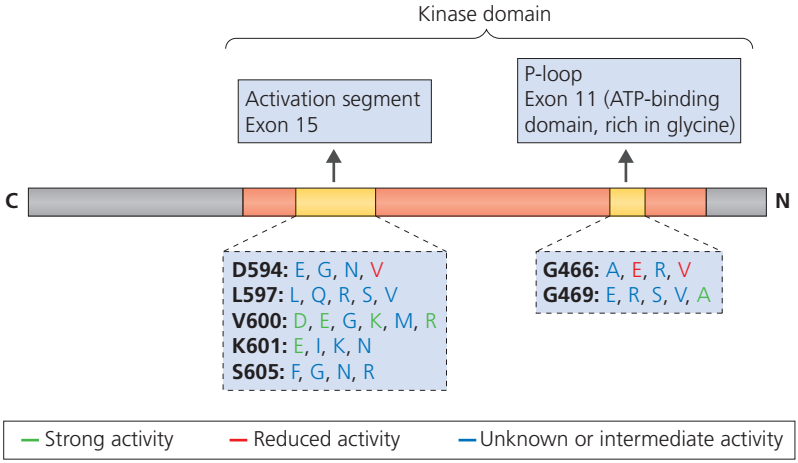

Figure 5.2 Common types of *BRAF* mutation in melanoma.[75]

correlates with distinct histopathological features, such as intraepidermal melanoma nest formation and a larger rounder border of the tumor with the surrounding skin, suggesting surrogate markers can be used to select patients for molecular testing.[78]

BRAF mutations also arise more commonly in patients who are younger at presentation and those with lymph node metastasis (rather than satellite tumors or visceral metastasis).[78]

BRAF inhibitors can lead to remarkable early tumor responses in melanoma, though these may be of short duration in some patients. Approximately 20% of patients with mutant BRAF melanoma show no response, and most patients treated with monotherapy relapse, with a median PFS of 8–9 months.

Vemurafenib is an orally available small molecule kinase inhibitor with activity against BRAF with the p.V600E mutation; its indication is restricted to melanoma patients with a demonstrated *BRAF* p.V600E mutation by an FDA-approved test. This agent was approved by the FDA in 2011 and by the EMA in 2012. Vemurafenib has shown an improvement in PFS and overall survival in patients with unresectable or advanced melanoma.[79]

Dabrafenib, an orally available, small molecule, selective BRAF inhibitor was approved by the FDA and EMA in 2013. This agent

demonstrated an improvement in PFS compared with dacarbazine (DTIC) in the international multicenter BREAK-3 trial.[80]

MEK inhibitors. Trametinib is an oral, small molecule, selective inhibitor of MEK1 and MEK2 that was approved by the FDA in 2013 for melanoma patients with unresectable or metastatic melanoma with *BRAF* p.V600E or p.V600K mutations. Trametinib is associated with improved PFS versus dacarbazine.[81]

Cobimetinib is an orally available, small molecule, selective MEK inhibitor approved by the FDA and EMA in 2015 for use in combination with the BRAF inhibitor vemurafenib for the same indication.[82]

c-KIT inhibitors. Early data suggest that mucosal or acral melanomas with activating mutations or amplifications in *KIT* may be sensitive to a variety of c-KIT inhibitors.[83]

BRAF and MEK inhibitors in combination. In 2014, the combination of dabrafenib and trametinib received accelerated approval from the FDA for patients with unresectable or metastatic melanomas that carry the *BRAF* p.V600E or p.V600K mutation. The combination demonstrated improved durable response rates over single-agent dabrafenib.[84] The treatment combination is also approved for use in the EU.

In 2015, the combination of vemurafenib and cobimetinib was approved by the FDA and the EMA for metastatic melanomas that carry the *BRAF* p.V600E or p.V600K mutation.[71]

Mechanisms of resistance to BRAF/MEK inhibitors. The majority of patients with metastatic melanoma who present with the activating *BRAF* mutation (p.V600E or p.V600K) respond to treatment. However, 20% of these patients are primarily refractory to selective BRAF inhibitors and do not respond.[85] The mechanisms of intrinsic resistance can include *RAC1* mutations, loss of PTEN, dysregulation of cell cycle proteins, and changes to the tumor microenvironment. These abnormalities are summarized in Table 5.4.

TABLE 5.4

Mechanisms of resistance to BRAF/MEK inhibition in melanoma

Intrinsic	Intrinsic or acquired
• Stromal secretion of HGF, which can activate MET and reactivate MAPK and PI3K/AKT pathways • Aberrations affecting the CDK4/cyclin D1 complex, which is important during the cell cycle • *HOXD8* mutations (expression of HOXD8 appears to suppress cancer)	• Mutations affecting the PI3K/AKT pathway • PTEN loss • *MEK* mutations • *NRAF* mutations • Loss of NF1

Acquired
• *BRAF* splicing • *BRAF* copy gains or amplification • RTK upregulation • ERK feedback

CDK4, cyclin-dependent kinase 4; HGF, hepatocyte growth factor; NF1, neurofibromin 1.

Tumor mutational burden. A high TMB augments tumor immunogenicity and increased numbers of tumor neoantigens; it may stimulate an immune response. DNA damage from exogenous factors is responsible for the high TMB seen in melanoma. The finding of high TMB is associated with higher response rates to immune checkpoint inhibitors.[86]

Sarcoma

Sarcomas are a highly heterogeneous group of malignant tumors showing differentiation toward adult mesenchymal tissue types. They are a divergent group of tumors morphologically, genetically and behaviorally. Although much is known about the individual molecular pathology of many soft-tissue tumors, this knowledge has been applied mainly in the diagnosis of sarcomas, with gene panels used mostly for diagnostic purposes. However, some tumors, such as

GISTs, have well-known molecular abnormalities that provide not only prognostic information but also serve as predictive biomarkers for targeted therapy. Examples are aberrations affecting c-KIT and platelet-derived growth factor receptor (PDGFR), which can support imatinib therapy.

Gastrointestinal stromal tumors. Around 80% of GISTs show mutations in *KIT* that result in constitutive activation of the RTK, c-KIT. Most commonly, there is mutation (SNV or indel) of exon 11, then exon 9, followed by exons 13 and 17 (Table 5.5). Up to 50% of *KIT*-mutation-negative GISTs have activating mutations of the *PDGFRA* gene, usually in exons 18, 12 and 14.[87]

A small subset of GISTS display mutations in *BRAF*, *KRAS*, *NF1*, *NRAS* or *SDH* .

GISTs with a deletion in exon 11 (codon 557–8) are more biologically aggressive than those with substitutions in the same codons.

Standard commercially available gene profiling chips (NGS) are highly appropriate to determine the genes associated with GISTs. The presence, type and location of the abnormality predict response to TKIs.[88]

Initially, imatinib was the drug of choice,[89] but the newer TKIs sunitinib and regorafenib have also shown efficacy, particularly for GISTs resistant to imatinib.[90] Avapritinib was approved by the FDA in early 2020 for GIST harboring a *PDGFRA* exon 18 mutation, including the D842V mutation, while the kinase inhibitor ripretinib was approved later in the same year as fourth-line therapy for adults with an advanced GIST.[91] Crenolanib, which selectively inhibits PDGFRA mutant protein, particularly that arising from *PDGFRA* D842 mutation, is in clinical trials for use in patients with GIST.[91,92] The second- and third-generation TKIs dasatinib, nilotinib and ponatinib, which target *BCR–ABL* products, have shown limited results.[93]

Infantile fibrosarcoma is caused by the fusion of *NTRK3* with *ETV6*, which results in the ETS transcription factor contributing its helix–loop–helix domain to the kinase domain of NTRK3 in the resulting product. Clinical trials of Trk TKIs, such as larotrectinib and entrectinib, are under way in a range of sarcomas.[94,95]

TABLE 5.5

Molecular classification of GIST

Genetic type	Relative frequency (%)	Site	Imatinib response
***KIT* mutation**	**80**		
Exon 8	Rare	Small bowel	May be sensitive
Exon 9	10	Small bowel, colon	Sensitive, may require higher dose
Exon 11	66	All sites	Sensitive
Exon 13	1	All sites	Moderately sensitive
Exon 17	1	All sites	Resistant
***PDGFRA* mutation**	**5–8**		
Exon 12	1	All sites	Sensitive
Exon 14 (SNV)	<1	Stomach	Sensitive
Exon 18 D842V	5	Stomach, mesentery	Resistant
Exon 18 other	1	All sites	Moderately sensitive
Wild type	**12–15**		
Carney triad NF1-related (epimutation of SDH subunit gene)	7.5	Stomach	Resistant
SDH deficient	4.5	Stomach	Resistant
BRAF (p.V600E), *HRAS*, *NRAS* or *PIK3CA* NF1-related sarcomas	Rare	Small bowel	Resistant

NF1, neurofibromin 1; SDH, succinate dehydrogenase.

Epithelioid sarcoma. The FDA granted accelerated approval to tazemetostat for the treatment of adults and young people (16 years or older) with metastatic or locally advanced epithelioid sarcoma who are not eligible for complete resection (surgical removal of the tissue). Tazemetostat inhibits enhancer of zeste homolog 2 (EZH2); the biomarker is loss of integrase interactor 1 (INI1).[96]

Inflammatory myofibroblastic tumor. In around 50% of cases, and particularly in children, these rare tumors harbor rearrangements of chromosome 2p23. This is the site of *ALK*, which encodes an RTK that is upregulated on appropriate fusion to one of more than ten potential translocation partners. Specific TKI inhibitors, such as crizotinib, may be used for patients with inoperable/disseminated tumors.[97]

Molecular targeting in other non-GIST sarcomas. Pazopanib, an oral anti-angiogenic drug targeting vascular endothelial growth factor receptor (VEGFR), PDGFR, fibroblast growth factor receptor (FGFR), c-KIT and many other tyrosine kinases, was associated with improved PFS in patients with soft-tissue sarcomas other than liposarcomas (mainly leiomyosarcoma and synovial sarcoma) in the Phase III PALETTE study.[98] There is, however, no biomarker to guide its use.

Other TKIs used for treating GISTs, such sorafenib, sunitinib and regorafenib, have some effect against non-GI sarcomas, particularly in tumors with *PDGFRA* mutations.

Other pathways investigated include the PI3K/AKT/mTOR pathway. The mTOR inhibitor ridaforolimus showed prolongation of PFS (though the drug was not approved by the FDA),[99] while the mTOR inhibitor sirolimus is effective in the treatment of perivascular epithelioid tumors (PEComas), which are known to show mTOR pathway activation.[100]

Well-differentiated and dedifferentiated liposarcomas show amplification of *MDM2* and *CDK4/6*. While the cyclin-dependent kinase 4/6 (CDK4/6) inhibitor palbociclib shows only modest results, there is some evidence that dual inhibition of CDK4/6 and MDM2 proto-oncogene (MDM2) may be synergistic.[101]

Summary. Summaries of molecular biomarkers and related therapies in sarcomas and targeted therapies in soft-tissue tumors are provided in Tables 5.6 and 5.7.

TABLE 5.6

Sarcomas with NGS-detectable biomarkers and targeted therapy*

Tumor type	Molecular biomarker	Targeted therapy
GIST	*KIT*	
	Sensitive: exon 9, 11, 13	Imatinib, sunitinib, regorafenib
	Resistant: exon 17	
	PDGFR	
	Sensitive: exon 12, 14, 18	Imatinib, sunitinib, regorafenib
	Resistant: exon 18 (D842V)	Avapritinib
Infantile fibrosarcoma	*ETV6–NTRK3*	Larotrectinib, entrectinib
Inflammatory myofibroblastic tumor	*ALK* fusion	Crizotinib
Epithelioid sarcoma	INI1 loss	Tazemetostat

*Examples of therapies; check appropriate sources for up-to-date approvals and prescribing information.

TABLE 5.7

Examples of targeted therapies for soft-tissue tumors*

Drug	Target	Tumor
Anlotinib	c-KIT, FGFR1–4, PDGFRA/B, RET, VEGFR2/3	Non-GIST STS
Cediranib	VEGFR1–3	Alveolar soft part sarcoma
Crizotinib	ALK	Inflammatory myofibroblastic tumor
Entrectinib	Trk fusions	Solid tumors with *NTRK* fusion
Imatinib	BCR–ABL, c-KIT, PDGFRA	GIST
Larotrectinib	Trk fusions	Infantile fibrosarcoma Solid tumors with *NTRK* fusion
Nilotinib	BCR–ABL, c-KIT, PDGFR	GIST
Palbociclib	CDK4/6	Liposarcoma
Pazopanib	BCR–ABL, c-KIT, PDGFR, VEGFR	Non-GIST STS other than liposarcoma Liposarcoma
Regorafenib	BCR–ABL, c-KIT, PDGFR, VEGFR	GIST Non-GIST STS
Sorafenib	c-KIT, FGFR1–4, PDGFR RET, VEGFR	Non-GIST STS
Sunitinib	c-KIT, FGFR1–4, PDGFR, VEGFR	GIST Non-GIST STS

*Some are approved and some are in clinical development – check appropriate sources for the up-to-date status.
STS, soft-tissue sarcoma.
Adapted from Nakano and Takahashi 2018.[93]

Key points – predictive and prognostic biomarkers

- The molecular status of, at least, *EGFR*, *ALK*, *ROS1* and *BRAF* must be tested in patients with advanced stage NSCLC to determine suitability for TKI therapy.
- PD-L1 expression also needs to be tested in patients with advanced NSCLC to guide immunotherapy decisions.
- Knowledge of mutations affecting *KRAS*, *MET*, *RET* and *NTRK* may also help select treatment for advanced NSCLC.
- Individuals with advanced breast cancer should undergo molecular testing to assess, at least, *PIK3CA* (ER+ mBC), germinal *BRCA* mutations (*HER2*– mBC), *HER2* amplification, MSI and *NTRK*.
- Knowledge of PD-L1 expression is also required to guide immunotherapy in TNBC.
- The utility of assessing several other genes (*ESR1*, *AKT1*, *PTEN* and *HER2*) and the value of assessing TMB are currently being investigated in breast cancer.
- Chromosomal instability accounts for around 85% of all CRCs, while MSI accounts for around 15%. CIMP, which shows overlap with the MSI pathway, is found in around 17% of CRCs.
- The minimum testing required to determine whether anti-EGFR therapy may be of benefit in CRC is extended RAS testing of *KRAS* and *NRAS*: codons 12 and 13 of exon 2; codons 59 and 61 of exon 3; and codons 117 and 146 of exon 4.
- Melanoma is a highly mutated malignancy, with mutations – including *BRAF*, *NRAS* and *KIT* – documented in all melanoma subtypes.
- Approximately half of melanomas show *BRAF* point mutations. BRAF inhibitors can lead to remarkable early tumor responses in melanoma; however, these responses may be short.
- The combination of BRAF and MEK inhibitors has demonstrated improved durable response rates over a single-agent BRAF inhibitor.
- Mucosal or acral melanomas with activating mutations or amplifications in *KIT* may be sensitive to c-KIT inhibitors.
- The majority of GISTs have *KIT* mutations that result in constitutive activation of the RTK, c-KIT.
- TKIs have some effectiveness in GISTs and non-GI sarcomas.

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6 Overcoming barriers to genotype-directed therapy

Preparing high-quality samples

In the era of personalized medicine, tissue samples (histological or cytological specimens) acquire a central role not only for morphological evaluation but also for molecular analyses. In this setting, careful attention should be paid to pre-analytic factors that can adversely affect the quality and quantity of nucleic acids in the sample, leading to uninterpretable molecular results.[1]

FFPE histological samples represent the gold standard starting material from which to extract nucleic acids for molecular analysis. FFPE specimens contain a high quantity of material for both morphological evaluation and ancillary techniques and do not require additional molecular validation, unlike cytological samples.[2]

A prolonged fixation time in formalin should be avoided, as this can induce C>T artifacts.[3] Such a change can mimic a mutation and may lead to a false-positive result. Other factors that may influence the integrity of nucleic acids include:

- cold ischemia time (the time between biospecimen removal from the body and its preservation)
- decalcification
- duration of storage of the paraffin block.

Data from the literature suggest some good-practice recommendations (Table 6.1).[4]

Cytological preparations (direct smears and liquid-based cytology), in contrast to FFPE samples, do not suffer the histological issues related to formalin fixation (cytological specimens are air dried or alcohol fixed), cold ischemia or paraffin storage (Table 6.2).[2] Direct smears are suitable for rapid on-site evaluation (ROSE) when fine needle aspiration (FNA) is performed.[5] This procedure supports the correct management of aspirated material for morphomolecular analysis. In addition, the possibility of targeting a neoplastic lesion

TABLE 6.1

Good practice for FFPE sample preparation

Step	DNA	RNA
Cold ischemia time	≤24 hours	≤12 hours
Formalin fixation time*	72 hours	8–48 hours
Tissue block storage time	≤5 years	≤1 year

*Room temperature or 4°C.
From Bass et al. 2014.[4]

with the fine needle allows cancer cell enrichment and avoids contamination with non-neoplastic elements.

The major limitation of cytological samples is the necessity for careful validation of techniques before they are implemented in clinical practice. Moreover, despite the high-quality sampling of nucleic acids, these preparations have low quantities of material available for analysis and there is a high risk that diagnostic material will be lost.

Cell blocks are cytological preparations that resemble histological samples. The aspirated material is processed in FFPE format. Consequently, cell blocks have advantages and disadvantages similar to those of histological samples.[2]

How to report

Molecular reports are a crucial part of molecular laboratories' workflow. They should contain all relevant information to support the clinician's management of the patient, particularly regarding the best treatment choice.[6,7] For this reason, the communication should be accurate and avoid any misinterpretation by molecular pathologists or clinicians, particularly oncologists or other physicians who request the molecular analysis.

Incomplete or difficult to understand reports may lead to errors in patient management. Molecular reports should be short and easy to interpret, focusing on the main information that could be of interest for the patient's clinical management. All reports should show the patient's unique identifiers (name, surname, date of birth and

TABLE 6.2

The pros and cons of sample preparations

Sample type	Pros	Cons
FFPE histological samples	• High quantity of material • Careful validation unnecessary • Morphology preserved	• Low-quality NAs (prolonged cold ischemia, formalin fixation and paraffin storage)
Direct smear	• High-quality NAs • ROSE possible	• Low quantity of material • Requires training in slide preparation • Requires rigorous validation • Loss of morphology
Liquid-based cytology	• High-quality NAs (when using methanol-based fixatives) • Standardized slide preparation	• Low quantity of material • Low-quality NAs (when using hemolytic fixatives) • Unsuitable for ROSE • Requires rigorous validation • Loss of morphology
Cell block	• High quantity of material • Careful validation unnecessary • Morphology preserved	• Low-quality NAs (prolonged formalin fixation and paraffin storage)

NA, nucleic acid.

identification number). In addition, it is important to report the:[6]

- ward or service
- date
- sample type
- name of the clinician who requested the molecular analysis.

The body of the text should report, in a brief and clear manner, the main characteristics of samples (percentage of neoplastic cells, fixation problems, presence of contaminant that can limit the analysis) and the information regarding the mutational status of the analyzed biomarkers.[6,7]

As recommended by the AMP, ASCO and CAP, a four-tiered system for the interpretation and reporting of variants in cancer should be adopted, in particular when tested using large NGS panels (Table 6.3).[7]

According to the guidance from the professional bodies, tiers I–III variants should be reported, but it is not always necessary to report tier IV variants. Mutations should be reported using Human Genome Variation Society (HGVS) nomenclature and following the scheme *gene*, p. (protein reference sequence) and c. (coding DNA reference sequence) annotations (for example, *KRAS* p.G12D c.35G>A). Gene rearrangements should be reported by indicating both genes involved (for example, *EML4*/*ALK* fusion). CNVs detected by NGS should be reported as copy number 'GAIN' or 'LOSS' (for example, *EGFR* copy number GAIN [copy number ratio 25]). It is important to indicate, where possible, the variant allelic frequency and coverage of detected alterations.

It is also important to report as 'wild type' the absence of mutations in clinically relevant genes (tier I).

TABLE 6.3

The four-tiered system for reporting cancer variants

Class	Type of variant
Tier I	Strong clinical importance
Tier II	Potential role in cancer patients
Tier III	Unknown significance, but associated with cancer
Tier IV	Known benign or likely benign significance

The report should contain a clinical interpretation of the detected variants to suggest treatment options, with literature citations where possible. Information about the methods – the type of test adopted, the reference range, the limit of detection (LOD) and the NGS parameters applied – should be included at the end of the report.[7]

Building knowledge

The increasing knowledge about genomic alterations involved in cancer development and the technological improvements represent a serious challenge for physicians making decisions about cancer treatment.[8] In particular, clinicians may have had limited training in molecular biology while molecular pathologists may have low awareness of clinical management. To overcome these limitations, it is very important that challenging cases are brought to, and discussed by, MTBs.[9,10]

Molecular tumor boards are multidisciplinary groups of professionals involved in the management of cancer patients, such as molecular pathologists, clinicians, surgeons, radiologists, geneticists, bioinformaticians and biologists. There are 6–40 members at each meeting.[10]

At each meeting, the MTB discusses the correct management of individual cancer patients. It is the role of the MTB to decipher the complexity of the cancer genomic landscape and translate it into information useful for patient management.

Although there are no guideline recommendations, the MTB should meet at least weekly to discuss cases, especially when particular issues emerge. As well as therapeutic strategies, the meeting provides an opportunity to solve other kinds of problems, such as the type of sample that should be analyzed, the molecular technique and gene panel that should be adopted and the interpretation of molecular results, which is particularly important when unclear or doubtful data have been reported.[10]

Owing to the complexity of each cancer patient, MTBs generally discuss two or three patients per meeting. In the absence of guidelines, van der Velden et al. propose recommendations to optimize and facilitate the MTB function.[10] First, there should be global harmonization in cancer sequencing practices and procedures. Second, MTBs should

comprise, as a minimum, clinicians (all specialists involved in the patient's cancer management), pathologists (including molecular pathologists), biologists, geneticists (particularly when considering the presence of germline variants) and bioinformaticians. Finally, MTBs should set out how they will handle unsolicited findings, in particular regarding germline variants.

Costs/reimbursement

Costs and reimbursements are crucially important for molecular pathology laboratories offering predictive assays. Owing to a high workload and the necessity of orthogonal technologies (validation process and confirmation of mutational results), laboratories may implement two or more different platforms.

Costs. Malapelle et al., in a validation study, demonstrated that the costs of NGS were affordable compared with Sanger sequencing (the gold standard).[11] In this study, patients with advanced CRC were analyzed for clinically relevant mutations in *KRAS* and *NRAS* exons 2, 3 and 4 and *BRAF* exon 15. As far as consumable costs are concerned, Sanger sequencing (a single-gene test) cost at least €28 per reaction, giving a total cost of €196 for the analysis of seven exons. With NGS testing using the Ion Torrent platform (see page 34), 504 mutational hotspot regions in 22 genes can be tested simultaneously for 8 patients using a single 316 chip. In this case, the cost for each patient's analysis was calculated as €187.23.[11]

In another study, Malapelle et al. reported that the cost of NGS analysis could be reduced by adopting a narrow custom gene panel covering 568 hotspot mutations in six genes (*EGFR*, *KRAS*, *NRAS*, *BRAF*, *KIT* and *PDGFRA*).[12] In addition, the protocol was optimized to run 16 patient samples in a single 316 chip.[12] These modifications reduced the cost to €98 per sample, similar to the cost of a traditional RT-PCR test.[12]

Reimbursement. Besides the different gene panels and platforms, it is important to consider the healthcare system in which these technologies are adopted and the reimbursement system for molecular tests.[13,14]

If there is a well-resourced reimbursement-based system, insurance coverage can guarantee the refund of the cost of wide tumor sequencing.

In this setting, NGS is fully adopted as a 'one-stop-shop' for all biomarkers.[13,14]

If there is universal healthcare, resources are limited. Careful attention should be paid to ensuring that the molecular data that can represent the standard of care for cancer patients, at the very least, can be gained. In addition, costs rise if a large number of laboratories perform molecular tests on a relatively low number of samples. In this setting, it may be helpful to centralize NGS to make it viable.[13,14]

Key points – overcoming barriers to genotype-directed therapy

- Different sample types (histological or cytological) have distinct advantages and disadvantages; these are a key issue in molecular analysis.
- Standardization in molecular reporting is very important.
- Discussing a patient's results and management at an MTB meeting may help to overcome challenging issues.
- Laboratories should pay careful attention to costs and reimbursement systems for molecular analysis.

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Useful resources

Organizations

American Society of Clinical Oncology (ASCO)
www.asco.org

Association for Molecular Pathology (AMP)
www.amp.org

College of American Pathologists (CAP)
www.cap.org

European Society for Medical Oncology (ESMO)
www.esmo.org

Human Genome Variation Society (HGVS)
www.hgvs.org/

International Academy of Cytology
www.cytology-iac.org

Databases

COSMIC database
COSMIC is the world's largest and most comprehensive resource for exploring the impact of somatic mutations in human cancer.
cancer.sanger.ac.uk/cosmic

OncoKB
Precision oncology knowledge base that provides information about the effects and treatment implications of specific cancer gene alterations. It is developed and maintained by the Memorial Sloan Kettering Cancer Center.
www.oncokb.org

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